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Molecular profiling of mitochondrial dysfunction in *Caenorhabditis elegans*

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Abstract

Cellular effects of primary mitochondrial dysfunction, as well as potential mitochondrial disease therapies, can be modeled in living animals such as the microscopic nematode, *Caenorhabditis elegans*. In particular, molecular analyses can provide substantial insight into the mechanism by which genetic and/or pharmacologic manipulations alter mitochondrial function. The relative expression of individual genes across both nuclear and mitochondrial genomes, as well as relative quantitation of mitochondrial DNA content, can be readily performed by quantitative Real-Time PCR (qRT-PCR) analysis of *C. elegans*. Additionally, microarray expression profiling offers a powerful tool by which to survey the global genetic consequences of various causes of primary mitochondrial dysfunction and potential therapeutic interventions at both the single gene and integrated pathway level. Here, we describe detailed protocols for RNA and DNA isolation from whole animal populations in *C. elegans*, qRT-PCR analysis of both nuclear and mitochondrial genes, and global nuclear genome expression profiling using the Affymetrix GeneChip *C. elegans* Genome Array.

Keywords

Total RNA; mitochondrial DNA; qRT-PCR; Taqman; Affymetrix GeneChip *C. elegans* Genome Array

1. Introduction

Primary mitochondrial disease results from mutations in either the mitochondrial DNA (mtDNA)-encoded genes or in nuclear genes whose products localize to the mitochondrion (1). In addition, a host of other genetic disorders and/or pharmacologic agents can cause secondary mitochondrial dysfunction. Bidirectional nuclear-mitochondrial 'cross-talk' is evident in the consistent pattern of nuclear and mitochondrial genome expression alterations that occur in the setting of mitochondrial dysfunction (2–3). Molecular analyses offer a critical means by which to elucidate the specific responses of both nuclear and mitochondrial genomes to mitochondrial dysfunction.

Caenorhabditis elegans offers a robust translational model animal in which to facilitate both the *in vivo* and *in vitro* study of mitochondrial disease (4). Its utility is largely rooted in the highly conserved nature of mitochondrial structure, composition, and function across

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evolution. Relative to wild-type (N2 Bristol) worms, a range of transgenic animals harboring mutations in nuclear-encoded respiratory chain subunits, assembly factors, enzymes, or other integral mitochondrial proteins can be compared (5–6). In addition, potential mitochondrial toxins and pharmacologic therapies for mitochondrial dysfunction can be systematically investigated in *C. elegans* mitochondrial mutant strains (7).

Here, we describe detailed protocols for RNA and DNA isolation from whole animal *C. elegans* populations, qRT-PCR analyses to determine the relative expression of nuclear-encoded mitochondrial genes (eg, *SOD2* and *SOD3*) as well as relative mtDNA content (eg, *ND4*) genes, and global nuclear genome expression profiling using the commercially-available Affymetrix GeneChip *C. elegans* Genome Array. The analysis of synchronous worm populations is described to provide sufficient starting material for diverse molecular analyses, although similar methods can be applied to isolate nucleic acids from single animals. In addition, utilization of a Taqman Gene Expression Assay for a common endogenous control gene (*drs-1*) is reported that has shown consistent performance in qRT-PCR expression analysis of primary mitochondrial dysfunction in *C. elegans* (7).

2. Materials

2.1 Total RNA isolation from synchronous worm populations

1. Nematode growth media (NGM) for worm growth plates (Bioworld) (8)
2. 10 cm plastic culture plates
3. OP50 *E. Coli* bacteria
4. S-basal, pH 7.0: Dissolve 3.4g of KH₂PO₄, 4.4 g of K₂HPO₄, and 5.85 g of NaCl in 800 ml of MilliQ water. Adjust pH with NaOH and add MilliQ water to a final volume of 1000ml. Sterilize by autoclaving for 30 minutes.
5. 1.5 ml RNase-free Eppendorf tube with tight fitting plastic pestle.
6. 15 ml centrifuge tubes
7. 5 M NaOH: Dissolve 200 g in approximately 800 ml of MilliQ water and bring to a final volume of 1000 ml.
8. Liquid bleach (Fisher Scientific) or commercially available bleach (Clorox).
9. Dimethyl sulfoxide (DMSO)
10. Kontes Pellet Pestle Cordless Motor (Fisher Scientific Inc., Pittsburgh, PA)
11. Trizol Reagent (Invitrogen Corporation, Carlsbad, CA)
12. Chloroform
13. 70% ethanol (EtOH)
14. RNase-free water included in RNeasy Mini Kit or MilliQ water.
15. RNeasy Mini Kit (Qiagen, Inc., Valencia, CA)
16. NanoDrop ND-1000 Spectrophotometer
17. Table centrifuge with capacity to accommodate 15 ml tubes at 1300 g at room temperature.
18. Two table centrifuges to accommodate 1.7 ml Eppendorf tubes for maximum speed of 16000g. Place one in cold room at 4°C and another one at room temperature.
19. Pharmacologic agent(s) to be studied

2.2 DNA isolation from synchronous worm populations

1. 10 cm NGM agar plates for worm maintenance (as per section 2.1)
2. OP50 *E. Coli* bacteria
3. S. basal, pH: 7.0: Dissolve 3.4g of KH₂PO₄, 4.4 g of K₂HPO₄ and 5.85 g of NaCl in 800 ml of MilliQ water. Adjust pH with NaOH, and add distilled water to a final volume of 1000 ml. Sterilize by autoclaving for 30 minutes.
4. 15 ml centrifuge tubes
5. Dissolve 200 g of 5 M NaOH in approximately 800 ml of what MilliQ water and bring to a final volume of 1000 ml.
6. Liquid bleach (Fisher Scientific) or commercially available bleach (Clorox).
7. Table centrifuge with capacity to accommodate 15 ml tubes at 1300 g.
8. 1.5 ml Eppendorf tubes with tight fitting plastic pestle
9. Kontes Pellet Pestle Cordless Motor (Fisher Scientific Inc., Pittsburgh, PA)
10. QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA)
11. NanoDrop ND-1000 Spectrophotometer

2.3 qRT-PCR analysis of relative mtDNA content in synchronous worm populations

1. Taqman Fast Universal PCR Master mix (Applied Biosystems, Foster City, CA)
2. MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA)
3. Optical adhesive covers (Applied Biosystems, Foster City, CA)
4. RNase-free water or MilliQ water
5. Taqman Gene Expression Assay for nuclear-encoded endogenous control gene, *drs-1* (Assay # Ce02451127_g1)
6. Taqman Gene Expression Assay for mtDNA-encoded complex I subunit gene, *nd4* (Assay # Custom Taqman Gene Expression Assay, Applied Biosystem, Foster City, CA. Forward primer sequence:
5'GAGGCTCCTACAACAGCTAGAATAC3'. Reverse primer sequence:
5'TCATACATTGTTGTGTACAAATCTTAAACTACCT3'
7. 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA)
8. 7500 Fast Real-Time PCR System Software v2.0.1 (Applied Biosystems, Foster City, CA)

2.4 qRT-PCR of nuclear gene relative expression in synchronous worm populations

2.4.1 DNase treatment of total RNA

1. Turbo DNA-free kit (Ambion Inc., Austin, TX)
2. Dry bath for 37°C temperature control
3. 0.5 ml RNase-free tubes
4. Table centrifuge (Spectrafuge 24D, Labnet)

2.4.2 RT-PCR reaction to generate cDNA

1. High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA)
2. PCR tubes
3. GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA)

2.4.3 qRT-PCR reaction to assay nuclear gene relative expression

1. Taqman Fast Universal PCR Master mix (Applied Biosystems, Foster City, CA)
2. MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA)
3. Optical adhesive covers
4. RNase-free water or MilliQ water
5. Taqman Gene Expression Assay for nuclear-encoded endogenous control gene, *drs-1* (Assay # Ce02451127_g1)
6. Taqman Gene Expression Assay for mtDNA-encoded complex I subunit (eg, *sod-3*, assay # Ce02404515_g1).
7. 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA)
8. 7500 Fast Real-Time PCR System Software v2.0.1 (Applied Biosystems, Foster City, CA)

2.5 Affymetrix GeneChip *C. elegans* Genome Array analysis

2.5.1 Microarray Performance

1. GeneChip *C. elegans* Genome Array (Catalogue # 900383 per 5 arrays, Affymetrix, Santa Clara, CA)
2. 100 ng of total RNA from four to six biological replicates per strain/condition
3. 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA)

2.5.2 Microarray Platform Description—Microarray technology permits the measurement of the expression level for thousands of transcripts simultaneously. We have previously used the GeneChip *C. elegans* Genome Array (Affymetrix, Inc.) to study the expression of most worm transcripts under different experimental conditions (2). This 3' expression array platform measures about 22,500 known *C. elegans* transcripts. Of note, *C. elegans* Tiling 1.0R is an alternative platform that has recently been developed to interrogate the whole worm genome, which offers the benefit of being able to measure the relative expression not only of known gene transcripts but also of unknown transcripts, such as microRNAs and other small non-coding RNAs (9–10). Both platforms use 25-base long oligomers that are spotted on the array surface as probes to hybridize biotin-labeled cDNA generated by reverse transcription. The signal strength of each spot after hybridization represents the abundance of cDNA, which is used to determine the expression level of the corresponding transcript.

3. Methods

3.1 *C. elegans* growth and treatment

1. *C. elegans* worm strains are described at www.wormbase.org and publicly available from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN).
2. Maintain worms at 20°C on NGM agar plates spread with OP50 *E. coli* bacteria as standard nematode food source.
3. Bleach gravid adult worms to obtain sufficient eggs for purposes of generating synchronous young adult populations (8) (*see Note 1*)
 - a. Wash gravid worms off from NGM agar plates with 3.5 ml of sterile water into a 15 ml centrifuge tube.
 - b. Make a fresh solution of 0.5 ml 5M NaOH and 1 ml bleach immediately prior to use.
 - c. Add bleach solution to worms in 15 ml centrifuge tube to achieve a final volume of 5 ml.
 - d. Shake tube well for several second, and then incubate on benchtop.
 - e. Repeat shaking every 2 minutes for a total of 10 minutes.
 - f. Centrifuge 15 ml tube in a table-top centrifuge for 1 minute at 1300 g to pellet the released eggs.
 - g. Aspirate the supernatant to 0.1 ml.
 - h. Add sterile water to 5 ml and shake well.
 - i. Repeat steps f and g.
 - j. Use a sterile Pasteur pipette to transfer eggs in the remaining 0.1 ml of liquid to a fresh NGM agar plate without bacteria.
4. Incubate eggs on unspread NGM agar plate (without bacteria) at 20°C overnight (*see Note 2*).
5. The next day, transfer L1-stage arrested animals onto fresh NGM agar plates spread with OP50 *E. coli*.
6. Maintain worms at 20°C until they have reached adult stage, as defined by the presence of eggs laid on the plate.
7. Wash worms off from NGM agar plates in 1.5 ml of S. basal, allowing adults to separate from eggs and larvae by gravity for 5 minutes.
8. Collect bottom layer of adults (pellet) for total RNA extraction.

3.2 Pharmacologic treatment of synchronous young adult or developing worms

3.2.1—If studying the effects of a study drug, add the desired drug or appropriate buffer control to a 10 cm NGM agar plate after the bacteria lawn has dried and prior to plating worms.

¹During the bleaching process several vigorous shaking steps are recommended. Wild-type (N2 Bristol) can tolerate this shaking, but mitochondrial mutant worms require much gentler shaking to yield viable eggs.

²Without bacteria, worm development is arrested at the L1 larval stage. When subsequently transferred to NGM agar plates spread with OP50 *E. coli*, their development resumes simultaneously to achieve stage synchrony, as is necessary for expression analyses.

1. For control (buffer only) plates, evenly spread 10 cm bacterial-spread NGM agar plate with 500 μ l of *S. basal* for water soluble drugs or with 20% DMSO (100 μ l DMSO and 400 μ l *S. basal*) for non-water soluble drugs.
2. For drug treatment plate, add desired drug of interest in 500 μ l total volume of *S. basal* or 20% DMSO, as depends on its solubility.

3.2.2—Worms can be treated with study drugs by feeding either **(A)** Young adults for 24 hours or **(B)** Developing larvae from the L1 arrested stage through the young adult stage.

3.2.2.1. Pharmacologic treatment of young adult stage worms

1. Transfer approximately 1,000 synchronous first day young adult stage worms to NGM 10 cm plates spread with OP50 *E. coli* and desired drug concentration or buffer control (*see* Note 3).
2. Incubate worms on drug treatment or buffer control 10 cm NGM agar plates at 20°C for 24 hours.
3. Wash worms off from NGM agar plates with 1.5ml of *S. basal* into 1.5ml Eppendorf tube.
4. Allow adults to separate from eggs and larvae by gravity for 5 minutes.
5. Collect bottom layer of adults (pellet) into 1.5 ml RNase-free Eppendorf tube for total RNA extraction.

3.2.2.2. Pharmacologic treatment of developing worms

1. Transfer approximately 1,000 synchronous, L1-arrested larvae from unspread NGM agar plates (no bacteria) to drug treated or buffer control plates (*see* Note 3).
2. Incubate worms on drug treatment or buffer control 10 cm NGM agar plates at 20°C for approximately 2–3 days until they reach adulthood (as defined by eggs laid on plate).
3. Wash adult worms off from NGM agar plates with 1.5ml *S. basal* into 1.5 ml Eppendorf tube.
4. Allow adults to separate from eggs and larvae by gravity for 5 minutes.
5. Collect bottom layer of adults (pellet) in 1.5 ml RNase-free Eppendorf tube for total RNA extraction.

3.3 Total RNA extraction from adult *C. elegans*

1. Wash adult worm pellet with 1 ml of *S. basal* to remove residual bacteria.
2. Pipette off supernatant to leave worm pellet.
3. Repeat wash 5–6 times.
4. After the final wash, leave worm pellet with approximately 100 μ l *S. basal* in the 1.5 ml RNase-free Eppendorf tube.
5. Add 400 μ l of Trizol reagent to the worm pellet in the 1.5 ml RNase-free Eppendorf tube.

³Estimate worm number by counting three 100 μ l aliquots. Adjust worm concentration to 1000 worms/ml of *S. basal*.

6. Grind worms for 1 minute with Kontes Pellet Pestle Cordless Motor with a fitted pestle. At this point the extraction can be stopped and the samples stored indefinitely at -80°C (*see* Note 4)
7. Freeze/thaw the sample by incubating first at 37°C for 10 minutes and then at -80°C for 10 minutes (*see* Note 5).
8. Repeat freeze/thaw cycle.
9. After the final thaw, add an additional $200\mu\text{l}$ of Trizol reagent to the sample.
10. Incubate sample at room temperature for 5 minutes.
11. Add $140\mu\text{l}$ of chloroform to the sample and shake vigorously for 15 seconds to mix.
12. Incubate sample at room temperature for 2 minutes.
13. Centrifuge sample at no more than 12,000 g for 15 minutes at 4°C in the cold room.
14. Remove the top aqueous phase to a fresh RNase-free 1.5 ml Eppendorf tube (*see* Note 6).
15. Slowly add an equal volume of 70% ethanol by pipetting.
16. The Qiagen RNeasy Mini Kit is used to finish the RNA extraction, as detailed below.
17. Transfer the mixture to a Qiagen RNeasy mini spin column.
18. Centrifuge column at maximal speed for 15 seconds and discard flow-through.
19. Add $700\mu\text{l}$ of Buffer RW1 centrifuge at maximal speed for 15 seconds, and discard flow-through.
20. Add $500\mu\text{l}$ of buffer RPE, centrifuge at maximal speed of 16,000g for 15 seconds, and discard flow-through.
21. Add an additional $500\mu\text{l}$ of buffer RPE, centrifuge at maximal speed for 2 minutes, and discard flow-through.
22. Centrifuge tubes again at maximal speed for 1 minute to remove any residual buffer.
23. Add $50\mu\text{l}$ of RNase-free water to the center of the filter.
24. After 1 minute, collect eluted RNA by spinning sample at maximal speed for 30 seconds.
25. Measure concentration and 260/280 ratio using NanoDrop spectrophotometer (*see* Note 7).
26. Store total RNA at -80°C until ready to use for quantitative Real-Time PCR or microarray expression studies.

⁴Trizol should be worked with only in a well-ventilated chemical hood.

⁵Make sure that the samples are well frozen. If necessary, allow extra time for freezing. One indication that the samples are well frozen is when the sample color turns light pink.

⁶When removing the top aqueous phase, carefully avoid touching the middle layer with the tip of the pipette to assure high quality of RNA.

⁷For quality assurance, we also analyze RNA samples by Agilent 2100 Bioanalyzer. RNA integrity number (RIN) above 8 is acceptable (maximum RIN is 10) for downstream applications requiring non-degraded RNA, such as microarray analysis.

3.4 DNA isolation from larval and adult *C. elegans*

Obtain synchronous worm populations as detailed in section 3.1

3.4.1 Preparation of adult stage worms for DNA isolation

1. Bleach gravid adult worms (8), as described in 3.1.
2. Transfer recovered eggs to NGM plates without bacteria (*see* Note 1)
3. The following day transfer the L1 arrested animals to OP50 *E. coli* seeded NGM plates and leave until reach young adult stage.
4. Wash synchronous young adults off with 1.5 ml of S. basal into 1.5 ml Eppendorf tubes.

3.4.2 Preparation of larval stage worms for DNA isolation

1. Bleach gravid adult worms (8), as described in 3.1.
2. Transfer recovered eggs onto NGM plates without bacteria (*see* Note 1).
3. The following day, wash L1-arrested animals off from the unspread NGM agar plates with 1.5 ml of S. basal.

3.4.3 DNA isolation from adult stage worms

1. To isolate DNA from synchronous L1 larvae and young adult worm population, the QIAamp DNA Mini Kit (Qiagen) is used per the recommended DNA purification from tissues protocol, with slight adaptation as detailed below for *C. elegans*.
2. Wash worms off from 10 cm NGM agar plates with 1.5 ml of S. basal into 1.5 ml Eppendorf tube.
3. Wash worms 5 to 6 times in 1 ml of S. basal in 1.5 ml Eppendorf tube to remove residual bacteria.
4. Allow worms to settle by gravity in approximately 100 μ l S. basal in a 1.5 ml Eppendorf tube.
5. Grind worms for 1 minute with Kontes Pellet Pestle Cordless Motor with a fitted pestle.
6. Follow the QIAamp protocol for DNA purification from tissues (Qiagen) to obtain genomic DNA, as follows:
 - a. To ground worms, add 180 μ l ATL lysis buffer and 20 μ l of Proteinase K from the QIAamp kit.
 - b. Vortex samples.
 - c. Incubate samples for 2 hours at 56°C to lyse worms, briefly vortexing twice per hour.
 - d. Follow the QIAamp protocol from step 5.b, as follows (taken from QIAamp DNA Mini and Blood Mini Handbook: add 200 μ l of Buffer AL to samples, mix by pulse-vortexing for 15 seconds, then incubate at 70°C for 10 minutes. Briefly centrifuge the tubes to remove drops from inside the lid.
 - e. Add 200 μ l of ethanol (96–100%) to the samples and mix by pulse-vortexing for 15 seconds. Briefly centrifuge the tubes to remove drops from inside the lid.

- f. Carefully apply the mixture from step e to the QIAamp Mini spin column in a 2 ml collection tube without wetting the rim. Close the cap and centrifuge at $6,000 \times g$ (8000) rpm for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube. Discard the tube containing the filtrate.
- g. Carefully open the QIAamp Mini spin column and add 500 μ l of Buffer AW1 without wetting the rim. Close the cap and centrifuge at $6,000 \times g$ (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube. Discard the tube containing the filtrate.
- h. Carefully open the QIAamp Mini spin column and add 500 μ l of Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed ($20,000 \times g$; 14000 rpm or depending on the table centrifuge you have. We use Spectrafuge 24D from Labnet with maximum speed of $16,000 \times g$) for 3 minutes.
- i. Place the QIAamp Mini spin column in a new 2 ml collection tube. Discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.
- j. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube. Discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μ l of Buffer AE. Incubate at room temperature for 1 minute and then centrifuge at $6,000 \times g$ (8000 rpm) for 1 minute.
- k. Determine the genomic DNA concentration using an ND-1000 Spectrophotometer.

3.5. qRT-PCR analysis of relative mtDNA content in *C. elegans*

1. Combine 40 ng of DNA per qRT-PCR reaction containing Taqman Gene Expression Assays for the housekeeping gene, *drs-1* (Ce02451127_g1) and the mtDNA-encoded complex I subunit gene, *nd4* (custom designed Taqman Gene Expression Assay from Applied Biosystems).
2. Add Taqman Universal PCR Master Mix per standard Applied Biosystems protocol. Table 1 details some of the Taqman Gene Expression Assays commonly used to study mitochondrial function in our laboratory.
3. Perform Real-time qPCR on a 7500 Fast Real-Time PCR System.
4. Analyze relative gene expression using the provided software.

3.6. qRT-PCR analysis of relative nuclear gene expression in *C. elegans*

3.6.1 DNase treatment of RNA—DNase-treat 10 μ g of total RNA using TURBO DNA-free kit (Ambion), per standard protocol.

3.6.2 RT-PCR reaction to generate cDNA from DNase-treated RNA—Using the High Capacity cDNA Reverse Transcription Kit and protocol from Applied Biosystems, perform RT-PCR on 1–2 μ g RNA (depending on concentration) in a 20 μ l reaction mixture.

3.6.3 qRT-PCR reaction

1. Use 40 ng of cDNA per qRT-PCR reaction.

- a. We use Taqman Gene Expression Assays for both the housekeeping gene *drs-1* and target genes of interest (see Table 1).
2. Prepare qRT-PCR plates per standard Applied Biosystems protocol.
3. Analyze data by comparative Ct (delta-delta Ct) quantitative analysis.

3.7 Affymetrix GeneChip *C. elegans* Genome Array Analysis

3.5.1 Probe Remapping—Affymetrix expression microarrays are organized by probes sets, which consist of multiple probes that map to a given transcript, where the expression level of that transcript is represented by multiple measurements among probes of the same probe set. Each Affymetrix probe set is annotated with a unique probe set identifier. However, such annotation becomes ambiguous and outdated over time. For example, it is not rare for the same transcript to be mapped to multiple probe sets. In addition, some predicted probe sequences turn out to be incorrect as the worm genome becomes more accurately sequenced. Independent resources such as BRAINARRAY are available that can remap and regroup original probes to provide the most up-to-date version of worm transcripts and remove probes that perform badly or have ambiguity (11). Remapping assures that each probe set is unambiguously annotated with a unique RefSeq, Entrez, or other formal gene identifier. The newest version of BRAINARRAY is what we have most recently used to map all probes on *C. elegans* Genome Array to 17,255 unique Entrez gene identifiers. Entrez IDs can be further associated with biological functions through gene categorization resources such as Gene Ontology (www.geneontology.org) and KEGG (www.genome.jp/kegg).

3.7.2. Data Normalization—Prior to analysis of relative expression levels between array groups, array data needs to be normalized to remove any systematic bias that may exist between individual arrays. Various sources of systematic bias exist, such as the amount of total RNA and the efficiency of hybridization. Robust multiarray analysis (RMA) is an algorithm commonly used to normalize and summarize array data (12). We use the RMA implemented by Bioconductor (<http://bioconductor.org>) to process raw, probe-level array data. The processed data is an N*M data matrix, where N is the number of transcripts and M is the number of arrays.

3.7.3. Gene Expression Analysis Methods—Both unsupervised and supervised analyses can be used to compare gene expression between sample groups. Unsupervised methods, such as hierarchical clustering and principal components analysis, evaluate the global difference of gene expression profiles without using sample grouping information. If samples in different groups can be separated by an unsupervised method (Fig. 1), one can conclude that sample groups will be distinguishable based on their expression profiles. In contrast, supervised methods use the sample grouping information to identify specific genes that are differentially expressed between groups. Differential expression can be either represented by fold change (or similar indexes) to indicate the magnitude of the group difference, or by p value of a statistical test to indicate the statistical significance of the group difference. One or both types of indexes can be used to sort and select differentially expressed genes between sample groups. For example, a list can be generated of all genes having at least a two-fold change between groups and a p value less than 0.05. Furthermore, the percentage of false positives, called false discovery rate, can be estimated in a selected gene list.

The biological interpretation of differential expression can substantially vary for specific genes. For example, slightly altered expression of a transcription factor may dramatically alter the expression of its downstream targets. However, most group comparisons will

generate hundreds to thousands of genes that have at least moderate expression changes, which makes it impractical for researchers to manually investigate all of their functions and relationships. Publicly available software such as Gene Set Enrichment Analysis (GSEA) allows researchers to analyze multiple genes as a set, such as genes belonging to the same KEGG metabolic pathway or Gene Ontology category (13). If most genes of a pathway analyzed by GSEA have relatively increased expression in a mutant worm strain relative to control, we can conclude that pathway is up-regulated in the mutant even if the fold-change of individual pathway genes may not be significant (Fig. 2). GSEA software provides a few ‘standard’ collections of gene sets within which genes are annotated using human gene symbols. To utilize this resource for *C. elegans*, we compile our own gene set collection of metabolic pathways from KEGG database and annotate genes with Entrez Gene IDs (2). Gene-pathway mapping information necessary to compile custom gene sets can be downloaded from ftp://ftp.genome.jp/pub/kegg/pathway/organisms/cel/cel_gene_map.tab.

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Abbreviations

mtDNA	mitochondrial DNA
qRT-PCR	quantitative real-time PCR
KEGG	Kyoto Encyclopedia of Genes and Genomes
GSEA	Gene Set Enrichment Analysis

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Figure 1. Unsupervised clustering of microarray expression data in mitochondrial complex I mutant worms

Separation of samples by an unsupervised clustering method into different groups that correlate with a known biological variable, such as mutation type, increases the likelihood that the sample groups will be distinguishable based on their gene expression profiles. Here, unsupervised clustering satisfactorily separated wild-type (N2 Bristol worms grown on OP50 *E. coli* bacteria) from complex I subunit mutant worms (*gas-1(fc21)* grown on OP50 *E. coli* bacteria) (2).

Figure 2. Gene Set Enrichment Analysis (GSEA) output showing relative expression of “oxidative phosphorylation” gene cluster in mitochondrial complex I subunit (*gas-1(fc2I)*) mutant worms (left side, red highlight) relative to wild-type N2 Bristol worms (right side, blue highlight) (2). Vertical black lines indicate the relative expression between mutant and control groups of individual gene members of the oxidative phosphorylation gene cluster, as defined in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Genes that fall to the left side of the green “peak” enrichment score are significantly enriched in the mitochondrial mutant worms.

Table 1

Taqman Gene Expression Assays used to study relative mtDNA content or relative expression of mitochondrial oxidative stress response.

<i>C. elegans</i> Gene	Gene name and function	AB Assay #
<i>drs-1</i>	aspartyl(D) tRNA synthetase (Housekeeping gene)	Ce02451127_g1
<i>act-3</i>	Actin (Housekeeping gene)	Ce02784145_s1
<i>nd4</i>	ND4 (mtDNA complex I subunit)	custom assay *
<i>sod-2</i>	SOD2 (manganese superoxide dismutase)	Ce02410777_g1
<i>sod-3</i>	SOD2 (manganese superoxide dismutase)	Ce02404515_g1

* forward primer sequence: 5'GAGGCTCCTACAACAGCTAGAATAC3'; reverse primer sequence: 5'TCATACATTGTTGTGTACAAATCTTAACTACCT3'