Video Article

Measuring Oxidative Stress Resistance of *Caenorhabditis elegans* in 96-well Microtiter Plates

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Abstract

Oxidative stress, which is the result of an imbalance between production and detoxification of reactive oxygen species, is a major contributor to chronic human disorders, including cardiovascular and neurodegenerative diseases, diabetes, aging, and cancer. Therefore, it is important to study oxidative stress not only in cell systems but also using whole organisms. *C. elegans* is an attractive model organism to study the genetics of oxidative stress signal transduction pathways, which are highly evolutionarily conserved.

Here, we provide a protocol to measure oxidative stress resistance in *C. elegans* in liquid. Briefly, ROS-inducing reagents such as paraquat (PQ) and H_2O_2 are dissolved in M9 buffer, and solutions are aliquoted in the wells of a 96 well microtiter plate. Synchronized L4/young adult *C. elegans* animals are transferred to the wells (5-8 animals/well) and survival is measured every hour until most worms are dead. When performing an oxidative stress resistance assay using a low concentration of stressors in plates, aging might influence the behavior of animals upon oxidative stress, which could lead to an incorrect interpretation of the data. However, in the assay described herein, this problem is unlikely to occur since only L4/young adult animals are being used. Moreover, this protocol is inexpensive and results are obtained in one day, which renders this technique attractive for genetic screens. Overall, this will help to understand oxidative stress signal transduction pathways, which could be translated into better characterization of oxidative stress-associated human disorders.

Video Link

The video component of this article can be found at https://www.jove.com/video/52746/

Introduction

In eukaryotes, oxidative phosphorylation taking place in the electron transport chain of the mitochondria is the main driver of energy production in the form of ATP. Reactive oxygen species (ROS) are a natural byproduct of this process. Despite their important role as signaling molecules, excessive ROS can lead to DNA damage, protein carbonylation, and lipid oxidation. An imbalance between ROS production and detoxification causes oxidative stress, which leads to energy depletion, cellular damage, and triggers cell death[1,2]. Oxidative stress contributes to aging and to the development of many life-threatening diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases[3-9].

Cells have evolved enzymatic and non-enzymatic defense strategies to maintain proper ROS levels and to protect their constituents against oxidative damage[1,2]. Superoxide dismutase (SOD) enzymes act first to convert superoxide to H_2O_2, which is later converted to water by catalase or peroxidase enzymes. Non enzymatic defense strategies include mostly molecules that react faster with ROS as compared to cellular macromolecules, protecting essential cellular components. Despite the protective role of ROS detoxifying enzymes, some ROS molecules escape the antioxidant defense mechanisms and lead to oxidative damage. Detection, repair, and degradation of the damaged cellular components are essential defense strategies during oxidative stress[1,2].

Signaling pathways involved in stress resistance and specifically oxidative stress are highly evolutionarily conserved[10,11]. Unlike cell culture experiments where organismal conditions are only partially reproduced, the study of oxidative stress in model organisms[12,13] has great significance. *C. elegans* is a free-living nematode that can be easily and inexpensively cultured on a bacterial lawn on agar media. It is small in size (about 1 mm in length) and normally grows as a self-fertilizing hermaphrodite, which facilitates genetic manipulations. It has a rapid life cycle and a high reproductive capacity, producing about 300 offspring per generation, making it a powerful tool to perform large-scale genetic screens[14]. The *C. elegans* genome is fully sequenced and 40-50% of the genes are predicted to be homologues of human disease-associated genes[15-18]. The knockdown of genes of interest using RNAi is rapid and easy in *C. elegans.* Gene down regulation could be achieved by feeding animals the *E. coli* bacteria that harbor a plasmid expressing the double-stranded RNA that targets the mRNA of interest[19]. Therefore, determination of gene function using large scale RNAi screens has great impact on understanding human diseases including cancer [20,21].

Studies of oxidative stress resistance in *C. elegans* have led to the identification of conserved mechanisms of resistance to oxidative stress[13,22]. Some pathways identified are common pathways that modulate longevity and resistance to other stresses as well such as hypoxia, heat, and osmotic stress. These pathways include the insulin signaling, TOR signaling, and autophagy. Other key pathways involve detoxification of ROS such as superoxide dismutase enzymes and catalase enzymes, or in damage repair such as heat shock and chaperone proteins[11,13,22].

This protocol describes how to determine the resistance to oxidative stress of *C. elegans* in liquid. We used *flcn-1(ok975)* and wild-type animals to demonstrate the protocol since we have previously shown an increased resistance to oxidative stress upon loss of *flcn-1(ok975)* in *C. elegans*[23]. We have also shown that this increased resistance depends on AMPK and autophagy, a signaling axis that improves cellular bioenergetics and promotes stress resistance [23]. PQ is an oxidative stressor that interferes with the electron transport chain to produce reactive oxygen species[24]. The same assay could be adapted and other ROS sources or ROS generating compounds could be used such as H_2O_2 and rotenone. Similar assays have been developed on plates where low concentrations of PQ are used[25,26]. The advantage of this assay is that it is very fast, and the results could be obtained in one day. Additionally, the total volume of liquid used to perform the oxidative stress resistance assay in 96 well plates is low as compared to the volume used to prepare PQ-containing plates. Therefore, the amount of PQ used is in the liquid assay is low, which renders the assay inexpensive and limits the production of toxic wastes. However, limitations of this assay as compared to plate assays include the lack of food in the liquid assay and the lower concentration of oxygen in liquid as compared to air. These are important factors that in some cases, might influence the results. Therefore, confirming reproducibility using other methods of oxidative stress resistance is recommended to support results obtained in this assay.

Protocol

1. Preparation of Reagents

1. Preparation of media for *C. elegans* growth (in this case, wild-type animals and *flcn-1(ok975)* mutant animals).
    1. Prepare Modified Youngren's Only Bacto-peptone (MYOB) dry mix containing 5.5 g of Tris HCl, 2.4 g of Tris base, 31 g of Bactopetone, 20 g of NaCl and 0.08 g of cholesterol. Mix well with shaking.
    NOTE: This mix is sufficient to prepare 10 L of MYOB medium.
    NOTE: Normal growth medium (NGM) plates could be used instead of MYOB plates[27]. However, the same type of plates should be used for valid comparisons between strains and between independent repeats. In this protocol, we only used MYOB plates.
    2. Prepare 1 L of MYOB medium by adding 6 g of MYOB dry mix, 17 g of agar and make up to 1 L with H_2O and autoclave for 45 min at 122 °C. Pour plates and let dry at RT for 2 days.
    3. Using sterile technique, inoculate 100 ml of culture of LB broth medium with *E. coli* OP50 bacteria and grow O/N at 37 °C.
    4. Seed MYOB plates with *E. coli* OP50 bacteria. Allow it to grow at RT for 48 hr.

2. Preparation for gene downregulation using RNAi
    1. Prepare MYOB RNAi-feeding plates. Proceed as in steps 1.1.1 and 1.1.2. After autoclaving, allow the medium to cool down to about 55 °C and add 1 ml of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1 ml of 100 mM ampicillin.
    2. Streak *E. coli* HT115 bacteria transformed with a plasmid, which expresses either control EV or *flcn-1* specific RNAi on Ampicillin (50 µg/ml)/tetracyclin (15 µg/ml) agar plates. Grow culture O/N at 37 °C.
    3. Pick colonies and inoculate *E. coli* HT115 bacteria transformed with a plasmid which expresses either control EV or *flcn-1* specific RNAi and grow culture in LB/Ampicillin (50 µg/ml) medium and O/N at 37 °C.
    4. Seed plates with *E. coli* HT115 EV bacteria or HT115 *flcn-1* RNAi bacteria.
    NOTE: Keep plates 48 hr at RT before usage. If RNAi knockdown by feeding is in effective, use other methods of delivery of the ds-RNA [28].

3. Inducing oxidative stress using paraquat (PQ):
    1. Prepare M9 buffer by mixing 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl and 0.25 g of $MgSO_4 \cdot 7\ H_2O$. Bring up to 1 L with distilled water. Autoclave solution for 45 min at 122 °C and store bottles at RT.
    2. On the day of the assay, prepare the M9/PQ-containing solution. For this protocol, use 100 mM PQ (0.1028 g of PQ dissolved in 4 ml of M9 buffer fills an entire 96 well plates with 40 µl of M9/PQ per well).
    CAUTION: PQ is a hazardous and highly toxic compound. Handle according to appropriate guidelines.
    NOTE: When first running a new strain or a new condition, it is recommended to assay survival in increasing concentrations of PQ to determine the best concentration to use in order to kill the animals within 7-10 hr.

2. Preparation of Age-synchronized L4 Population

1. Transfer 20 gravid adult hermaphrodites to a fresh MYOB plate seeded with *E. coli* OP50 bacteria. Prepare several plates based on the number of worms needed during the assay.
2. Allow them to lay eggs for 6 hr and then using a platinum wire worm pick remove the mothers. Check the plates under the microscope to assess the number of eggs laid.
3. Allow the eggs to hatch and grow at 20 °C for 48 hr. At this time, the animals are in a late L4/young adult stage. Pick same stage animals and transfer to the PQ solution-containing wells.
NOTE: Since 48 hr incubation only applies to mutants that grow similarly to wild-type animals, it is important to monitor developmental rate of newly tested mutants to ensure it conforms to the wild-type development rate, otherwise, other timelines should be used.
4. Alternatively, use synchronization techniques. Generate synchronized population of L1 larvae worms using hypochlorite solution (25 ml water, 125 ml of 5.25% sodium hypochlorite, 50 ml of 4 M NaOH; wrap bottle with aluminum foil to isolate from light). However, for this assay, it is not recommended, since intense bleaching might affect the behavior of the animals during the assay.
CAUTION: Handle hypochlorite solution according to guidelines.

NOTE: When using RNAi to down regulate the gene of interest, synchronize the hermaphrodites as described in section 2, with the exception of the transfer of the mothers to RNAi plates seeded with the specific RNAi bacteria. When the knockdown with the RNAi is weak, feeding for multiple generations is recommended.

3. Performing the Oxidative Stress Resistance Assay

1. Pipette 40 µl of the 100 mM PQ solution (prepared fresh) to every well of a 96 well plate. Use at least 12 wells as replicates of every condition (treatment, mutant, *etc.*). Using a platinum wire worm pick, transfer 5-8 L4 larvae animals to every well indicating the start time and the end time.
2. When starting another condition, note the start time and the end time. Place the plate at 20 °C in the incubation time between transferring the worms and scoring dead animals an hour later from the start time.
3. Using the dissecting microscope, count survival every hr. Gently shake the plate before starting to count. Indicate worms that do not move, even after spotting a high-intensity light, as dead animals. Also indicate the number of animals alive.
   NOTE: Looking at worm shape, tails and head movement at high magnification, help determine dead worms from alive.
4. Repeat this step every hour until most worms are dead.

4. Determination of Survival Percentage at Every Time Point

1. Calculate the total number of animals per condition by adding the total number of animals transferred to every well. Ignore the animals that are damaged or killed upon transfer.
2. For every time point, calculate the total number of dead animals per condition (sum of dead animals in the 12 wells). For every time point, calculate percentage of death (number of dead animals divided by total number of animals and multiplied by 100) and percentage of survival (100 minus percent death).
3. Repeat this assay at least 3 independent times.

Representative Results

Comparing wild-type *C. elegans* to *flcn-1(ok975)* mutant animals

Here we used 100 mM PQ to determine the resistance of wild-type *C. elegans* animals compared to *flcn-1(ok975)* which has been shown to resist oxidative stress, heat, and anoxia[23]. After 4 hr of treatment, 48.3% of wild-type survived as compared to 77.8% survival in *flcn-1(ok975)* animals. As expected, *flcn-1(ok975)* mutant animals were more resistant to 100 mM PQ compared to wild-type (**Figure 2** and **Table 1**).

Comparing wild-type animals fed with control EV or *flcn-1* RNAi bacteria

Similar to the result presented in the previous section, down regulation of *flcn-1* using RNAi increased the resistance to 100 mM PQ. This result demonstrates that down regulation of gene function using RNAi mimics loss-of-function mutation (**Figure 2** and **Table 1**).

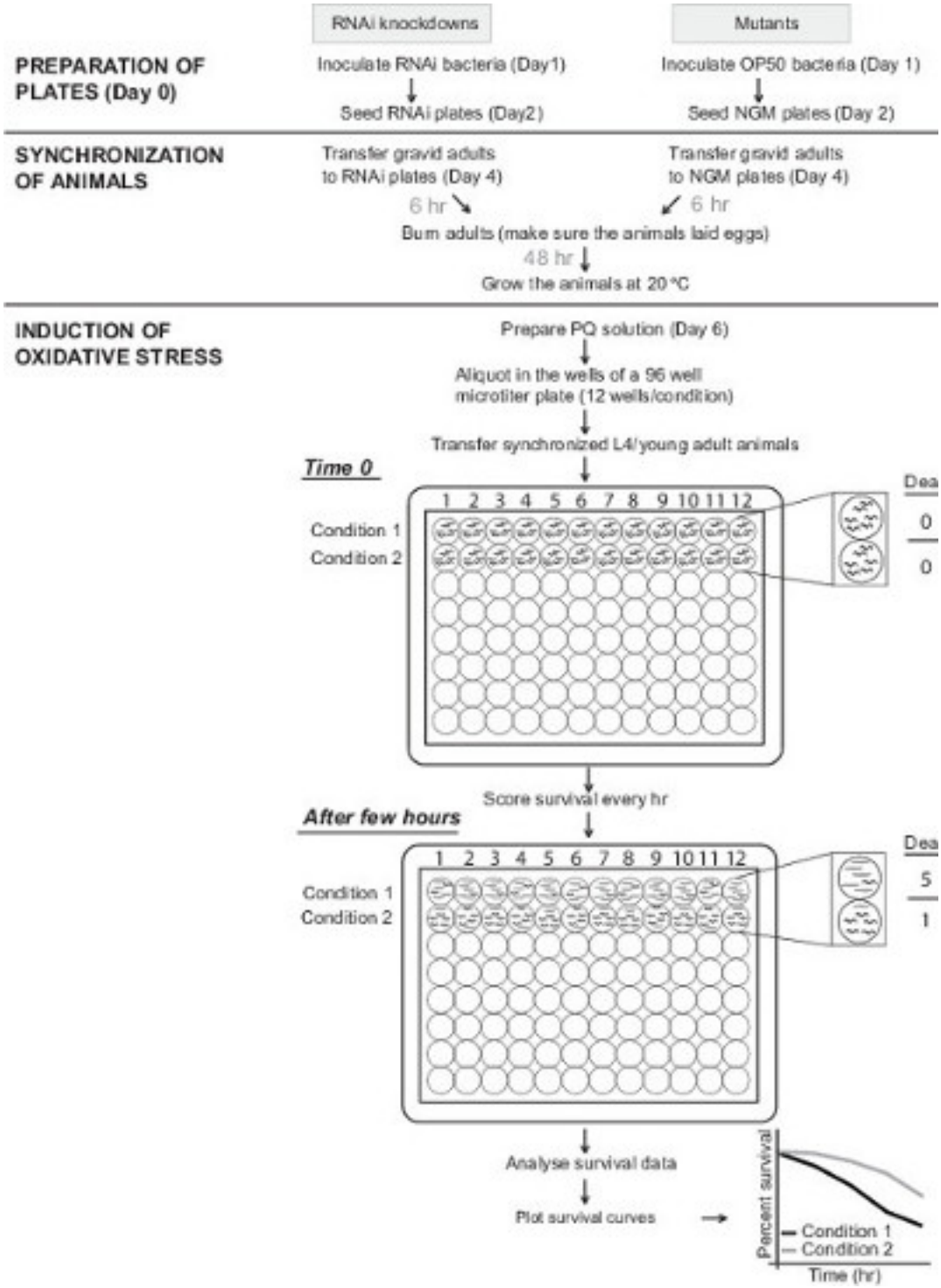

Figure 1. Schematic figure of the method of oxidative stress resistance determination in 96 well plates in *C. elegans*.

Synchronized L4/young adult animals grown on MYOB plates and fed with E. *coli* OP50 bacteria are transferred to the wells of a 96 well microtiter plate containing 100 mM PQ and survival is measured hourly until a large number of worms are dead. In the case of RNAi knockdowns, the same procedure is followed except that the synchronized animals are grown on plates supplemented with IPTG, and are fed with the *E. coli* HT115 bacteria harboring the plasmid that will knockdown the target gene upon expression.

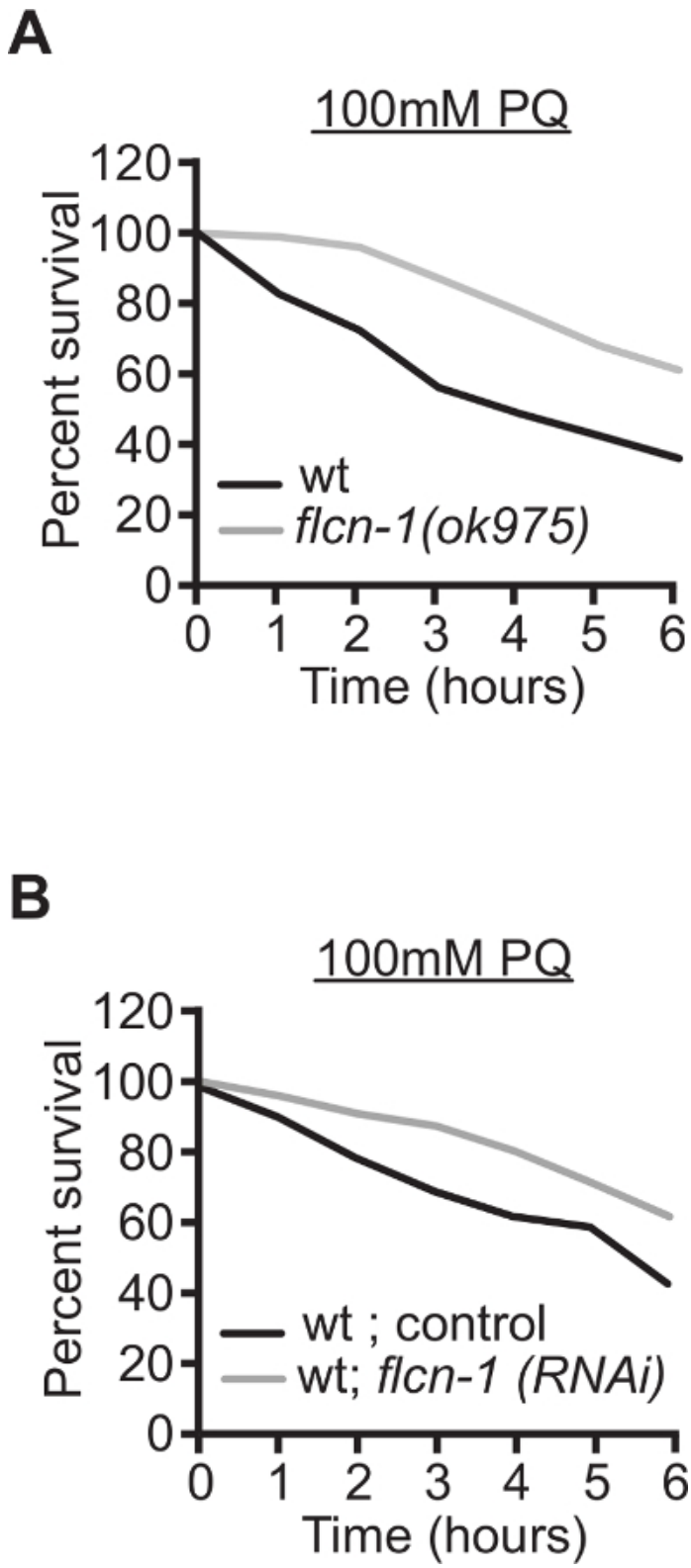

Figure 2. Loss of FLCN-1 increases resistance to oxidative stress in *C. elegans*. (**A**-**B**) Percent survival to 100 mM PQ of (A) w.t. and *flcn-1(ok975)* mutant animals and (**B**) wt animals treated with control or *flcn-1*RNAi.

	Percent survival to 100 mM PQ				
1 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	82.38 ± 1.31	0.0002	3	210

	flcn-1(ok975)	99.03 ± 1.67		3	224
	wt; control	91.70 ± 0.55	0.0462	3	202
	wt; *flcn-1*(RNAi)	95.93 ± 2.48		3	207
2 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	72.13 ± 7.13	0.005	3	210
	flcn-1(ok975)	96.33 ± 2.28		3	224
	wt; control	80.27 ± 5.34	0.0261	3	202
	wt; *flcn-1*(RNAi)	91.23 ± 1.42		3	207
3 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	55.37 ± 4.72	0.0004	3	210
	flcn-1(ok975)	87.00 ± 1.36		3	224
	wt; control	70.77 ± 3.50	0.0027	3	202
	wt; *flcn-1*(RNAi)	87.63 ± 2.67		3	207
4 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	48.30 ± 6.39	0.002	3	210
	flcn-1(ok975)	77.80 ± 3.12		3	224
	wt; control	63.77 ± 0.66	0.0122	3	202
	wt; *flcn-1*(RNAi)	80.57 ± 6.68		3	207
5 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	41.60 ± 8.33	0.0062	3	210
	flcn-1(ok975)	67.43 ± 1.42		3	224
	wt; control	60.53 ± 2.58	0.0313	3	202
	wt; *flcn-1*(RNAi)	71.53 ± 5.24		3	207
6 hr	Strain, RNAi	Percent Survival (±SD)	P Value	Number of experiments	Total Number of worms
	wt	34.86 ± 5.88	0.0021	3	210
	flcn-1(ok975)	60.34 ± 2.05		3	224
	wt; control	44.43 ± 3.93	0.0042	3	202
	wt; *flcn-1*(RNAi)	62.13 ± 3.44		3	207

Table 1. Summary of results and statistical analysis of the oxidative stress resistance from Figure 2.

Discussion

C. elegans is an attractive model organism to study genetically oxidative stress resistance *in vivo* since it can be easily cultured, and rapidly leads to a large number of genetically identical offspring. Multiple methods to measure oxidative stress resistance have been previously described and they are based on the supplementation of culture plates with various ROS sources such as PQ, rotenone, H_2O_2, and juglone[25,26,29-32]. Here we describe a protocol that measures oxidative stress resistance in liquid in 96 well plates using 100 mM PQ. A similar assay has been performed by Greer *et al.*[33]. This assay could be further adapted to study oxidative stress in liquid using other ROS sources. For instance, we have shown that loss of *flcn-1* increases resistance to several H_2O_2 concentrations as well[23].

This assay could be technically challenging for some strains that display motility defects or swimming-induced paralysis phenotypes such as strains carrying a mutation in the dopamine transporter *dat-1*[34]. In this case, the inability to move is confusing since it does not necessarily mean decreased oxidative stress resistance but rather a motility defect. Furthermore, counting of dead animals should be carefully completed and researchers have to pay attention to *C. elegans* behavioral details such as tail movements, and head movements, or even slow body movements. If the PQ concentration is too high, and the death kinetics of the animals is very fast, one might consider decreasing the

concentration of PQ in order to get slower death rates. Some animals are extremely sensitive to oxidative stress such as *aak-2* mutant animals[26,33,35,36] while others are highly resistant such as *daf-2* mutant animals[37]. In this case, assaying survival with different concentrations of PQ should be considered.

For RNAi experiments, negative results could be due to an inefficiency of the RNAi treatment. In this case, measurement of mRNA levels is essential to determine whether the gene knockdown is successful.

This protocol could be further adapted for high throughput screening of oxidative stress resistance using a detector that tracks the swimming behavior of *C. elegans* in liquid[38,39]. This would potentially enable the monitoring of the endurance of *C. elegans* and the swimming behavior such as frequency of body bending under oxidative stress. Higher rates of body movement could indicate higher worm fitness under stress.

Pathways that lead to oxidative stress resistance in lower eukaryotes are highly conserved across evolution and are linked to oxidative stress-associated diseases and aging in humans. Mitohormesis and the balanced generation of ROS are essential to extend lifespan and improve health span. However, excessive ROS is highly damaging to cells, tissues/organs, and organisms. Finding pathways, that if altered, provide an optimal ROS balance is thus essential and the screens for drugs that modulate the resistance to oxidative stress could help the development of cures for multiple diseases with the common denominator: oxidative stress. Performing these screenings in *C. elegans* is an advantageous fast, inexpensive, and reliable method that has great potential and value for the understanding and treatment of human diseases linked to oxidative stress.

Disclosures

The authors declare that they have no competing financial interests.

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