We thank the editorial team and all the reviewers for an exceptionally comprehensive review of the manuscript. We have put in considerable efforts to address all the points brought up by the editorial team and the reviewers, and believe this has significantly strengthened the manuscript. We believe that some points brought up by reviewer 3 were beyond the scope of this manuscript and reflected more what would be appropriate for a literature review, but have addressed the concerns to the best of our abilities for what would be appropriate for a protocol/methods manuscript.

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Done.

3. Please avoid the use of personal pronouns (you, your, we).

Done.

4. Please include email addresses for all authors in the manuscript itself.

Done.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sigma-Aldrich, Biometrica, BioSorter, etc.

Done – microscope names were still included. Should these be removed as well?

Protocol:
1. There are several long, non-instructional, paragraphs in the protocol; these should be moved to the Introduction, Results, or Discussion, as appropriate.

Done.

2. The 'Materials' sections are not necessary-all information there should be in the Table of Materials, in numbered protocol steps as instructions in the imperative, or possibly in separate tables.

All materials have been moved to Table 1, and all commercial product names have been removed within the manuscript and is available only in the table of materials.

3. The content ‘Notes and Troubleshooting’ should either be notes immediately after the relevant steps or be in the Discussion section.

All notes have been moved to their relevant sections.

4. There is a (roughly) 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing; see formatting guidelines above) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have attempted to minimize the protocol and highlighted sections as much as possible. Upon re-review, we will work directly with the editor (Phillip Steindel) to further decrease the length.

5. For each protocol step/substep, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Done.

Tables:
1. Please remove the embedded Tables from the manuscript. Please instead upload as .xls/.xlsx-formatted files.

All tables have been converted to xls format.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

This is confirmed.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript Bar-Ziv et al. present protocols for examining stress response and stress resistance in C. elegans. They describe how to use fluorescent reporter strains to quantify the mitochondrial unfolded protein response, the heat shock response and the ER unfolded protein response. They also provide protocols for examining resistance to mitochondrial/ROS, heat and ER stress. Overall, this is a well written, meticulously prepared manuscript that will be useful for those examining stress in C. elegans.

Major Concerns:
No major concerns.

Minor Concerns:
1. It would be important for the authors to note that it is possible for these reporters to be activated by

other pathways aside from the stress pathways being tested. For example gst-4 can be activated by EGF signaling (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5190145/).

A statement has been added to the discussion reviewing examples of where stress response reporters are not specific to each individual paradigm as a caution to readers.

2. The authors describe the use of the COPAS biosorter to quantify fluorescence in the fluorescent reporter strains. However, most laboratories do not have access to this equipment. It would be useful to provide a protocol for quantifying fluorescence from images of the worms. This can be done using free software such as ImageJ.

We have added a section for quantification using ImageJ.

3. The authors mention an increased sensitivity to stress for worms grown on the standard OP50 bacteria. It would be useful for the authors to provide a reference. If unpublished, perhaps the authors could add a figure comparing stress resistance in worms grown on OP50 versus HT115 as this would be useful information for the C. elegans community.

A reference is provided in the following sentence, which describes the increased stress sensitivity is due to vitamin B12 deficiency (Revtovich, A.V., Lee, R., Kirienko, N.V. Interplay between mitochondria and diet mediates pathogen and stress resistance in Caenorhabditis elegans. *PLOS Genetics*. **15** (3), e1008011, doi: 10.1371/journal.pgen.1008011 (2019).)

4. Line 135. Why is it recommended to transfer young animals? In a sick strain these could become sterile and fail to produce progeny.

A sentence has been added to chunk animals when dealing with sterility or sickness issues.

5. Line 252 and multiple other places. 3-4 days seems long for worms to reach adulthood. On OP50, WT worms typically develop from hatch to adult in just under 2 days. Is this slower development because of the RNAi plate? Is it because of the RNAi treatment? This would be worth mentioning.

In the WormAtlas, it is described that animals take about 65-96 hours to make it to Day 1 of adulthood, which we describe as “gravid adults” or “egg-laying maximal.” We have made it more clear in the protocol that this is what we describe as a Day 1 adult.

DEVELOPMENT AT DIFFERENT TEMPERATURES

	"16°C" (16.0 ± 0.3°C)	"20°C" (19.5 ± 0.5°C)	"25°C" (25.0 ± 0.2°C)
Egg laid	0 hr	0 hr	0 hr
Egg hatches	16-18 hr	10-12 hr	8-9 hr
First-molt lethargus	36.5 hr	26 hr	18.0 hr
Second-molt lethargus	48 hr	34.5 hr	25.5 hr
Third-molt lethargus	60.0 hr	43.5 hr	31 hr
Fourth-molt lethargus	75 hr	56 hr	39 hr
Egg-laying begins	~90 hr	~65 hr	~47 hr
Egg-laying maximal	~140 hr	~96 hr	~62 hr
Egg-laying ends	~180 hr	~128 hr	~88 hr
Length at first molt	360 µm	370 µm	380 µm
Length at second molt	490 µm	480 µm	510 µm
Length at third molt	650 µm	640 µm	620 µm
Length at fourth molt	900 µm	850 µm	940 µm
Length at egg-laying onset	1150 µm	1060 µm	1110 µm
Maximal egg-laying rate	5.4/hr	9.1/hr	8.1/hr
Total eggs laid	275	280	170

6. It was not always clear whether the superscript number referred to a note or a reference.

All notes were moved directly into their respective sections, and now all superscripts are references.

7. Line 272. "translocates" This is a bit misleading. ATFS-1 is in cytoplasm and MTS allows for import into mitochondria. Under stress, this import is blocked leaving ATFS-1 in cytoplasm. This allows NLS to bring ATFS-1 to nucleus.

The wording has been fixed.

8. Section 2.2.2.1 is essentially the same as 2.2.1.1 except the gene being knocked down is different. Perhaps the authors could just mention that the procedure is the same except for the RNAi clone.

Done.

9. Line 293-298 It was unclear why there is a specific materials section for this subsection but not the others. Could these materials be included in the list at the start of section 2?

All materials sections have been removed and have been turned into Table 1.

10. Line 343, 355 Why is there a 16-24 hour recovery for TBHP exposure, and 2 hour recovery for PQ exposure?

These are the times that we saw the most robust induction of *gst-4p::GFP*. It is possible that because TBHP is much more stable than PQ, there is a longer-term effect.

11. Line 351 "THBP" should be "PQ"

Done.

12. Line 357 It might be worth putting RNAi before compound exposure for consistency with previous sections.

Done.

13. Line 397 Is 100 mM correct? We use 10 mM which is sufficient to immobilize long enough to image.

Yes – we had a note that stated that sodium azide can be brought down to 10 mM and now this has been moved directly to the section.

14. Line 657 The time points for 34 degrees are noted in the main protocol while the critical time points for 37 degrees are found in the note. It might be worth having them in the same place.

Done.

15. Note 23 and note 33 are identical. Please check.

All notes have been removed.

16. Line 847 5 days should be 5 hours

Done.

17. Table 2. It would be useful to include a reference for each strain.

A reference for each strain is included both in the introduction and the references section.

18. Table 3. The duration of exposure is only included for heat stress. It would be useful to include this information for all.

Done.

19. In table 2 and table 3, sod-3 is indicated as an output for the insulin-IGF1 signaling pathway. In table 5, sod-3 is indicated as an output measure for OxSR mediated by SKN-1. This could be confusing to readers and should be clarified.

In Table 2, it has been put in as an output for both OxSR and insulin-IGF signaling. In table 3, the parentheses is to explain that *daf-2* is the gene encoding the insulin receptor. It is not describing what the *sod-3* reporter measures.

20. Figure 5. It would be helpful to label the stress on the figure e.g. paraquat, heat, TM.

Done.

Reviewer #2:
The authors have done a terrific job and a service to the field in assembling these assays and protocols to evaluate organelle-specific stress responses in nematodes. The protocols are beautifully described and will be of use to scientists with or without expertise in C. elegans techniques. The manuscript is beautifully written and comprehensive. Thus, I only have several minor suggestions.

Page 6. Please include a sentence indicating that the mechanism by which induction of hsp-4::gfp during ER stress is regulated (IRE-1 and XBP-1). The other stress response reporters indicate the regulators (SKN-1, ATFS-1, etc). filleted

We have described a more detailed mechanism of how each of the stress responses are activated in their respective sections of the introduction & results.

What is tag-335?

All RNAi recommendations have now been paired to a description of what the gene encodes and what the function of the protein is.

Line 368. Please use either HSF1 or HSF-1.

Everything has been standardized to HSF-1 (the *C. elegans* nomenclature).

Line 748. As mentioned, tunicamycin is expensive. DTT can be used as a less expensive alternative as it also induces the hsp-4::gfp reporter due to ER stress.

We have had very little success with DTT in inducing *hsp-4p::GFP* and thus have not included this method in our protocols.

The word "filleted" is used throughout and may be confusing as it is not clear to me what it means in context of the manuscript? A Google search definition indicates it means "to remove bones from "a fish."

This is a colloquial *C. elegans* term for lifespans and was originally defined during both of its uses in the manuscript. However, this word has been removed.

Reviewer #3:
The authors describe assays to study various stress responses in C. elegans, focusing on transcriptional readouts studied by means of GFP reporter assays and functional assays using population survival. The methods are fairly standard in the field and there aren't any major issues with them (see below for some specific questions/comments). The protocols provided herein are well described and clear, and should

be therefore be useful to a lab not familiar with them.
However, I'd like to authors to more specifically consider some of the general points discussed below; as is, the manuscript has adopted a fairly proteostasis centric view, and could better explain the non-linearity and exclusivity of transcription factors in gene regulation.

We understand that this protocol has taken a fairly proteostatic viewpoint, but this was intentional as describing all the functions of transcription factors in gene regulation is beyond the scope of this protocol.

- General (this is noted below in specific instances): The authors should clarify that their chosen reporters merely report on a fraction of the underlying response pathways and in no way reflect the organisms' complete response to a stress. I.e., hsp-4 is not equal to the UPR, gst-4 is not equal to the OSR, etc. Highlighting the organisms' response complexity and redundancy is an important point that needs to be cleared up. Otherwise, an uninformed reader may adopt an overly simplistic view of these stress responses. Further below, line 1010, text such as "can all be suppressed by knockdown of the primary transcription factors upstream" should be revised for that reason - the view of the "primary/master" regulator is outdated and a factor being genetically required to induced a gene in stress buy no means makes it "primary/master". Indeed, it's quite likely that many factors are required to induce the 4 reporter genes described here.

In the discussion, we have dedicated an entire section to describing the limitations of the reporters and alternative methods that can be used to supplement all reporter assays have been provided. We have adjusted all references to “primary” transcription factors to state either “one of the primary transcription factors” or “an important transcription factor.”

- General; Throughout the manuscript, the authors appear to equate "stress responses" with "protein quality control" (e.g. p2 line 29, p2 line 44 and below, etc). The two terms should not be conflated. Please, state up front that proteostasis is merely a part of any one stress response, and may not be of equal importance in all stresses. Further, please clarify that stress response goes well beyond ensuring protein folding, modification, and degradation. More extensive referencing in the intro would help.

We have edited the introduction to reflect that this protocol is dedicated to protein quality control.

- Some statements are poorly supported by references. For example P2L53 and below, "Some cellular stress responses are not dedicated to a specific cellular compartment, but rather focus on dealing with a particular type of stress. For example, the OxSR serves to alleviate the toxic effects of reactive oxygen species (ROS)." Please provide citations that provide evidence that the OxSR (which is what, exactly, anyway?) is mounted in all cellular compartments. If not available, please temper this statement; the two possibilities are not mutually exclusive. Indeed, the whole paragraph is supported by only one citation; more extensive referencing would be appropriate.

This statement has been removed. OxSR has been described as oxidative stress response in its first mention, as have all the other stress responses.

- P3L69 revise "Additionally, the relatively quick physiological response to stress (between hours and a few weeks)" - weeks?

The word “weeks” has been removed.

- P4L1128: "1. Grow a culture of OP50 in LB or equivalent media of choice for 24-48 hours in ambient temperature (~22-25 °C)." Why? We always do this overnight at 37. Much easier. Is there is reason not to? Then please state so. Otherwise, suggest both, as preferred by the experimenter.

The reason is stated in the notes, which have now been included directly in the text.

- P6L158: "7. Invert the bleach and worm mixture for ~5 minutes (do not exceed 10 minutes). Vigorous shaking will help to dissolve worm carcasses faster and is recommended for optimal preservation of eggs." We find that many mutants, especially stress sensitive mutants (of interest here), are sensitive to bleaching. I'd suggest to the authors to suggest to readers that bleaching time may be shorter for mutants/transgenic strains.

Again, this was already in the notes, which have now been added directly into the text.

- P6L195, recipe/source for IPTG missing

The IPTG was in the table of materials, but we have also now included this in our new Table 1.

- P7L233 and below: the view described here of the UPR as solely a proteostasis response is outdated. It is now clear that the UPR also monitors ER membrane status. The authors should clarify this and reference pertinent papers, especially because used the same hsp-4 reporter. By comparison, the authors' description of the UPR-mito below is more comprehensive in its inclusivity. Indeed, the authors observation of hsp-4::gfp expression fluctuating at various temperatures (L713) may be an indirect consequence of temperature induced lipid alterations (saturation).

We have modified our description of UPR^{ER} to include its role in ER membrane homeostasis (line 707). However, to keep within the scope of this JOVE collection, our manuscript focuses on the role of UPR^{ER} in protein homeostasis, as all of our assays utilize tunicamycin.

Our recent paper has shown that *hsp-4* is a direct readout for protein homeostasis as a downstream target of UPR^{ER}, and that other genes are involved in ER membrane status. Moreover, these two phenomenon can be distinctly separated (https://www.biorxiv.org/content/10.1101/471177v1). However, we focused this protocol on protein homeostasis and thus we believe addressing this is outside the scope of the manuscript.

- P7L239 and below: it is my understanding that hsp-4 is a specific readout of the ire-1/xbp-1 branch of the UPR, but isn't under control of pek-1/atf-4 or atf-6. The authors should clarify this; it's important to delineate specificities of transcriptional reporters.

It had been described both in the introduction and the results section that *hsp-4* is a direct target of XBP-1.

- P7L255 and below, "2.2.2.2 Inducing ER stress using a chemical agent". The authors surprisingly describe a protocol using tunicamycin in liquid culture; a reason for this is not described, and other chemicals below are described as being used on solid media. The authors should clarify that solid/liquid worm culture transition may cause gene expression changes that may affect genes under study. They

should further clarify that many labs have used Tunicamycin in solid culture, reference pertinent papers, and describe the corresponding protocol. In addition, it is not clear to me what the 15-20hr or even o/n recovery step does - are animals that don't recover eliminated? If so that should be stated. Otherwise, what's the point of the recovery step?

We do not believe that it is within the scope of this manuscript to describe all the available methods to performing tunicamycin assays. We have provided both a liquid treatment assay and a solid agar survival assay. Moreover, we have provided a solid agar RNAi method. Finally, we have added an extra line within the protocol to describe that the tunicamycin reporter assay can also be performed on solid agar.

We use a 16-20 hour recovery because this is necessary to accumulate enough GFP to be visible. This has been explicitly stated in the text.

- P9L314 and below. The description of oxidative stress response is incomplete. It is especially surprising that the authors propose using TBHP as an inducer of the "SKN-1 response". Recent papers show that a substantial fraction of TBHP regulated genes are SKN-1 independent (and that gst-4 is regulated by other TFs in oxidative stress); these papers should be cited and this clarified. Further, TBHP and paraquat are non-equivalent, which should be clarified.

We understand that TBHP and paraquat are not equivalent, and that is why both assays have been provided. Moreover, we had originally cited a paper that shows a much more comprehensive review of the protocols available for oxidative stress (Senchuk, M.M., Dues, D.J., Van Raamsdonk, J.M. Measuring Oxidative Stress in Caenorhabditis elegans: Paraquat and Juglone Sensitivity Assays. *Bio-protocol*. **7** (1), doi: 10.21769/BioProtoc.2086 (2017)). This reference has been moved to the introduction for ease of finding.

- P15L575: It's not clear to me why paraquat was chosen for oxidative stress assays when both PQ and TBHP were used to induce gst-4? TBHP is just as fine and has been used quite a bit for OSR studies. Please, again, clarify that PQ is merely one way of causing oxidative stress. A brief attempt to do this is made in L583, but please expand.

Again, a comprehensive overview of oxidative stress response assays have been provided in the introduction. It is out of the scope of this manuscript to provide this.

- P15L602 and below. I don't think the experiment described here is an appropriate readout of ER status. The concentration of 25 ng/µL tunicamycin seems very low. Does it really have an effect (life span effect in the figure could easily just represent short life pan of xbp-1 mutant)? In our hands, Tunicamycin wears off after a few days and any effects after that likely have nothing to do with ER stress, but rather simply reflect life span phenotypes, which may or may not be connected to stress response phenotypes. To truly monitor ER stress responses, higher concentrations should be used in my opinion. The fact that the other chemicals in 4.2 are used in a more classical, short-term stress survival assays would agree with this view. Also, P16L621 "5. At day 1, lifespans are "filleted". These are not (should not be, anyway) lifespans, but stress assays. Also, remove "filleted".

In our assay, the animals are always on tunicamycin plates. Therefore, they are always in the presence of the ER stress and the effect does not wear off. We have shown in previous work that *xbp-1* RNAi does not decrease lifespan in basal conditions. Thus, sensitivity of these *xbp-1* RNAi animals to tunicamycin (shorter survival compared to N2) is not due to them having an inherently short lifespan. Our data

presented here is not novel, but merely representative (e.g. Taylor, R.C., Dillin, A. XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity. *Cell*. **153** (7), 1435–1447, doi: 10.1016/j.cell.2013.05.042 (2013).).

Due to the solubility of tunicamycin, we have found 25 ng/µL to be a high enough level without causing toxicity from DMSO concentration. We have gone as low as 5 ng/µL of tunicamycin and see a dose-dependent effect on lifespan and *hsp-4p::GFP* induction, and have only included the most robust concentration in this protocol for clarity and ease.

In addition, we have tested tunicamycin plates that were greater than 2 months old (stored in the dark at 4C), and found that these can induce *hsp-4p::GFP* and cause L1 arrest of larvae, giving us confidence that the plates we are using have long-term functionality – at least enough for a 15-day assay as we describe. We unfortunately have not been able to adapt a "short-term" tunicamycin protocol that is robust, although that would certainly be an interesting area of investigation for a future study.

- P16L629: Why is paraquat assay in liquid media? No justification is given. I see no reason not to do it on solid media.

We have attempted to do a paraquat assay on solid agar and had very inconsistent results with lower concentrations and extremely high censorship in higher concentrations. To avoid discouraging this assay, we have provided a reference that covers many of the available oxidative stress response assays.

- P17L716: The authors observation on starvation inducing ER stress is not novel; they should cite pertinent literature, particularly Jo H et al., Cell Metab 9: 440-448, 2009.

We have removed this statement from the text during revisions.

- P20L813: "chemical oxidant, TBHP, results in a milder, but still significant, activation of gst-4p::GFP (Figure 3)." The Blackwell lab has shown that arsenite produces a much higher induction of gst-4

Again, a reference for a full list of oxidative stress response assays has been provided.

- P25 table 2: "and bolded strains are those where representative data are provided in this manuscript." No strains were bolded. Further, the authors should provide citations for the sod-3::gfp and T24B8.5p::GFP strains.

This statement has been removed. Citations have been added.

- P30L1040: "Such information cannot be obtained from qRT-PCR or RNA-seq, as it uses whole worm extracts". Inaccurate. Please consider mentioning scRNA-seq and several methods for tissue-specific gene expression profiling (from Waterston, Moerman, Riddle, Kim, Murphy labs, most recently Kaletsky et al. PLoS Genetics 2018).

Citation has been added.

Reviewer #4:
Manuscript Summary:

Here, the authors describe a series of assays and tools for analyzing different QC systems using reporters in C. elegans. In addition to the detailed protocols for each assays, the authors are making extra effort to guide readers so that even non-nematodologists should be able to use these tools. Overall, this is an excellent and thorough resource for the community.

Major Concerns:
None

Minor Concerns:
There are part of the manuscript that are in highlighted yellow.

Apologies, this is solely for the editorial staff.

In Figure 5B, author should highlight that RNAi against daf-2 is not necessarily as effective to show phenotypes as long-lived alleles of daf-2.

In our hands, the *daf-2* RNAi was sufficient to show major differences in phenotypes, and thus it was not essential to use the *daf-2* mutant.

It would be useful for authors to highlight the details of the protocol associated with lining up worms for imaging. This is a relatively challenging technique for beginners, but this method allows readers to clearly observe the reproducilibilty phenotypes (a variation across a cohort), which is quite valuable. This should hopefully become standard in the field and authors could highlight the benefits of this approach.

This is a very good point, and we have full intentions to capture the importance of this section in the video – unfortunately, this is a difficult thing to do in writing.

Reviewer #5:
Manuscript Summary:
In their manuscript „Measurements of physiological stress responses in C. elegans", Bar-Ziv et al. describe routine protocols for examining proteotoxic stess responses in the nematode C. elegans in response to important environmental stressors, such as heat and oxidative stress. The authors are „key opinion leaders" in the field of research into proteotixic stress and aging in C. elegans, such that I expect great interest of new and established C. elegans labs in their precise protocols, which they normally do not/cannot share in their original publications. The level of detail is such that no extensive pre-exisiting knowledge in C. elegans methods/techniques is necessary to perform these protocols such that they will be extremely helpful for new undergraduate/graduate students, in particular when considering that a video will be provided in addition. Lastly, in their discussion, the authors point to complementary assays (e.g. qPCR) and highlight particular strength/limitations of the assays they describe, such that researchers can make an „informed decision" on which assay to use. Accordingly, I only have very few specific comments/questions on the manuscript which I suggest to address before publication in JOVE (which I definitely recommend), with the intention to make the manuscript even more helpful to researchers new to C. elegans.

Major Concerns:
none

Minor Concerns:
Section 2.1: specify the number and stage of worms to be transferred on RNAi-plates and give a

recommendation at which stage/age worms should be examined; double-check step (5): do you intend to point the reader to section 2.2.1?

In Table 2, we have specified the number of eggs that can be transferred onto RNAi plates. Moreover, we have more accurately described the stages at which experiments should be performed – we describe "Day 1" adults as 65-96 hours of development, which is a "gravid" adult described by WormAtlas as an "egg-lay maximal" worm where the entire egg sac is full of eggs. We hope this would give some more direct guidance.

Section 2.2.1.1. The time window provided to give the reader a rough idea how long it takes for worms cultured at 20 °C to reach day 1 of adulthood is larger than 24 h. Why? I guess this is because some RNAi-constructs may slow down development. Please explain why you provide such a broad time window. Provide instructions on how to set up the experiment if worms develop at different speed (e.g. staggered plating or staggered analysis).

A section has been added to incorporate all the information for this.

Section 2.2.3. Ptypo: TBH should read TBHP

Done.

Section 2.2.2.2: The protocol provided assesses the ability to recover from ER-stress, rather than the activation of the ER-stress response, as „promised" in the introduction to this section. Clarify.

We have added additional information that this specific assay requires recovery in order to detect the induction of *hsp-4*. We also offered an alternative method to induce *hsp-4*, which is a chronic tunicamycin exposure.

Section 2.2.3.1. and 2.2.4. Similar to comment on 2.2.2.2: recovery, rather than activation?

Again, the recovery time is required for GFP accumulation to detectable levels. All these notes have been added into the protocols in their respective sections.

Section 3: The COPAS-BioSort is not a standard-device in many labs. Provide a clear recommendation of how to quantitatively analyze images if such a device is not available, even though these alternatives are much lower throughput (e.g. manual outlinig of worms in ImageJ)

We have added a section on quantification using ImageJ.

Section 4.1. Provide an idea of how long it takes for wildtype worms to die under the conditions described. For plate assays, why not use FUDR to avoid the additional stress to worms during frequent manual transfer? Also for plate assays, specify the number of worms per plate and the number of paltes per condition, similar to how you specify the number of wells per condition and number of worms per well for the paraquat assay in liquid. For the assay in liquid (which my lab does not perform because of the particular concern that I want to rise here): are there cases (mutants/RNAi-treatments) where survival is that long that one has to fear that worms may experience starvation in addition to oxidative stress? Can this risk be reduced by on purpose transferring lots of bacteria into the well, together with the worms?

The average expected survival was added into the section. While FUDR might minimize stresses from manual transfer, it comes with its own set of issues. For all survival assays, suggested numbers of worms have been recommended. We are uncertain of whether starvation or the actual oxidative stress itself has a greater impact, but we have provided a reference that gives a comprehensive overview of all the pros and cons of oxidative stress response protocols in the introduction.

Comment on Table 2: In some studies it may be of interest to assess the effect of an RNAi-treatment of interest on multiple reporters. In this case, the reporter constructs need to be crossed into a common wildtype background.

All of the reporters are on the Bristol N2 background. This information has been added to Table 2 (now Table 3 in the revised version).

Comment on Table 5. For a qPCR-experiment, one also needs reference genes; thus, for reasons of completeness, also recommend specific reference genes and primers.

We apologize for this oversight and reference genes have been added.

Reviewer #6:

Manuscript Summary:

The manuscript by Bar-Ziv, Frakes et al. describe protocols related to the assessment of stress responses using the model organism C. elegans, including the ER UPR, mitochondrial UPR, cytoplasmic heat shock response and the oxidative stress response. A thorough set of methods have been provided including growth conditions of C. elegans and bacterial cultures, imaging techniques, conditions to induce each stress response, quantification of stress response reporter strength using either small sample sizes (microscopy) or larger quantifications using a COPAS biosorter. The authors also provide physiological assays to compliment the readouts obtained using the stress reporters. Sufficient resources are also provided for the listed protocols including transgenic C. elegans strain IDs, microscopy settings, qPCR gene primer sets, and manufacturer information. The discussion also offers sufficient considerations to be made when using stress reporters as readouts of these cellular pathways as well as suggestions to further validate findings derived from these type of experiments. Overall, this is a very well written and thorough overview describing this method.

Major Concerns:

None.

Minor Concerns:

Two minor changes are suggested:

1. Line 568: I would suggest changing "...tunicamycin, which blocks N-linked glycosylation and subsequent accumulation of misfolded proteins in the ER" to "...tunicamycin, which blocks N-linked glycosylation causing accumulation of misfolded proteins in the ER."

Done.

2. Line 738 and 766 seem to be identical statements which can be condensed into one.

All the notes have been removed and placed into their appropriate sections, thus resolving this issue.