Visualizing the organization and differentiation of the male-specific nervous system of C. elegans

Tessa Tekieli, Eviatar Yemini, Amin Nejatbakhsh, Chen Wang, Erdem Varol, Robert W Fernandez, Neda Masoudi, Liam Paninski and Oliver Hobert
DOI: 10.1242/dev.199687

Editor: Swathi Arur

Review timeline
Original submission: 7 April 2021
Editorial decision: 10 May 2021
First revision received: 9 July 2021
Accepted: 16 August 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199687

MS TITLE: Visualizing the organization and differentiation of the male-specific nervous system of C. elegans

AUTHORS: Tessa Tekieli, Eviatar Yemini, Amin Nejatbakhsh, Erdem Varol, Robert W Fernandez, Neda Masoudi, Liam Paninski, and Oliver Hobert

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees are excited about the work, and the utility of the work for the field at large. The reviewers also offer some recommendations to improve the manuscript, especially with clarity in figures, writing and as reviewer 2 points out specific analysis in wild type males rather than just through the use of the mutations as is the present case. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper by Tekieli et al. is a follow-up on previous work describing the NeuroPAL tool for neuronal cell identification in C. elegans. Like the mammalian brainbow system, NeroPAL distinguishes adjacent neurons by color coding them using 4 distinct reporters. Instead of mitotic

recombination, NeuroPAL uses a combination of dozens of prescreened reporters, with known cell expression patterns. This work extends the use of this system to C. elegans males, showing that male specific neurons can all be distinguished through the 4-color combinatorial code. The authors use the system to demonstrate lineage variability in males, to identify roles of a number of transcription factor genes in generating male specific neurons, and reveal an interesting wave of differentiation of male tail neurons at the L4 stage, despite variability in the timing of production of these cells.

The nearly invariant lineage of C. elegans has been useful in deciphering gene activity in specific cells, by allowing reproducible observation and manipulation of the same cells across many animals. The NeuroPAL tool allows this now to be done more easily, by crossing a reporter for a gene of interest into the marked strain, and examining overlap with the colors of a particular cell at a particular location. The system also allows one to deduce an overview of neuronal differentiation.

Comments for the author

The manuscript is very well written, the figures and table are clear, and I have no issue with the science. I think the paper will be an important reference for anyone studying the male neuroanatomy of C. elegans.

Reviewer 2

Advance summary and potential significance to field

NeuroPal (Yemeni et al, 2021) is a transgene-based technology to label every single neuron in the entire C elegans nervous system with a unique fluorescent color code.
Here, Tekieli et al build on the initial study, and extend usage of NeuroPal to interrogate the nervous system of the C. elegans male. This work demonstrates that NeuroPal can be used to label the male nervous system and uniquely identifying male-sex specific neurons. The utility of this is further validated by confirming/extending previous studies by others, including on three well-characterized mutants (lin-4, lin-32, and egl-5) on the development of male-specific neurons. Finally, this study provides the novel finding that male-specific neurons born early in development remain undifferentiated until late L4 larva, such that all sex-specific neurons differentiate coordinately.
This study advances the field in two ways. One, it provides cellular accessibility to study male-specific neurons, for which unique cell-specific markers have been largely lacking. This should be a useful tool to study sex-differences in neural circuits. Two, the finding of coordinated differentiation is of general interest to systems biology and neuronal development. This finding sets the stage, and tools, for future mechanistic studies. Finally, extension of NeuroPal to males enables study of circuit-level sexual dimorphism with cell-level resolution, as well as examine how late-born neurons integrate into pre-existing circuits.
Thus, this study is of broad interest. However, the following points need to be addressed to make this manuscript suitable for publication.

Comments for the author

Major Comments:
1. The authors use him-8 and him-5 mutants to generate males. It is possible that these mutations contribute to the phenotypes observed. Evaluation of natural males in at least one mutant (e.g. lin-4) is needed to exclude this possibility. If not, then global analyses of sex-shared neuron concordance in hermaphrodites with/out him mutations may provide an indirect measure.

2. It is confusing whether in some mutants lack of fluorescence reflects undifferentiated or non-existent cells. UPN-labeled /DIC images, or further clarification is needed (Fig 5, 6E, 6F)

3. Additional insight into the biology of coordinated timing is warranted to strengthen this observation. At least one of these studies is suggested at minimum:
-does intrinsic sex-identity of a cell drive this program (cell-specific rescue of him-5)

-evaluation of wave onset in unc-86 mutants.
-evaluation of wave onset in some members of the heterochronic pathway.

Minor and organizational comments:
1. Typos:
- Pg.7: concluision by closely considering
- Pg 8: we observer a similar extent
- Pg.8: Fig 3 is coded red text and lacks panel reference.
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-Pg 10: figure mention for lin-4 lineage should be Figure 5B not 5A.

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3. Pg. 6: “4 distinct, but fused panneuronal promoters”. While published information, it will be helpful to briefly describe this for stand-alone clarity in this manuscript.

4. Multiple figures:
- Some figures (eg. Fig 5, 6) do not follow text-flow, making for choppy reading. Re-organization recommended.
- neurons noted in text should be marked in the figures (eg. RMF, PCB in Fig 7).
- Many images are hard to interpret. Suggest use of arrows/pointers for all cells of interest.
- Many figure labels are too small to read on print, or on screen without significant zoom. Larger fonts/ reconfigured images are recommended (eg. Fig 2C, 6A, 6B, 7 head panels).
Reference/schematics (eg. in Fig 2) and DIC (Fig 7) can be smaller if space is a limitation.
- Some of the figure legend text is useful description to understand the mutant biology and should be discussed in the body of the manuscript (eg. Fig 6).

5. Fig. 1: If needed for space limitation, lineage diagram can be a supplemental figure without loss of readability

6. Fig. 2: It will be helpful to mark male-specific features on each panel and/or provide the hermaphrodite equivalent region for comparison. Also, orientation labels will be helpful in 2A.

7. Fig. 3: It will be helpful to highlight the DX and EF labels. DX was hard to find.

8. Fig. 5: 5D and 5E are not referenced in text.

9. Fig. 6:
-dat-1:RFP and UPN is not discussed in text or legend, making its inclusion in labels and experiment confusing to understand.
-Closeup images for egl-5 (Figure 6B) and explanation for lin-32 mutants (Figure 6D, for eg. PDC, PGA) is needed to understand the results clearly.
10. Figure 7: Head panels are too small, and cell-labels in tail images hard to see. The figure overall is hard to follow the point on. Suggest restructuring and resizing the panels, and adding arrows.

Reviewer 3

Advance summary and potential significance to field

NeuroPAL (neuronal polychromatic atlas of landmarks) is a tool for neuronal identification in C. elegans (Yemini 2021). The C. elegans is a hermaphrodite species. Major C. elegans research is conducted in the hermaphrodite. The C. elegans male receives less attention but has its unique place in biology, especially in sexually dimorphic development and behavior regulation. Tekieli et al. now expand the original NeuroPAL work to introduce neuronal identification in the C. elegans male, a heroic effort for the C. elegans research community.

Comments for the author

NeuroPAL (neuronal polychromatic atlas of landmarks) is a tool for neuronal identification in C. elegans (Yemini 2021). The C. elegans is a hermaphrodite species. Major C. elegans research is conducted in the hermaphrodite. The C. elegans male receives less attention but has its unique place in biology, especially in sexually dimorphic development and behavior regulation. Tekieli et al. now expand the original NeuroPAL work to introduce neuronal identification in the C. elegans male, a heroic effort for the C. elegans research community. In general, the manuscript is well written, and experiments largely support conclusions.

We have three major concerns about the current MS. Firstly, the C. elegans males have 94 male-specific neurons. Yet, the authors did not modify the original transgenic animals to accommodate the male- specific neurons. Many male specific neurons are not visible. For example, in figure 4, the CAn neurons are missing. Secondly, the micrographs presented in the figures generally are of low quality compared to the original Yemini 2021. The vague nuclear outline and the discolored images make neuronal identification hard—which hinders the central goal of the MS. Thirdly, throughout the figures, all neurons are labeled with a name without supporting data or landmarking. We have suggestions to address these three major concerns that will improve this manuscript as a user's guide for NeuroPAL in male

1. NeuroPAL was developed for the hermaphrodite nervous system and requires modification to completely cover the male-specific neurons. Authors should considering add a Table showing which male neurons are not identifiable and discuss the current limitations of the system. As currently written, this is not apparently. For example, a complete male neuroPAL needs additional transgenes. While not required for this manuscript, authors should address the shortcomings of the NeuroPAL system that was optimized/designed for the hermaphrodite.
2. Higher quality images are necessary. Image quality not as good as original 202 paper. Even in uncompressed figures, images are poor resolution with fuzzy edged-nuclei. Please improve imaging and consider adding accompanying DIC. This will improve practicality and usability for researchers wanting to identify male neurons.
3. Difficulty in interpreting figures without clear diagrams and landmarks.
    a. Figures were difficult to navigate, interpret, and understand male development and anatomy without familiarity, even for a C. elegans male aficionado. To understand blast cells and resulting neurons, we suggest modify/reproducing Figure 4 from Emmons' 2005 WormBook chapter.
    b. Define landmarks as in Yemini 2021. This is essential to help futures users orient their male tail images. These landmarks could be the smaller circles (those neurons with invariant positions) in Figure 3B

Specific comments

Figure 2B

The shape of the nuclei in the images appears fuzzy, without a clear-cut border. The images here appear of low quality. The nuclei look discolored comparing to the Yemini 2021. Also, the texture of the nuclei seems different from the figures in the Yemini 2021, where the discrete chromosomes in the nuclei are readily visible.

Figure 2C

We do not understand the term "Real images." Please define.

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In Figure 2 C, many neurons in the micrographs appear not correlated to the atlas cartoon, misleading for identifying neuron purpose.

Figure 2 DE

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Figure 3A

The quality of the images is low. Many other neurons in the left and the right are not symmetric. How do authors know if the neurons are not present or if there is no expression of the NeuroPAL? Pairing with DIC may be helpful.
Figure 3B

Consider using smaller circles as landmarks

Breaking into smaller panels to be useful. The current overlay is difficult to read.

Change color-coded text to black and white. For example, orange and yellow are not easily visible.

Table S2: cubic microns difficult unit to comprehend/not useful for Figure 3 intepretation. X-Y location may be more useful.

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The quality of the images is low. Micrograph of the worm body region would be helpful. The overall ganglion and the gangliar landmarks are not shown.

Figure 4A. CA1-4 appear not visible in the neuroPAL channel. Scale bar labeling doubled.

Figure 4B. CA8-9 appear not visible in the neuroPAL channel.

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Comparison of micrographs of the worm body region would be helpful.

Again, the wild type and the lin-4 mutant images are not correlated. If they are correlated, the differences between wild type and the lin-4 mutant are much more than what the authors described.

Figure 6.

Comparison of micrographs of the worm body region would be helpful.

Again, the wild type and the lin-32 mutant images are not correlated. If they are correlated, the differences between wild type and the lin-32 mutant are much more than what the authors described.

Figure 7.

Micrographs of the worm head from where the insets are taken would be helpful.

Again, the images are of low quality. The neurons are discolored and without a clear nuclear outline comparing to Yemini 2021. The neuronal landmarks aiding neuron identity are not shown.

General: Does him-5 or him-8 have any affect on the health of the strain or on the behavior/mating efficiency of males? This is an important consideration when introducing into other backgrounds where one is looking specifically at male development, anatomy, physiology, etc.

First revision

Author response to reviewers' comments

RESPONSE TO REVIEWERS COMMENTS (our responses in red)

Reviewer #1

This paper by Tekieli et al. is a follow-up on previous work describing the NeuroPAL tool for neuronal cell identification in C. elegans. Like the mammalian brainbow system, NeroPAL distinguishes adjacent neurons by color coding them using 4 distinct reporters. Instead of mitotic recombination, NeuroPAL uses a combination of dozens of prescreened reporters, with known cell expression patterns. This work extends the use of this system to C. elegans males, showing that male specific neurons can all be distinguished through the 4-color combinatorial code. The authors use the system to demonstrate lineage variability in males, to identify roles of a number of transcription factor genes in generating male specific neurons, and reveal an interesting wave of differentiation of male tail neurons at the L4 stage, despite variability in the timing of production of these cells.

The nearly invariant lineage of C. elegans has been useful in deciphering gene activity in specific cells, by allowing reproducible observation and manipulation of the same cells across many animals. The NeuroPAL tool allows this now to be done more easily, by crossing a reporter for a gene of interest into the marked strain, and examining overlap with the colors of a particular cell at a particular location. The system also allows one to deduce an overview of neuronal differentiation.

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This study advances the field in two ways. One, it provides cellular accessibility to study male-specific neurons, for which unique cell-specific markers have been largely lacking. This should be a useful tool to study sex-differences in neural circuits. Two, the finding of coordinated differentiation is of general interest to systems biology and neuronal development. This finding sets the stage, and tools, for future mechanistic studies. Finally, extension of NeuroPal to males enables

study of circuit-level sexual dimorphism with cell-level resolution, as well as examine how late-born neurons integrate into pre-existing circuits. Thus, this study is of broad interest. However, the following points need to be addressed to make this manuscript suitable for publication.

Major Comments:
1. The authors use him-8 and him-5 mutants to generate males. It is possible that these mutations contribute to the phenotypes observed. Evaluation of natural males in at least one mutant (e.g. lin-4) is needed to exclude this possibility. If not, then global analyses of sex-shared neuron concordance in hermaphrodites with/out him mutations may provide an indirect measure.

We wish to point out that in the 3 mutant cases we describe (egl-5, lin-32, lin-4/heterochronic mutant) classic lineage/phenotyping studies have used him mutants. We are confirming previously reported defects, again using him mutants. That's all internally consistent. The reasons this has been done in the past (and why we do it here again) is that males of these strains are not competent for mating; it's therefore difficult to establish a large enough population of males for scoring (i.e. one can not simply take males derived from heat-shock and cross them back to hermaphrodites, to establish a population of males).
This being said, we do see the point of the reviewer and we chose to go the alternate route that the reviewer suggest: We compared NeuroPAL color codes in naturally induced males vs. him-5 males (ALL neurons, i.e. sex-shared and male-specific) – and there are no differences in color codes. We now mention this in the Methods.

2. It is confusing whether in some mutants lack of fluorescence reflects undifferentiated or non-existent cells. UPN-labeled /DIC images, or further clarification is needed (Fig 5, 6E, 6F)
UPN-labeled images were added to these figures.

3. Additional insight into the biology of coordinated timing is warranted to strengthen this observation. At least one of these studies is suggested at minimum:
does intrinsic sex-identity of a cell drive this program (cell-specific rescue of him-5)
The reviewer probably means cell-specific tra-1/2 (rather than him-5) manipulations to affect sexual identity; this is difficult because that pathway is involved involved in sex-specific proliferation; i.e. the elegant, "simple" sex-identity change that has been done in postmitotic, sex-shared neurons, will have more pleiotropic, earlier defects in sex-specific neurons. This being said, we have conducted a simple and straight-forward experiment to address the question of intrinsic sexual identity: Previous studies have shown that the male-specific, embryonically born CEM neuron activates marker gene expression, e.g. the pkd-2::gfp reporter, only in the L4 stage, i.e. long after their birth. CEM therefore provides an example for coordinated timing, equivalent to what we have shown throughout the entire male-specific nervous system with NeuroPAL. The experiment we now did is to abrogate the sexual specificity of CEM existence in ced-3 mutants. In these mutants, the CEM neurons survive in hermaphrodites and we can now ask whether the temporal control of pkd-2 expression still observed in hermaphrodites. As we show in the new Supp. Fig. S3, this in indeed the case. We now mention this result in p. 14 in the manuscript.
-evaluation of wave onset in unc-86 mutants.
The sex-specific neurons that show unc-86 expression and show just-in-time differentiation are the male-specific CEM neurons and the hermaphrodite-specific HSN neurons. Previous, published work has already shown that unc-86 affects the temporally controlled markes in the CEM and HSN neurons: In unc-86 mutants, the temporally controlled markers pkd-2 (Shaham/Bargmann paper) and neurotransmitter markers (Pereira et al. paper) will not get turned on in the CEM neurons. The temporally controlled HSN marker (e.g. tph-1) are known to not be expressed in unc-86 mutants (Horvitz, Ruvkun labs). Notably, previous expression analysis has shown that unc-86 is expressed in CEM and HSN neurons from embryogensis onward (Finney/Ruvkun and our lab). We can hence conclude that unc-86 is permissively required for the temporally delayed onset of marker gene expression, but that unc-86 is either prevented from turning on these markers in early stages or, alternatively, is lacking a critical co-factor until the L4 stage. We have now clarified this in the Discussion on page 17/18.
evaluation of wave onset in some members of the heterochronic pathway.
This has also been done for the CEM neurons by the Portman lab (Lawson, eLife 2019). They show that the heterochronic pathway has a PARTIAL effect on the timing of CEM differentiation (we mention this in the Discussion on page 17). Hence, there must be other, additional mechansims that control differentiation timing, at least for the CEMs. Testing heterochronic mutants for their impact

in male-specific neurons of the tail is furthermore complicated by the impact that heterochronic mutants have on the proliferation of sex-specific neurons (with the exception of CEMs, which are born in the embryo; hence the heterochronic mutants don't affect their lineage patterns). This is all clearly a very interesting problem that we will plan to address in future studies.

In addition to what the reviewer suggested, we have further flashed out the just-in-time differentiation description by considering markers other than NeuroPAL. We have now also examined NeuroPAL-transgene independent fosmid and CRISPR-tagged genes and found them all to confirm the just-in-time differentiation in the L4 stage. This includes three fosmid transgenes for the panneuronal rab-3 gene, the synaptic organizer oig-1 and the vesicular transporter unc-47. In addition, we have used three loci that we endogenously tagged with gfp using CRISPR/Cas9: The nlp-51 and flp-27 neuropeptide encoding genes, and the eat-4/VGLUT locus, each expressed in a number of non-overlapping male-specific neurons. All of these reporters show a coordinated onset of expression during the L4 stage, at least one larval stage after the respective neuron has been born. This new data is now shown in a new Figure 8 and described in p. 13/14 in the manuscript.

Minor and organizational comments:
1. Typos:
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- Pg 8: we observer a similar extent
- Pg.8: Fig 3 is coded red text and lacks panel reference.
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- Figure 4: legend says him-8 and figure says him-5.
-Pg 10: figure mention for lin-4 lineage should be Figure 5B not 5A.
All of the above typos have been corrected.

2. Pg 4: integration of male-specific neurons into existing circuitry is an exciting question but is not addressed in this manuscript. Suggest this is more appropriately placed in the Discussion section.
This has been removed.

3. Pg. 6: "4 distinct, but fused panneuronal promoters". While published information, it will be helpful to briefly describe this for stand-alone clarity in this manuscript.
Additional information about the panneuronal promoters has been added to the description in the manuscript.

4. Multiple figures:
- Some figures (eg. Fig 5, 6) do not follow text-flow, making for choppy reading. Re-organization recommended.
- neurons noted in text should be marked in the figures (eg. RMF, PCB in Fig 7).
- Many images are hard to interpret. Suggest use of arrows/pointers for all cells of interest.
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Reference/schematics (eg. in Fig 2) and DIC (Fig 7) can be smaller if space is a limitation.
- Some of the figure legend text is useful description to understand the mutant biology and should be discussed in the body of the manuscript (eg. Fig 6).
All of the above are fixed.

5. Fig. 1: If needed for space limitation, lineage diagram can be a supplemental figure without loss of readability

6. Fig. 2: It will be helpful to mark male-specific features on each panel and/or provide the hermaphrodite equivalent region for comparison. Also, orientation labels will be helpful in 2A.
The changes have been incorporated. Male-specific neurons are outlined in a blue dashed circles and ganglia are labeled in all images to help with orientation.

7. Fig. 3: It will be helpful to highlight the DX and EF labels. DX was hard to find.
Added arrows to indicate DX and EF and highlighted the corresponding labels.

8. Fig. 5: 5D and 5E are not referenced in text.

Figures 5D and E are now referenced in the text.

9. Fig. 6:
-dat-1:RFP and UPN is not discussed in text or legend, making its inclusion in labels and experiment confusing to understand.
Now included.

-Closeup images for egl-5 (Figure 6B) and explanation for lin-32 mutants (Figure 6D, for eg. PDC, PGA) is needed to understand the results clearly.
Better image for egl-5 now included. Re lin-32, there are no defects observed, as stated in the legend.

10. Figure 7: Head panels are too small, and cell-labels in tail images hard to see. The figure overall is hard to follow the point on. Suggest restructuring and resizing the panels, and adding arrows.
Full micrographs of the heads have been added with inset images of the head regions in which the male neurons are located. The size of the tail images have been increased. Male-specific neurons are indicated with blue dashed circles and neuron name and sex-shared neurons are indicated with red dashed circles. Two sex-shared neurons, VA11 and PVQ, are labeled with neuron name to allow for orientation without labeling all sex-shared neurons to increase the size of labels on male-specific neurons.

REVIEWER #3

NeuroPAL (neuronal polychromatic atlas of landmarks) is a tool for neuronal identification in C. elegans (Yemini 2021). The C. elegans is a hermaphrodite species. Major C. elegans research is conducted in the hermaphrodite. The C. elegans male receives less attention but has its unique place in biology, especially in sexually dimorphic development and behavior regulation. Tekieli et al. now expand the original NeuroPAL work to introduce neuronal identification in the C. elegans male, a heroic effort for the C. elegans research community. In general, the manuscript is well written, and experiments largely support conclusions.
We have three major concerns about the current MS. Firstly, the C. elegans males have 94 male-specific neurons. Yet, the authors did not modify the original transgenic animals to accommodate the malespecific neurons. Many male specific neurons are not visible. For example, in figure 4, the CAn neurons are missing. Secondly, the micrographs presented in the figures generally are of low quality compared to the original Yemini 2021. The vague nuclear outline and the discolored images make neuronal identification hard—which hinders the central goal of the MS. Thirdly, throughout the figures, all neurons are labeled with a name without supporting data or landmarking. We have suggestions to address these three major concerns that will improve this manuscript as a user's guide for NeuroPAL in male

1. NeuroPAL was developed for the hermaphrodite nervous system and requires modification to completely cover the male-specific neurons. Authors should considering add a Table showing which male neurons are not identifiable and discuss the current limitations of the system. As currently written, this is not apparently. For example, a complete male neuroPAL needs additional transgenes. While not required for this manuscript, authors should address the shortcomings of the NeuroPAL system that was optimized/designed for the hermaphrodite.
Supp Table S1 has the requested info, i.e. shows the limitations of the system, meaning that we do not know for each individual neuron where the disambiguating color code comes from. We now added a few sentences in the Result section where we emphasize this shortcoming and refer to that Table.

2. Higher quality images are necessary. Image quality not as good as original 202 paper. Even in uncompressed figures, images are poor resolution with fuzzy edged-nuclei. Please improve imaging and consider adding accompanying DIC. This will improve practicality and usability for researchers wanting to identify male neurons.
Done throughout all figures.

3. Difficulty in interpreting figures without clear diagrams and landmarks.

a. Figures were difficult to navigate, interpret, and understand male development and anatomy without familiarity, even for a C. elegans male aficionado. To understand blast cells and resulting neurons, we suggest modify/reproducing Figure 4 from Emmons' 2005 WormBook chapter.
b. Define landmarks as in Yemini 2021. This is essential to help futures users orient their male tail images. These landmarks could be the smaller circles (those neurons with invariant positions) in Figure 3B
All figures have now been completely labeled with neuron IDs to aid in identification/orientation of images.

Specific comments:

Figure 2B: The shape of the nuclei in the images appears fuzzy, without a clear-cut border. The images here appear of low quality. The nuclei look discolored comparing to the Yemini 2021. Also, the texture of the nuclei seems different from the figures in the Yemini 2021, where the discrete chromosomes in the nuclei are readily visible.
Included improved images in this panel

Figure 2 C: We do not understand the term "Real images." Please define.
An overall micrograph corresponding to the midbody region would be more helpful. The inset images should come from the overall micrograph, not the cartoon, which looks disorganized.
Real images has been corrected to micrographs. Cartoon was swapped for an overall micrograph.

In Figure 2 C, many neurons in the micrographs appear not correlated to the atlas cartoon, misleading for identifying neuron purpose.
The cartoon has been swapped out for an overall micrograph.

Figure 2 DE: An overall micrograph corresponding to the male region would be more helpful. The inset images should come from the overall micrograph, not the cartoon, which looka disorganized.
Cartoon was swapped for an overall micrograph.

In Figure 2 DE, some male-specific neurons are not visible, which will significantly hinder the usefulness of the male NeuroPAL. The male tail is dense with neurons. Maybe micrographs of male worms on dorsal or ventral view should also be presented to help delineate the individual neurons.
Some neurons are marked by panneuronal only, so images with panneuronal are now included in these figures to aid in neuronal ID.

Figure 3A: The quality of the images is low. Many other neurons in the left and the right are not symmetric. How do authors know if the neurons are not present or if there is no expression of the NeuroPAL? Pairing with DIC may be helpful.
Included higher quality images and paired with DIC to show that the neurons are simply not marked by NeuroPAL but indeed aren't there.

Figure 3B: Consider using smaller circles as landmarks. Breaking into smaller panels to be useful. The current overlay is difficult to read. Change color-coded text to black and white. For example, orange and yellow are not easily visible.
We want to keep the color coding of labels consistent across hermaphrodites and males, so here we stick with the convention established in the Yemini 2021 paper. Labels have been adjusted to improve readability.

Table S2: cubic microns difficult unit to comprehend/not useful for Figure 3 intepretation. X-Y location may be more useful.
We used this to stay consistent with the original NeuroPAL paper.

Figure 4. The quality of the images is low. Micrograph of the worm body region would be helpful. The overall ganglion and the gangliar landmarks are not shown.
Figure 4A. CA1-4 appear not visible in the neuroPAL channel. Scale bar labeling doubled.
Figure 4B. CA8-9 appear not visible in the neuroPAL channel.
Images including the panneuronal have now been added to this figure to aid in the identification of CA neurons. Micrograph of the male tail region has been added to aid in the identification of the region pictured.

Figure 5. Comparison of micrographs of the worm body region would be helpful. Again, the wild type and the lin-4 mutant images are not correlated. If they are correlated, the differences between wild type and the lin-4 mutant are much more than what the authors described.
Micrographs of the representative body region (posterior VNC and male tail) comparing WT and *lin-4* mutant are shown now in Fig 5A with boxed regions referring to regions pictured in Fig 5C-F.

Figure 6. Comparison of micrographs of the worm body region would be helpful. Again, the wild type and the lin-32 mutant images are not correlated. If they are correlated, the differences between wild type and the lin-32 mutant are much more than what the authors described.
Micrographs of representative worm body region (male tail) comparing WT and *egl-5* are included in Fig 6A with boxed regions referring to regions pictured in Fig 6B&C. Micrographs of representative worm body region (male tail) comparing WT and *lin-32* are included in Fig 6D with boxed regions referring to regions pictured in Fig 6E-H.

Figure 7. Micrographs of the worm head from where the insets are taken would be helpful. Again, the images are of low quality. The neurons are discolored and without a clear nuclear outline comparing to Yemini 2021. The neuronal landmarks aiding neuron identity are not shown.
Full micrographs of the heads have been added with inset images of the head regions in which the male neurons are located. The size of the tail images has been increased. Male-specific neurons are indicated with blue dashed circles and neuron name and sex-shared neurons are indicated with red dashed circles. Two sex-shared neurons, VA11 and PVQ, are labeled with neuron name to allow for orientation without labeling all sex-shared neurons to increase the size of labels on male-specific neurons.

General: Does him-5 or him-8 have any affect on the health of the strain or on the behavior/mating efficiency of males? This is an important consideration when introducing into other backgrounds where one is looking specifically at male development, anatomy, physiology, etc.

Second decision letter

MS ID#: DEVELOP/2021/199687

MS TITLE: Visualizing the organization and differentiation of the male-specific nervous system of C. elegans

AUTHORS: Tessa Tekieli, Eviatar Yemini, Amin Nejatbakhsh, Chen Wang, Erdem Varol, Robert W Fernandez, Neda Masoudi, Liam Paninski, and Oliver Hobert
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have done a good job addressing reviewer comments. I have no further issues.

Comments for the author

The authors have done a good job addressing reviewer comments. I have no further issues.

Reviewer 2

Advance summary and potential significance to field

Tekieli et al extend NeuroPal (Yemeni et al, 2021), a transgene-based technology to fluorescently label all C. elegans neurons uniquely, to males. This tool now provides cell-accessibility to study male-specific neurons. By extending NeuroPal to mutant animals of select and well-characterized genes (lin-4, lin-32, and egl-5), the study also finds that male-specific neurons, born through animal life, execute coordinated (just-in-time) differentiation in the late larval L4 stage. Thus, this work sets the stage and tools for further mechanistic studies of male neural circuit development, and sex-dimorphism with single-cell resolution, in C. elegans.

Comments for the author

In the revised manuscript, the authors have provided data, rephrased sections, and/or explained their rationale satisfactorily. The current version reads well and is recommended for publication.