nature research

Peer Review File

Manuscript Title: A multiscale brain map derived from whole-brain volumetric reconstructions

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The MS by Brittin et al. describes an analysis of the neuropil in the nerve ring of Caenorhabditis elegans in terms of the distributions of neighbourhoods and synaptic contacts of individual nerve processes. Neighbourhoods are defined as the set of adjacent processes that surround a given nerve process together with their contact areas. Use was made of the EM serial sections of two nerve rings on which the original connectome was based (ref 5). These sections have been recently re-scored by the authors and an updated connectome published (ref 3).

Based on their application of cluster analysis to the neighbourhood data, the authors claim the nerve ring can be described as 5 roughly parallel regions and discuss the constraints this might have on the connectome.

By comparing given neurons in different animals, and on laterally symmetric sides of the same animal, the authors were able to look at the collection of synapses and neighbourhoods of four different examples of each neuron class. From these data they came to the interesting conclusion that connectivity in C. elegans consists of an invariant core circuit embedded in a variable background. They then went on to show how this core circuit could be described by a Modular Residual Network.

This MS has potential interest as it describes synaptic connectivity in the context of the complete set of adjacent neighbours that each nerve processes possesses. Synaptic connections are limited to a subset of these neighbouring processes. This work extends earlier studies of neighbourhoods (ref 16) and provides new insights to how a nervous system is organised and how it might develop. However, I do not consider this MS suitable for publication in a journal such as Nature for the following reasons:

1. The presence of a synapse is inferred from images seen on EMs of serial sections. Unfortunately, there are rarely any post-synaptic specialisations visible at putative chemical synapses in C. elegans. Whilst many synapses are nevertheless unambiguous, there are cases, particularly with polyadic synapses, where there are varying degrees of uncertainty as to the identity of all post-synaptic partners. Gap junctions, because of their symmetrical configuration, do not have this particular problem. However, there are still uncertainties in some cases, particularly when two membranes from closely adjacent neurites are closely apposed but there is little increase in stain visible in these regions. These problems lead to an inevitable level of subjectivity in calling synapses as was explained in the original connectome reconstruction (ref 5). This limitation in synapse identification has to be acknowledged particularly when discussing connectivity data. It is striking that the authors called significantly more synapses than the original reconstructions when the synapses on the same animals were rescored (ref 3). Recently a non-peer reviewed reconstruction of a different adult nerve ring (Witvliet et al. (2020) bioRxiv preprint) described a similar number of chemical synapses to that described in the original. There are insufficient data to determine which one of these reconstructions is more “correct”. This situation therefore behoves authors drawing conclusions from connectivity data to clearly state caveats

when interpreting these data.

2. The potentially interesting conclusion that the neural connectivity can be described as a core circuit that is embedded in a variable background lacks credibility given the failure to acknowledge the inevitable level of ambiguity in calling synaptic contacts - it could be that the variability is due to miss-called contacts. Identification of adjacencies is not subject to the ambiguities of chemical synapse or gap junction calling and It is striking that in Fig. 2a the A1 is smaller than A4 whereas C1 is larger than C4 and G1 is larger than G4. This to me is suggestive of over enthusiastic calling of chemical synapses and gap junctions. Ultimately ambiguities in chemical synapses might be resolved when there is a knowledge of a match of receptor and transmitter between the partners. For gap junctions, it would be worth appending a confidence level for each contact based on the visibility of the structure in the EM images. I would be interested to know how the additional synaptic contacts scored when the authors re-called these two datasets distribute between core and the variable contacts.

3. I do agree with the authors on the demarcation of a core circuit as one that includes only C4 and G4 synapses. This is a fairly unambiguous circuit and, as such, should be focussed upon by investigators who are trying to model behaviour or developmental mechanisms. One of the most interesting figures in this MS for me is buried in the supplement (S23) where it is shown that the size distributions of C4 and G4 synapses are striking larger than those of the 3, 2 or 1 cases. There could well be a separate variable component, but it would be less than that claimed due to the inevitability of a level of false positives in synapse calling.

4. Although I am enthusiastic about the notion of a core circuit defined by connections that exist in all four symmetrical regions, I was surprized that the authors did not use other information that may imply a strong synaptic connection. Some chemical synapses are large structures in the EM but are only seen single at the point where two processes may cross. Some gap junctions are much more obvious than others. It would be good to incorporate a measure of the "strength" of a class of synaptic connection as determined by its multiplicity and/or size.

5. The original reconstruction the nerve ring as consisting of three layers (ref 5). Recently an un-peer reviewed study clamed that using cluster analysis the nerve ring could be divided into four layers (Moyle et al. (2020) bioRxiv). In this study, a different cluster analysis algorithm has been used to show that the nerve ring is a five-layered structure. I am yet to be convinced that efforts to divide the nerve ring up into ever smaller domains is a worthwhile exercise. To then go on and characterise neurons as being intrinsically different depending whether they lie in a domain interior or on a boundary strikes me as like building a house of cards. All the neurons in the nervous system have been reconstructed in three dimensions and their visible synaptic connections annotated. This is essentially a complete EM anatomical description of the structure of the C. elegans nervous system. I am not convinced that trying to chop this structure into arbitrary domains based on adjacency is a worth-while exercise. Indeed, giving names to these domains that imply function could eventually prove to be misleading.

6. The authors fail to explain why they think the core connectome can be described by a residual learning network. These networks have to do with AI strategies for learning and image classification. I fail to see the relevance of these networks to the core connectome from the authors' narrative. While I can understand the motivation for trying to understand the connectome in terms of computational structures that have been developed in AI, I think this could be dangerous. The connectome is what it is - it was not designed by data scientists. We are rapidly obtaining knowledge of the dynamic activity of neurons when animals are performing particular behaviours and their individual repertoires of neurotransmitters and receptors. Computer modelling will have an important role to play when sufficient information has been obtained about the nature of the interconnections (both focal and humeral) between all the component neurons. I am less enthusiastic about network analysis, but it might have a role.

7. The Jaccard index described on line 677 is incorrect. The authors appear to have confused the symbols for set union and set intersection. Also on line 684.

8. I found some of the figures and their legends hard to figure out. For example, Fig. S3 legend "Neighbourhood size (…) does not vary with neighbourhood size" ??

9. Maybe I missed it, but what is a "survival distribution"?

10. I could not understand how the adjacency data were normalised from the narrative. This made it difficult from me to evaluate Fig. S6.

11. I don't like the use of the word axon throughout the text to describe any neuron process. Axon generally implies a pre-synaptic process, which is not necessarily the case. Neurite would be a better word to use.

12. The use of 10 point rather than the usual 12 point font for this MS made reviewing this study less enjoyable than it might have been.

Referee #2 (Remarks to the Author):

The manuscript by Brittin et al. segments more than 700 legacy EM images of the C. elegans nerve ring to create for the first time a volumetric reconstruction of what is effectively the worm brain. Although the worm brain with only 181 neurites is very simple compared to the brains of other organisms, its complexity has frustrated deeper understanding for decades. To create new avenues of understanding the authors have segmented the legacy EMs of White et al. from L4 and adult animals and used those results to generate a space-filling model of the neuropil. They show that left-right versions of homologous neurons are similar in adjacency and in the absence of additional series use this assumption of equivalence to evaluate the reproducibility of neuron adjacencies, synapses and gap junctions. They then go on to define a set of adjacencies, synapses and gap junctions that are found in all 4 representations of each neuron and by modeling support the hypothesis that these sets represent largely conserved sets and most of the conserved connections. They then used these reproducible sets to evaluate the organization of the neurons within the neuropil. By a two-layered clustering approach, they find 5 bundles of neurons, ordered along the AP axis of the worm. By examining the neurons in each bundle, they label the bundles, using a mixture of anatomical and functional terms. Different types of sensory input enter the ring from the anterior and posterior, whereas interneurons are located in the more central bundles. They then classify each of the neurons by the contacts made, within bundle, boundary and trans-bundle neurons, recognizing that the trans-bundle neurons are particularly well-positioned to link different portions of the neuropil. Finally, they use the various levels of organization they have found to propose a 3-layered brain map, with sensory input at the top layer, with local integrations of the sensory information in layer 2 and finally with integration across the different inputs in layer 3 before providing output to the muscles of the worm.

General Comments
This paper represents a major step forward in understanding worm neurobiology. By creating a digital, volumetric representation of the neuropil, they and others (there is a related paper from other groups working with the same segmented data set) can now examine relationships of neurons to each other in ways not possible from synapse and gap junction information alone. Using their resource they reveal basic levels of organization that help us understand how the organization of the neuropil on multiple levels allows the integration of sensory input to produce worm behavior. It summaries a huge amount of work, makes important inroads into understanding of the worm "brain" and will be of wide interest.

There are some concerns, however. Foremost is their division of the neurites into bundles. As the authors state in the methods "it should come as no surprise that different algorithms will impose slightly different community partitions onto the network." Comparing the partitions in this work with those of Moyle et al (BioRxiv, doi: https://doi.org/10.1101/2020.03.15.992222), the "anterior" bundle of this work contains almost exactly the same neurons as stratum 1 in the Moyle work; their avoidance bundle is contained entirely within stratum 3; and many of their lateral bundle is contained within stratum 2 and others are unassigned. But their taxis bundle is split between strata 3 and 4 as well as unassigned and their sublateral bundle is split between S2 and S3 as well as unassigned. The exceptions in anterior (RMD) and avoidance (BAG) bundles may be instructive. In the force-directed layout map RMD is centered in the midst of the lateral bundle neurons and BAG is outside the main cluster of avoidance neurons. Their assignments in Moyle are more consistent with the apparent topology in this layout. The methods used to partition the neurons differ (I don't know which is better), but a simple-minded interpretation of the comparison of the two results might be that, while the neuropil clearly shows an anterior to posterior organization as well as a radial one, there are only three robust partitions. It would be nice if the different groups could agree on a common set of partitions (and nomenclature), but more likely as the authors state, the complexity may not readily resolve into clear bundles. If so, the authors should be more candid about the limits of their analysis, not just bury their skepticism in the methods, but deal with it openly in the main text. A resultant question is, what is the meaning of boundary neurons, if the boundaries are different in different attempts at partitioning? Are boundary neurons really different from intra-bundle neurons? Or simply an artifact of classification? Perhaps, there are better ways to describe the differences in neurons, such as the number of different neurons contacted, or spatial coherence of those contacts.

A second concern is that the manual segmentation is treated as correct. Given the challenges of defining the boundary of a cell where the plane of the membrane is oblique to the EM slice and other possible problems is this a fair assumption? In the methods the authors point out that given the areas of contact under consideration, compared to the contribution of any one section, any such problems will likely be ironed out. Is it worth resegmenting a region to see how reproducible it is? Perhaps, I am needlessly concerned.

Other concerns center on the adjacency/neighborhood analysis. The authors should offer some clearly stated reasons for their emphasis of adjacency over synapses and gap junctions, which are presumably the functional endpoint. On the use of adjacency to show that bilateral symmetry of homologous neurons extends to the nanoscale, isn't the principal question the extent (total area of contact) to which those neurons contact specific other neurons in similar locations? Eventually the authors show that large contact patches are conserved between specific neurons, but the issue of the location of those interactions didn't seem to be addressed. Preservation of contact location might have implications for how the contacts are established. Finally, beyond using the contact area to eliminate small, less reproducible contacts, it is unclear if the authors used the total area of contact to weight contacts in the further analyses.

The construction of the brain map is interesting but beyond my expertise. I would note that they speak of a three layer map in the text, but Figure 4 refers to a "4-layer map of synaptic information flow in the nerve ring". Since the fourth layer is muscle and is outside the nerve ring, the authors just need to be consistent. The section also has more jargon, e.g. "fan-in", "small world".

Specific comments:
l.19 It might be good to add "of 300 and 400 sections respectively" to give readers a sense of the scale of the undertaking.
l.29 the loop in IL1 looks different. Which pair of IL1s is shown?
Fig 1 and Fig S1 While Fig S1 is labeled as from the L4 series, I can only assume by default that Fig 1 is from the adult, although it is perhaps the L4 without superficial neurons removed. In any case comparable representations of the two volumetric reconstructions would be helpful. And given

how different the two representations look, some comment is appropriate.
l.29 Simple neighbor size seems a meaningless measure as the authors themselves state. See general comment
l.38 "the conserved degree (axon contact sites in Aδ occur in $1 \leq \delta \leq 4$ datasets)" since "conserved degree" is not used again, why simply say "where δ represents the number of datasets in which the contact site occurs".
l.82 Reference?
l.89 Is there a list of the neurons assigned to each bundle? If not please provide one. If so, please reference.
l.114 Again, is there a list somewhere of the neurons in these different classes? How were the transbundle neurons assigned to a specific bundle? The Moyle et al paper had an unassigned class, which makes some sense.
l.117 and elsewhere. Is axon the best term here? Why not the more neutral term "neurite"?
l.119 "consistent with this" What does "this" refer to?
l.126 Again is there a list of these neurons?
l.204 "large number … present" > large number … presents
Fig 4 Where is SIBD? Some single neurons are stated to be omitted, but SIBD is symmetric

l.255 to generated > generate
l.264 resulted primarily from poor segmentation -- raises issues about segmentation fidelity

l.326 "Intra-bundle contact" might be better titled "bundle contacts"
l.343 356 is not 2*173.
l.348 RMF is another indication that there are errors.
l.368 "but make" > but makes
l.368 "a … connections"

Fig S1 Why are there major discontinuities in moving A to P? This figure looks quite different from Fig 1. Why?
Here and elsewhere the supplemental figure legends have various errors. I highlight just a few I caught.
In the figure itself one neuron is labeled ILV? What is this? A typo? Also, I suspect non-worm readers will be confused by the IL1, IL1D, IL1V etc., terminology, not realizing that these are distinct pairs. Perhaps a brief explanation of terminology would be helpful.
"make each axons" > axon Can the authors speculate as to why White underscored scored here?
Fig S4 This portion of S4 legend needs work: "… for low, medium and high axon contact groups, as measure by the Jaccard index. on the survival distribution of surface area contact. (b) Survival distribution …"
Fig S11 eddges > edges

Author Rebuttals to Initial Comments:

Note: Author Rebuttals in RED

We thank the reviewers for their extensive comments. Below, we include our replies, in red. We also refer the reviewers to a summary of changes we have included and two versions of the resubmission – one with changes marked (in red) and a second, clean resubmission.

Referee #1 (Remarks to the Author):

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on which the original connectome was based (ref 5). These sections have been recently re-scored by the authors and an updated connectome published (ref 3).

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1. The presence of a synapse is inferred from images seen on EMs of serial sections. Unfortunately, there are rarely any post-synaptic specialisations visible at putative chemical synapses in C. elegans. Whilst many synapses are nevertheless unambiguous, there are cases, particularly with polyadic synapses, where there are varying degrees of uncertainty as to the identity of all post-synaptic partners. Gap junctions, because of their symmetrical configuration, do not have this particular problem. However, there are still uncertainties in some cases, particularly when two membranes from closely adjacent neurites are closely apposed but there is little increase in stain visible in these regions. These problems lead to an inevitable level of subjectivity in calling synapses as was explained in the original connectome reconstruction (ref 5). This limitation in synapse identification has to be acknowledged particularly when discussing connectivity data. It is striking that the authors called significantly more synapses than the original reconstructions when the synapses on the same animals were rescored (ref 3). Recently a non-peer reviewed reconstruction of a different adult nerve ring (Witvliet et al. (2020) bioRxiv preprint) described a similar number of chemical synapses to that described in the original. There are insufficient data to determine which one of these reconstructions is more “correct”. This situation therefore behoves authors drawing conclusions from connectivity data to clearly state caveats when interpreting these data.

Reply: We thank the reviewer for bringing these issues up and for their insistence that we make clear the limitations for our dataset. We agree with the reviewer that there is some level of subjectivity when scoring synapses (particularly polyadic synapses) and gap junctions from EM. The questions raised by the reviewer – regarding polyads, gap junctions and the significant increase in synapses scores – were previously addressed by Cook *et al*. (Ref. 3), which is why we did not revisit those issues here. However, we agree with the reviewer's point that while these scoring issues may not have been critical to any biological conclusions drawn in Ref. 3, they have the potential to affect our conclusions about the variable circuit in the current manuscript. We now acknowledge these limitations in the manuscript as well as perform additional validation tests to address the contribution of few-EM section synaptic scores and polyads, as well as comparisons with White *et al*. and with Witvliet *et al*. datasets (details below).

We have confidence in our gap junctional annotations, but acknowledge a likely higher rate of false negatives (missed, small gap junctions) that may be functional. The small numbers preclude an analysis that is as extensive as the one we present for synapses, but the general principle of a core circuit embedded in a variable background appears very robust to any small error rates. We again refer the reviewer to the additional analysis (details below).

2. The potentially interesting conclusion that the neural connectivity can be described as a core circuit that is embedded in a variable background lacks credibility given the failure to acknowledge the inevitable level of ambiguity in calling synaptic contacts - it could be that the variability is due to miss-called contacts. It is striking that in Fig. 2a the A1 is smaller than A4 whereas C1 is larger than C4 and G1 is larger than G4. This to me is suggestive of over enthusiastic calling of chemical synapses and gap junctions.

Reply: There are multiple points here that we wish to address:

1) the credibility of our scoring by comparison to other datasets;
2) the contributions of admittedly more subjective scoring of small (in #EM sections) synaptic and gap junction contacts;
3) the contribution of admittedly more subjective scoring of polyads,

And consequently (combining 1-3):

4) the sensitivity of our results on the core and variable circuits to the above scoring issues.

To ensure our results are robust to the details of the annotation, in the first submission, we already attempted to minimize risk of false positives (or over enthusiasm, in the words of the reviewer) by

- Excluding small membrane contacts <0.4 μm^2. With respect to the specific comment on the low A1 count (now denoted M1) in Figure 2a: this is in part due to eliminating these small membrane contacts from the analysis (comprising <2% of membrane contact area but 35% of the count of membrane contacts; in this way, we also excluded ~35% of the least reproducible, C1, synaptic contacts (Figure S5)).

(Incidentally note also that Witvliet *et al.* G1 count > G4 count among our A4 (M4) contacts).

- Limiting the synaptic analysis to neurons with reproducible membrane contact (previously denoted A4, now M4, with validation on less reproducible membrane contacts in the Supplementary materials, Figure S8); and
- For this restricted set of synapses, comparing models for synaptic contacts with membrane contacts below and above average size (updated Figure S7).

To additionally address these points, we have now performed a number of new validation tests using the same model framework (see updated Figure S7):

- We fit our model to a dataset where 1 EM section synaptic contacts have been removed.
- We fit our model to a dataset where non-reproducible polyadic synapses (all polyadic targets appearing only in C1) have been removed.
- We fit our model to the original score by White *et al.* (1986) connectome (limited to our A4 contact sites, now called M4, to allow comparison with our Figure 2).
- We fit our model to the two adult synaptic reconstructions provided by Witvliet *et al.* (2020) bioRxiv preprint (again, limited to our A4/M4 contact sites), to allow a comparison with our Figure 2.

It is not surprising that the number of synaptic contacts found by White *et al.* (Ref. 5) is much smaller, nor that the ones "missed" (or additional ones scored by us) tend to be smaller (spanning fewer EM sections). This might be in part due to different scoring criteria, but more likely to be because White *et al.* simply did not have access to digitized images. For example, White *et al.* also scored synapses that appear in only 1EM section (in our volumetric scoring). More generally, while the number of EMs tends to correlate with how reproducible the contacts are (Figure S10, previously Figure S23), synaptic contacts with few EM sections in White *et al*. (using our volumetric reconstruction), are distributed across C1-C4 contacts. This also applies to synapses we scored in addition to those of Ref 5 – additional small or few-EM synaptic contacts are evenly distributed across C1-C4 (see Figure S10). Therefore across scoring methods, some few EM synaptic contacts are highly reproducible, while most are variable.

Next, fitting our model to White *et al*. synaptic contacts, we find qualitatively similar model fits. Therefore the interpretation of a substantial variable circuit is robust to the difference in scoring between White *et al*. and Cook *et al*. The same cannot be said for White *et al*. gap junctions (we were not able to reliably fit our model there, suggesting, if the model applies to gap junctions, that some variable gap junctions were missed by White *et al*.).

We cannot assess specific scoring criteria for Witvliet *et al.*, as we have no access to their volumetric data. We can, however, compare the actual synaptic contacts scored.

- Fitting our model to Witvliet *et al*. synaptic contacts (as above, on our set of A4/M4 membrane contacts), we find qualitatively similar model fits. The same is true for gap junctions. Therefore the interpretation of a substantial variable circuit is robust to the difference in scoring between these diverse datasets.
- All but one of our C4 contacts were also scored by Witvliet et al. (including 107/108 contacts with <5 EMs).
- To address false positives, we computed the fraction of synaptic contacts (on A4/M4 membrane contacts, i.e., the set of membrane contacts in Figure 2b) that would be expected to be 'absent' in two new animals (assuming the same scoring criteria were

applied and assuming the membrane contacts were perfectly reproduced in the 2 new animals, see new Methods subsection on validation). We find that Witvliet *et al*. missed only ~50 contacts more than the average number predicted by our model. Given the differences due to variability in process placement, the fact that Witvliet *et al*. reconstructed 2 adults (as compared to one L4 and one adult in our dataset) and the parsimony of our model, we find that this level of agreement provides confidence that the differences in scoring do not alter any of our conclusions.

When we eliminate all 1-EM synaptic contacts from our dataset, our C1 count drops well below that of Witvliet *et al*. And yet, the model still holds and still predicts a substantial variable circuit. The same holds true if we remove variable polyads.

Putting all these validations together, we conclude that while specific model parameters may vary across these different datasets, the key result of a variable and core component consistently shows up across all tested datasets (see updated Figure S7 and validation section in Methods).

Ultimately ambiguities in chemical synapses might be resolved when there is a knowledge of a match of receptor and transmitter between the partners.

Reply: Indeed, in the absence of functional or physiological data, all structural connectomic studies can do at this stage is the validation across data sets and scoring criteria, which we have now done.

For gap junctions, it would be worth appending a confidence level for each contact based on the visibility of the structure in the EM images.

Reply: We are not aware of a way of doing this that is not itself subjective. See however validations in the updated Figure S7.

I would be interested to know how the additional synaptic contacts scored when the authors re-called these two datasets distribute between core and the variable contacts.

Reply: We believe the comparison with White *et al*. and Witvliet *et al*. addresses this request. Indeed, the reviewer's intuition is correct: when we consider more conservative datasets (either by restricting our data set or by analyzing datasets scored by others), we do find that the number of variables contacts decreases (Figures S10, S12). However, the main result of a reproducible, core circuit, embedded in a variable component still holds (see above). In fact, the variable circuit is estimated to be >50% across all datasets (even after suppressing small synapses to a level well below the fraction annotated either by White *et al*. or by Witvliet *et al*.). Our basic conclusion from these additional tests is that imposing a cutoff for synaptic contacts based on size affects the estimated "fraction" of core versus variable synapses.

Therefore the exact numerical value of that fraction (model parameter, *f*), while useful as a rough measure, and for comparisons within the same dataset (e.g., between inter- and intra-bundle synapses), should not be interpreted as a precise quantitative prediction. (And in fact, we had been careful not to over-interpret the fraction in our initial submission.) We have made sure to be conservative in our wording in the manuscript.

3. I do agree with the authors on the demarcation of a core circuit as one that includes only C4 and G4 synapses. This is a fairly unambiguous circuit and, as such, should be focussed upon by investigators who are trying to model behaviour or developmental mechanisms. One of the most interesting figures in this MS for me is buried in the supplement (S23) where it is shown that the size distributions of C4 and G4 synapses are striking larger than those of the 3, 2 or 1 cases. There could well be a separate variable component, but it would be less than that claimed due to the inevitability of a level of false positives in synapse calling.

Reply: We are glad that the reviewer agrees with our choice to focus on C4 and G4 contacts for purposes of a reference core connectome. However, to be clear, while our analysis predicts that C4 and G4 synaptic contacts are almost certainly part of a core circuit (~99% likelihood), the C4 and G4 themselves do not represent the complete core circuit. This can be seen by comparing our contacts with the new connectomes of Witvliet *et al.* (2020). Almost all of C4 contacts in each of the respective datasets was also scored in the other dataset. However, the C4 contacts in one dataset can appear as C3, C2 and (rarely) even C1 synaptic contacts. Thus, core synapses can present as variable. Conversely, some C1 and C2 contacts scored in one dataset are scored as C4 in the other dataset. Thus, synapses that present as variable in one dataset may actually be part of the core circuit. We now detail this level of nuance within the supplementary materials. Our model and analysis are not intended to capture all of this level of nuance, but rather the fundamental new result about the core and variable circuit, and for this purpose, C4 is a good reference core and C1-C2 provide a first approximation for the variable circuit. We even use the model to predict how many reconstructions would be needed to fully disambiguate the core and variable components.

Returning to Figure S10, we expect that the measurement of size via number of EM sections adds power to the analysis but breaks down when we get to the smaller synaptic contacts which can be real, functional, variable or reproducible. Furthermore, we caution that the number of EMs does not have a direct biological interpretation. We conclude that while we cannot ascertain the functionality of individual synapses, nor would we be justified in imposing a strict cutoff, e.g., eliminating all small synaptic contacts from our analysis. Indeed, excluding all small synaptic contacts risks losing important information about some functional (even if more likely variable) synapses that may carry significant information flow in *C. elegans*.

4. Although I am enthusiastic about the notion of a core circuit defined by connections thas the t exist in all four symmetrical regions, I was surprized that the authors did not use other information that may imply a strong synaptic connection. Some chemical synapses are large structures in the EM but are only seen single at the point where two processes may cross. Some gap junctions are much

more obvious than others. It would be good to incorporate a measure of the "strength" of a class of synaptic connection as determined by its multiplicity and/or size.

Reply: We are glad that the reviewer is enthusiastic about the notion of a core circuit. With respect either to membrane contact area or EM section count, this is addressed in our replies above. To reiterate: we found that these are not great predictors of core and variable contacts. While it is possible to define a threshold (~5 EM sections) above which synaptic contacts are likely to be core, there is no biological interpretation to this threshold and it will break down for smaller functional synapses. Furthermore, imposing such a threshold would neglect synaptic contacts across C1 to C4 for which – based strictly on size –would be impossible to discern as core/variable. We expect the same to be found with respect to multiplicity of synapses (because multiplicity correlates with the number of EM sections). As previously acknowledged, really the only way to move forward is from additional connectomes. We believe that our methodology will be useful in addressing these questions as additional connectomes and volumetric reconstructions become available.

That said we stress that we do use the membrane contact area in modularity analysis (to generate Figures S11-S12, to obtain bundles); for visualization (arrow thickness in brain map, Figure 4b and associated figures including a new figure – S21); and for validation (reproducibility analysis broken down into small/large membrane contact area in Figure S7; eliminating 1 EM section synapses also in Figure S7; and the statistics of C1-C4 as a function of membrane contact area in Figure S10, previously S23).

5. The original reconstruction the nerve ring as consisting of three layers (ref 5). Recently an un-peer reviewed study clamed that using cluster analysis the nerve ring could be divided into four layers (Moyle et al. (2020) bioRxiv). In this study, a different cluster analysis algorithm has been used to show that the nerve ring is a five-layered structure. I am yet to be convinced that efforts to divide the nerve ring up into ever smaller domains is a worthwhile exercise. To then go on and characterise neurons as being intrinsically different depending whether they lie in a domain interior or on a boundary strikes me as like building a house of cards. All the neurons in the nervous system have been reconstructed in three dimensions and their visible synaptic connections annotated. This is essentially a complete EM anatomical description of the structure of the C. elegans nervous system. I am not convinced that trying to chop this structure into arbitrary domains based on adjacency is a worth-while exercise. Indeed, giving names to these domains that imply function could eventually prove to be misleading.

Reply: We appreciate the reviewer's skepticism that the nerve ring cannot be subdivided into ever smaller organizational units. We query, however, the claim that White *et al*. (Ref 5) divided the nerve ring into 3 layers. We note that process bundles are indeed referred to but those are not numbered to our knowledge. We would like to address two separate questions: first the importance of roughly parallel process bundles, including our methodology for

obtaining them and the results on single, boundary and trans-bundle processes. The second concerns the comparison with Moyle *et al*.

We fully acknowledge that different clustering methods would give rise to slightly different classification of bundles (in numbers and in the assignments of specific neurons). Nor are we motivated to increase subdivisions of the nerve ring. Our approach, here, was based on spatial adjacency. We have gone to some effort to enhance robustness, eliminating the smallest contacts, and selecting only the most reproduced A4 (M4) contacts for the analysis. We also checked for robustness of our results (eliminating the anterior loop for example). The results clearly indicated that some neurons stay in the same neighborhood along the entire process, whereas others do not. This basic observation was already made (and described, e.g. for AIB, in great detail) by White *et al*. (1983). Having now revisited that paper, we suppose that it might have been precisely this neighborhood organization that led White *et al.* to use the term 'process bundles'. However, without a complete volumetric reconstruction, that paper stopped short of any enumeration of bundles, or classification, or any systematic or quantitative analysis of neighborhoods, bundles, or process types in the nerve ring. We would argue that our results are therefore not inconsistent with White *et al*. and also completely agree with the Reviewer that the specific number of bundles is not an end in itself.

In revisiting our modularity analysis, we noticed that trans-bundle neurites slightly mask the bundle organization because, by spanning different domains across the nerve ring, they bias and obscure the spatial clusters of nerve ring processes. By removing the trans-bundle neurites from the analysis and then repeating the modularity analysis, the bundle organization is more discernible (new Figure S17). Specifically we find that the taxis, avoidance, and anterior bundles are clearly separate and each contains both single-bundle neurons, and boundary neurons separating between them and other spatially distinct bundles. While the laterals and sublaterals are clearly distinct from taxis/avoidance/anterior bundles, the analysis does show some overlap. We believe that this is because sublaterals comprise mostly of boundary and trans-bundle processes yet have few synaptic connections. Thus, while they are both spatially and developmentally distinct, they are not distinct in their synaptic connectivity. Therefore for information processing purposes (in particular for the brain map in Figure 4), we now treat them collectively. We thank the reviewer for pushing us to revisit this point.

Our detailed classification of bundles was previously in the online spreadsheets and we had provided the breakdown of membrane contacts per cell class. To ensure this is more accessible and transparent, we have now

- Included more explanation in the Methods.
- Included a table with a step by step delineation of the protocol we followed to assign the bundles.
- Included an updated spreadsheet in SOM 4 following the step by step assignments for each cell class.
- Updated the membrane contact distributions to show the breakdown of membrane contacts into each bundle.

Finally, we would like to clarify that the classification of single/boundary/trans-bundle processes is not derived from whether the trajectories lie in the center or boundary of a domain, but, in most cases, from the distribution of membrane contacts across different neighborhoods. These results were extensively validated by repeating the analysis for different bundle assignments. A small

number of neurons can indeed be classified differently (and those are noted in the spreadsheet we included, see SOM 4), but all our results are robust to such re-assignments. Importantly, we found that subjectivity in the bundle assignment or process classification does not apply to the vast majority of processes (we refer the reviewers to Table S4 and to the membrane contact distributions provided in SOM 5). As an exception to this, merging sublaterals and laterals leads to 7 boundary neuron classes being reclassified from boundary to single-bundle (ALN, AVK, RIG, SAAD, SDQ, SIAD, SMDD). As those neurons carry few synaptic connections, and 5 of the 7 classes (all but RIG and SMDD) lack synaptic or morphological specializations along their trajectories, they indeed fit better with our definition of single-bundle processes.

With respect to Moyle *et al*., it is difficult for us to comment as they use both a different methodology, and different pre-processing of the data (we do not normalize or use dimensionality reduction techniques on the raw data, nor did they apply any of our processing steps. However, we note that

1) 16 of their processes are "unassigned" which makes a direct comparison difficult.
2) Reviewer 2 has pointed out overlaps and differences between our bundle classification and the strata of Moyle *et al.* (see our reply below). We now acknowledge this in the manuscript as well.

Put together, the distinction between 3 (if avoidance and taxis were merged, as well as if laterals and sublaterals were merged), 4 (with laterals and sublaterals merged only), or 5 distinct bundles is not a key result in our minds, and is open to interpretation, but the spatial organizational concept, including these basic regions, and the existence of different process types with different information processing roles supported within this organization are both important and novel contributions. These two results (the finding of spatially distinct neighborhoods that are organized into parallel bundles; and the finding of different process types) hold independently, and together, we believe they provide a very compelling description of the spatial organization of the nerve ring.

6. The authors fail to explain why they think the core connectome can be described by a residual learning network. These networks have to do with AI strategies for learning and image classification. I fail to see the relevance of these networks to the core connectome from the authors' narrative. While I can understand the motivation for trying to understand the connectome in terms of computational structures that have been developed in AI, I think this could be dangerous. The connectome is what it is - it was not designed by data scientists. We are rapidly obtaining knowledge of the dynamic activity of neurons when animals are performing particular behaviours and their individual repertoires of neurotransmitters and receptors. Computer modelling will have an important role to play when sufficient information has been obtained about the nature of the interconnections (both focal and humeral) between all the component neurons. I am less enthusiastic about network analysis, but it might have a role.

Reply: We agree with the reviewer that the connectome cannot be understood in terms of AI structures, nor can the brain be treated as an engineering design, nor neurons as logic gates. We do not believe that we have done that. We also did not obtain the brain map from network analysis. Rather, we use the insight gained from the preceding results to postulate the layered structure and find that the strong alignment with bundles provides a framework for

visualizing circuit architecture and information flow in the nerve ring. We have reworded the relevant sentences to reflect this.

7. The Jaccard index described on line 677 is incorrect. The authors appear to have confused the symbols for set union and set intersection. Also on line 684.

Reply: We thank the reviewer for this level of detailed review. This is now fixed.

8. I found some of the figures and their legends hard to figure out. For example, Fig. S3 legend "Neighbourhood size (…) does not vary with neighbourhood size" ??

Reply: We apologize for this oversight. This now reads "Variability in neighbourhood size (…) does not vary with neighbourhood size".

9. Maybe I missed it, but what is a "survival distribution"?

Reply: We apologize that we omitted this definition. The survival distribution (or reverse cumulative distribution) for some dataset $\{x(i)\}$ plots the fraction of x that 'survives' (i.e., larger than) a particular value of x. We now include a definition in the methods in the relevant figure captions.

10. I could not understand how the adjacency data were normalised from the narrative. This made it difficult from me to evaluate Fig. S6.

Reply: The normalization is a standard way of taking non-normally distributed data and mapping them to a zero-mean normal distribution. This is only used in the supplementary materials (not in the main paper) for two purposes;

1) For ease of visualizations, when plotting survival distributions (Figures S4, S6).
2) For determining a meaningful "average" in order to separate "above average" and "below average" membrane contact areas (updated Figure S7).

We now add a brief explanation of this normalization to the relevant figure caption (same Figure).

11. I don't like the use of the word axon throughout the text to describe any neuron process. Axon generally implies a pre-synaptic process, which is not necessarily the case. Neurite would be a better word to use.

Reply: The reviewer is correct. Where appropriate, 'axon' has now been replaced by 'process' (following terminology by White *et al*.) or by 'neurite' (typically in a developmental context).

12. The use of 10 point rather than the usual 12 point font for this MS made reviewing this study less enjoyable than it might have been.

Reply: To aid readability the resubmitted manuscript is now in 12 point font, as is this reply.

Referee #2 (Remarks to the Author):

The manuscript by Brittin et al. segments more than 700 legacy EM images of the C. elegans nerve ring to create for the first time a volumetric reconstruction of what is effectively the worm brain. Although the worm brain with only 181 neurites is very simple compared to the brains of other organisms, its complexity has frustrated deeper understanding for decades. To create new avenues of understanding the authors have segmented the legacy EMs of White et al. from L4 and adult animals and used those results to generate a space-filling model of the neuropil. They show that left-right versions of homologous neurons are similar in adjacency and in the absence of additional series use this assumption of equivalence to evaluate the reproducibility of neuron adjacencies, synapses and gap junctions. They then go on to define a set of adjacencies, synapses and gap junctions that are found in all 4 representations of each neuron and by modeling support the hypothesis that these sets represent largely conserved sets and most of the conserved connections. They then used these reproducible sets to evaluate the organization of the neurons within the neuropil. By a two-layered clustering approach, they find 5 bundles of neurons, ordered along the AP axis of the worm. By examining the neurons in each bundle, they label the bundles, using a mixture of anatomical and functional terms. Different types of sensory input enter the ring from the anterior and posterior, whereas interneurons are located in the more central bundles. They then classify each of the neurons by the contacts made, within bundle, boundary and trans-bundle neurons, recognizing that the trans-bundle neurons are particularly well-positioned to link different portions of the neuropil. Finally, they use the various levels of organization they have found to propose a 3-layered brain map, with sensory input at the top layer, with local integrations of the sensory information in layer 2 and finally with integration across the different inputs in layer 3 before providing output to the muscles of the worm.

General Comments

This paper represents a major step forward in understanding worm neurobiology. By creating a digital, volumetric representation of the neuropil, they and others (there is a related paper from other groups working with the same segmented data set) can now examine relationships of neurons to each other in ways not possible from synapse and gap junction information alone. Using their resource they reveal basic levels of organization that help us understand how the organization of the

neuropil on multiple levels allows the integration of sensory input to produce worm behavior. It summaries a huge amount of work, makes important inroads into understanding of the worm "brain" and will be of wide interest.

There are some concerns, however. Foremost is their division of the neurites into bundles. As the authors state in the methods "it should come as no surprise that different algorithms will impose slightly different community partitions onto the network." Comparing the partitions in this work with those of Moyle et al (BioRxiv, doi: https://doi.org/10.1101/2020.03.15.992222), the "anterior" bundle of this work contains almost exactly the same neurons as stratum 1 in the Moyle work; their avoidance bundle is contained entirely within stratum 3; and many of their lateral bundle is contained within stratum 2 and others are unassigned. But their taxis bundle is split between strata 3 and 4 as well as unassigned and their sublateral bundle is split between S2 and S3 as well as unassigned. The exceptions in anterior (RMD) and avoidance (BAG) bundles may be instructive. In the force-directed layout map RMD is centered in the midst of the lateral bundle neurons and BAG is outside the main cluster of avoidance neurons. Their assignments in Moyle are more consistent with the apparent topology in this layout. The methods used to partition the neurons differ (I don't know which is better), but a simple-minded interpretation of the comparison of the two results might be that, while the neuropil clearly shows an anterior to posterior organization as well as a radial one, there are only three robust partitions. It would be nice if the different groups could agree on a common set of partitions (and nomenclature), but more likely as the authors state, the complexity may not readily resolve into clear bundles. If so, the authors should be more candid about the limits of their analysis, not just bury their skepticism in the methods, but deal with it openly in the main text. A resultant question is, what is the meaning of boundary neurons, if the boundaries are different in different attempts at partitioning? Are boundary neurons really different from intra-bundle neurons? Or simply an artifact of classification? Perhaps, there are better ways to describe the differences in neurons, such as the number of different neurons contacted, or spatial coherence of those contacts.

Reply: We appreciate the Reviewer raising these important issues concerning the partition of the nerve ring into bundles. We have identified 3 important points in the Reviewer's comment, which we address below:

1. The biological validity of our partitioning of the nerve ring.

2. Interpreting the roles of specific cells within a given framework.

3. Converging on a consensus among different partitioning methods.

The qualitative observation of process bundles dates back to White *et al.* (1983) but there, the use of the term bundles predominantly refers to extensions to and from the nerve ring and along the body, with only anecdotal descriptions of aligned processes/process bundles in the nerve ring itself, and no clear delineation or partitioning of those bundles. We are also not aware of other delineation or counting of partitions of the nerve ring since (until this study and the study of Moyle *et al.*). Clearly the absence of any physical separation between neuron

processes within the nerve ring makes identification of distinct bundles challenging, which is why we have resorted to computational methods.

Spatially, the 5 bundles that we see appear repeatedly for related NR-wide spatial analyses that we have run. To validate and gain insight into this partitioning, we detail how each cell fits within the partitioning as single, boundary and trans-bundle processes. In arriving at our classifications we considered a number of factors – membrane contacts between cells, process trajectories, spatial coherence between processes – which we now more fully articulate in the Methods and supplementary results. From precisely this detailed inspection of how processes fit within their and neighboring partitions, it is immediately evident that trans-bundle processes such as RMD and BAG can appear misplaced in simple graph visualizations. And yet, based on their process trajectories, we are confident in their bundle assignment. We now also repeat the cluster analysis without the trans-bundle processes (new Figure S17). Biologically, we are encouraged by the follow up study (Brittin *et al.*, 2020) which identifies 5 embryonic structures that appear consistent with the later development of our bundles, but we agree that this is just one view of a much more complex structure. We would not wish to 'bury our skepticism' within the methods. To better address the limitations of our partitioning algorithm in the manuscript itself, we now note the difficulty of partitioning the nerve ring in the discussion, and cite Moyle *et al.* for an alternative partitioning. Specifically we note in the Discussion that a more conservative functional partitioning would be into 3, merging the laterals with sublaterals (which we have now done for synaptic analysis) and Avoidance with Taxis (though we believe we have presented strong evidence to support a functional partition there).

One useful way to look at partitioning is to focus on determining the boundaries, and hence the boundary processes. The reviewer's observation that classification of processes is sensitive to the partitioning methods is correct. We find this is especially true for boundaries, and less so for single and trans-bundle processes. We have performed a number of checks, and for all those, the key conclusions of the paper are not affected by re-classifying the processes whose assignment is least certain. Nonetheless, this is precisely why boundary processes need to be laid bare for the broader scientific community to scrutinize. If bundles are more than an abstraction, then the boundary cells may provide a direct handle for testing any partitioning model. We therefore include all our classifications steps in Table 4 and the accompanying commented spreadsheet as well as data for accompanying methods (membrane contact distributions).

There are important distinctions between the methodologies in this manuscript and that of Moyle *et al.* Our methodologies integrate both a bottom-up (topological clustering of contacts) and top-down (consideration of overall process trajectories) approach. We (but not Moyle *et al.*) remove the anterior loops when classifying the bundles, identify and then manually check the assignment of trans-bundle processes (SOM 4, 5). We (but not Moyle *et al.*) also opt for robustness rather than completeness of raw data, by restricting our analysis to M4 contacts. We do not, however, perform any PCA decomposition of our data prior to clustering. These differences may account for some of the differences in our clusters or bundles.

In contrast to our clustering predictions, Moyle *et al.* argue that the papillary sensory processes mark the boundaries between some of their strata. However, from a developmental perspective, papillary processes grow into the nerve ring after pioneer bundles have already innervated the nerve ring, indicating that any papillary "boundary" is responding to already existing molecular demarcation between bundles. Also, as far as we can see from Moyle *et*

al., it seems that these papillary "boundaries" occur at a handful of discrete points and we do not see how they could account for the complete clustering of process bundles.

Taken together, we agree that it would indeed be nice if different clustering methods arrived at a unanimous partitioning, but we believe this is premature. Rather, and especially given the challenge and subjectivity of any clustering approach, we consider that collectively, our two studies demonstrate 1) a large level of agreement, pointing to common, clear organizing principles, and, at the same time, 2) the richness and subtlety of the spatial organization of the nerve ring. Ultimately, we take the view that models are most useful when they provide detailed predictions that allow future researchers to test, and potentially disprove, parts or all of the model. From this perspective we see no issue with different studies making disparate, even inconsistent predictions. Therefore, while we empathize with the Reviewer's frustrations that different groups are unable to converge on a single model of nerve ring organization at this time, we have discussed these matters internally, and feel that the broader scientific community would not benefit from our respective groups forcing a convergence prematurely. We feel that our partitioning model when paired with the brain map presents a distinct perspective on *C. elegans* nervous system development and function. Future experiments are necessary to determine the extent to which the details of our bundle classification are biologically meaningful.

A second concern is that the manual segmentation is treated as correct. Given the challenges of defining the boundary of a cell where the plane of the membrane is oblique to the EM slice and other possible problems is this a fair assumption? In the methods the authors point out that given the areas of contact under consideration, compared to the contribution of any one section, any such problems will likely be ironed out. Is it worth resegmenting a region to see how reproducible it is? Perhaps, I am needlessly concerned.

We appreciate the reviewer's concerns with regards to the quality of the segmentation which is the foundation of the entire study. First, we would like to speak more broadly to quality of the segmentation data which has been considered complete for over 3 years now (Brittin *et al.* (2018), biorxiv, https://doi.org/10.1101/485771). We have shared the segmentation data with other labs – indeed, Moyle *et al.* (2020) is based on our segmentation (membrane contact) data. We believe this is indeed an important resource for the community. Over this time, no gross errors have been uncovered and we find the adult and L4 segmentations are mostly consistent with each other. The one exception, which Reviewer 2 has pointed out below, are the RMF processes which have branching processes in the L4 but not the adult. However, these differences were originally noted in the supplemental sections of White *et al.* (1986). As such, these differences may very well reflect individual or developmental variability.

More specifically, the Reviewer raises 2 technical concerns – oblique slicing and other minor segmentation contacts in specific sections – which we address in turn.

Within the context of the entire reconstruction, missed information due to the plane of the membrane being oblique to the EM slice is considered very minor. For all cells, most of (in many cases the entire) process runs transverse to the EM slice, which makes identification of membranes straightforward. In general, we observe that only very small segments of a neurite are oblique to the EM slice. The main points where membrane contact information may be

lost due to the membrane being oblique are (i) at the dorsal midline of the nerve ring commissure and (ii) for a few processes that exhibit a looping/turning morpholog (e.g., RIA and SIAV). In these instances, it is possible that an oblique process could abut a neighboring process in a subsequent EM section. However, we observe that such contact areas are extremely small – much smaller than the contact thresholds used in this study.

As we stated in the methods, minor errors either occur from process segmentation not being extended fully to a neighboring process or erroneously being extended to a neighboring process. We do not believe that these minor errors adversely affect the analysis for the following reasons:

1. As validation we have manually scored 2 EM sections and found that the automated scoring outperformed the manual scoring (see Methods). Additionally, errors observed for a process in one EM section are not generally observed in subsequent EM sections.
2. It is easy to scan through the EM stack and visually identify errors. The segmentation has been shared with other labs over the past few years and a handful of minor errors have been addressed through this process.
3. We have designed our analysis so as not to be sensitive to minor errors in segmentation. By setting a conservative membrane threshold (0.4 μm^2), we are confident that we also suppress any (or almost all) noise due to segmentation errors.

Other concerns center on the adjacency/neighborhood analysis. The authors should offer some clearly stated reasons for their emphasis of adjacency over synapses and gap junctions, which are presumably the functional endpoint. On the use of adjacency to show that bilateral symmetry of homologous neurons extends to the nanoscale, isn't the principal question the extent (total area of contact) to which those neurons contact specific other neurons in similar locations? Eventually the authors show that large contact patches are conserved between specific neurons, but the issue of the location of those interactions didn't seem to be addressed. Preservation of contact location might have implications for how the contacts are established.

This is a valid point. We have added a number of localization panels to Figure S3 to address examples and statistics of reproducibility of membrane and synaptic contacts at the nanoscale, with respect to their location along the process. First, we analyze reproducibility of membrane contacts for different resolution of "bin sizes" along the AP axis (full data available online, SOM 2). For high spatial resolution (bins sizes ~0.7 µm), there is ~50% rate of a contact occurring across at least 3 of the 4 datasets per bin. Bins increased to about 1.4 µm increases this rate to ~65%. That said, most M4 cell pairs share at least 1 bin along the AP axis in which all 4 contacts are observed. We then repeated this analysis for synaptic localization (only C4 on M4 data). On average, the chance that 3-4 synaptic contacts will form in a specific conserved membrane contact location is low (slightly over 50% for bins sizes of ~1.4 µm, diminishing quickly for smaller bin sizes). And the chance that a C4 contact will co-localize (in 3 or 4 datasets) at least once along the 4 processes is also much lower than for membrane contacts. The most parsimonious interpretation seems to be consistent with our approach in the paper: membrane contacts are highly constrained spatially and therefore tend to be

more conserved. Within the range of existing membrane contacts, the specific locations of most synapses (including at least a large proportion of conserved ones) do not appear to be conserved at the micron/sub-micron resolution, though it is likely that many are constrained to form on specific branches or spatial domains along the process. We now mention this in the text.

Consistently with the above, our emphasis remains on adjacency and this is rooted in the simple realization that spatial adjacency is the constraint for a synapse forming. As the consensus membrane adjacency set is much more likely to be developmentally conserved, it is particularly useful, for identifying the core connectome, for studies of precision and specificity and for constructing the brain map.

Finally, beyond using the contact area to eliminate small, less reproducible contacts, it is unclear if the authors used the total area of contact to weight contacts in the further analyses.

The total area of membrane contact is used for clustering analysis, for membrane contact distribution analysis, for model validation and in visualizations of information flow. We have slightly reworded the methods and hope this is clearer now.

The construction of the brain map is interesting but beyond my expertise. I would note that they speak of a three layer map in the text, but Figure 4 refers to a “4-layer map of synaptic information flow in the nerve ring”. Since the fourth layer is muscle and is outside the nerve ring, the authors just need to be consistent. The section also has more jargon, e.g. “fan-in”, “small world”.

We have remove the reference to layer 4 (designating only as motor output). We have added a supplementary figure to describe and illustrate the network connectivity features/motifs such as fan-in and small world and interpret them within the framework of our brain map.

Specific comments:

l.19 It might be good to add “of 300 and 400 sections respectively” to give readers a sense of the scale of the undertaking.

Thank you. Done.

l.29 the loop in IL1 looks different. Which pair of IL1s is shown?

Figure S2 shows all members of IL1 class (IL1L, IL1VL, IL1DL and IL1R, IL1VR, ILDR). In the initial submission, perceived differences may have been due to slight differences in orientation between the right and left side. In the resubmission, we have tried to better orient the right and left sides so that the two sets of processes appear more similar. We have also clarified the caption.

Fig 1 and Fig S1 While Fig S1 is labeled as from the L4 series, I can only assume by default that Fig 1 is from the adult, although it is perhaps the L4 without superficial neurons removed. In any case comparable representations of the two volumetric reconstructions would be helpful. And given how different the two representations look, some comment is appropriate.

Both Figure 1 and Figure S1 (b) show the L4. Both figure captions have now been clarified.

l.29 Simple neighbor size seems a meaningless measure as the authors themselves state. See general comment

We include neighborhood size, first for completeness, and second because it was unknown that the variability is independent of size and seems even bilaterally. We agree this is not a key result.

l.38 “the conserved degree (axon contact sites in Aδ occur in $1 \leq \delta \leq 4$ datasets)” since “conserved degree” is not used again, why simply say “where δ represents the number of datasets in which the contact site occurs”.

Done.

l.82 Reference?

We now cite Ware *et al.* (1975) and Figure 1(a).

l.89 Is there a list of the neurons assigned to each bundle? If not please provide one. If so, please reference.

A spreadsheet with bundle assignments was provided with the initial submission. This spreadsheet (SOM 4) has been updated and referenced in the manuscript (now line 118).

l.114 Again, is there a list somewhere of the neurons in these different classes? How were the transbundle neurons assigned to a specific bundle? The Moyle et al paper had an unassigned class, which makes some sense.

We thank the reviewer for bringing this up. We previously made available a spreadsheet with justifications and accompanying membrane contact distributions that were used for this classification. We are sorry if the reviewers did not find the link to the online resources (see SOM 4 – unfortunately on line 113 of the previous submission we referenced the Supplementary Results but not the online materials). We now references the spreadsheet here as well (line 154). We differ with Moyle *et al.* on some unclassified cells (e.g. AWA). We also note (as the reviewer has noticed) that 9/16 unclassified neuron classes (AIB, AIZ, AVA, AVE, RIM, RIR, RIS, RMG, URX) are classified by us as trans-bundle processes. Because these processes are trans-bundle, they may indeed be more difficult to classify within Moyle *et al.*'s computational framework. We believe that these are useful insights achieved through our methodology.

l.117 and elsewhere. Is axon the best term here? Why not the more neutral term "neurite"?

This was not ideal. We now use 'process', following White *et al.* ('neurite' used occasionally).

l.119 "consistent with this" What does "this" refer to?

Thank you. Fixed.

l.126 Again is there a list of these neurons?

Yes, this was all in the spreadsheet. We now reference the supplementary spreadsheet (SOM 4, lines 174, 175).

l.204 "large number … present" > large number … presents

Done.

Fig 4 Where is SIBD? Some single neurons are stated to be omitted, but SIBD is symmetric

Figure 4 only displays C4 contacts. SIBD makes no C4 synaptic contacts, therefore it is not included in the brainmap. We have now reiterated in the caption that panel b only includes C4 contacts.

l.255 to generated > generate

Thank you. Fixed.

l.264 resulted primarily from poor segmentation -- raises issues about segmentation fidelity

Please see our response to Reviewer 2's second point and extended treatment in the manuscript.

l.326 "Intra-bundle contact" might be better titled "bundle contacts"

Thank you. Fixed.

l.343 356 is not 2*173.

We apologize for the oversight, this has now been corrected.

l.348 RMF is another indication that there are errors.

Differences in RMF between the adult and the L4 were originally noted by White *et al.* (1983). Our reconstruction is consistent with these previous observations.

l.368 “but make” > but makes

l.368 “a … connections”

Thank you. Fixed.

Fig S1 Why are there major discontinuities in moving A to P? This figure looks quite different from Fig 1. Why?

We thank the reviewer for raising this. We now explicitly note the differences between the figures. Specifically, Figure S1 shows the complete reconstruction volume and all processes, while Figure 1 is just the nerve ring with superficial processes removed. In Figure S1, we now added a box around the portion that corresponds the Figure 1. Discontinuities are due to points of spatial misalignment in the reconstruction. While these discontinuities affect the smoothness of the visualization, they do not affect the contact analysis because we are able to accurately trace the cell identities using White *et al.*’s markings within the EMs. In the updated Figure S1, we have tried to smooth out the discontinuity.

Here and elsewhere the supplemental figure legends have various errors. I highlight just a few I caught.

In the figure itself one neuron is labeled ILV? What is this? A typo?

Thank you. This is now fixed (IL1V).

Also, I suspect non-worm readers will be confused by the IL1, IL1D, IL1V etc., terminology, not realizing that these are distinct pairs. Perhaps a brief explanation of terminology would be helpful.

Thank you for the suggestion. We now open the Methods with a paragraph on terminology and nomenclature.

“make each axons” > axon

This now states: “To visually distinguish between processes, each is assigned a random color.”

Can the authors speculate as to why White underscored scored here?

We can only suppose that White *et al.* (1986) underscored membrane contacts because they did not have access to digitized images. There is also a bit of confusion in the literature as to whether only every 5th EM section was scored (Durbin, 1987) or all of them.

Fig S4 This portion of S4 legend needs work: "… for low, medium and high axon contact groups, as measure by the Jaccard index. on the survival distribution of surface area contact.

We apologize for the typos in this caption which we now fixed.

(b) Survival distribution …"

We now provide a definition of the survival distribution in the caption. See also our reply to Reviewer 1.

Fig S11 eddges > edges

Thank you. Fixed.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The revised manuscript resubmitted by Brittin et al. satisfactorily addresses concerns that I expressed in my previous review about the section of their study that deals with the variability of synaptic contacts i.e. those seen between symmetrical homologues in the same animal and between the same neuron class in different animals. The authors provide convincing arguments for the existence of a conserved core circuit embedded in a looser variable circuit. This obviously has ramifications for individualistic behaviours among isogenic animals. The inclusion of neighbourhood data in this study give useful indications of how much of this variability is due to structural stability (i.e. differences in the availability of putative synaptic partners) or intrinsic propensity to make connections to an adjacent partner. I find their simple mathematical model convincing and revealing of an important feature of the organisation of the C. elegans nervous system, one that

demands an eventual biological explanation. One simple addition to this study that could be made would be to list all process pairs that have significant adjacency (i.e. M4) yet make no synaptic connections in all four regions. This might be indicative of inhibitory interactions regulating synaptogenesis.

Unfortunately, the authors still fail to convince me that the use of cluster analysis on the adjacency data to define 5 layers is a worthwhile exercise or indeed valid. While I certainly agree that the nerve ring has three discernible layers as originally stated by White et al. 1986 (Fig 20 legend), I find the claims that cluster analysis of adjacency data reveals five unambiguous layers unconvincing. I might be more convinced had their applied their analysis to both the N2U and JSH datasets and got the same 5 domain classifications in both cases with no individual manual tweaking. Even more convincing would be if they could apply their analysis to the two adult animals recently reconstructed by Witvliet et al. (2020) and get the same result. I note that Moyle et al. (2020) recently tried a differing clustering strategy on the same datasets and came out with four rather than five domains with different partition boundaries. They at least did try to compare N2U with JSH but there seemed to be differences. I agree that the basic three-layer structure may have significant functional ramifications as the mechano-receptor circuitry is predominantly in the anterior layer whereas the chemo-receptor circuity is predominantly in the posterior layer. Also, recent studies have shown that the sublateral neurons in the center layer act as pioneers for ring neurogenesis indicating that the layered structure probably results from sequential directed pathfinding. Nevertheless, I am sceptical about the insights that further sub-divisions by cluster analysis might provide. I presume the authors hope to reveal hidden bundles, but this might be unnecessary as complete 3D reconstructions are now publicly available for 3 adult and several larval nerve rings. These data can be selectively visualised using computer graphic renderings. The graphics tools available are improving rapidly, so it is possible (or soon will be) to visualise a 3D rendering of a neuron and selected neighbours together with their synaptic contacts. Simple tools could be provided to provide adjacency values and synapse number. In this way the bundle organisation of the nerve ring will be made readily apparent to the investigator.

I urge the authors to consider cutting down their manuscript by eliminating their cluster analysis and to focus on the study of core/variable synapses that they have undertaken. I think this is an excellent study and would make a tight, focussed paper which has something significant to say about the organisation of the C. elegans nervous system - something which may prove generalizable to all nervous systems.

Referee #2 (Remarks to the Author):

The authors have largely addressed my concerns with their revisions. However, I would suggest that, rather than waiting until the discussion, when they first introduce the 5 bundles (l. 119) that they would be more forthright about the subjective nature of this division. Their new Table S4 makes clear the process involved, which is helpful, but it also makes clear the several subjective steps involved. And later in constructing their brain map (l. 189) they elect to merge two bundles and elsewhere acknowledge that the two more posterior bundles could be considered one. Also, while the removal of the transbundle processes in Figure 17b strengthens the division into anterior, avoidance and taxis bundles, the division of the laterals from the sublaterals looks even more arbitrary. Maybe something like "We find that dividing the processes into 5 groups is most consistent with the force-directed graph and the known biology of the neurons … " .

Minor items I happened to pick up:
Figure S13a and S17a both purport to be
"Force-directed layout of the conserved degree 4 (M4) set of membrane contacts in the nerve ring. Nodes and edges represent cell classes and membrane contacts, respectively."
And while the two representations are similar, they differ in important ways, e.g. the

interdigitation of lateral and sublaterals. What is different? Also, S13a lacks the legends present in 17a that explain the shapes and colors. Finally, S13a still labels the set A4, rather than M4.

l. 30 (see Methods).
l. 59 p should follow "connectivity" to maintain a consistent structure in the sentence.
l. 138 Should this be "opposing sides"?
l. 200-204. With nested parenthetical remarks, this needs a rewrite.
Fig. S17b "Trans-bundle"

Author Rebuttals to First Revision:

Note: Author Rebuttals in RED

We thank the reviewers for their constructive comments. Below, we include our replies, in red. We also refer the reviewers to a summary of changes we have included and two versions of the resubmission – one with changes marked (in red) and a second, clean resubmission.

Referees' comments:

Referee #1 (Remarks to the Author):

The revised manuscript resubmitted by Brittin et al. satisfactorily addresses concerns that I expressed in my previous review about the section of their study that deals with the variability of synaptic contacts
i.e. those seen between symmetrical homologues in the same animal and between the same neuron class in different animals. The authors provide convincing arguments for the existence of a conserved core circuit embedded in a looser variable circuit. This obviously has ramifications for individualistic behaviours among isogenic animals. The inclusion of neighbourhood data in this study give useful indications of how much of this variability is due to structural stability (i.e. differences in the availability of putative synaptic partners) or intrinsic propensity to make connections to an adjacent partner. I find their simple mathematical model convincing and revealing of an important feature of the organisation of the C. elegans nervous system, one that demands an eventual biological explanation.

Reply: We are glad that Reviewer 1 finds our extended analysis convincing. Our revisions to the manuscript focus on the spatial analysis of the nerve ring (addressing comments below), but are now tightly linked to these core-variable results. We emphasize that our clustering analysis is based on the core membrane contacts, and the brain map, on core synapses. These therefore follow directly from this section. We have also changed our clustering methodology in the same spirit: developing population models to ensure robustness, generalizability and amenability to extensive validation.

One simple addition to this study that could be made would be to list all process pairs that have significant adjacency (i.e. M4) yet make no synaptic connections in all four regions. This might be indicative of inhibitory interactions regulating synaptogenesis.

Reply: We agree that significant adjacency with no synaptic connections could be indicative of inhibitory interactions (captured phenomenologically in our model by the "specificity" parameter). In SOM 3, we now include a list that shows which M4

membrane contacts yield the requested contacts with no synapses as well as those with C1, C2, C3 and C4 synapses.

Unfortunately, the authors still fail to convince me that the use of cluster analysis on the adjacency data to define 5 layers is a worthwhile exercise or indeed valid. While I certainly agree that the nerve ring has three discernible layers as originally stated by White et al. 1986 (Fig 20 legend), I find the claims that cluster analysis of adjacency data reveals five unambiguous layers unconvincing.

Reply: We appreciate Reviewer 1's skepticism and are grateful to the reviewer for pushing us on this point, as they have led us to probe deeper and obtain new and stronger results. We grant that our previous multiple-step clustering approach was difficult to follow, and included a number of subjective choices. The comment speaks to a number points, which we address in turn: we must demonstrate the
(i) utility and (ii) validity of our cluster analysis; we should also (iii) communicate measure of uncertainty and (iv) avoid any implication of physically distinct layers where there are none.

(i) Utility. We have addressed this in a number of ways in the paper:

a. The core-variable section of the paper is not spatially grounded. This section provides this spatial grounding. We now make this much more explicit.
b. The core component of the nerve ring (membrane contacts, synapses, etc.) does not bring us closer to constructing an anatomically and functionally meaningful brain map (our ultimate aim, and one of the main challenges of systems neuroscience). The latter requires insight into organization across scales, and hence a quantitative understanding of the high level spatial organization of the nerve ring. Again, we believe this is much stronger and clearer now.
c. Our spatial analysis is the glue of the paper – bringing together spatial organization that supports the core circuit and hence the brain map. To bring this out more clearly, we now absorb this analysis into the first section, and present it as the transition between the first section on the core-variable analysis and the second section, on the brain map.

(ii) Validity. We recognize that the 5 clusters in our analysis are not easily discernible from visual inspection. Indeed quantitative analysis of complex data can be very sensitive to the input data. While we are confident in the validity of the 5 clusters because we have manually scrutinized the contact profiles and process trajectories of each cell (see Table S4 of previous submission), we agree that the validity of such an approach must be demonstrated, and we now do this using a population model framework (details below). This approach gives us confidence values and control parameters to validate our data, to compare the L4 and adult, etc. This new analysis leads to new insight and novel predictions at the population level (see below).

(iii) Uncertainty: The population models we developed are based on randomly perturbed membrane contacts (Figures 3 and S7). The noise distribution and amplitude are dictated by the data so we have no free parameters (i.e. no

subjectivity). Cluster assignments are now based on a consensus both within and among population datasets. For each population dataset (1000 individuals generated from the L4, adult and M4 datasets, each), we count how frequently 2 cell classes cluster together across a population. We have high confidence in cluster assignments of groups of cells that frequently cluster together and low confidence in cluster assignments of cells that do not frequently cluster together. A small number of neurons (7 cell classes) do not meet our stringent consensus criterion and we leave these neuron classes unassigned to any cluster, rather than forcing a cluster assignment. This spatially grounded population model approach allows us to identify spatial organization that specifies the core circuit.

(iv) Language and interpretation: As there are no physical separations within the nerve ring, naturally groups of cells have no distinct "boundaries", so perhaps our language was too strong. In line with this, we have softened our language with regard to the nerve ring organization. We have done away with the concept of "boundary" and "trans-bundle" processes which might wrongly imply a distinct physical separation between clusters. In fact we would like to clarify that modularity/community finding algorithms extract "modules" or "communities" that are defined as having more intra-group connections (edges of a graph) than among groups.

To avoid over-interpreting or over-fitting the data, we have limited our organizational claims about the neuropil to a narrower definition of spatial clusters: groups of neurites with high spatial affinity across a population of worms. Therefore, we use clustering to identify groups of neural processes with consistent (high frequency) spatial affinity. We have dropped all analysis of spatial separation between such groups.

We are very aware of the limitations of our data (with only 2 animals at our disposal) and the importance of general results that are valid across different animals. By adopting a population approach, and focusing on the core contacts, we now demonstrate consistency between the L4 and adult, and also predict the robustness of our clusters across the species.

I might be more convinced had their applied their analysis to both the N2U and JSH datasets and got the same 5 domain classifications in both cases with no individual manual tweaking.

Reply: We now use 1 step clustering, and our cluster assignment involves no individual or manual tweaking. We provide clustering results for population models of M4, N2U and JSH (Figure 3c and Figure 7). All population models grossly give the same 5 clusters: Indeed, there are only 7 cell classes with discrepant cluster assignment between the three population models and we do not attempt to assign these cells a cluster classification (though for the purposes of the brain map, Figure 4, we place unclassified cells in modules based on their process trajectories). We also perform several other validation tests where the noise amplitude is varied in the population model, including much larger perturbations than those empirically observed, as well as varying the spatial domain, i.e. the volume of the nerve ring over which the clustering is performed (Figure S7 and S8). The 5-domain classification is robust across all these validation tests.

However, we also note where our clustering fails: While robust for all M4 population models, as well as populations of bilaterally conserved N2U and JSH membrane contacts, clustering utterly fails when we consider M1-M3 datasets. Therefore, we infer that the most reproducible contacts provide the relevant data for uncovering robust organization of the nerve ring, whereas the variable contacts largely mask this organization. This also explains why 1 or 2 specimen (JSH, N2U) will somewhat differ, and why the 5 clusters, despite their robustness, are not easily discernible from the aggregate datasets (including all M1-M4 contacts). We now include this result in Figure S8.

Even more convincing would be if they could apply their analysis to the two adult animals recently reconstructed by Witvliet et al. (2020) and get the same result.

Reply: We reached out to Witvliet *et al.* for their volumetric dataset, but received no reply. Moreover, our understanding is that the Witvliet *et al.* volumetric data were extrapolated from neurite skeletons and therefore do not include direct measurements of membrane contact between neurites. If so, these data would not be amenable to a spatial analysis based on physical contacts (which forms the entire basis of our work).

I note that Moyle et al. (2020) recently tried a differing clustering strategy on the same datasets and came out with four rather than five domains with different partition boundaries.

Reply: There are a number of difference between our methodologies which could contribute to our different clustering results.

We have ruled out the following factors for the discrepancy:

1. We, but not Moyle *et al.,* threshold the membrane contacts areas for clustering by removing the smallest 35% of contacts. When we repeated our clustering without any size threshold of core contacts, we found that our clustering results are robust to this change (Figure S7).
2. We, but not Moyle *et al.*, analyze the entire nerve ring neuropil (Moyle *et al.* restricts their anal- ysis to the commissure portion of the nerve ring, excluding its synaptically dense posterior lobe). When we repeated our clustering on core contacts within the restricted volume used by Moyle *et al.*, we again found very similar clusters to those we obtained from the full volume (Figure S7).

Therefore, we expect the biggest reasons are likely due to

1. Our focus on M4 and bilaterally conserved, i.e. likely reproducible contacts, whereas Moyle *et al.* include variable contacts: We, but not Moyle *et al.*, restrict our clustering to the conserved M4 contacts. Our clustering results of the M1-M3 datasets (Figure S8) suggest that clustering on the aggregate data could obscure the organization of the core circuit (see discussion above). Related to this, our final clustering assignments are now based on consensus among M4, L4 and adult population models of conserved contacts (see above). Moyle *et al.*'s clustering assign- ments are based on consensus among L4 left, L4 right, adult left and adult right after clustering on the aggregate raw contacts in each dataset (including variable contacts).
2. Our approach (topological clustering based on modularity optimization) operates on the membrane contacts directly and is the recommended approach when the

network data represents pairwise relations (membrane contacts are pairwise relations between cells). By comparison, Moyle *et al.* use a diffusion based approach that does not operate directly on the membrane contacts. Instead diffusive clustering is a community finding approach that uses random walkers (Markov processes) on the network to infer communities and is therefore the recommended approach when network data represents network flow (e.g. information flow or transport networks). Below we cite Ref. 42 (a seminal paper on diffusive algorithms) summarizing Ref. 42's authors' position regarding when topological clustering methods (such as the one we use) might be preferred over diffusion (i.e., random walk) based approaches:

> "Which method should a researcher use? It depends on which of the two senses of network, described above, that the researcher is studying. For analyzing network data where links represent patterns of movement among nodes, flow-based approaches such as the map equation are likely to identify the most important aspects of structure. For analyzing network data where links represent not flows but rather pairwise relationships, it may be useful to detect structure even where no flow exists. For these systems, topological methods such as modularity (11) or cluster-based compression (16) may be preferable."

We refer the reviewers to the same reference for specific examples of well documented failures of diffusion based algorithms for flow-less problems (indeed it is precisely these issues that motivated the community to develop better topological clustering approaches). In particular, two issues pertain to Moyle *et al.*: first, row normalization will impose an uneven flow on some locations in the network which will bias the clustering; second, in the absence of flow, spatial features will be entirely missed.

[Moyle *et al.*] at least did try to compare N2U with JSH but there seemed to be differences.

We now provide clustering results for bilaterally conserved contact in the N2U and JSH datasets (Figure 1). We find that the perturbed population models of these datasets (Figure S7) both result in 5 largely similar clusters. We note that we obtain 5 clusters for the unperturbed dataset of JSH but 6 clusters for the unperturbed N2U dataset (Figure S8). The additional cluster in N2U splits the taxis cluster in two. However, the merging of these sub-clusters in the perturbed population (even when we halve the perturbation amplitude) suggests that subdivision of the taxis cluster is not robust to natural variability in membrane contact areas. Therefore, our 5 clusters are parsimonious, robust, objective solutions for clustering of the core, reproducible contacts of the nerve ring. In fact, even though N2U and JSH were taken at different developmental time points, we still observe a highly conserved core circuit between the two animals (approximately 80% overlap between bilaterally conserved L4 and adult membrane contacts, with a similar overlap between the adult and M4). In this context, the similarity of the clusters across the two populations models is, in our view, compelling.

I agree that the basic three-layer structure may have significant functional ramifications as the mechano-receptor circuitry is predominantly in the anterior layer whereas the chemo-receptor circuity is predominantly in the posterior layer.

Reply: We believe that our population-level definition of clusters addresses this. We now apply a 1- step, objective graph clustering algorithm to each individual sampled from our population models. Our analysis follows directly from the first section of the paper on the core/variable analysis of contacts and the connectome that these contacts support. As discussed above, our analysis suggests that aggregate data, including variable contacts, significantly obscure the finer features of the nerve ring organization. Therefore, without defining a reference dataset, this would not have been possible. We now cite White *et al.*'s observation in this context.

Also, recent studies have shown that the sublateral neurons in the center layer act as pioneers for ring neurogenesis indicating that the layered structure probably results from sequential directed pathfinding.

Indeed this is an exciting time for understanding the early development of the nerve ring. We believe that the spatial analysis we present can only help to provide more insight as well as constrain and inform models of development, whether limited to simple sequential pathfinding, or consisting of additional mechanisms, but this is beyond the scope of the present paper.

Nevertheless, I am sceptical about the insights that further sub-divisions by cluster analysis might provide. I presume the authors hope to reveal hidden bundles,

We address insights gained throughout the paper but especially in the Discussion. Constraints on development are one important result. The reliance on reproducible spatial features provides another novel insight: if we wish to uncover structure in neural tissue at this resolution, focusing on conserved structures may be essential. Other insights come from using our spatial cluster results to probe the synaptic organization. Finally, the spatial analysis allows us to identify cells that preferentially synapse with other neighborhoods across the nerve ring. At least some of these neurons have previously been identified as crossing neighborhoods in *C. elegans*, but we are not aware of anyone making a general observation that these non-local neurons tend to adopt strategies to this end (synaptic specializations, for example). A similar observation has recently been made in mammals (Morgan *et al.*, Ref 30). Multi- scale spatial analysis can therefore help identify generalizable principles of coordination across different neural circuits or regions of a neuropil. This was one of the insights that we needed to posit the brain map. Previous attempts (there have been numerous!) have largely focused on sensorimotor pathways, rather than on parallel pathways that converge onto a distributed circuit with extensive non- local connections.

In summary, we are not looking for 'hidden bundles'. We simply wish to identify groups of neurites with consistently high spatial affinity. We then use our results to draw insight about how the spatial organization supports the synaptic organization and information flow.

but this might be unnecessary as complete 3D reconstructions are now publicly available for 3 adult and several larval nerve rings. These data can be selectively visualised using computer graphic renderings. The graphics tools available are improving rapidly, so it is possible (or soon will be) to visualise a 3D rendering of a neuron and selected neighbours together with their synaptic contacts. Simple tools could be provided to provide adjacency values and synapse number. In this way the

bundle organisation of the nerve ring will be made readily apparent to the investigator.

Reply: We agree that visualization tools are incredibly valuable for developing an intuition about volumetric data. We refer the reviewer to our graphical resources on wormwiring.org. This paper focuses on quantitative analysis and data-driven modeling. To this end, data need to be reduced to a measurable space. We are not aware of visualization tools that give objective quantitative results, matching the high bar set by the reviewer for our clustering analysis. Regardless, our clustering results on M1-M3 (Figure S8) suggests ascertaining organization structure directly from aggregate data would likely be difficult, and indeed, beyond, the grossest features (e.g., White *et al.*'s 3 layers) may not be biologically meaningful. While there is clear and consistent organization among core contacts, this organization appears to become quickly obscured by the sea of variability in which the core circuit is embedded (Figure S8).

I urge the authors to consider cutting down their manuscript by eliminating their cluster analysis and to focus on the study of core/variable synapses that they have undertaken. I think this is an excellent study and would make a tight, focussed paper which has something significant to say about the organisation of the C. elegans nervous system - something which may prove generalizable to all nervous systems.

Reply: We appreciate the Reviewer's positive comments. We have considerably shortened the manuscript. We have also improved and considerably shortened the cluster analysis, but rather than eliminating it, we now ground our spatial analysis in the study of core/variable membrane contacts and synapses. We hope that the result is indeed a much tighter and focused paper. Once again, we thank the reviewer for their thoughtful and constructive critique.

Referee #2 (Remarks to the Author):

The authors have largely addressed my concerns with their revisions. However, I would suggest that, rather than waiting until the discussion, when they first introduce the 5 bundles (l. 119) that they would be more forthright about the subjective nature of this division. Their new Table S4 makes clear the process involved, which is helpful, but it also makes clear the several subjective steps involved.

Reply: We thank Reviewer 2 for raising concerns about clarity and transparency about any subjectivity in our previous clustering methodology. As we have now removed subjectivity from our methodology (see our reply to Reviewer 1) most of these comments no longer apply to the current resubmission. The only *potentially* subjective choices are in the choice of noise profile to generate our model population. We remove this subjectivity by constraining our population model to match the observed variability in the empirical data (Figure S7). Thus our population models have no free parameters.
Subjectivity was also eliminated from clustering assignments which are now dictated by consensus across population datasets (Figure 3). Where there is lack of consensus among datasets, we simply label the cells as "unclassified" and do not force any classification. As such, Table S4 of the previous submission is now obsolete and has been removed.

And later in constructing their brain map (l. 189) they elect to merge two bundles and elsewhere acknowledge that the two more posterior bundles could be considered one. Also, while the removal of the transbundle processes in Figure 17b strengthens the division into

anterior, avoidance and taxis bundles, the division of the laterals from the sublaterals looks even more arbitrary. Maybe something like "We find that dividing the processes into 5 groups is most consistent with the force-directed graph and the known biology of the neurons … " .

We acknowledge that the multiple steps taken in our previous submission came across as subjective and perhaps arbitrary. We now apply a 1-step clustering approach (see reply to Reviewer 1 above).

We revisited the question of laterals and sublaterals using our spatial population models and consistently found these neurons separated into two clusters (which we label lateral and sublateral). We generated a number of population models across datasets, noise amplitudes and spatial domains (Figure S8). The separation between laterals and sublaterals is consistent across these population models (again with no subjective input from us into those assignments). These results combined with the known biology of these neurons supports the notion that these cells can be considered as two subgroups of neurites with relatively high spatial affinity. We note that the spatial separation does not extend to a clear separation in synaptic connectivity, hence their merger in the brain map. This is now explicitly noted in the first section of the results.

In this resubmission, we have also minimized subjectivity in constructing the brain map: With the exception of 1 cell (CEPD) and unclassified cells, we adhere to the results of the clustering assignment. For CEPD and 7 unclassified cell classes, we use the process trajectories to place these cells on the brain map. The neurite trajectories of CEPD cells are straightforward – they follow those of other papillary sensory cells. It is therefore reasonable to place CEPD with the other papillary sensory cell classes. (This choice is vindicated by CEPD synaptic connectivity with anterior sensory cells.) For the sake of transparency, we keep the sublateral assignment color of CEPD within the brain map, even though we have placed CEPD among the anterior layer 1 cells. We also explicitly note this in the caption. The placement of the 7 unclassified cells is admittedly more challenging. To minimize subjectivity, we are aided by defining fiducial points in and around the nerve ring. In the caption of Figure 4, we explicitly state that unclassified cells were placed based on process trajectory and refer to methods. We repeat this only applies to the unclassified cells.

Minor items I happened to pick up:
Figure S13a and S17a both purport to be
"Force-directed layout of the conserved degree 4 (M4) set of membrane contacts in the nerve ring. Nodes and edges represent cell classes and membrane contacts, respectively."
And while the two representations are similar, they differ in important ways, e.g. the interdigitation of lateral and sublaterals. What is different? Also, S13a lacks the legends present in 17a that explain the shapes and colors. Finally, S13a still labels the set A4, rather than M4.

Reply: We greatly appreciate Reviewer 2's scrupulous reading of our supplemental figures. However, in line with the request to simplify and streamline our methodology, we no longer rely on force directed graphs and these figures have therefore been removed.

l. 30 (see Methods).

Corrected

l. 59 p should follow “connectivity” to maintain a consistent structure in the sentence.

Changed “target connectivity” to “target contacts”

l. 138 Should this be “opposing sides”?

Due to space limitations, we have removed this paragraph.

l. 200-204. With nested parenthetical remarks, this needs a rewrite. Thank you. Reworked

Fig. S17b “Trans-bundle” Figure has been removed.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

I find this version of the Brittin et al. manuscripts to be much improved over previous versions. It is more concise, and the new clustering methodology used is better described and more convincing than that used previously. To me the most significant aspect of this study is the identification and definition of core and variable components within the connectome. In particular, how the neighbourhoods of neurites have core and variable inhabitants and the discussions of how neighbourhood variability relates to the variable connections observed in the connectome. I think the authors have done a good job in working up methods to analyse and define the neighbourhood organisation of a connectome which should be generally useful as more reconstructed connectomes become available.

Although I still have reservations about the utility of cluster analyses, particularly given the limited number of datasets available, I think the authors have demonstrated that, using well established hierarchical clustering algorithms, five clusters can be discerned. These results are in contrast to the four clusters described in the recent preprint by Moyle et al. which were obtained using a novel diffusion condensation strategy. When more datasets become available, it should be possible to better compare these two approaches.

The speculations on the functional consequences of the spatial organisation of the neuropil on connectivity are of interest in that they provide insights into how structure may constrain and define function.

I think there are still some, mostly minor, issues that need to be dealt with in order for this study to be suitable for publication:

1. Although I appreciate that the identification of the members of the five putative classes suggested by the cluster analysis is tentative given the limited data currently available, I think the authors need to clearly state which processes make up each of their five clusters in both N2U and JSH datasets. I could not find this comparison even though I requested it in my previous review. Simple tables in the supplementary data would suffice. I get the impression from Fig. S7j that, although five clusters were found both in JSH and N2U, the clusters have different compositions.

2. I was sometimes confused when the authors refer to contacts as to whether synaptic or neighbourhood contacts were being specified (e.g. line 72). I suggest that the terms adjacency contact, synaptic contact, or gap junction contact are used explicitly, as appropriate, every time a contact is mentioned.

3. I was not sure what exactly the M4 consensus dataset is – more clarity is needed. Is it simply the set that includes all given process pairs that have an adjacency area threshold of greater than one third the maximum adjacency in that region along with equivalent thresholded pairs in the other three regions? Also, what do the M values mean that are specified in the SOM3 connectivity edge list?

4. There are several C. elegans neurons that abruptly switch neighbourhoods in specific regions (e.g. AVE). Would it not be better to consider the proximal and distal regions of these neurites as two separate entities for the purpose of defining neurite bundles by cluster analysis?

5. Having argued the case for the existence of a conserved core embedded in a background of variable connections I think the authors need to include a connectivity matrix (including synaptic weights) for what they consider the core connectome even though this is currently tentative given the limited data available. Many students of the C. elegans nervous system would be interested in these data.

6. Line 22 – White et al. describe the N2U animal as an "old hermaphrodite".

7. Corrections are needed to the labelling of panels described in the legend of Fig. 3, e.g. there is no panel f or h included in the figure. Also, there is no panel g in Fig. S7 although it is mentioned in the legend.

8. I was puzzled as to how gap junction data from Witvliet et al. are displayed in Fig. S5i. To my knowledge, this group has not released any gap junction data from their reconstructions as yet.

9. More information should be given in the "read me" files associated with the supplementary data. For example, what applications are needed to read the .xml or the .graphml files? What do the values specified for M in the connectivity edge list in SOM3 represent?

10. Fig. S10 legend line 6 – surrounded.

Author Rebuttals to Second Revision:

Note: Author Rebuttals in RED

Referees' comments:

Referee #1 (Remarks to the Author):

I find this version of the Brittin et al. manuscripts to be much improved over previous versions. It is more concise, and the new clustering methodology used is better described and more convincing than that used previously. To me the most significant aspect of this study is the identification and definition of core and variable components within the connectome. In particular, how the neighbourhoods of neurites have core and variable inhabitants and the discussions of how neighbourhood variability relates to the variable connections observed in the connectome. I think the authors have done a good job in working up methods to analyse and define the neighbourhood organisation of a connectome which should be generally useful as more reconstructed connectomes become available.

Although I still have reservations about the utility of cluster analyses, particularly given the limited number of datasets available, I think the authors have demonstrated that, using well established hierarchical clustering algorithms, five clusters can be discerned. These

results are in contrast to the four clusters described in the recent preprint by Moyle et al. which were obtained using a novel diffusion condensation strategy. When more datasets become available, it should be possible to better compare these two approaches.

The speculations on the functional consequences of the spatial organisation of the neuropil on connectivity are of interest in that they provide insights into how structure may constrain and define function.

I think there are still some, mostly minor, issues that need to be dealt with in order for this study to be suitable for publication:

1. Although I appreciate that the identification of the members of the five putative classes suggested by the cluster analysis is tentative given the limited data currently available, I think the authors need to clearly state which processes make up each of their five clusters in both N2U and JSH datasets. I could not find this comparison even though I requested it in my previous review. Simple tables in the supplementary data would suffice. I get the impression from Fig. S7j that, although five clusters were found both in JSH and N2U, the clusters have different compositions.

Cell names were listed in Figure 1b but we have now removed these as the font was clearly too small. The corresponding table (including the unperturbed $\mathbb{M}^4$ as well as the 3 populations ($\mathbb{M}^4$, L4, adult) datasets and the final "consensus" cluster assignments) was also available in SOM 4. We have now added the unperturbed L4 and adult cluster assignments and all additional cluster assignments from the validations performed (see Supplementary Information 4 and associated Source Data for Fig. 1 and Extended Data Fig. 5).

2. I was sometimes confused when the authors refer to contacts as to whether synaptic or neighbourhood contacts were being specified (e.g. line 72). I suggest that the terms adjacency contact, synaptic contact, or gap junction contact are used explicitly, as appropriate, every time a contact is mentioned.

We agree and now eliminate all ambiguity by using the terms membrane contact, synaptic contact and gap junction(al) contact to distinguish the three types of contact. The only times we have omitted this is when the contact is symbolically defined (e.g. as $\mathbb{M}^4$) or when used in a generic sense (referring to membrane, synapse or gap junction contacts).

3. I was not sure what exactly the M4 consensus dataset is – more clarity is needed. Is it simply the set that includes all given process pairs that have an adjacency area threshold of greater than one third the maximum adjacency in that region along with equivalent thresholded pairs in the other three regions? Also, what do the M values mean that are specified in the SOM3 connectivity edge list?

To avoid ambiguity, we have removed all mentions of consensus in reference to $\mathbb{M}^4$. The term "consensus" is now only used in reference to clustering. Additionally, we

have provided further clarification for SOM3 (now Supplementary Information 3) in the "SI guide".

4. There are several C. elegans neurons that abruptly switch neighbourhoods in specific regions (e.g. AVE). Would it not be better to consider the proximal and distal regions of these neurites as two separate entities for the purpose of defining neurite bundles by cluster analysis?

We accept this suggestion and have added a panel to Extended Data Fig. 8 listing a selection of such cells, their current cluster assignment and the neighborhoods they traverse. We also added a section to the Methods ("Mapping neighborhood changes of neurites") describing the procedure used to determine neighborhoods and neighborhood changes.

5. Having argued the case for the existence of a conserved core embedded in a background of variable connections I think the authors need to include a connectivity matrix (including synaptic weights) for what they consider the core connectome even though this is currently tentative given the limited data available. Many students of the C. elegans nervous system would be interested in these data.

$\mathbb{M}^4$ datasets were available in SOM 3 including membrane contact areas and the synaptic reproducibility index and the gap junctional reproducibility index (i.e. delta from 0 to 4) for each contact, but without the synaptic weight. We apologize if any of this was not clear, or easily accessible.

Previously, we provided a single edge list file that integrated all $\mathbb{M}$, $\mathbb{C}$ and $\mathbb{G}$ contacts. For simplicity, we now split each set of contacts into separate sheets within an xlsx file (Supplementary Information 4; Sheets M, C and G, respectively. Each edge list includes the weight, which is described in the SI guide. Further explanation is given in the SI guide.

6. Line 22 – White et al. describe the N2U animal as an "old hermaphrodite".

We thank the reviewer for this (correct) comment. We removed the adjective "young". We note that Cook et al. 2019 estimate it as 3 day old adult and we have now clarified that the meaning was 3 days from adulthood. To clarify this estimate, we were fortunate that the same animal N2U was carefully imaged not only in the head for the synaptic details, but in the midbody in order to pursue the ventral cord, dorsal cord down to the vulval region. This included both higher power images of the nerve cords, but lower power images of vulval muscles, uterine contents, etc. Parallel work by one of us (DHH) in characterizing the anatomy of aging adults with the Driscoll lab and others has included much work on animals reaching either 7 days or 15 days of adulthood, and a lesser number of animals at 4 days of adulthood. By viewing the contents of the anterior gonad in N2U, it became clear that this animal was most likely a 3 day old adult, or possibly a 4 day adult, but not older. In older animals, when the germline is becoming exhausted of fertilized oocytes, the luminal contents instead begins to fill with odd debris from decaying

unfertilized germline cells. Animal N2U still holds viable fertilized embryos, but no signs of debris. Thus it fits best as either a 3 day or 4 day old adult, but surely not older than 4 days (since adulthood) [cf Toth et al 2012; Herndon et al, 2002]. Note this age estimate is for animals grown at 20 degrees C and may require adjustment for animal N2U depending on its growth conditions.

7. Corrections are needed to the labelling of panels described in the legend of Fig. 3, e.g. there is no panel f or h included in the figure. Also, there is no panel g in Fig. S7 although it is mentioned in the legend.

We apologize for these slips. These have been fixed.

8. I was puzzled as to how gap junction data from Witvliet et al. are displayed in Fig. S5i. To my knowledge, this group has not released any gap junction data from their reconstructions as yet.

Witvliet *et al.* scored gap junctions were made available with their synaptic dataset. However, they note that these scores are "by no means exhaustive". For purposes of assessing core/variable distribution, we felt the comparison was valid.

9. More information should be given in the "read me" files associated with the supplementary data. For example, what applications are needed to read the .xml or the .graphml files? What do the values specified for M in the connectivity edge list in SOM3 represent?

We have now written a detailed Supplementary Information Guide. In addition, we now provide the files in plain text (csv format), for readers not familiar/comfortable with xml.

10. Fig. S10 legend line 6 – surrounded.

Thank you. Fixed.