EMBO reports

A combinatorial panel for flow cytometry-based isolation of enteric nervous system cells from human intestine

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Johnathan,

Thank you for the submission of your manuscript to EMBO reports. We have now received the comments from 2 referees, which are pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. While referee 1 raises more minor issues, referee 2 asks for more data. We think that all referee comments need to be addressed.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (15th Nov 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised

manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:

https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

This paper describes a new way to isolate human enteric nervous system (ENS) cells from the bowel via flow-cytometry. Many prior groups have tried this, but prior methods were very inefficient making it impossible obtain RNAseq data (for example) from many enteric neurons. This new method is therefore a very welcome addition to the field and will facilitate future studies. The approach may be valuable to study Hirschsprung disease, chronic intestinal pseudo-obstruction, gastroparesis, irritable bowel syndrome, diabetic bowel disease, Parkinson's disease of the bowel, and other disorders.

The manuscript is very well written, and figures are beautifully annotated and presented. I commented on many "Minor issues" below that will enhance data presentation, but none of these detract from the value of the work.

They took advantage of decades of work and new RNAseq data to identify new markers for flow cytometry that are highly ENS specific. They then used CD56/CD90/CD24 co-expression to isolate ENS cells and found that intact live neurons and glia express the highest levels of these markers. They do a great job showing expression of these markers in cell populations of interest (i.e., overlap) and then follow up by showing that the cells isolated can be evaluated by RNAseq.

They demonstrate nicely that cell fragments need to be excluded from analyses, and that some cells (e.g. glia) may be attached to other cell components (e.g., neurites). They found a way to eliminate these potential confounding cells with attached cell fragments.

They also compare several cell dissociation methods and show that one does a better job maintaining the markers that they use for flow cytometry than others.

Authors provide data for 22,549 "high quality" cells evaluated by scRNAseq. Remarkably, even after sorting cells, only 351 (1.6%) were classified as enteric neurons and many in the "neuronal" cluster were classified as "Differentiating neurons".

As a comparison another recent paper that employed myenteric plexus region microdissection yielded only 48 enteric neurons our of 20,167 cells sequenced (Cell Mol Gastroenterol Hepatol 2021; 11:1548-1593; https://doi.org/10.1016/j.jcmgh.2020.12.014). This new method therefore enriched for enteric neurons 7.3-fold compared to the microdissection strategy. A separate study also using RNAseq from whole bowel isolated very few neurons from post-natal samples (DOI: 10.1038/s41586-021-03852-1). The other major advantage of this paper's strategy is that it does not require cell culture and the approach is used on cryopreserved tissue, dramatically simplifying logistics coordinating surgery and tissue analysis. Finally, they isolate whole cells instead of just nuclei, which may be valuable for RNAseq or other analyses.

Important issues: I provide many suggestions for improvement, but very few involve experiments. Very nice work!

Authors argue (probably correctly) that the CD24hi/TIP cell subcluster is almost all neurons based on ELAVL and this seems to be supported by FACS (Figure 4A). Given this, it is a little surprising that the qRT-PCR analyses in Figure 4C show statistically non-significant differences in ELAVL4 and SNAP25 mRNA in the CD24low and CD24TIP cell populations. Is there a way to explain why the ELAVL4 protein analyses (Figure 4A) and ELAVL4 mRNA analysis (Figure 4C) seem to differ?

Line 279: Authors say that for the first time human enteric neurons can be distinguished from enteric glia by FACS, but Figure 4C and Figure 5 suggest that they did not cleanly separate enteric neurons from glia for molecular analyses. It would be valuable to show that a large population of enteric neurons could be cleanly isolated and used for RNAseq (or shown to be neurons in another (less expensive) way.

Because this is primarily a methods paper, details in the methods are especially important. It would be nice to have tables or reagents, and other details like a "STAR Methods" section in Cell Press journals. I will list examples where methods could be more clearly delineated.
-Line 340: Clarify if tissue was full thickness biopsy.
-Line 340: How was tissue handled in the operating room or in pathology on the way to the lab? Was it kept on ice, in media or buffer? How long did it take between the time the tissue was removed from the patient to the time the tissue was minced or the minced tissue frozen?

-Line 341: How carefully were luminal contents removed? It says "rinsed in ice cold PBS" but was this a brief rinse, vigorous vortexing, multiple rinses? What exactly is done? What volumes of PBS are used?
-Line 345: Does the entire 2-3 cm2 piece of tissue end up in a single cryovial with 1.5 mL CryoStor media?
-Line 348 forward: How much tissue was added to how much digestion solution?
-Lines 350 to 352: Are these final concentrations of the enzymes? What stock solutions are used for the tissue digesting enzymes? How are these stock solutions stored?
-Lines 354: Approximately how many times do you triturate? This is important to know since shear stress can damage cells. Is this trituration done gently or vigorously? Is there a way to define what you are doing this so others can replicate.
-Line 356: What cell strainer was used?
-Line 356: When cells are washed with HBSS/FBS, what does that mean? Were the cells that passed through the 40 micron strainer centrifuged? If so, what speed, what temperature, and how long? What volume of HBSS/FBS was used for the wash?
-Line 357: What temperature was centrifugation?
-Line 358: What are centrifugation conditions?
-Line 359: What volume of washing buffer was used? What is the "Washing buffer" This is the first time this term is used. Do authors mean HBSS without Ca Mg plus 2% FBS?
-Line 364: Mentions supplemental Table 1 listing antibodies. I cannot find supplemental tables.
-Line 374: Mentions Table 1. I cannot find table 1.
-Lines 363 to 370: Add more details. I assume the "missing" Supplemental Table 1 includes antibody dilutions, catalog and RRID numbers. What does "washed twice" mean"?
-Line 368: How long were cells incubated with LIVE/DEAD dye.
-Line 369: Cells were permeabilized in 0.2% Triton X-100. Was there anything else in this solution or just Triton and water?
-Line 370: How was intracellular staining done?
-Line 385-389: What cells were used for this? Where is Supplemental Table 1
-Lines 390 to 404: How much tissue did they start with to yield 22,549 high quality cells (cm2 or weight of tissue and tissue region)? Was this full thickness bowel and if so, how were cells sorted exactly for this experiment?
-Lines 390 to 404: Information could be provided elsewhere, but we need information about the origin of these RNAseq data? How old was the person whose cells were sequenced? What bowel region was used? What was the indication for surgery? Was tissue examined and looked normal or was tissue inflamed?
-Line 408 to 409: What statistical approach was used if data were not normally distributed?

Minor issues:

Line 82 and 83: Not an accurate description of the location of ENS cells and their projections. For example many myenteric neurons have projections within the myenteric plexus (i.e., interneurons) or to the muscle layers of the bowel (excitatory and inhibitory motor neurons). Also, this ignores submucosal neurons. Consider revising for accuracy, but OK to keep simple.

Line 88: Consider "The most well understood serious enteric neuropathy is Hirschsprung disease", since arguably at least some cases of irritable bowel syndrome or slow transit constipation are likely to be due to enteric neuropathy and affect >20% of the population in Western countries.

Introduction: Consider adding references to support statements.

Line 98: Last word should be "activated" instead of "activate".

Supplemental Figure 1B and 1C: I like the labeling on the bottom image axes (CD56 APC and CD271-FITC). When I saw the top panels that just say APC or FITC it required more thought to understand what was being analyzed. Please consider all axes indicating fluorophore and marker (e.g., CD56 APC). Also make pattern of spacing and dashes uniform (i.e., now it says CD 56 APC (no dash), CD271-FITC (dash), and CD- Alexa700 (dash and space)).

Line 122: Did authors use whole ileum or just muscle layer?

Lines 127, 128 and other places: Superscripts are very small so it is difficulty to see + signs, and especially "minus" signs. Consider using a larger font for the superscripts.

Figure 1D: I am confused by Y axis on this graph. I think they are graphing "% CD271+ cells that are also CD56+/CD271+" for the green bar, but instead the Y-axis label says "%purity vs CD56+/CD271+ neural cluster". If I am correct, consider changing Y-axis label to "% of cells that are CD56+ CD271+". Note too that CD56+/CD271+ might be interpreted as a fraction instead of indicating double positive cells.

Figure 1, Supplemental Figure 1, associated text (and throughout the manuscript): All figures would benefit from consistent labeling. Here are a few examples from these Figures.
-What is the difference between CD56+/high and CD56+? If "+" is different from "+/high" designation, please demonstrate those differences in the FACS plots.
-The cells in Figure 1B labeled "CD56+" (I think although font is tiny) appear to be the same cells labeled "CD56+/high" in

Supplemental Figure 1D.
-In Supplemental Figure 1D, the X-axis label in the top panel second column says "NCAM APC-A", while the label for the plot says "CD56+/high". Since CD56 = NCAM1 I understand what this means, but please make all the labeling consistent. Here I would probably change X-axis to "CD56-APC-A" or "CD56-APC" (like in Supplemental Figure 1B)
-What is the difference between FITC-A (Supplemental Figure 1D top) and FITC (the X-axis label just below)? What is the difference between "APC" and "APC-A"? What is the difference between "CD90 Alexa Fluor 700-A" (Supplemental Figure 1D top) and CD90- Alexa700" (Supplemental Figure 1D bottom)?
-Text line 134 refers to "CD56+/High CD271+" in Figure 1A. The label in Figure 1A says "CD56+ CD271+". Figure 1 legend says "CD56+/hi/CD271+". Spacing, dashes, backslashes, "+" versus "+/high" versus "+/hi" should only be different if they are intended to designate something different. The inconsistent labeling is not only confusing, but will make it more difficult to search the text.

Lines 140-142: Redundant "qRT-PCR for neuronal and glial markers" used 2x in the same sentence.

Supplemental Figure 3A: X-axis label is upside down, possibly backwards and has superimposed words. Also does Hoechst-A differ from Hoechst?

Figure 5: Please include a flow cytometry (or other) schematic to indicate clearly what cell populations underwent scRNAseq. For example, were these simply dissociated cells, or were they flow sorted, and if so, for what markers? Did they sequence the CD56high/CD90high/CD24 high pool or something else? This is important so we can understand the RNAseq outcome data.

Line 391: Please clarify what was done to these cells prior to scRNAseq? It says "After tissue dissociation" but were these cells flow sorted for CD56/CD90/CD24 and if so, which flow pool was sequenced? This is important since the analyses yielded 22,549 cells with high quality data, of which 20,750 were glia and 351 were neurons (lines 402-403). This is remarkable enrichment for ENS, but as in other reported analyses only a small percentage of cells were enteric neurons.

Lines 241 to 243 and Figure 5E: Authors call neuron subtypes "peripheral XX neurons", but it is probably better to call these gut-derived neurons "enteric XX neurons" to distinguish (for example) form other "peripheral sensory neurons" like dorsal root ganglion cells.

Figure 5: There are two parts to this figure labeled "E" in both the figure itself and in the figure legend. Please label the violin plots "F" and change the figure legend. Surprisingly, Figure 5E is not referenced at all in the text (Lines 243 to 248) and instead they reference the missing Figure 5F. Please review all figure references.

Figure 5E/F: It is interesting that many of the cells in the "neuron" cluster are classified as "Differentiating neurons". While I see a little RET and SNAP25 in the violin plots, it would be nice to know more about these cells, since this may be a surprising finding. In particular, there is debate among ENS scientists about the extent and frequency of ENS neurogenesis that occurs after birth and in adults. Pseudo-time analysis of cell in Figure 5A would be interesting, as would a separate pseudo-time analysis of just the neuronal cluster. For example, murine literature suggests Schwann cells can become neurons after birth or that a glial subset or a Nestin+ subset of cells might act as adult ENS precursors. Are these "Differentiating neurons" also the cells in Figure 5C that express PLP1, SOX10 and S100B? Are these really Schwann cells? Are the EDNRB+ cells the same as the NOTCH2+ cells?

Discussion and throughout the text: Authors discuss isolation of "neural cells", but actually recover >90% glia. Please consider revising the text to discuss neuron and glia isolation, recognizing prior strategies also isolate large numbers of glia. For example, line 262 talks about neural isolation, but unless I'm confused, CD90 and CD56 were used to isolate cells used for scRNAseq and only 1.6% of those cells are neurons.

Line 281: Authors speculate that the function of CD24 in ENS is similar to function in CNS based on data showing expression of CD24 in ENS cells. I would omit this comment since there are no data presented about CD24 function and no earlier discussion of what CD24 function is in the CNS.

Line 289: Consider adding https://doi.org/10.1016/j.jcmgh.2020.12.014, a paper where ENS was dissected out and then used for RNAseq.

Line 291: There are also many myenteric neurons in the human bowel submucosa, including close to the bowel epithelium.

Line 292: This is the first time I can tell that the scRNAseq was performed on "sorted CD56+/CD90+ neural cells". I would not call these "neural cells" since 90% are glia and 1.6% are neurons based on scRNAseq. I would call them "ENS cells" or "enteric nervous system cells". Please show a FACS plot indicating precisely which cell population was used for scRNAseq (ideally in Figure 5).

Lines 299 to 302: In this paper, scRNAseq data demonstrate a 59 to 1 ratio of glia to neurons. While I do not know of reliable data for human infants, in adult human colon the glia to neuron ratio is 3-5.6 to 1 (See Figure 2 in

https://doi.org/10.1053/j.gastro.2020.02.035). To the best of my knowledge all the other RNAseq papers from the bowel showed very high glia to neuron ratios, consistent with the idea that neurons are much more fragile than glia (e.g. 10x more fragile). Not sure text change is needed here, but the magnitude of these cell ratios based on cell counting versus scRNAseq might be worth emphasizing.

Line 308: I think the referenced studies are about mice. How many neurons were evaluated in any of the prior published human scRNAseq data for children?

Line 315 suggests cells isolated by flow cytometry could be cultured. Are there any data to support the idea that cells isolated using this strategy will survive in culture?

Line 318 Says prior studies did not attempt to enrich for ENS. This paper microdissected myenteric plexus https://doi.org/10.1016/j.jcmgh.2020.12.014 before snRNAseq.

Line 339: Should be "ileostomy" and "colostomy" instead of "ileostoma" and "colostoma".

Figure 1 and other Figure legends: Since most of the isolated cells end up being glia, please refer to them as "enteric nervous system cluster". The same issue applies throughout the text and figure legends. If the clusters are "not just neurons", please characterize them more accurately.

Line 435: What marker is meant by "for this marker"?

Line 452: "D)" says representative sorted neural cells, but the image says "Putative glia". I suspect this should be D-F) instead of just D, but please review carefully.

Figure legend 3D-F: I like the representative images, but an explanation of features that define for this group the different types of cells would be valuable. Does debris simply mean DAPI negative?

Figure legend 3 D-F: Indicate that cells are DAPI stained.

Figure 4: The purple ELAVL4 stained cells on the FACS plots have an interesting pattern. While most FACS analyzed cells form "clouds" of cells in a cluster (each dot apparently randomly located relative to others in the cluster), the purple "cells" form multiple short lines, each 6 dots long in a zig-zag line. I am guessing this is what happens when you plot "in bold to improve visibility" but this is not really "bold". Is there a better way to describe this symbol or a better way to plot?

Supplemental Figure legend 3: Says standard cell cycle plot is on the left and Hoechst + versus negative are on the right. I think left and right are reversed.

Referee #2:

This manuscript addresses an important question in the ENS field, which is how human ENS cells (which represent a very small minority of all cells in the gut) can be enriched to facilitate targeted analyses such as scRNAseq. However, this reviewer feels that a significant amount of work would be needed before considering it as a good fit for EMBO Reports. There are a number of key questions that would need to be addressed:
1- In the end, what is the added value of using a combination of CD56/CD90 vs CD56 alone to sort ENS cells? Hard to see how this is needed for scRNAseq.
2- Where are all of these markers (also including CD24 and CD271) expressed in the developing human ENS? Cytometry data should be accompanied by immunofluorescence data for that cohort.
3- Why are there so few neurons recovered? They should represent about 14% of recovered ENS cells (based on 1:7 ratio), not only 2%. Perhaps CD56/NCAM is expressed in only a subset of them? Alternatively, as pointed out by the authors, neurons might be harder to recover using enzymatic-based dissociation. Immunofluorescence would again be helpful to address all of these questions.
4- There is a profound lack of details about the samples that were analyzed. How many N per condition? Is it needed to pool multiple samples from different individuals? How many ENS cells are contained within a single sample? What is the exact age of all individuals involved in this study?

Referee 1:

This paper describes a new way to isolate human enteric nervous system (ENS) cells from the bowel via flow-cytometry. Many prior groups have tried this, but prior methods were very inefficient making it impossible obtain RNAseq data (for example) from many enteric neurons. This new method is therefore a very welcome addition to the field and will facilitate future studies. The approach may be valuable to study Hirschsprung disease, chronic intestinal pseudo-obstruction, gastroparesis, irritable bowel syndrome, diabetic bowel disease, Parkinson's disease of the bowel, and other disorders. The manuscript is very well written, and figures are beautifully annotated and presented. I commented on many "Minor issues" below that will enhance data presentation, but none of these detract from the value of the work.

We thank the reviewer for the positive overall evaluation of our manuscript. We have considered all the points raised and performed manuscript editing as described below. Under each reviewer's point we have indicated the changes applied.

Important issues: I provide many suggestions for improvement, but very few involve experiments. Very nice work!

We again thank the reviewer for the compliments. We have taken the suggestions at heart, and have carefully evaluated and answered all the reviewer's points. We found the comments relevant and appropriate and we believe that their implementation has significantly contributed to improve the quality and readability of the manuscript.

Authors argue (probably correctly) that the CD24hi/TIP cell subcluster is almost all neurons based on ELAVL and this seems to be supported by FACS (Figure 4A). Given this, it is a little surprising that the qRT-PCR analyses in Figure 4C show statistically non-significant differences in ELAVL4 and SNAP25 mRNA in the CD24low and CD24TIP cell populations. Is there a way to explain why the ELAVL4 protein analyses (Figure 4A) and ELAVL4 mRNA analysis (Figure 4C) seem to differ?

The reviewer makes a valid comment. Initially, qRT-PCR failed to reach significance due to a limited number of replicates and a large degree of variance between measurements. Additionally, the number of $CD24^{TIP}$ cells was small in some samples, leading to very low amount of RNA compared with the other subpopulations, and thus inevitably producing lower quality qRT-PCR data. We have increased our technical replicates and could reach statistical significance for all the single markers, as shown in the updated graph (Figure 5C). Moreover, as confirmatory evidence of the predominantly neuronal identity of $CD24^{TIP}$, we have improved our analysis of ganglionic vs aganglionic colon in Hirschsprung patients, by adding more samples and increasing the technical quality of the data. In particular, low CD24 levels were not well visible due to loss of CD24 staining intensity after room temperature incubation (for cell cycle dye saturation), combined with spillover from the Cycle Green nuclear dye. The new round of analysis was based on Hoechst 33242 staining, as shown in Figure 3 and 5B (previous 4B), leading to the visualization of differences in ganglionic vs aganglionic colon, which were not properly appreciated before. We have also made sure that the pieces of aganglionic colon we used were not contaminated by surrounding ganglionic regions, and vice versa. We clearly show in the revised manuscript, that aganglionic preparations miss two ENS subpopulations, $CD24^{TIP}$ and $CD24^{LOW}$, in both nucleated and non-nucleated fractions (Figure 5D, E; Suppl. Figure 4E-I), which raises interesting hypotheses for further analyses. Please, about all these changes see lines 248-259, 666-670, 782-790.

Line 279: Authors say that for the first time human enteric neurons can be distinguished from enteric glia by FACS, but Figure 4C and Figure 5 suggest that they did not cleanly separate enteric neurons from glia for molecular analyses. It would be valuable to show that a large population of enteric neurons could be cleanly isolated and used for RNAseq (or shown to be neurons in another (less expensive) way.

We appreciate the reviewer's comment and agree that the separation of enteric neurons from glia by our current method might not be absolute, due to considerable overlap of surface marker expression, and as a result of adherence of debris to cellular membranes. Importantly, we would like to emphasize that our

scRNAseq experiments were not intended to show a validation of glia vs. neuron separation, but as a validation of the quality and cellular identity of collected ENS cells in general.

However, we expect that our method, provided multiple samples sorted for $CD24^{TIP}$ are pooled, can be implemented to enrich for neurons in numbers amenable for scRNAseq. Unfortunately, due to time and resource constraints, we were not able to provide these data in the current manuscript. Nonetheless, after adding more replicates, we show that the level of neuronal enrichment based on expression of neuronal markers obtained by qRT-PCR between $CD24^{TIP}$ ENS cells and the rest of the ENS cluster, is statistically significant, as reported in Figure 5C in the revised version. This suggests that whilst not entirely clean, our method is a valuable improvement over current protocols.

Noteworthy, the identification of neurons in fixed samples does provide a non-equivocal discrimination capable of segregating neurons from glia (as evidenced by ELAVL4 staining). However, the usage of fixed samples precludes downstream validation of cellular identity by qRT-PCR or scRNAseq. Ongoing developments in scRNAseq field, enabling researchers to sequence fixed samples, will potentially be able to address these issues.

Because this is primarily a methods paper, details in the methods are especially important. It would be nice to have tables or reagents, and other details like a "STAR Methods" section in Cell Press journals. I will list examples where methods could be more clearly delineated.

-Line 340: Clarify if tissue was full thickness biopsy.

As suggested by the reviewer, we adjusted the text to provide clarity. We added: “In the operating room, full thickness tissue biopsies of approximately 2-3 cm^2…”. Please see lines 443-444.

-Line 340: How was tissue handled in the operating room or in pathology on the way to the lab? Was it kept on ice, in media or buffer? How long did it take between the time the tissue was removed from the patient to the time the tissue was minced or the minced tissue frozen?

As suggested by the reviewer, we adjusted the text to provide clarity. We added: “… were taken from discarded tissue of resection specimens, placed in 25 mL of ice-cold PBS and transported within 30 minutes to our laboratory.”. Please see lines 444-445.

-Line 341: How carefully were luminal contents removed? It says "rinsed in ice cold PBS" but was this a brief rinse, vigorous vortexing, multiple rinses? What exactly is done? What volumes of PBS are used?

As suggested by the reviewer, we adjusted the text to provide clarity. We added: “Next, the tissue was vigorously shaken for 10 seconds and the PBS was decanted. These washing steps were repeated until the tissue was macroscopically devoid of feces.” Please see lines 445-447.

-Line 345: Does the entire 2-3 cm^2 piece of tissue end up in a single cryovial with 1.5 mL CryoStor media?

As suggested by the reviewer, we adjusted the text to provide clarity. We added: “Aliquots of 500 mg of minced tissue were stored in cryovials containing 1.5 ml Cryostor cell cryopreservation media (C2874, Sigma).” Please see lines 455-456.

-**Line 348 forward: How much tissue was added to how much digestion solution?**

As suggested by the reviewer, we adjusted the text to provide clarity. We added: “Cryopreserved tissue (500 mg) was rapidly thawed in a 37°C water bath, washed with ice cold PBS, and placed in a gentleMACS C-tube (130-093-237, Miltenyi Biotec) with 5 ml of digestion solution…”. Please see lines 455-456.

-Lines 350 to 352: Are these final concentrations of the enzymes? What stock solutions are used for the tissue digesting enzymes? How are these stock solutions stored?

As suggested by the reviewer, we have added the requested information: “… with 5 mL digestion solution [[DMEM/F12 (11320-074, Gibco) containing 10 mM HEPES (15630106, Thermo Fisher), and 100 µg/mL DNase I

(11284932001, Sigma)]. Collagenase II (3 mg/mL; 17101-015, Gibco) + Dispase (1.25mg/mL; 17105-041, Gibco), Collagenase D (2.5mg/mL; 11088858001, Roche) or Liberase TM (0.42mg/mL; 5401119001, Roche) were also added to the digestion solution. Liquid stocks of HEPES (1M) were stored at 4 degrees, whilst stocks of DNAse I (10 mg/ml) were kept at –20 degrees. Collagenase II, Dispase and Collagenase D were freshly prepared from powder (stored at -20 degrees). Please see lines 456-462.

-Lines 354: Approximately how many times do you triturate? This is important to know since shear stress can damage cells. Is this trituration done gently or vigorously? Is there a way to define what you are doing this so others can replicate.

As suggested by the reviewer, we have added the requested information: "… the tissue suspension was triturated gently for ten times, with a 19G needle and syringe, followed by ten times with a 22G needle, until a homogeneous suspension was achieved.". Please see lines 464-465.

-Line 356: What cell strainer was used?

As suggested by the reviewer, we have added the requested information: "The suspension was filtered through a 70 µm cell strainer (130-098-458, Miltenyi Biotec)". Please see line 467.

-Line 356: When cells are washed with HBSS/FBS, what does that mean? Were the cells that passed through the 40 micron strainer centrifuged? If so, what speed, what temperature, and how long? What volume of HBSS/FBS was used for the wash?

As suggested by the reviewer, we have added the requested information: "The suspension was filtered through a 70 µm cell strainer (130-098-458, Miltenyi Biotec) and mixed 1:10 with HBSS buffer without Ca2+ Mg2+ (14175095, Life Technologies), and 2% Fetal Bovine Serum. Afterwards, cells were washed by pelleting using a swing bucket pre-cooled (4 degrees) centrifuge, at 540 rpm for 3 minutes, with maximum acceleration and deceleration.". Please see lines 464-470.

-Line 357: What temperature was centrifugation?

We have added the requested information: "Afterwards, cells were washed by pelleting using a swing bucket pre-cooled (4 degrees) centrifuge, at 540 rpm for 3 minutes". Please see line 468-469.

-Line 358: What are centrifugation conditions?

As suggested by the reviewer, we have added the requested information: "Afterwards, cells were pelleted using a swing bucket pre-cooled (4 degrees) centrifuge, at 540 rpm for 3 minutes, with maximum acceleration and deceleration.". Please see lines 468-470.

-Line 359: What volume of washing buffer was used? What is the "Washing buffer" This is the first time this term is used. Do authors mean HBSS without Ca Mg plus 2% FBS?

As suggested by the reviewer, we adjusted the text to provide clarity. We added: "Cells were then centrifuged, resuspended in 1 mL of HBSS without Ca^{2+} Mg^{2+} and 2% Fetal Bovine Serum, and…". Please see line 472.

-Line 364: Mentions supplemental Table 1 listing antibodies. I cannot find supplemental tables.

We accidentally forgot to include supplemental table 1. The list of antibodies used can now be found in Table 1.

-Line 374: Mentions Table 1. I cannot find table 1.

We accidentally forgot to include Table 1, but has now been included in the manuscript.

-Lines 363 to 370: Add more details. I assume the "missing" Supplemental Table 1 includes antibody dilutions, catalog and RRID numbers. What does "washed twice" mean"?

Indeed, the reviewer is correct. Such information was available in Supplemental table 1, now added as Table 1. Regarding the "washed twice', we have now modified the sentence to make it clearer: "For dead cell exclusion during FACS analysis, cells were resuspended in 100 uL of HBSS with 2% FBS and pelleted twice and subsequently resuspended in …". Please see lines 472-480.

-Line 368: How long were cells incubated with LIVE/DEAD dye.

As suggested by the reviewer, we have added the requested information: "… and then incubated for ten minutes with a LIVE/DEAD fixable viability dye…". Please see line 481.

-Line 369: Cells were permeabilized in 0.2% Triton X-100. Was there anything else in this solution or just Triton and water?

As suggested by the reviewer, we have added the requested information: "… double wash and permeabilization with 0.2% PBS-Triton X-100 (15', RT), were performed.". Please see lines 483-484.

-Line 370: How was intracellular staining done?

As suggested by the reviewer, we have added the requested information: "Cells were then stained with intracellular markers in PBS with 0.2% Triton X-100, 1% BSA. For conjugated markers, cells were stained on ice for 90 minutes and subsequently washed using 1 ml PBS with 2% FBS and centrifuged at 4 degrees, 540 rpm for 3 minutes, followed by resuspension in 100 uL PBS. For unconjugated markers cells were stained on ice with primary antibodies for 90 minutes in 100 uL PBS, subsequently washed using 1 ml PBS with 2% FBS and centrifuged at 4 degrees, 540 rpm for 3 minutes. Cells were then resuspended in 100 uL of PBS and stained for 1 hour on ice with secondary antibodies. Afterwards, cells were washed with 1 mL PBS with 2% FBS, centrifuged as described above and resuspended in 100 uL of PBS." Please see lines 482-491.

-Line 385-389: What cells were used for this? Where is Supplemental Table 1

As suggested by the reviewer, we have added the requested information: "RNA from $CD56^{+}CD90^{+}$, $CD24^{LOW}CD24^{HI}$ and $CD24^{TIP}$ sorted cells,…". Please see line 517. Supplemental Table 1 is now added as Table 1.

-Lines 390 to 404: How much tissue did they start with to yield 22,549 high quality cells (cm2 or weight of tissue and tissue region)? Was this full thickness bowel and if so, how were cells sorted exactly for this experiment?

As suggested by the reviewer, we have added the requested information: "For scRNAseq, four control patients were analysed (Table 2). One frozen vial containing approximately 500 mg of minced full thickness intestinal biopsy, was used per patient. After tissue dissociation, cells were stained for CD56/CD90/CD24/Lin/DAPI and cycle green. $Live/Lin^{-}/CycleGreen^{+}/CD56^{+}/CD90^{+}$ cells were sorted and concentrated in 25 µL of PBS.". Please see lines 523-526.

-Lines 390 to 404: Information could be provided elsewhere, but we need information about the origin of these RNAseq data? How old was the person whose cells were sequenced? What bowel region was used? What was the indication for surgery? Was tissue examined and looked normal or was tissue inflamed?

As suggested by the reviewer, we have added the requested information in the new Table 2.

-Line 408 to 409: What statistical approach was used if data were not normally distributed?

As suggested by the reviewer, we have added the requested information: "Data was tested for normality, using the Shapiro-Wilk test. If data passed this test either as non-transformed or log-transformed values,

differences were analyzed using multiple unpaired t tests. The Mann-Whitney U test was used if data were not normally distributed." Please see lines 543-544.

Minor issues:

Line 82 and 83: Not an accurate description of the location of ENS cells and their projections. For example many myenteric neurons have projections within the myenteric plexus (i.e., interneurons) or to the muscle layers of the bowel (excitatory and inhibitory motor neurons). Also, this ignores submucosal neurons. Consider revising for accuracy, but OK to keep simple.

As suggested by the reviewer, we have changed this sentence. It is now simplified and reads: "Neuronal cell bodies are localized within ganglia between the muscle layers of the intestine.". Please see line 82.

Line 88: Consider "The most well understood serious enteric neuropathy is Hirschsprung disease", since arguably at least some cases of irritable bowel syndrome or slow transit constipation are likely to be due to enteric neuropathy and affect >20% of the population in Western countries.

We have revised the sentence as suggested by the reviewer. Please see lines 89-89.

Introduction: Consider adding references to support statements.

As suggested by the reviewer, additional references were added throughout the manuscript to support statements.

Line 98: Last word should be "activated" instead of "activate".

We have revised the sentence as suggested by the reviewer. Please see line 99-100.

Supplemental Figure 1B and 1C: I like the labeling on the bottom image axes (CD56 APC and CD271-FITC). When I saw the top panels that just say APC or FITC it required more thought to understand what was being analyzed. Please consider all axes indicating fluorophore and marker (e.g., CD56 APC). Also make pattern of spacing and dashes uniform (i.e., now it says CD 56 APC (no dash), CD271-FITC (dash), and CD- Alexa700 (dash and space)).

As suggested by the reviewer, the figures have been revised, clarifying fluorochrome and marker associated with each of the axes and/or plots.

Line 122: Did authors use whole ileum or just muscle layer?

As suggested by the reviewer, we have added the requested information: " … we prepared live single-cell suspensions from full thickness human ileum samples." Please see lines 160-161. To make this immediately clear for the readers, we have added data showing the expression of EpCAM, as evidence of the presence of the epithelial layer in our samples. Please see lines 167-168 and Supplemental Figure 1E.

Lines 127, 128 and other places: Superscripts are very small so it is difficult to see + signs, and especially "minus" signs. Consider using a larger font for the superscripts.

We have made the suggested modifications. Larger font for superscripts is now used throughout the manuscript.

Figure 1D: I am confused by Y axis on this graph. I think they are graphing "% CD271+ cells that are also CD56+/CD271+" for the green bar, but instead the Y-axis label says "%purity vs CD56+/CD271+ neural cluster". If I am correct, consider changing Y-axis label to "% of cells that are CD56+ CD271+". Note too that CD56+/CD271+ might be interpreted as a fraction instead of indicating double positive cells.

The reviewer is correct. We have made the suggested modifications to the now Figure 1E.

Figure 1, Supplemental Figure 1, associated text (and throughout the manuscript): All figures would benefit from consistent labeling. Here are a few examples from these Figures.
-What is the difference between CD56+/high and CD56+? If "+" is different from "+/high" designation, please demonstrate those differences in the FACS plots.

The reviewer makes a valid comment. The reason why we used "high" and not only "+", is because we thought this was a better definition for the selection of clusters that are not only positive for a marker, but also distant from negative. In other words, the selection of the gate is made based on the presence of a cluster of cells and not on simple positivity. In Huizer *et al* 2017 (cited in our manuscript), and simplified in our more recent paper (Sacchetti *et al*, 2021), we have defined as "high" the populations that are at least 1 log above negative. It seems this applies to our neural cluster in live samples, but not in all fixed samples. Therefore, the use of "+/high" was a way to include both types of samples, but indeed we did not explain this point clearly. For the sake of clarity, we decided to change all the "+/high" labels to simply "+", except when referring to the levels of CD24. In this case, we think is necessary to distinguish between +/high to define the heterogeneity of CD24 levels. However, we have now specified this distinction in the methods section. Please see lines 509-514.

-The cells in Figure 1B labeled "CD56+" (I think although font is tiny) appear to be the same cells labeled "CD56+/high" in Supplemental Figure 1D.

The reviewer is correct. As mentioned in the previous comment, we have changed all the "+/high" labels to simply "+".

-In Supplemental Figure 1D, the X-axis label in the top panel second column says "NCAM APC-A", while the label for the plot says "CD56+/high". Since CD56 = NCAM1 I understand what this means, but please make all the labeling consistent. Here I would probably change X-axis to "CD56-APC-A" or "CD56-APC" (like in Supplemental Figure 1B)

As suggested by the reviewer, all labels have been unified.

-What is the difference between FITC-A (Supplemental Figure 1D top) and FITC (the X-axis label just below)? What is the difference between "APC" and "APC-A"? What is the difference between "CD90 Alexa Fluor 700-A" (Supplemental Figure 1D top) and CD90- Alexa700" (Supplemental Figure 1D bottom)?

The reviewer makes an interesting remark. There is no real difference, it's just an editing issue that we have now explained in the methods section (please see lines 506-508) and figure legends. FITC-A, APC-A, etc., appear on the original FACS plots to indicate that we are reading the area (A) of the fluorescent signal, that is, the area that is under the curve of the fluorescent emission, resulting from: width (W) of the signal (or duration) on X, and H= height (H) of the signal on Y. Unless differently specified, the use of simple labels as FITC, PE, APC, etc., refers to the area of the fluorescent emission. Thus "A" is generally omitted in most of the published papers, and we have now done the same in the figures for which this is possible. However, for the channels where "W" and "H" are used for specific purposes, it is essential to identify and label them properly, to avoid confusion. Thus, we had to specify "A" and "W" when referring to parameters that are also used for single cell gating, i.e., Hoechst-A vs Hoechst- W and FSC-A/SSC-A vs FSC-W/SSC-W. Please see Supplemental Figure 1A and Supplemental Figure 4.

-Text line 134 refers to "CD56+/High CD271+" in Figure 1A. The label in Figure 1A says "CD56+ CD271+". Figure 1 legend says "CD56+/hi/CD271+". Spacing, dashes, backslashes, "+" versus "+/high" versus "+/hi" should only be different if they are intended to designate something different. The inconsistent labeling is not only confusing, but will make it more difficult to search the text.

We agree with the reviewer. As mentioned above, we have now unified all labels using $CD56^{+}$, $CD90^{+}$, etc., leaving only for CD24 a definition of different levels, since these refer to separate clusters.

- Lines 140-142: Redundant "qRT-PCR for neuronal and glial markers" used 2x in the same sentence.

As suggested by the reviewer, we corrected this mistake. Please see line 176.

- Supplemental Figure 3A: X-axis label is upside down, possibly backwards and has superimposed words. Also does Hoechst-A differ from Hoechst?

The reviewer has again correctly noticed some imperfections and omissions. Concerning the first point, we have edited the figure to avoid the confusing backward plot and the superimposed words have been removed. Essentially, we have moved the histogram plot to the right side, and, although 90 degrees rotated, it is with a forward orientation. The reason of the rotation has been mentioned in the supplemental figure legend. Please see lines 756-758.

Regarding the Hoechst-A, Hoechst nomenclature, we have explained in reply to previous points that FSC-A, SSC-A, Hoechst-A indicate the total area (A) of the detected signal (resulting from scattering or fluorescence), resulting from integration of the curve produced by H (height) and W (width) of the fluorescent signal. Simple labeling of the axes with "marker + fluorochrome" normally refers to the "-A" parameter, and the definition of A is skipped. However, the specification of FSCA as FSC-A, FSC-W, FSC-H, SSC as SSC-A, SCC-W, SSC-H, Hoechst as Hoechst-A, Hoechst-W, Hoechst-H is used when the W and H parameters are introduced with a specific application, that is, the separation of single from non-single cells. For the sake of clarity, when referring to FSC, SSC, and Hoechst, we have always specified "A" and "W" in our plots.

- Figure 5: Please include a flow cytometry (or other) schematic to indicate clearly what cell populations underwent scRNAseq. For example, were these simply dissociated cells, or were they flow sorted, and if so, for what markers? Did they sequence the CD56high/CD90high/CD24 high pool or something else? This is important so we can understand the RNAseq outcome data.

As suggested by the reviewer, we tried to make this point clear by adding a schematic panel in the beginning of the figure to show how the samples were processed. We sorted the full nucleated ENS cluster based on CD56/CD90 staining and Cycle Green for live DNA staining. This information has also been added in the figure legend. To be noticed, Figure 5 has become Figure 6 in the revised version of the manuscript.

- Line 391: Please clarify what was done to these cells prior to scRNAseq? It says "After tissue dissociation" but were these cells flow sorted for CD56/CD90/CD24 and if so, which flow pool was sequenced? This is important since the analyses yielded 22,549 cells with high quality data, of which 20,750 were glia and 351 were neurons (lines 402-403). This is remarkable enrichment for ENS, but as in other reported analyses only a small percentage of cells were enteric neurons.

As suggested by the reviewer, we have clarified this sentence by adding: "After tissue dissociation, cells were stained for CD56/CD90/CD24/Lin/DAPI and cycle green. Live/Lin^-/$CycleGreen^+$/$CD56^+$/$CD90^+$ cells were sorted and concentrated in 25 µL of PBS.". Please see lines 524-526. Moreover, we have added a schematic panel in Figure 5 that has become Figure 6, to show how cells were selected for scRNAseq.

- Lines 241 to 243 and Figure 5E: Authors call neuron subtypes "peripheral XX neurons", but it is probably better to call these gut-derived neurons "enteric XX neurons" to distinguish (for example) form other "peripheral sensory neurons" like dorsal root ganglion cells.

We have made the suggested change in the manuscript. Please see lines 285-289 and Figure 6E.

- Figure 5: There are two parts to this figure labeled "E" in both the figure itself and in the figure legend. Please label the violin plots "F" and change the figure legend. Surprisingly, Figure 5E is not referenced at all in the text (Lines 243 to 248) and instead they reference the missing Figure 5F. Please review all figure references.

The reviewer is totally correct. We have now corrected this mistake (see new Figure 6). We've also reviewed all figure references.

- Figure 5E/F: It is interesting that many of the cells in the "neuron" cluster are classified as "Differentiating neurons". While I see a little RET and SNAP25 in the violin plots, it would be nice to know more about these

cells, since this may be a surprising finding. In particular, there is debate among ENS scientists about the extent and frequency of ENS neurogenesis that occurs after birth and in adults. Pseudo-time analysis of cell in Figure 5A would be interesting, as would a separate pseudo-time analysis of just the neuronal cluster. For example, murine literature suggests Schwann cells can become neurons after birth or that a glial subset or a Nestin+ subset of cells might act as adult ENS precursors. Are these "Differentiating neurons" also the cells in Figure 5C that express PLP1, SOX10 and S100B? Are these really Schwann cells? Are the EDNRB+ cells the same as the NOTCH2+ cells?

The reviewer raises interesting points which merit further investigation. For this particular manuscript however, we opted not to pursue a more in-depth analysis of these topics for two reasons. Firstly, the neuronal data included in this manuscript lacks sufficient cell numbers, and thus power, to perform valid pseudo-time analysis. Moreover, the single cell data included in this manuscript was intended as a proof of concept, rather than a detailed investigation of the ENS transcriptomic landscape and as such, an exhaustive analysis was not the objective. We are currently preparing a separate paper in which we (using the isolation method described in this work) aimed to increase the dataset size as well as include single cell data from Hirschsprung patients in order to tackle some of the questions raised by the reviewer.

- Discussion and throughout the text: Authors discuss isolation of "neural cells", but actually recover >90% glia. Please consider revising the text to discuss neuron and glia isolation, recognizing prior strategies also isolate large numbers of glia. For example, line 262 talks about neural isolation, but unless I'm confused, CD90 and CD56 were used to isolate cells used for scRNAseq and only 1.6% of those cells are neurons.

As suggested by the reviewer, we have changed throughout the text all the definitions of “neural cluster” and “neural cells” to “ENS cluster” and “ENS cells”, sometimes “neurons and glia”.

- Line 281: Authors speculate that the function of CD24 in ENS is similar to function in CNS based on data showing expression of CD24 in ENS cells. I would omit this comment since there are no data presented about CD24 function and no earlier discussion of what CD24 function is in the CNS.

As suggested by the reviewer, we have deleted this sentence.

Line 289: Consider adding https://doi.org/10.1016/j.jcmgh.2020.12.014, a paper where ENS was dissected out and then used for RNAseq.

As suggested by the reviewer, we have incorporated this reference in the manuscript. Please see lines 370 and 393.

- Line 291: There are also many myenteric neurons in the human bowel submucosa, including close to the bowel epithelium.

As suggested by the reviewer, we have now rewritten the sentence: “The latter procedure can result in a biased sampling of EG, which are known to be distributed across the entire gut wall axis, but also of enteric neurons present in the submucosa and adjacent to the epithelium (Seguella & Gulbransen, 2021).” Please see lines 371-373.

- Line 292: This is the first time I can tell that the scRNAseq was performed on "sorted CD56+/CD90+ neural cells". I would not call these "neural cells" since 90% are glia and 1.6% are neurons based on scRNAseq. I would call them "ENS cells" or "enteric nervous system cells". Please show a FACS plot indicating precisely which cell population was used for scRNAseq (ideally in Figure 5)

These two points mentioned by the reviewer, have already been accounted for in the previous comments. All definitions of “neural cluster” and “neural cells” were changed throughout the text to “ENS cluster” and “ENS cells”, sometimes “neurons” and “glia”. We have also clearly defined the cell population used for scRNAseq in Figure 5, that is now Figure 6.

- Lines 299 to 302: In this paper, scRNAseq data demonstrate a 59 to 1 ratio of glia to neurons. While I do not know of reliable data for human infants, in adult human colon the glia to neuron ratio is 3-5.6 to 1 (See Figure 2 in https://doi.org/10.1053/j.gastro.2020.02.035). To the best of my knowledge all the other RNAseq papers from the bowel showed very high glia to neuron ratios, consistent with the idea that neurons are much more fragile than glia (e.g. 10x more fragile). Not sure text change is needed here, but the magnitude of these cell ratios based on cell counting versus scRNAseq might be worth emphasizing.

The reviewer makes a valid comment as indeed, current scRNAseq data appear to show a high glia to neuron ratio. Whilst this appears to be partly due to the delicate nature of neurons, we also have included statements in the manuscript which emphasize that quantification studies of the ENS not only have yet to be performed in children, but also have thus far neglected the extra-ganglionic glia presence. Including glia present in the muscularis propria, lamina propria and in the nerve fibers traversing the gut might impact the known ratios significantly. Please see lines 380-384.

- Line 308: I think the referenced studies are about mice. How many neurons were evaluated in any of the prior published human scRNAseq data for children?

The reviewer makes a valid comment. We are not aware of any scRNAseq data for children including enteric neurons. A recent review published by Guyer et al. (https://doi.org/10.3390/biom12030452) summarized all single-sequencing studies of the ENS, and here are only reported fetal and adult human data (Guyer *et al*, 2022).

- Line 315 suggests cells isolated by flow cytometry could be cultured. Are there any data to support the idea that cells isolated using this strategy will survive in culture?

The reviewer makes a valid point. We have not tried to cultured sorted ENS cells and have thus refrained from mentioning this in the manuscript.

Line 318 Says prior studies did not attempt to enrich for ENS. This paper microdissected myenteric plexus https://doi.org/10.1016/j.jcmgh.2020.12.014 before snRNAseq.

We thank the reviewer for evidencing this paper. We have changed the text accordingly. Please see lines 368-370.

- Line 339: Should be "ileostomy" and "colostomy" instead of "ileostoma" and "colostoma".

As suggested by the reviewer, we have corrected this point. Please see line 442.

- Figure 1 and other Figure legends: Since most of the isolated cells end up being glia, please refer to them as "enteric nervous system cluster". The same issue applies throughout the text and figure legends. If the clusters are "not just neurons", please characterize them more accurately.

As mentioned in previous comments, we have changed the definition from “neural” to “ENS” throughout the text.

- Line 435: What marker is meant by "for this marker"?

As suggested by the reviewer, we have modified the text to make it clear that we are referring to CD24.

- Line 452: "D)" says representative sorted neural cells, but the image says "Putative glia". I suspect this should be D-F) instead of just D, but please review carefully.
Figure legend 3D-F: I like the representative images, but an explanation of features that define for this group the different types of cells would be valuable. Does debris simply mean DAPI negative?

As suggested by the reviewer, we have modified the text accordingly Please see lines 222-225. Regarding the figure legend 3D-F, debris was used as equivalent of DAPI or Hoechst negative. Nuclear staining on live cells was here based on Hoechst33342. Fixed cell staining for FACS and microscopy was based on DAPI. We have added these remarks in the legends of Figure 3 and Figure 4.

- Figure legend 3 D-F: Indicate that cells are DAPI stained.

As suggested by the reviewer, we have added the DAPI stain to the figure description. In addition, for the sake of clarity, we have divided microscopy from Figure 3 and made a separate Figure 4 for microscopy, in which pre-stained cells (Lin, Live-fixable violet, CD56, CD90, CD24) were fixed with formalin and sorted out. DAPI was added together with the mounting medium.

- Figure 4: The purple ELAVL4 stained cells on the FACS plots have an interesting pattern. While most FACS analyzed cells form "clouds" of cells in a cluster (each dot apparently randomly located relative to others in the cluster), the purple "cells" form multiple short lines, each 6 dots long in a zig-zag line. I am guessing this is what happens when you plot "in bold to improve visibility" but this is not really "bold". Is there a better way to describe this symbol or a better way to plot?

We agree with the reviewer, that the shape of the dots is a bit “peculiar”. However, this is what the DIVA software which we used for analysis, allowed. For the sake of clarity, we have also used density plots and normal dots in new Figure 5D and Suppl. Figure 4A-H, to show how the plots look like using these alternative options. However, we have kept the enlarged dots in main figure 5A, B to allow better visibility of the small $CD24^{TIP}$ population. We have renamed these “dots” as “enlarged dots” (big size dots according to DIVA software) instead of “bold”. Please see lines 654-655, 660-662, (all from legend Figure 5), 775-777 (legend Suppl. Figure 4),

- Supplemental Figure legend 3: Says standard cell cycle plot is on the left and Hoechst + versus negative are on the right. I think left and right are reversed.

The reviewer makes a valid comment. This was indeed an error. Furthermore, we noticed that the sentence had to be better explained. However, following a previous comment on the same figure, we have performed editing of the indicated elements of Supplemental Figure 3. To fix the backward orientation of cell cycle plot, we have moved it to the right side. So now, standard cell cycle plot is on the right and Hoechst + versus negative are on the left.

Referee 2:

This manuscript addresses an important question in the ENS field, which is how human ENS cells (which represent a very small minority of all cells in the gut) can be enriched to facilitate targeted analyses such as scRNAseq. However, this reviewer feels that a significant amount of work would be needed before considering it as a good fit for EMBO Reports. There are a number of key questions that would need to be addressed:

We thank the reviewer for the positive evaluation of the relevance of our work. We have made an effort to address (to our ability) all the key questions raised by the reviewer.

1- In the end, what is the added value of using a combination of CD56/CD90 vs CD56 alone to sort ENS cells? Hard to see how this is needed for scRNAseq.

The reviewer has raised here a relevant point that deserves further explanation, as well as some editing of the manuscript, to be clarified. We are aware that in our method, the major improvement in ENS cell purity comes from the use of CD56 as ENS-selective marker. For the study of the ENS by scRNAseq, the reviewer is partially correct, as the addition of CD90 to live samples, only brings a small improvement (from 97 to ~100%). However, there are cases, in particular in fixed samples, where signal to noise ratio of CD56 might lead to lower discriminatory capacity of CD56 as a single marker (due to different types of background and lower live/dead/Lin discrimination). To make this point clear, in Supplemental Figure 2H we have added a graph that shows the match of different markers or marker, combinations vs TUBB3. It becomes evident that CD56 alone has a certain loss of resolution, dropping to ~92% match, whereas CD56/CD271 and CD56/CD90 equally perform near 100%. In the new graph in Figure 2C, the $CD56^{+}/CD90^{+}$ cluster analyzed vs TUBB3 and SOX10, produces almost 100% match with both markers. As we intended to design a protocol that could be used for a variety of downstream applications beyond scRNAseq, where high purity may become a major issue (e.g., qRT-PCR, western blotting, proteomics, metabolomics, imaging flow cytometry, and mass cytometry), we wanted it to offer the best purification possible of the populations and subpopulations of interest. In the revised manuscript, we have tried to reduce in the text an excessive focus on scRNAseq as the sole potentially valuable application of our method. Please see lines 404-419 and the Abstract, which has been revised according to this point.

Lastly, we would like to emphasize another added value of the inclusion of CD90 (and in parallel of CD24), to our panel that goes beyond sample purity. In our manuscript we show that by introducing these markers to the staining panel, we were able to identify heterogeneity within the ENS cluster (please see lines 174-175). We illustrate this as well with the improved comparison of ganglionic vs. aganglionic tissue in Hirschsprung patients (please see Figure 5D, E and Supplemental Figure 4E-I). We show that two subpopulations ($CD24^{LOW}$ and $CD24^{TIP}$, the latter corresponding to $CD90^{L}$) are absent in aganglionic tissue, and provide additional insights on the subdivision of the ENS cluster (please see lines 246-259, and legends relative to Figure 5 and Suppl. Fig 4). Whilst yet further work is necessary to better characterize these populations, we believe our analyses, and thus the inclusion of these markers, opens the way to a more refined flow cytometric analysis and sorting of ENS cells.

2- Where are all of these markers (also including CD24 and CD271) expressed in the developing human ENS? Cytometry data should be accompanied by immunofluorescence data for that cohort.

The reviewer's comment is well appreciated. To clarify this point, we have added in Figure 1A our immunostaining on human pediatric intestinal tissue, analogous to the samples used in our flow cytometry experiments. Interestingly, CD56 and CD271 were expressed in the ENS throughout all layers of the gut, from ganglia to submucosa to nerve fibers within the lamina propria. However, CD90 appeared to be preferentially expressed within ganglia, whilst CD24 was localized both within ganglia, as on neuronal fibers in the muscularis mucosae, evidencing their neuronal specificity. These data are now presented in the manuscript. Please see lines 156-160.

3- Why are there so few neurons recovered? They should represent about 14% of recovered ENS cells (based on 1:7 ratio), not only 2%. Perhaps CD56/NCAM is expressed in only a subset of them? Alternatively, as pointed out by the authors, neurons might be harder to recover using enzymatic-based dissociation. Immunofluorescence would again be helpful to address all of these questions.

The reviewer's point is quite relevant and focused on one of the main points that may evidence a bias in our ENS cell preparation and analysis. However, the 1:7 ratio is based on quantifications performed in ganglia, rather than the entirety of the gut (Hoff *et al*, 2008). It is thus probable that using full-thickness biopsies, this ratio might be higher as there are a plethora of glia present within the muscularis as well as nerve fibers traversing the gut wall (Please see lines 380-382). Furthermore, these analyses have been performed in adult tissue and murine models, rather than pediatric tissue, from which data on glia vs neuron quantification is lacking (see lines 382-384).

Moreover, the recovery rate of cells (in this case enteric neurons) following tissue digestion and sorting is inherently biased by viability and dissociation method, rendering a direct comparison to immunohistochemical quantifications unfair. Our scRNAseq data also showed a lower average ratio neurons/ENS cells (1.6%) than the ratio predicted by FACS (2.9% according to $CD24^{TIP}$ and 2.5% according to $ELAVL4^{+}$) thus, corroborating that the sorting procedure and post-sorting processing for scRNAseq may introduce an additional bias against neurons. This is made clear in the new added Table 2, where, in addition to patient characteristics, we also show that the recovery of cells for scRNAseq is much lower than the number of sorted cells, thus indicating cell loss during the procedure. We have now included these points in the manuscript. Please see lines 387-391 and Table 2 (which makes clear that the number of cells successfully sequenced is significantly lower than the number of sorted cells).

Regarding the eventual presence of subsets of neurons that do not express CD56, we believe this is highly unlikely to occur. The existing literature (as cited in our manuscript) indicates CD56 as universally expressed in neurons. Moreover, we show a representative staining of pediatric tissue where all neurons are $CD56^{+}$ (please see Figure 1A). We have also inserted in the revised manuscript, Figure 2C, which shows validation of our ENS cluster against TUBB3 and SOX10, indicating complete match of our CD56/CD90 selected cells with these markers. In non-ENS cells $TUBB3^{+}$ events are only 0.15% (Figure 2C), thus much lower than expected for eventual $CD56^{-}$ neurons in a 1:7 ratio with ENS cells. Moreover, these $CD56^{-}TUBB3^{+}$ events do not form any definite cluster compatible with a neuronal population, but are scattered around with higher density at the lowest TUBB3 levels. Thus, such events are only compatible with some (really minimal) background noise, ruling out the presence of neurons that are not detected by our markers. For the sake of clarity, we have also added Supplemental Figure 2H, where, among different markers and combinations of markers, we show that the $CD271^{+}CD56^{-}$ events are totally TUBB3 negative, again ruling out the presence of potential $CD271^{+}CD56^{-}$ neurons. These observations are now discussed in the manuscript. Please see lines 201-203, Figure 2C legend, Supplemental Figure 2H legend.

4- There is a profound lack of details about the samples that were analyzed. How many N per condition? Is it needed to pool multiple samples from different individuals? How many ENS cells are contained within a single sample? What is the exact age of all individuals involved in this study?

The reviewer has raised valid points, and we realize that we had initially not provided sufficient details. Below, is a point-by-point response to the comments raised.

How many N per condition?

As suggested by the reviewer, we have specified the number of replicates/samples per condition where applicable. We have also updated the statistics reported in many plots and graphs not only by specifying the N per condition, but also by increasing the N replicates. Please see all the lines highlighted in Figure legends. In particular, as also mentioned in reply to reviewer 1, regarding Hirschsprung colon samples, we have extended our preliminary analysis (N=1) to more samples (N=4) and improved the resolution of the subpopulations. In particular, low CD24 levels were not well visible due to loss of CD24 staining intensity after room temperature incubation (for cell cycle dye saturation), combined with spillover from the Cycle Green nuclear dye. The new round of analysis was based on Hoechst 33242 staining, as shown in Figure 3 and 5B (previous 4B), leading to the visualization of differences in ganglionic vs aganglionic colon, which were not properly appreciated before. We have also made sure that the pieces of aganglionic colon we used were not contaminated by surrounding

ganglionic regions, and vice versa. Please, about all changes related to Hirschsprung patients see lines 248-259, 666-670, 782-790.

Is it needed to pool samples from different individuals? How many cells are contained within a single sample?

Pooling of samples from different individuals for scRNAseq was not necessary. We have added Table 2 to the manuscript which describes how many cells were obtained from each individual sample. Similarly, for our flow cytometric experiments, each sample always yielded sufficient cells for an individual assay. Multiple samples were used whenever we needed to perform biological replicates, antibody titrations, negative controls, and re-iterations due to technical issues with the flow cytometer.

What is the exact age of all individuals involved in this study?

The age of the individuals included for the scRNAseq experiments in the study, can now be seen in Table 2. We did not specify in our manuscript the exact age of the samples (n > 50) used for all the flow cytometric experiments (we collected biopsies from children younger than 1 year of age, as described in the Methods section of the manuscript, line 442). Our study design sought to ascertain a constant reproducible pattern of ENS cell identification rather than to assess staining pattern as a function of age and thus, this parameter was not recorded systematically for all samples used for flow cytometric experiments.

References

Guyer RA, Mueller JL, Goldstein AM (2022) Applications of Single-Cell Sequencing Technology to the Enteric Nervous System. *Biomolecules* 12

Hoff S, Zeller F, von Weyhern CW, Wegner M, Schemann M, Michel K, Ruhl A (2008) Quantitative assessment of glial cells in the human and guinea pig enteric nervous system with an anti-Sox8/9/10 antibody. *J Comp Neurol* 509: 356-371

Sacchetti A, Teeuwssen M, Verhagen M, Joosten R, Xu T, Stabile R, van der Steen B, Watson MM, Gusinac A, Kim WK *et al* (2021) Phenotypic plasticity underlies local invasion and distant metastasis in colon cancer. *Elife* 10

Seguella L, Gulbransen BD (2021) Enteric glial biology, intercellular signalling and roles in gastrointestinal disease. *Nat Rev Gastroenterol Hepatol*

Dear Dr. Alves,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. As you will see, both referees still have a few more minor suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript. Please also co-submit a point-by-point response with your final ms.

A few other editorial requests also need to be addressed:

- Please correct the conflict of interest subheading to "Disclosure and Competing Interests statement".

- Please add the FUNDING INFO to the ms and also enter it in our online ms submission system when you submit the final ms.

- The Appendix Figure S3C callout is missing, please add.

- A table of content with page numbers is missing from the APPENDIX FILE, please add. The figure legends need to be moved to below the figure in the Appendix file, and should be deleted from the ms file.

- Please remove the list of Abbreviations from the ms.

- "Methods" needs correcting to 'Materials & Methods'.

- The manuscript sections are in the wrong order, please correct.

- Table 2 is missing the name and legend/title, please add.

- Please remember and confirm that your deposited data needs to be publicly available upon the online publication of your ms.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms.

I would like to suggest some minor changes to the title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

A combinatorial panel for flow cytometry-based isolation of enteric neural cells from human intestine

Efficient isolation of neurons and glia from the human enteric nervous system (ENS) is challenging, because these rare and delicate cells have to be obtained from scarce patient material. Here, we developed a panel to enrich for ENS cells from human intestine by fluorescence-activated cell sorting (FACS). Based on CD56/CD24/CD90 co-expression, we label ENS cells with higher specificity and resolution than previous methods. Surprisingly, neuronal (CD24, TUBB3) and glial (SOX10) selective markers appear co-expressed by all ENS events [What do you mean by "event"? Please re-write]. We demonstrate that this staining pattern is mainly driven by neuronal fragments, either free or attached to glial cells, and that live intact neurons express the highest CD24 and CD90 levels. We apply our protocol to isolate ENS cells for single-cell RNA sequencing and show that these cells can be obtained with high quality, enabling interrogation of the human ENS transcriptome. Taken together, we developed a new FACS protocol that is very selective for human ENS cells and allows discrimination of neurons and glia, opening up new avenues to study this complex system in health and disease.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

This manuscript was extensively and thoughtfully revised, taking into consideration all prior reviewer comments. The work is beautifully designed and well-illustrated. There are a few remaining very minor issues that the authors should consider. This will be a very valuable addition to the literature, providing the best method to date to isolate ENS cells from human small bowel and colon. I congratulate the authors on this really nice work!

Minor issues:

Line 111: Should be "reviewed" instead of "revised", I suspect.

Figure 1E: The Y-axis label should refer to CD271 instead of to CD217?

Figure 1F: Grey bars are labeled "non-neural cells" but is there a more specific designation that might make sense? For example, are cells in the grey bar CD56- CD90- CD271- or are they defined in another unambiguous way? In part the current labeling is confusing since the CD56+ CD90+ cells appear to include neurons and glia, whereas "non-neural cells" group has low levels for neuronal or glial markers but should be where glia are found! "Non-ENS cells" used in the Figure legend might make more sense.

Figure 2A legend: Text should say "are additionally stained" instead of "are additional stained".

Figure 3D: Legend says this is supposed to be a FSC vs SSC plot. Instead, it appears that the plot used in Figure 3B is also shown in Figure 3D. Please insert the correct image.

Figure 3D: The separated "Non-nucleated", "Nucleated" and "Merge" plots do show FSC versus SSC, but are poorly labeled (no labeling on the top, with axes labeled "FSC-A" and "SSC-A" instead of "FSC" and "SSC"). I'm not sure which labeling is correct but the figure legend and the figure should match.

Figure 5A: In the box at the bottom left indicating the color legend, the purple bar is shifted to the right compared to the other two bars. Data are still interpretable, but consider aligning the bars. Similar issue for Figure 6A. On further review, I see that this indenting in intentional, but left my comments so you see my confusion. I like the annotation in Figure 2A and 3A with the small arrow indicating that the indentation is there because this is a subset of the non-indented cells. There are many other places where the small arrows showing sub-sets are a nice touch.

Figure 6B: Are these only CD56+ CD90+ or are the cells shown really Live Lin- CD56+ CD90+?

Appendix Figure S1: Part A top 3 graphs have different font on Y-axes compared to the rest of the figure.

Appendix Figure S2: Part G refers to SSC in text and SSC-A in graph Y-axis labels. Please make sure figure legend and graph labels match to avoid ambiguity.

Appendix Figure S4: Part D. What is the purpose of the tan/green/red bar at the bottom of the page? Were bars in the graph supposed to be multi-color instead of black?

Please review for uniformity. Sometimes there are extra "/" for example CD56+/CD90+ versus CD56+ CD90+. The same convention should be used throughout the text and figures, as well as in figure legends.

Line 189: I'm confused. How could one provide quantitative data on glia to neuron ratio "outside myenteric and submucosal plexuses"? There are NO NEURONS outside the plexus (right?) so glia:neuron ratio outside the plexus is infinite if you count any glia outside of ganglia as outside the plexus. Arguably enteric glia are part of the "plexus" even when outside of ganglia, but there are many glia associated with small nerve fibers that some might not consider part of the plexus. Glia to neuron ratios in adults (including those in myenteric ganglia) are reported in this manuscript: 10.1053/j.gastro.2020.02.035. Consider revising lines 189 to 192 to provide simpler text "Ratios of neurons to glia in human bowel are not well established, especially in children." The manuscript referenced was adult and provides neuron and glia numbers after 3D imaging only in the layers near myenteric plexus.

Line 197: "CD24 was expected to be exclusive to neurons". Do authors mean within the ENS CD56+ CD90+ pool, CD24 was expected to be in neurons and not in glia? CD 24 is expressed in most B cells and in developing neuroblasts.

Line 264: Indicated that their sorting protocol enriches for "viable" neuronal and glial populations. Viable means "living" cells. What data in this section shows that the isolated cells are alive or could be cultured? What method was used to show the cells are alive?

Line 295: Authors refer to RET as a "more differentiated neuronal marker" but RET is also expressed in fetal ENS precursors

shortly after these cells begin to colonize the bowel. RET expression might be higher in a subset of mature enteric neurons and lost in other enteric neurons and in glia. Consider selecting a different "differentiated neuron marker" or omitting this comment about RET.

Line 371: Should be "from" instead of "form" at the end of this line.

Authors note that ENS cells isolated may have fragments of other cells attached, yet the neuron and glial UMAP clusters appear quite separate in Figure 6B and 6G. Does scRNAseq suggest that cell fragments adhering to ENS cells did not significantly affect sequencing data? This is important since many cells were TUBB3+ and SOX10+ (Figure 2C). Did the additional steps to remove cells without nuclei solve this problem? An extra sentence or so in the Discussion about how the clear separation of neuron from glia in the scRNAseq data was achieved when the "cells" in Figure 2C that have both neuron and glial markers would be helpful.

Line 475: Please provide centrifugal force in "x g" instead or in rpm units, or provide the exact centrifuge rotor (and diameter) so one could calculate "x g" (less preferred option). Also, I think they mean "swinging bucket rotor" instead of "swing bucket . . . centrifuge". Line 492 and 495 also give rpm instead of "x g". Please provide "x g" in all places where centrifugation occurs.

Table 1: Consider adding RRID numbers antibodies (https://scicrunch.org/resources). Add information for Hoechst 33342.

Referee #2:

Most of my previous comments have been properly addressed with the exception of immunofluorescence data that are now included in Fig.1A. These data show that all markers are expressed in neurons (TUBB3+), but what about glia? Both low and high magnification views should be shown for all stainings. A pan-glia marker should also be included in theses analyses (SOX10 is listed in Table 1 but not used here; S100b would be a good alternative). Moreover, in contrast to what the authors state, CD90 does not look more specific to ganglia compared to the other markers (there seems to be many other cells/projections that are also positive; but perhaps these are blood vessel-associated endothelial cells?). Please clarify.

Other minor points that should be addressed to improve clarity:

-Enteric glial cells should be abbreviated EGCs, not EG.

-qRT-PCR abbreviation and meaning is not correct; it should be RT-qPCR for Reverse Transcriptase quantitative PCR.

-Alternative name(s) should be mentioned for CD24, as already indicated for all other CD markers in the Introduction.

-The color-coded legend in Fig1B is unnecessary/confusing;

-Bars in some of the color-coded legends are not of the same size

-All axis titles should be carefully revised for accuracy (e.g., CD217 in Fig1E; PIMN/PSN/PEMN in Fig.6G; NCAM-APC in FigS5A...)

-Number of replicates are still not systematically indicated, either in text, legend or figures.

-Usage of "neural" (designing both neurons and glia) vs "neuronal" (designing neurons only) should also be carefully revised throughout the manuscript; the second subtitle of the Results section suggest that fragments of both neurons and glia can be found whereas the corresponding figure only shows neuronal fragments.

-The authors should also be cautious when referring to the overlap between CD24-LOW/HI and CD90-R/L clusters, which is not a perfect match according to the percentages shown in Fig.S2D-E.

-UCHL1 is misspelled UHCL1 on line 288.

Response to the editorial comments

- Please correct the conflict of interest subheading to "Disclosure and Competing Interests statement".

- As pointed out by the editorial office, we have corrected this point.

- Please add the FUNDING INFO to the ms and also enter it in our online ms submission system when you submit the final ms.

- As pointed out by the editorial office, we have added the funding information to the manuscript.

- The Appendix Figure S3C callout is missing, please add.

- As pointed out by the editorial office, we have corrected this point. Figure S3C callout was missed due to a typing error. Now we refer to Appendix Figure S3C-E (line 219).

- A table of content with page numbers is missing from the APPENDIX FILE, please add. The figure legends need to be moved to below the figure in the Appendix file, and should be deleted from the ms file.

- As pointed out by the editorial office, we have corrected this point.

- Please remove the list of Abbreviations from the ms.

- As pointed out by the editorial office, we have corrected this point.

- "Methods" needs correcting to 'Materials & Methods'.

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- The manuscript sections are in the wrong order, please correct.

- As pointed out by the editorial office, we have corrected this point.

- Table 2 is missing the name and legend/title, please add.

- As pointed out by the editorial office, we have corrected this point.

- Please remember and confirm that your deposited data needs to be publicly available upon the online publication of your ms.

- The data has been uploaded to the Gene Expression Omnibus server. We are awaiting approval of the files and will include the record number in the final proofreading. Moreover, we will communicate the GEO accession number to the editor as soon as we receive it. We expect this to take no longer than 7 days from the date of re-submission (31st of January).

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms.

- As pointed out by the editorial office, we have addressed these points.

- I would like to suggest some minor changes to the title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

A combinatorial panel for flow cytometry-based isolation of enteric neural cells from human intestine

Efficient isolation of neurons and glia from the human enteric nervous system (ENS) is challenging, because these rare and delicate cells have to be obtained from scarce patient material. Here, we developed a panel to enrich for ENS cells from human intestine by fluorescence-activated cell sorting (FACS). Based on CD56/CD24/CD90 co-expression, we label ENS cells with higher specificity and resolution than previous methods. Surprisingly, neuronal (CD24, TUBB3) and glial (SOX10) selective markers appear co-expressed by all ENS events [What do you mean by "event"? Please re-write]. We demonstrate that this staining pattern is mainly driven by neuronal fragments, either free or attached to glial cells, and that live intact neurons express the highest CD24 and CD90 levels. We apply our protocol to isolate ENS cells for single-cell RNA sequencing and show that these cells can be obtained with high quality, enabling interrogation of the human ENS transcriptome. Taken together, we developed a new FACS protocol that is very selective for human ENS cells and allows discrimination of neurons and glia, opening up new avenues to study this complex system in health and disease.

- We have considered the editor comments and have made the adequate changes to the title of the manuscript. However, due to previous comments of the reviewers, especially reviewer 1, we think that is more correct to refer to enteric neurons and enteric glia as enteric nervous system cells and not as enteric neural cells. Throughout the manuscript, this is how we refer to these cells and thus, we decided to keep the initial terminology. We have also corrected the abstract accordingly.

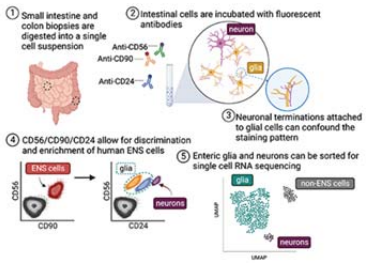
- **EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.**

  - As suggested by the editorial office, the required information can be seen below. The synopsis image was uploaded in the submission system and can be seen below as well.

A) Short summary:
This work describes flow-cytometry based enrichment of human enteric neurons and glia using a combination of cell surface markers (CD56/CD90/CD24), which enable studies of the human ENS.

B) Bullet points:
- Based on CD56/CD90/CD24 co-expression, enteric nervous system cells can be enriched by flow cytometry with higher specificity and resolution than previous methods
- Enteric neurons representing a minority of the ENS cluster, can be resolved from enteric glial cells based on CD24 levels.
- CD56/CD90/CD24 positive cells can be further processed for downstream analysis, such as scRNAseq, enabling interrogation of the human ENS transcriptome.

C) Synopsis image:



Response to Referees

Referee #1:

This manuscript was extensively and thoughtfully revised, taking into consideration all prior reviewer comments. The work is beautifully designed and well-illustrated. There are a few remaining very minor issues that the authors should consider. This will be a very valuable addition to the literature, providing the best method to date to isolate ENS cells from human small bowel and colon. I congratulate the authors on this really nice work!

- We are grateful to Referee #1 comments and his very positive evaluation of our work.

Minor issues:

Line 111: Should be "reviewed" instead of "revised", I suspect.

- As suggested by the reviewer, we have corrected this point.

Figure 1E: The Y-axis label should refer to CD271 instead of to CD217?

- As suggested by the reviewer, we have corrected this point.

Figure 1F: Grey bars are labeled "non-neural cells" but is there a more specific designation that might make sense? For example, are cells in the grey bar CD56- CD90- CD271- or are they defined in another unambiguous way? In part the current labeling is confusing since the CD56+ CD90+ cells appear to include neurons and glia, whereas "non-neural cells" group has low levels for neuronal or glial markers but should be where glia are found! "Non-ENS cells" used in the Figure legend might make more sense.

- As suggested by the reviewer, we have corrected this point.

Figure 2A legend: Text should say "are additionally stained" instead of "are additional stained".

- As suggested by the reviewer, we have corrected this point.

Figure 3D: Legend says this is supposed to be a FSC vs SSC plot. Instead, it appears that the plot used in Figure 3B is also shown in Figure 3D. Please insert the correct image.

- We have now removed the repetition of the CD56/CD90 plot for the sake of clarity. Figure 3D only shows the FSC vs SSC plot mentioned in the Figure Legend.

Figure 3D: The separated "Non-nucleated", "Nucleated" and "Merge" plots do show FSC versus SSC, but are poorly labeled (no labeling on the top, with axes labeled "FSC-A" and "SSC-A" instead of "FSC" and "SSC"). I'm not sure which labeling is correct but the figure legend and the figure should match.

- We agree with the reviewer. As suggested, Figure 3D was corrected and we have now made sure that the figure legend and the figure match.

Figure 5A: In the box at the bottom left indicating the color legend, the purple bar is shifted to the right compared to the other two bars. Data are still interpretable, but consider aligning the bars. Similar issue for Figure 6A. On further review, I see that this indenting in intentional, but left my comments so you see my confusion. I like the annotation in Figure 2A and 3A with the small arrow indicating that the indentation is there

because this is a subset of the non-indented cells. There are many other places where the small arrows showing sub-sets are a nice touch.

- As suggested by the reviewer, a small arrow was added to Figure 5A and Figure 6A indicating indentation.

Figure 6B: Are these only CD56+ CD90+ or are the cells shown really Live Lin- CD56+ CD90+?

- The reviewer is correct, the cells shown are Live Lin-/CD56+ CD90+. As described in Appendix Figure S1A the cells are always first selected - using a single exclusion gate on the same channel - as DAPI negative (live) and Lin negative. After this pre-selection, ENS cells are specifically selected as CD56+ CD90+.

Appendix Figure S1: Part A top 3 graphs have different font on Y-axes compared to the rest of the figure.

- As suggested by the reviewer, we have corrected Appendix Figure S1.

Appendix Figure S2: Part G refers to SSC in text and SSC-A in graph Y-axis labels. Please make sure figure legend and graph labels match to avoid ambiguity.

- As suggested by the reviewer, we have corrected the figure legend of Appendix Figure S2.

Appendix Figure S4: Part D. What is the purpose of the tan/green/red bar at the bottom of the page? Were bars in the graph supposed to be multi-color instead of black?

- The reviewer makes a valid point. The colors in the figure indicate: total ENS cluster particles (red), nucleated ENS particles (green), and non-nucleated ENS particles (dark yellow). We have restructured the graph to make this more evident and have explained this in the figure legend of the Appendix Figure S4.

Please review for uniformity. Sometimes there are extra "/" for example CD56+/CD90+ versus CD56+ CD90+. The same convention should be used throughout the text and figures, as well as in figure legends.

- As suggested by the reviewer, we have removed the extra "/", and have revised the manuscript for uniformity.

Line 189: I'm confused. How could one provide quantitative data on glia to neuron ratio "outside myenteric and submucosal plexuses"? There are NO NEURONS outside the plexus (right?) so glia:neuron ratio outside the plexus is infinite if you count any glia outside of ganglia as outside the plexus. Arguably enteric glia are part of the "plexus" even when outside of ganglia, but there are many glia associated with small nerve fibers that some might not consider part of the plexus. Glia to neuron ratios in adults (including those in myenteric ganglia) are reported in this manuscript: 10.1053/j.gastro.2020.02.035. Consider revising lines 189 to 192 to provide simpler text "Ratios of neurons to glia in human bowel are not well established, especially in children." The manuscript referenced was adult and provides neuron and glia numbers after 3D imaging only in the layers near myenteric plexus.

- The reviewer makes a valid point. As suggested, we have revised lines 185-186 in the manuscript and have also included reference to the work of Graham et al., 2020.

Line 197: "CD24 was expected to be exclusive to neurons". Do authors mean within the ENS CD56+ CD90+ pool, CD24 was expected to be in neurons and not in glia? CD24 is expressed in most B cells and in developing neuroblasts.

- The reviewer makes a valid point. Indeed, we refer to the ENS cluster only. We have revised the text to make this point clear.

Line 264: Indicated that their sorting protocol enriches for "viable" neuronal and glial populations. Viable means "living" cells. What data in this section shows that the isolated cells are alive or could be cultured? What method was used to show the cells are alive?

- The reviewer raises a valid point. Throughout our manuscript 'live' or 'viable' cells have been defined as cells with preserved membrane integrity and thus capable of excluding viability dyes such as DAPI and trypan blue. Moreover, potential $DAPI^-$ apoptotic cells, with fragmented DNA (sub-G1), were also excluded by means of live DNA staining (this point was added to the results, referring to Appendix Figure S3A,B. (lines 215-216). Of course, some degree of loss of viability is to be expected post-sorting, which is inherent to the stress endured during sorting. Regardless, re-analysis by FACS of the sorted cells shows 86±4% viability according to DAPI staining (line 275) and also shows that no additional DNA fragmentation occurs. Moreover, scRNAseq demonstrated the presence of a high number of cells with a good RNA expression pattern, which means they were likely intact, i.e, with conserved membrane integrity. However, we agree that, viable in this context, does not necessarily imply that these cells can be cultured. We have not attempted to culture our sorted cells, and thus cannot answer this question. We have made clear in the manuscript that our definition of post-sorting viability is limited to the abovementioned.

Line 295: Authors refer to RET as a "more differentiated neuronal marker" but RET is also expressed in fetal ENS precursors shortly after these cells begin to colonize the bowel. RET expression might be higher in a subset of mature enteric neurons and lost in other enteric neurons and in glia. Consider selecting a different "differentiated neuron marker" or omitting this comment about RET.

- The reviewer makes a valid point. To avoid confusion, we decided to omit RET in this sentence, as suggested.

Line 371: Should be "from" instead of "form" at the end of this line.

- As suggested by the reviewer, we have corrected this point.

Authors note that ENS cells isolated may have fragments of other cells attached, yet the neuron and glial UMAP clusters appear quite separate in Figure 6B and 6G. Does scRNAseq suggest that cell fragments adhering to ENS cells did not significantly affect sequencing data? This is important since many cells were TUBB3+ and SOX10+ (Figure 2C). Did the additional steps to remove cells without nuclei solve this problem? An extra sentence or so in the Discussion about how the clear separation of neuron from glia in the scRNAseq data was achieved when the "cells" in Figure 2C that have both neuron and glial markers would be helpful.

- The reviewer's comment is appreciated. We did not perform any additional steps to remove cell fragments attached to neurons or glia. However, the reason we think we were able to get a clear separation of these two cell types (and of a few other cell types carrying neuronal fragments as well) in scRNAseq is due to the fact that cell fragments are largely devoid of intact mRNA, and therefore are not targeted for sequencing. We included and extra sentence in the discussion addressing this. Please see lines 375-376.

Line 475: Please provide centrifugal force in "x g" instead or in rpm units, or provide the exact centrifuge rotor (and diameter) so one could calculate "x g" (less preferred option). Also, I think they mean "swinging bucket rotor" instead of "swing bucket . . . centrifuge". Line 492 and 495 also give rpm instead of "x g". Please provide "x g" in all places where centrifugation occurs.

- As suggested by the reviewer, we have corrected the points raised. We also realized that we had mistakenly written rpm instead of rcf. We have corrected this now.

Table 1: Consider adding RRID numbers antibodies (https://scicrunch.org/resources). Add information for Hoechst 33342.

- The reviewer comment is appreciated. However, we noticed that some antibodies, ex: EpCAM antibody ESA214, appears as discontinued in the database, but is still produced by GeneTex (Cat No. GTX30708). Therefore, since we don't know if the database is regularly updated, we decided that providing the catalog numbers was the best option. We have added information for Hoechst 33342.

Referee #2:

Most of my previous comments have been properly addressed with the exception of immunofluorescence data that are now included in Fig.1A. These data show that all markers are expressed in neurons (TUBB3+), but what about glia? Both low and high magnification views should be shown for all stainings. A pan-glia marker should also be included in theses analyses (SOX10 is listed in Table 1 but not used here; S100b would be a good alternative). Moreover, in contrast to what the authors state, CD90 does not look more specific to ganglia compared to the other markers (there seems to be many other cells/projections that are also positive; but perhaps these are blood vessel-associated endothelial cells?). Please clarify.

- We thank the reviewer for the valuable suggestions and tried to address them in the manuscript. The following changes were made to Figure 1A:
  1) We have substituted the neuronal marker TUBB3 with ELAVL4, the latter being specific to the soma of neurons, which we believe provides a more precise indication of co-localization of our markers of interest to the neuronal cell body, than TUBB3 which is also expressed in neurites and axons.
  2) We have performed multi-color confocal immunostainings including the pan-glia marker S100B as well as ELAVL4, in the same assay to demonstrate co-localization with our markers of interest.
  3) Both low and high magnification views are provided for all stainings.
  4) We have corrected our description of CD90 specificity and now state in the manuscript that all markers co-localize to both neurons and glia in the pediatric ENS. Please see lines 157-159.
  5) We have elected to remove CD271 immunostainings from Figure 1A, as this marker is not included in our final marker panel, and thus, we believe that this information is irrelevant for our studies.

Other minor points that should be addressed to improve clarity:

- Enteric glial cells should be abbreviated EGCs, not EG.

- As suggested by the reviewer, enteric glial cells were abbreviated to EGCs in the manuscript.

- qRT-PCR abbreviation and meaning is not correct; it should be RT-qPCR for Reverse Transcriptase quantitative PCR.

- As suggested by the reviewer, we have corrected the point raised.

- Alternative name(s) should be mentioned for CD24, as already indicated for all other CD markers in the Introduction.

- As suggested by the reviewer, we added the alternative name for CD24. Please see lines 114-115.

-The color-coded legend in Fig1B is unnecessary/confusing;

- As suggested by the reviewer, we have removed the legend in Figure 1B.

-Bars in some of the color-coded legends are not of the same size

- As suggested by the reviewer, we have corrected the point raised.

-All axis titles should be carefully revised for accuracy (e.g., CD217 in Fig1E; PIMN/PSN/PEMN in Fig.6G; NCAM-APC in FigS5A...)

- As suggested by the reviewer, we have corrected these inaccuracies. We also want to mention that we decided to refer to neuronal subtypes as Enteric Inhibitory Motor Neurons (EIMN), Enteric Sensory Neurons (ESN) and Enteric Excitatory Motor Neurons (EEMN), as we believe these represent the clearest terminology for these cells.

-Number of replicates are still not systematically indicated, either in text, legend or figures.

- The reviewer is right, sometimes this point was missed. We have now checked throughout the text and systematically indicated the number of replicates in figure legends. Also, we have revised all the statistics and added more replicates when possible.

-Usage of "neural" (designing both neurons and glia) vs "neuronal" (designing neurons only) should also be carefully revised throughout the manuscript; the second subtitle of the Results section suggest that fragments of both neurons and glia can be found whereas the corresponding figure only shows neuronal fragments.

- We have unified the expression of “neuronal” throughout the manuscript as we have only demonstrated neuronal fragments being attached to glia in our experiments.

-The authors should also be cautious when referring to the overlap between CD24-LOW/HI and CD90-R/L clusters, which is not a perfect match according to the percentages shown in Fig.S2D-E.

- The reviewer is correct; indeed, this overlap is not a perfect match. To avoid misunderstandings, we have addressed this in the text and mention that the CD90-L and CD90-R clusters are very close to each other, with minimal gap and inevitable partial overlap. Please see lines 196-198. Regardless, we believe the observed percentages of match between CD24-LOW/HI and CD90-R/L clusters (around 90 %), are quite good considering this major technical limit. To this we should add that also CD24 levels may be not 100% resolved in all samples, depending on CD24 staining quality. We have improved statistics by adding more replicates resulting in a percentage of match closer to 90% for the CD24 HI cluster in both ileum and colon (Appendix Figure S1D).

-UCHL1 is misspelled UHCL1 on line 288.

- As suggested by the reviewer, we have corrected the point raised.

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The data shown in figures should satisfy the following conditions:
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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