High-resolution dynamic mapping of the *C. elegans* intestinal brush border

Aurélien Bidaud-Meynard, Flora Demouchy, Ophélie Nicolle, Anne Pacquelet, Shashi Kumar Suman, Camille N Plancke, François B Robin and Grégoire Michaux
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MS TITLE: High resolution dynamic mapping of the C. elegans intestinal brush border

AUTHORS: Aurelien Bidaud-Meynard, Flora Demouchy, Ophelie Nicolle, Anne Pacquelet, Camille Plancke, Shashi Kumar Suman, Francois Robin, and Gregoire Michaux

I have now received all the referees reports on the above manuscript, and have reached a decision. The overall evaluation is positive, and the reviewers provide recommendations for further improving the clarity and more accurately reflect the analysis. I invite you to submit a revised manuscript. The revised manuscript will be re-reviewed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

While the structure and function of absorptive microvilli are well characterized, the processes regulating and mediating the morphogenesis of microvilli are almost completely opaque. The detailed, focused, and well presented studies by Bidaud-Meynard et al. are significant by establishing C. elegans as a simple, genetically tractable, in vivo system by which microvilli morphogenesis can be studied using TEM of fixed samples and most importantly using light microscopy methods of living organisms. While the work is descriptive, the author's observations offer significant developmental and molecular insights into the assembly and maintenance of microvilli. Bidaud-Meynard et al. show that microvilli are quite dynamic; during embryogenesis microvilli are created de novo at the apical membrane and then microvilli are added, elongate, and enlarge during post-embryonic development. The authors make a key contribution by defining the short period (between the 1.5 and 2.5 fold stages) when microvilli are generated and the brush border is established within intestinal cells. Importantly, the work identifies key differences and similarities in protein localization between C. elegans and mammalian microvilli, making a comparative approach key in defining fundamental mechanisms of microvilli morphogenesis.

Comments for the author

Many of the author's conclusions are well supported by the data presented. The conclusions drawn from TEM, the gold standard in the field, are well justified. Nearly all of the microvilli fluorescent markers used are expressed from genomically modified loci minimizing concerns about expression level and providing confidence in the observations of temporal and spatial patterns. Importantly, super-resolution light microscopy approaches are used to show that PLST-1, FLN-2, HUM-5, MLC-5, EPS-8, ERM-1, and IFB-2 truly represent microvilli associated proteins in adults. Finally, the authors carried out robustly quantified and well-controlled FRAP experiments in pre and post microvilli assembly stages. However, the authors analysis of 1.5-fold embryos by FRAP does not represent a microvilli assembly stage but rather a pre-microvilli assembly stage.
The authors TEM analysis in Fig 1 suggest that bean to 1.5-fold embryos are not engaged in significant levels of microvilli assembly. Unlike 2-fold and later stages, there is no quantification of microvilli characteristics at these stages. Yet, Bidaud-Meynard et al. conclude that 1.5-fold stage embryos represent an early microvilli assembly stage in their FRAP studies. It would be better to characterize E16 to 1.5-fold embryos as pre-assembly stage. It is possible that the rapid FRAP recovery seen with many markers at these early stages are linked to microvilli assembly, but it is equally plausible that they represent functions of these proteins in different apical membrane processes.

- Additional minor comments.
1. It would be useful to non-C. elegans researchers to include at the start of the Results section a short description of how embryos are staged based on length and the time of development this encompasses (in min or hours).
2. Given the limitations of examining single thin sections by TEM, to aid in interpreting the results in Fig 1, it would be useful to have a short description of how lumen diameter, microvilli density/length/width were quantified in the methods.
3. In 2J the labels are not well-aligned with the data.
4. There are some minor spelling errors throughout.

Reviewer 2

Advance summary and potential significance to field

The C. elegans intestine has been a useful model for many aspects of development and function of the gut. The gut lumen must allow internalization of macromolecules for digestion, plus excretion of waste products, while serving as a barrier to prevent infection by pathogens.
Over the years, many gene products have been found that localize to the brush border, the undulated surface of enterocytes that faces the gut lumen, through which bacteria, fungi and other nutrients (and pathogens) pass. In this work, the authors use fluorescence, transmission electron microscopy, and genetic tools, to model the formation and maintenance of the C. elegans intestinal brush border, and in particular, frame the various gene products in terms of a model for how the brush border is formed and how the various components are arranged.
The authors use transmission EM to describe the formation of the brush border during embryogenesis. They then examine various markers known to localize to the brush border, including actin, ezrin, etc. They identify major F-actin organizing factors, PLST-1 and FLN-2, and a specific set of myosin orthologs, HUM-5, MLC-5, from their mammalian orthologs. The localization/expression is specific for these as other putative orthologs do not show either expression in gut cells. They then examine expression of these factors during establishment of the brush border, and propose (based on the order of expression and analysis of expression changes in some mutant backgrounds), 'pre-assembly', 'assembly' and 'mature' protein modules.
Microvilli are at the edge of detection for light-based microscopy methods. The authors test applications of different fluorophores and super-resolution methods and can image individual microvilli. Applying these methods, they localize the various proteins within microvilli during development. Using fluorescence recovery (FRAP), they investigate the dynamics of microvilli proteins to find differential stabilities among proteins in maturing, and mature, microvilli.
Much of the work is necessarily descriptive because there is no other way to set up this system. However, the work collectively represents a big leap in our understanding of the brush border, and it is long overdue for these details to have been worked out. The quality of imaging, precision,

presentation, and data analysis sets a high bar for future work in this system but also establishes a highly useful reference for the anatomy and development of the brush border. I support publication of this work in Development with minor comments.

Comments for the author

Minor comments Line 64 – the introduction ends with a description of the work, but it would be nice if it had a simple one-sentence conclusion, such as 'We show that...'
Line 153 – allowed to – allowed us to?
Figure 3A-3I – where blue is used, can it be cyan or a lighter shade of blue be used instead?

Reviewer 3

Advance summary and potential significance to field

The manuscript reports on the development of the apical domain in the C.
elegans intestine with a focus on the microvillar brush border using high-resolution imaging (electron microscopy, super-resolution light microscopy) in combination with quantitative expression and turnover analyses. It describes sequential steps associated with microvilli outgrowth and maturation. Some of the images are of exceptional and breath-taking quality, especially the super-resolution light microscopic co-localizations of different markers. Although no mechanistic data are provided, the manuscript describes highly useful reporter strains and offers a wealth of information that will be of interest for future, more mechanistic in vivo studies of microvillus morphogenesis.

Comments for the author

Some points should be considered prior to publication:
- I do not understand, what the authors mean by "new mapping tool". Is it a method or a specific reporter strain? And if it is either, what is new about it? I would suggest to avoid the term (or alternatively talk about a "tool box"). [The argument is also relevant to p. 9, 179, where a "novel approach" is advertised, and to p. 11, 216, where the approach is labeled as a "new methodology".]
- Fig. 1F,G: I do not see the relevance of the data. Furthermore, the density of microvilli is not homogenous along the anterior-posterior axis and may vary during adulthood. The age of the animal is not provided. I would therefore recommend to delete the data.
- This study, in accordance with other previous studies, nicely shows that the apical actin-rich layer is positioned on top of the intermediate filament-containing endotube. For the sake of clarity, I suggest to make this distinction by referring to the submembraneous actin cortex as the terminal web and to the intermediate filament layer below as the endotube.
This should also be included in Fig. S5.
- p. 8, line 152 and Fig. 3C: An image of PGP-1 detection should be included.
- The ultrastructure of the microvillar actin core differs from that seen in mammals. Could this account for some of the differences that are observed in C. elegans?
- Table S1: Precise information or, much better, gene sequences of the new alleles created in this study should be provided. There are also some glitches in nomenclature, which require correction (e.g., genes should not be capitalized).
- Legend to Fig. 2 A-I: The order of the description does not correspond to the order of images, which is again different from the heat map in J and the graph in S. It is also different from the order in K-R. All of this is confusing. Please, harmonize it and with the order in Fig. S2.

Minor:
- Fig. 3F, G: The ERM-1 label cannot be seen in the image.
- p. 9, 164: I do not consider IFB-2 to be a classical brush border marker.
- p. 9, 165: IFB-2 is a component of the endotube.
- p.9, 168 Make clear that ACT-5 was not endogenously tagged.
- p.6, 101 Figs 2D, S3 -> Figs 2C, S2

Very minor:
- p.3, 41 a in vivo model -> an in vivo model

- p. 3, 46 components -> component
- p. 5, 88 factor in mammalian cells brush -> factors in mammalian brush

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First revision

Author response to reviewers' comments

We would like first to thank the editor as well as the three reviewers for their positive appraisal of our work and for their constructive comments to improve our manuscript. Please find below the point-by-point response to the reviewer's comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
While the structure and function of absorptive microvilli are well characterized, the processes regulating and mediating the morphogenesis of microvilli are almost completely opaque. The detailed, focused, and well presented studies by Bidaud-Meynard et al. are significant by establishing C. elegans as a simple, genetically tractable, in vivo system by which microvilli morphogenesis can be studied using TEM of fixed samples and most importantly using light microscopy methods of living organisms. While the work is descriptive, the author's observations offer significant developmental and molecular insights into the assembly and maintenance of microvilli. Bidaud-Meynard et al. show that microvilli are quite dynamic; during embryogenesis microvilli are created de novo at the apical membrane and then microvilli are added, elongate, and enlarge during post-embryonic development. The authors make a key contribution by defining the short period (between the 1.5 and 2.5 fold stages) when microvilli are generated and the brush border is established within intestinal cells. Importantly, the work identifies key differences and similarities in protein localization between C. elegans and mammalian microvilli, making a comparative approach key in defining fundamental mechanisms of microvilli morphogenesis.

Reviewer 1 Comments for the Author:
Many of the author's conclusions are well supported by the data presented. The conclusions drawn from TEM, the gold standard in the field, are well justified. Nearly all of the microvilli fluorescent markers used are expressed from genomically modified loci minimizing concerns about expression level and providing confidence in the observations of temporal and spatial patterns. Importantly, super-resolution light microscopy approaches are used to show that PLST-1, FLN-2, HUM-5, MLC-5, EPS-8, ERM-1, and IFB-2 truly represent microvilli associated proteins in adults. Finally, the authors carried out robustly quantified and well-controlled FRAP experiments in pre and post microvilli assembly stages. However, the authors analysis of 1.5-fold embryos by FRAP does not represent a microvilli assembly stage but rather a pre-microvilli assembly stage.

The authors TEM analysis in Fig 1 suggest that bean to 1.5-fold embryos are not engaged in significant levels of microvilli assembly. Unlike 2-fold and later stages, there is no quantification of microvilli characteristics at these stages. Yet, Bidaud-Meynard et al. conclude that 1.5-fold stage embryos represent an early microvilli assembly stage in their FRAP studies. It would be better to characterize E16 to 1.5-fold embryos as pre-assembly stage. It is possible that the rapid FRAP recovery seen with many markers at these early stages are linked to microvilli assembly, but it is equally plausible that they represent functions of these proteins in different apical membrane processes.
> We thank the reviewer for this remark on early stages of microvilli assembly. We fully agree that, according to TEM data (Fig. 1), the microvilli are more in a pre-assembly than an assembly state at the 1,5-fold stage. In the revised version, it is now stated that FRAP performed at the 1,5-fold stage reflect the dynamics of brush border components during microvilli pre-assembly: "*While ERM-1 was very dynamic in 1,5-fold embryos, which likely reflects its involvement in microvilli pre-assembly, it became surprisingly stable in established brush border (adult worm)…*".

- Additional minor comments.
1. It would be useful to non-C. elegans researchers to include at the start of the Results section a short description of how embryos are staged based on length and the time of development this encompasses (in min or hours).
> We thank the reviewer for her/his suggestion that will broaden the public of our work. A brief description of *C. elegans* embryonic and larval development is now provided at the beginning of the results section.

2. Given the limitations of examining single thin sections by TEM, to aid in interpreting the results in Fig 1, it would be useful to have a short description of how lumen diameter, microvilli density/length/width were quantified in the methods.
> As requested, the methods used for TEM measurements have been updated in the Mat&Met relevant section (p13).

3. In 2J the labels are not well-aligned with the data.
> The labelling has been correctly aligned in the revised version of the figures.

4. There are some minor spelling errors throughout.
> We made our best to correct any errors.

Reviewer 2 Advance Summary and Potential Significance to Field:

The C. elegans intestine has been a useful model for many aspects of development and function of the gut. The gut lumen must allow internalization of macromolecules for digestion, plus excretion of waste products, while serving as a barrier to prevent infection by pathogens.

Over the years, many gene products have been found that localize to the brush border, the undulated surface of enterocytes that faces the gut lumen, through which bacteria, fungi and other nutrients (and pathogens) pass. In this work, the authors use fluorescence, transmission electron microscopy, and genetic tools, to model the formation and maintenance of the C. elegans intestinal brush border, and in particular, frame the various gene products in terms of a model for how the brush border is formed and how the various components are arranged. The authors use transmission EM to describe the formation of the brush border during embryogenesis. They then examine various markers known to localize to the brush border, including actin, ezrin, etc. They identify major F-actin organizing factors, PLST-1 and FLN-2, and a specific set of myosin orthologs, HUM-5, MLC-5, from their mammalian orthologs. The localization/expression is specific for these as other putative orthologs do not show either expression in gut cells. They then examine expression of these factors during establishment of the brush border, and propose (based on the order of expression and analysis of expression changes in some mutant backgrounds), 'pre-assembly', 'assembly' and 'mature' protein modules. Microvilli are at the edge of detection for light-based microscopy methods. The authors test applications of different fluorophores and super-resolution methods and can image individual microvilli. Applying these methods, they localize the various proteins within microvilli during development. Using fluorescence recovery (FRAP), they investigate the dynamics of microvilli proteins to find differential stabilities among proteins in maturing, and mature, microvilli. Much of the work is necessarily descriptive because there is no other way to set up this system. However, the work collectively represents a big leap in our understanding of the brush border, and it is long overdue for these details to have been worked out. The quality of imaging, precision, presentation, and data analysis sets a high bar for future work in this system but also establishes a highly useful reference for the anatomy and development of the brush border. I support publication of this work in Development with minor comments.

Reviewer 2 Comments for the Author:
Minor comments
Line 64 – the introduction ends with a description of the work, but it would be nice if it had a simple one-sentence conclusion, such as 'We show that...'
> We added the following conclusion sentence: "*In particular we show that intestinal microvilli form and grow throughout embryonic and larval development but are highly stable once formed.*"

Line 153 – allowed to – allowed us to?
> The sentence has been corrected to “allowed us” in the revised manuscript.

Figure 3A-3I – where blue is used, can it be cyan or a lighter shade of blue be used instead?
> This has been corrected as suggested except in panel E as none of the various colour combinations we tried made the triple staining easier to assess, as determined by the colour-blind first author

Reviewer 3 Advance Summary and Potential Significance to Field: The manuscript reports on the development of the apical domain in the C. elegans intestine with a focus on the microvillar brush border using high-resolution imaging (electron microscopy, super-resolution light microscopy) in combination with quantitative expression and turnover analyses. It describes sequential steps associated with microvilli outgrowth and maturation. Some of the images are of exceptional and breath-taking quality, especially the super-resolution light microscopic co-localizations of different markers. Although no mechanistic data are provided, the manuscript describes highly useful reporter strains and offers a wealth of information that will be of interest for future, more mechanistic in vivo studies of microvillus morphogenesis.

Reviewer 3 Comments for the Author:
Some points should be considered prior to publication:

- I do not understand, what the authors mean by "new mapping tool". Is it a method or a specific reporter strain? And if it is either, what is new about it? I would suggest to avoid the term (or alternatively talk about a "tool box"). [The argument is also relevant to p. 9, 179, where a "novel approach" is advertised, and to p. 11, 216, where the approach is labeled as a "new methodology".]
> To improve the clarity of the manuscript, the text has been corrected and the term “toolbox” has been preferred to define our work.

- Fig. 1F,G: I do not see the relevance of the data. Furthermore, the density of microvilli is not homogenous along the anterior-posterior axis and may vary during adulthood. The age of the animal is not provided. I would therefore recommend to delete the data.
> We wish to maintain these panels as it illustrates the likely hexagonal packing of microvilli, like in mammalian cells. We have now provided the age of the animals in Fig1F-G; in fact, G was calculated in 2 animals, not just one; it is now stated in the Fig1 legend. Additionally, using pictures as shown in Fig3D, we did not observe obvious variations in density along the AP axis (GM personal observation).
It is a classical approach in cell culture to use scanning EM to examine microvilli density; as this method cannot be exploited in C. elegans, we used these transversal sections. Moreover, these measurements are essential to validate our approach detailed in the super-resolution imaging section (lines 145-147).

- This study, in accordance with other previous studies, nicely shows that the apical actin-rich layer is positioned on top of the intermediate filament-containing endotube. For the sake of clarity, I suggest to make this distinction by referring to the submembraneous actin cortex as the terminal web and to the intermediate filament layer below as the endotube. This should also be included in Fig. S5.
> We modified the text throughout the manuscript accordingly, and IFB-2 has been positioned underneath ACT-5 in Fig. S5.

- p. 8, line 152 and Fig. 3C: An image of PGP-1 detection should be included.
> We already published the microvillar localization of endogenously tagged PGP-1 in our latest manuscript (Fig4C, Bidaud-Meynard et al, *Development*, 2019 Jun 5;146(11):dev174508). We therefore decided not to include new images in this manuscript and only quote this previous article. However, we can provide a new image of PGP-1 if the reviewer thinks that it is needed in this manuscript as well.

- The ultrastructure of the microvillar actin core differs from that seen in mammals. Could this account for some of the differences that are observed in C. elegans?

> Yes, it is possible that actin organization is different. However, the level of details we can get from high pressure freezing is not good enough for any robust statement. Moreover, we need to keep the manuscript within the wordlimit of a report. We therefore decided not to comment that point.

- Table S1: Precise information or, much better, gene sequences of the new alleles created in this study should be provided. There are also someglitches in nomenclature, which require correction (e.g., genes should not be capitalized).
> We have corrected the nomenclature and added the sequence of the new alleles created as supplementary files SF1-SF10.

- Legend to Fig. 2 A-I: The order of the description does not correspond to the order of images, which is again different from the heat map in J and the graph in S. It is also different from the order in K-R. All of this is confusing. Please, harmonize it and with the order in Fig. S2.
> We thank the reviewer for pointing out the misleading organization of images in Figs 2 and S2. The figure organization has been harmonized with the text in the revised manuscript and figures.

Minor:
- Fig. 3F, G: The ERM-1 label cannot be seen in the image.
> In Figs 3F-G, the upper panels only show the PLST-1 and FLN-2 channels to better assess their apical signal organization, while the colocalization with ERM-1 is shown only in lower panels. For better clarity, the markers' labelling is now in white in the upper panel (1 channel) and in the corresponding color in the lower panel(colocalization).

- p. 9, 164: I do not consider IFB-2 to be a classical brush border marker.
- p. 9, 165:IFB-2 is a component of the endotube.
> For better clarity, the sentence has been changed to "*Using a strain co-expressing endogenously taggedversions of two classical brush border markers, ERM-1 and, EPS-8, and the endotube's intermediate filament component IFB-2 …*".

- p.9, 168Make clear that ACT-5 was not endogenously tagged.
> The fact that ACT-5 is exogenously expressed is now clearly defined in the revised manuscript (lines 126 &174).

- p.6, 101Figs 2D, S3 -> Figs 2C, S2
> This mistake has been corrected in the revised version of the manuscript.

Very minor:
- p.3, 41a in vivo model -> an in vivo model
- p. 3, 46components -> component
- p. 5, 88factor in mammalian cells brush -> factors in mammalian brush
> All these spelling mistakes have been corrected in the revised version of the manuscript.

Second decision letter

MS ID#: DEVELOP/2021/200029

MS TITLE: High resolution dynamic mapping of the C. elegans intestinal brush border

AUTHORS: Aurelien Bidaud-Meynard, Flora Demouchy, Ophelie Nicolle, Anne Pacquelet, Shashi Kumar Suman, Camille Plancke, Francois Robin, and Gregoire Michaux
ARTICLE TYPE: Techniques and Resources Report

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

Reviewer 1

Advance summary and potential significance to field

While the structure and function of absorptive microvilli are well characterized, the processes regulating and mediating the morphogenesis of microvilli are almost completely opaque. The detailed, focused, and well presented studies by Bidaud-Meynard et al. are significant by establishing C. elegans as a simple, genetically tractable, in vivo system by which microvilli morphogenesis can be studied using TEM of fixed samples and most importantly using light microscopy methods of living organisms. While the work is descriptive, the author's observations offer significant developmental and molecular insights into the assembly and maintenance of microvilli. Bidaud-Meynard et al. show that microvilli are quite dynamic; during embryogenesis microvilli are created de novo at the apical membrane and then microvilli are added, elongate, and enlarge during post-embryonic development. The authors make a key contribution by defining the short period (between the 1.5 and 2.5 fold stages) when microvilli are generated and the brush border is established within intestinal cells. Importantly, the work identifies key differences and similarities in protein localization between C. elegans and mammalian microvilli, making a comparative approach key in defining fundamental mechanisms of microvilli morphogenesis. Reviewer 1 Comments for the author Many of the author's conclusions are well supported by the data presented. The conclusions drawn from TEM, the gold standard in the field, are well justified. Nearly all of the microvilli fluorescent markers used are expressed from genomically modified loci minimizing concerns about expression level and providing confidence in the observations of temporal and spatial patterns. Importantly, super-resolution light microscopy approaches are used to show that PLST-1, FLN-2, HUM-5, MLC-5, EPS-8, ERM-1, and IFB-2 truly represent microvilli associated proteins in adults. Finally, the authors carried out robustly quantified and well-controlled FRAP experiments in pre and post microvilli assembly stages.

Comments for the author

All of my relatively minor concerns have been adequately addressed by the authors in the revised manuscript and I am fully in support of publishing this excellent work.

Reviewer 2

Advance summary and potential significance to field

The paper advances a high-resolution model for the assembly and maintenance of the brush border of the C. elegans intestine, and will be very useful as a basis for future studies.

Comments for the author

The revised version of the manuscript does a nice job of addressing the reviewer comments. I recommend its acceptance.

Reviewer 3

Advance summary and potential significance to field

I refer to my previous statement.

Comments for the author

I am satisfied with the manuscript version as is.