# High-resolution ultrasound and speckle tracking: a non-invasive approach to assess *in vivo* gastrointestinal motility during development

Pierre Sicard, Amandine Falco, Sandrine Faure, Jérome Thireau, Stéphanie E. Lindsey, Norbert Chauvet and Pascal de Santa Barbara
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**Original submission**

First decision letter

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MS TITLE: High-Resolution Ultrasound and Speckle Tracking: a non-invasive approach to assess in vivo gastrointestinal motility during development

AUTHORS: Pierre Sicard, Amandine Falco, Sandrine Faure, Jerome Thireau, Stephanie E Lindsey, Norbert Chauvet, and Pascal de Santa Barbara

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

Reviewer 1

*Advance summary and potential significance to field*

This innovative study by Sicard et al. describes a unique assessment of fetal GI motility in vivo through the use of advanced imaging ultrasound and light sheet imaging techniques. The detailed report describes the development of GI motility synchronization in various GI tract segments and characterizes the functional role of both the ENS and smooth muscle layers. The ability to monitor the development of GI tract function in fetuses before birth could lead to early detection of disease and inform management. Additional studies into fetal motility both in avian and human models are clearly needed before GI tract monitoring commonly used before birth. The authors have written a comprehensive manuscript describing both novel imaging methods and therapeutic studies that provide mechanistic insight into GI development. Below is a list of major and minor comments aimed at improving the text.

*Comments for the author*

Major Comments:
1. The use of chick embryos in this study is justified and useful. That being said, it must be assumed that GI development of chickens is likely different than that in humans and other species. A section acknowledging species differences and other limitations would be beneficial to this manuscript.
2. The intervention studies summarized in Figure 3 add mechanistic insight that improves the impact of this work. That said, no data is reported in embryos where an injection occurred, but neither CoCl2 or TTX was administered. Without this is it possible the reductions in circumferential velocity and displacement were due to the injection procedure itself and not the administration of CoCl2 or TTX? Have the authors tried sham procedures where saline or other relevant control compounds are administered to rule out damage simply due to the injection procedure?
3. The lightsheet microscopy images in Figure 4 are beautiful, but only qualitative assessment is provided. Is there a method to quantitatively assess the smooth muscle orientation and neural neuronal network differences rather than qualitative observations?

Minor Comments:
4. The strain data is likely presented as a percentage (%) where the raw data is multiplied by 100. If this is correct, recommend adding % to all strain values in the text and figures.
5. Recommend adding either standard error or standard deviation to all data where appropriate throughout the text. This would help the reader get a sense of data variance.
6. Introduction: The ENS originates from vENCDCs which populate the GI tract. Please clarify how the cell migrate by either "AP migration wave" or "migration along the GI tract". The two phrases introduce some confusion.
7. Suggest slightly rewording "it is not known when and how digestive motor skills appear and develop during development, mainly due to the limitations of in vivo embryo assessment." Sase et al. demonstrate that human fetal gastric peristalsis appears as early as 14 weeks gestation and the frequency and duration of peristaltic waves increase until 32 week's gestation. https://doi.org/10.1046/j.1469-0705.1999.13050323.x
8. Materials and Methods: Were all three images acquired for each embryo used in the analyses to obtain measurements of area, diameter, strain, etc…?
Were the three image results averaged into one reported measurement. Please clarify.
9. Statistics: Were the assumptions of one-way ANOVA validated. I.e. was the equality of variance assessed for the strain, area, and diameter analysis?
10. How many sections of the small intestine were evaluated? Figure 2 shows multiple cross sections that vary in size and could lead to variable areas and strains.
11. Can the authors provide context as to why embryonic days E8 to E15 selected to monitor GI motility? Chevalier et al. demonstrate motility as early as E5 and E6 in the stomach and duodenum (https://doi.org/10.1371/journal.pone.0172511).
12. Additional details about how strain dyssynchrony is calculated would be helpful in the methods/results section. Is the cardiac resynchronization index applicable to this model for quantification of dyssynchrony?

13. Statistics: Why was an unpaired t-test selected for comparison between the pre- CoCl2, and TTX? A paired t-test could be considered as the pre and post timepoints reflect the same group in two separate scenarios.
14. The stomach was assessed at E8, E13, and E15 for synchronized movements.
It was then assessed at E13 for neuronal network and smooth muscle orientation effects. What is the rationale behind the selection of E13 as a timepoint for assessment? Could the two days less of development also contribute to the "less dense and less interconnected" neuronal mesh as the synchronized movement of the stomach due to ENS does not appear until E15?
15. BAPX1 leads to gastric expansion which is highlighted in Figure 4C. Were the differences in size assessed and could they be related to the level of fiber structure disorganization.
16. The colon diameter was measured in the longitudinal orientation. Were short axis measurements also acquired?
17. Statistics: Sample size n=5 in the CoCl2 and TTX? Did an embryo in this phase of the study meet the exclusion criteria outlined in the materials and methods? Please clarify.
18. Suggest clarifying abbreviation KIT.
19. Results: Suggest mentioning the assumption that through the use of the Lagrangian linear definition of strain is only valid for strains around 5% or less. This metric is likely an underestimation of strain when 20-30% is observed.
20. Page 11: Recommend moving the first five sentences of the "Smooth muscle layer organization…" section to the discussion. This section is not really a result.
21. Page 14: Suggest replacing "in-vivo" with "in vivo".
22. Page 16: Recommend splitting the last sentence of the Discussion section into two sentences. The current version is difficult to understand. Additional minor editorial assistance could also be helpful to correct grammatical errors and improving sentence syntax.
23. Figure 1: Is the vertical axis deformation or strain? And are these data percentages (%)? Also, why is "Radial strain" listed in the top right of these?
The label on the left has 3D strain.
24. Figure 2H: Were the authors able to quantify radial strain in the longitudinal images as well? If so, how do these values compare to the radial strain estimates reported in 2E?
25. Figure 3D: Should heart rate in the right subpanel be bpm (beats per minute) instead of bbp?
26. Figure 4D: Can scale bars be added to these lightsheet panels?

Reviewer 2

*Advance summary and potential significance to field*

In this manuscript Sicard et al., analyzed in ovo the motor activity dynamics during avian gastrointestinal tract development. The authors applied echography technique combined with speckle tracking analysis, pharmacological inhibitors specific for smooth muscle and enteric neurons, and RCAS-mediated BAPX1 gene overexpression methods to characterize the functional and coordinated gastrointestinal motility before hatching. Greater understanding of the intrauterine visceral movement, emphasizing the complex nature of the neural-smooth muscle interactions are a great value to both developmental biology and physiology field. This study is well put together and clearly written. The presented findings provide mechanistic insight into how enteric nervous system synchronize the muscle contractions in the stomach.

*Comments for the author*

Comments and recommendations.:
1) Page 5, last sentence: please define the other plexus in the gut wall.
2) I would recommend for the authors to cite these original papers (Lecoin L, Gabella G, Le Douarin N., 1996. Development 122:725–733; Young HM, Ciampoli D, Southwell BR, Newgreen DF. 1996. Origin of interstitial cells of Cajal in the mouse intestine. Dev Biol 180:97–107) for ICC origin instead of Guérin et al., 2020; Le Guen et al., 2015.
3) Correct Evan Blue to Evans Blue
4) Inorganic calcium channel blocker CoCl2 also inducing hypoxia. Hypoxia effects the smooth muscle force, a rapid attenuation of force occurs in smooth muscles of stomach (Huang,

Chowdhury, Kobayashi & Tomita, 1993). Organic Ca-channel blockers, such as verapamil, nisoldipine, or nifedipine (see Huycke et al., 2019) might help to overcome this side effect.
5) How was the effective concentration of TTX defined?
6) BAPX1 negatively regulates BMP4 expression. Intra-oral administration of pharmacological BMP4-inhibitor (Noggin) might be used to directly investigate the deregulation of the BMP pathway activity on visceral smooth muscle contraction.
7) Sukegawa et al reported (2000) that noggin misexpression using RCAS vector inhibited BMP signaling in stomach mesenchyme and induced ectopic enteric nervous system. Do the authors observe ectopic ganglia in the stomach wall or small/large intestine sections?
8) In BMP4 blocked RCAS-Noggin-infected avian embryos (Nerurkar et al., 2017), the entire small intestine remained outside the body cavity. Do the authors have data on malformations of injected embryos?
9) How the intestinal proliferation and apoptosis rate changes after in ovo treatments?
10) Author acknowledge that BAPX1-RCAS infection induced minor morphological defects in stomach. What about other organs of the embryos? How the rest of GI tract formed?
11) For their in ovo pharmacological inhibition experiments novel intraoral administration method was developed. It would be appropriate to show with a series of images how the vital dye enter and reach the stomach. This would be also very helpful for those who would recapitulate the technique.
12) This comment is related to no:11. Detailed description of intrabeak administration, access to the embryo through extraembryonic membranes of chicken embryo is missing.
13) HH10 stage early embryo was injected with multiple RCAS viruses. Only the stomach showed anti-3C2 immunoreactivity or other organs were infected? Mesenchymal or epithelial cells.
14) The authors conclude that in BAPX-expressing stomachs, the smooth muscle bundles were disorganized. However, based on panel D in figure 4 it seems that the majority of actin+ cells display a similar directionality and not “patchy” as 3c2 immunoractivity appears in Fig4A in bottom right pivture? Is it possible that muscle orientation depends from infection rate of the mesenchyme? Please comment.

Reviewer 3

*Advance summary and potential significance to field*

The authors present high-resolution ultrasound and speckle tracking in the chicken embryo to determine fetal gastrointestinal (GI) tract movements. They then test the effects of pharmacological inhibitors and BAPX1 gene overexpression on GI motility. This is an interesting new way of measuring GI movements, but I have several concerns as detailed below.

*Comments for the author*

1) Abstract/Introduction: the authors state that GI motor activity has rarely been studied during embryo development and that this paper provides the first recording of fetal gastrointestinal motiluty in living embryos. This feels a bit like an overstatement because it does not take the rich literature of observing gut motility in living embryos/larvae in fish species, particularly in zebrafish. Different studies have been done on fish species using live imaging to determine different gut parameters etc. The paper should be reworked to incorporate and compare their findings to the entire literature on gut motility.
2) The authors need to provide more details for their methods and in their figures to make their approach easier to understand to the broad readership of Development that is not familiar with ultrasound images and speckle tracing. What is speckle tracing and how does it work to follow gut movements? How are velocity, displacement, and strain determined by the speckle tracking analysis software? Even though software is used, these details must be provided so the reader can better understand what is exactly measured. Also, for Fig 1, 2 & 3 a schematic would be great to orient the reader and it’s clear what the figure shows. For example, it is very hard to understand where the contractions happen in Fig. 1A. How is the 3D strain calculate from 2D images in Fig. 1C. What does the segmentation entail in Fig. 1E? These are just examples, the authors should carefully go through the manuscript to ensure that a non-expert can follow the data displayed.
3) What is the control for BABX1 activity? It is not clear from the methods how it is determined that BABX1 overexpression results in changes in BMP activity in the stomach. How were

the BABX1:GFP levels determined in the stomach to show that you have even overexpression of BABX1?
4) Fig. 4: the smooth muscle cell staining is really hard to see or interpret. For Fig 4D-F, it is not obvious that smooth muscle cells are different in BABX1 stomachs. Showing the staining in a close-up or a better way to show the changed morphology/fiber structure would be helpful to ascertain what the phenotype is.
5) The movie length is very short and only 3 separate 1 min movies were taken per sample. This is quite short to capture different gut movements. What information can you provide that these movies represent the extent of gut movements at that developmental stage? what is the variability between the different movies? How many embryos were images? That number should be included.

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**First revision**

Author response to reviewers' comments

We would like to thank the reviewers for their constructive and pertinent comments on our manuscript. We have carefully considered all reviewer's remarks and have modified our manuscript accordingly. The reviewer's remarks are in bold typeface; our responses appear in red in the letter and in the manuscript.

Reviewer: 1
This innovative study by Sicard et al. describes a unique assessment of fetal GI motility in vivo through the use of advanced imaging ultrasound and light sheet imaging techniques. The detailed report describes the development of GI motility synchronization in various GI tract segments and characterizes the functional role of both the ENS and smooth muscle layers. The ability to monitor the development of GI tract function in fetuses before birth could lead to early detection of disease and inform management. Additional studies into fetal motility both in avian and human models are clearly needed before GI tract monitoring commonly used before birth. The authors have written a comprehensive manuscript describing both novel imaging methods and therapeutic studies that provide mechanistic insight into GI development. Below is a list of major and minor comments aimed at improving the text.

Major Comments:
1.The use of chick embryos in this study is justified and useful. That being said, it must be assumed that GI development of chickens is likely different than that in humans and other species. A section acknowledging species differences and other limitations would be beneficial to this manuscript.
RE: We agree with the reviewer's comment. The chick GI tract present some anatomical differences, compared with mammals, linked to the absence of teeth for food breakdown. Chicken use mechanical breakdown in the digestive system. Most GI variations among species concern the stomach morphology and can be correlated with the different diets. However, the global molecular patterning of the GI tract is remarkably similar among the different vertebrate lineages (Smith et al., 2000).
To consider the reviewer's remark in the revised version of the manuscript, we commented in the Introduction and Discussion sections on the similarity and difference of the chick embryo model compared to humans/mice (the similar timing of ENS colonization and smooth muscle differentiation in human and chick embryos in contrast to mouse) and the advantages of using chick embryos to evaluate fetal GI motility without the interference of the mother's movement and the use of anesthesia that affects intestinal motility in humans and animal models. We also added the limitation of our approaches that we estimated to be mainly due to the whole-body embryo movement (in the Material and Method section).

2.The intervention studies summarized in Figure 3 add mechanistic insight that improves the impact of this work. That said, no data is reported in embryos where an injection occurred, but neither CoCl2 or TTX was administered. Without this is it possible the reductions in circumferential velocity and displacement were due to the injection procedure itself and not the administration of CoCl2 or

TTX? Have the authors tried sham procedures where saline or other relevant control compounds are administered to rule out damage simply due to the injection procedure?
RE: We agree with the reviewer's comment on the importance of assessing the potential impact of the injection procedure on GI motility. In the first version of the manuscript, we only presented data obtained before and after intra-oral administration of TTX, cobalt chloride, or imatinib all diluted in PBS (Figure 3, Supplemental Figure 2). In the revised version of the manuscript, we present also the experiment assessing the innocuity on contraction (circumferential velocity and radial displacement) and on heart rate of intra-oral administration of PBS alone). As now presented in Supplemental Figure 2A and commented in the Materials & Methods and Results sections, intra-oral injection of PBS did not alter GI motility vs pre-treatment condition.

3.The lightsheet microscopy images in Figure 4 are beautiful, but only qualitative assessment is provided. Is there a method to quantitatively assess the smooth muscle orientation and neural neuronal network differences rather than qualitative observations?
RE: Thanks for the comments concerning the light-sheet microscopy experiment that required a lot of technical development in order to image the different layers of the digestive smooth muscles with the appropriate antibodies. We agree with the reviewer that the observations presented are currently only qualitative. Due to the E13 chick stomach size (5 mm length x 5 mm width) and the dense organization of the smooth muscle fibers, we used the UltraMicroscope Blaze to image the entire stomach with a 2X objective (MVPLAPO Olympus) and 0.5X numerical aperture. This prevented us to obtain sufficient precision to use these data for quantification. We are aware that this quantification will be necessary for future studies and we are now evaluating the use of light-sheet microscopy with more powerful objectives to improve the image quality with the aim of determining the smooth muscle cell angles, size and connection. We are working on this to develop a new method to evaluate smooth muscle organization.

Minor Comments:
4.The strain data is likely presented as a percentage (%) where the raw data is multiplied by 100. If this is correct, recommend adding % to all strain values in the text and figures.
RE: We agree with the reviewer's comment and corrected the strain data accordingly in Figures 2E and 2H.
5.Recommend adding either standard error or standard deviation to all data where appropriate throughout the text. This would help the reader get a sense of data variance.
RE: We agree with the reviewer's comment and added standard errors throughout the text for all figures concerned.
6.Introduction: The ENS originates from vENCDCs which populate the GI tract. Please clarify how the cell migrate by either “AP migration wave” or “migration along the GI tract”. The two phrases introduce some confusion.
RE: We corrected the sentence to avoid confusion.
7.Suggest slightly rewording “it is not known when and how digestive motor skills appear and develop during development, mainly due to the limitations of in vivo embryo assessment.” Sase et al. demonstrate that human fetal gastric peristalsis appears as early as 14 weeks gestation and the frequency and duration of peristaltic waves increase until 32 week’s gestation.
https://doi.org/10.1046/j.1469-0705.1999.13050323.x
RE: We agree with the reviewer's comment and in the revised version of the manuscript, we incorporated Sase et al as reference and also modified the sentence.
8.Materials and Methods: Were all three images acquired for each embryo used in the analyses to obtain measurements of area, diameter, strain, etc…? Were the three image results averaged into one reported measurement. Please clarify.
RE: As we performed ultrasound of the embryos without anesthesia, embryos can move within the shell which complicates the measurement of digestive motility. As commonly done for ultrasound imaging acquisition (cardiac, obstetrical, vascular…), we recorded movies for several minutes, and then we analyzed the GI tract movements using the part of the movies where the whole embryo did not move. To better explain our approach, we now included the precise description of image acquisition and analyses in the Materials & Methods and Results sections.
9.Statistics: Were the assumptions of one-way ANOVA validated. I.e. was the equality of variance assessed for the strain, area, and diameter analysis?
RE: We analyzed the stomach area and asynchrony results with the Kolmogorov-Smirnov test for testing data distribution (Fig. 1D & 1F) and distribution was normal.

10.How many sections of the small intestine were evaluated? Figure 2 shows multiple cross sections that vary in size and could lead to variable areas and strains.
RE: On average, we analyzed (and reported in Figure 2) 3-5 small intestine sections per embryo. As it is a dynamic evaluation, we measured the maximum lumen for each small intestine section. We included in the revised version of the manuscript this comment in the Materials and Methods section.
11.Can the authors provide context as to why embryonic days E8 to E15 selected to monitor GI motility? Chevalier et al. demonstrate motility as early as E5 and E6 in the stomach and duodenum (https://doi.org/10.1371/journal.pone.0172511).
RE: Chevalier and colleagues showed using a dissected digestive tract that motility was present at E7, but also found that at this stage, motility was still variable in amplitude in the jejunum, ileum and colon. The amplitude in these areas shows a strong increase from E8. As we had shown that digestive smooth muscle differentiation (expression of CALPONIN, regulator of smooth muscle contraction) was present from E7 onwards in the stomach (Faure et al, 2015; Notarnicola et al, 2012), we started our measurements from E8. We now included an explanation on why we started to analyze the GI motility from E8 in the Results section.
12.Additional details about how strain dyssynchrony is calculated would be helpful in the methods/results section. Is the cardiac resynchronization index applicable to this model for quantification of dyssynchrony?
RE: In cardiology, UltraSound (US) and strain analyses are routinely used to assess the consequences of arrhythmia on cardiac function. In addition, this technique is used to detect contraction synchrony during peristalsis (Mittal et al, Am J Physiol Gastrointest Liver Physiol, 2006, 290(3):G431-8). Here, we hypothesized that US coupled with strain analysis could be used to determine gastric asynchrony during development in chick embryos. We quantified stomach strain dyssynchrony from the standard derivation of the maximum radial time-strain curves of the six segments delineated in the developing stomach and the time-strain curves generated for each segment. Because we did not evaluate the electrical waves during embryo development, it is probably too early to conclude that our measurements are similar to cardiac dyssynchrony and cardiac resynchronization index. To avoid any misunderstanding, we removed the reference on cardiac resynchronization. We now added in the revised version of the manuscript a new reference and in the Results section: " We quantified stomach motility asynchrony, from the standard derivation of the maximum radial time-strain curves of the six gastric segments delineated in the developing stomach (Fig. 1E) and the time-strain curves generated for each segment by adapting a previously described synchrony index used to study peristalsis (Mittal et al, 2006).".
13.Statistics: Why was an unpaired t-test selected for comparison between the pre- CoCl2, and TTX? A paired t-test could be considered as the pre and post timepoints reflect the same group in two separate scenarios.
RE: We agree with the reviewer's comment on the paired t-test and applied it to our analyses. These are now presented in Figure 3 and Supplemental Figure 2 and commented in the Materials and Methods section. Statistics were reinforced with this test for all experiments. In addition, we added in new Supplementary Figure 2 the changes for each sample before and after treatment.
14.The stomach was assessed at E8, E13, and E15 for synchronized movements. It was then assessed at E13 for neuronal network and smooth muscle orientation effects. What is the rationale behind the selection of E13 as a timepoint for assessment? Could the two days less of development also contribute to the "less dense and less interconnected" neuronal mesh as the synchronized movement of the stomach due to ENS does not appear until E15?
RE: We found that stomach motility control through ENS regulation is present at E15 (Figure 3). For this reason, we chose to analyze the functional and morphological consequence of BAPX1 overexpression at E13 before the contraction regulation through ENS. Moreover, as at E13 the magnitude of stomach deformation was high (Fig.1C), this will help us to monitor the impact of BAPX1-induced dysfunction.
15. BAPX1 leads to gastric expansion which is highlighted in Figure 4C. Were the differences in size assessed and could they be related to the level of fiber structure disorganization.
RE: We did not evaluate in close detail the size difference of dissected stomach between the control and BAPX1 misexpresssion conditions. We agree that the disorganization and change of density of smooth muscle fibers could explain the size change and more importantly the changes in the tissue mechanical properties. To answer this interesting question, we plan to develop approaches adapted from Butcher et al (2007) to evaluate stomach tissue stiffness.
16. The colon diameter was measured in the longitudinal orientation. Were short axis measurements also acquired?

RE: As we observed the gastrointestinal tract into the eggs, we were constrained in the possibility of orienting the transducer. Therefore, the colon, which is centralized in the avian model, was observable in its entirety longitudinally. We could not permanently observe the colon in cross section and for this reason we only presented the colon diameter and longitudinal evaluation.
17. Statistics: Sample size n=5 in the CoCl2 and TTX? Did an embryo in this phase of the study meet the exclusion criteria outlined in the materials and methods? Please clarify.
RE: We thank the reviewer for this remark. We apologize and we now described that 5 embryos were analyzed per drug injection without exclusion criteria in the Materials & Methods section. We now added in Supplemental Figure 2 the fate of all treated embryos.
18. Suggest clarifying abbreviation KIT.
RE: In the new version of the manuscript, we defined the KIT abbreviation in the Results section.
19. Results: Suggest mentioning the assumption that through the use of the Lagrangian linear definition of strain is only valid for strains around 5% or less. This metric is likely an underestimation of strain when 20-30% is observed.
RE: We thank the reviewer for this excellent comment about the fact that Lagrangian strain will give lower absolute values, especially between -20 to -30%, compared to those obtained with Eularian strain calculation. We added a limitation sentence "Our strain results are likely to be underestimate due to the use of Lagrangian linear methods" in the Materials and Methods section.
20. Page 11: Recommend moving the first five sentences of the "Smooth muscle layer organization…" section to the discussion. This section is not really a result.
RE: We agree with the reviewer's comment. We now incorporated in the revised version of the manuscript experiments showing that BAPX1 overexpression leads to the inhibition of BMP4 expression (in situ hybridization) and BMP activity (western blotting) (Supplemental Figure 3). With the incorporation of these data we rewrote this part of Results accordingly.
21. Page 14: Suggest replacing "in-vivo" with "in vivo".
RE: We corrected this typo, thanks.
22.Page 16: Recommend splitting the last sentence of the Discussion section into two sentences. The current version is difficult to understand. Additional minor editorial assistance could also be helpful to correct grammatical errors and improving sentence syntax.
RE: As suggested, we modified the last sentence of the Discussion. Minor editorial errors were improved with editing of the manuscript by an English native colleague.
23.Figure 1: Is the vertical axis deformation or strain? And are these data percentages (%)? Also, why is "Radial strain" listed in the top right of these? The label on the left has 3D strain.
RE: We thank the reviewer for this comment. We modified Figure 1 to better explain the obtained results and changed the y axis: radial strain (%).
24. Figure 2H: Were the authors able to quantify radial strain in the longitudinal images as well? If so, how do these values compare to the radial strain estimates reported in 2E?
RE: We were not able to quantify radial strain in the colon mainly due to anatomic features (the colon thickness is faint).
25.Figure 3D: Should heart rate in the right subpanel be bpm (beats per minute) instead of bbp?
RE: We corrected this typo in the Figure 3 and added the bpm abbreviation in the Results and Abbreviations sections, thanks.
26.Figure 4D: Can scale bars be added to these lightsheet panels?
RE: We added scale bars in Figure4D of the revised version.

Reviewer: 2
In this manuscript Sicard et al., analyzed in ovo the motor activity dynamics during avian gastrointestinal tract development. The authors applied echography technique combined with speckle tracking analysis, pharmacological inhibitors specific for smooth muscle and enteric neurons, and RCAS-mediated BAPX1 gene overexpression methods to characterize the functional and coordinated gastrointestinal motility before hatching. Greater understanding of the intrauterine visceral movement, emphasizing the complex nature of the neural-smooth muscle interactions are a great value to both developmental biology and physiology field. This study is well put together and clearly written. The presented findings provide mechanistic insight into how enteric nervous system synchronize the muscle contractions in the stomach.

Comments and recommendations:
1) Page 5, last sentence: please define the other plexus in the gut wall.
RE: We now define the submucosa plexus in the Introduction section.

2) I would recommend for the authors to cite these original papers (Lecoin L, Gabella G, Le Douarin N., 1996. Development 122:725–733; Young HM, Ciampoli D, Southwell BR, Newgreen DF. 1996. Origin of interstitial cells of Cajal in the mouse intestine. Dev Biol 180:97–107) for ICC origin instead of Guérin et al., 2020; Le Guen et al., 2015.
RE: As suggested, we modified the references as recommended.
3) Correct Evan Blue to Evans Blue
RE: We corrected this typo, thanks
4) Inorganic calcium channel blocker CoCl2 also inducing hypoxia. Hypoxia effects the smooth muscle force, a rapid attenuation of force occurs in smooth muscles of stomach (Huang, Chowdhury, Kobayashi & Tomita, 1993). Organic Ca-channel blockers, such as verapamil, nisoldipine, or nifedipine (see Huycke et al., 2019) might help to overcome this side effect.
RE: CoCl2 rapidly blocks extracellular Ca2+ entry through L-type voltage-dependent Ca2+ channels and receptor-operated channels. Thus, it was used to pharmacologically abolish contraction of embryonic mouse and chick intestine in organ culture (Roberts et al, 2010; Chevalier et al, 2017). However, we agree with the reviewer's comment that CoCl2 could induce hypoxemia. Nevertheless, this effect occurs with incubation of tissue sample or cells at high concentration and/or for days through the modulation of the expression of the HIF-1a transcription factor (for review Munoz-Sanchez and Chanez-Cardenas, 2018). As we evaluated the consequence of intraoral administration of CoCl2 solution after 1 hour, we are closer to the experimental procedure described by Roberts (2010) and Chevalier (2017) and we only targeted contraction activity. In the revised version of the manuscript, we now added in the Results section more detail about the experimental procedure.

5) How was the effective concentration of TTX defined?
RE: In mouse and chick dissected intestines, the involvement of enteric neurons in motility is assessed using TTX concentrations between 1 and 5 µM (Roberts et al, 2010; Chevalier et al, 2017). As TTX cannot be given intravenously in sufficient doses to obtain neural blockade without causing toxic systemic effects, we decided to administer intra-orally 100 µl of solution with increasing concentration of TTX (1, 10, 25, and 50 µM). We determined that 25 µM was the optimal concentration to obtain reproducible stomach motility inhibition without impact on the heart rate. We added more detail in the Materials & Methods section.

6) BAPX1 negatively regulates BMP4 expression. Intra-oral administration of pharmacological BMP4-inhibitor (Noggin) might be used to directly investigate the deregulation of the BMP pathway activity on visceral smooth muscle contraction.
RE: According to the reviewer's comment, we decided to evaluate the consequence of BMP inhibition on the functionality of the developing stomach using retrovirally misexpressed BAPX1. One main reason is that the trophism of avian retroviruses in the developing stomach is specific of mesenchymal cells and does not directly target vENCDCs or epithelium (Faure et al, 2015, Supplemental Figure 3; Moniot et al, Figure 6). We now included one enlargement of 3C2 staining in Figure S3D showing the specific presence of 3C2 staining in the stomach mesenchyme. In addition, as BAPX1 is a homeobox-containing transcription factor, the inhibition of the BMP pathway activity is related to the mesenchyme targeting. As Noggin could diffuse from the mesenchyme to the vENCDCs or epithelium, we would not be able to monitor specific mesenchymal alterations, but rather a broad phenotype. In the revised version of the manuscript, we added a comment on the mesenchyme target specificity of our approach in the Materials & Methods section and in Supplemental Figure S3D.

7) Sukegawa et al reported (2000) that noggin misexpression using RCAS vector inhibited BMP signaling in stomach mesenchyme and induced ectopic enteric nervous system. Do the authors observe ectopic ganglia in the stomach wall or small/large intestine sections?
RE: Thanks for this interesting question. We did not find ectopic ganglia extending in the stomach mesenchyme in BAPX1-overexpressing stomachs, as nicely described in Sukegawa et al (2000). As commented above (no6), the main difference is that we inhibited the BMP pathway directly in the stomach mesenchyme with the avian retroviral approach, which has high trophism for the mesenchyme, and by targeting the transcription factor BAPX1 directly in the infected mesenchymal cells, unlike Noggin, a morphogen that can target adjacent cells (e.g. vENCDCs or epithelium).

8) In BMP4 blocked RCAS-Noggin–infected avian embryos (Nerurkar et al., 2017), the entire small intestine remained outside the body cavity. Do the authors have data on malformations of injected embryos?
RE: In line with our previous response, we did not observe any alteration in the body wall closure in BAXP1-infected embryos. We used the retroviral misexpression approach in chick embryos to target different parts of the gastrointestinal tract and using organ maps (Matsuhita, S, 1995). Therefore, we could specifically target the stomach mesenchyme by injecting in the splanchnic mesenchyme located between somite 3 and 6 (Moniot et al 2004; Faure et al 2015). In addition, as previously commented, BAPX1 inhibits the BMP pathway activity in the digestive mesenchyme, whereas Noggin activity, as a morphogen, can spread to other tissues or organs.

9) How the intestinal proliferation and apoptosis rate changes after in ovo treatments?
RE: This is an interesting point that we now evaluated. We previously showed that ablation of vENCDCs leads to aberrant expression of BMP4 and activation of NOTCH activity in the stomach mesenchyme at E6.5. This was associated with no significant change in the number of cleaved caspase 3-positive cells, but a decreased rate of cell proliferation (using PH3, a standard marker of G2/M transition) (Faure et al, 2015). Using a similar method, we evaluated the cell proliferation and apoptosis in the E13 stomach smooth muscle layer. We did not detect any significant change in the number of cleaved caspase 3-positive cells, but in the smooth muscle layer of BAPX1 stomachs, proliferation was increased (48% increase of Phospho-Histone H3 (PH3)-positive cells/SMCs, P<0.05). These data are now presented in the revised version of the manuscript (Supplemental Figure 3C) and commented in the Results section.

10) Author acknowledge that BAPX1-RCAS infection induced minor morphological defects in stomach. What about other organs of the embryos? How the rest of GI tract formed?
RE: Using specific injection of retroviruses in the splanchnic mesoderm located between somite level 3-5 at HH10, we specifically misexpressed BAPX1 retrovirus in the stomach mesenchyme (Moniot et al., 2004; Faure et al., 2015). Using such approach, we did not observe malformations in other organs, particularly the small intestine and colon.

11) For their in ovo pharmacological inhibition experiments novel intraoral administration method was developed. It would be appropriate to show with a series of images how the vital dye enter and reach the stomach. This would be also very helpful for those who would recapitulate the technique.
RE: We agree with the reviewer's comment and in the revised version of the manuscript, we added in Supplemental Figure 1 images of Evans blue solution in the gizzard one hour after intra-oral injection.

12)This comment is related to no:11. Detailed description of intrabeak administration, access to the embryo through extraembryonic membranes of chicken embryo is missing.
RE: We thank the reviewer for this comment indicating that we were not clear on how we administered the drug solution in the beak. To have access to the beak of E15 embryos, we used a High Temperature Cautery Handle (Bovie Medical Corporation, Fine Scientific Tools) to open a small window without bleeding in the extraembryonic membranes close to the beak. All these comments are now included in the Materials & Methods section.

13) HH10 stage early embryo was injected with multiple RCAS viruses. Only the stomach showed anti-3C2 immunoreactivity or other organs were infected? Mesenchymal or epithelial cells.
RE: Thank for this question. As answered to comments n. 8 &10, due to the injection of the splanchnic mesenchyme at HH10 stage, we only observed 3C2 positivity in the stomach mesenchyme (Moniot et al, 2004; Faure et al, 2015). We now included one enlarged image in Figure S3D.

14)The authors conclude that in BAPX-expressing stomachs, the smooth muscle bundles were disorganized. However, based on panel D in figure 4 it seems that the majority of actin+ cells display a similar directionality and not "patchy" as 3c2 immunoractivity appears in Fig4A in bottom right picture? Is it possible that muscle orientation depends from infection rate of the mesenchyme? Please comment.
RE: We thanks the reviewer for this remark. In our experiment, we observed strong to moderate infection level, but in all conditions, infection led to strong functional alteration (Figure 4B) and also to the change in muscle orientation. As it has been suggested that BAPX1 is involved in the

left/right asymmetry (Nielsen et al, 2000), we hypothesized that this axis is randomized, as observed for the stomach morphology (de Santa Barbara et al, 2005). We added in the revised version of the manuscript new 3C2 staining images (Supplementary Figure S3D) showing homogenous BAPX1 overexpression in the stomach mesenchyme.

Reviewer: 3
The authors present high-resolution ultrasound and speckle tracking in the chicken embryo to determine fetal gastrointestinal (GI) tract movements. They then test the effects of pharmacological inhibitors and BAPX1 gene overexpression on GI motility. This is an interesting new way of measuring GI movements, but I have several concerns as detailed below.

1) Abstract/Introduction: the authors state that GI motor activity has rarely been studied during embryo development and that this paper provides the first recording of fetal gastrointestinal motility in living embryos. This feels a bit like an overstatement because it does not take the rich literature of observing gut motility in living embryos/larvae in fish species, particularly in zebrafish. Different studies have been done on fish species using live imaging to determine different gut parameters etc. The paper should be reworked to incorporate and compare their findings to the entire literature on gut motility.
RE: We agree with the reviewer's comment that in fish species intestinal motility in living larvae was nicely described (Holmberg et al., 2004; Ganz et al., 2018). However, in contrast to our approach that evaluated the digestive motility directly in chick embryos, anesthetic treatment is needed in zebrafish. As in humans, anesthesia alters intestinal motility (De Corte et al., 2012), we think that our approaches are of high interest and robust. To better explain why we used the chick embryo in this study, we added in the revised version of the manuscript some sentences on the similarity in the timing of ENS colonization between humans and the avian model, and the direct evaluation of fetal digestive motility in chick embryo without interference by the mother's movement and without the use of anesthetic.

2) The authors need to provide more details for their methods and in their figures to make their approach easier to understand to the broad readership of Development that is not familiar with ultrasound images and speckle tracing. What is speckle tracing and how does it work to follow gut movements? How are velocity, displacement, and strain determined by the speckle tracking analysis software? Even though software is used, these details must be provided so the reader can better understand what is exactly measured. Also, for Fig 1, 2 & 3 a schematic would be great to orient the reader and it's clear what the figure shows. For example, it is very hard to understand where the contractions happen in Fig. 1A. How is the 3D strain calculate from 2D images in Fig. 1C. What does the segmentation entail in Fig. 1E? These are just examples, the authors should carefully go through the manuscript to ensure that a non-expert can follow the data displayed.
RE: We thanks the reviewer for this comment concerning the global accessibility to the whole Developmental community of our results obtained using ultrasound.
- Through the revision of this manuscript, we expended the Materials and Method section to better explain the ultrasound/strain approach to the readers. We described the methodology based on the speckle tracking analysis. More precisely, we used the Vevo stain software (VevoLab 5.6.1, Visualsonics) to analyze the motion of gut tissues by tracking natural acoustic reflection interference also called speckle pattern. The speckle tracking algorithm used in this study allows us to calculate the velocity, displacement, and strain from E8 to E15 of each region of the GI tract including stomach, intestine and colon. All these comments are now included in the Material and Method section.
- In the first version, we included diagrams that represented the different speckle tracking evaluated in small intestine, colon and stomach (Figures 2D, 2G and 3B, respectively). We now added more arrows, labelling and dotted lines in Figures 1, 2 & 4 to explain the stomach deformations associated with the dynamic opening and closure of its lumen and the anatomic observation obtained with ultrasound.
- We better explained how we obtained 3D strain of the E8, E13 and E15 stomach in the new version by including details in the Figure legends. "Fig 1C represents a 3D heat map of strain percentage (z) for each individual segment of the stomach (y) during 5-8 sec of acquisitions (x)".

3) What is the control for BABX1 activity? It is not clear from the methods how it is determined that BABX1 overexpression results in changes in BMP activity in the stomach. How were the BABX1:GFP levels determined in the stomach to show that you have even overexpression of BABX1?
RE: We thank the reviewer for this comment related to the impact of BAPX1 overexpression on BMP activity in the stomach. BAPX1 inhibits the expression of BMP4 in the developing stomach (Nielsen et al 2001), and our team showed that BAPX1 expression in the intestine leads to the decrease of PSMAD1 expression in the intestine (de Santa Barbara et al, 2005). We carried out in control and BAPX1-expressing stomachs in situ hybridization and western blot analyses and found that BAPX1 overexpression leads to BMP4 and BMPRII mRNA decrease that is associated with inhibition of activated phosphorylated SMAD1 (PSMAD1) (Figure S3A and S3B).

4) Fig. 4: the smooth muscle cell staining is really hard to see or interpret. For Fig 4D-F, it is not obvious that smooth muscle cells are different in BABX1 stomachs. Showing the staining in a close-up or a better way to show the changed morphology/fiber structure would be helpful to ascertain what the phenotype is.
RE: We agree with the reviewer only partially. The images showing the change in orientation of the bundles or smooth muscle fibers in Figures 4D and 4E are quite convincing. It is true that our data are only qualitative and that a quantification could lead to a better characterization of the phenotype. However, due to the E13 chick stomach size (5 mm length x 5 mm width) and the dense organization of the smooth muscle fibers, we used the UltraMicroscope Blaze to image the entire stomach with a 2X objective (MVPLAPO Olympus) and 0.5X numerical aperture, which prevented us from obtaining sufficiently precise data for quantification. We are aware that this quantification will be necessary for future studies and we are now evaluating the use of light-sheet microscopy with more powerful objectives to improve the images in order to determine the smooth muscle cell angles, size and connection. Once validated, this new approach and analysis could be described in another work focusing on smooth muscle layer organization. However, to take into consideration the reviewer's remark, we now present other cross-sectional plans showing differences between the control and BAPX1 stomach that suggest an alteration in the fiber orientation and the smooth muscle bundle organization in the BAPX1 stomach (Figure S4E).

5) The movie length is very short and only 3 separate 1 min movies were taken per sample. This is quite short to capture different gut movements. What information can you provide that these movies represent the extent of gut movements at that developmental stage? What is the variability between the different movies? How many embryos were images? That number should be included.
RE: We thank the reviewer for this question. We added some explanations concerning images obtained by ultrasonography in the Materials & Methods section. We stated that movies of the digestive tract motor activity were taken over several minutes, which enabled us to evaluate and quantify contractions. To record larger sequences (as usually used in cardiology) allowed us to focus on clear sequence of GI motility by excluding periods during which the embryo moved and interfered with the analyses (because embryos were not anesthetized). In the Movie files, we presented as example only the part of these videos where the motor activity was not contaminated by the embryo movements to qualitatively present our observation. For each condition, we monitored movies from 5 to 6 embryos without exclusion criteria. All these comments are now included into the Materials & Methods section.

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Second decision letter

MS ID#: DEVELOP/2022/200625

MS TITLE: High-Resolution Ultrasound and Speckle Tracking: a non-invasive approach to assess in vivo gastrointestinal motility during development

AUTHORS: Pierre Sicard, Amandine Falco, Sandrine Faure, Jerome Thireau, Stephanie E Lindsey, Norbert Chauvet, and Pascal de Santa Barbara
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Reviewer 3 had a minor request to clarify a statement in your manuscript about live imaging in other species.

Reviewer 1

*Advance summary and potential significance to field*

The authors have done a wonderful job both responding to the previous critiques from all three reviewers and revising the manuscript. I have no further suggestions or questions.

*Comments for the author*

N/A

Reviewer 2

*Advance summary and potential significance to field*

Although the postnatal intestinal motility is well studies, this paper provides novel insight into the GI motor activity dinamics during avian embryonic development. These observations are of significance both to understanding myogenesis in embryonic gut wall, and potentially to comprehending the pathogenesis of disorders, many still-to-be defined, that result from birth defects of the visceral smooth muscle cells.
I received satisfactory answers to all my questions/comments and therefore I recommend the manuscript for publication in Development.

*Comments for the author*

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Reviewer 3

*Advance summary and potential significance to field*

The authors present high-resolution ultrasound and speckle tracking in the chicken embryo to determine fetal gastrointestinal (GI) tract movements. They then test the effects of pharmacological inhibitors and BAPX1 gene overexpression on GI motility. This is an interesting new way of measuring GI movements, but I have several concerns as detailed below.

*Comments for the author*

The authors have addressed most of my comments – this is a really nice manuscript - and I only have one small remaining comment:
1) I agree with the reply by the authors about the need to study gut motility also without anesthetics and agree with the changes that have been made in the text. However, in the introduction, the authors still write: “Until now, embryonic gut motility has been studied only in organ culture systems.”
As embryonic gut motility has also been studied in live animals such as zebrafish but just with anesthesia, this should be added as information, or, alternatively, it should be clarified that the authors mean Until now, embryonic gut motility has been studied only in organ culture systems without anesthesia or add a qualifier so that it is clear why the zebrafish data is not included in that statement.