nature portfolio

Peer Review File

Real-Time Nanoscale Investigation of Spore Coat Assembly in *Bacillus subtilis*

Corresponding Author: Dr Rut Carballido-Lopez

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Lablaine et al. reports the visualization of spore coat assembly in Bacillus subtilis using time lapse lattice-SIM, which provides increased spatial and temporal resolution of the localization patterns of individual coat proteins. In general, the manuscript was well written, and the experiments were well controlled. I imagine that this paper will be widely cited by sporulation researchers. I have only relatively cosmetic suggestions which the authors may or may not wish to incorporate.
Sincerely,
Kumaran Ramamurthi

Major comments:

1. The use of TMR-star as a marker for late stages of sporulation is a valuable contribution of this report. However, I think that the conclusion that TMR-star specifically marks inner coat assembly is not entirely justified by the data presented. As I understand, the conclusion was based on the reported pattern of inner coat assembly in B. cereus, the coincident staining pattern of TMR-star and several late-synthesized inner coat proteins, and the partial mis-localization of TMR-star in the absence of SafA). Identifying the target of TMR-star is certainly beyond the scope of this report. However, the authors may choose to delete one or more genes encoding a coat basement layer protein like SpoIVA (by historical definition, to be considered a “coat” protein, its localization around the forespore must be dependent on SpoIVA; other proteins at the forespore surface that do not depend on SpoIVA are not technically classified as “coat proteins”) and testing TMR-star localization. In the absence of demonstrating such a dependence, I suggest softening the conclusion (line 106 of the intro, line 210, for example) to simply state TMR-star staining is *coincident* with inner coat assembly.

2. Line 22 (first use of ‘refractile spores’). Consider more explicitly stating the challenges associated with identifying intermediate assembly stages after the forespore becomes refractile using phase contrast optics. The refractility of the forespore is itself a landmark event, and there are several membrane-permeable dyes that permit staining the forespore membrane at this stage. I assume the authors are suggesting that it would be helpful to have an independent landmark event to delineate spore maturation after forespores become refractile and before it is released as a mature spore?

3. Line 293: “Extensive displacement of molecules on the forespore surface has not been previously described in spore forming bacteria.” Forespore surface proteins YabP, YheD, and YutH have been previously described as exhibiting dynamic localization patterns (PMID 15231775). Consider softening this claim, as doing so would not impair any novelty of the present study.

4. Fig. 5C. Consider more clearly showing the orientation of the forespore schematic. Given the placement of SigE and SigK, and the localization of CotE, I assume that this is a lateral view with the mother cell to the left?

Minor comments:

1. Line 89, "…only been reported…via computational image analysis methods." A) please provide references. B) Technically, structured illumination, which is employed in this report, is also based on a computational foundation (image reconstruction using Fourier transform). Consider rephrasing this statement?

2. Fig. 2B. Do the colors used to denote CotY, SpsI, YmaG, and YhaX correspond to anything? If not, consider simply labeling all of them in green (to reflect the GFP fusion of each) or simply black.

3. Fig. S2. Typo: "interval", not "intervale", correct?

4. I realize that space in the main text may be precious, but consider moving Fig. S2 to the main text, since it is the first instance in the manuscript where the authors' central technique is highlighted in an intuitive fashion.

5. Fig. 2 legend, elsewhere. Consider defining statistically not significant ("ns") as $P > 0.05$.

Reviewer #2

(Remarks to the Author)
In this article, Lablaine et al. report a significantly updated analysis of the process of spore coat assembly in the model bacterium Bacillus subtilis. The process has been investigated in detail before, but due to technical limitations of classic fluorescence microscopy, some questions remained unanswered. It is also a highly complex process involving at least 80 different proteins that are distributed to different concentric coat layers (basement layer, inner coat, outer coat and crust) with different stages occurring in a period of several hours under the control of coat morphogenetic proteins and sporulation transcription factors. Here, the use of Structured Illumination Microscopy (SIM) resulted in an appreciable gain in resolution (70 nm, i.e., nanoscale). Perhaps even more importantly, data were collected for single cells in time lapse experiments, which unambiguously captured localization dynamics for a selection of coat proteins during the assembly process. Remarkably, new patterns of dynamic localization were observed, primarily for a class of inner coat proteins. These proteins first appear to localize to the midspore region until they are redistributed to fully encase the maturing spore. Interestingly, this behavior is also observed with the TMR-star red fluorescent stain, which the authors introduce as a specific marker of the inner spore coat layer. This will have important implications for future research in the field, since there was a lack of late-stage morphological sporulation stains. The authors also show that the localization of TMR star is dependent on the morphogenetic protein of the inner coat SafA and the regulators of gene expression in the mother cell at late stages of sporulation, SigK and GerE. Another important observation is that formation of the outer coat influences the redistribution of this class of inner coat proteins, which is also a new concept considering that assembly of the inner and outer coat layers were viewed as essentially independent processes. In general, the experiments are well done, and the images are superb and truly informative. I have listed a few points for discussion below.
Major point:
Is TMR-star truly a marker of the inner coat or is it more appropriately defined as a stain that colocalizes with a class of inner coat proteins, i.e. those that localize late (such as YmaG or YutH) or even later (CotD and YeeK)? It does not seem that TMR-star localization dynamics match that of well-defined early-localizing inner coat proteins such as YaaH or YuzC (kinetics of localization based on McKenney and Eichenberger, 2012, Mol Micro) .
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l. 59 and elsewhere in the paper: "undercoat (or basement layer)". It would be informative to know why the authors prefer the formulation "undercoat" to the term "basement layer", which has been commonly used in several reviews of the spore coat assembly process. This layer is definitely a critical component of the coat structure, which is implied by the term "basement layer", the term "undercoat" suggests that it is a separate structure that happens to be localized under the coat.
l. 63 "and CotYZ are required for crust formation", it would be more accurate to say that "CotXYZ are required for crust formation"
l.232-234: designation of LipC as an inner coat protein. In Subtiwiki and sources listed therein, LipC is designated as a basement layer protein (undercoat). The original designation might be wrong (since LipC-GFP appears to colocalize with TMR-star), but this should be mentioned in case LipC needs to be reclassified.
l.263-264:" Previous diffraction-limited studies indicated that YutH, its paralogue YsxE, and YisY initially localize at the forespore pole, but left unclear their localization during later sporulation stages." And heading:" Early-synthesized inner coat proteins YutH and YisY are dynamically redistributed on the forespore surface". I think this characterization of the earlier work is not entirely accurate. Ref 28 already indicated that the localization of YutH was dynamic (it is in the title of the paper), whereas details of YisY-GFP localization were provided in Ref 23 (in the supplements), including late time points. It is clear, however, that the new data presented in the current paper provide additional details and much improved resolution, both spatially and temporally.
L.681: "Driven by σE synthesis". It might be more accurate to say "Driven by σE-dependent synthesis"

Reviewer #3

(Remarks to the Author)
Overall this is a solid paper that establishes labelling and imaging approaches to look at Bacillus subtilis spores and uses this to tease out the positions and relationships between various coat proteins. Important strengths of the paper are (i) the use of TMR-star, previously shown in B. cereus, to label the spore inner coat, (ii) the use of super-resolution imaging methods to distinguish between GFP-tagged proteins and the TMR label at different layers of the spore and (iii) the use of vertical positioning to better image perpendicular structures in sporulating bacteria.
The paper is well written and clearly presented.

I have only minor comments.
1. Page 7, line 19, I think this only refers to Fig 2B-C
2. Page 8, lower paragraph. How can the TMR signal be “thicker” or “thinner”? Can they elaborate more on what they think is happening? The dye itself is not going to get thicker – do they think it is binding to a structure that is crumpling up on itself to become thicker? I can see how a dye-bound structure would get more sparsely distributed if its target is reduced, but it is not intuitive to understand how it would become thinner.
3. In general the figures are very well designed and easy to read, with channels clearly labelled. However in Fig 4A the green/magenta are not labelled on the figure itself and the reader needs to go to the figure legend. It would be good to have this info on the panel itself
4. None of the data in Fig 4 has been quantified. This information should be included.
5. Line 422, typo “braising”

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors did an excellent job addressing all the points raised by the reviewers.

Reviewer #3

(Remarks to the Author)

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Lablaine et al. reports the visualization of spore coat assembly in Bacillus subtilis using time lapse lattice-SIM, which provides increased spatial and temporal resolution of the localization patterns of individual coat proteins. In general, the manuscript was well written, and the experiments were well controlled. I imagine that this paper will be widely cited by sporulation researchers. I have only relatively cosmetic suggestions which the authors may or may not wish to incorporate.
Sincerely,
Kumaran Ramamurthi

We thank the reviewer for his positive feedback.

Major comments:

1. The use of TMR-star as a marker for late stages of sporulation is a valuable contribution of this report. However, I think that the conclusion that TMR-star specifically marks inner coat assembly is not entirely justified by the data presented. As I understand, the conclusion was based on the reported pattern of inner coat assembly in B. cereus, the coincident staining pattern of TMR-star and several late-synthesized inner coat proteins, and the partial mis-localization of TMR-star in the absence of SafA). Identifying the target of TMR-star is certainly beyond the scope of this report. However, the authors may choose to delete one or more genes encoding a coat basement layer protein like SpoIVA (by historical definition, to be considered a "coat" protein, its localization around the forespore must be dependent on SpoIVA; other proteins at the forespore surface that do not depend on SpoIVA are not technically classified as "coat proteins") and testing TMR-star localization. In the absence of demonstrating such a dependence, I suggest softening the conclusion (line 106 of the intro, line 210, for example) to simply state TMR-star staining is *coincident* with inner coat assembly.

Thank you for raising this point regarding whether TMR-star specifically stains the inner coat, which was also raised by Reviewer 2.

Following the reviewer's suggestion, we performed an additional experiment in which we labeled Δ*spoIVA* sporangia with TMR-star (the new strain is now listed in Table S1). As shown in the corresponding, new panel 'ΔIVA' of Fig. 2F, TMR-star localized abnormally in Δ*spoIVA* sporangia. Localization around the forespore was lost and TMR labelled non-adherent material within the mother cell cytoplasm. This localization pattern is reminiscent of the localization of inner coat material previously described with TEM in Δ*spoIVA* sporangia (Bauda et al. 2024, *Nat. Commun*.). This result provides further support for a localization of TMR-star in the inner part of the *B. subtilis* coat and has been added to the revised manuscript:

Lines 187-189: "In a *ΔspoIVA* mutant, the TMR-star signal was not anchored to the forespore surface and, instead, labelled non-adherent material in the mother cell cytoplasm (Fig. 2F, red asterisk)."

To summarize, our results show that:

i) TMR-star colocalizes (at the nanoscale level) with a class of late assembling inner coat proteins (YmaG, YxeE, LipC, YeeK, CotD) but not with early-synthesized inner coat proteins (YutH, YsxE, YisY)

ii) TMR-star localization/intensity around the forespore shows dependencies on SpoIVA, SafA and GerE -but not on CotE and CotZ

In line with the reviewers' comments, we have revised our conclusions and acknowledge that TMR-star does not report on inner coat assembly *sensu stricto*, as it does not colocalize with early-synthesized inner coat proteins. Thus, we agree that we cannot postulate that "TMR-star […] specifically labels the inner coat of *B. subtilis*" as indicated in the original version of the manuscript. **We now describe the TMR-star as a dye that can be used "to monitor the late stages of inner coat development".**

We have revised the manuscript accordingly, including at the end of the Introduction:

Original version: "We also demonstrated that TMR-star, a cell-permeable red fluorescent ligand typically used to label the SNAP-tag, which was recently shown to bind to the coat of *B. cereus* species[35,39], ~~specifically labels the inner coat of *B. subtilis*.~~"

⇨ Lines 89-91: "We also demonstrated that TMR-star, a cell-permeable red fluorescent ligand typically used to label the SNAP-tag, which was recently shown to bind to the coat of *B. cereus* species[35,39], can also be used in *B. subtilis* to monitor the late stages of inner coat development."

2. Line 22 (first use of 'refractile spores'). Consider more explicitly stating the challenges associated with identifying intermediate assembly stages after the forespore becomes refractile using phase contrast optics. The refractility of the forespore is itself a landmark event, and there are several membrane-permeable dyes that permit staining the forespore membrane at this stage. I assume the authors are suggesting that it would be helpful to have an independent landmark event to delineate spore maturation after forespores become refractile and before it is released as a mature spore?

Thank you for this constructive comment. We agree with the reviewer and have modified the text as follows:

Original version: "Furthermore, while early and intermediate sporulation stages can be monitored using commercial membrane dyes and phase-contrast microscopy; respectively, ~~no physiological markers are currently available to monitor later stages once the forespore becomes refractile.~~"

⇨ Lines 76-79: "Furthermore, while early and intermediate sporulation stages can be monitored using commercial membrane dyes and phase-contrast microscopy; respectively, it would be valuable to identify an independent landmark event to delineate spore maturation after forespores become refractile and before they are released as mature spores."

3. Line 293: "Extensive displacement of molecules on the forespore surface has not been previously described in spore forming bacteria." Forespore surface proteins YabP, YheD, and YutH have been previously described as exhibiting dynamic localization patterns (PMID

15231775). Consider softening this claim, as doing so would not impair any novelty of the present study.

Thank you for pointing this out. This was also raised by *reviewer* #2. We agree and we have removed this sentence and revised the section title as follows:

Original version: "**Early-synthesized inner coat proteins YutH and YisY ~~are dynamically redistributed on the forespore surface~~**"

⇨ Lines 233-234:" **Early-synthesized inner coat proteins YutH and YisY are displaced from the midspore region towards the poles**. "

We also updated the text to acknowledge previous findings:

Previous version: "Previous diffraction-limited studies indicated that YutH, its paralogue YsxE, and YisY initially localize at the forespore pole, ~~but left unclear their localization~~ during later sporulation stages[23,28]".

⇨ Lines 233-234: "Previous diffraction-limited studies indicated that YutH, its paralogue YsxE, and YisY initially localize at the forespore pole and suggested that YutH is dynamic on the spore surface[23,28]"

And we adapted the conclusions:

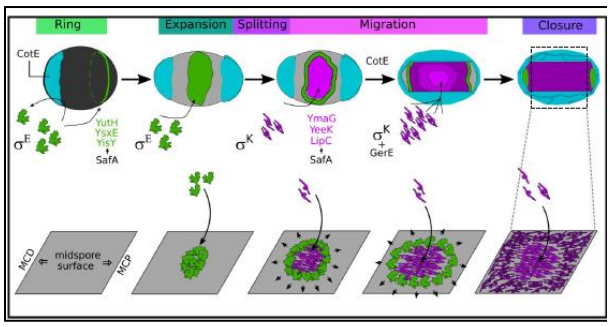
Original version: "In conclusion, our reconstituted 4D-sequence ~~revealed dynamic redistribution~~ of YutH-GFP along the forespore cylinder during late stages of sporulation, spatiotemporally intertwined with TMR-star deposition."

⇨ Lines 277-279: "In conclusion, our single-cell analysis coupled to the reconstituted 4D-sequence revealed a tight spatiotemporal coupling between YutH-GFP redistribution and TMR-star deposition along the forespore cylinder during late stages of sporulation."

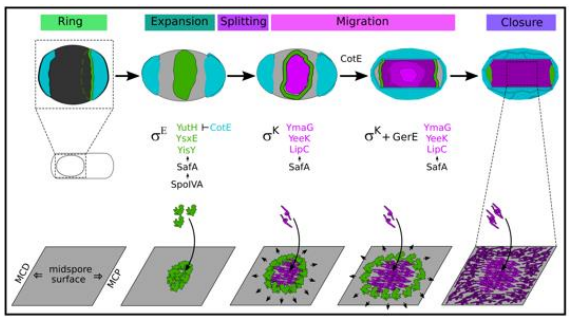
4. Fig. 5C. Consider more clearly showing the orientation of the forespore schematic. Given the placement of SigE and SigK, and the localization of CotE, I assume that this is a lateral view with the mother cell to the left?

We acknowledge that the schematic in Fig. 5C was confusing. The correct orientation places the mother cell on the right. The CotE layer on the mother cell pole (MCP) was omitted initially to highlight the localization of the YutH "ring". We now include a sporangium schematic to help orient the reader and have restored the CotE cap on the MCP in our model. We also simplified the labelling of proteins incorporation for clarity.

Original version



Revised version:



Minor comments:

1. Line 89, "…only been reported…via computational image analysis methods." A) please provide references. B) Technically, structured illumination, which is employed in this report, is also based on a computational foundation (image reconstruction using Fourier transform). Consider rephrasing this statement?

We deleted part of the sentence and added references:

Original version: "Sub-diffraction localization of individual coat proteins ~~has only been reported~~ in sporangia at a late sporulation stage or in mature spores ~~via computational image analysis methods.~~"

⇨ Lines 75-76 "Sub-diffraction localization of individual coat proteins has mainly been reported in sporangia at a late sporulation stage or in mature spores[22,23,41]"

2. Fig. 2B. Do the colors used to denote CotY, SpsI, YmaG, and YhaX correspond to anything? If not, consider simply labeling all of them in green (to reflect the GFP fusion of each) or simply black.

Colors were referring to the Schematic in Fig. 1B showing the different coat sublayers. To avoid confusion, they are now in black in all figures.

3. Fig. S2. Typo: "interval", not "intervale", correct?

This typo was also present in Fig S7 and S8 and are now corrected. Thanks for spotting it!

4. I realize that space in the main text may be precious, but consider moving Fig. S2 to the main text, since it is the first instance in the manuscript where the authors' central technique is highlighted in an intuitive fashion.

We had actually discussed and considered this before our original submission. In line with the reviewer's suggestion, we have included a slightly reduced version of Fig. S2 (with the first row of snapshots -where no signal is visible yet- removed) as panel D of the main Fig. 2.

To emphasize the novelty of our approach, we have also added labels on top of panels C and D indicating "population level" and "single cell level', respectively. Finally, we have generated two new supplemental movies (Supplementary Movie 1 and 3) that show, respectively:

i) sporulation development across a microcolony imaged with brightfield microscopy
ii) single cell dynamics of YutH-GFP/TMR-star localization during sporulation

5. Fig. 2 legend, elsewhere. Consider defining statistically not significant ("ns") as $P > 0.05$.

Done. Thanks. "ns" is now defined in the figure legend as $P > 0.05$.

Reviewer #2 (Remarks to the Author):

In this article, Lablaine et al. report a significantly updated analysis of the process of spore coat assembly in the model bacterium Bacillus subtilis. The process has been investigated in detail before, but due to technical limitations of classic fluorescence microscopy, some questions remained unanswered. It is also a highly complex process involving at least 80 different proteins that are distributed to different concentric coat layers (basement layer, inner coat, outer coat and crust) with different stages occurring in a period of several hours under the control of coat morphogenetic proteins and sporulation transcription factors. Here, the use of Structured Illumination Microscopy (SIM) resulted in an appreciable gain in resolution (70 nm, i.e., nanoscale). Perhaps even more importantly, data were collected for single cells in time lapse experiments, which unambiguously captured localization dynamics for a selection of coat proteins during the assembly process. Remarkably, new patterns of dynamic localization were observed, primarily for a class of inner coat proteins. These proteins first appear to localize to the midspore region until they are redistributed to fully encase the maturing spore. Interestingly, this behavior is also observed with the TMR-star red fluorescent stain, which the authors introduce as a specific marker of the inner spore coat layer. This will have important implications for future research in the field, since there was a lack of late-stage morphological sporulation stains. The authors also show that the localization of TMR star is dependent on the morphogenetic protein of the inner coat SafA and the regulators of gene expression in the mother cell at late stages of sporulation, SigK and GerE. Another important observation is that formation of the outer coat influences the redistribution of this class of inner coat proteins, which is also a new concept considering that assembly of the inner and outer coat layers were viewed as essentially independent processes. In general, the experiments are well done, and the images are superb and truly informative. I have listed a few points for discussion below.

We sincerely thank the reviewer for their appreciation of our work and the constructive remarks addressed.

Major point:
Is TMR-star truly a marker of the inner coat or is it more appropriately defined as a stain that colocalizes with a class of inner coat proteins, i.e. those that localize late (such as YmaG or YutH) or even later (CotD and YeeK)? It does not seem that TMR-star localization dynamics match that of well-defined early-localizing inner coat proteins such as YaaH or YuzC (kinetics of localization based on McKenney and Eichenberger, 2012, Mol Micro).

Thank you for raising this point, also raised by reviewer #1. We agree and now describe TMR-star as a dye that can be used “to evaluate the late stages of inner coat development,” which more accurately reflects our findings. Please see our response to Reviewer 1’ comment n°1 for further details.

Minor points:
l. 59 and elsewhere in the paper: “undercoat (or basement layer)”. It would be informative to know why the authors prefer the formulation “undercoat” to the term “basement layer”, which has been commonly used in several reviews of the spore coat assembly process. This layer is definitely a critical component of the coat structure, which is implied by the term

“basement layer”, the term “undercoat” suggests that it is a separate structure that happens to be localized under the coat.

To align with current literature, we now exclusively use the term “basement layer” throughout the manuscript.

l. 63 “and CotYZ are required for crust formation”, it would be more accurate to say that “CotXYZ are required for crust formation”

Corrected as suggested. Thank you.

l.232-234: designation of LipC as an inner coat protein. In Subtiwiki and sources listed therein, LipC is designated as a basement layer protein (undercoat). The original designation might be wrong (since LipC-GFP appears to colocalize with TMR-star), but this should be mentioned in case LipC needs to be reclassified.

Thank you very much for this thoughtful suggestion! The localization of LipC-GFP was originally described as dependent of SpoVID, SafA and CotE (Masayama et al., 2007) and, based on those results, LipC has been indeed classified as a basement protein (Driks and Eichenberger 2016).

Based on the strong nanoscale colocalization of LipC-GFP with TMR-star and its assembly dependence on SafA (Masayama et al., 2007), we suggest reclassifying LipC as an inner coat protein. This is now discussed in the Results and marked with an asterisk in Fig. 3.

Lines 205-206 “We also performed this analysis for LipC, a SafA, σ^K- and GerE-dependent protein, currently classified as a basement protein[5] [...]

Lines 223-225 The dependency of LipC assembly[54] and the strong nanoscale colocalization of LipC-GFP with TMR-star (Fig. 3A-C and Supplementary Fig. S5C) suggest that the original designation of LipC as a basement layer protein may be inaccurate, and instead support its localization within the inner coat.”

l.263-264:” Previous diffraction-limited studies indicated that YutH, its paralogue YsxE, and YisY initially localize at the forespore pole, but left unclear their localization during later sporulation stages.” And heading:” Early-synthesized inner coat proteins YutH and YisY are dynamically redistributed on the forespore surface”. I think this characterization of the earlier work is not entirely accurate. Ref 28 already indicated that the localization of YutH was dynamic (it is in the title of the paper), whereas details of YisY-GFP localization were provided in Ref 23 (in the supplements), including late time points. It is clear, however, that the new data presented in the current paper provide additional details and much improved resolution, both spatially and temporally.

This point was also raised by reviewer #1 - comment n°3. We have revised the heading as follows:

Original version: “**Early-synthesized inner coat proteins YutH and YisY ~~are dynamically redistributed on the forespore surface~~**”

⇨ Lines 231-232:" **Early-synthesized inner coat proteins YutH and YisY are displaced from the midspore region towards the poles**. "

We have also revised the text to better reflect prior studies and acknowledge that the dynamic nature of YutH was previously reported/suggested, although our study provides higher spatial and temporal resolution. Please see our answer to reviewer #1 - comment n°3 for more details

L.681: "Driven by σE synthesis". It might be more accurate to say "Driven by σE-dependent synthesis"

Changed to: "Driven by σE-dependent synthesis." Thanks

Reviewer #3 (Remarks to the Author):

Overall this is a solid paper that establishes labelling and imaging approaches to look at Bacillus subtilis spores and uses this to tease out the positions and relationships between various coat proteins. Important strengths of the paper are (i) the use of TMR-star, previously shown in B. cereus, to label the spore inner coat, (ii) the use of super-resolution imaging methods to distinguish between GFP-tagged proteins and the TMR label at different layers of the spore and (iii) the use of vertical positioning to better image perpendicular structures in sporulating bacteria.
The paper is well written and clearly presented.

We thank the reviewer for their positive evaluation of our work.

I have only minor comments.
1. Page 7, line 19, I think this only refers to Fig 2B-C

Thanks for spotting this. Corrected as suggested.

2. Page 8, lower paragraph. How can the TMR signal be "thicker" or "thinner"? Can they elaborate more on what they think is happening? The dye itself is not going to get thicker – do they think it is binding to a structure that is crumpling up on itself to become thicker? I can see how a dye-bound structure would get more sparsely distributed if its target is reduced, but it is not intuitive to understand how it would become thinner.

As discussed in response to reviewers #1 and #2, our results show that the TMR-star signal localization mirrors the localization of inner coat material described with TEM in previous studies, in a WT condition as well as in all the mutant tested in the present study.

In TEM images, the mature inner coat appears as a thick structure consisting of a juxtaposition of three to five thin laminae. Thus, the TMR-star, at a late stage of inner coat development, likely binds to all these closely packed layers (laminae). While the thickness of a single lamina is below the resolution of our SIM² system, the signal emanating from TMR-star molecules fixed to all these closely packed layers is above the resolution of our system.

On the basis of this laminar view of the mature inner coat; it has been shown by electron microscopy that the structure of the inner coat is strongly impaired and thinner in a *gerE* mutant. Notably, the various laminae were not visible (Moir 1981). In the Δ*gerE* background, we observed a reduction of the "thickness" of the forespore outline stained with TMR-star signal, which likely represents binding of TMR-star to this unmatured/incomplete inner coat material. In other words, in this mutant the substrate of the TMR-star is reduced. We adapted the text in consequence:

Original version: "~~suggesting abnormal organization of the structure to which TMR-star binds.~~"

⇨ Line 187: "indicating that the target of the TMR-star is reduced in this mutant."

Concerning the thicker signal of the TMR around the forespore in a Δ*safA* mutant, the inner coat is not correctly anchored on the forespore surface and appears partially disorganized (Takamatsu et al., 1999). The laminae of the inner coat, which are not well anchored, are likely partly dissociating and thus generate a 'looser' structure.

We now discuss this point in the section:

⇨ Lines 193-200: "Importantly, the localization of TMR-star signal observed in the various conditions closely mirrors the features of inner coat assembly previously described in transmission electron microscopy studies[15,18,52,53]. In wild-type forespores the TMR-star signal can be interpreted as the binding of the dye to the various, densely packed, laminae that compose the inner coat. Our results suggest that in *ΔsafA* sporangia; these laminae are partially decompacted around the forespore and partially detached in the mother cell cytoplasm. Altogether, our nanoscale colocalization analysis (Fig. 2B-E and Supplementary Fig. S4) and genetic analysis (Fig. 2F) show that TMR-star binds to a substrate within the inner coat that depends on late mother cell transcription factors."

3. In general the figures are very well designed and easy to read, with channels clearly labelled. However in Fig 4A the green/magenta are not labelled on the figure itself and the reader needs to go to the figure legend. It would be good to have this info on the panel itself

Channel labels for green/magenta have now been added directly to the figure panels.

4. None of the data in Fig 4 has been quantified. This information should be included.

We believe that quantitative analysis would not significantly enhance the conclusions due to the transient nature of the key localization patterns. We now indicate that all 4D sequences were reproduced in at least two biological replicates, and that the data presented reflect patterns consistently observed across replicates. These details are presented in the reporting summary and we have also included them in the Material and Methods section 'Statistics and reproducibility':

Lines 478-480: "Data supporting the 4D assembly of YutH-GFP were performed at least in triplicate for the WT condition and in duplicate for the mutant strains. All phenotypes described, including 3D localizations, were successfully replicated in at least three fields of view per replicate."

5. Line 422, typo "braising"

Corrected. Thanks!