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A new mechanism of fibronectin fibril assembly revealed by live imaging and super-resolution microscopy

Darshika Tomer, Cecilia Arriagada, Sudipto Munshi, Brianna Alexander, Brenda French, Pavan Vedula, Valentina Caorsi, Andrew House, Murat Guvendiren, Anna Kashina, Jean Schwarzbauer and Sophie Astrof

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MS TITLE: A new mechanism of fibronectin fibril assembly revealed by live imaging and super-resolution microscopy

AUTHORS: Darshika Tomer, Cecilia Arriagada, Sudipto Munshi, Brianna Alexander, Brenda French, Pavan Vedula, Valentina Caorsi, Andrew House, Murat Guvendiren, Anna Kashina, Jean Schwarzbauer, and Sophie Astrof
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the referee reports are somewhat divergent. Based on the enthusiasm of two of the referees, I would be amenable to considering a revised version of the paper that responds to the issues raised as much as is possible. Please note in particular the comments in the "feedback" section from referee #1 and consider these in revising your paper. We would then return it to the reviewers.

Please 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The authors make a strong argument that assembly of fibronectin on cell surfaces occurs stepwise such that fibronectin dimers polymerize into discreet globules comprising 6-11 dimers with a diameter of approximately 28 nm and the globules (called “nano-domains”) are transported linearly over the cell surface to form fibrils in which the globules have approximately 100-nm periodicity. The findings are not compatible with the widely accepted model of assembly in which the 54-58 modules of a fibronectin dimer are extended in a linear array and interact side-to-side via defined overlapping segments. Because FUD, a bacterially inspired polypeptide that interacts with the 70K N-terminal region of fibronectin, blocks the formation of the fibrillar array of globules but not the formation of globules per se, it is proposed that fibrillar array formation is driven by engagement of 70K regions of fibronectins in one globule with fibronectin in immediately adjacent globules or with molecules that are not fibronectin. There is a paucity of epitopes for anti-fibronectin antibodies in regions between globules, and therefore the authors give serious credence to the second possibility and in particular that the non-fibronectin molecules are the large apparent molecular weight molecules (LAMMs) to which surface-bound fibronectin or 70K can be cross-linked.
The argument is based on supra-resolution microscopic localization of multiple anti-fibronectin antibodies to periodically placed globules in fibrils formed under a variety of conditions, including in vivo. Results are reported visually and quantitatively in ways that make it easy relate the two. As controls to characterize fibronectin globules and their spacing in fibrils, nuclear pore proteins, which form complexes of known stoichiometry and spacing, have been imaged and analyzed in parallel. Various image analysis programs were employed to remove observer bias. The paper seems like an excellent model for others seeking to make the same types of arguments.

Comments for the author

1. Alpha5beta1 integrins colocalize with globules at the edge of the cell and in linear arrays, implying that clustered integrins bind fibronectin and drive globule formation. Are the authors ready to state that explicitly? If so is there information about the number of integrins clustered in adhesive complexes?
2. It would be helpful to see a back-of-the-envelope calculation about whether the 54-58 modules, for which dimensions are known, per dimer of 10 fibronectin dimers would fit easily into a globules with a diameter of 28 nm.
3. It also would be helpful to describe the 70K region, FUD, and polypeptides with activities like FUD (Mauer et al, 2012; Ma et al, 2015) in more detail. Isolated 70K in a strong inhibitor of fibronectin assembly (McKeown and Longo, 1985). How do the authors fit that observation in their model? FUD and the other polypeptides are disordered and engage multiple type I modules within 70K by a specialized type of concerted anti-parallel beta-strand addition called beta-zipper formation. Although one can point out similarities in sequences in active polypeptides, there is plenty of room for degeneracy. Do the authors think that LAMMs have disordered regions and bind the 70K region by the same mechanism?
4. Finally, it would be helpful if the authors speculate on how the 100-nm periodicity is enforced.
5. The paper contains a vast amount of information and for the most part seems clean. It would benefit from a final review to insure that the figures are as the authors want. I.e., is the length bar in Fig 2B2 correct, and should not there be lengths for the bars in the A2, B2, C2, and D2 panels of Fig. 4 and the promised vertical bar in panel D of that figure? The authors also should decide which of the many abbreviations are needed, be sure that the abbreviations are defined the first time used, and consider a convention in which abbreviations are independently defined in figure and table legends so that these can be interpreted without searching through the main text.

Reviewer 2

Advance summary and potential significance to field

The authors offer an interesting hypothesis of a non fibronectin protein involved in the assembly of the fibronectin matrix. However, there are no data presented which demonstrate the existence of

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such a protein. The data in the paper are largely consistent with earlier studies on the mechanism of Fn fibrillogenesis with little by way of new insights in matrix assembly. Were the authors able to provide some biochemical and functional data to support this hypothesis, such a finding would have significant impact on the field.

Comments for the author

Tomer, Arriagada, Munshi et al. JCS
The data presented in this manuscript from Tomer et al., represent a microscopic analysis of the stages of fibronectin fibrillogenesis. While the imaging is state of the art, the findings essentially corroborate what we have learned about Fn assembly over the past 40 years.
The authors show that fibrillogenesis proceeds from the formation of "nanodomains" containing 6-11 Fn dimers which are initially associated with the a5b1 integrin. The authors propose that Fn assembly initiates in peripheral a5b1 integrin adhesion sites which move rearward with the flow of actin and become organized into longer linear "beaded fibrillar adhesions". There have been several earlier studies showing that Fn fibrillogenesis initiates in adhesion sites which are drawn inward and elongated into fibrils in association with actin (Zamir et al, Nature Cell Biol 2000; Pankov et al., J Cell Biol. 2000; Ohashi J Cell Sci 2002; Geiger and Yamada. Cold Spring Harb Perspect Biol 2011).
The authors use several antibody preparations which recognize epitopes along the Fn molecule to stain the developing matrix. The Fn fibers have a beaded staining pattern which is seen regardless of the antibody used. The "beads" or "nanodomains" stain with antibodies directed against epitopes across the entire Fn molecule. Based on the size and staining patterns, the authors conclude that the nanodomains consist of 6-11 Fn dimers. The authors propose that the lack of antibody staining in the linear regions of the fibrils indicate that this region of the fiber is composed of a non-Fn protein which serves as a linker between the Fn nanodomains. This conclusion is inconsistent with several earlier studies which have also documented the "beads on a string" appearance of Fn fibrils…both in fibrils assembled by cultured cells and in fibrils generated in cell-free systems by surface-initiated assembly of purified fibronectin (Chen et al, Scanning 1997; Nylea and Kaartinen J Struc Bio 2010; Fruh et al., 2015; Syzmanski et al., Biomat Sci 2017).
As further support for their hypothesis, the authors note that the linear region of the fiber does not consist of Fn because the antibody cocktail "would uniformly label Fn fibrils". An alternative explanation would be that the absence of uniform staining between the nanodomains could arise from changes in the display of antibody epitopes. The Fn matrix functions as a scaffold for the binding of several molecules including matrix molecules, integrins, growth factors and cytokines. The association of these molecules with the Fn matrix would be expected to block the accessibility of antibody binding sites.
Additionally, increases in mechanical force can cause the unfolding of Fn's Type III domains. Earlier studies have shown that Fn is under constant tension in the ECM which leads to the unfolding the Type III domains which would also be expected to disrupt the display of antibody epitopes (Ohashi et al., PNAS 1999).
Fn's amino-terminus is known to be required for matrix assembly and consistent with this, the authors show that FUD, a peptide which binds to Fn's amino-terminus prevents fibril formation, does not disrupt the nanodomains, but does disrupt the fibrillar structure releasing the individual nanodomains from the fiber. Based on these data, the authors propose that the amino terminal region of the Fn molecules in the nanodomains are mediating the attachment of the nanodomains to the putative non-Fn linker protein. However, it should also be noted that the fibers assembled by surface-initiated Fn fibrillogenesis in a cell-free system are also dependent on the Fn amino-terminus (Salmeron-Sanchez Biomaterials 2011), obviating the need for a non-Fn linker protein. These previous studies indicate that beaded Fn fibers can assemble in cell free systems and that this depends on homophilic interactions mediated by Fn's amino terminus.
The authors propose, but provide no evidence for, the idea that LAMM (Large Apparent Molecular Mass molecule) might be the protein linking the nanodomains into fibers. This hypothesis is based on a single study from 1996 demonstrating that the amino-terminal fragment of Fn could be cross-linked to a large molecule on cell surfaces by Factor XIII (Zhang and Mosher JBC 1996). LAMM was never identified nor have subsequent papers on this model been published.
Are the authors also proposing novel aspects of the early steps in fibrillogenesis? Are the nanodomains thought to form by the clustering of integrins bound to substrate attached Fn? Would this involve homophilic interactions among the Fn molecules? Is the linker protein thought to be

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continually synthesized and secreted? Such a model would be inconsistent with earlier studies showing that incorporation of exogenous Fn into matrix does not require protein synthesis.

Reviewer 3

Advance summary and potential significance to field

The submitted manuscript uncovers a novel paradigm in of fibronectin fibrillogenesis. Using a combination of tools and methods ranging from old well established and documented fibronectin domain specific antibodies and modern super resolution techniques the authors provide strong evidence for Fn fibrillogenesis occuring via assembly on small FN nanodomains into linear fibrillary arrays. The authors demonstrate that FN fibrils are periodic in vivo in the cardiac tissue as well as when produced by cells in vitro and provide a wealth of controls for the imaging and the probes used to rule out that this is an imaging artefact. The ECM field is mature and it is rather remarkable that such a novel paradigm is uncovered for a protein that has been investigated for nearly half a century. This is an exciting discovery, the quality of the experiments is excellent and well controlled.
The authors demonstrate carefully that their endogenously tagged FN is functional in vitro and in vivo suing the key gold-standard experiments in the field. This is important and validates the relevance of their data.
The authors experimental data challenge the current model “that thinnest Fn1 fibrils are made of extended single Fn1 molecules that are periodically aligned in a regular, end-to-end fashion with regions containing N-termini alternating with regions containing C-279 termini (Chen et al.,1997; Dzamba and Peters 1991; Fruh et al., 2015).” This is an important finding and the antibody staining data indicating that instead the FN fibers are formed of FN nanodomains that contain full-length Fn1 dimers is convincing.

Comments for the author

All the data presented are from mouse tissue and cells. The impact of the study would be increased further if the authors were able to show with some of their antibody stainings that their main finding “Fn fibrillogenesis occuring via assembly on small FN nanodomains into linear fibrillary arrays “ holds true also across species in human cells.
The authors present nice data showing that the bacterial FUD peptide inhibits the fibrillogenesis but does not interfere with the nanodomain assembly. These data would be even more convincing if the authors could include experiments imaging Fn1 proteins lacking the N-terminal assembly domain as these have been shown to be unable to form fibrils (Schwarzbauer, 1991),

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First revision

Author response to reviewers' comments

Response to Reviewers’ Critiques
We would like to thank the reviewers for carefully reading the manuscript and providing critical comments and insights. We have addressed each of the reviewers’ comments below and modified our manuscript accordingly. We have re-written and re-organized the discussion section. The modifications made in response to reviewers’ comments are highlighted and referred to as their line numbers below.

Reviewer 1.

1. *Alpha5beta1 integrins colocalize with globules at the edge of the cell and in linear arrays, implying that clustered integrins bind fibronectin and drive globule formation. Are the authors ready to state that explicitly? If so, is there information about the number of integrins clustered in adhesive complexes?*

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Understanding how, when, and where Fn1 nanodomains form and the role of integrins in this process is an exciting future direction of this research. Careful experimentation will be needed to determine whether α5β 1 or any other integrins are involved in nanodomain formation. We included a description of our proposed model for Fn1 fibrillogenesis in the first paragraph of the Discussion, lines 443-453, and later in the Discussion where we refer to **Figure 8E**.

2. *It would be helpful to see a back-of-the-envelope calculation about whether the 54-58 modules, for which dimensions are known, per dimer of 10 fibronectin dimers would fit easily into globules with a diameter of 28 nm.*

We did the following “back-of-the-envelope” calculation and, to our surprise, the number of Fn1 dimers was similar to that determined by image analysis. This calculation is for the reviewer only and is not included in the manuscript.

Each type III is about 2 nm x 1.5 nm x 3.6 nm (Sharma et al., 1999) = volume of 10.8 nm^3. FN has 15-17 type III, 12 type I, 2 type II = 29-31 repeats. We used 30 individual repeats per subunit and the same volume for all repeats.
10.8 x 30 repeats = 324.0 nm^3 = estimated volume for 1 FN subunit (not accounting for packing of spheres).

28 nm diameter nanodomain. r = 14 nm. Volume = 11488.21 nm^3. If we assume a FN subunit is a sphere, we can apply the estimated efficiency of 64% for packing small spheres within a larger sphere.
11488.21 x 0.64 = 7352.46 nm^3 for one nanodomain.

7352.46 ÷ 324 = 22.7 FN subunits = 11.35 FN dimers.

3a. *It also would be helpful to describe the 70K region, FUD, and polypeptides with activities like FUD (Mauer et al, 2012; Ma et al, 2015) in more detail. Isolated 70K is a strong inhibitor of fibronectin assembly (McKeown and Longo, 1985). How do the authors fit that observation in their model?*

We mention 70K and FUD as inhibitors in the Results section (lines 410-416) and in the Discussion section (lines 477-479). We have revised our Discussion to better explain a potential mechanism linking nanodomains through Fn1-Fn1 interactions. We realized that by using LAMMs as an example of one potential mechanism for nanodomain organization, we were giving the impression that this was our favored model, and the inclusion of LAMMs was also making the description of a nanodomain array more complicated than necessary at this stage of the research. We now focus our model on connections via Fn1-Fn1 interactions **(Fig. 8E)** and have reduced the emphasis on LAMMs including removing diagrams that rely on LAMMs for nanodomain organization.

3b. *FUD and the other polypeptides are disordered and engage multiple type I modules within 70K by a specialized type of concerted anti-parallel beta-strand addition called beta-zipper formation. Although one can point out similarities in sequences in active polypeptides, there is plenty of room for degeneracy. Do authors think that LAMMs have disordered regions and bind the 70K region by the same mechanism?*

From the referees’ comments, it seems that we gave the impression that LAMMs are our favored model to explain nanodomain assembly. LAMMs have been previously suggested by others and cannot be excluded by our results. Note that in the rewritten Discussion (lines 480-482) we suggest that extended Fn1 dimers between nanodomains provide the link in fibrils. Fn1 links could explain the ~100 nm periodicity of nanodomains. This is also shown in **Figure 8E**.

4. *Finally, it would be helpful if the authors speculate on how the 100-nm periodicity is enforced.*
See response to point 3.

5. *The paper contains a vast amount of information and for the most part seems clean. It would benefit from a final review to ensure that the figures are as the authors want. I.e., is the length bar in Fig 2B2 correct, and should not there be lengths for the bars in the A2, B2, C2, and D2 panels of Fig. 4 and the promised vertical bar in panel D of that figure? The authors also should*

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decide which of the many abbreviations are needed, be sure that the abbreviations are defined the first time used, and consider a convention in which abbreviations are independently defined in figure and table legends so that these can be interpreted without searching through the main text.

We appreciate this comment, and we have now carefully examined all figures and dealt with abbreviations, in particular, the ones mentioned by the reviewer:

The scale bar in Fig. 2B2 is correct. The scale bar lengths in Fig. 4A2, B2, C2, and D2 are listed in the legend to avoid obscuring the image. We made the bar in Fig. 4D thicker and hope it is more visible now.

We have also reread the text and made minor edits throughout to improve clarity and consistency.

Reviewer 2.

1) *The authors offer an interesting hypothesis of a non-fibronectin protein involved in the assembly of the fibronectin matrix. However, there are no data presented that demonstrate the existence of such a protein. The data in the paper are largely consistent with earlier studies on the mechanism of Fn fibrillogenesis with little by way of new insights into matrix assembly.*

We respectfully disagree with the reviewer, the current model of Fn1 fibrillogenesis posits that Fn1 fibrils are composed of aligned and extended Fn1 molecules such that Fn1 fibrils look like ropes made of LEGO pieces, in which each Fn1 protein domain is a LEGO piece of a different color. The novelty is now explicitly stated in the first paragraph of the Discussion.

Our model is different. In our model, Fn1 fibrils are like arrays of pearls. Each pearl is made of multiple Fn1 dimers. In the Discussion (**lines 450-453**), we provide examples from the literature of Fn1 structures that could also be described as “nanodomains”.

To the best of our knowledge, no one prior to us has shown that Fn1 fibrils as linear arrays of nanodomains, each containing multiple Fn1 dimers.

2) As further support for their hypothesis, the authors note that the linear region of the fiber does not consist of Fn because the antibody cocktail “would uniformly label Fn fibrils”. ***An alternative explanation would be that the absence of uniform staining between the nanodomains could arise from changes in the display of antibody epitopes****. The Fn matrix functions as a scaffold for the binding of several molecules including matrix molecules, integrins, growth factors and cytokines. The association of these molecules with the Fn matrix would be expected to block the accessibility of antibody binding sites. Additionally, increases in mechanical force can cause the unfolding of Fn’s Type III domains. Earlier studies have shown that Fn is under constant tension in the ECM which leads to the unfolding the Type III domains which would also be expected to disrupt the display of antibody epitopes (Ohashi et al., PNAS 1999).*

We agree that less staining between nanodomains does not mean there is no Fn1 in that region. We now state in the Results (**lines 326 - 330**) that there is Fn1 localized between nanodomains but that it is much less. Epitope accessibility and Fn1 conformational changes are two reasons. Another is that there may be only a few Fn1 molecules between nanodomains which would result in limited staining. We have modified our description of these data and now make it clear that our favored model is linkage by extended Fn1 molecules between nanodomains (see Fig. 8E, and **lines 480-485**). We refer to the work of Ohashi et al. in the Discussion (**line 508**).

3) *Fn’s amino terminus is known to be required for matrix assembly and consistent with this, the authors show that FUD, a peptide that binds to Fn’s amino- terminus prevents fibril formation, does not disrupt the nanodomains, but does disrupt the fibrillar structure releasing the individual nanodomains from the fiber. Based on these data, the authors propose that the amino-terminal region of the Fn molecules in the nanodomains are mediating the attachment of the nanodomains to the putative non-Fn linker protein. However, it should also be noted that the fibers assembled by surface-initiated Fn fibrillogenesis in a cell-free system are also dependent on the Fn amino terminus (Salmeron-Sanchez Biomaterials 2011), obviating the need for a non-Fn linker protein.*

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These previous studies indicate that beaded Fn fibers can assemble in cell-free systems and that this depends on homophilic interactions mediated by Fn's amino terminus.

We realized that by using LAMMs as an example of one potential mechanism for nanodomain organization, we were giving the impression that this was our favored model. We have now reduced the emphasis on LAMMs and removed diagrams that rely on LAMMs for nanodomain organization. Our favored model is interactions between Fn1 molecules extending from adjacent nanodomains. Our speculation on the possible involvement of LAMMs in fibrillogenesis was based on existing literature and has now been toned down.

4) *The authors propose, but provide no evidence for, the idea that LAMM (Large Apparent Molecular Mass molecule) might be the protein linking the nanodomains into fibers. This hypothesis is based on a single study from 1996 demonstrating that the amino-terminal fragment of Fn could be cross-linked to a large molecule on cell surfaces by Factor XIII (Zhang and Mosher JBC 1996). LAMM was never identified nor have subsequent papers on this model been published.*

See response to point 3.

4a) *Are the authors also proposing novel aspects of the early steps in fibrillogenesis?*

Our data are consistent with reports that Fn1 fibrillogenesis occurs concurrently with the rearward actin flow of fibrillar adhesions. The novel aspects include: Centripetally- translocating Fn1 nanodomains containing multiple Fn1 dimers become organized into linear arrays of periodically-spaced nanodomains. The co-localization with α5β1 integrins suggests that nanodomains are associated with fibrillar adhesions. Our live imaging data indicate that the interactions mediated by the N-terminus of Fn1 are important for the organization of mobile Fn1 nanodomains into arrays. When these interactions are blocked by FUD, the nanodomains still move centripetally but they are no longer hooked up into organized arrays. The transmembrane and cytoskeletal molecules involved in organizing mobile nanodomains into arrays will need to be further investigated in the future to compare these with focal and fibrillar adhesions (lines 500- 505).

4b) *Are the nanodomains thought to form by the clustering of integrins bound to substrate attached Fn?* ***4c)*** *Would this involve homophilic interactions among the Fn molecules?* ***4d)*** *Is the linker protein thought to be continually synthesized and secreted?* ***4e)*** *Such a model would be inconsistent with earlier studies showing that incorporation of exogenous Fn into matrix does not require protein synthesis.*

4b-c) As reviewer 2 suggests, integrins such as α5β1 are likely to be clustered and connect Fn1 nanodomains with the actin cytoskeleton. We show that α5β1 co-localizes with the beads of Fn1 in TIRF images (Fig. 2). The discovery that Fn1 fibrils can arise from the organization of mobile Fn1 nanodomains into arrays is being reported in this manuscript. We are currently developing and testing hypotheses about the mechanism(s) by which nanodomains form and the molecules mediating their formation.

4d-e) Our data are consistent with several models, one which has an extended Fn1 dimer as the linker and the other involving a putative linking protein or complex. Our data do not contradict any biochemical data on Fn1 fibrillogenesis collected over the years.

Reviewer 3.

1. *All the data presented are from mouse tissue and cells. The impact of the study would be increased further if the authors were able to show with some of their antibody stainings that their main finding "Fn fibrillogenesis occurring via assembly on small FN nanodomains into linear fibrillary arrays " holds true also across species in human cells.*

In our discussion section, we mention that the dotted appearance of human Fn1 fibrils has been noticed before, e.g., see **Fig. 11A-B** in (Furcht et al., 1980). In this study, Furcht stained ECM synthesized by human cells with polyclonal antibodies to Fn1 and noted that the staining looked periodical. To our eyes, the pattern of staining observed in **Fig. 11A-B** in this paper looks very much like ours. The author of this work did not follow up on this observation. This observation was mentioned in Richard Hynes "Fibronectins" book, but otherwise, it appears that it has become

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forgotten and the significance of this observation has gone unexplained. We cite this study in our Discussion.

Fn1 synthesized by human lung fibroblasts imaged by cryoEM also appears nodular (Lansky et al., 2019). In both studies, the authors don't go any further than simply stating these observations. We have cited these studies in our Discussion, **line 461 (the reference to Lansky) and lines 261-263 (references to Furcht)**. Together with our work, these studies show that the nanodomain architecture of Fn1 fibrils is observed in species other than mouse.

2. *The authors present nice data showing that the bacterial FUD peptide inhibits fibrillogenesis but does not interfere with the nanodomain assembly. These data would be even more convincing if the authors could include experiments imaging Fn1 proteins lacking the N-terminal assembly domain as these have been shown to be unable to form fibrils (Schwarzbauer, 1991).*

Understanding the role of Fn1 domains and features (e.g., N-term domain and C-term disulfide bonds) in Fn1 fibrillogenesis is part of our experimental plan for further mechanistic studies to understand the process of Fn1 fibrillogenesis. We are planning to use N-terminus deletion mutants together with other mutants from (Schwarzbauer, 1991) in our live imaging and super-resolution studies in the future.

Feedback on other Reports (from Reviewer 1):

RE: Reviewer 2
"Reviewer 2 suggests ways that epitopes could be lost. The epitopes for the polyclonal epitopes were mapped by overlapping short linear peptides, so this explanation seems unlikely to me. However, the explanation could be included in the discussion of a revised paper."

We now mention in the Discussion (**lines 469-485**) the simplest explanation for reduced detection of Fn1 between nanodomains. This discussion accompanies our description of how extended Fn1 dimers could provide the linkage between adjacent nanodomains.

"… because the N-terminal modules of fibronectin arose de novo in chordates and are able to engage intrinsically disordered stretches of a number of different proteins, I worry that LAMMs may be multiple proteins serving the same function, which could be very difficult to prove. The finding of nanodomains provides a strong rationale to initiate a search but the paper should not be held up pending the results of the search."

We agree with the reviewer's assessment. We mention in the Discussion (**lines 486- 499**) that more research is needed to determine the mechanism that connects nanodomains into arrays.

"Finally, reviewer 2 notes that plasma fibronectin assembles into fibrils in cell-free systems, something that could be added to the discussion."

We mention in vitro self-assembly studies in the Discussion (**lines 486-489**). RE: Reviewer 3

"I do not think studies of fibronectin assembled by cultured human (as opposed to murine) cells would add much. The mouse cells were cultured under a number of conditions, and importantly, the same results were seen in mouse embryonic tissues."

In our response to reviewer 3, we point out two examples from the literature that report Fn1 structures similar to nanodomains. These are cited (**lines 261-263** and **lines 461**).

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Second decision letter

MS ID#: JOCES/2022/260120

MS TITLE: A new mechanism of fibronectin fibril assembly revealed by live imaging and super-resolution microscopy

AUTHORS: Darshika Tomer, Cecilia Arriagada, Sudipto Munshi, Brianna Alexander, Brenda French, Pavan Vedula, Valentina Caorsi, Andrew House, Murat Guvendiren, Anna Kashina, Jean Schwarzbauer, and Sophie Astrof
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Strong fluorescence microscopic evidence is presented that fibronectin fibrils comprise discrete aggregates of fibronectin held together by material sparse in fibronectin antigenic epitopes. The evidence is contrary to currently accepted models of fibronectin fibrils and raises interesting questions about the control of aggregate formation and how the aggregates are held together.

Comments for the author

The changes made in response to the three reviews are appropriate and improve the paper.

Reviewer 3

Advance summary and potential significance to field

This is a very exciting paper and has the potential to open many new directions in the field

Comments for the author

The authors have addressed my minor comments in a satisfactory manner.

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