Journal of Clinical and Translational Research
Peer review process file 03.201702.003

FITC-linked Fibrin-Binding Peptide and real-time live confocal microscopy as a novel tool to visualize fibrin(ogen) in coagulation

Nikolaj Weiss, Bettina Schenk, Mirjam Bachler, Cristina Solomon, Dietmar Fries, Martin Hermann

Corresponding author:
Martin Hermann
Department of Anesthesiology and Critical Care Medicine, Innsbruck Medical University, Innrain 66, 6020 Innsbruck, Austria

Handling editor:
Michal Heger
Department of Experimental Surgery, Academic Medical Center, University of Amsterdam, the Netherlands

Review timeline:

Received: 31 May, 2016
1st Editorial decision: 29 July, 2016
Revision received: 9 September, 2016
2nd Editorial decision: 12 February, 2017
Revision received: 22 May, 2017
3rd Editorial decision: 24 May, 2017
Published online: 24 May, 2017

1st editorial decision
Date: 29-Jul-2016

Ref.: Ms. No. JCTRes-D-16-00014
FITC-linked Fibrin-Binding Peptide and real-time live confocal microscopy as a novel tool to visualize fibrin(ogen) in coagulation
Journal of Clinical and Translational Research

Dear Dr. Hermann,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Aug 28, 2016.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Editor's comments:

Dear authors,

Four reviewers have critically appraised your work and relayed a generally positive outlook regarding your study but also provided constructive criticism that should help you improve the paper. One reviewer is recommending a reject while three reviewers are recommending major revisions to be made before the paper is acceptable. After deliberation with an associate editor we have decided to render a major revision verdict.

I therefore kindly ask you to implement the requested modifications to the fullest possible extent and supply a response to every point raised in a separate document. Also, in the instances where you deem a rebuttal is necessary, please provide sound argumentation that is in support of your position. Although we have asked experts in the field to peruse over your work, their critical points may sometimes stem from miscommunication in your paper or incomplete understanding of how certain things were done. All these factors will weigh on the final decision, so it is imperative that your rebuttal is equally solid as the work itself.

The JCTR editorial board also would like to emphasize the following points:

1) Our image manipulation software has picked up on Figure 4B and C as being possibly manipulated with software. Please indicate explicitly in the figure legend or text that images were modified, if this is indeed the case, and provide a rationale in the rebuttal letter why this was done.

2) A general consensus amongst the reviewers is that the Materials and Methods require more elaborate elucidation of the methods used as well as the addition of appropriate controls. Please ensure that others can reproduce the methods in exactly the same manner as you conducted the experiments (by being more elaborate in the Methods text) and that the obtained results are not skewed by technical problems (this can be resolved by the addition of control experiments).

3) The authors added a fluorescent label to a previously described fibrin-binding peptide for use in confocal microscopy with potential future applications in in vivo imaging. However, the same peptide (with a different fluorescent label) has already been used to visualize fibrin in murine thrombi in vivo (Hara et al. 2012 JACC: Cardiovascular Imaging). Please make a reference to the prior art in the paper and address the novelty of your method in juxtaposition to what has already been published (e.g., in the Discussion).

4) The authors repeatedly state that labeling fibrin with the fibrin-binding peptide is superior to the established use of pre-labeled fibrinogen, yet provide no references or experiments to justify this

conclusion. Please explicitly justify or cite the appropriate references to corroborate your claims.

5) The methods used for each experiment are unclear, statements in the results do not match what is described in the figure legends, and it is difficult to interpret what is being shown in some of the figures. Please tend to this and make sure that the presented data are unequivocal and devoid of incongruences.

6) The introduction should clearly formulate the research question.

Thank you for submitting your work to JCTR and we look forward to your revised paper.

Kindest regards,

Michal.

Reviewers' comments:

Reviewer #1: General Comments
The authors claim to develop a novel biochemical tool for microscopy based visual analysis of fibrin clot structure. For this purpose the authors conducted an in vitro study using a commercially available method and compared that with their new method. The research question is an important one in view of the clinical significance of the clot dynamics, however, the methods used lack some important control groups. Therefore, additional experiments are required before the claims made by authors could be believed. Also, there are no quantitations (micrograph quantitative data, number of experiments, sample size, statistics) performed on any of the images.
Specific points -
1. The authors have overlooked the available anti-Fibrin antibodies (such as the one offered by Fitzgerald Industries, 10-2523) which are used to stain polymerized fibrin, these antibodies can be used after conjugation with fluorophores using commercial conjugation kits, so no need of adding secondary antibodies. The authors may show the comparison with those antibodies or else should convincingly explain how, other than just fixation step, FFBP is better.
2. In Figure 2, a control well without fibrin or coagulation mix, but with any non-specific protein (BSA or collagen) and FFBP is required to assess the specificity of FFBP to Fibrin. This is easy to perform.
3. There is no data to show staining with different concentrations of FFBP since it is a new fibrin labelling tool as they claim.
4. The methods require more clarity, at many points the language is confusing. In last para, as they say they have used two different pre-labelled fibrinogens, first, the Alexa-647, from sigma, and later they mention one from thermo as well, however the data from the latter is missing.
5. Figure 4 panel results are not clear, the angles/plains used for imaging should be explained

clearly, apart from this, Figure 4B and 4C needs to be submitted in raw/unedited form.

6. In the future directions section in text, the authors claim this model of theirs to be more representative of traumatic bleeding than the injury based in vivo models, this point has no rationale as the in vitro model used by authors is no close to traumatic bleeding either, the ideal way would be to stain the natural clot formed after a cut to the animal, thus, this point should be omitted from manuscript.

7. The title claims a new tool i.e. real time live confocal microscopy); do they mean it has not been used before for similar purpose? Also, they have not provided any such live clot formation images, all the images are taken after the clotting. Therefore, the title needs to be reframed.

Reviewer #2: In your materials and methods,you omitted to state how you collected the blood - later on in results you stated citrated whole blood was used. Please include the exact collection and preparation of the blood in the M/M.

Please clarify your thoughts on the effect of the citrate on the fibrin and your new marker. You do not reverse the effect of the citrate with Calcium or create clot with addition of e.g. thrombin. Please elaborate clearly how your fibrin strands are then formed. As this is a methods paper, I think you need to EXACTLY STATE in a point by point description WHAT you did with volumes, as clear as possible. Remember that some readers will not be familiar with coagulation, and if you want your method to be used in the future it is important to make it as easy and clear as possible. Nice work!

Reviewer #3: The authors added a fluorescent label to a previously described fibrin-binding peptide (FFBP) and used this peptide to label fibrin for confocal microscopy. The authors showed that FFBP stained fibrin in clots under both static and flow conditions. The authors conclude that FFBP is superior to pre-labeled fibrinogen for fibrin visualization in confocal microscopy and that FFBP may be a powerful tool in new intravital models of bleeding.

Major Concerns

1. The authors repeatedly emphasize that using FFBP to visualize fibrin is superior to using pre-labeled fibrinogen. However, few reasons for this conclusion are discussed and no publications regarding an effect of pre-labeling fibrinogen on fibrin formation are cited. The authors should either cite publications to support this conclusion or perform experiments (fibrin polymerization assays) demonstrating that the established method results in abnormal fibrin formation.

2. The peptide to which FITC was conjugated to form FFBP has been previously described, and in fact a fluorescent version of this peptide has been used to visualize mouse thrombi in vivo (Hara et al. 2012 JACC: Cardiovascular Imaging). The authors cite this paper, but do not discuss how a fluorescently-labeled fibrin binding peptide has already been used in intravital microscopy. The authors should discuss this and explain how FFBP is different or superior to the previously described peptide used in intravital imaging.

3. The Methods are confusing throughout. Was FFBP added to all reactions in Figures 1-3 prior to the initiation of clotting? Was pre-labeled fibrinogen used in all of these experiments or just in Figure 1? Was whole blood used in Figure 4 or simply plasma? The methods should be clarified and written

in a more systematic manner with each specific experimental setup described.

4. In Figure 1, both the Results and figure legend state that clots were formed from citrated human plasma, yet panels C and D clearly show platelets and RBCs. Was whole blood used in these experiments? This should be clarified.
5. In Figure 2, why is the fibrin network denser in panel A relative to panels E and I? They are all controls, and thus should have similar structures. Please discuss this.
6. In the Results, the findings presented in Figure 2 should be more thoroughly described. The statement "revealed differences in clot morphology" is vague and should be expanded.
7. In Figure 3, the numerous, round objects stained by wheat germ agglutinin are much too small to be RBCs (typically 6-8 μm in diameter) based on the scale bar (10 μm). Are these platelets? Or is this typical of staining with wheat germ agglutinin? This should be discussed.
8. The authors state that oxygen-mediated activation of coagulation is leading to the increased fibrin density at the air-clot interface shown in Figure 4A. However, the legend states tissue factor and calcium were used to trigger clotting in these experiments. Moreover, oxygen is not an activator of coagulation. This increased density near the clot surface is likely due to platelet-mediated clot contraction, which increases fibrin density at the clot margin. This should be discussed.
9. The presence of cells in whole blood reduces the permeability of whole blood clots. Have the authors determined if FFBP can penetrate into a pre-formed blood clot to visualize the fibrin network within? The manuscript would be strengthened by additional experiments examining FFBP staining of existing plasma and whole blood clots.
10. A new intravital mouse model of hemostasis that involves fibrin visualization has been recently described (Getz et al. 2015 J Thromb Haemost). The authors should discuss this and how FFBP could potentially be used in this model.

Minor Concerns

1. The Introduction is overly wordy and repetitive. It should be shortened.
2. How many times was each experiment repeated? This should be indicated in each figure legend.
3. Figure 2: the fibrinogen concentrations listed in the Figure proper do not match what is listed in the figure legend. This should be remedied.
4. Figure 2: the units on the RBC and platelet counts should be specified in the legend.
5. Figure 4: the dotted line described in the legend is not shown in panel A. This should be added.
6. Page 8, Results/Discussion: the statement, "Together with platelets and RBCs, [fibrinogen] belongs to the quantitatively most prominent proteins involved in coagulation" makes it seem that platelets and RBCs are proteins, which they are not. This should be re-worded.

Reviewer #4: Potentially, the peptide that is tested is interesting, since staining of fibrin structure with this method gives possibilities in research. My main concern with the paper is the selection of experiments that are used in the validation. The method of making the peptide seems good. With the approach to test the peptide (labelled with FITC) I miss a lot of necessary steps, such as

* Peptide (during or after clotting) versus labelled fibrinogen
* Specificity (labelling other (coagulation) protein)
* Searching for optimal concentration peptide
* Effect of peptide on fibrin matrix (with varying concentrations of the peptide)
* etc…..

Introduction, Line 14-24 : [..] it has been difficult to study the role of fibrin formation following traumatic bleeding, as current in vivo models are primarily based on induction of thrombus formation with laser or ferric chloride, and therefore do not accurately resemble traumatic bleeding [5]. The importance of developing tools for studying fibrinogen and fibrin formation in the context of traumatic bleeding was recently highlighted [6]. The main problem, according to the authors, is to study the role of fibrin formation following traumatic bleeding, but nowhere in the experiments there is a link with traumatic bleeding, thus not answer to the question is given. After traumatic bleeding, fibrinogen is often given nowadays, is the proposal to label that before giving it. If first a clot is formed and then labelling, what does it answer? It has to be certain that labelling afterwards is the same clotting in the presence of the labelled peptide. For studying fibrin formation visualization needs to be possible during the clotting. There is a mismatch in the hypothesis in these sentences. The first one focusses on how the thrombus (fibrin clot) is formed, the second one focusses on how to study fibrin. Consequently, this is not an example for why labeling fibrin is important. Furthermore, this paper does not give a better model system to study traumatic bleeing.

Materials and methods: I miss a section about how the all of the fibrin clots are formed; this should be described in more detail.

Results (and discussion), Line 57-2:

Initial imaging in 8-well chambered cover slides showed excellent overlap between FFBP and Alexa Fluor 647-labeled fibrinogen in citrated human plasma (Fig. 1) demonstrating that the fibrin-binding characteristics of the original peptide were not affected by fusion with FITC. No cross reactivity was observed. As far as I know, pre-labeled fibrinogen can only be used up to 10% of the entire matrix. Does this peptide label all of the fibrin? If so, you wouldn't expect a 100% overlap, but more green (FFBP) than red (Alexa Fluor 647-labeled fibrinogen).

Line 7-14: Visualization of fibrin via FFBP under static conditions - Further experiments with Alexa Fluor 647- labeled fibrinogen revealed differences in clot morphology after the addition of varying concentrations of fibrinogen (1-50 μg/ml), RBCs (5.5×104-2.7×106/well) and platelets (1.5×104-7×105/well) (Fig. 2). This image only shows a different fibrin structure (labeled with FFBP) with different fibrinogen concentrations, which is known, and platelets/RBC's. Where is the Alexa Fluor 647-labeled fibrinogen here?

Line 33-38: Next, we investigated fibrin formation in response to a natural and prominent trigger of clot formation under conditions of traumatic bleeding, namely exposure to oxygen. How does this work? Only adding oxygen to fibrinogen will not trigger clotting.

(Results and) discussion, Line 9-14: Our results highlight the importance of fibrinogen and fibrin in the clot formation process, in particular under conditions relevant to traumatic bleeding, such as exposure to oxygen or the contact to surfaces. How? This study only labels fibrin, but cannot say anything other than 'a fibrin clot can be formed under certain conditions, containing RBC's and platelets'.

It is unclear in the text where the fibrinogen is pre-labelled or postlabelling in the paper. This should be much more clear.

Fig 2: What concentration of fibrinogen was used in experiments with Platelets and RBC's?

In figure add: the labeled peptide may also influence fibrin network structure. If there is such a perfect overlap (please test whether this is true) than both methods are equally good and the new method has the advantage of adding after fibrin is formed.

I also do not understand " as current in vivo models are primarily based on induction of thrombus formation with laser or ferric chloride, and therefore do not accurately resemble traumatic bleeding´. This suggests that another model is made for traumatic bleeding, this is not done.

Page 7: Figure 1A and 1B show exactly the same fibrin structure. There must be something wrong, because you cannot make two independent clot that look exactly the same. No cross reactivity was observed. How is this tested?
It is unclear what the use is of the mixing experiments with erythrocytes or platelets. You can see that the fibrin matrix is different, but there is no control. This is unclear in the paper.
Page 7. "to visualize fibrin in conditions relevant to traumatic and perioperative bleeding." It is still unclear how these experiment are relevant for studying bleeding, this needs to be explained in detail.
Page 8: in particular under conditions relevant to traumatic bleeding, such as exposure to oxygen or the contact to surfaces. I do not see how this statement is supported by results.

****

2nd editorial decision
Date: 12-Feb-2017

Ref.: Ms. No. JCTRes-D-16-00014R1
FITC-linked Fibrin-Binding Peptide and Real-Time Live Confocal Microscopy as a Versatile Tool to Visualize Fibrin(ogen) in Coagulation
Journal of Clinical and Translational Research

Dear Dr. Hermann,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Mar 14, 2017.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1:

The authors have, up to some extent, addressed most of the concerns raised. However, two major points still need to be addressed by the investigators. First, a negative control group which uses labeled FFBP used on a collagen coated surface needs to be included (with and without plasma/cells) to rule out binding of FFBP peptide to protein fibers other than Fibrin polymers. This is important for increasing the application spectra of this peptide, as, collagen is an important constituent of sub-endothelial matrix and thus also provides a surface which is closer to natural condition for clotting. Secondly, the authors have attempted to justify not performing quantitation of images (in terms of total or per unit area fluorescence intensity) and to use subjective approach which is not the appropriate one

in this case. Quantitations (in acceptable forms) not only provide improved form of results but also presents a referable data for comparison for other investigators studying a similar phenomenon in future.

Reviewer #2: Authors have addressed all comments succesfully.

Reviewer #3: The authors present a revised manuscript investigating the use of a fluorescent fibrin-binding peptide (FFBP) in confocal microscopy. Although changes to the manuscript have been made, further clarifications and experiments are necessary to help readers interpret and understand the novelty of the data presented.

1. In the Introduction, the authors make a statement regarding possible effects of pre-labeled fibrinogen on fibrin formation. As this reviewer mentioned previously, an experiment comparing the effects of both pre-labeled fibrinogen and FFBP on fibrin polymerization would strengthen the manuscript.
2. Several aspects of the Methods section still need to be clarified:
a. How were the RBCs used in Figure 1 and 2 isolated? How many RBCs and platelets were added to the clots shown in Figure 1? In Figure 2, how were volume changes following addition of varying platelets or RBCs accounted for?
b. On page 6, the authors mention that whole blood was pipetted into wells and clotted. What experiments used whole blood?
c. For the flow experiments, what was the flow rate? Were these clots formed in the presence of FFBP or was the clot stained after clot formation? How were star-tem and ex-tem added to the flow system to trigger clotting?
d. The overall organization of the Methods section is confusing, and out of order. The numbered experiment list appears near the end of the section after the methods for the experiment have already been described earlier, and the detailed Methods for Figure 2 appear near the end after discussion of the flow experiments presented in Figure 3.
e. The authors state that in some experiments FFBP was added to clots 15 minutes after clotting was initiated. How was the FFBP added to the clot? How long did it take for FFBP to penetrate the pre-formed clot, and was staining more intense near the clot margins, and less intense in the core of the clot? How did the authors know that fibrin polymerization was fully complete within 15 minutes?
3. In the Figure 1 legend, a reference is made to panels E and I. Figure 1 only contains panels A-D. This should be changed.
4. The authors state that addition of RBCs to clots reduced network density (Figure 2E-H). Is there truly a change in fibrin density, or are the RBCs simply interfering with visualization of the fibers? The same can be said about Supplemental Video 2. This should be discussed.
5. In Figure 2, only one panel is shown where FFBP was added after clotting (Figure 2M), which the authors state is the primary advantage of FFBP over pre-labeled fibrinogen. However, this image appears to be of lower quality, with much higher background fluorescence than clots formed in the presence of FFBP. Is this high background typical of pre-formed clots stained with FFBP? This should be discussed, and more images of clots stained after clotting should be included.
6. In addition to clearly visible fibrin fibers, small, round objects stain with FFBP in Figure 3. What are these objects? This should be discussed.
7. In Figure 4A, the authors report that there is increased fibrin density at the air-clot interface. Although there is increased signal at the margin, it appears that fibrin density increases closer to the center of the clot. Also, is it possible that the differing refractive indices of air and the clot could influence imaging at this interface? Furthermore, the difference in fibrin density the authors report is difficult to discern in panels B and C. Lastly, why is the network density/staining intensity so much greater in panel A relative to panels B and C? These points should be addressed.
8. In the Discussion, the authors re-emphasize that the benefit of the FFBP over pre-labeled fibrinogen is that FFBP can stain existing clots, which could be valuable in vivo. This staining would be dependent on the FFBP being able to penetrate the clot. Are the authors concerned that a reduction of clot permeability by RBCs and other cells may prevent staining with FFBP, limiting its utility? This

should be discussed.

9. The supplemental figures are inadequately discussed/referenced in the manuscript proper. Specific reference to each should be made in the text. The videos uploaded for S3 and S4 are also the same video.

Reviewer #4: All our comments have been incorporated in the new version, the objective is adjusted and the conclusion is in line with the data.

********Authors response*********

Reviewers' comments:

Reviewer #1:

The authors have, up to some extent, addressed most of the concerns raised. However, two major points still need to be addressed by the investigators. First, a negative control group which uses labeled FFBP used on a collagen coated surface needs to be included (with and without plasma/cells) to rule out binding of FFBP peptide to protein fibers other than Fibrin polymers. This is important for increasing the application spectra of this peptide, as, collagen is an important constituent of sub-endothelial matrix and thus also provides a surface which is closer to natural condition for clotting…

Response: The reviewer is correct. We therefore coated 8-well dishes with type I collagen (rat tail) and performed experiments as before. The result is comparable to the ones without type I collagen. The only difference we see is the enhanced clotting behaviour between platelets, which might be attributed to the collagen type I. Until now we have not observed any other interaction/staining of FFBP besides the one with fibrin. The new control Figures have been added to the manuscript (Panels P and Q in Figure 2).

…Secondly, the authors have attempted to justify not performing quantitation of images (in terms of total or per unit area fluorescence intensity) and to use subjective approach which is not the appropriate one in this case. Quantitations (in acceptable forms) not only provide improved form of results but also presents a referable data for comparison for other investigators studying a similar phenomenon in future.

Response: Herein we present an alternative staining method which has several advantages compared to other fibrins staining methods. It was not the aim of this publication to address other questions such as the quantification of the fibrin networks.

Reviewer #3:

1. In the Introduction, the authors make a statement regarding possible effects of pre-labeled fibrinogen on fibrin formation. As this reviewer mentioned previously, an experiment comparing the effects of both pre-labeled fibrinogen and FFBP on fibrin polymerization would strengthen the manuscript.

Response: We performed the experiments with normal plasma for which both stains yielded the same results. We discussed in the manuscript that per definition, addition of a component which is part of the resulting network might per definition “rescue” any potential problems in plasma samples with low/absent levels of fibrinogen or with not/less functional fibrinogen. The statement regarding the potential influence of pre-labeled fibrinogen on coagulation has now been removed from the introduction (although it is still mentioned in the discussion). This is only one of the potential benefits, however, which also include it being a more simple method for post-staining existing fibrin networks that does not require fixation and may be more suitable for in vivo use.

2. Several aspects of the Methods section still need to be clarified:

a. How were the RBCs used in Figure 1 and 2 isolated? How many RBCs and platelets were added to the clots shown in Figure 1? In Figure 2, how were volume changes following addition of varying platelets or RBCs accounted for?

Response: For the experiments presented in Figure 1 we did not quantify RBC nor platelets. We added an aliquot of each fraction obtained after the centrifugation steps described in the materials and methods section. The intention of figure 1 is to show the potential of FFBP to specifically visualize fibrin.

Regarding Figure 2, the reviewer is right and I apologize, we did not mention how we isolated the red blood cells, the following text has been added to the manuscript:

Blood/erythrocyte/platelet-rich plasma/plasma samples:
… Platelet number was determined using a Coulter Hematology Analyzer. Volumes were equalized for all samples with autologous plasma (i.e. same volumes for all samples).

b. On page 6, the authors mention that whole blood was pipetted into wells and clotted. What experiments used whole blood?

Response: The reviewer is correct; whole blood was not used. The sentence has been amended as follows: “For this purpose, 200 µl of either citrated plasma only or with added erythrocytes/platelets were pipetted into each well.“

c. For the flow experiments, what was the flow rate? Were these clots formed in the presence of FFBP or was the clot stained after clot formation? How were star-tem and ex-tem added to the flow system to trigger clotting?

Response: The flow rate was 60 dyne/cm^2. Clots were formed in the presence of FFBP. Star-tem and ex-tem were added to the left chamber which is placed before the vessel.

This information has been included in the respective methods section.

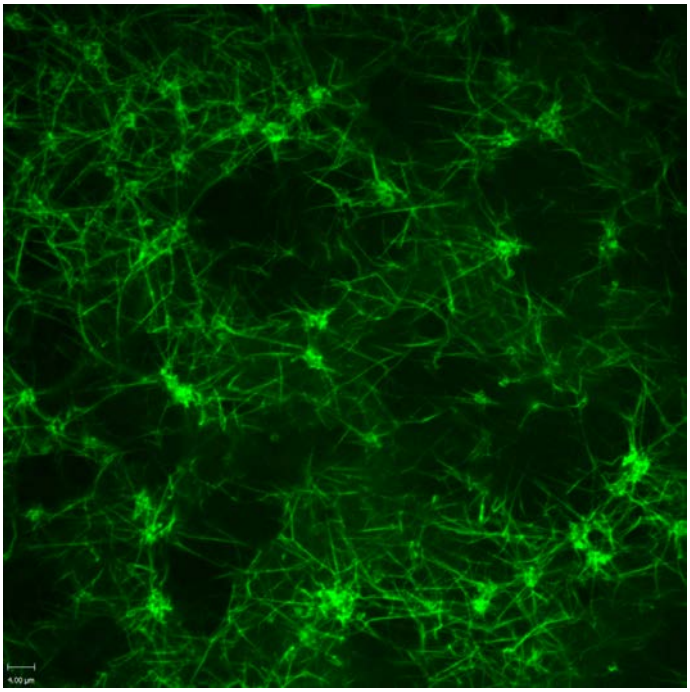
d. The overall organization of the Methods section is confusing, and out of order. The numbered experiment list appears near the end of the section after the methods for the experiment have already been described earlier, and the detailed Methods for Figure 2 appear near the end after discussion of the flow experiments presented in Figure 3.

Response: We agree with the reviewer and have revised the methods section by moving the overview of experiments to the beginning of the section and adding further details on methods used.

e. The authors state that in some experiments FFBP was added to clots 15 minutes after clotting was initiated. How was the FFBP added to the clot? How long did it take for FFBP to penetrate the pre-formed clot, and was staining more intense near the clot margins, and less intense in the core of the clot? How did the authors know that fibrin polymerization was fully complete within 15 minutes?

Response: The clots were overlayed with FFBP (dissolved in PBS in a concentration of 4.5 ng/µl). Due to limitations in the working distance of the objectives used for this kind of confocal microscopy, we can only visualize the borders and approx. 30 µm of the clot. We do not reach the core of the clot. This might be addressed with two photon laser microscopy.

We do not exclude that polymerization might still be going on after 15 minutes. If needed, of course also longer time periods might be analysed using our approach. This could be of particular interest when issues such as clot contraction or lysis are addressed. The image below shows a similar staining performed 30 minutes after initiation of coagulation:

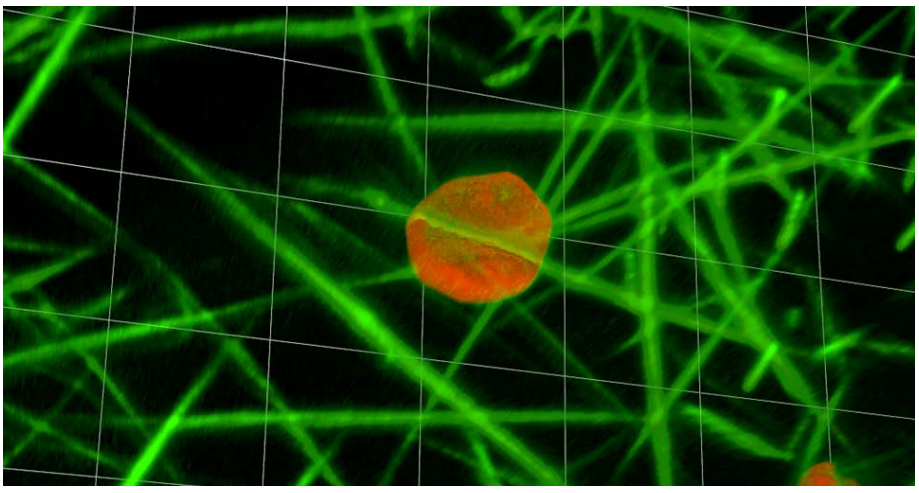


3. In the Figure 1 legend, a reference is made to panels E and I. Figure 1 only contains panels A-D. This should be changed.

Response: This has now been corrected.

4. The authors state that addition of RBCs to clots reduced network density (Figure 2E-H). Is there truly a change in fibrin density, or are the RBCs simply interfering with visualization of the fibers? The same can be said about Supplemental Video 2. This should be discussed.

Response: The volume which is occupied by the presence of RBCs cannot be filled with fibrin fibers. They can only be formed in the plasma surrounding the RBCs. See image below, which shows a fibrin fiber close to the RBC. Fibrin fibers do not penetrate the membrane of RBCs. A note of this has been made in the manuscript in the respective section where this result is discussed.



5. In Figure 2, only one panel is shown where FFBP was added after clotting (Figure 2M), which the authors state is the primary advantage of FFBP over pre-labeled fibrinogen. However, this image appears to be of lower quality, with much higher background fluorescence than clots formed in the presence of FFBP. Is this

high background typical of pre-formed clots stained with FFBP? This should be discussed, and more images of clots stained after clotting should be included.

Response: The reviewer is right. The imaging quality is better when FFBP is applied while the clots are being formed. However we think the quality is still good enough, even when FFBP is added after clot formation. A lesser quality is not surprising as the stain has to diffuse through the already formed and nearly solid clot. A note of this has been made in the manuscript in respective section where the result is discussed. An additional image has been added (Fig. 2P), which shows post-staining with FFBP when added 30 minutes after the induction of coagulation.

6. In addition to clearly visible fibrin fibers, small, round objects stain with FFBP in Figure 3. What are these objects? This should be discussed.

Response: Fibrin is formed around platelets, however, besides platelet aggregation, also the release of microparticles might trigger fibrin formation and thereby explain this staining seen in figure 3. A note of this has now been made in the manuscript.

7. In Figure 4A, the authors report that there is increased fibrin density at the air-clot interface. Although there is increased signal at the margin, it appears that fibrin density increases closer to the center of the clot. Also, is it possible that the differing refractive indices of air and the clot could influence imaging at this interface? Furthermore, the difference in fibrin density the authors report is difficult to discern in panels B and C. Lastly, why is the network density/staining intensity so much greater in panel A relative to panels B and C? These points should be addressed.

Response: The network density/staining intensity is so much greater in panel A relative to panels B and C because A shows a z-stack consisting of several optical planes and B and C only show one optical plane. The differences are discernible in the original image files but appear to have undergone some quality loss in the PDF document provided for reviewing purposes.

8. In the Discussion, the authors re-emphasize that the benefit of the FFBP over pre-labeled fibrinogen is that FFBP can stain existing clots, which could be valuable in vivo. This staining would be dependent on the FFBP being able to penetrate the clot. Are the authors concerned that a reduction of clot permeability by RBCs and other cells may prevent staining with FFBP, limiting its utility? This should be discussed.

Response: The reviewer is correct. Of course cellular components such as RBCs will have an influence on post coagulation staining of fibrin networks via FFBP. Nevertheless such questions can be addressed by adding other stains such as fluorescently labeled wheat germ agglutinin in order to show how good or bad the clot can be permeabilized with fluorescent stains. This discussion has now been included in the manuscript.

9. The supplemental figures are inadequately discussed/referenced in the manuscript proper. Specific reference to each should be made in the text. The videos uploaded for S3 and S4 are also the same video.

Response: The referencing of supplementary materials has been improved as suggested by the reviewer. The duplicate reference to one of the videos has been removed.

3rd editorial decision

Date: 22-May-2017

Ref.: Ms. No. JCTRes-D-16-00014R2
FITC-linked Fibrin-Binding Peptide and Real-Time Live Confocal Microscopy as a Versatile Tool to Visualize Fibrin(ogen) in Coagulation
Journal of Clinical and Translational Research

Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

Dear authors,

The JCTR editorial board has considered the modifications you made to the manuscript, which were in line with the reviewers' comments raised after your first resubmission. We have reached the conclusion that you have sufficiently addressed the issues with the conduction of new experiments and modifications of the text and consequently find your manuscript suitable for publication.

The editor has made a few minor modifications which will be incorporated in the proofs (see attached document). The proofs you will receive shortly. We kindly ask you to go through these proofs thoroughly and indicate in the PDF, using the comment functions, where modifications need to be implemented.

Thank you for submitting your work to JCTR and congratulations on the nice work.

Kindest regards, also on behalf of the JCTR editorial board,

Michal.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.
****