Editorial comments:
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52019_R3_082114.docx) is located in your Editorial Manager. Please download the .docx file and use this updated version for any future revisions.

Response:
Done

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response:
Done

Please make the following minor edits to your text to clarify certain aspects of your protocol:

1) How are the isolated RBCs labeled in step 3.3? Please provide a reference or additional details.

Response: The following has been added to 3.3 to clarify the details on labeling the RBCs.

3.3.1 Response: Suspend cells at a density of 1 x 10^6/mL in any chosen serum-free culture medium.

3.3.2 Add 5 µL/mL of the cell suspension to the cell-labeling solution. Mix well by gentle pipetting gently.

3.3.3 Incubate for 20 minutes at 37°C.

3.3.4 Centrifuge the labeled suspension tubes at 1500 rpm for 5 minutes at 37°C.

3.3.5 Remove the supernatant and gently re-suspend the cells in 37°C medium.

3.3.6 Repeat the wash procedure (3.3.4 and 3.3.5) two more times.

2) When allowing the fibrin to polymerize, what temperature is used throughout?

Response: The fibrin was allowed to polymerize between 21-23°C (room temperature).

The following has been added in Sections 2, 3, and 4: "Allow the fibrin to polymerize at 21-23°C for 2 hr before confocal imaging."

3) In step 4.4, what volume is pipetted into the chamber? Only a concentration is provided.

Response:
"25 µL of plasmin was pipetted into the chamber." This information was added to the manuscript."

4) Please specify what a damp environment is in step 4.2.

Response:
The following has been added to step 4.2: "Do not seal the ends of the chamber with clear adhesive. Allow the fibrin clots to polymerize for 1.5 hr at 21-23°C in a sealed enclosure containing 1 ml of water to prevent dehydration."

5) What statistical analysis is performed in step 6? You go in to further details of the statistical analysis in the discussion, but mentioning the methods used here would be helpful, as would providing a reference for the reader if possible.

Response:
The following are in the references section of the manuscript:
44) Kuehl, R. O. *Design Of Experiments : Statistical Principles Of Research Design And Analysis*. 2nd ed. Cengage Learning, Pacific Grove, CA (2000).
45) Lindman, H. R. *Analysis Of Variance In Experimental Designs*. Springer Texts in Statistics, New York, N.Y (1992).

In addition, the following has been added at Step 6:

1.1 Analyze the lysis data using statistical analysis software. Perform a one-way ANOVA and a *post hoc* Tukey-Kramer test[44,45].

As you edit your protocol please keep the length limit of 2.75 pages for highlighted portions in mind.

Response:
This has been corrected.

Please provide high resolution images for the Figures for publication. The file must be at least 1440x480 pixels or 300 dpi.

Response:
The images are now at a resolution of 300 dpi.

Peer review comments regarding image analysis and fibrinolysis

1) The reviewers' provided some feedback regarding your image analysis and fibrinolysis that should be further addressed in the manuscript text.

Reviewer #1 stated: "The major concern about the submitted manuscript is that fibrin network image analysis is missing."

Additionally, reviewer #2 stated "Example data would be illustrative and easier for other researchers to learn this technique if this article would present representative data for the fibrinolysis rates plotted in Figure 5. There is insufficient information about how to choose at what time the lysis is completed without some representative examples."

Overall, the details and direction for the image analysis is lacking in the manuscript text itself. Please add more details to the text regarding how to monitor the time lapse video to determine full lysis. Please include a video (or possibly a multi-panel figure) of lysis progression indicating the expected results for clot lysis. Additionally examples and guidelines for how to visually identify a fully lysed clot would be very helpful.

Response:
The time lapse indicating full lysis is important in this analysis. It would be very difficult to physically measure how a fully lysed clot evolves. However, a time lapse video has been included that shows lysis taking place on one of the clot samples. Full clot lysis identification is not the goal here. It is assumed that the lysis rate is constant and does not vary with distance or time. A video showing a representative lysis of a clot has been uploaded.

2) Reviewer #2 stated "The disadvantage is that the methods result in descriptive and non-quantitative results; one exception to this is the lysis measurement which provides as number in units of 'area per unit time,' but I have reservations about the assumptions that lead to measurement of lysis with such units." and continues "It doesn't make sense to me that the time at which the lysis appears to be 'complete' would depend upon the area of examination. It is not clear what exactly they are measuring – is it rate-limited by the plasmin diffusion into the clot, or is all of the clot interacting at once? The measure of lysis as an 'area per unit time' would need to be supported by an appropriate model"

You replied to this with details and a reference for the model on which your assumptions are based. Please incorporate this into the manuscript text. While the entire model does not need to be added, discussion of the assumptions, parameters, considerations and limitations would clarify the procedure for measuring the fibrinolysis rate. Additional discussion of how the area of observation is related to the activity and lysis ability of the plasmin applied should be included.

Response:
Information pertaining to the model has been added to the manuscript in the Discussion section.

3) A major concern of reviewer #3, also mentioned by reviewer #2, was how the density of the clots was analyzed. In your rebuttal you mention that this was observed qualitatively from confocal images. Please clarify this throughout the text.

Response:
The density of the clots was analyzed qualitatively. Experimentally, one could count the number of fibers per unit area (fibers/area). This methodology is what was performed in our vitro simulations visually. In the manuscript, it has been clarified to include: density (number of fibers per unit area).