

# Journal of Visualized Experiments

## Experimental and Imaging Techniques for Examining Fibrin Clot Structures in Normal and Diseased States

--Manuscript Draft--

<b>Manuscript Number:</b>	JoVE52019R4
<b>Full Title:</b>	Experimental and Imaging Techniques for Examining Fibrin Clot Structures in Normal and Diseased States
<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Keywords:</b>	fibrin; clot; disease; confocal microscopy; diabetes; sickle cell; glycation; erythrocyte
<b>Manuscript Classifications:</b>	1.11.118.290: Erythrocytes; 3.15.378: Hematologic Diseases; 3.19.246: Diabetes Mellitus; 4.12.776.124.270: Fibrin; 4.12.776.124.50.250: Fibrinogen; 5.1.370.350.515.395: Microscopy, Confocal
<b>Corresponding Author:</b>	Rodney D. Averett, Ph.D. Georgia Institute of Technology Atlanta, Georgia UNITED STATES
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	rdaverett@gatech.edu
<b>Corresponding Author's Institution:</b>	Georgia Institute of Technology
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Natalie Fan, BS
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Natalie Fan, BS Philip Keegan Manu O Platt, Ph.D.
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	Fibrin is an extracellular matrix protein that is responsible for maintaining the structural integrity of blood clots. Much research has been done on fibrin in the past years to include the investigation of synthesis, structure-function, and lysis of clots. However, there is still much unknown about the morphological and structural features of clots that ensue from patients with disease. In this research study, experimental techniques are presented that allow for the examination of morphological differences of abnormal clot structures due to diseased states such as diabetes and sickle cell anemia. Our study focuses on the preparation and evaluation of fibrin clots in order to assess morphological differences using various experimental assays and confocal microscopy. In addition, a method is also described that allows for continuous, real-time calculation of lysis rates in fibrin clots. The techniques described herein are important for researchers and clinicians seeking to elucidate comorbid thrombotic pathologies such as myocardial infarctions, ischemic heart disease, and strokes in patients with diabetes or sickle cell disease.
<b>Author Comments:</b>	None
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.

Rodney D. Averett, Ph.D., Research Engineer  
The George W. Woodruff School of Mechanical Engineering  
Parker H. Petit Institute for Bioengineering & Bioscience  
315 Ferst Drive, N.W., Suite 3306  
Atlanta, GA 30332-0363

October 14<sup>th</sup>, 2014

Dear Dr. Nandita Singh:

I am writing to submit the revised article entitled: "Experimental and Imaging Techniques for Examining Fibrin Clot Structures in Normal and Diseased States" (JoVE 52019) to the Journal of Visualized Experiments. All of the Editorial comments have been addressed and are reflected via the "Track Changes" function in the Word document. In addition, a point-by-point response has been created and is available.


In this research article, a unique method is presented in which fibrin clot structures are imaged in normal and representative disease conditions. This work is important, as it elucidates the morphology differences in clot structures due to hyperthrombotic complications such as diabetes and sickle cell disease. The author contributions are as follows: Natalie K. Fan, Philip Keegan, Manu O. Platt, and Rodney D. Averett (Corresponding Author).

I am suggesting the following peer reviewers to review the aforementioned article:

- 1) Prof. Sergey S. Shevkoplyas ([sshevkop@central.uh.edu](mailto:sshevkop@central.uh.edu)), University of Houston
- 2) Maribel Vazquez ([vazquez@ccny.cuny.edu](mailto:vazquez@ccny.cuny.edu)), CCNY
- 3) Falvo, Michael R ([falvo@physics.unc.edu](mailto:falvo@physics.unc.edu)), University of North Carolina Chapel Hill
- 4) Gorkun, Oleg ([oleg\\_gorkun@med.unc.edu](mailto:oleg_gorkun@med.unc.edu)) University of North Carolina Chapel Hill
- 5) Ariens, Robert A.S. ([r.a.s.ariens@leeds.ac.uk](mailto:r.a.s.ariens@leeds.ac.uk)), University of Leeds
- 6) Christine Helms ([chelms@richmond.edu](mailto:chelms@richmond.edu)), University of Richmond

I look forward now to the peer review of this paper and working to get the article published in JOVE. If you have any questions, please do not hesitate to contact me at: [rdaverett@gatech.edu](mailto:rdaverett@gatech.edu), (404)385-0243. Thank you for your consideration.

Best regards,



Rodney D. Averett

**TITLE:**

Experimental and Imaging Techniques for Examining Fibrin Clot Structures in Normal and Diseased States

**AUTHORS:**

Fan, Natalie K.  
Wallace H. Coulter Department of Biomedical Engineering  
Georgia Institute of Technology & Emory University School of Medicine  
Atlanta, GA USA  
[nfan7@gatech.edu](mailto:nfan7@gatech.edu)

Keegan, Philip  
Wallace H. Coulter Department of Biomedical Engineering  
Georgia Institute of Technology & Emory University School of Medicine  
Atlanta, GA USA  
[pkeegan@gatech.edu](mailto:pkeegan@gatech.edu)

Platt, Manu O.  
Wallace H. Coulter Department of Biomedical Engineering  
Parker H. Petit Institute for Bioengineering & Bioscience  
Georgia Institute of Technology & Emory University School of Medicine  
Atlanta, GA USA  
[manu.platt@bme.gatech.edu](mailto:manu.platt@bme.gatech.edu)

Averett, Rodney D.  
George W. Woodruff School of Mechanical Engineering  
Parker H. Petit Institute for Bioengineering & Bioscience  
Georgia Institute of Technology  
Atlanta, GA USA  
[rdaverett@gatech.edu](mailto:rdaverett@gatech.edu)

**CORRESPONDING AUTHOR:**

Rodney D. Averett, Ph.D.  
[rdaverett@gatech.edu](mailto:rdaverett@gatech.edu)

**KEYWORDS:**

fibrin; clot; disease; confocal microscopy; diabetes; glycation; erythrocyte; sickle cell

**SHORT ABSTRACT:**

In this manuscript, experimental techniques, including blood preparation, confocal microscopy, and lysis rate analysis, to examine the morphological differences between normal and abnormal clot structures due to diseased states are presented.

**LONG ABSTRACT:**

Fibrin is an extracellular matrix protein that is responsible for maintaining the structural integrity of blood clots. Much research has been done on fibrin in the past years to include the

investigation of synthesis, structure-function, and lysis of clots. However, there is still much unknown about the morphological and structural features of clots that ensue from patients with disease. In this research study, experimental techniques are presented that allow for the examination of morphological differences of abnormal clot structures due to diseased states such as diabetes and sickle cell anemia. Our study focuses on the preparation and evaluation of fibrin clots in order to assess morphological differences using various experimental assays and confocal microscopy. In addition, a method is also described that allows for continuous, real-time calculation of lysis rates in fibrin clots. The techniques described herein are important for researchers and clinicians seeking to elucidate comorbid thrombotic pathologies such as myocardial infarctions, ischemic heart disease, and strokes in patients with diabetes or sickle cell disease.

## **INTRODUCTION:**

Injury to a blood vessel's endothelial lining is repaired through the hemostatic response, or the formation of a blood clot. When blood permeates into the extracellular matrix, tissue factors activate platelets in the blood stream that facilitate initiation of the coagulation cascade. The key mechanical component of this healing process is the fibrin matrix, composed of fibrin fibers that are highly elastic, and can sustain large forces<sup>1-4</sup>. Many researchers have studied the formation structure, and function of fibrin extensively in the past decades<sup>5-13</sup>.

Patients with diseases such as diabetes mellitus and sickle cell have an increased risk of developing thrombotic complications such as myocardial infarctions, ischemic heart disease, and strokes<sup>14-19</sup>. Over 2 million people are newly diagnosed with diabetes mellitus each year in the United States. There are two types of diabetes: Type I, where the body fails to produce adequate amounts of insulin, and Type II, where the body becomes resistant to insulin. Among diabetic patients, cardiovascular disease (CVD) is the cause for 80% of the morbidity and mortality associated with the disease<sup>20,21</sup>.

Sickle cell disease (SCD) is a genetic blood disorder that affects more than 100,000 people in the United States<sup>22</sup>. SCD is a point-mutation disease that causes red blood cells to become crescent-shaped, making it difficult for the cells to pass through the blood vasculature<sup>23</sup>. Both of these disease states increase the chances of developing atherothrombotic conditions in the body. One of the reasons for this is a result of altered fibrin structure and function in diseased states<sup>14,24-26</sup>.

In both diabetes and sickle cell disease, there is hypercoagulation and hypofibrinolysis activity that induces atherothrombosis and cardiovascular disease (CVD) as compared to healthy patients<sup>17,27,28</sup>. It is known that hypofibrinolysis promotes atherosclerosis progression and engenders recurrent ischemic events for patients with premature coronary artery disease<sup>29</sup>. In the current manuscript, we investigated the role of fibrin physical properties in this particular setting. Fibrin clot structures in non-diseased patients are composed of thin fibers, larger pores, and generally less dense<sup>14,24</sup>. The increased porosity and less dense fibrin clots in healthy patients have been found to facilitate fibrinolysis<sup>16</sup>. In hyperthrombotic conditions such as diabetic and sickle cell disease, there is an increase in fibrinogen production, causing the fibrinogen concentration to increase from normal levels of 2.5 mg/ml in healthy patients<sup>30-33</sup>. Fibrin clots formed in diabetic patients have been found to be less porous, more rigid, have more branch points, and denser when compared to healthy, non-diabetic patients<sup>14,24,33-35</sup>. The altered fibrin structure is a result

of glycation mechanisms that occur in the proteins involved in clot formation. Nonenzymatic (irreversible) glycation occurs when glucose molecules bind to lysine residues on the fibrinogen molecule, which inhibits human factor XIIIa (FXIIIa) from properly cross-linking glutamine and lysine residues<sup>33,36,37</sup>.

The structural analysis of fibrin networks has been studied extensively recently. In particular, researchers have utilized electron microscopy and 3D reconstruction of fibrin networks<sup>38</sup>, investigated how both intravascular (endothelial) cells and extravascular (fibroblasts and smooth muscle) cells affect fibrin structure<sup>39</sup>, utilized viscoelastic and spectral analysis to analyze fibrin structures<sup>40</sup>, and developed correlations between fibrin structure and mechanical properties using experimental and computational approaches<sup>41</sup>. The focus of the current study was to formulate clot structures under simulated diabetic and sickle cell thrombosis conditions and to use confocal microscopy for examination of the structure and function of the clots in diseased states. Fibrin clots were formed from human fibrinogen, human thrombin, and FXIIIa. The clots were lysed using plasmin. To simulate diabetic conditions, increased concentration of fibrinogen was incubated in glucose solution to induce *in vitro* fibrinogen glycation. To simulate sickle cell disease clotting conditions, increased fibrinogen concentrations were mixed with sickle cell hematocrit collected from patients as done previously by our group<sup>42</sup>. These methods were used to examine the structure and functions involved in fibrin clot formation and fibrinolysis under diseased conditions, as well as the mechanisms that induce CVD. Based on current information about these diseases, the glycated fibrin clot structures were denser with fewer and smaller pores. The fibrin clots with red blood cells from sickle cell patients (RBCs) were also denser and displayed aggregation of the RBCs and agglomerated fibrin clusters. This is a well-established phenomenon that has been determined previously<sup>43</sup>. It was also hypothesized that the fibrinolysis rate would be significantly lower in glycated fibrin clots with and without reduced plasmin compared to healthy, normal fibrin. The results showed that for glycated fibrin clots, significantly different lysis rate results were observed only under conditions of reduced plasmin concentration. This experimental technique of using confocal microscopy offers significant advantages over other imaging methods because the cells and proteins remain in their native state, which enables the capturing of real-time video of the clotting activity. This method of synthetically inducing clotting is also cheaper and more time efficient than obtaining patient samples and filtering out individual proteins and enzymes. Furthermore, by using separated proteins and enzymes to synthesize clots, the clots were standardized so that there was not variability between samples as a result of other proteins in the plasma.

## **PROTOCOL:**

The following protocol adheres to the guidelines set by the Institutional Review Board (IRB) at Georgia Tech.

### **1. Blood Collection and Red Blood Cell Isolation Procedure**

1.1 Collect 40-120 ml of blood from donors in 10 ml heparinized vacutainer tubes. Start PBMC (peripheral blood mononucleated cell) isolation within 4 hr of collection. During this time, keep blood at room temperature.

Note: Overnight storage of blood in room temperature or in 4 °C is **NOT** recommended as this will result in lower PBMC yield.

- 1.2 Dilute whole blood 1:1 in ice cold (4 °C) sterile PBS.
- 1.3 Pipette 10 ml of ice cold hydrophilic polysaccharide into an empty, sterile 50 ml conical tube. Gently transfer diluted whole blood on top of the hydrophilic polysaccharide.
- 1.4 Use a 25 ml pipette in slow setting and pipette blood along the side of the tube very slowly. Ensure that the blood is not mixed with the saccharide prior to centrifugation because the hydrophilic polysaccharide is toxic to cells and otherwise, the PBMC yield will be lower.
- 1.5 Centrifuge blood samples for 30 min at 400 x g at 4 °C. If available, reduce the brake speed of the centrifuge to half of maximum for better separation after centrifugation.
- 1.6 Aspirate off the plasma/platelet fraction of the centrifuged blood sample. Carefully aspirate off the hydrophilic polysaccharide layer directly above the packed RBC layer.
- 1.7 Collect 8-10 ml of packed RBCs and dilute 1:1 with unsterile 0.9% NaCl saline.

## **2. Confocal Microscopy Evaluation of Glycated Clot Structures (Simulated Diabetic Clots)**

- 2.1 Defrost human fibrinogen and fluorescently labeled human fibrinogen conjugate at 37 °C.
- 2.2 Pipette the following into a 0.5 ml graduated microcentrifuge tube.
  - 2.2.1 For the healthy, normal fibrinogen clots, mix human fibrinogen with 10% fluorescently labeled human fibrinogen conjugate in a 50 mM Tris/100 mM NaCl buffer at a concentration of 5 mg/ml.
  - 2.2.2 For artificially glycated fibrinogen (to simulate diabetic clots), incubate human fibrinogen and 10% fluorescently labeled fibrinogen conjugate in a 5 mM solution of glucose dissolved in 50 mM Tris/100 mM NaCl for a resulting concentration of 6.8 mg/ml.
- 2.3 Cover the micro-centrifuge tubes using an opaque material to avoid exposure to light. Incubate the tubes in a 37 °C water bath for 48 hr.
- 2.4 Make a chamber on a glass microscope slide by affixing a thin adhesive on 2 sides of the slide.
- 2.5 After the 48 hr incubation period, prepare reagents for fibrin formation by defrosting FXIIIa and human alpha thrombin at room temperature.
- 2.6 Dilute FXIIIa to 80 U/ml in 5 mM CaCl<sub>2</sub> buffer.

2.7 Dilute thrombin to 4 U/ml in 5 mM CaCl<sub>2</sub> buffer.

2.7.1 For normal clots, ensure that the final concentrations of fibrinogen, FXIIIa, and thrombin are 2.5 mg/ml, 20 U/ml, and 1 U/ml, respectively at a volume of 50 µl.

2.7.2 For the glycosylated clots, ensure that the final concentrations of fibrinogen, FXIIIa, and thrombin are 3.4 mg/ml, 20 U/ml, and 1 U/ml, respectively at a volume of 50 µl.

2.8 Immediately pipette 30 µl of the fibrin solution into the chamber formed by the adhesive.

2.9 Place a 22 mm x 22 mm glass cover slip with thickness of 0.15 mm on top of the 2 layers of adhesive. Seal the open sides with clear adhesive to prevent the fibrin clot from drying out. Ensure that the adhesive does not interact with the fibrin clot.

2.10 Allow the fibrin clots to polymerize at 21-23°C for 2 hr before confocal imaging.

2.11 Take confocal microscopy images of the clots using a confocal microscope with a 40x/1.30 Oil M27 lens with a 488 nm Argon laser.

### 3. Confocal Microscopy Evaluation of Fibrin Clots Containing RBCs from Sick Cell Patients

3.1 Defrost human fibrinogen and fluorescently labeled human fibrinogen conjugate at 37 °C.

3.2 Pipette the following into a 0.5 ml graduated microcentrifuge tube.

3.2.1 For the healthy, normal fibrin clot, mix human fibrinogen with 10% fluorescently labeled human fibrinogen conjugate in a 50 mM Tris/100 mM NaCl buffer at a concentration of 5 mg/ml.

3.2.2 For the clots containing SCD RBCs, mix human fibrinogen and 10% fluorescently labeled human fibrinogen conjugate in a 50 mM Tris/100 mM NaCl such that the resulting concentration of the fibrinogen is 10 mg/ml.

3.3 Label the isolated RBCs (both normal and those from sickle cell patients obtained from the RBC isolation protocol) using a cell-labeling solution.

3.3.1 Suspend cells at a density of  $1 \times 10^6$ /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.

3.4 Dilute FXIIIa to 80 U/ml in 5 mM CaCl<sub>2</sub> buffer.

3.5 Dilute thrombin to 4 U/ml in 5 mM CaCl<sub>2</sub> buffer.

3.5.1 For normal clots, ensure that the final concentrations of fibrinogen, FXIIIa, and thrombin are 2.5 mg/ml, 20 U/ml, and 1 U/ml, respectively at a volume of 50 µl.

3.5.2 For the clots containing SCD RBCs, ensure that the final concentrations of fibrinogen, FXIIIa, and thrombin are 5 mg/ml, 20 U/ml, and 1 U/ml, respectively at a volume of 50 µl.

3.6 Pipette 10 µl of the labeled RBCs into the fibrin solution. Pipette 30 µl of the fibrin with RBCs onto the microscope glass (refer to slide preparation for glycated clots).

3.7 Allow the fibrin to polymerize at 21-23°C for 2 hr before confocal imaging.

3.8 Take confocal images of the clots using the confocal microscope, and utilize excitation lasers with wavelengths of 488 nm and 633 nm to excite the fluorescently labeled fibrin and RBCs.

#### **4. Confocal Microscopy Evaluation of Fibrinolysis Rates in Glycated Clot Structures**

4.1 Repeat the confocal microscopy protocol of glycated clots (Protocol Step 2) for the fibrinolysis rate evaluation in glycated clots.

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 250 ml of water to prevent dehydration.

4.3 After polymerization, obtain images of the clots using the confocal microscope.

4.4 Pipette plasmin into the open end of the chamber and disperse the plasmin through the clot via capillary action.

4.4.1 For the normal and glycated fibrinolysis experiments with normal concentrations, pipette 25 µl at 200 µg/ml of plasmin into the opening of the chamber.

4.4.2 For the glycated clots with reduced concentrations, pipette 25 µL at 50 µg/ml into the opening of the chamber.

4.5 Use the time series feature (see Figure 1) in the confocal microscope software to capture real-time video footage of the plasmin lysing the clot.

4.5.1 Click acquisition mode and then select “Time Series.” Select the number of cycles and the recording time interval.

## 5. Calculating Fibrinolysis Rates

5.1 Determine the lysis rate by setting an area (2,500  $\mu\text{m}^2$ ) and calculating the elapsed time required to dissolve a fixed area of the clot. Calculate the lysis rate using the following equation (Figure 2):

$$\text{Plasmin lysis rate} = \frac{\text{clot area}}{\text{time to lyse the clot area}}$$

## 6. Statistical Analysis of Fibrinolysis Rates

6.1 Analyze the lysis data using statistical analysis software. Perform a one-way ANOVA and a *post hoc* Tukey-Kramer test<sup>44,45</sup>.

## REPRESENTATIVE RESULTS:

### Confocal Microscopy Analysis of Glycated Fibrin Clot Structures

The confocal microscopy images of normal and glycated clots are presented in Figure 3. Confocal microscopy analysis of the normal and glycated clots reveals that glycated clots are denser with smaller pores than the normal clots both with and without the addition of FXIIIa during clot polymerization. In Figure 3a and 3b, there is a lower fibrin concentration which created a less dense structure than the glycated clots with higher fibrin concentration (Figures 3c and 3d).

The greater density observed (number of fibers per unit area) in the glycated clots is a result of the increased fibrinogen concentration that occurs in people with diabetes. In the normal clots, the fibrin fibers were more linear and formed longer fibers upon polymerization with FXIIIa, which is likely a result of the FXIIIa forming cross-links between the fibers. In the case of the glycated clots, the fibers appeared to be shorter, even with addition of FXIIIa. This is likely the result of the polymerization process that was altered due to the presence of glycated regions on the fibrinogen molecule. In addition, from analyzing Figure 3, it is likely that the fiber thickness was also reduced as a result of glycation, which is likely why the fluorescence from the glycated fibers appear to have a lower intensity.

### Confocal Microscopy Analysis of Fibrin Clots Containing RBCs from Sick Cell Patients

The confocal microscope images of the fibrin clot structure of clots with normal and RBCs from sickle cell patients are presented in Figure 4. As predicted, the confocal images of the fibrin clots containing RBCs from sickle cell patients had denser structures than those of healthy fibrin clots. Figure 4a shows a homogenous distribution of fibrin and normal RBCs. However, in Figure 4b there are clumps of fibrin and voids where no fibrin network formed. The RBCs from sickle cell patients were not homogeneously dispersed, but rather were embedded in the fibrin clumps. The increased density (number of fibers per unit area) was a result of the increased fibrinogen concentration that occurs as a result of hypercoagulation in SCD patients. Furthermore, the natural tendency of the RBCs from sickle cell patients to form aggregates engendered clumps of

RBCs that became interwoven into the fibrin network. This resulted in the formation of aberrant clot structures that are markedly different in morphology than those in normal patients. From Figure 4b, it is observed that RBCs from sickle cell patients caused greater heterogeneity in the fibrin sample and also caused a wider spatial distribution of RBCs in the fibrin sample, when compared to the healthy fibrin clots with normal RBCs. In contrast, the healthy fibrin clots with normal RBCs displayed a more homogenous dispersion of cells throughout the fibrin network.

It is unknown how much HbS (sickle hemoglobin) was polymerized in the erythrocytes from sickle cell patients and is contained in the samples. What is known, however, is that the presence of any amount of HbS causes greater aggregation of cells in number of HbS molecules per unit volume vs. normal HbA molecules. Pan et al.<sup>46</sup> have demonstrated this phenomenon in a study where they compared the aggregation/clustering effects of deoxy-HbS, and, for comparison, of oxy-HbS and oxy-normal adult hemoglobin, HbA. The clusters formed within a few seconds after solution preparation and their sizes and numbers remained relatively steady for up to 3 hours. One of the conclusions from the study was that both oxy-HbS and deoxy-HbS molecules clustered at a higher number density than oxy-HbA (normal) molecules as a function of temperature. Pertaining to the current study, this means that the RBCs from sickle cell patients were likely to cluster together, and trapped fibrin as it was being polymerized from the fibrinogen precursor.

### **Confocal Microscopy Lysis Rate Analysis of Normal and Glycated Clots**

It was hypothesized that the fibrinolysis rates would be greater for normal fibrin clots compared to glycated fibrin clots with decreased plasmin concentration. To test this hypothesis, confocal microscopy was used to assess the lysis rates of fibrin clots with the addition of plasmin. The average (mean) lysis rates of normal fibrin with the addition of 200 µg/ml of plasmin was  $43.2 \pm 26.5 \mu\text{m}^2/\text{s}$  and the average (mean) lysis rates of glycated fibrin with the addition of 200 µg/ml of plasmin was  $29.7 \pm 11.2 \mu\text{m}^2/\text{s}$ . For the glycated fibrin clot lysed with 50 µg/ml of plasmin, the average lysis rate was  $12.8 \pm 3.1 \mu\text{m}^2/\text{s}$ . A total of 10 samples each (30 samples) were used to determine the average and standard deviation values. These values are presented in Figure 5. A p-value of 0.05 was used to determine significance between the independent experimental groups. Analysis of the fibrinolysis rates demonstrated that the value for the normal clots compared to the glycated (simulated diabetic) clots with decreased plasmin were significantly different.

**Figure 1:** Sample screenshot from confocal microscope software detailing method for obtaining real-time, continuous imaging of fibrin clot lysis.

**Figure 2.** Example confocal image with fixed area used to calculate lysis rate of fibrin clot sample.

**Figure 3:** Confocal images of healthy and glycated (diabetic) fibrin clots. Green fluorescence represents the fibrin network. The unglycated fibrin represents clots formed in healthy patients, whereas glycated fibrin represents clots formed in diabetic patients. a) Unglycated fibrin without the addition of FXIIIa b) Unglycated fibrin with FXIIIa c) Glycated fibrin without FXIIIa and d) Glycated fibrin with FXIIIa. The red scale bar indicates 50 µm.

**Figure 4:** Confocal images of healthy fibrin clots with normal RBCs and fibrin clots with RBCs from sickle cell patients. Green fluorescence represents the fibrin network and magenta represents labeled healthy and RBCs from sickle cell patients. a) Healthy RBCs in normal fibrin concentration with FXIIIa and b) Increased fibrin with FXIIIa and RBCs from a sickle cell patient. The white scale bar indicates 50  $\mu\text{m}$ . The white circles in image b) indicate recognizable sickled RBCs.

**Figure 5:** Fibrinolysis rates of normal fibrin clots compared to glycated (diabetic) fibrin clots and glycated clots with reduced plasmin concentration. Statistical significance was determined based on a one-way ANOVA and a *post hoc* Tukey-Kramer multiple comparison test. The ANOVA analysis showed that there was a significant difference between lysis rate means. The Tukey-Kramer test showed that there was a significant difference in means between the normal clots and clots with decreased plasmin with a  $p < 0.05$ . The \* indicates a significant difference from normal clots.

## DISCUSSION:

To obtain meaningful data about the structure of clotting mechanisms in disease states, it is important to isolate the factors involved in clotting to determine the effects of the proteins and cells in these conditions. This protocol was developed for the purposes of investigating the structure of the fibrin clot in diabetic and SCD states *in vitro*.

It is necessary to understand the mechanisms involved in fibrin formation and fibrinolysis in disease states since altered conditions cause hypercoagulation, atherothrombosis, and CVD. From the confocal images obtained in this experiment, it is evident that hypercoagulation activity occurs in diabetic and SCD states, which causes an increased fibrinogen concentration. An increased fibrinogen concentration results in a denser fibrin network with more branch points and smaller pores throughout the network. In fibrin networks of SCD patients, not only is there more fibrin present, but the aggregating nature of the erythrocytes from sickle cell patients also changes the structure of the network as it entraps fibrin into clusters. The conglomeration of RBCs from sickle cell patients also reduces porosity of the fibrin clusters in the network, but appears to increase void concentration in the overall network. The results of the fibrinolysis analysis of the glycated clots supported the hypothesis of the lysis rate in glycated clots being lower with reduced plasmin than that of healthy, normal clots. However, the results did not provide conclusive evidence about lysis rates of glycated clots with normal concentrations of plasmin. Thus it can be concluded that for diabetic patients with normal plasmin concentration, there may be sufficient fibrinolysis activity to degrade fibrin clots in an efficient manner.

For the experiments performed in this research study, isolated proteins and RBCs were used, instead of whole blood and plasma. By using separated proteins to synthesize the fibrin clots, the causes for altered structures between the normal vs. diseased clots could be determined. This greatly reduced the possibility of having other plasma proteins interfere with the structure and morphology of the fibrin clots. However, by using isolated proteins, it is crucial to store and handle the cells and proteins properly, as they are more vulnerable to cell death and protein degradation.

The confocal microscopy imaging technique chosen for this study was due to the ability to obtain images of the fibrin clot structures while retaining the natural thrombotic properties of the cells and proteins. Confocal microscopy has also been shown to give improved contrast and optical resolution of the specimen with reduced noise compared to traditional light microscopy. The laser light is directed into a pinhole that blocks unfocused light from entering. Utilization of confocal microscopy obviated the need for the addition of fixative chemicals; thus the cells and proteins were able to maintain their natural protein activity of forming fibrin clots. As a result the cells, proteins, and enzymes functioned in their native state, allowing the use of confocal microscopy for capturing images and video of real-time activity. With confocal microscopy, 3D images of the fibrin clots were captured and videos of plasmin digestion of fibrin clots were obtained in real-time. When adding plasmin to the clot to capture a time series video of the clot lysis process, it was important to use capillary action via plasmin to homogenously disperse the enzyme through the fibrin matrix. This was done to ensure that the added plasmin concentration was accurate and allowed the enzyme to lyse the entire clot, in contrast to the local area that the plasmin was added.

The method and the results obtained in this study were similar to a study done by Dunn *et al*, who found that clot lysis rates were significantly lower in diabetic subjects than control subjects<sup>25</sup>. In both studies, there were significant differences in lysis rates of diabetic (glycated) clots; however, the current protocol may accurately represent diabetic states because of the reduced plasmin levels. Additionally, in this research study fibrinolysis was captured continuously in real-time using the confocal microscope, in contrast to analyzing discrete sections at different time intervals. Wooton *et al.*<sup>47</sup> have investigated fibrin clot lysis and employed a lysis front velocity (cm/s) to investigate the rate of lysis for clots. They used confocal image analysis in their study to determine the front velocity. In the current study, instead of a lysis front velocity, a lysis area velocity was used (because of the way the image was analyzed on the confocal). To further validate the method developed in our study, the authors in<sup>48</sup> have described a mathematical model of fibrin clot lysis by plasmin, in which they prescribe the lysis front velocity as a function of the effective rate constant of fibrinolysis. The equation is given as:

$$\frac{dX}{dt} = \frac{1}{\rho_{Fn}} \left( \frac{k_{eff} K_a C_{pm}}{1 + K_a C_{pm}} \right).$$

Here,  $\frac{dX}{dt}$  represents the effective rate constant of fibrinolysis,  $K_a$  is the adsorption constant,  $C_{pm}$  is the plasmin concentration, and  $\rho_{Fn}$  is the density of fibrin. If the measured quantity  $\frac{dA}{dt}$  (measured from the confocal analysis in the current study) is utilized and the chain rule is employed, this leads to:

$$\frac{dA}{dt} = x \frac{dy}{dt} + y \frac{dx}{dt}$$

Assuming only one dimension is changing per unit time leads to:

$$\frac{dA}{dt} = y \frac{dx}{dt}$$

$$\frac{1}{y} \frac{dA}{dt} = \frac{dx}{dt}$$

Thus the effective rate constant of fibrinolysis for the experiment leads to:

$$k_{eff} = \rho \frac{1}{y} \frac{dA}{dt} \left( \frac{1 + K_a C_{pm}}{K_a C_{pm}} \right)$$

This relationship infers that the effective rate constant of fibrinolysis ( $k_{eff} \left[ \frac{mg}{cm^2 \cdot min} \right]$ ) is a function of the plasmin concentration ( $C_{pm}$ ) and the area lysis rate  $\frac{dA}{dt}$ .

This technique is also important for the study of thrombosis in SCD patients, since there is little known about fibrin and clotting mechanisms with respect to this disease. There have been very few studies conducted on the effects of SCD on the structure of fibrin clots; thus the confocal images collected from this study provides rare visualization of the clot structure with incorporated sickled RBCs.

To compare the differences in means between the groups, use a one-way ANOVA and a *post hoc* Tukey-Kramer test. A one-way ANOVA is a statistical test used to determine a statistically significant difference in the means between independent groups. The Tukey-Kramer post-test is used to compare the means from each independent group and determine which groups' means are statistically different from each other. Before performing a one-way ANOVA, there are six assumptions that must be met to ensure that the results are valid. The six assumptions are: a continuous dependent variable (lysis rate), an independent variable consisting of two or more independent categorical groups (normal, glycated, and glycated with reduced plasmin), and independently observed data in each group such that one event occurring does not affect another event (This assures that there is no overlap in data.), the dataset not containing any significant outliers, normally distributed data verified by the Shapiro-Wilk statistical test, and homogeneity of variances in the data verified by the Levene statistical test. Once these assumptions were met, a one-way ANOVA and a *post hoc* Tukey-Kramer test were performed with a p-value of 0.05 as the threshold for determining statistical significance. For a rigorous detail of the one-way ANOVA and post-hoc Tukey-Kramer test, refer to <sup>44,45</sup>.

Overall, this method allows for imaging of normal and abnormal (due to diseased states) fibrin clots in their native states. The method also provides details for real-time imaging of clot lysis as well as statistical analysis of lysis rates. The ease with which the thrombus can be produced and imaged *in vitro* is useful for a wide range of applications in cellular and tissue engineering.

#### ACKNOWLEDGMENTS:

The authors would like to thank the Lam Lab at Georgia Tech for many helpful discussions in developing the experimental assays. Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under Award Number K01HL115486 and by New Innovator Grant 1DP2OD007433-01 from the Office of the Director, National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## DISCLOSURES:

The authors have nothing to disclose.

## REFERENCES:

- 1 Averett, Rodney D. *et al.* A Modular Fibrinogen Model that Captures the Stress-Strain Behavior of Fibrin Fibers. *Biophysical Journal* **103**, 1537-1544, <http://dx.doi.org/10.1016/j.bpj.2012.08.038> (2012).
- 2 Carlisle, C. R. *et al.* The mechanical properties of individual, electrospun fibrinogen fibers. *Biomaterials* **30**, 1205-1213, <http://dx.doi.org/10.1016/j.biomaterials.2008.11.006> (2009).
- 3 Falvo, M. R., Gorkun, O. V. & Lord, S. T. The molecular origins of the mechanical properties of fibrin. *Biophysical Chemistry* **152**, 15-20, <http://dx.doi.org/10.1016/j.bpc.2010.08.009> (2010).
- 4 Guthold, M. & Cho, Samuel S. Fibrinogen Unfolding Mechanisms Are Not Too Much of a Stretch. *Structure* **19**, 1536-1538, <http://dx.doi.org/10.1016/j.str.2011.10.008> (2011).
- 5 Gerth, C., Roberts, W. W. & Ferry, J. D. Rheology of fibrin clots II: Linear viscoelastic behavior in shear creep. *Biophysical Chemistry* **2**, 208-217, [http://dx.doi.org/10.1016/0301-4622\(74\)80046-3](http://dx.doi.org/10.1016/0301-4622(74)80046-3) (1974).
- 6 Nelb, G. W., Kamykowski, G. W. & Ferry, J. D. Rheology of fibrin clots. v. shear modulus, creep, and creep recovery of fine unligated clots. *Biophysical Chemistry* **13**, 15-23, [http://dx.doi.org/10.1016/0301-4622\(81\)80020-8](http://dx.doi.org/10.1016/0301-4622(81)80020-8) (1981).
- 7 Roberts, W. W., Kramer, O., Rosser, R. W., Nestler, F. H. M. & Ferry, J. D. Rheology of fibrin clots. I.: Dynamic viscoelastic properties and fluid permeation. *Biophysical Chemistry* **1**, 152-160, [http://dx.doi.org/10.1016/0301-4622\(74\)80002-5](http://dx.doi.org/10.1016/0301-4622(74)80002-5) (1974).
- 8 Ryan, E. A., Mockros, L. F., Weisel, J. W. & Lorand, L. Structural Origins of Fibrin Clot Rheology. *Biophysical Journal* **77**, 2813-2826, [http://dx.doi.org/10.1016/S0006-3495\(99\)77113-4](http://dx.doi.org/10.1016/S0006-3495(99)77113-4) (1999).
- 9 Weisel, J. W. Fibrin assembly. Lateral aggregation and the role of the two pairs of fibrinopeptides. *Biophysical Journal* **50**, 1079-1093, [http://dx.doi.org/10.1016/S0006-3495\(86\)83552-4](http://dx.doi.org/10.1016/S0006-3495(86)83552-4) (1986).
- 10 Weisel, J. W. The mechanical properties of fibrin for basic scientists and clinicians. *Biophysical Chemistry* **112**, 267-276, <http://dx.doi.org/10.1016/j.bpc.2004.07.029> (2004).
- 11 Wolberg, A. S. Thrombin generation and fibrin clot structure. *Blood Reviews* **21**, 131-142, <http://dx.doi.org/10.1016/j.blre.2006.11.001> (2007).
- 12 Wolberg, A. S. & Campbell, R. A. Thrombin generation, fibrin clot formation and hemostasis. *Transfusion and Apheresis Science* **38**, 15-23, <http://dx.doi.org/10.1016/j.transci.2007.12.005> (2008).
- 13 Yeromonahos, C., Polack, B. & Caton, F. Nanostructure of the Fibrin Clot. *Biophysical Journal* **99**, 2018-2027, <http://dx.doi.org/10.1016/j.bpj.2010.04.059> (2010).
- 14 Dunn, E. J. Fibrinogen and fibrin clot structure in diabetes. *Herz* **29**, 470-479 (2004).
- 15 Gladwin, M. T. & Sachdev, V. Cardiovascular abnormalities in sickle cell disease. *Journal of the American College of Cardiology* **59**, 1123-1133 (2012).
- 16 Collet, J. P. *et al.* Altered Fibrin Architecture Is Associated With Hypofibrinolysis and Premature Coronary Atherothrombosis. *Arteriosclerosis, thrombosis, and vascular biology* **26**, 2567-2573, 10.1161/01.ATV.0000241589.52950.4c (2006).
- 17 Carr, M. E. Diabetes mellitus: a hypercoagulable state. *Journal of Diabetes and its*

*Complications* **15**, 44-54 (2001).

18 Ajjan, R. A. & Ariëns, R. A. S. Cardiovascular disease and heritability of the prothrombotic state. *Blood Reviews* **23**, 67-78, <http://dx.doi.org/10.1016/j.blre.2008.07.001> (2009).

19 Standeven, K. F., Ariëns, R. A. S. & Grant, P. J. The molecular physiology and pathology of fibrin structure/function. *Blood Reviews* **19**, 275-288, <http://dx.doi.org/10.1016/j.blre.2005.01.003> (2005).

20 American Diabetes Association, <<http://www.diabetes.org/>> (2014).

21 Alzahrani, S. & Ajjan, R. Review article: Coagulation and fibrinolysis in diabetes. *Diabetes and Vascular Disease Research* **7**, 260-273, 10.1177/1479164110383723 (2010).

22 Sickel Cell Disease Association of America, Inc., <<http://www.sickelcelldisease.org/>> (2014).

23 Stuart, M. J. & Nagel, R. L. Sickel-cell disease. *The Lancet* **364**, 1343-1360 (2004).

24 Dunn, E. J., Ariëns, R. A. S. & Grant, P. J. The influence of type 2 diabetes on fibrin structure and function. *Diabetologia* **48**, 1198-1206, 10.1007/s00125-005-1742-2 (2005).

25 Dunn, E. J., Philippou, H., Ariëns, R. A. S. & Grant, P. J. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia* **49**, 1071-1080, 10.1007/s00125-006-0197-4 (2006).

26 Famodu, A. & Reid, H. Plasma fibrinogen levels in sickel cell disease. *Tropical and geographical medicine* **39**, 36-38 (1987).

27 Richardson, S., Matthews, K., Stuart, J., Geddes, A. & Wilcox, R. Serial Changes in Coagulation and Viscosity during Sickel-Cell Crisis. *British journal of haematology* **41**, 95-103 (1979).

28 Ataga, K. I. & Orringer, E. P. Hypercoagulability in sickel cell disease: a curious paradox. *The American Journal of Medicine* **115**, 721-728, <http://dx.doi.org/10.1016/j.amjmed.2003.07.011> (2003).

29 Collet, J. P. *et al.* Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arteriosclerosis, thrombosis, and vascular biology* **26**, 2567-2573, 10.1161/01.ATV.0000241589.52950.4c (2006).

30 Barazzoni, R. *et al.* Increased Fibrinogen Production in Type 2 Diabetic Patients without Detectable Vascular Complications: Correlation with Plasma Glucagon Concentrations. *The Journal of Clinical Endocrinology & Metabolism* **85**, 3121-3125, 10.1210/jcem.85.9.6779 (2000).

31 Ceriello, A. Coagulation activation in diabetes mellitus: the role of hyperglycaemia and therapeutic prospects. *Diabetologia* **36**, 1119-1125, 10.1007/bf00401055 (1993).

32 Mayne, E. E., Bridges, J. M. & Weaver, J. A. Platelet adhesiveness, plasma fibrinogen and factor VIII levels in diabetes mellitus. *Diabetologia* **6**, 436-440, 10.1007/bf01212078 (1970).

33 Weisel, J. W. Fibrinogen and fibrin. *Advances in protein chemistry* **70**, 247-299 (2005).

34 Collet, J. *et al.* Influence of Fibrin Network Conformation and Fibrin Fiber Diameter on Fibrinolysis Speed Dynamic and Structural Approaches by Confocal Microscopy. *Arteriosclerosis, thrombosis, and vascular biology* **20**, 1354-1361 (2000).

35 Jörneskog, G. *et al.* Altered properties of the fibrin gel structure in patients with IDDM. *Diabetologia* **39**, 1519-1523, 10.1007/s001250050607 (1996).

36 Svensson, J. *et al.* Acetylation and glycation of fibrinogen in vitro occur at specific lysine residues in a concentration dependent manner: A mass spectrometric and isotope labeling study. *Biochemical and Biophysical Research Communications* **421**, 335-342,



<http://dx.doi.org/10.1016/j.bbrc.2012.03.154> (2012).

37 Pieters, M. *et al.* Glycation of fibrinogen in uncontrolled diabetic patients and the effects of glycaemic control on fibrinogen glycation. *Thrombosis Research* **120**, 439-446,

<http://dx.doi.org/10.1016/j.thromres.2006.10.016> (2007).

38 Baradet, T. C., Haselgrove, J. C. & Weisel, J. W. Three-dimensional reconstruction of fibrin clot networks from stereoscopic intermediate voltage electron microscope images and analysis of branching. *Biophysical journal* **68**, 1551-1560, 10.1016/s0006-3495(95)80327-9 (1995).

39 Campbell, R. A., Overmyer, K. A., Selzman, C. H., Sheridan, B. C. & Wolberg, A. S. Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability. *Blood* **114**, 4886-4896, 10.1182/blood-2009-06-228940 (2009).

40 Curtis, D. J. *et al.* A study of microstructural templating in fibrin-thrombin gel networks by spectral and viscoelastic analysis. *Soft Matter* **9**, 4883-4889, 10.1039/C3SM50263E (2013).

41 Kim, E. *et al.* Correlation between fibrin network structure and mechanical properties: an experimental and computational analysis. *Soft Matter* **7**, 4983-4992, 10.1039/C0SM01528H (2011).

42 Keegan, P. M., Surapaneni, S. & Platt, M. O. Sick cell disease activates peripheral blood mononuclear cells to induce cathepsins k and v activity in endothelial cells. *Anemia* **2012**, 201781, 10.1155/2012/201781 (2012).

43 Allison, A. Properties of sickle-cell haemoglobin. *Biochemical Journal* **65**, 212 (1957).

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-Verlag Publishing, New York, N.Y., USA, 1992).

46 Pan, W., Galkin, O., Filobelo, L., Nagel, R. L. & Vekilov, P. G. Metastable Mesoscopic Clusters in Solutions of Sickle-Cell Hemoglobin. *Biophysical Journal* **92**, 267-277, <http://dx.doi.org/10.1529/biophysj.106.094854> (2007).

47 Wootton, D. M., Popel, A. S. & Alevriadou, B. R. An experimental and theoretical study on the dissolution of mural fibrin clots by tissue-type plasminogen activator. *Biotechnology and bioengineering* **77**, 405-419 (2002).

48 Sazonova, I. Y. *et al.* 127 Mathematic model of fibrin clot lysis by plasmin. *Fibrinolysis and Proteolysis* **12 Supplement 1**, 45, [http://dx.doi.org/10.1016/S0268-9499\(98\)80156-2](http://dx.doi.org/10.1016/S0268-9499(98)80156-2) (1998).

Figure 1

[Click here to download high resolution image](#)

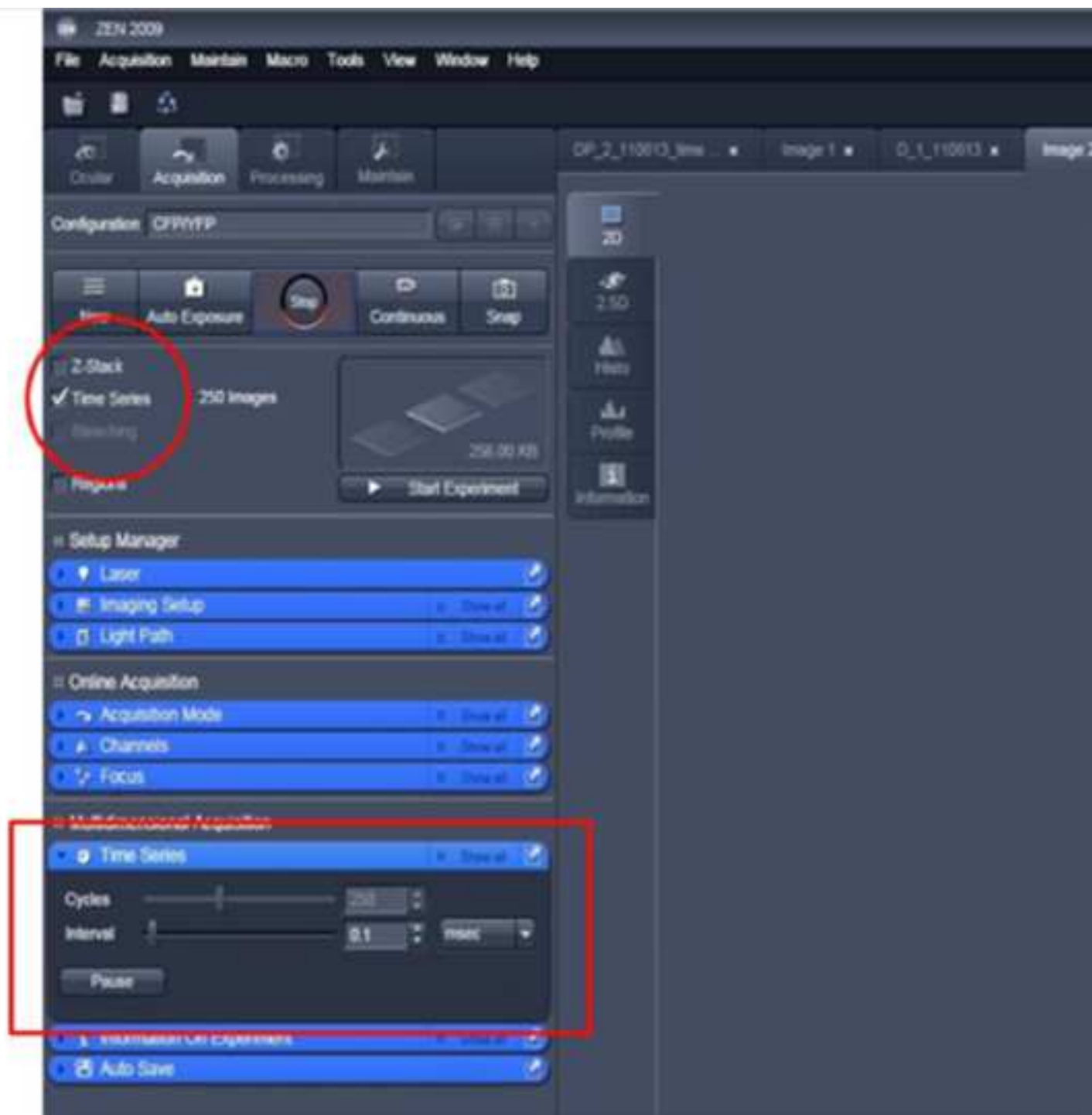


Figure 2  
[Click here to download high resolution image](#)

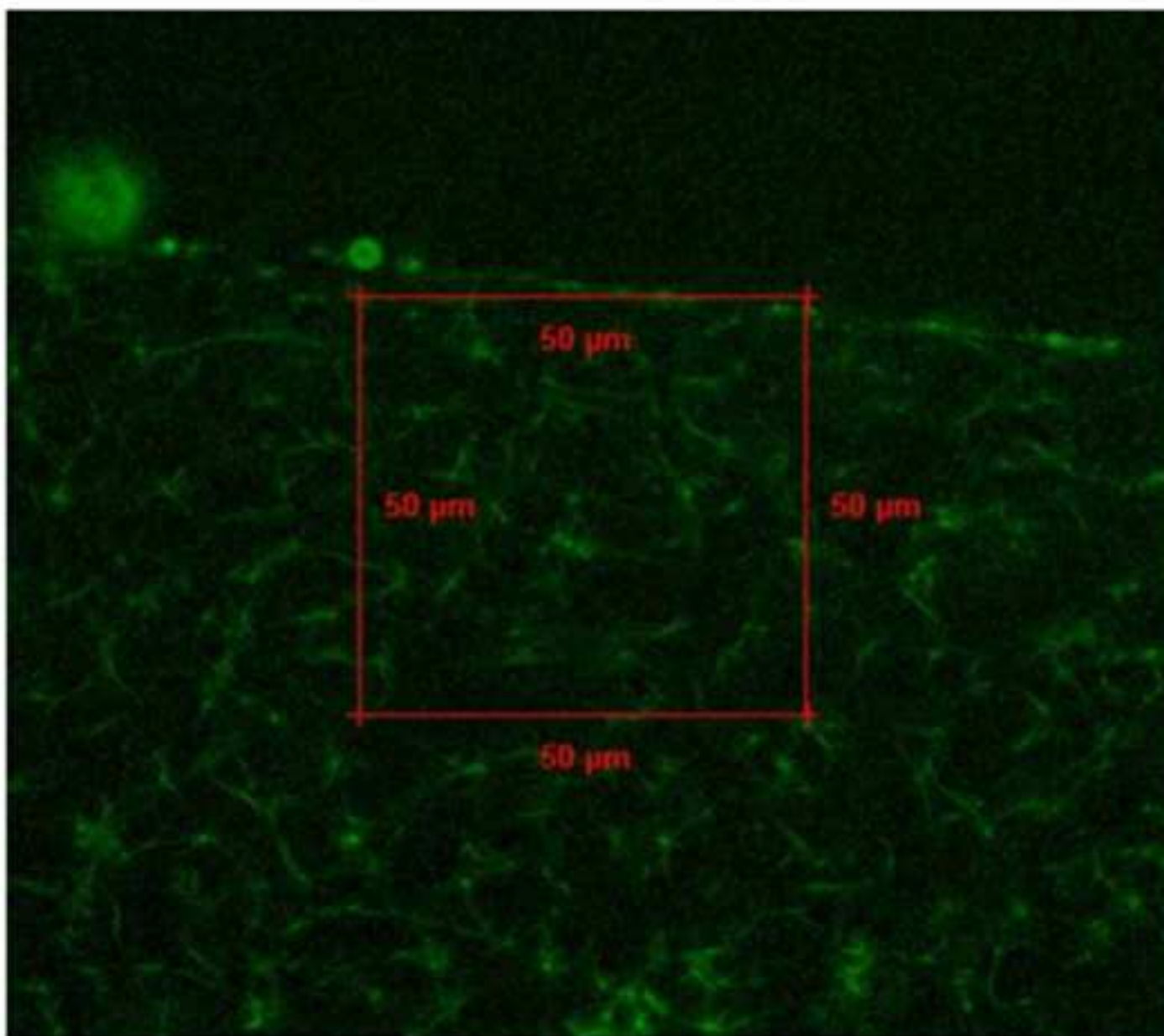


Figure 3

[Click here to download high resolution image](#)

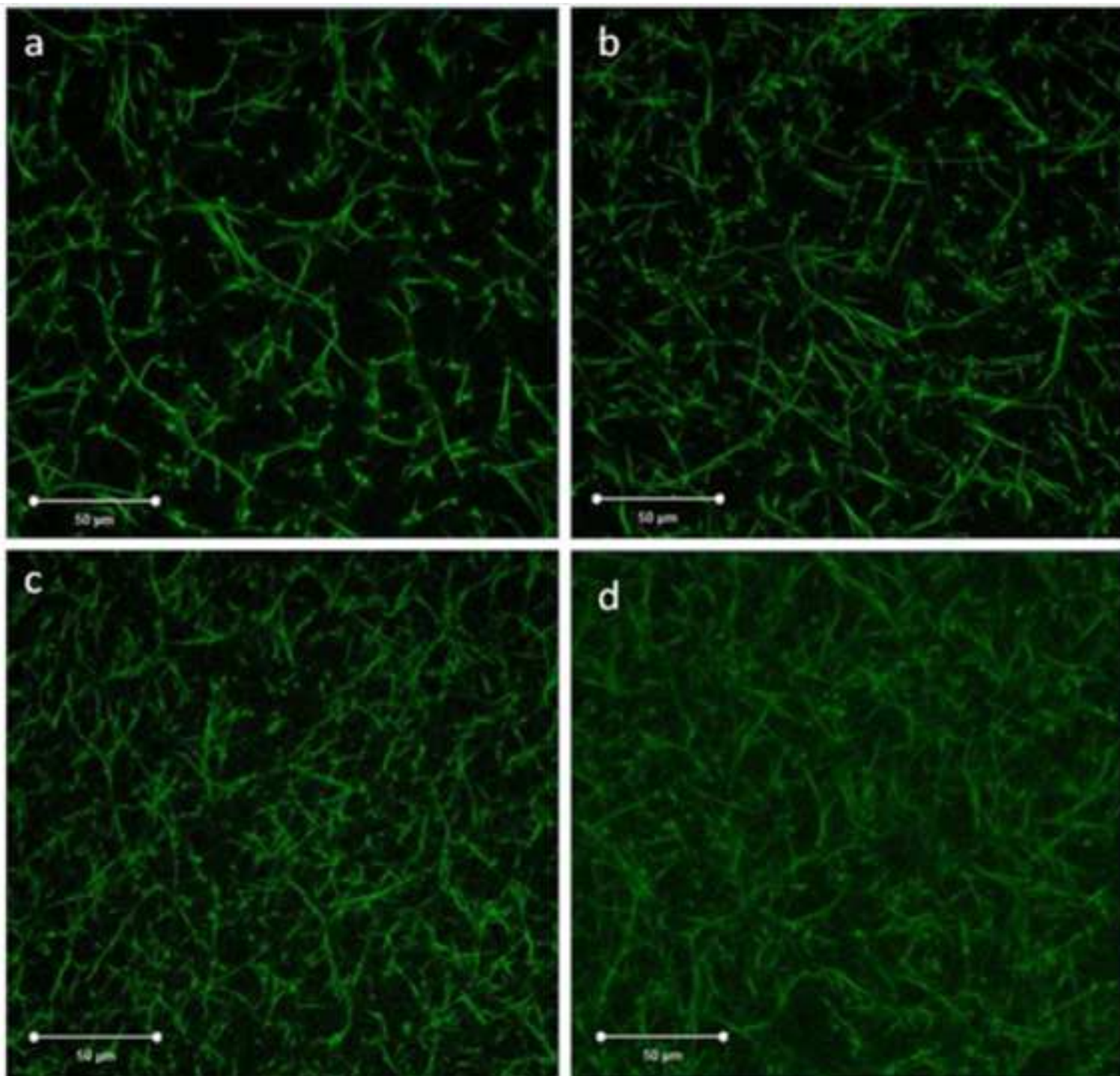




Figure 4  
[Click here to download high resolution image](#)

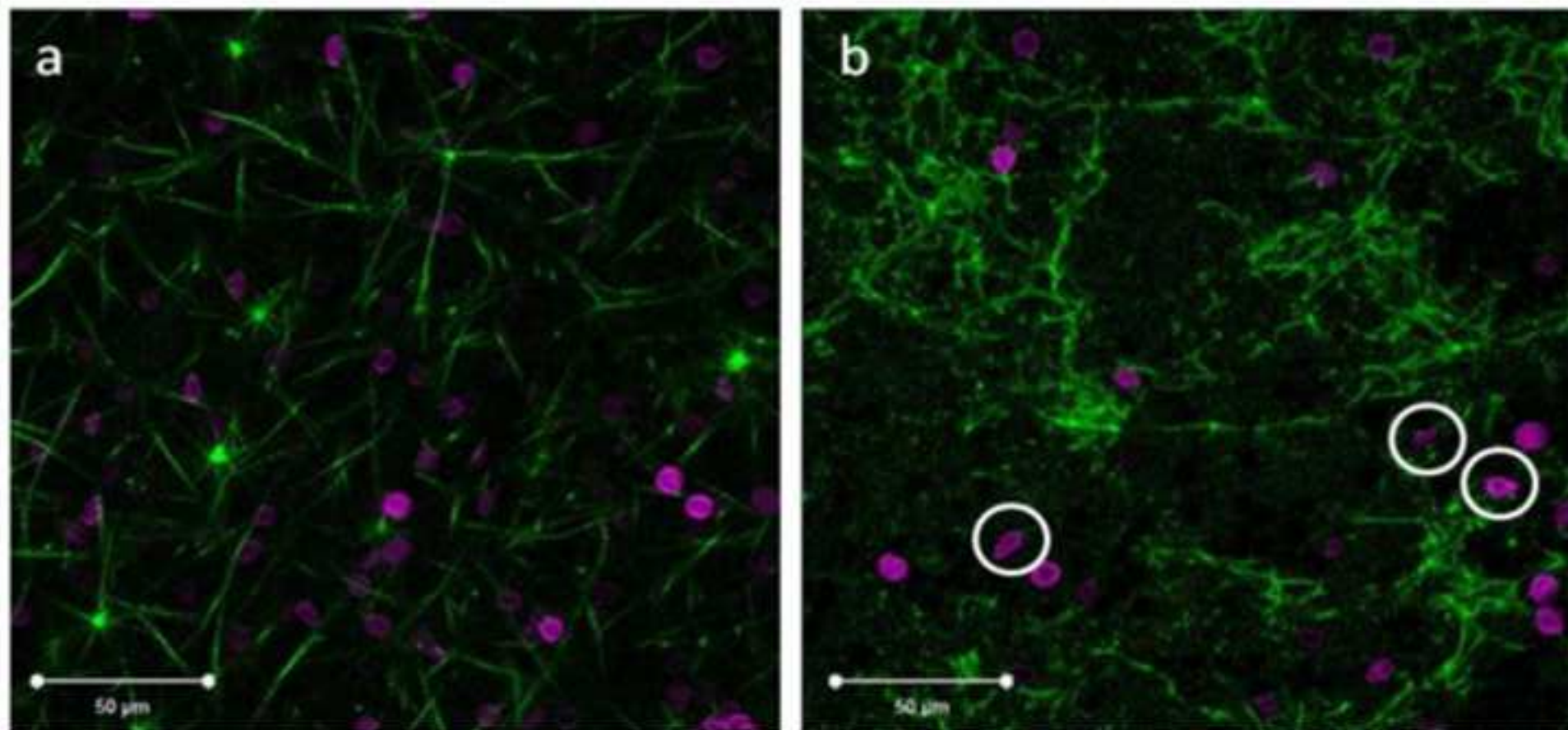
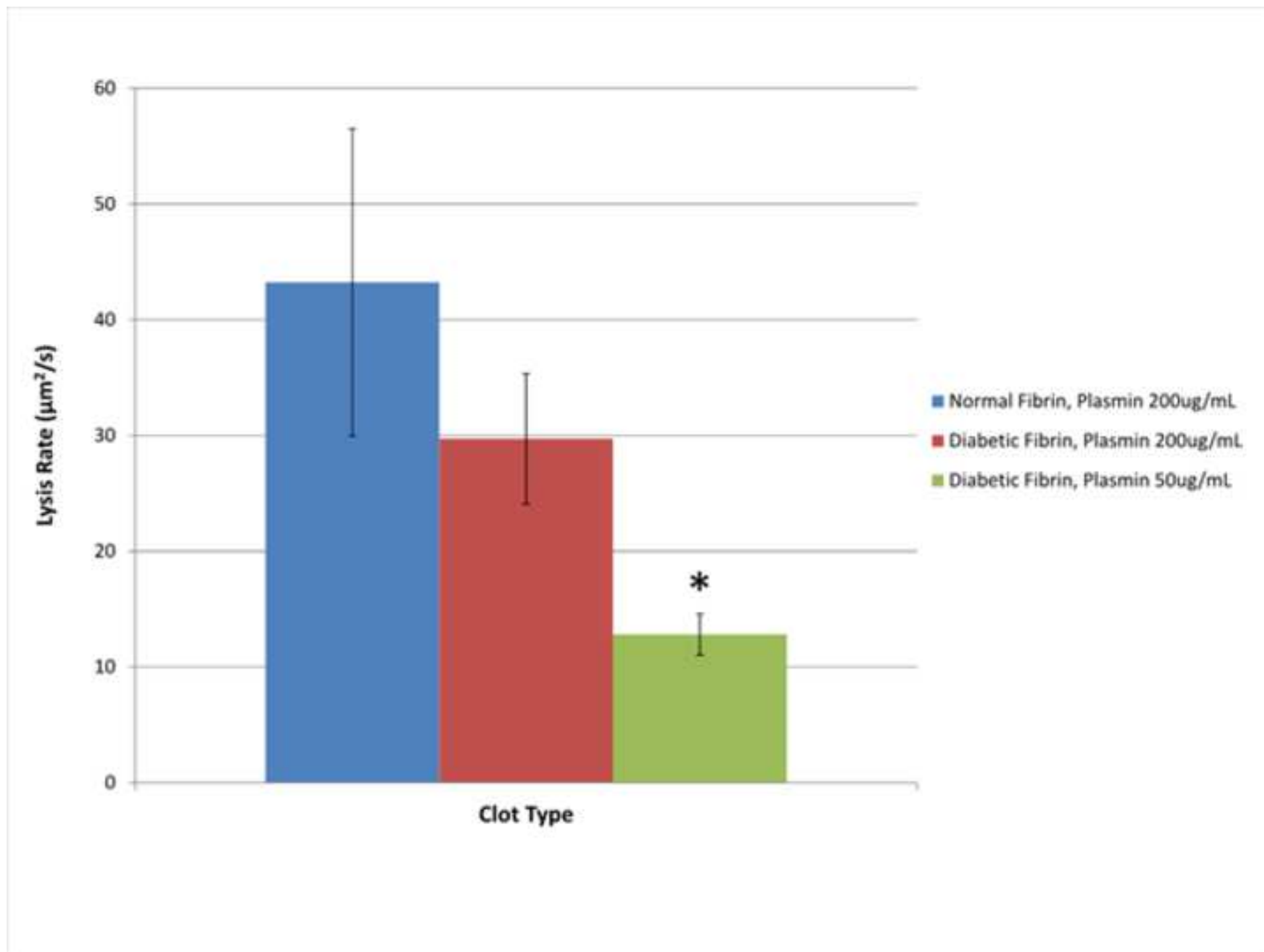


Figure 5  
[Click here to download high resolution image](#)



Name of Material/ Equipment	Company	Catalog Number
PBS	Life Technologies	10010031
Ficoll-Paque (hydrophilic polysaccharide)	GE Healthcare	45-001-749
10 ml heparinized vacutainer tubes	BD Biosciences	366643
Human Fibrinogen	Enzyme Research Laboratories	N/A
Alexa Fluor 488 human fibrinogen conjugate	Molecular Probes	F13191
0.5 mL graduated microcentrifuge tube	Fisher Scientific	05-408-120
Glucose powder	Life Technologies	15023-02
FXIIIa	Enzyme Research Laboratories	N/A
VIS Confocal Microscope	Zeiss LSM 510	LSM 510
50 mM Tris	Lonza	S50-642
Calcium Chloride (CaCl <sub>2</sub> )	Sigma Aldrich	449709-10G
Vybrant DiD cell-labeling solution	Life Technologies	L7781
Plasmin	Enzyme Research Laboratories	N/A
Sodium Chloride (5 M NaCl)	Life Technologies	AM9759
Statistical Modeling Software	IBM	SPSS Statistics 22

**Comments/Description**



Lysis video

[Click here to download Animated Figure \(video and/or .ai figure files\): 20141002-071710\\_clip.avi](#)



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Experimental and Imaging Techniques for Examining Fibrin Clot Structure in Normal and Diseased States

Author(s): Natalie K. Fan, Philip Keegan, Manu O. Platt, Rodney D. Averett

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

## ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name:	Rodney D. Averett	
Department:	George W. Woodruff School of Mechanical Engineering	
Institution:	Parker H. Petit Institute for Bioengineering & Bioscience	
Article Title:	Georgia Institute of Technology	
	Experimental and Imaging Techniques for Examining Fibrin	
	Clot Structure in Normal and Diseased States	
Signature:		Date: 2/6/2014

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

**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  $1 \times 10^6$ /mL in any chosen serum-free culture medium.
- 3.3.2 Add 5  $\mu$ L/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  $\mu$ L 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<sup>44,45</sup>.

*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).