Measurement of Platelet Clot Volume in a Microscale Thrombosis Screening Device

Laura Seaman
Biological Engineering, Massachusetts Institute of Technology

NNIN REU Site: Nanotechnology Research Center, Georgia Institute of Technology, Atlanta, GA
NNIN REU Principal Investigator: Dr. Craig Forest, Mechanical Engineering, Georgia Institute of Technology
NNIN REU Mentor: Melissa Li, Biomedical Engineering, Georgia Institute of Technology
Contact: laseaman@mit.edu, cforest@gatech.edu, melissa_li@gatech.edu

Introduction:

Cardiovascular disease (CVD) is the leading cause of death in the United States. In patients with CVD, plaque builds up in arteries creating local constrictions called stenoses. These stenoses increase shear rates within arteries causing platelets to form clots. This process, known as thrombosis, can result in the occlusion of the artery causing stroke or heart attack.

We have developed a microfluidic device to measure clot formation under restricted flow. Clot formation is monitored using simultaneous readings of laser light transmission through the stenosis region and mass flow. During each trial, the light transmission increases due to the increased scattering absorption of light in red blood cells compared to platelets. To calibrate this changing light transmission to a clot volume, an estimate of the final clot volume in the device was needed.

The volume was initially estimated to be around 30 nL. This presented a challenge because it was too small for weighing or displacement measurements. Additionally, the clot was too fragile to remove from the device. Previous methods have used only light microscopy or confocal microscopy. Light microscopy has excellent edge detection in single-plane images, but poor z-direction depth resolution [1]. Conversely, confocal microscopy has excellent z-direction resolution, but poor edge finding abilities [2]. Thus we sought to create a more accurate volumetric calculation through the combination of these methods.

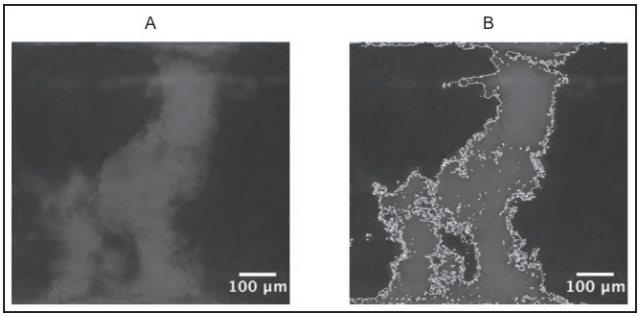


Figure 1: A) Brightfield image of the top of the clot. B) Selected clot region.

Experimental Procedure:

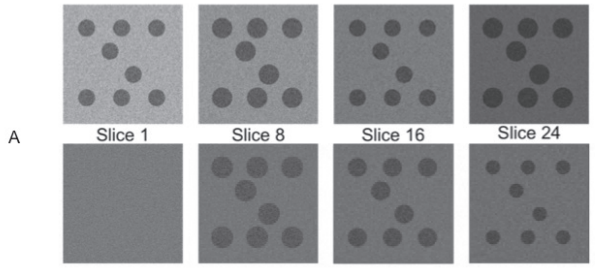
In our procedures, we first estimated the dimensions of the platelet clot using light microscopy. Next, we created estimates of density within the clot using confocal measurements at multiple planes.

First, the Keyence digital microscope was used as a 250x brightfield microscope to obtain single plane images of the top and bottom of the clot as shown in Figure 1. We were able to select for the clot areas using ImageJ color thresholding, and subsequently calculate the clot's area. These measurements were then averaged and multiplied by the channel depth in order to obtain measurements of clot volume.

Next, we conducted confocal studies to obtain density measurements. To contrast between the clot and surrounding red blood cells, we used Mepacrine, a fluorescent platelet stain with excitation and emission at 488 and 520 nm respectively at a final concentration of 0.24 M. Images of the fluorescently stained clot were acquired using a Zeiss laser scanning confocal microscope with a 20x objective at 1 μm z-axis resolution through a 71 μm pinhole and a dwell time of 1.61 μs.

In confocal microscopy, the deeper into the tissue the images are taken, the less intense the image is. This decay causes a maximum depth penetration of 60 μm, preventing direct volume calculation. The decay also must be corrected before a threshold can be applied since the same intensity naturally selects a much larger area in the deeper images because they are naturally darker.

To correct the depth-related decrease in intensity, ImageJ was utilized to threshold the images using Shangabg's algorithm. The threshold was applied to each picture and the number of bright, or clot, pixels were counted. The density was calculated by standardizing the areas of the clot in each picture by dividing by the area in the top picture. Then these standardized areas were averaged within each stack, resulting in a density. To verify the efficacy of the method, controls were created. They went through the process as the



B

	Real Clot	Test 1	Test 2 (no intensity decay)
calculated density		86.31	86.31
measured density	82.63	100.95	88.99
% Error		18.50	3.30

Figure 2: A) Control images before and after thresholding. B) Results using ImageJ.

real clots and the error between the known density and that measured by the process was calculated, as shown in Figure 2. The error of 18.5% indicates that the process failed to accurately select the clot regions.

Instead, a custom MATLAB® script was used to apply a noise reduction filter, wiener2, and adjust the images using imadjust, so that in each one 1% of the pixels were at the maximum and minimum value. It then calculated a single threshold and applied it to all the pictures in a stack.

Figure 3 shows the clot before any changes, after the intensity is adjusted, and after the threshold is applied. Controls were run then through the same script as the real clots. Results, shown in Figure 4, showed errors under 1.4%. The average density of the clots was calculated to be 76.86%.

As shown in Figure 4, these measurements were used to calculate three different volumes for comparison using the known height of the stenosis region. The first volume estimate used just the brightfield image of the bottom of the clot. The second version involved averaging the areas found from the top and bottom brightfield images. The averaged the top and bottom areas then scaled it by the density.

A

	Test 1	Test 2	Test 3 (more noise)
calculated density	93.36	92.04	93.36
measured density	93.97	91.84	94.62
% Error	0.65	0.21	1.34

B

	Clot 1	Clot 2	Average
Density	74.74	78.98	76.86

C

	clot 1	clot 2	clot 3	clot 4	clot 5	clot 6
Volume (μm^3) bottom only	3.80E7	7.80E7	4.47E7	4.56E7	3.67E7	4.81E7
Volume (μm^3) top & bottom	2.95E7	3.95E7	4.39E7	3.77E7	3.47E7	4.28E7
Volume (μm^3) with density	2.27E7	3.04E7	3.37E7	2.89E7	2.67E7	3.29E7

D

	Avg Volume	Avg Volume - % of Stenosis region	% Difference
Bottom only	4.85E7	83.06	66.0
Top & Bottom with Density	3.80E7 2.92E7	65.10 50.04	30.1

Figure 4: A) Clot density control results. B) Density of real clots. C) Clot volumes. D) Clot volume average.

Results and Conclusions:

The thrombus platelet density was 76.86%, which compares with measurements from a previous study of 80%. The overall platelet thrombus volume was estimated as 30 million mircometers [2], full results in Figure 4. This corresponds with the experimental recorded increase of 24% light transmission and the theoretical increase of 18.8% calculated in previous work [3].

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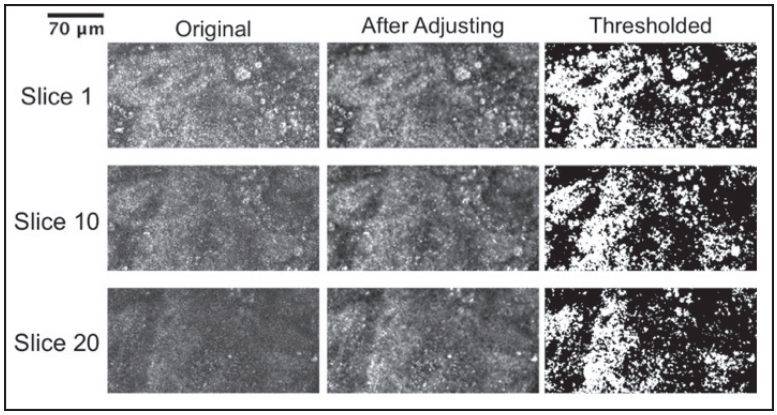

Figure 3: Images of a real clot before processing, after adjusting the intensity, and after thresholding.

References:

[1] Ku, D., Para, A., et al; "Rapid Platelet Accumulation Leading to Thrombotic Occlusion"; Annals of Biomedical Engineering, Vol. 39, 1961-1971 (2011).

[2] Ruggeri, Z., Savage, B., et al; "Specific Synergy of Multiple Substrate-Receptor Interaction in Platelet Thrombus Formation under Flow"; Cell, Vol. 94, 657-666 (1998).

[3] Li, M., Ackerman, J., et al; "Microfluidic System for Multichannel Optical Measurement of Shear Induced Platelet Thrombosis in Unfractionated Blood"; (2011).