Effects of Fluorescent Labelling and Deformability and Geometric Properties of Red Blood Cells

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1.0 INTRODUCTION

Erythrocytes are highly deformable cells[1]. This important property allows them to compress and change their shapes in order to go through vessels and capillaries that are smaller than their average diameter. While blood flows through vessels, blood cells generate friction among one another creating resistance and parabolic flow. If blood was not a non-Newtonian fluid, then blood over fast velocity vessels would be very viscous; thus creating high friction and slow blood flow[1]. Deformability is important because it allows blood to stay fluid even at high shear rates. Blood flow is a main factor in determining heterogeneous blood cell distribution at bifurcations and is highly regulated. Hemodynamics is the study of blood flow, and a major research area is studying blood flow in the skeletal tissue. Directly measuring blood flow in capillaries can be accomplished because of the slow single file blood cells, but directly measuring blood flow in high velocity blood vessels such as arterioles is still being researched. A technique to measure and trace velocity and hemodynamics in arterioles is currently under study. By injecting fluorescently labelled red blood cells into an in vivo rat specimen, blood flow could be traced by viewing the labelled cells. The advantage of injecting fluorescent cells allows single blood cells to stand out against the rest of the blood cells in fast flowing arterioles. A single labelled cell could be traced and represent the entire population of blood cells. Fluorescent cells are effective tracers; however it is important to preserve the properties of dyed cells because increase of blood viscosity will impede blood flow. The effects of fluorescently labelling on the blood cell diameter, surface area, volume and sphericity index are unknown. The objective of this study is to study the effects of fluorescent labelling on red blood cell deformability and geometry as well as the effects of albumin on deformability.

2.0 THEORY

Deformability of a blood cell is the ability to deform the shape of the cell to compress their shape to fit into smaller vessels[1]. Without deformability, cells would plug capillaries because they cannot deform their shape to squeeze through. This is possible because they have approximately 25% more membrane surface area than the minimum required to enclose the volume [2]. Red blood cells have the ability to remain fluid over a hematocrit of 90%, but only if they are healthy [1]. They can also undergo shear stress without lysing. Deformability of a red blood cell is important to blood flow throughout the body. Blood is also a Newtonian fluid, which allows hematocrit to increase up to 90% before changing the viscosity of the blood[1]. This makes blood hemodynamic important to study.

Geometry of a blood cell can give many indications of how healthy it is. Diameter, surface area, volume, and sphericity index are parameters that can be measured and compared to healthy cells to determine if there is any change between unstained and stained cells. Sphericity index is the measure of how spherical an object is[3], thereby giving an indication of how relatively rigid the red blood cell is. It is a unitless measurement from 0 to 1; 0 is infinitely planar, and 1 is perfectly spherical. Red blood cells cannot endure a surface area increase of more than 5-10% without hemolysis, but can have a volume increase up to 30% before hemolysis[3].

The stain used is Fluorescein isothiocyanate (FIT-C) dye. It is a synthetic organic molecule that binds to membrane proteins, and fluoresces under fluorescent light [5]. Tris-Buffered Ringer's Solution (TR) (appendix 7.2) is used to wash blood cell sample via centrifuge during staining process. Tris-Buffered Ringer's Albumin Solution (TRA) (appendix 7.3) is used to maintain healthiness of cells for overnight storage. The use of albumin is to maintain the biconcave shape of a normal erythrocyte [2].

2.1 Hang Test

Hang Test allows blood cell profiles to be taken when blood cells are hung from their edge, and allows calculation of surface area, volume, and sphericity index [2]. A simple brightfield microscope is used to view prepared slides after Kohlering the scope; brings the specimen into focus by optimizing the amount of light used. When analysing results, Image J is used, and it is an imaging program used to view and analyze via measuring cells. Calculations and statistical analysis is run using Excel.

2.2 Calculations

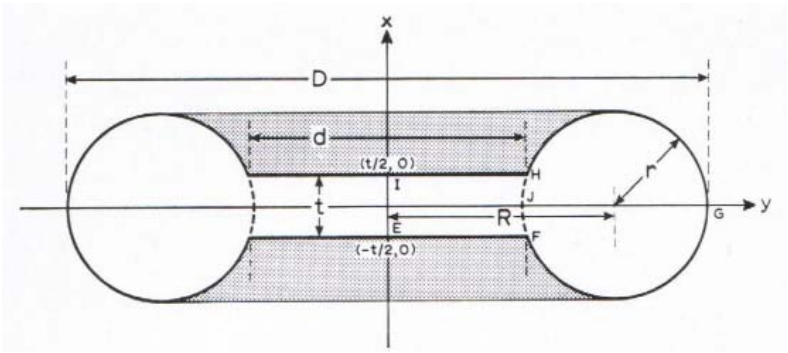

Fig 1. Schematic diagram of the red cell on edge [6].
D is the diameter of the toroid, d is the inner diameter up to inside the toroid, t is the inner thickness of the inner disc. From D, d, and t, the following were computed.

$$r = \frac{t^2 + (D - d)^2}{4(D - d)}$$

Equation 1. Radius of toroid[6].

$$R = \frac{D}{2} - r$$

Equation 2. Radius of middle of cell to middle of toroid [6].

$$V_T = 2\pi^2 R r^2$$

Equation 3. Volume of a toroid [6].

$$V_D = \pi\left[(R^2 + r^2)t - \frac{t^3}{12} - Rt\sqrt{r^2 - \frac{t^2}{4}} - 2Rr^2 \sin^{-1}\left(\frac{t}{2r}\right)\right]$$

Equation 4. Volume of the inner disc minus the indentation caused by the toroid integrated by surface of revolution [6].

Equation 5. Total volume = $V_T + V_D$

$$A_T = 4\pi r\left[\pi R - R\sin^{-1}\left(\frac{t}{2r}\right) + \frac{t}{2}\right]$$

Equation 6. Surface area of the toroid minus the area generated by HJF rotated around the axis[6].

$$A_D = 2(\pi(d/2)^2)$$

Equation 7. Surface area of the top and bottom disc [6].

Equation 8. Total surface area = $A_T + A_D$

$$\text{Sphericity Index} = \frac{4.84(V_T)^{2/3}}{A_T}$$

Equation 9. Sphericity Index[3].

3.0 METHODS

First, blood sample must be extracted from the specimen (rat) after the specimen has been put through anaesthetics. After, the blood sample needs to be washed. Washing the cells after staining or extraction is done with a centrifuge. By spinning down the blood sample, the red blood cells will be differentiated to the bottom of the tube; leaving the blood plasma, white blood cell layer, or buffer at the top for removal. Then the sample is split into two portions, one for staining, and one for unstained. Unstained sample is put into isotonic TR and TRA buffer for testing, and stained sample follows the FIT-C staining protocol; where DMSO and FIT-C stain is mixed with blood sample. These cells are to be stirred and incubated for two hours, for further details refer to appendix 7.1.

Once both unstained and stained sample have been made, the hang test is conducted. Where a slide with a chamber-well attached to it is prepared, paraffin oil is placed into the chamber to act as a suspension fluid for the blood sample. An inverted coverslip is prepared with 10µL of rat blood sample, and sits for 2 minutes. The coverslip is then flipped back and placed on top of the paraffin oil filled well. View and record images of the slide under a brightfield microscope at 40x objective. Finally, process through Image J and run statistical analysis on results in Excel.

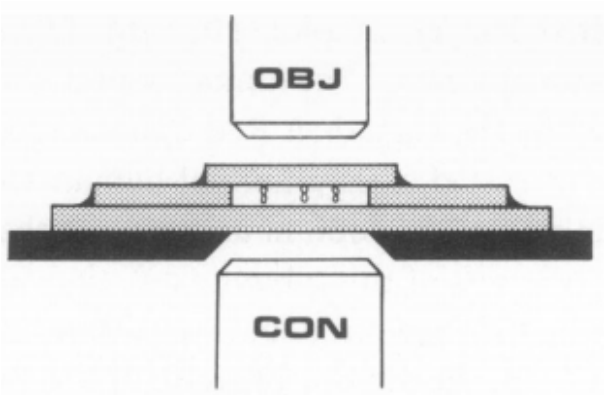

Fig 2. Diagram of the microscope chamber[2].

Prime cells chosen have to fulfill the criteria of them hanging edge on.

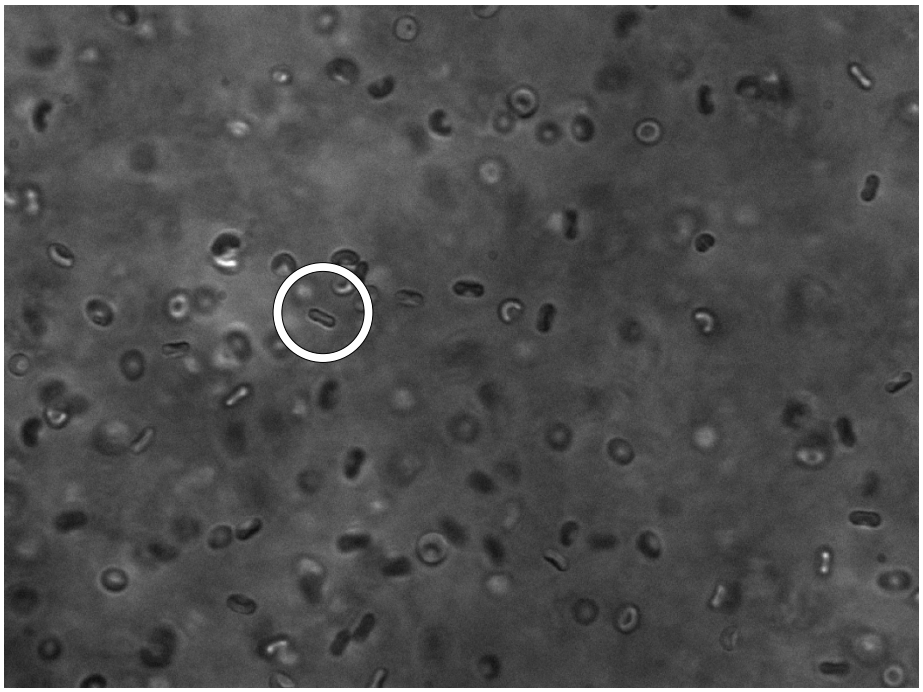

Fig 3. Stained erythrocytes from a rat under 40x objective lens.

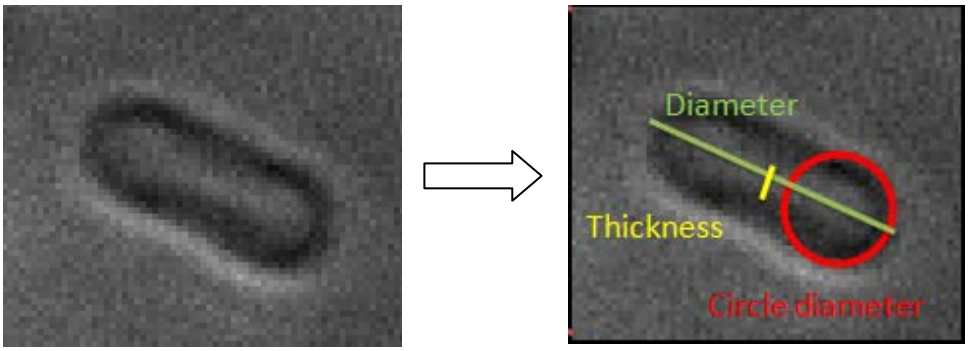

Fig 4. 300x zoomed stained erythrocyte under bright field microscope.

Each measurement is measured by hand with the line and circle tool. Diameter is from the outer dark boundary to the other outer dark boundary along the length of the cell. Thickness is measured from the inner dark boundary to the other inner dark boundary in the middle of the cell. The inner diameter is approximated with a circle tool tracing the outer dark edges of the toroid; where $d = D - 2*diameter_{circle}$.

3.1 Complementary Study Method

After blood extraction and washing the cells 4 times, unstained cells were placed in albumin (TRA: 0.5% per 500mL) buffer and albumin free buffer (TR). The stained cells were also placed in albumin buffer (TRA), and because of the protocol, TR blood sample was not prepared for analysis.

4.0 RESULTS

Average Measurements of Geometric Parameters for Rat Red Blood Cells					
	Unstained	Standard Deviation	Stained	Standard Deviation	% Increase
Diameter, µm	7.62	± 0.47	7.44	± 0.37	-2.36
Surface Area, μm^2	130.89	± 15.71	125.49	± 12.74	-4.12
Volume, μm^3	48.54	± 10.02	46.16	± 4.61	-4.90
Sphericity Index	0.49	± 0.02	0.50	± 0.04	2.00

Table 1. Results for mean measurements of geometric parameters for unstained and stained rat red blood cells ± standard deviation. Percentage increase is also presented as an increase (or decrease) from unstained to stained measurements. Diameter in stained was less than stained by 2.36%, surface area for stained cells decreased from unstained by 4.12%, volume in stained decreased from unstained by 4.9%, and sphericity index in stained increased from unstained by 2%.

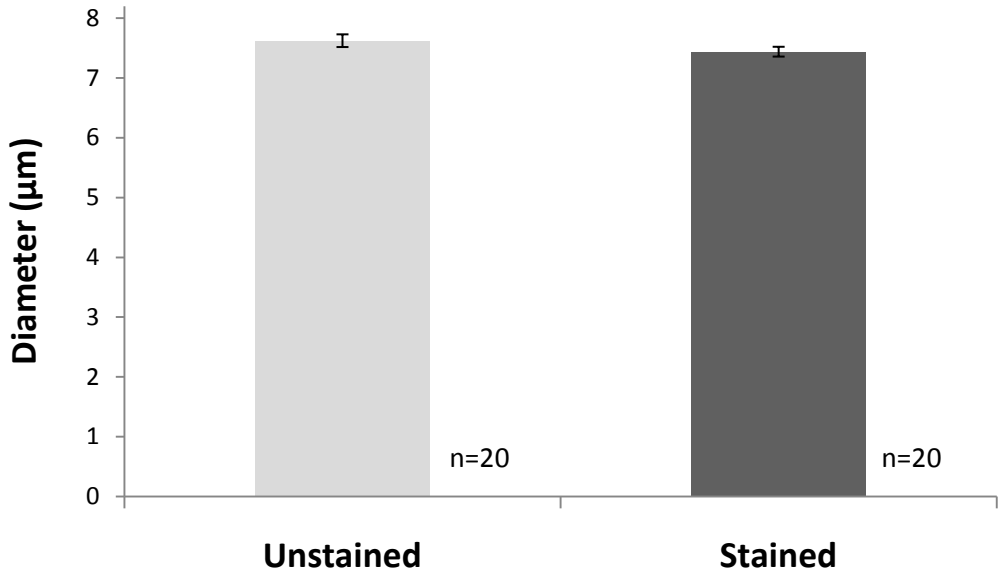

Fig 5. Diameter of unstained and stained rat red blood cells. T-test was performed; p-value > 0.05. Data presented as mean ± standard error. There was no significant difference between average unstained and stained values with a sample of 20 each. The measured stained average (7.44 µm) was smaller than the unstained (7.62 µm) by 2.36%

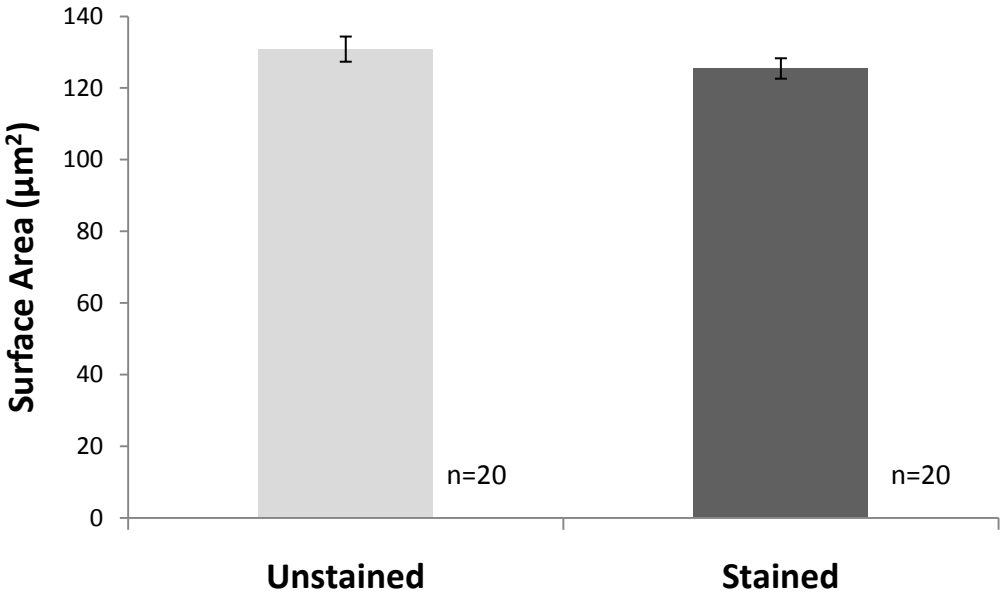

Fig 6. Surface area of unstained and stained rat red blood cells. T-test was performed; p-value > 0.05. Data presented as mean ± standard error. There was no significant difference between average unstained and stained values with a sample of 20 each. The measured stained average surface area (125.49 μm^2) was smaller than the unstained (130.89 μm^2) by 4.12%.

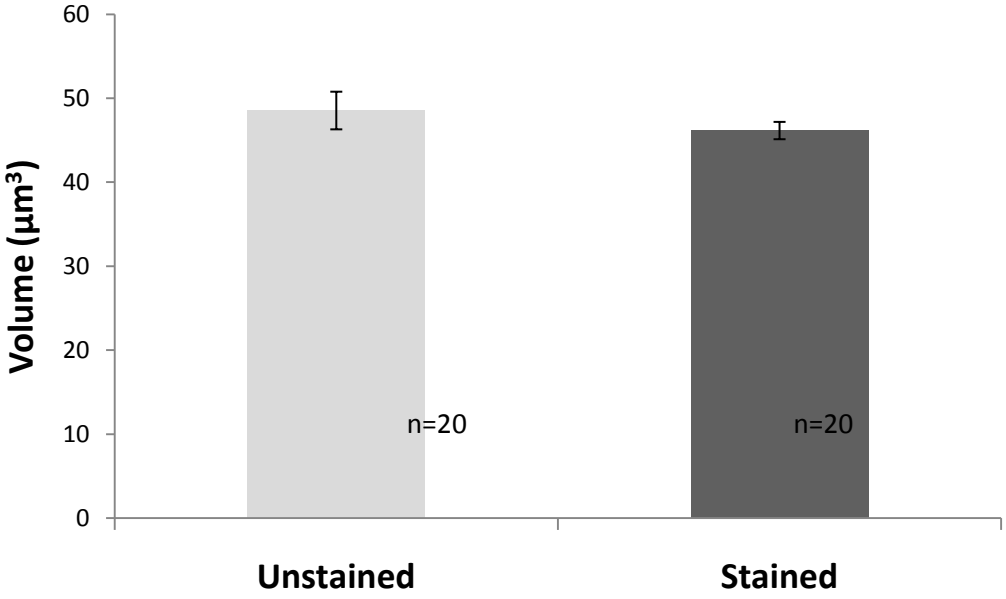

Fig 7. Volume of unstained and stained rat red blood cells. T-test was performed; p-value > 0.05. Data presented as mean ± standard error. There was no significant difference between average unstained and stained values with a sample of 20 each. The measured stained average volume (46.16μm^3) was smaller than the unstained (48.54μm^3) by 4.90%.

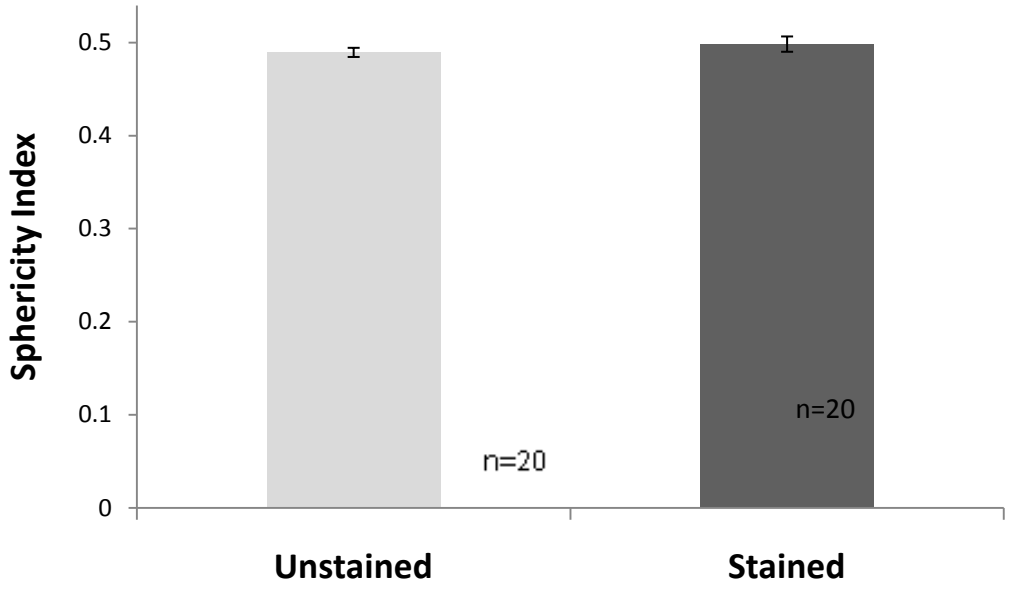

Fig 8. Sphericity index value for unstained and stained rat red blood cells. T-test was performed; p-value > 0.05. Data presented as mean ± standard error. There was no significant difference between average unstained and stained values with a sample of 20 each. The measured stained average sphericity index (0.50) was larger than the unstained (0.49) by 2%.

Effects of Albumin on Red Blood Cell Shape		
	Unstained	Stained
No Albumin, abnormal shaped	~5%	-
Albumin (0.5% per 500mL), abnormal shaped	~95%	~30%

Table 2. Qualitative analysis on the effects of albumin on red blood cell shape. Abnormal shaped means the cells look like a C-shape or cup shaped. In the control (no albumin), around 5% were C-shaped. In unstained with albumin, around 95% were C-shaped, and stained with albumin, around 30% were C-shaped.

5.0 DISCUSSION

This experiment chose to use the simple geometric math model over the initial method of hand tracing boundaries of a red cell. Having technology and computer imaging programs allowed computation of more cells and accurately than the hand tracing method. Although, the geometry model does not finely account for all portions of an erythrocyte, it can be accounted for by the large number of sample taken and averaged out.

The calculated values for unstained rat blood cells were much larger than Canham results[4]. Diameter (by 9.45%), surface area (by 27.08%), and sphericity index (by 13.95%) were all larger than literature measurements, however volume was smaller by 25.21%[4]. The accounted difference may be because of the difference in measurement technique and algorithm used to calculate these parameters. The simple geometry does not take into account the smooth transition from the edge thickness to the middle thickness, thus underestimating the volume and overestimating the surface area of the cell. Consequently, this overestimates the sphericity index of the cell. Although the results are different from published values, it is still possible to compare unstained cells and stained cells as long as the analysis technique is consistent.

The measurements taken from the samples were between unstained in TR and stained in TRA buffer. This was necessary because the sample in TRA unstained were mostly abnormally shaped (cup shaped). The sample in TR unstained had less abnormally shaped cells, and thus were used as the control as they represent live blood cells. Although the stained cells were in TRA buffer, there were enough cells samples that were normal shaped and could be used for analysis.

There were no significant differences between unstained and stained cells for the four parameters measured, diameter, surface area, volume, and sphericity index. All parameters had a decrease in value from unstained cells (Table 1). Volume decreased more than surface area by 0.78 % suggesting more fluid escaped from the blood cell causing there to be more surface area than usual to enclose the volume of fluid. Osmotic imbalance between inside of the cell and the buffer solution causes fluid from the cell to leak out, and this is facilitated by the use of DMSO from the staining protocol. DMSO dissolves cell membranes to make blood cells more permeable to allow FITC dye to pass through and bind to membrane bound proteins. The loss of volume content in blood cells was not great enough to change the properties of the cell. There was a sphericity index increase from unstained to stain of approximately 2% (Table 1). The upper limits of a red cell can increase the sphericity index by 8-12.5% according to the fact that red cells can undergo 5-10% increase of surface area and 30% increase of volume before lysing.

Unstained Cells upper limit sphericity index:

Surface area - $130.89\mu m^2 \times 110\% = 137.43\mu m^2$

Volume - $48.54\mu m^3 \times 130\% = 63.10\mu m^3$

Sphericity Index = $(4.84 \times (63.10\mu m^3)^{(2/3)})/ 137.43\mu m^2 = 0.56$

According to the results, the stained cell parameters did not change enough to reach the upper limits of geometry change. Also, from qualitative in-vivo studies, fluorescently labelled cells do not plug capillaries or reduce blood flow velocity. They are still capable of deforming their shape to compress through smaller capillaries. Also, during the hang test, both unstained and stained samples would react the same, i.e. cells were still hung on their edge, and profiles were caught on view. If the stained cells had different properties (more rigid), then their shape would change when viewed. If it happened that the stained cells become rigid and spherical, then during the hang test, there should have been no profiles taken and in-vivo measurement, they would plug capillaries and change blood flow.

However, these results were purely based on cells that looked normal (straight). Some cells had a different shape conformation, and looked cup shaped. Alfred Jay showed the effects of albumin in isotonic solution on red blood cell. Albumin binds to cell membrane and changes the shape of the cell by decreasing diameter, inner thickness, and increasing maximum thickness at the rim [2]. A complementary study was done to qualitative effects of albumin on our cell samples. The unstained sample with albumin present compared to the sample in albumin free buffer was 19 times more cup shaped. This implies that albumin is the culprit and is responsible for causing these abnormally shaped cells. In the stained sample, because of the staining protocol, an albumin free sample was not prepared. The stained sample had approximately 3 times as less cup shaped than the unstained sample. This difference could be accounted for the repeated washes the stained cells underwent. While the unstained TRA blood sample was washed only 4 times, the stained cells were washed a total of 14 times. From this observation, the effects of albumin on the cell shape could be washed away, but further testing needs to be conducted before this is supported.

Although the results showed no significant differences in parameters between unstained and stained cells, there are many errors that should be taken into consideration. The hang test was performed on freshly taken and stained cells; usually they are left over night for the in-vivo experiments. The difference in time was a limitation on this experiment; it was also a limitation in the complementary experiment. Also, while analyzing and taking measurements of each selected cell profile, a zoomed picture of the cell had to be used. Pixelation of the cell could have caused an inaccurate estimation of initial measurements of diameter, thickness and inner diameter.

For future studies, the staining protocol may be changed to eliminate the effects of albumin on the shape conformation of the cells. The amount of albumin could be changed, or washing the stained cells more times could wash away the effects of albumin. Another albumin source could be used, because the staining protocol uses bovine albumin, but there could be a biological incompatibility between the two species; possibly rat albumin may be used.

6.0 CONCLUSIONS

The initial purpose of this paper was to determine if there was a difference in deformability and geometry from staining and albumin between unstained and stained erythrocytes. It was hypothesized there would be no difference in deformability and geometry given by the parameters of diameter, surface area, volume and sphericity index, in order for this tracing technique to be used in-vivo studies. The findings of this study showed there to be no significant differences between the four geometric parameters of a blood cell, and the sphericity index stayed well below the upper limit of a cell lysing. The results were also supported by in-vivo studies, as stained erythrocytes did not slow down blood flow or plug capillaries. However, albumin was found to be a major abnormal shape conformation factor. Overall, staining the cell with FIT-C dye does not change deformability, but albumin was found to be responsible for most of the abnormal shape configurations. Further studies will show effects of albumin and how the staining protocol can be changed to eliminate the effects of albumin on the blood cells.

7.0 Appendix

7.1 RBC FITC Labeling

1. Draw 1ml of blood via cardiac puncture in 0.17-ml heparin
2. Bring buffer solutions to room temperature and adjust pH to 7.4
3. Centrifuge 1 ml of donor blood for 2.5min and discard plasma layer
4. Wash packed cells in TR 4X
5. Prepare a fresh solution of dye: 4-mg FITC in 0.1-ml DMSO, add 10-ml of TRA
6. Adjust pH to 7.4 (0.1M HCL or 0.1M NaOH)
7. Add washed RBCs (1ml) and incubate at room temperature for 2 hrs, stir and maintain pH
8. Wash ~10X in TRA to remove excess dye. Adjust HCT to ~30% with TRA
9. Incubate overnight at $4^{o}C$
10. Bring TRA buffer to room temperature and wash cells until supernatant appears clear
11. View cells on the fluorescence microcope to ensure adequate intensity
12. Suspend in TRA, adjust HCT to ~30%
13. Inject cells via the jugular vein

7.2 Tris-Buffered Ringer's Solution (TR)

For 1L:

Sodium Chloride (NaCl)	7.708g
Potassium Chloride (KCL)	0.350g
Calcium Chloride ($CaCl_2*2H_2O$)	0.294g
Magnesium Sulfate ($MgSO_4*7H_2O$)	0.288g
Tris	2.544g

The pH is about 10.25. Titrate with 1 M HCL to pH 7.4.

7.3 Tris-Buffered Ringer's Albumin Solution (TRA)

1. Heat 500-ml of TR to $37^{o}C$
2. Add 2.5-g of Bovine Serum Albumin (BSA, Sigma) and dissolve by constant stirring
3. Adjust pH to 7.4 with 1 M NaOH

8.0 References

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