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Corresponding Author:	Amir Farnoud UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	farnoud@ohio.edu
Order of Authors:	Parnian Bigdelou
	Amir Farnoud
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DEPARTMENT OF CHEMICAL AND BIOMOLECULAR ENGINEERING
Stocker Center 161
Athens, OH 45701
T: (740) 593-1426
F: (740) 593-0873
farnoud@ohio.edu

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Dear Dr. Cao,

Enclosed, please find a copy of our revised manuscript "*JoVE60659: Induction of eryptosis using a calcium ionophore"* for consideration for publication in the *Journal of Visualized Experiments* (JoVE) in the Biochemistry section.

We have addressed the editorial and reviewers' comments. The revised manuscript is uploaded with Track Changes to facilitate the review. A point by point response to all the comments is also uploaded.

This manuscript has not been submitted to *JoVE* previously, has not been published in any language, is not currently under consideration by another journal, and will not be submitted elsewhere while under review by *JoVE*.

Sincerely,

Amir M. Farnoud, Ph.D.
Assistant Professor
Department of Chemical and Biomolecular Engineering
Ohio University
farnoud@ohio.edu

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TITLE:
Induction of Eryptosis Using a Calcium Ionophore

AUTHORS AND AFFILIATIONS:
Parnian Bigdelou[1], Amir M. Farnoud[1]
[1]Department of Chemical and Biomolecular Engineering, Ohio University, Athens, OH, USA

Corresponding Author:
Amir M. Farnoud (Farnoud@ohio.edu)

Email Address of Co-author:
Parnian Bigdelou (pb551318@ohio.edu)

KEYWORDS:
erythrocyte, eryptosis, phosphatidylserine, annexin V, ionomycin, cell membrane

SUMMARY:
A protocol for the induction of eryptosis, programmed cell death in erythrocytes, using the calcium ionophore, ionomycin, is provided. Successful eryptosis is evaluated by monitoring the localization phosphatidylserine in the membrane outer leaflet. Factors affecting the success of the protocol have been examined and optimal conditions provided.

ABSTRACT:
Eryptosis, erythrocyte programmed cell death, occurs in a number of hematological diseases and during injury to erythrocytes. A hallmark of eryptotic cells is the loss of compositional asymmetry of the cell membrane, leading to the translocation of phosphatidylserine to the membrane outer leaflet. This process is triggered by increased intracellular concentration of Ca^{2+}, which activates scramblase, an enzyme that facilitates bidirectional movement of phospholipids between membrane leaflets. Given the importance of eryptosis in various diseased conditions, there have been efforts to induce eryptosis *in vitro*. Such efforts have generally relied on the calcium ionophore, ionomycin, to enhance intracellular Ca^{2+} concentration and induce eryptosis. However, many discrepancies have been reported in the literature regarding the procedure for inducing eryptosis using ionomycin. Herein, we report a step-by-step protocol for ionomycin-induced eryptosis in human erythrocytes. We focus on important steps in the procedure including the ionophore concentration, incubation time, and glucose depletion, and provide representative result. This protocol can be used to reproducibly induce eryptosis in the laboratory.

INTRODUCTION:
Programmed cell death in erythrocytes, also known as eryptosis, is common in many clinical conditions and hematological disorders. Eryptosis is associated with cell shrinkage and the loss of phospholipid asymmetry in the cell plasma membrane[1,2]. Loss of asymmetry results in the translocation of phosphatidylserine (PS), a lipid normally localized in the inner leaflet[3,4], to the cell outer leaflet, which signals to macrophages to phagocytose and remove defective

erythrocytes[5–8]. At the end of the normal life span of erythrocytes, removal of eryptotic cells by macrophages ensures the balance of erythrocytes in circulation. However, in diseased conditions, such as sickle cell disease and thalassemia[9–11], enhanced eryptosis may result in severe anemia[2]. Due to its importance in hematological diseases, there is significant interest in examining the factors inducing or inhibiting eryptosis and the molecular mechanisms underlying this process.

The plasma membrane of healthy erythrocytes is asymmetric, with different phospholipids localizing at the outer and inner leaflets. Membrane asymmetry is primarily regulated by the action of membrane enzymes. Aminophospholipid translocase facilitates the transport of aminophospholipids, PS and phosphatidylethanolamine (PE), by directing these lipids to the cell inner leaflet. On the other hand, floppase transports the choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), from the inner to the outer leaflet of the cell membrane[12]. However, unlike healthy cells, the membrane of eryptotic erythrocytes is scrambled. This is due to the action of a third enzyme, scramblase, which disrupts phospholipid asymmetry by facilitating the bidirectional transport of aminophospholipids[13–16]. Scramblase is activated by elevated intracellular levels of Ca^{2+}. Therefore, calcium ionophores, which facilitate the transport of Ca^{2+} across the cell membrane[12], are efficient inducers of eryptosis.

Ionomycin, a calcium ionophore, has been widely used to induce eryptosis in erythrocytes[12,17–26]. Ionomycin has both hydrophilic and hydrophobic groups, which are necessary to bind and capture Ca^{2+} ion, and transport it to the cytosolic space[27–29]. This leads to the activation of scramblase and translocation of PS to the outer leaflet, which can be easily detected using annexin-V, a cellular protein with a high affinity to PS[12]. Although triggering eryptosis by ionomycin is commonly reported, there is considerable method discrepancy in the literature (**Table 1**). The population of erythrocytes undergoing eryptosis depends on different factors such as ionophore concentration, treatment time with ionophore, and the sugar content of extracellular environment (glucose depletion activates cation channels and facilitates the entry of Ca^{2+} into the cytosolic space)[30,31]. However, there is little consistency in these factors in the literature, making it difficult to perform eryptosis reproducibly *in vitro*.

In this protocol, we present a step-by-step procedure to induce eryptosis in human erythrocytes. Factors affecting successful eryptosis including Ca^{2+} concentration, ionophore concentration, treatment time, and pre-incubation in glucose-depleted buffer are examined and optimal values are reported. This procedure demonstrates that pre-incubation of erythrocytes in a glucose-free buffer significantly increases the percentage of eryptosis compared to glucose-containing buffer. This protocol can be used in the laboratory to produce eryptotic erythrocytes for various applications.

PROTOCOL:

All human blood samples used in the protocol described below were purchased as de-identified samples. No human subjects were directly involved or recruited for this study. The guidelines of the Declaration of Helsinki should be used when research involves human subjects.

1. Erythrocyte isolation from whole blood

1.1. Add 500 µL of whole blood in acid citrate dextrose (ACD) (stored at 4 °C) to a microcentrifuge tube.

NOTE: Whole blood was purchased in ACD. According to the company, 1.5 mL of ACD is added to 7 mL of whole blood (8.5 mL total volume).

1.2. Centrifuge the whole blood at 700 x *g* for 5 min at room temperature (RT) and remove the clear plasma and the thin buffy coat using a pipette to leave the red erythrocyte layer.

1.3. Prepare 1 L of Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, and 1 mM $CaCl_2$. Adjust the pH to 7.4 by adding 2 µL drops of 1.0 M NaOH. To prepare glucose-free Ringer solution, follow the same protocol, but do not include glucose in the solution.

1.4. Wash the erythrocytes 2x in Ringer solution by suspending the cell pellet in 1.5 mL of Ringer solution, centrifuging at 700 x *g* for 5 min at RT, and removing the supernatant.

1.5. Make a 0.4% hematocrit by resuspending 40 µL of the erythrocyte pellet in 9,960 µL of glucose-free Ringer solution to reach a final volume of 10 mL.

NOTE: Hematocrit is a term used to refer to the volume fraction of erythrocytes in suspension. A 0.4% hematocrit is a suspension containing 0.4% erythrocytes.

1.6. Incubate the cell suspension at 37 °C for 7 days.

2. Treatment of erythrocytes with ionomycin and measurement of hemolysis

2.1. Dissolve 1 mg of ionomycin calcium salt in 630 µL of dimethyl sulfoxide (DMSO) to reach a final concentration of 2 mM. Aliquot and store at -20 °C.

2.2. Take 1 mL of the 0.4% hematocrit from step 1.5 and add 0.5 µL of 2 mM ionomycin to reach a final concentration of 1 µM. Incubate for 2 h at 37 °C.

2.2.1. Use 1 mL of the hematocrit with no ionomycin treatment as a negative control.

2.3. Centrifuge the ionomycin-treated and untreated hematocrits at 700 x *g* for 5 min at RT, and remove their supernatants to leave the cell pellets at the bottom of the tubes.

2.4. Wash the cells 3x with Ringer solution by suspending the cell pellets in 1.5 mL of Ringer solution, centrifuging at 700 x *g* for 5 min at RT and discarding the supernatants.

2.5. To measure hemolysis, add 1 mL of the untreated 0.4% hematocrit from step 1.5 to a microcentrifuge tube and incubate for 2 h at 37 °C as the negative control for hemolysis (0%).

2.6. Add 1 mL of the untreated 0.4% hematocrit from step 1.5 to a microcentrifuge tube and centrifuge at 700 x *g* for 5 min at RT. Remove the supernatant and add 1 mL of distilled water to the cell pellet and incubate for 2 h at 37 °C as the positive control for hemolysis (100%).

2.7. Add 1 mL of the ionomycin-treated 0.4% hematocrit from step 2.2 to a microcentrifuge tube.

2.8. Centrifuge the untreated cells, treated cells, and the cells in distilled water at 700 x *g* for 5 min at RT.

2.9. Take 200 µL of the supernatants and add to a 96-well plate.

2.10. Measure the absorbance at 541 nm using a microplate reader.

2.11. Calculate the hemolysis using Equation 1[32]:

$$\%\text{Hemolysis} = (A_T - A_0)/(A_{100} - A_0)*100 \qquad \text{Equation 1}$$

where A_0 is the absorbance of erythrocytes in Ringer solution, A_{100} is the absorbance of erythrocytes in water, and A_T is the absorbance of treated erythrocytes by ionomycin.

3. Annexin-V binding assay

3.1. Dilute 2 mL of the 5x annexin V binding buffer in 8 mL of phosphate-buffered saline (PBS) to obtain 1x binding buffer.

3.2. Resuspend the ionomycin-treated and untreated cell pellets from step 2.4 in 1 mL of 1x binding buffer.

3.3. Take 235 µL of the cell suspensions in the binding buffer and add 15 µL of Annexin V-Alexa Flour 488 conjugate.

3.4. Incubate the cells at RT for 20 min in a dark place. Centrifuge at 700 x *g* for 5 min at RT and remove the supernatant.

3.5. Wash the cells 2x with 1x binding buffer, by suspending the cell pellet in 1.5 mL of the binding buffer, centrifuging at 700 x *g* for 5 min at RT and removing the supernatant.

3.6. Resuspend the cell pellets in 250 µL of 1x binding buffer for flow cytometry measurements.

4. Flow cytometry

4.1. Transfer 200 µL of the annexin-V stained erythrocytes to 1 mL round bottom polystyrene tubes compatible with flow cytometry.

4.2. Login to the flow cytometry software and click on the **new experiment** button. Click on the **new tube** button. Select the **global sheet** and choose the **apply analysis** to measure the fluorescence intensity with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

4.3. Set number of cells to 20,000 to be collected for fluorescence-activated cell sorting (FACS) analysis.

4.4. Select the desired tube and click on **load** button. Click on **record** button for forward scatter and side scatter measurements. Repeat for all samples.

4.5. Right click on **specimen** button and click on **apply batch analysis** to generate the result file.

4.6. Right click on **specimen** button and click on **generate FSC files**.

4.7. Add the flow cytometry data (FSC files) into the workplace of flow cytometry software.

4.8. Analyze the control data by selecting the cell population of interest and adding statistics for eryptosis value.

4.8.1. Double click on control and select histogram versus fluorescence intensity.

4.8.2. Click on gate button to draw a gate on the histogram which indicates the percentage of eryptosis.

4.9. Apply the same statistics for all other experimental tubes to obtain the eryptosis values. Right click on **control** and select **copy analysis** *to group*.

4.10. After properly gating all samples, transfer the analyzed data by dragging and dropping them into the layout editor.

4.10.1. Overlay the analyzed data with control in layout editor.

4.10.2. Set the desired histograms and intensities by changing the x and y axis of the overlaid graphs.

4.10.3. Export image files by clicking on **export** button and save the graphs in desired location.

5. Confocal microscopy

5.1. Transfer 5 µL of annexin-V-stained cells on a microscope slide and cover it with a cover slip. Keep in a dark place to prevent photobleaching.

5.2. Use Argon laser of the confocal fluorescence microscope to observe the cells excited at 488 nm with desired magnifications.

NOTE: A confocal microscope is not necessarily needed and any microscope with fluorescence capabilities can be used to obtain fluorescence images that demonstrate annexin-V binding.

5.3. Obtain fluorescence images of the control (non-treated cells) and treated cells.

NOTE: Non-treated cells are expected to show very weak fluorescence signals, whereas treated cells are expected to show bright green fluorescence on their membranes.

REPRESENTATIVE RESULTS:

Optimization of ionomycin concentration

While ionomycin is required to induce eryptosis, increased ionomycin concentrations can lead to hemolysis (i.e. lysis of erythrocytes and release of hemoglobin), which needs to be avoided. Treatment of erythrocytes with 1 µM ionomycin in Ringer solution for 2 h is enough to induce eryptosis, as evidenced by successful labeling with annexin-V Alexa Flour 488 conjugate and quantification by FACS analysis (**Figure 1A**). Higher concentrations of ionomycin (5 and 10 µM) result in a slight increase in eryptosis (**Figure 1A–D**). However, such concentrations also enhance hemolysis (**Figure 1E**), which is not desired. In order to stay below 5% hemolysis, 1 µM ionomycin should be used.

Treatment time with ionomycin

Incubation of erythrocytes with ionomycin in Ringer solution for as little as 30 min is enough to induce eryptosis (**Figure 2A**). Increased incubation time increases the level of eryptosis, as measured by the annexin V-binding assay, for up to 2 h (**Figure 2B,C**). However, further incubation time results in a slight decrease in the level of eryptosis (**Figure 2D**). Maximum eryptosis was obtained after 2 h of treatment with 1 µM ionomycin, and for all other treatment times, lower eryptosis was obtained (**Figure 2E**). Representative flow cytometry histograms are presented in **Figure 2A–D**. In addition, average percentage eryptosis and hemolysis, for various treatment times with 1 µM ionomycin, are presented in **Figure 2E** and **Figure 2F**, respectively. The higher value of hemolysis after 180 min explains the reduction in eryptosis after the same amount of incubation (**Figure 2E**) as less viable cells exist upon 180 min of treatment with ionomycin.

Moreover, cells were treated with low concentrations of ionomycin including 0, 0.25, 0.5, and 1 µM for longer treatment times including 6 and 12 h, and eryptosis was measured (**Figure 3**). Cells treated with ionomycin concentrations of lower that 1 µM for 6 and 12 h show lower

eryptosis compared to the cells treated with 1 µM ionomycin (**Figure 3**). Since decreasing the concentration and increasing the exposure time did not enhance eryptosis, 1 µM was used to trigger eryptosis.

Eryptosis is dependent on incubation time and extracellular glucose concentration

Extracellular glucose concentration affects the outcome of the process. Higher eryptosis values are observed when erythrocytes are pre-incubated in glucose-free Ringer solution compared to glucose-containing Ringer solution prior to incubation with 1 µM ionomycin for 2 h. The highest eryptosis values are obtained after 7 days of pre-incubation in both solutions. However, eryptosis is higher after pre-incubation in glucose-free Ringer solution compared to normal Ringer solution, which contains 5 mM glucose (see **Figure 4A** for representative plots and **Figure 4B** for comparison of global means). In addition, forward scatter histograms indicate the effect of glucose depletion on erythrocyte shrinkage (**Figure 5A–D**). Forward scatter is a measure for cell size based on the light refraction, and the level of light scattered is directly proportional to the size of cells[33]. The cells incubated in glucose-free Ringer solution show less forward scatter compared to the cells incubated in glucose-containing buffer (**Figure 5E**), indicating cell shrinkage in the glucose-free environment.

In addition to flow cytometry measurements, cells were observed under a confocal fluorescence microscope to confirm eryptosis. Erythrocytes with no treatment (**Figure 6A**) and with ionomycin treatment (**Figure 6B**) were labeled with annexin-V Alexa Flour 488 conjugate and observed under microscope. Treated cells showed a bright fluorescence signal (**Figure 6B**) due to the binding of annexin-V to PS in the outer leaflet. In contrast, cells with no treatment showed a very weak fluorescence signal (**Figure 6A**) indicating very low eryptosis. Further example images of eryptotic erythrocytes labeled with annexin-V with high fluorescence signal are shown in **Figure 6C**.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative graphs of the effect of various ionomycin concentrations on eryptosis and hemolysis. Flow cytometry histograms of erythrocytes treated with (**A**) 1 µM, (**B**) 5 µM, and (**C**) 10 µM ionomycin (gray) at 37 °C at 0.4% hematocrit in Ringer solution for 2 h. Black line indicates non-treated cells. Percentage of eryptosis is indicated in each figure. Phosphatidylserine exposure was measured using annexin-V binding. (**D**) Arithmetic means ± SD (n = 3) of the percentage eryptosis of cells treated with different concentrations of ionomycin after 2 h treatment, and (**E**) arithmetic means ± SD (n = 3) of hemolysis of erythrocytes by different concentrations of ionomycin under same conditions.

Figure 2: Representative figures on the effect of various ionomycin treatment times on eryptosis. Flow cytometry histograms of erythrocytes treated with 1 µM ionomycin (gray) at 37 °C for (**A**) 30 min, (**B**) 60 min, (**C**) 120 min, and (**D**) 180 min at 0.4% hematocrit in Ringer solution. Black line indicates non-treated cells. Percentage of eryptosis is indicated in each figure. Phosphatidylserine exposure was measured through annexin-V binding. (**E**) Arithmetic

means ± SD (n = 3) of percentage eryptosis of cells treated with 1 µM ionomycin for different times. The highest eryptosis was obtained after 120 min treatment. (**F**) Arithmetic means ± SD (n = 3) of percentage hemolysis of cells treated with 1 µM ionomycin for different times. For statistical analysis, one-way non-parametric ANOVA with Kruskal-Wallis test was performed, and eryptosis after 120 min treatment was significantly higher than control as indicated in panel E. * is for $p < 0.05$.

Figure 3: Effect of various ionomycin concentrations and treatment times on eryptosis. Arithmetic means ± SD (n = 3) of the percentage eryptosis of cells treated with different concentrations of ionomycin is shown after various treatment times. The cells were treated with low concentrations of ionomycin including 0, 0.25, 0.5, and 1 µM for longer exposure (6 h and 12 h). Higher concentrations and longer treatments resulted in higher eryptosis values.

Figure 4: Effect of energy depletion on eryptosis. (**A**) Flow cytometry histogram for erythrocytes treated with 1 µM ionomycin (gray) at 37 °C for 2 h at 0.4% hematocrit, after pre-incubation in glucose-free Ringer solution (top figures) and Ringer solution (bottom figures) from 1 to 7 days, reveals that energy depletion facilitates eryptosis. Black line indicates non-treated cells. Percentages of eryptosis are indicated in the graphs for each day. (**B**) Arithmetic means ± SD (n = 3) of the percentage eryptosis of erythrocytes treated with 1 µM ionomycin at 37 °C for 2 h at 0.4% hematocrit, after pre-incubation in Ringer solution (black bars) and glucose-free Ringer solution (white bars) from 1 to 7 days.

Figure 5: Effect of energy depletion on cell size. Forward scatter histogram for erythrocytes treated with 1 µM ionomycin at 37 °C for 2 h at 0.4% hematocrit, after pre-incubation in glucose-free Ringer solution (gray) and Ringer solution (black line) for (A) 1 day, (B) 3 days, (C) 5 days, and (D) 7 days. The forward scatter histogram over time indicates erythrocyte shrinkage in glucose-free buffer. (**E**) Arithmetic means ± SD (n = 3) of forward scatter intensities of erythrocytes treated with 1 µM ionomycin at 37 °C for 2 h at 0.4% hematocrit, after pre-incubation in Ringer solution (black bars) and glucose-free Ringer solution (white bars) from 1 to 7 days.

Figure 6: Confocal fluorescence microscopy images of erythrocytes treated with (A) 0 µM, (B) and (C) 1 µM ionomycin at 37 °C for 2 h at 0.4% hematocrit. 40x objective magnification was used for images in panels A and B, and 100x objective magnification was used to take images for panel C. PS in healthy erythrocytes is located on the inner leaflet of the cell membrane, therefore there is no fluorescence signal in panel A. In panels B and C erythrocytes have been induced for eryptosis and there is a bright fluorescence signal resulting from the binding of annexin-V to PS translocated to the outer leaflet of the cell membrane.

Table 1: Various protocols used in the literature to induce eryptosis using ionomycin.

DISCUSSION:

The goal of this procedure is to provide optimal values for ionophore concentration, treatment time, and extracellular glucose concentration, which are important factors in ensuring

successful induction of eryptosis. A critical step in the protocol is the depletion of extracellular glucose, which, despite its importance, has not been sufficiently emphasized in the literature. The sugar content in normal Ringer solution (5 mM) has an inhibitory effect on eryptosis. Glucose depletion in the extracellular environment induces cellular stress and activates protein kinase C (PKC), resulting in the activation of calcium and potassium channels. This results in an increase in the entry of Ca^{2+} in the cytosolic space[30,31,34] and ultimately activates the scramblase[16], which increases eryptosis. Activation of potassium channel also results in potassium chloride leakage out of the cell, which leads to erythrocyte shrinkage[35].

The procedure outlined above needs to be performed with specific attention to hemolysis. It is important to use an optimized ionophore concentration, which is high enough to induce eryptosis, and low enough to prevent hemolysis. Similarly, incubating erythrocytes with ionomycin for a short period of time results in low eryptosis while very long incubation may lead to cell membrane disruption and hemolysis. It should also be noted that while the presented protocol is highly reliable when performed on the same erythrocyte sample, cells from different individuals respond differently to ionomycin and there might be inter-subject variability between different samples.

Particular attention should be paid to data analysis from flow cytometry. The percentage eryptosis obtained from the flow cytometer indicates the percentage of cell population with PS on their outer leaflet. However, cells with different intensities of annexin-V binding cannot be distinguished based on this number. Annexin-V binds to the PS exposed on cell surface, with a very high affinity and high specificity to PS[36-38]. However, as shown in the microscopy images in this report, different cells show differences in annexin-V binding intensity. The cells with low PS on their membranes have low fluorescence intensities, whereas higher PS occupancy on cell membrane results in higher fluorescence intensities.

The protocol presented in this paper can be modified by increasing the extracellular Ca^{2+} concentration. In this protocol, ionomycin was used to induce eryptosis in the presence of 1 mM $CaCl_2$; higher Ca^{2+} concentrations might lead to enhanced intracellular calcium levels and may induce more eryptosis. In addition, different calcium ionophores, such as selectophore and calcimycin, might have different ability to enhance the intracellular concentration of Ca^{2+}, compared to ionomycin, and could result in different eryptosis values. However, consistent eryptosis of erythrocytes can be achieved using ionomycin with the outlined protocol and can be used in the laboratory to examine the molecular mechanisms of eryptosis, mimic diseased conditions[39,40] in vitro, and screen potential therapeutics that inhibit eryptosis, among other applications.

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DISCLOSURES:

The authors have nothing to disclose.

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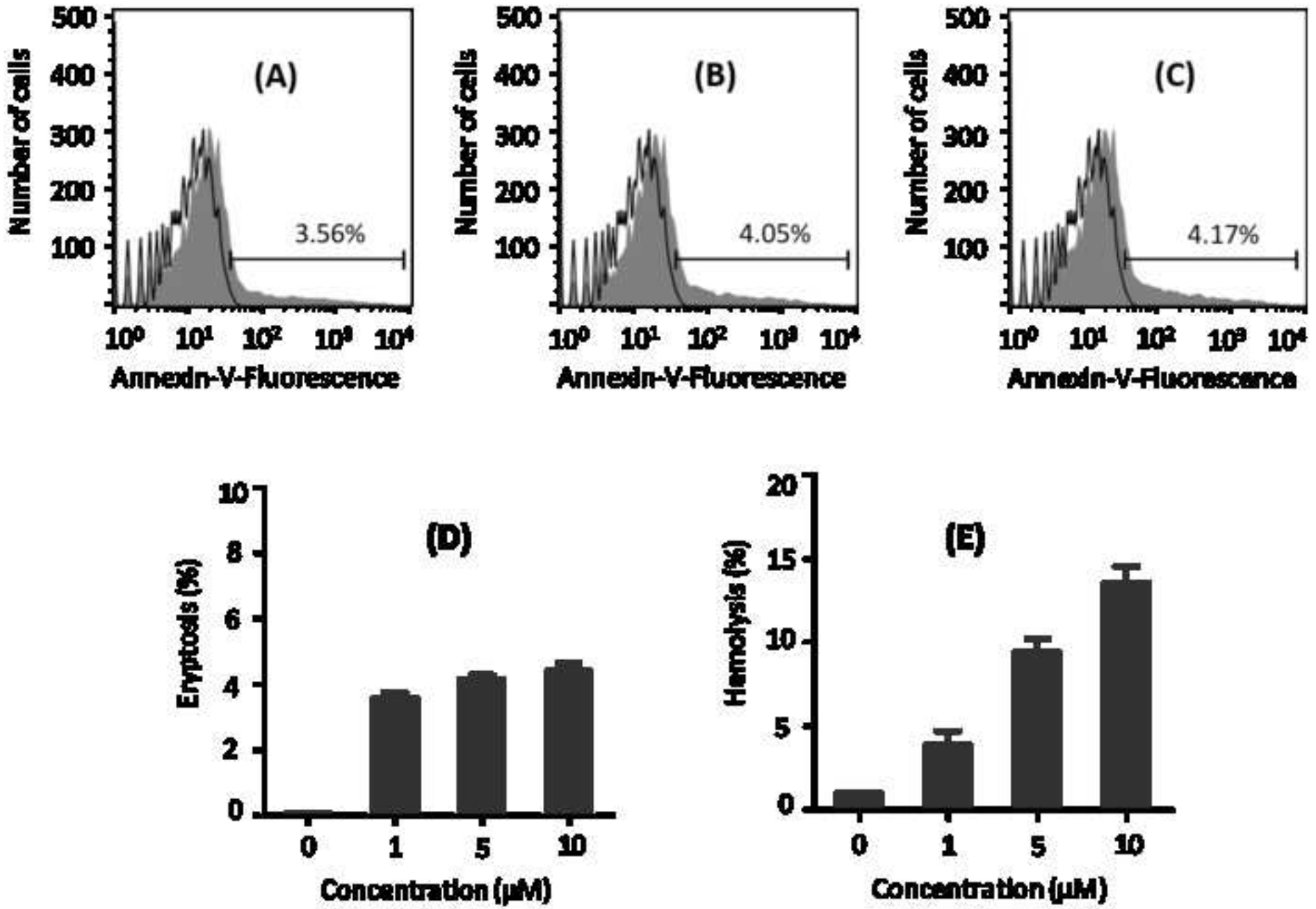

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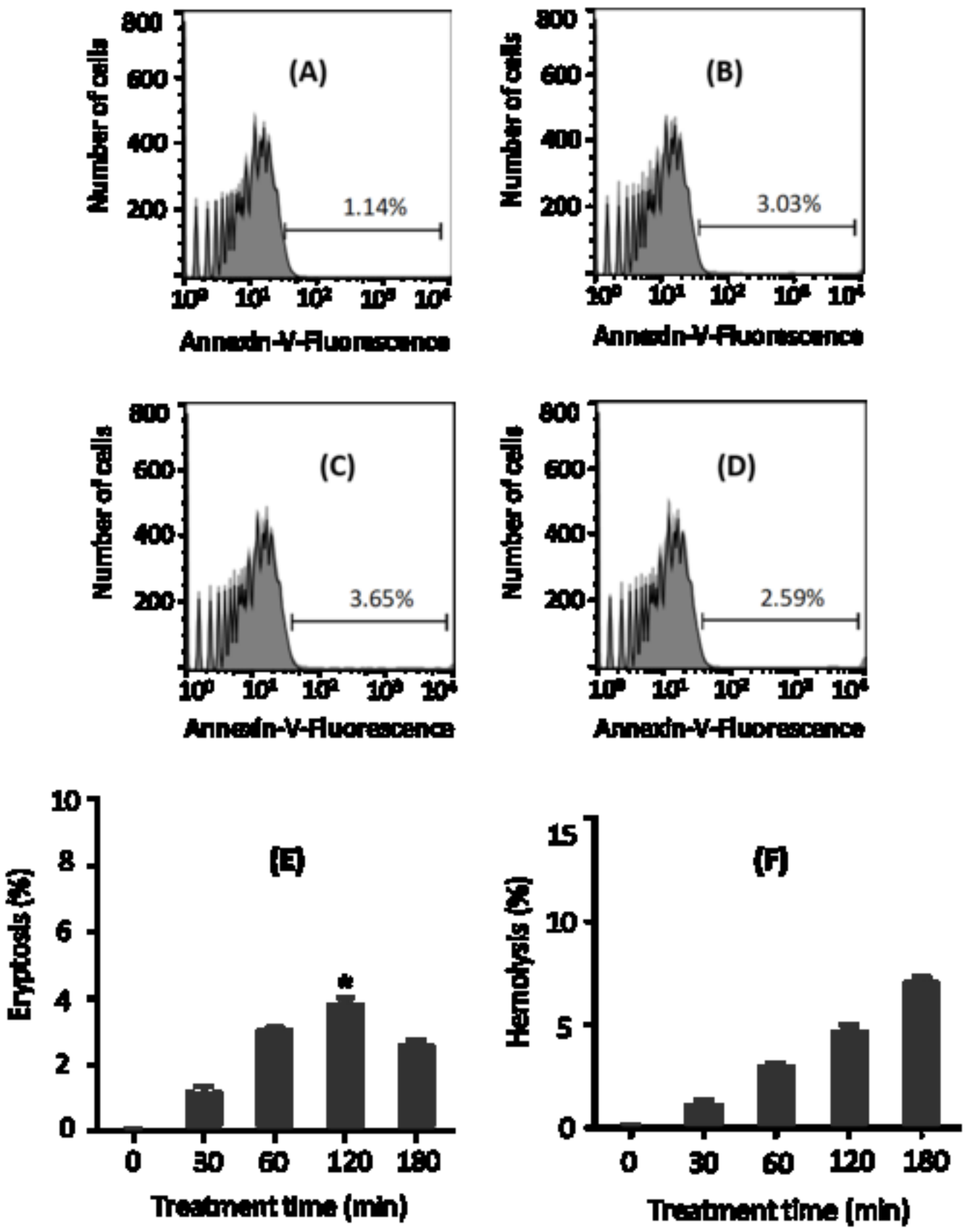

Figure 3-PNG File

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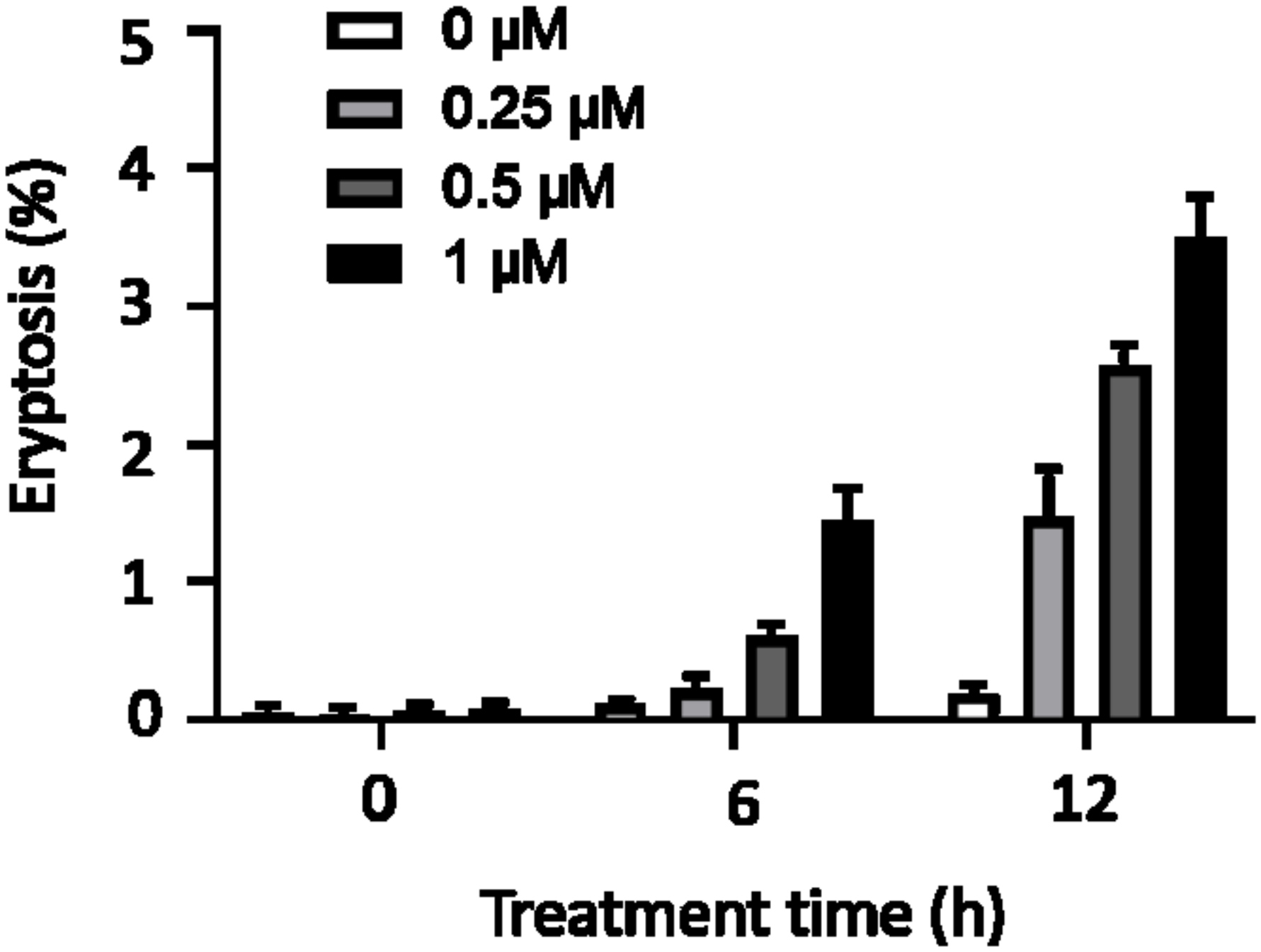

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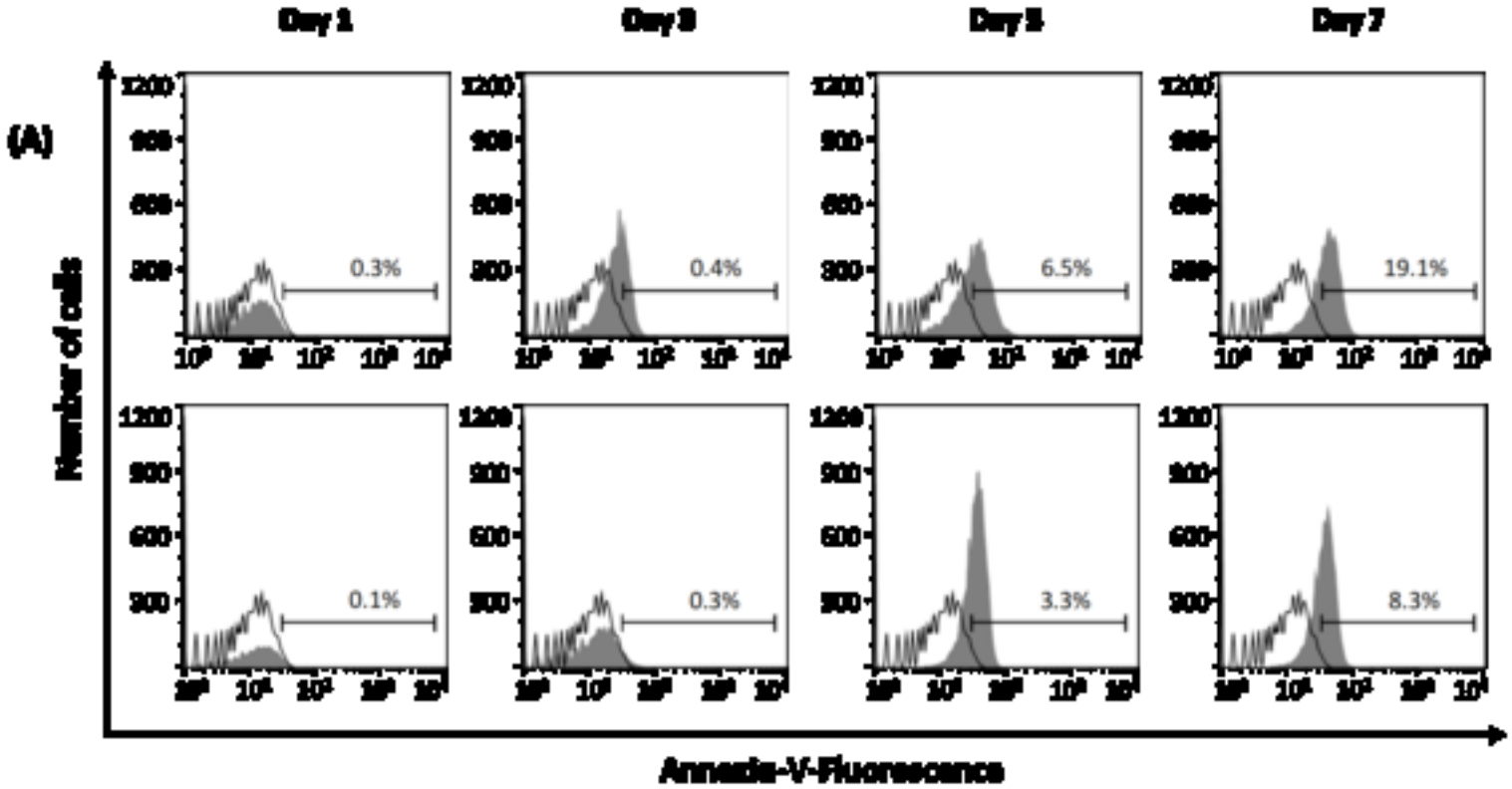

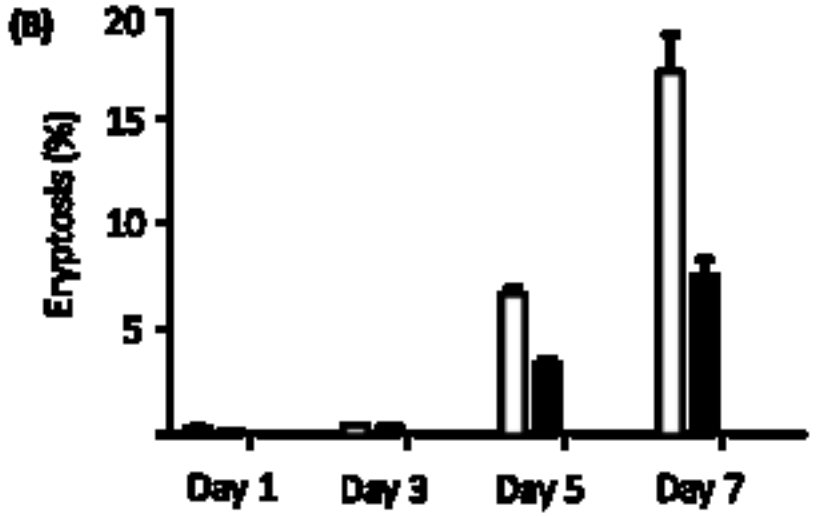

Figure 5-PNG

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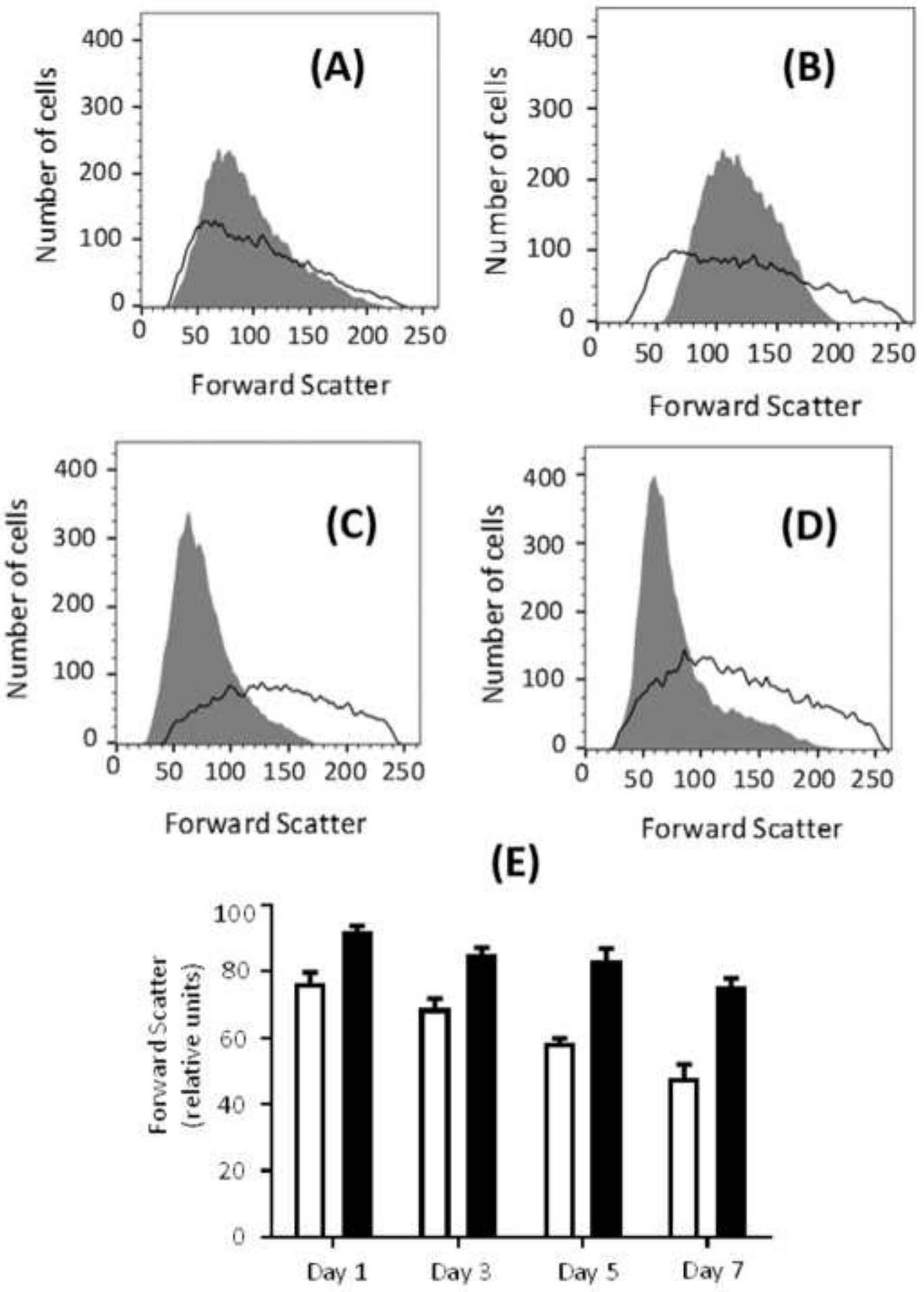

Figure 6-PNG File

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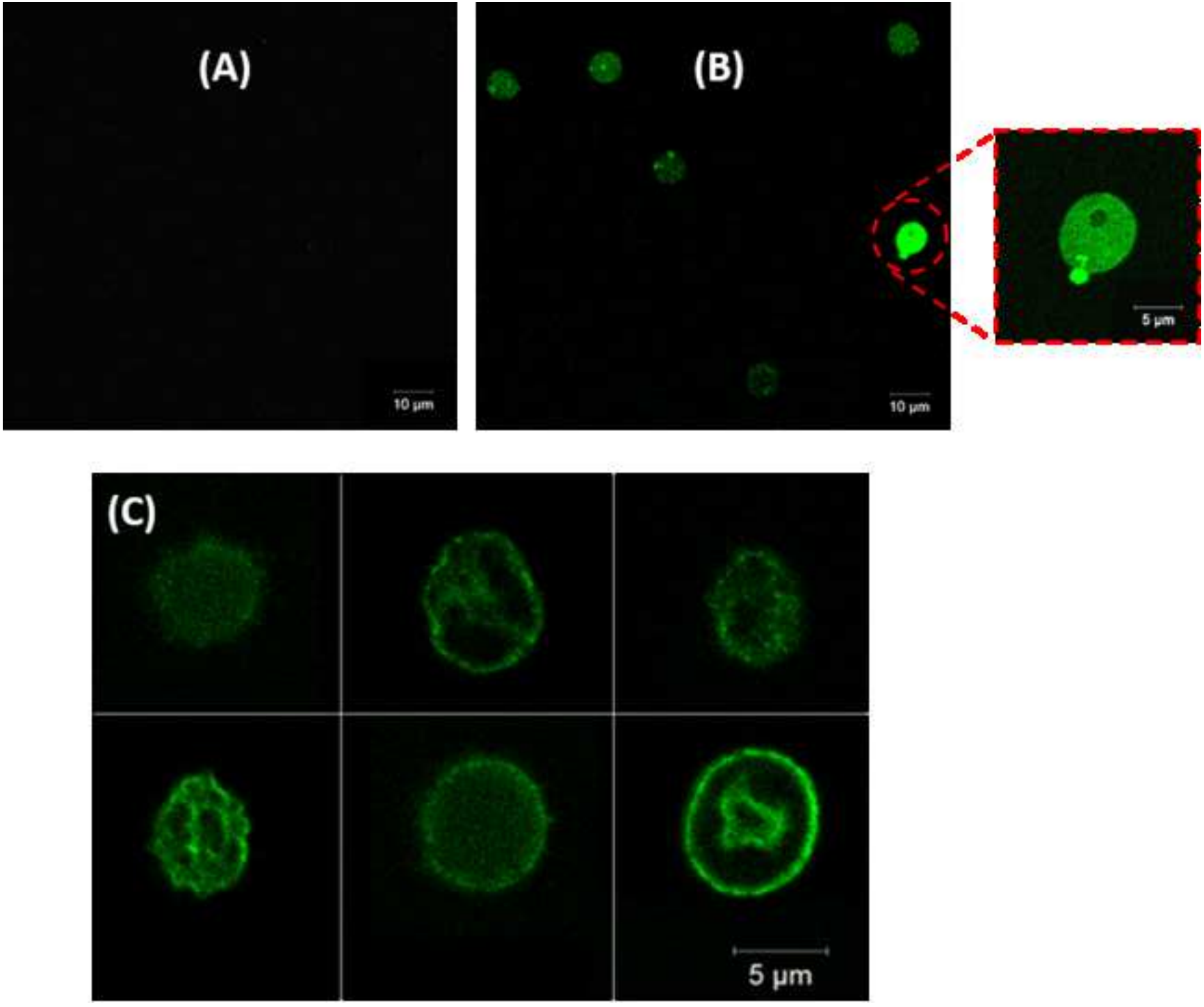

Click here to access/download;Table;60659_Table 1.xlsx

Cell density /hematocrit	Ionomycin concentration	Buffer	Pre-incubation	Treatment time with ionomycin	Detection method
1.65 x 10^8 cells/mL	0.3 mM	Buffer A*	36 h in buffer A	1 h	Annexin V
0.40%	1 mM	Ringer solution	48 h in Ringer	1 h	Annexin V
50%	10 mM	Buffer B**	-	3 h	Merocyanine 540
0.40%	1 mM	Ringer solution	48 h in Ringer	1 h	Annexin V
0.40%	1 mM	Ringer solution	48 h in Ringer	1 h	Annexin V
2%	1 mM	Ringer solution	-	4 h	Annexin V
0.40%	1 mM	Ringer solution	-	0.5 h	Annexin V
10%	1 mM	Ringer solution	-	3 h	Annexin V
0.40%	10 mM	Ringer solution	-	0.5 h	Annexin V
0.40%	1 mM	Ringer solution	48 h in Ringer	0.5 h	Annexin V
2 x 10^6 cells/mL	1 mM	HEPES-buffered saline (HBS)	-	0.5 h	Annexin V

*Buffer A: 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, and 1

**Buffer B: 5 mM Tris, 100 mM KC1, 60 mM NaCl, and 10 mM glucose

Reference
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1.8 mM $CaCl_2 \cdot 2H_2O$

Click here to access/download;Table of Materials;60659_Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
96-well plate	Fisher Scientific	12-565-331	
Annexin V Alexa Fluor 488 - apoptosis kit	Fisher Scientific	A10788	Store at 4 °C
BD FACSAria II flow cytometer	BD Biosciences	643177	
$CaCl_2$	Fisher Scientific	C79-500	
Centrifuge	Millipore Sigma	M7157	Model Eppendorf 5415C
Confocal fluorescence microscopy	Zeiss, LSM Tek Thornwood		Model LSM 510, Argon laser excited at 488 nm for taking images
Cover glasses circles	Fisher Scientific	12-545-100	
Disposable round bottom flow cytometry tube	VWR	VWRU47729-566	
DMSO	Sigma-Aldrich	472301-100ML	
DPBS	VWR Life Science	SH30028.02	
Glucose monohydrate	Sigma-Aldrich	Y0001745	
HEPES Buffer (1 M)	Fisher Scientific	50-751-7290	Store at 4 °C
Ionomycin calcium salt	EMD Milipore Corp.	407952-1MG	Dissolve in DMSO to reach 2 mM. Store at -20 °C
KCl	Fisher Scientific	P330-500	
$MgSO_4$	Fisher Scientific	M65-500	
Microcentrifuge tube	Fisher Scientific	02-681-5	
NaCl	Fisher Scientific	S271-500	
Plain glass microscope slides	Fisher Scientific	12-544-4	
Synergy HFM microplate reader	BioTek		
Whole blood in ACD	Zen-Bio		Store at 4 °C and warm to 37 °C prior to use

Click here to access/download;Rebuttal Letter;Response to reviewer comments.docx

We would like to thank the editor and the reviewers for their thorough review of the manuscript. Below, please find each comment followed by our response.

Editorial comments:

1. 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.

We have performed a thorough proofreading of the manuscript.

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

We follow the guidelines of the Declaration of Helsinki for experiments dealing with human subjects. However, all human blood used in the experiments were purchased as de-identified samples and no human subjects were directly involved or recruited for this study. We have noted this point at the start of the protocol.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

The numbering has been revised.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Eppendorf, FlowJo software, Tree Star, Inc., etc.

All commercial names are removed and general terms are used.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please

ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

More details are added in various steps of the protocol.

6. Lines 93-94: Please specify the source of whole blood. What volume of ACD is used here?

Both points have been addressed in the revised manuscript.

7. Line 97, 107: Please list an approximate volume to prepare.

Volumes are included in these sections.

8. Line 100: What volume is used to wash? What are the centrifugation parameters (force in x g, time and temperature)?

Volume, force, time, and temperature are included.

9. Lines 112, 141: What volume is used to wash?

Volumes are included.

10. Line 122: Please describe how the treatment is done and specify the temperature.

More details are added to explain how the treatment was done and temperature is included.

11. Lines 154-160: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks or menu selections for software actions, numerical values for settings, etc.).

More details are added in software steps.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Steps are revised accordingly.

13. Please include single line spacing between each numbered step or note in the protocol.

Single line spacing has been used.

14. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Protocol steps are highlighted in yellow to be featured in the video.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Complete sentences are now highlighted.

16. References: Please do not abbreviate journal titles; use full journal name.

Journal titles are revised and full names are used.

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

Table of materials has been revised.

Reviewer #1:

Manuscript Summary:

In this article, the authors have presented an easy to follow, easily adaptable protocol to induce eryptosis. I found the article to be clearly written, and I have only minor concerns, as listed below.

We thank the reviewer for their positive comments.

Major Concerns:

N/A

Minor Concerns:

1. In the protocol, section 1, step 2, the authors state "remove the plasma and buffy coat using a pipette." To make the protocol even more readily accessible to someone who might be new to this field, a little more detail might be helpful. For example: "remove the clear plasma and the thin buffy coat using a pipette, to leave the red erythrocyte layer." I do realize that this point is less important, given that the article will be accompanying a video.

The sentence is revised and more details are added.

2. When the authors describe centrifuge speeds, they often write, for example, "700 g." This should more accurately be "700 x g".

All centrifuge speeds are revised.

3. In the protocol, the authors switch back and forth between "erythrocytes" and "hematocrit". For example, in step 2, the authors state "Add 1 mL of the 0.4% hematocrit" but in step 3, "treat erythrocytes". Is it possible to use a single term throughout the protocol for clarity?

Erythrocytes refer to cells while hematocrit refers to the cell suspension. For example, 0.4% hematocrit means 0.4 vol% of erythrocytes in 100 total vol% of buffer.

4. There are two step 2s in section 3 of the protocol.

The numbering is changed.

Reviewer #2:

Major Concerns:

I have one very important questions, What is the need of this project if many other papers already present in which ionomycin used as a inducer of eryptosis. I have worked alot in this field and used ionomycin in my experiments with same concentration and duration of treatment. Please compare your work with other people who used ionphores to treat erythrocytes and induce erythrocytes. Before proceeding to review, please the Clear me novality of your work with examples.

We thank the reviewer for their comment. However, we would like to note that we have not claimed novelty in this manuscript. In fact, we provided a table referring to studies in which ionomycin is used. Rather, we have tried to provide a step-by-step protocol for eryptosis, which is a seemingly simple procedure, but has many small roadblocks.

Reviewer #3: Manuscript Summary:

In this manuscript the authors present a new step-by-step procedure to induce eryptosis in human erythrocytes. The authors state that the use of ionomycin is widely carried out and that there is a lot of discrepancy in the methodology used. Then, they design a protocol to induced eryptosis in human erythrocytes using ionomycin.

Major Concerns:

Although this is an interesting idea, the manuscript suffers from lack of scientific rigor. Studies to publish protocols should include a large number of trials, which does not happen in this work.
The paper needs more quality of the research process.

1) The statistical test used in this study (t-test) is not correct. Can the authors ensure that their sampling has a normal distribution? They also have a very low "n". They should use a non-parametric test.

We thank the reviewer for raising this important point. All statistical analyses were repeated and replaced with non-parametric ANOVA. Accordingly, the figures and legends for Figures 2, 4, and 5 were updated.

2) Do the control treatments have DMSO added to avoid any effect of it?

We did not add DMSO to control. This is because DMSO was diluted 2000 times for all samples and was not expected to affect the controls. However, if the reviewer believes such an experiment is necessary, we would be happy to perform it.

3) Page 6: Studies of time and concentration should not be independent. Better results could be obtained by lowering the amount of ionomycin and increasing the exposure time, while more closely resembling a pathological situation (Fig 1 and 2).

We thank the reviewer for this suggestion. We repeated the experiments with lower concentrations of ionomycin (0, 0.25, 0.5, and 1 µM) and for longer treatment times (6 and 12 hours). These experiments (shown in Figure 3) did not result in a higher level of eryptosis and the rest of the figures were kept at original values of 1 µM and 2 hours of incubation. We have noted this point in the revised manuscript.

4) Figure 2: What percentage of eryptosis showed time 0? The authors should include it in the figure.

The figure is revised and eryptosis at time 0 is included.

5) Figure 2: What percentage of hemolysis exists at 120 min?

Percentage of hemolysis at all time points are included in Figure 2F.

6) Figure 2: Why does the percentage of eryptosis decrease at 180 min? Is there a lot of hemolysis at that time?

Yes. A higher level hemolysis was observed at 180 minutes of treatment (Figure 2F). We have noted this point in the manuscript.

7) Figure 3 y 4: The data plotted in Figures 3 and 4 come from the same analysis. Authors should bring them together into a single figure.

These figures are now combined in Figure 4.

8) Figure 5: Perform GM intensity statistics.

GM intensity statistic is performed and the results are presented in Figure 5E.

9) Legend of Figure 6: Is the magnification reported correct? Are the eyepiece and objectives taken into account?

These are objective magnifications. Caption for Figure 6 is corrected.

10) Authors should perform a statistical analysis with the results of different trials to ensure: "The cells incubated in glucose-free Ringer solution show less forward scatter compared to the cells incubated in glucose-containing buffer (Figure 5), indicating extensive cell shrinkage in the glucose-free environment."

Statistical analysis is preformed and the results are presented in Figure 5E.

Minor Concerns:

Legend of Figure 4: remove the sentence "No indication is for p>0.05."from the figure.

It is removed.

Author Bio
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Biosketch

Parnian Bigdelou obtained her Bachelor's in Chemical Engineering from Sharif University of Technology (Tehran, Iran). She then completed a Master's degree in Chemical Engineering at Cleveland State University working on high throughput immunoassays. She is currently completing a second Master's degree in Biomedical Engineering at Ohio University with her work focused on the role of membrane asymmetry in nanoparticle-cell membrane interactions.

Author Bio

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Biosketch

Amir Farnoud obtained his Ph.D. in Chemical Engineering from the University of Iowa followed by postdoctoral training in Microbiology at SUNY-Stony Brook. Dr. Farnoud joined Ohio University in August 2015 where he is currently an Assistant Professor in the department of Chemical and Biomolecular Engineering and a member of the Biomedical Engineering program. Research in Dr. Farnoud's group is primarily focused on the interactions of engineered nanomaterials with biological membranes and is currently supported by the NIH and the NSF.