In vitro alterations of erythrocyte aggregation by action of *Trichinella spiralis* newborn larvae

Ponce de León Patricia[a], Toderi Martín[b], Castellini Horacio[b] and Riquelme Bibiana[b,*]

[a] *Facultad Cs. Bioquímicas y Farmacéuticas (UNR), Suipacha 535, 2000 Rosario, Argentina.*

[b] *Facultad de Cs. Exactas, Ingeniería y Agrimensura (UNR), Pellegrini 250, 2000 Rosario, Argentina.*

[c] *Óptica Aplicada a la Biología, Instituto de Física Rosario (CONICET-UNR), Boulevard 27 de febrero 210 bis, 2000 Rosario, Argentina.*

Abstract.

Possible changes in the erythrocyte membrane, by *in vitro* interaction with newborn larvae of *T. spiralis* (NL), were evaluated analyzing the alterations in erythrocyte aggregation by digital image analysis and laser transmission in a new optical chip aggregometer. NL were obtained from CBi mice infected with *T. spiralis*. RBCs samples from healthy donors where *in vitro* exposed to NL (concentration (3000 ± 500) larvae/mL) to assess its effect on RBC aggregation. Individual cell Coefficient (C_{CA}) and aggregation parameter (S) were calculated by digitally processing RBC aggregate images, indicating the amount and size of the erythrocyte aggregates present. Also, size distribution of aggregate was analyzed. Kinetic aggregation parameters (Amp_{750} and $t_{1/2}$) were calculated with a new optical chip aggregometer. Results show significant alterations in erythrocyte aggregability due the *in vitro* action of *T. spiralis* larvae increasing incubation time. These results are possibly related to the loss of surface sialic acid as it is captured by NL. Obtained results suggest that NL could produce hemorheological alterations in the host, which could be related to thrombosis and anemia reported in some patients with trichinosis.

[*]Corresponding author: Bibiana D. Riquelme, Pasaje Salamanca 1021, (2000) Rosario, Santa Fe, Argentina. Tel.: +54 341 4853222; E-mail: riquelme@fir-conicet.gov.ar; briquel@fbioyf.unr.edu.ar

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1. Introduction

Trichinellosis is a major zoonosis in Latin America and represents a problem to human and animal health [1]. Despite the high overall prevalence of intestinal worms in humans, and the economic impact they cause in the production of livestock, knowledge of the host-parasite relationship is still incomplete [2].

Trichinellosis is caused by eating raw or undercooked meat of animals infected with the larvae of a species of nematode called *Trichinella*. Infection commonly occurs in certain wild carnivorous or omnivorous animals. Humans are accidentally infected when eating improperly processed meat of these animals or eating food contaminated with such meat [3] [4]. The infection is acquired by ingesting cysts (encysted larvae) of *Trichinella spp*. After exposure to gastric acid and pepsin, muscle larvae are released from the cysts and invade the small bowel mucosa where they develop into adult worms. Except for the brief period in which the infective larvae are released from the muscle fibers, the only period in which *T. spiralis* is out of the cells is the migration stage NL to the striated muscle [5], a long period in which the larvae establish intimate contact with its host erythrocytes.

In a previous work [6] [7], in which the Polybrene method was used, it was reported that the contact of erythrocytes with *T. spiralis* larvae decrease erythrocyte sialic acid, indicating that the parasite can capture it. These results suggest a decrease in the erythrocyte surface electric charge, causing alteration in erythrocyte aggregation.

Red blood cells (RBC, erythrocytes) are the most abundant cells in the bloodstream and contain hemoglobin, the compound that carries oxygen through the body [8]. Any alteration in red blood cells, in either quantity, shape, size, structure or life cycle, can affect the oxygen-carrying capacity of the blood. Due to their mechanical properties and abundance, RBCs are mainly responsible for blood rhe-

ological behavior because of their influence on blood viscosity, which depends on erythrocyte deformation and aggregation [9] [10]. Increments in blood viscosity induce an increase in flow resistance and could produce obstructions of microcapillaries, altering microcirculation [11] [12] [13]. Consequently, several techniques were developed to analyze the RBC aggregation characteristics, being the transmitted light measurement through a blood sample widely used [14].

In addition, Kaneko and Anosa [15] have reported that a decrease in erythrocyte sialic acid alters the erythrocyte surface, modifying it antigenically and biochemically. These alterations could lead to increased phagocytosis of liver cells, contributing to the anemia status reported in this infection [16]. Moreover, depletion of sialic acid promotes abnormal erythrocyte aggregation, leading to the formation of clusters of aggregates with a consequent decrease in blood flow, increase in blood viscosity and possible microcapillary obstruction [17]. In consequence, the observed *in vitro* decrease in sialic acid on RBCs could produce hemorheologic alterations in the *in vivo* infection of the host, which could be related to thrombosis reported in some patients with trichinosis [18] [19].

In this work, possible changes in the erythrocyte membrane by the *in vitro* interaction with newborn larvae of *T. spiralis* were evaluated by analyzing the alterations in erythrocyte aggregation by digital image analysis and laser transmission in a new optical chip.

2. Materials and methods

The study was approved by the Bioethics Committee of the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (Res. Nº 1072/2014 on December 19th, 2014) and all donors gave informed written consent to participate.

2.1. Newborn larvae T. spiralis

Newborn larvae (NL) were obtained from CBi mice infected with *T. spiralis*, which were provided by the animal research facility of the Experimental Genetics Institute (Faculty of Medical Sciences, National University of Rosario). CBi is an inbred mouse strain derived from an outbreed population generated by crossing BALB/c, Rockland, NIH and Swiss mice. It was generated to be used as a base population of broad genetic basis and as the control line of an experiment of artificial body-conformation selection which gave rise, among others, to CBi− and CBi/L mice lines [20] [21].

Between 6 and 13 days post-infection, gravid females were obtained by surgery from the small intestine of mice. Females were incubated in 100 µL of RPMI-1640 medium (Sigma- Aldrich) supplemented with fetal bovine serum and antibiotics for 18 hours at 37 °C in atmosphere of 5% CO_2. NL were subsequently separated from adult females and collected in saline solution. Larvae concentrates were prepared at 24 hours, resulting in (3000 ± 500) larvae/mL [22].

2.2. Red blood cells

Fresh group O blood samples were collected from healthy donors (n=5) by venipuncture in sterile vials containing EDTA as anticoagulant, stored at 4 ºC and analyzed within 24 h. The blood samples were centrifuged at 1,100 g (25 ºC, 5 min). After removing plasma and buffy-coat, RBCs were washed with saline solution (SS: 0.90% w/v of NaCl, 308 mOsm/L) three times. Collection and processing of samples were performed within 24 hours from extraction time as recommended in the "New guidelines for hemorheological laboratory techniques" [23].

2.3. Erythrocyte Treatment

Treatment involves incubating 100 µL of globular sediment with an equal volume of NL concentrate (Treated RBCs). Control RBCs were incubated in the same way with saline solution only. In order to evaluate the effect of NL on erythrocyte aggregation, Treated and Control RBCs were incubated for 2

hours at 37 °C. Aliquots of each sample of the RBC suspension were taken at the initial time (t = 0), 60 and 120 minutes. Then, RBCs from each aliquot were washed in SS and re-suspended in autologous plasma at 0.3% of hematocrit to be analyzed by digital image analysis and at 40% of hematocrit to be measured in the optical chip.

2.4. Erythrocyte aggregation by Digital Image Analysis

RBCs were suspended in autologous plasma (to induce rouleaux formation) at 0.3 % hematocrit and poured into an excavated slide, which was placed on the stage of an Optical Inverted Microscope (Union Optical, Japan) [24] [25]. After 5 minutes, aggregation of cells was attained and microscopic images of RBCs aggregate populations were registered by triplicate using a 40x objective and a digital camera (Mikova DCM500 USB2.0). Each image was instantaneously stored in a computer file. Figure 1 shows an example of images registered for one sample (control and treated with NL for 60 and 120 minutes). Then, Image J software was used to study alteration in erythrocyte aggregation by analyzing the distribution of aggregate size and by determining the following parameters:

Erythrocyte aggregation parameter (S parameter) was defined as the difference between the total cell number and the number of isolated cells related to the number of total cells counted in each image [26]:

$$Sparameter = \frac{(n°totalcells - n°isolatedcells)}{n°totalcells} \quad (1)$$

This parameter ranges from 0 (fully disaggregated after treatment) to 1 (completely aggregated after treatment). Thus, the rate of the amount of aggregated cells to the total number of cells for each treatment was directly quantified for each sample.

Individual cell Coefficient (C_{CA}) was defined in previous work [27] as:

$$C_{CA} = \frac{CA_{initial} - CA_{final}}{CA_{initial}} \quad (2)$$

where $CA_{initial}$ is the percentage of the individual cell number before larva treatment (Control RBC) and CA_{final} is the individual cell percentage after larva treatment (Treated RBC). This coefficient varies between 0 (no differences in aggregation before and after treatment) and 1 (complete aggregation after treatment).

A study of ***aggregated size distribution*** was also carried out. To do this, erythrocyte aggregates were counted and classified in four categories:

- individual cells;
- aggregates of two, three, and four cells;
- aggregates of five, six and seven cells;
- aggregates of more than eight cells.

Then, the percentages corresponding to each category and each erythrocyte sample (control and treated) were calculated.

2.5. Optical chip erythrocyte aggregometer

Changes induced by the action of larvae on erythrocyte aggregation were evaluated by an optical chip, based on the analysis of laser transmission through a blood sample recorded in real time [13][14]. In this new optical chip, RBCs were suspended in autologous plasma at 40 % hematocrit and only 15 µL of this suspension was used to assess erythrocyte aggregation kinetics within a few minutes. The optical chip is the physical support for the blood sample, whose base was a common glass slide with a piece of double-sided plastic tape. The measurement device sets the sample on a horizontal plane, a diode laser beam (λ= 670 nm, 5 mW – Melles Griot) goes vertically through the sample and a photomultiplier records variations in the incident light intensity (see Fig. 2). Graphics of light intensity as a function of time called Syllectograms were obtained, and aggregation parameters were calculated. The parameters provided are:

Amp_{750}: the extent of light intensity at 750 s indicating the amount of RBC aggregates,

$t_{1/2}$: the time required to reach half of the total transmitted light intensity, in this case at 1500 s, indicating a characteristic time constant for the average level of aggregation.

Triplicate determinations on blood samples were performed for controls and RBCs incubated for 60 and 120 minutes with NL.

2.6. Statistical Analysis

Statistical comparison was made between the control and treated samples in order to evaluate the possible alterations. ANOVA (two-factor / threefactorial) was applied to percentage size distribution, C_{CA} and S parameter variables to study the possible variation in aggregation parameters by the action of NL and the effect of incubation time (0, 60 and 120 minutes) [28].

To study the aggregation kinetics phenomena, 4 measurements for each type of sample (control, 60 and 120 minutes of incubation with NL) were performed within 48 h from blood extraction and sample preparation. Aggregation parameters were calculated for every blood sample and then an average value and standard deviation of the parameters for each case were obtained.

All the values obtained were presented as mean ± Standard Deviation (SD).

3. Results and discussion

3.1. Erythrocyte aggregate parameters by analysis of digital images

Fig. 1 shows an example of the microscopic images of erythrocyte aggregates from a RBC control sample and the same RBCs incubated with NL for 60 minutes and 120 minutes. As seen in these images, samples from control donors had a greater RBC aggregability after larvae treatment with evidence of greater presence of rouleaux.

Tables 1 and 2 show S and C_{CA} parameters obtained by digital analysis of images from controls and RBCs incubated with NL at initial time and for 60 and 120 minutes. Statistical analysis of S and C_{CA}

values from treated RBCs shows a significant increase in both parameters with the incubation time ($p<0.0001$). The corresponding mean values of parameter S were more than 50% higher in RBCs treated for 60 and 120 minutes than in controls.

Table 3 shows the distribution values of RBC aggregate sizes for the control and RBCs incubated with NL at initial time and after 60 and 120 minutes. Figure 3 shows the corresponding mean values, which evidence a decrease in the number of individual cells and an increase in the number of aggregates of 5 or more cells. These results indicate an increase in erythrocyte aggregability with the incubation time.

3.2. Erythrocyte aggregation kinetics

Table 4 shows erythrocyte aggregation kinetic parameters for RBCs incubated with NL at initial time and after 60 and 120 minutes. Analysis of these results indicate that the amplitude of transmitted light at 750 s (Amp_{750}) and the time to reach 50% of aggregation ($t_{1/2}$) varied through the tests, indicating that the erythrocyte aggregation process was significantly altered in all samples incubated with NL ($p<0.05$). Amp_{750} increased with the incubation time, which evidences the presence of larger and more globular aggregates, while $t_{1/2}$ diminished, which implies faster aggregation kinetics. In the syllectogram (Fig. 4) it can be seen how aggregation kinetics is modified according to incubation time. A longer incubation time will present curves with greater slopes at the beginning of the phenomenon, which gives a longer $t_{1/2}$. This could be attributed to a decrease in the surface charge of the cells, favoring the formation of aggregates due to decreased electrostatic repulsion among erythrocytes. In addition, it contributes to the formation of globular aggregates, which allows greater transmission of light through the blood sample. For the same reason, Amp_{750} values were modified and presented an increasing behavior.

4. Conclusion

The digital image analysis showed that erythrocytes incubated with NL, presented a decrease in the percentage of the isolated cells and a greater size of the aggregates formed in relation to the controls. Statistical analysis concluded that aggregation in the treated RBCs was higher with increasing incubation time. This is possibly related to the loss of surface sialic acid as it is captured by NL, which is in agreement with the results of previous research [29].

As shown in a previous work [13], the amplitude of transmitted light at 750 s (Amp_{750}) and the time to reach 50% of aggregation ($t_{1/2}$) are sensitive to changes in the surface electric charge of the erythrocyte. The behavior observed through the tests indicates that Amp_{750} increased with incubation time, which is consistent with the presence of larger and more globular aggregates. Also, $t_{1/2}$ diminished, indicating faster aggregation kinetics probably related to an alteration in the surface electric charge produced by the action of larvae. A longer incubation time is closely related to a reduction in the RBC surface electric charge and, consequently, to alterations in the aggregation process yielding anomalous aggregates.

As a conclusion, larvae alter the erythrocyte membrane, which favors the RBC aggregation process accelerating it, and modified the traditional linear structures of rouleaux to produce globular erythrocyte aggregates similar to those described by Schmid-Schönbein, who gave the first description of "clump aggregation" in 1977 [30].

The results observed *in vitro* suggest that hemorheological alterations in the host are produced *in vivo* infection. Moreover, these hemorheologic alterations could be related to thrombosis and anemia reported in some patients with trichinosis [18] [19].

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Table 1: S parameter values from control and RBCs incubated with NL at initial time and after 60 and 120 minutes. Mean value ± SD

		0 minutes	**60 minutes**	**120 minutes**
Sample 1	Control	0.44 ± 0.03	0.48 ± 0.01	0.48 ± 0.01
	Treated	0.46 ± 0.04	0.69 ± 0.01	0.85 ± 0.01
Sample 2	Control	0.46 ± 0.01	0.44 ± 0.01	0.43 ± 0.01
	Treated	0.46 ± 0.05	0.76 ± 0.02	0.89 ± 0.01
Sample 3	Control	0.54 ± 0.02	0.54 ± 0.01	0.56 ± 0.03
	Treated	0.58 ± 0.01	0.86 ± 0.01	0.95 ± 0.02
Sample 4	Control	0.59 ± 0.04	0.55 ± 0.02	0.59 ± 0.02
	Treated	0.63 ± 0.02	0.70 ± 0.01	0.90 ± 0.01
Sample 5	Control	0.38 ± 0.01	0.42 ± 0.03	0.38 ± 0.02
	Treated	0.40 ± 0.02	0.78 ± 0.01	0.92 ± 0.02

Table 2: C_{CA} values for each sample of RBCs incubated with NL at initial time and after 60 and 120 minutes. Mean value ± SD

	0 minutes	**60 minutes**	**120 minutes**
Sample 1	0.045±0.001	0.42±0.02	0.73±0.02
Sample 2	0.060±0.001	0.57±0.03	0.81±0.01
Sample 3	0.080±0.028	0.69±0.01	0.88±0.07
Sample 4	0.080±0.028	0.33±0.03	0.76±0.02
Sample 5	0.055±0.021	0.62±0.02	0.87±0.03

Table 3: Distribution of RBC aggregate sizes for control and RBCs incubated with NL at initial time and after 60 and 120 minutes. Mean values ±SD

	Control	**0 minutes**	**60 minutes**	**120 minutes**
% individual cells	51 ±7	48 ±7	24 ±6*	8 ±4**
% groups of 2-4 cells	19 ±8	22±6	10 ±3*	3 ±1**
% groups of 5-7 cells	14 ±4	14 ±5	13 ±9	3 ± 1*
% groups ≥ 8 cells	15 ±11	15 ±9	53 ± 10**	81 ± 10**

* $p < 10^{-5}$; ** $p < 10^{-7}$

Table 4: Erythrocyte aggregation kinetic parameters for RBCs incubated with NL at initial time and after 60 and 120 minutes

Parameter	**Amp_{750} [a.u.]**			**t_{50} [s]**		
Incubation time	**0 min**	**60 min**	**120 min**	**0 min**	**60 min**	**120 min**
Sample 1	69.4 ± 0.9	78.1 ± 1.6	78.5 ± 1.5	500 ± 30	425 ± 20	350 ± 30
Sample 2	75.9 ± 1.6	81.2 ± 1.6	86.5 ± 1.8	420 ± 28	325 ± 10	312 ± 20
Sample 3	80.6 ± 0.3	83.8 ± 1.4	86.0 ± 0.4	350 ± 10	270 ± 10	220 ± 10
Sample 4	74.3 ± 0.9	80.5 ± 0.8	82.2 ± 1.8	380 ± 28	337 ± 10	310 ± 14

FIGURE CAPTIONS

Figure 1: Section of images showing erythrocyte aggregates from control and treated RBCs registered for one sample at initial time (0 minutes of incubation) and incubates with NL for 60 and 120 minutes.

Figure 2: Simplified diagram of the measuring device used in this study.

Figure 3: Mean values corresponding to distribution of RBC aggregate sizes from control and RBCs incubated with NL at initial time and for 60 and 120 minutes.

Figure 4: Syllectogram obtained of RBCs from control and treated with newborn larvae.

Figure 1

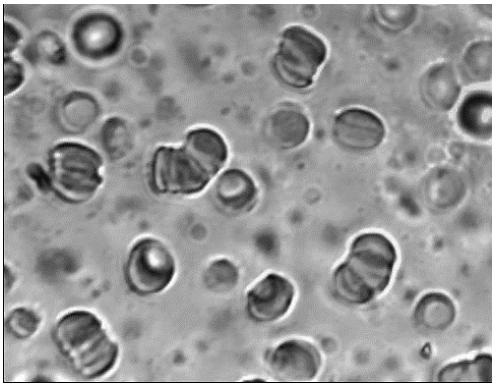
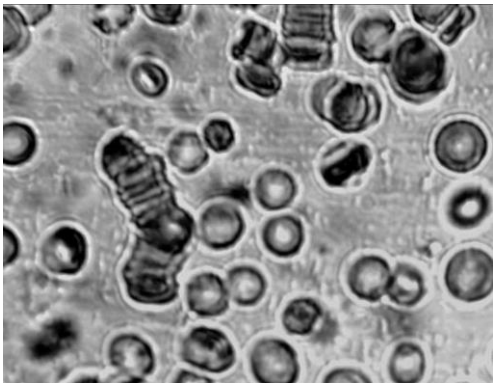
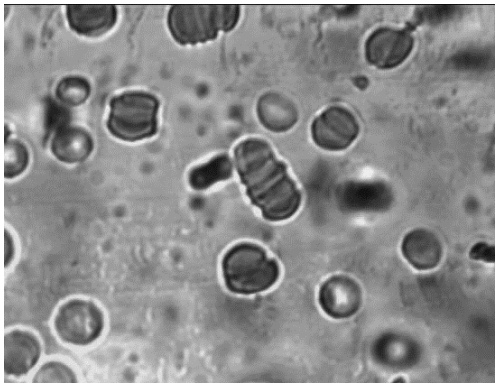
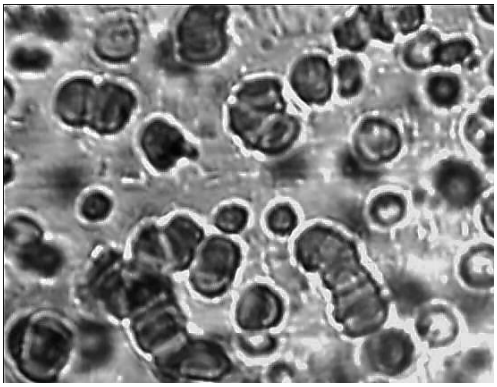
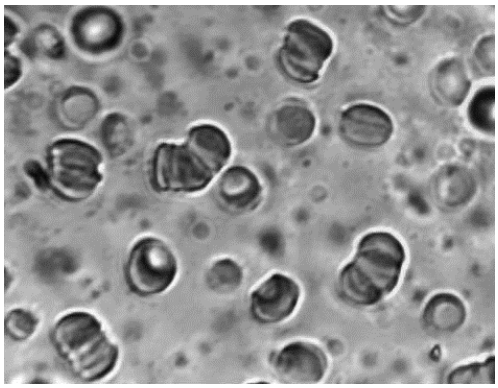
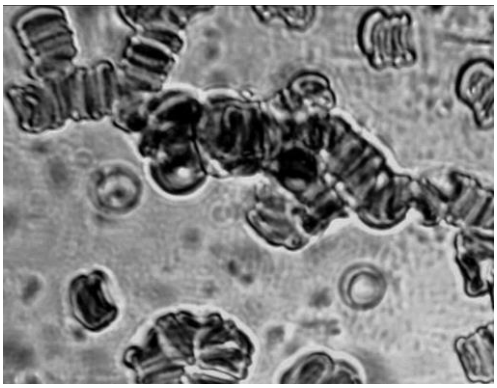
 Control RBC at 0 minutes	 RBC treated with NL at 0 minutes
 Control RBC at 60 minutes	 RBC treated with NL at 60 minutes
 Control RBC at 120 minutes	 RBC treated with NL at 120 minutes

Figure 2

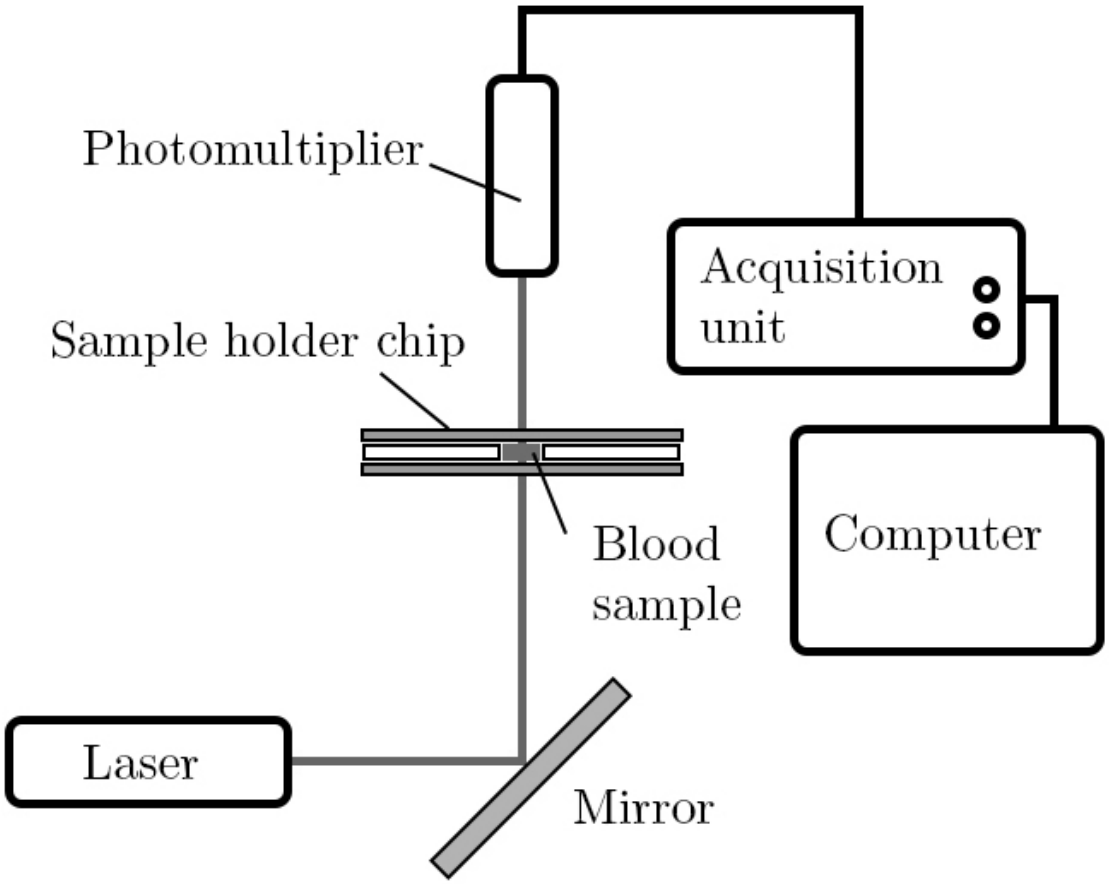

Photomultiplier
Acquisition unit
Sample holder chip
Computer
Blood sample
Laser
Mirror

Figure 3

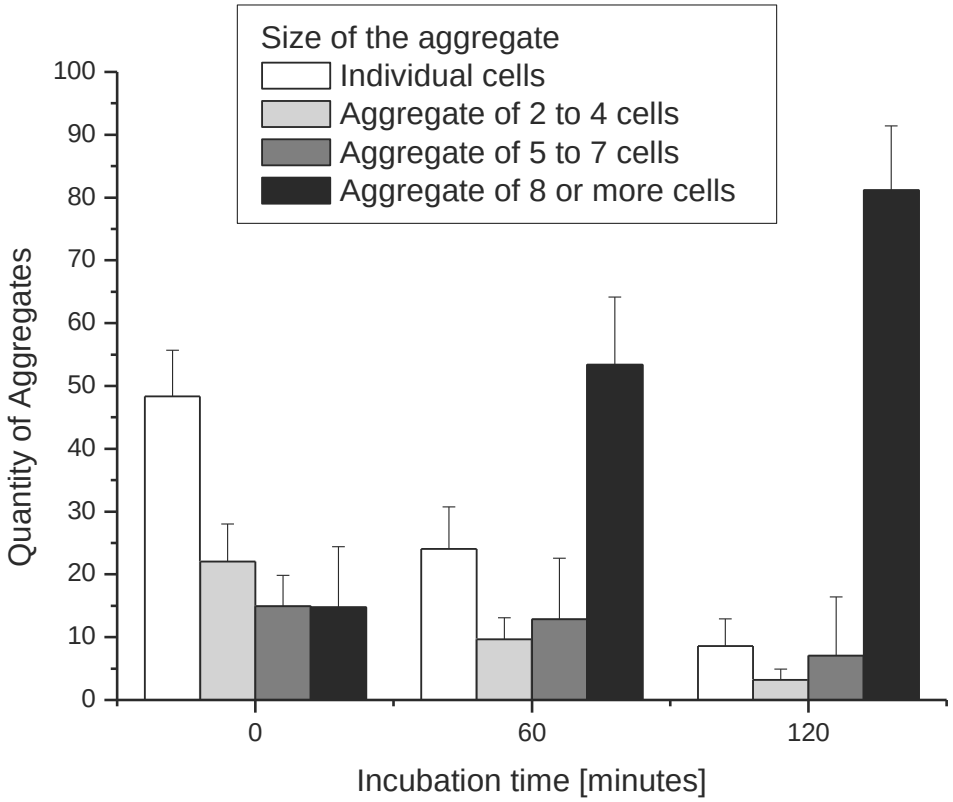
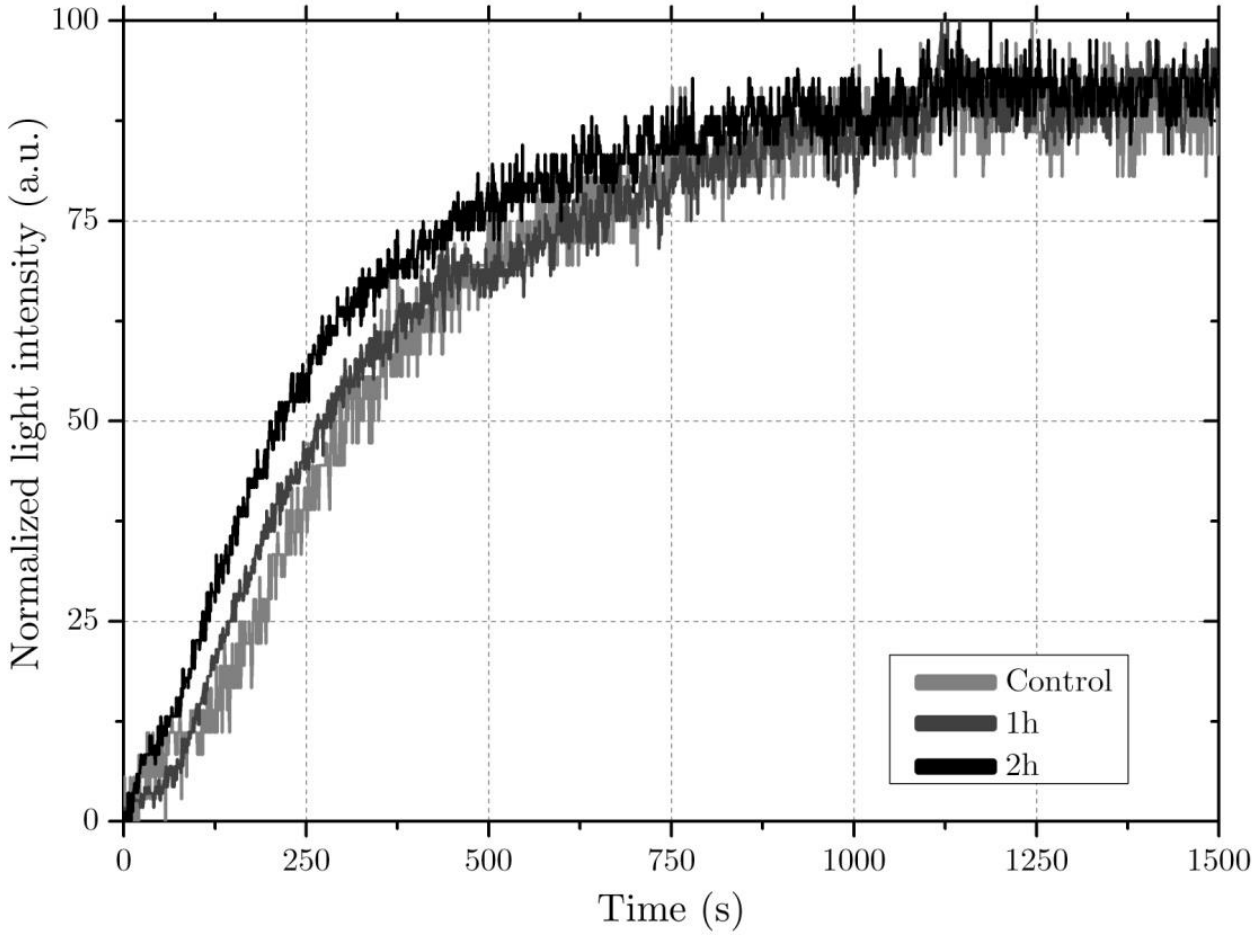

Size of the aggregate
Individual cells
Aggregate of 2 to 4 cells
Aggregate of 5 to 7 cells
Aggregate of 8 or more cells
Quantity of Aggregates
100
90
80
70
60
50
40
30
20
10
0
0
60
120
Incubation time [minutes]

Figure 4


Normalized light intensity (a.u.)
100
75
50
25
0
0
250
500
750
1000
1250
1500
Time (s)
Control
1h
2h