CELL BIOLOGY

# The preparation of samples for studying neutrophils without their isolation

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## Abstract

Neutrophils are the primary cells of the innate immune system. They destroy pathogenic microorganisms carrying out the oxidative stress mechanism through phagocytosis and extracellular traps (NETs). There is no doubt about the need to study neutrophils' functional activity, but there are some methodological problems with the nativity of conditions in such experiments. The aim of this work is to propose a method of preparing a sample of peripheral blood neutrophils for study by spectral-optical methods (such as confocal microscopy) providing an increased level of the nativity of the conditions.

**Keywords:** neutrophils, reactive oxygen species, respiratory burst, confocal microscopy.

## Introduction

Neutrophils are the most abundant cells of the innate immunity system, arousing the active interest of researchers in recent years. For a long time, they were perceived exclusively as phagocytic cells (Nathan, 2006; Soehnlein, 2009; Nordenfelt and Tapper, 2011), but now a lot of studies have been accumulated that expand the understanding of the functions of neutrophils (Mantovani, Cassatella, Costantini, and Jaillon, 2011; Tillack, Breiden, Martin, and Sospedra, 2012; Yipp et al., 2012; Muraro, De Souza, Gallo, and De Silva, 2018; Mozzini and Girelli, 2020; Poh, Loh, Friedland, and Ong, 2022). So, there is a need for sample preparation protocols that would ensure the maximum possible nativity of conditions for the studied cells, since neutrophils are extremely sensitive to the microenvironment (Varfolomeeva et al., 2016; Andryukov, Bogdanova, and Lyapun, 2019; Fedorova et al., 2021). Previously, we presented a method of evaluating the functional activity (namely, the ability to generate reactive oxygen species (ROS) of peripheral blood neutrophils under native conditions by flow cytometry (Filatov, Varfolomeeva, and Ivanov, 1995; Varfolomeeva et al., 2010), demonstrating the difference between the functional activity of neutrophils in whole blood and those isolated from it. Notably, functional activity of neutrophils is not only their activated or not activated state, but also their potential capacity to generate ROS (Varfolomeeva et al., 2010). Moreover, neutrophils are active cells whose function supposes their movement from blood to tissue to the inflammation. This potential motility is another type of neutrophils' functional activity that can be modified by microenvironment (Berton, Yan, Fumagalli, and Lowell, 1996). Microenvironment in terms of neutrophils is not only plasma (or any other media) biochemical composition. In addition to the influence of media itself, there is a huge impact of other cells surrounding neutrophils. Their elimination from the

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sample can modify neutrophils' activity. It means that any kind of neutrophils' isolation may alter their functional activity. In this article, we propose a protocol for preparing a sample of peripheral blood neutrophils without isolating them by modifying the generally accepted method of sample preparation by centrifugation in the histopaque-1077 density gradient. There is a more modern immunomagnetic method of neutrophils' isolation (Blanter et al., 2022) that makes the isolation step less stressful for the cells. Nevertheless, this method does not provide more native conditions of the resulting samples. The elimination of the isolation step increases the nativity of the conditions and reduces the stress effect on neutrophils accompanying isolation. We suggest using this protocol for studying morphology and functional activity of peripheral blood neutrophils by spectral-optical methods (such as confocal microscopy).

## Materials and methods

### Preparation of human neutrophils using isolation procedure

Venous blood samples were obtained from healthy donors, blood was taken from a vein (3–4 ml) into a vacuum heparin tube Lind-Vac (Estonia).

Neutrophils were isolated by centrifugation in the histopaque-1077 density gradient as in (Gorudko et al., 2018). Cells were suspended in blood plasma. All isolation procedures were performed at room temperature.

### Preparation of human neutrophils without isolation procedure

The whole blood of healthy donors was used. Peripheral blood in vacuum heparin tubes Lind-Vac (Estonia) was centrifuged at 252 g for 15 minutes at room temperature. After that, the plasma layer was put in an eppendorf tube and centrifuged under the same conditions to eliminate thrombocytes. A white band of leukocytes with a small number of erythrocytes was combined with the centrifuged plasma in the new eppendorf tube and stained with Hoechst 33342 (at a concentration of 10 mkg/ml). The aim of staining the sample was to visually separate neutrophils from the other blood cells by fluorescence and the nucleus morphology.

Further, the cell suspension was placed on the surface of an optical Petri dish for confocal microscopy pretreated for 3–4 hours with 0.001 % poly-L-lysine solution (Sigma-Aldrich, Merck). After 30–40 minutes of incubation, which is necessary for the neutrophils' adhesion, the sample was ready for the experiment. Before the experiment, the red blood cells should be carefully selected, and the sample should be filled with plasma (single red blood cells may remain in the sample).

The scheme of the described procedure is presented in the Supplementary materials (Fig. S1).

### Preparation of fluorescently labelled protein (fibrinogen)

Fibrinogen was obtained from sodium citrate-stabilized donor blood plasma. The protein was isolated by fractionation with glycine and ammonium sulfate, followed by purification by chromatography on UNOsphere Q. The preparations of fibrinogen were fluorochromated with Alexa488 (AF488) using N-hydroxysuccinimide ethers of dyes (Lumiprobe, #1820). Adhering to the ratio of 4 moles of dye per 1 mole of protein, 20 ml of 1 M solutions of dye ethers in DMSO were added per 1 ml of 5 mkM proteins in 200 mM NaHCO3. The reaction was carried out in the dark for 2 hours at +4 °C. Then the protein solutions were placed in centrifuge cells for protein concentration (Sartorius, VivaSpin 6, retention limit 30 kDa), at least 5 cycles of 10-fold concentration and dilution with PBS were conducted, traces of dye were removed from the solution and the buffer was replaced. The obtained preparations were analyzed for the absence of aggregates using disc electrophoresis in a polyacrylamide gel without detergents (Davis, 1964). The inclusion of labels and the concentration of labeled proteins were evaluated by absorption at 495 nm for AF488 ($\varepsilon_{495} = 71800\ M^{-1} \times sm^{-1}$), using correction factors at 280 nm — 0.10.

### Localization of the fibrinogen-neutrophil interaction

In these experiments neutrophils' samples prepared with and without the isolation procedure were used. For the sample prepared using isolation, fibrinogen-Alexa488 was added to the neutrophils isolated from peripheral blood at a concentration of 8 mkg/ml for 20 minutes. Then the sample was stained with Hoechst 33342 (at a concentration of 10 mkg/ml) to visualize cells' nuclei. Thereafter, the cell suspension was placed on a slide glass and covered with a cover glass.

For the sample prepared without using isolation, fibrinogen-Alexa488 was added to the cell suspension in an optical Petri dish after neutrophils' adhesion at a concentration of 8 mkg/ml for 20 minutes.

### Visualization of the reactive oxygen and chlorine species generation by neutrophils

In these experiments neutrophils' samples prepared with and without the isolation procedure were used.

Both types of samples were placed on the surface of an optical Petri dish for confocal microscopy pretreated for 3–4 hours with 0.001 % poly-L-lysine solution (Sigma-Aldrich, Merck) for the 30–40 minutes necessary for the neutrophils' adhesion. Also, both types of samples were stained with Hoechst 33342 (at a concentration of 10 mkg/ml). For the qualitative evaluation of the reac-

tive chlorine species (namely, HOCl) generation intensity and their localization, the fluorescent dye Celestine Blue (20 mkM) and taurine (20 mM) were used (Lutsenko et al., 2018; Reut et al., 2020). For $O_2^-$ visualization the fluorescent dye Gallocyanin (5 mkM) was used (Lutsenko et al., 2018). Phorbol ether (PMA) at a concentration of 10 mkg/ml was used for the neutrophils' activation (Filatov, Varfolomeeva, and Ivanov, 1995).

### Confocal microscopy scanning of the samples

Images were obtained with an inverted confocal laser-scanning microscope TCS SP5 SMD FLCS, Leica. Celestine Blue was excited at wavelength 488 nm, and emission was registered using a filter cutting off the waves below 505 nm. Fibrinogen-Alexa488 was excited at wavelength 488 nm, and emission was registered using a filter cutting off the waves below 505 nm. Hoechst 33342 was excited at 405 nm and its fluorescence was registered at a range of 420–475 nm. The scanning was made within 1.5 hours with a start point 15 minutes after PMA adding. No chamber with gas control was used, the temperature of 37 °C was maintained using the table with temperature control. Images were processed by the software coming with the microscope.

### Flow cytometry estimation of viability of neutrophils samples

To estimate the viability of neutrophils samples prepared with and without the isolation procedure we used propidium iodide (PI) staining. This dye increases its fluorescence intensity when interacting with DNA. DNA becomes accessible for interaction with PI only if the cell is damaged, so one can estimate the number of the dead cells by the fluorescence intensity. PI was added to the samples and then the fluorescence intensity was measured with a flow cytometer Cell Lab Quanta Beckman Coulter (USA). The measurements were carried out several times (1 minute, 30 minutes and 90 minutes after the addition of PI) to follow the dynamics of neutrophils' viability.

## Results and discussion

To compare the efficiency of the alternative neutrophils' preparation protocol, we conducted three experiments, which differed only in the sample preparation protocol (with the isolation procedure and without it). We used the method of neutrophils' sample preparation by centrifugation in the histopaque-1077 density gradient for this comparison because we formed the suggested in this work protocol by modifying this method. Also, it is the most common method of neutrophils' isolation.

In the first experiment we observed the interaction between neutrophils and fibrinogen-Alexa488 using confocal microscopy. It can be seen in Fig. 1 that there is no significant difference in the obtained images. But what matters is that the use of the isolation procedure leads to the reduction in neutrophils' viability and alters their functional activity. So, we face some shortcomings of this method: firstly, we are restricted in time due to the reduced cells' viability. Isolation process itself takes quite a large amount of time leading to the loss of viability of neutrophils. Moreover, multiple steps of the isolation procedure suggest removing neutrophils from their native microenvironment from the very beginning. It accelerates the process of viability loss. In our experiments, isolated neutrophils were viable for the first 30 minutes of the experiment and started to die after that. This problem can be partly solved if the neutrophils are fixed but fixation makes it impossible to observe the dynamics of the studied interaction. Moreover, fixation with glutaraldehyde is a non-specific process that means some amount of fibrinogen can be fixated on the neutrophils' surface by accident. Secondly, large changes in the microenvironment inevitably take place when isolating neutrophils from the whole blood. These changes influence the functional activity of neutrophils, which means the result of the experiment is less relatable for peripheral blood neutrophils.

On the contrary, using the sample preparation protocol without the isolation procedure makes the experiment conditions much closer to *in vivo* conditions. It allows one to obtain an image (Fig. 1b) of the same quality as with isolated neutrophils (Fig. 1a). Using this protocol reduces the number of steps needed to get a sample due to the absence of multiple centrifugations on the ficoll layer. It preserves the native microenvironment much better, which solves both problems of viability and functional activity of peripheral blood neutrophils.

Of course, there is an obvious disadvantage of this technique: the sample contains not only neutrophils. We suggest using Hoechst 33342 staining to distinguish neutrophils from other blood cells by their nuclei. One should not be concerned about possible toxicity of the dye, as the concentration we used (10 mkg/ml) is less damaging to neutrophils than the isolation procedure. In our experiments, non-isolated neutrophils (samples prepared according to the suggested protocol) were viable for 1.5 hours from the start of the experiment and even longer in some cases (up to 3 hours). In addition, lymphocytes simply do not attach to poly-L-lysine, as they do not have enough adhesion molecules on their surface. Therefore, after a couple of media replacements, there are only neutrophils left (of white blood cells fraction).

Another example of using the new technique we propose is an experiment to study the dynamics of the functional activity of neutrophils activated by phorbol ether.

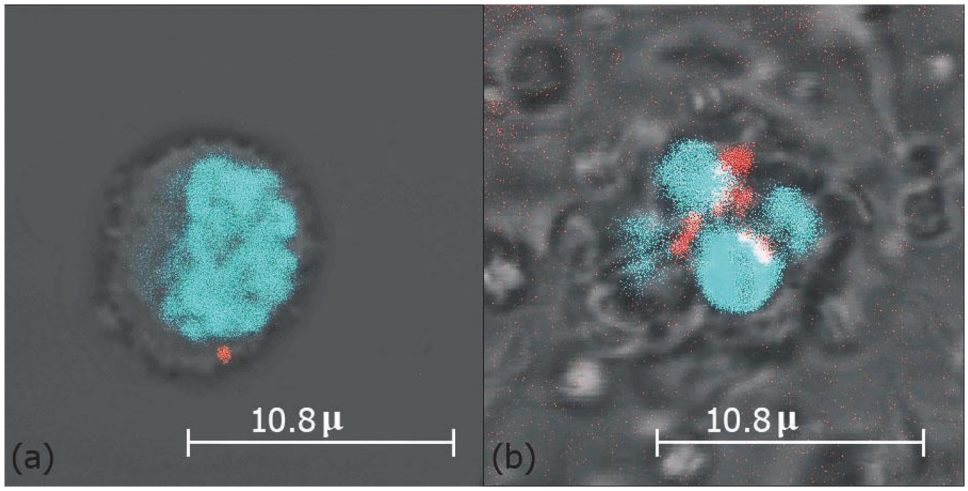


**Fig. 1.** Localization of fibrinogen-neutrophil interaction using (a) isolated and (b) non-isolated neutrophils from peripheral blood. Blue indicates DNA in neutrophils' nuclei (Hoechst 33342 is used), red indicates fluorescently labeled fibrinogen (Alexa488 is used). Objects without nuclei in (b) are erythrocytes.

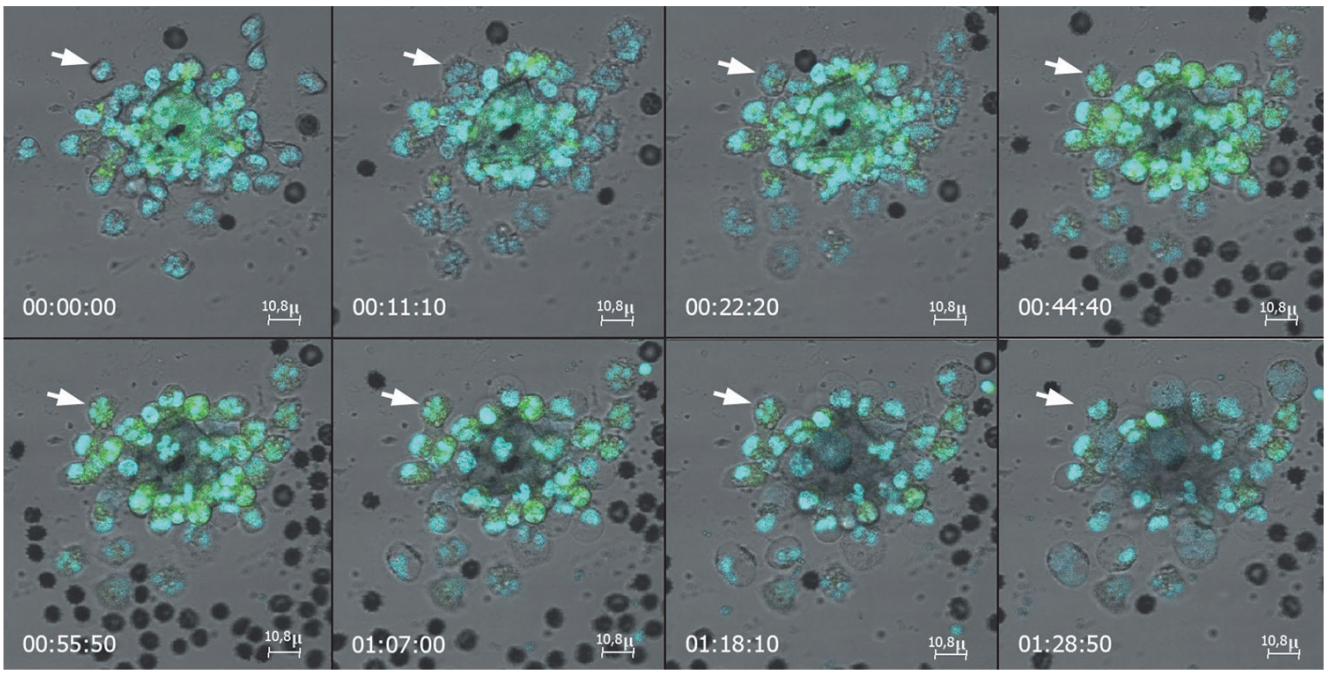


**Fig. 2.** Neutrophils interact with a foreign particle. Blue indicates DNA in neutrophils' nuclei (Hoechst 33342 is used), green indicates HOCl generation (Celestine Blue is used). The arrow indicates the neutrophil demonstrating changes in morphology and different types of functional activity: the movement to the foreign particle, ROS generation. Black little cells without nuclei are erythrocytes.

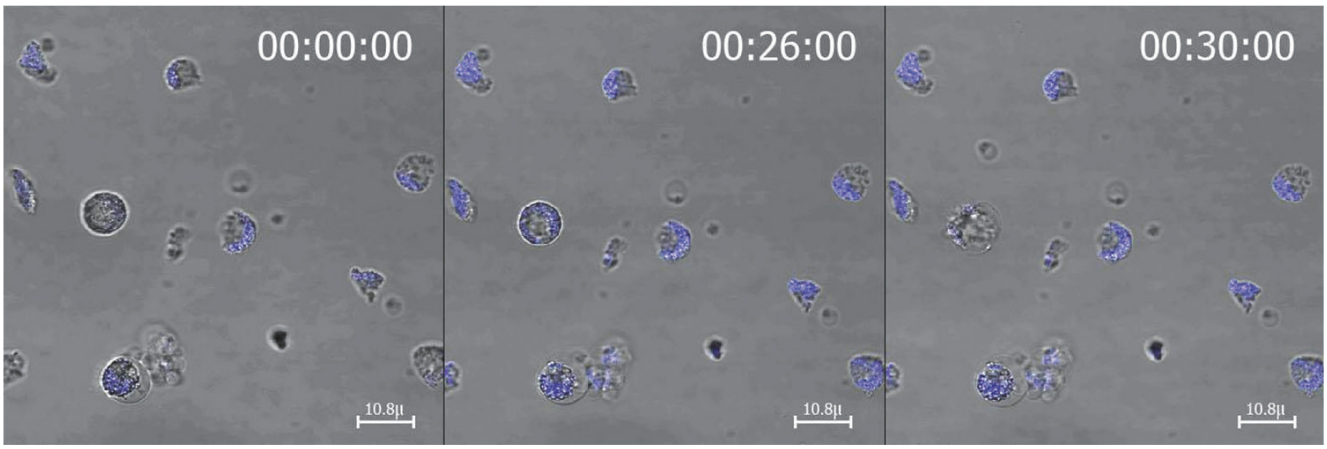


**Fig. 3.** Isolated peripheral blood neutrophils. Blue fluorescence indicates $O_2^-$ generation (Gallocyanine is used).

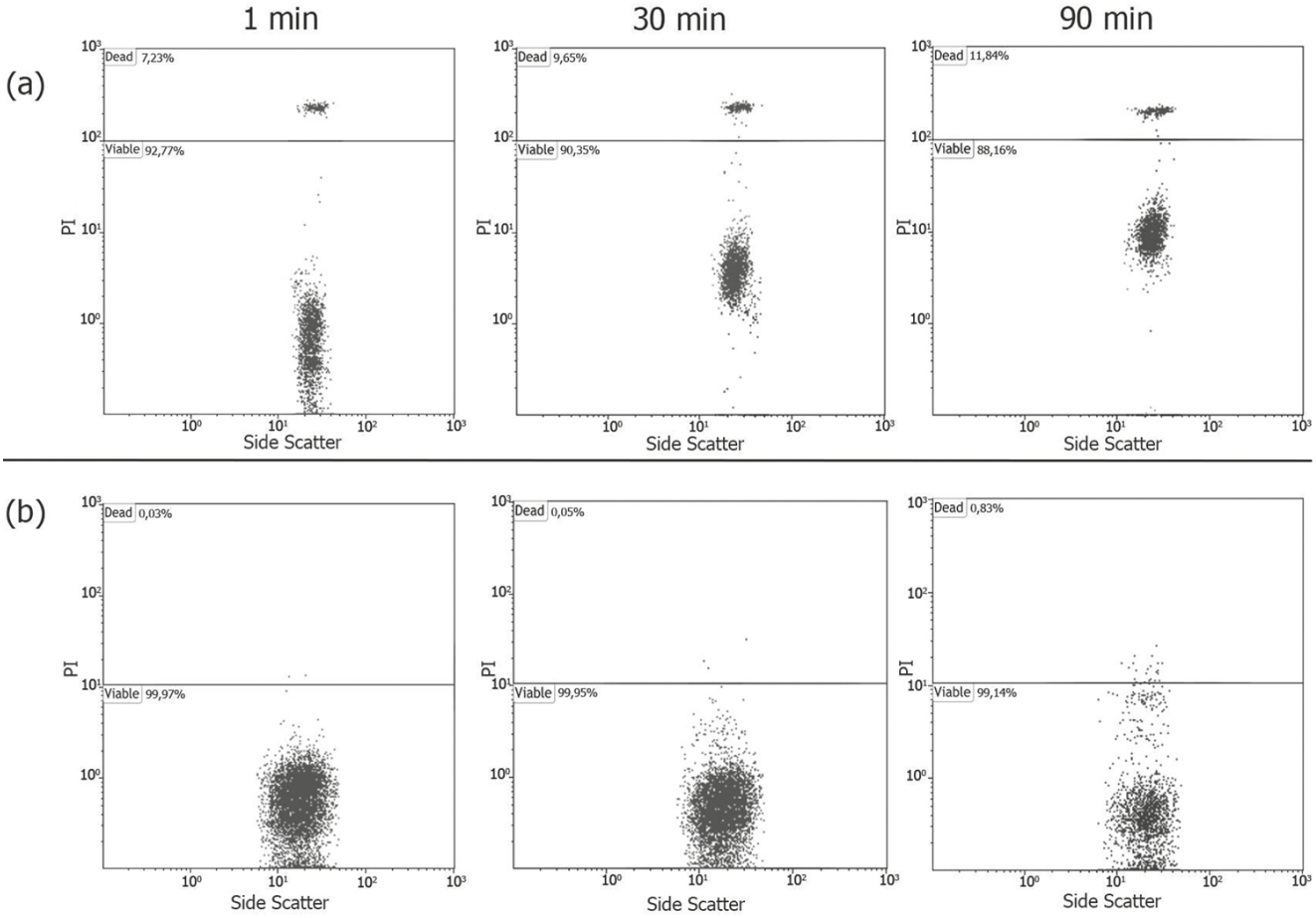


**Fig. 4.** The comparison of viability dynamics of neutrophils in samples prepared using isolation procedure (a) and without isolation (b). (Propidium iodide is used).

In Fig. 2, the interaction process between neutrophils and a foreign particle is shown. One can see there the manifestation of various functions of neutrophils, such as ameboid movement across the field (towards and away from the foreign particle), generation of HOCl and attempts to destroy the foreign particles using HOCl. Moreover, the changes in neutrophils' morphology can also be seen clearly. There is a larger-scale image of one frame from Fig. 2 in the Supplementary materials (Fig. S2) which demonstrates that the cells' nuclei are segmented.

Such an experiment is more difficult to carry out using the sample preparation technique with the neutrophils' isolation procedure. Fig. 3 shows that the lifespan of the isolated neutrophils is notably shorter than that of the not-isolated ones (Fig. 2). One can see in Fig. 2 that all neutrophils in the field stay alive after 1.5 hours of the experiment. Meanwhile, in Fig. 3 it can be seen that isolated neutrophils are deformed from the beginning, and start to collapse in 30 minutes. Moreover, this difference in viability can be registered by flow cytometry using PI staining (late apoptosis and necrosis estimation) (Fig. 4). Fig. 4a shows that there is a notable number of dead neutrophils in the sample even in the beginning of the experiment. After 1.5 hours of measurements the number of alive cells in the sample decreases from 93 to 88 %. Meanwhile, Fig. 4b demonstrates the presence of 99 % of alive cells in the sample during 1.5 hours of the experiment. Therefore, the investigations of the dynamics of the functional activity of isolated neutrophils appear to be a rather questionable perspective.

## Conclusion

The proposed method of preparing samples of peripheral blood neutrophils is meant for studying by spectral-optical methods and has several advantages over the widely used neutrophil isolation. It provides a higher nativity of conditions for neutrophils, shortens the time between the blood collection and the beginning of the experiment and reduces the stress inevitably arising when neutrophils are isolated from the blood. A higher nativity of conditions (including the presence of the other blood cells) means the closest possible to the physiological microenvironment, which is important when studying the functional activity of these innate immunity cells. Shortening the time between the blood collection and the start of the experiment minimizes the decrease in

the viability of neutrophils, which is observed for this type of cells as early as three hours after blood collection. Reducing the level of stress exposure is achieved by the absence of a standard procedure of isolating neutrophils from the blood and allows virtually eliminating additional mechanical and chemical stress, which also has a beneficial effect on the viability of neutrophils in the resulting sample.

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