Towards optimal neutrophil purification

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Introduction

The challenge of studying neutrophils is their short lifetime and their fragility. Therefore, there is a high demand for a fast and convenient purification protocol of neutrophils from whole blood. Currently, density gradient centrifugation is still the "gold standard" for neutrophil isolation, although it is time consuming and elaborate, and the achieved purities and yields are relatively low. Besides immunomagnetic separation using beads, flow sorting offers a promising alternative. However, it is difficult to sort viable and non-activated neutrophils with conventional droplet-based sorters. The MACSQuant® Tyto® is a microchip-based cell sorter, which requires no sheath fluid and droplet formation, thus applying very low shear forces and no decompression. With these characteristics, the MACSQuant Tyto offers gentle cell sorting conditions and is a well-suited candidate instrument for neutrophil isolation.

Here we compared the sort performance of three techniques for the isolation of human neutrophils from whole blood: i) flow sorting with the MACSQuant Tyto, ii) magnetic separation with the MACSxpress® Neutrophil Isolation Kit, and iii) Percoll® density gradient centrifugation. We analyzed viability, functionality, and activation status of the cells.

Results

1 Neutrophil separation

Human neutrophils were separated from whole blood with the three different methods. In the MAC SQuant Tyto sorting strategy, neutrophils were stained with CD45-APC and CD16-PE-Vio® 770, and SSC^{hi}CD16^{+} cells were sorted. The MACSxpress Neutrophil Isolation Kit is based on the depletion of non-neutrophil leukocytes by a cocktail of magnetic beads coupled to specific antibodies and the simultaneous sedimentation of erythrocytes. These two methods were compared to a standard density gradient centrifugation protocol, which is composed of two steps. First, Histopaque® 1119 medium is used to separate granulocytes from PBMCs and erythrocytes. A subsequent discontinuous Percoll gradient enables further purification of neutrophils. All isolated neutrophil fractions were analyzed with the MACSQuant Analyzer 10 (fig. 1; a trigger was set on CD45-APC and viable cells among CD45^{+} cells were gated. Neutrophils were identified as SSC^{hi}CD16^{+} cells).

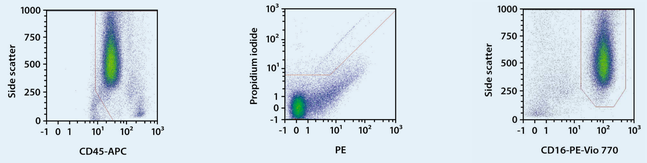

Figure 1

2 Determination of the optimal sort performance

In order to compare the different purification methods, we developed a metric to evaluate the sort performances with regard to the number of sorted cells and cell purity, as well as the time it took to process the sample (fig. 2B). With this sort performance index (SPI) it is now possible to quickly compare distinct experimental setups, different cell sorting instruments, or even different sorting technologies[1].

With a standard Percoll density gradient centrifugation, the achieved purity was around 93%, but only 30% of the target cells could be recovered and the protocol took around 1–1.5 h to complete (table 1). The MACSxpress Neutrophil Isolation Kit allowed a high cell purity and yield, based on a 20-minute protocol. Neutrophils that were sorted with the MACSQuant Tyto showed a comparable purity. The yield however was lower and the separation time was around 1.5 h. Therefore, MACSxpress Separation had the highest SPI, whereas the SPIs of density gradient centrifugation and MACSQuant Tyto were in a similar range. Nevertheless, the MACSQuant Tyto protocol is much easier to perform, smaller blood volumes are needed, and higher purities can be achieved.

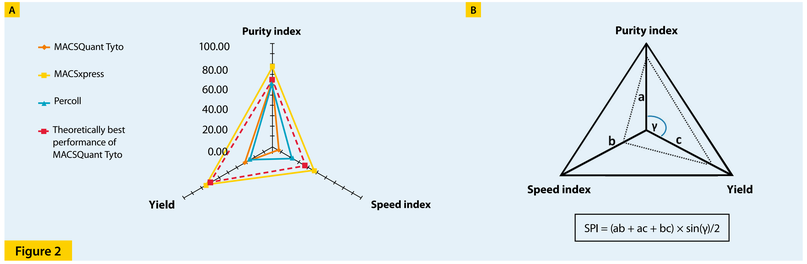

Figure 2

	MACSQuant Tyto	MACSxpress	Percoll
Purity (%)	95	97	93
Yield (%)	31	75	25
Processed cells/min	1.31×10^5	9.00×10^5	4.03×10^5
Speed index*	7	47	22
Purity index**	65	78	59
Sort performance index	34	66	35

* Speed index = (Mean processed cells/min)/(Max. processed cells/min)
** Purity index = [2 × log (100 – mean purity)] × 50

Table 1

3 Cell viability and activation status

Cell viability was assessed by using annexin V as a marker for apoptotic cells and PI as a marker for dead cells. After 4 h of incubation in medium at 37 °C and 5% CO_2, neutrophils isolated with MACSxpress Technology showed the highest viability, followed by cells sorted with the MACSQuant Tyto. Neutrophils that were isolated with the density gradient showed the highest percentages of apoptotic and dead cells (fig. 3A). In addition, different activation markers were analyzed. Fresh whole blood was used as negative control and PMA-stimulated, heparin-treated or EDTA-treated whole blood samples were used as positive controls. There was no up-regulation of CD11b (fig. 3B), CD66b (fig. 3D), and CD63 (fig. 3E) in neutrophils isolated with MACSxpress Technology and only minimal activation in cells isolated with the MACSQuant Tyto or density gradient compared to the negative controls. In addition, no down-regulation of CD62L could be detected in neutrophils isolated with MACSxpress Beads (fig. 3C), and only a slight decrease in the MFI could be detected in cells that were obtained using a density gradient or the MACSQuant Tyto.

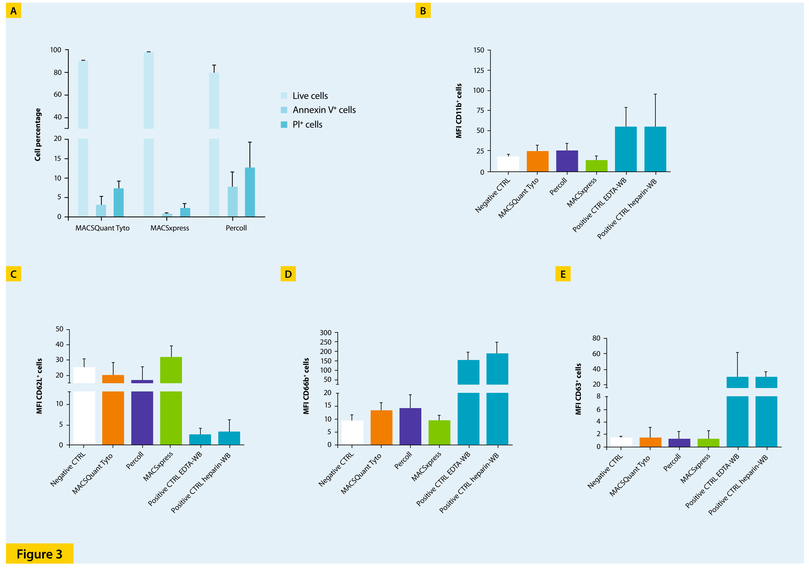

Figure 3

4 Cell functionality

The ability to phagocytose particles and to produce reactive oxygen species (ROS) are hallmarks of neutrophils. Thus, separated neutrophils were incubated with pHrodo™ Red *E. coli* BioParticles® and the percentage of cells that were able to phagocytose these particles was analyzed (fig. 4A). Although the percentage of phagocytic cells among isolated neutrophils was slightly lower than in the control, neutrophils isolated with all three techniques clearly showed phagocytic activity.

ROS generation was assessed by stimulating the cells with *E. coli*, fMLP, or PMA in the presence of the fluorogenic substrate DHR123, which is cleaved into Rhodamine 123 in the presence of an oxidative burst (fig. 4B, top). All isolated neutrophils showed a low oxidative burst after fMLP and a high oxidative burst after PMA stimulation. The enzymatic activity was comparable to the control (fig. 4B, bottom). The ability to migrate along an fMLP gradient was assessed in a 2D chemotaxis assay by using slides from Ibidi®. Figure 4C shows the traces of cell migration towards a 1 µM fMLP source after 30 minutes. Cell migration was recorded with an IN Cell Analyzer microscope with a 10× objective. Frames were captured every 30 s.

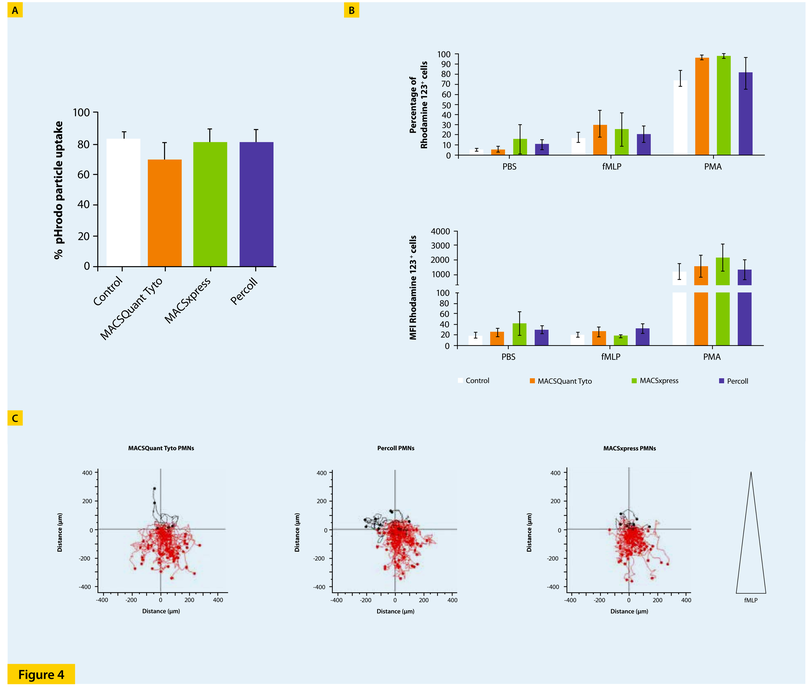

Figure 4

Conclusion

- The MACSxpress Neutrophil Isolation Kit shows the best sort performance with regard to cell purity and yield and sorting speed. Cells displayed a high viability and preserved functionality.
- Both magnetic cell separation and fluorescence-based flow sorting of neutrophils with the MACSQuant Tyto offer a more convenient and sensitive purification protocol than the current gold standard, i.e., density gradient centrifugation.
- A combination of the MACSxpress Neutrophil Isolation Kit and a subsequent sort on the MACSQuant Tyto could provide a fast method to isolate neutrophil subsets.

References

1. A. Bartholomäus *et al.* (2015) Proposal for a Sort-Performance Index. Poster Cyto 2015, Glasgow, Scotland.