BECKMAN COULTER
Life Sciences

Red Blood Cell (RBC) Microparticle Analysis by Flow Cytometry

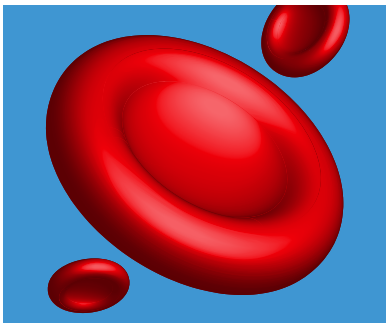
Data kindly provided by Albert Donnenberg, PhD, University of Pittsburgh Cancer Center.

Introduction

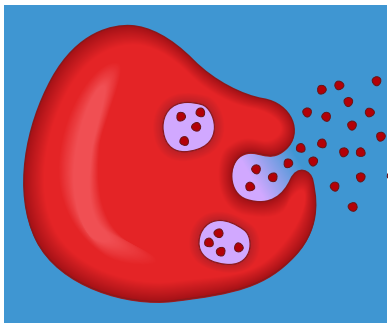
Storage of blood products is a medical necessity, ensuring the safety and availability of human blood for patient transfusion. Longer term storage of RBCs is associated with poorer patient outcomes which are thought to be due to the presence of bioactive exocytic microparticles.[1] Until recently, detecting and quantitating microparticles has been difficult, due to their size.

Objectives

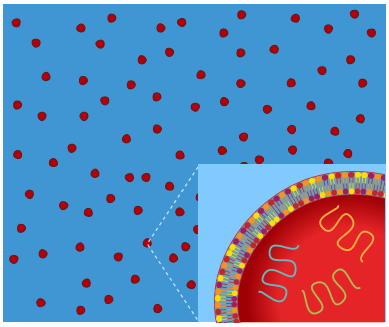
- Learn how to prepare microparticles from red blood cells
- Discover how to stain microparticles for flow cytometric analysis
- Investigate RBC microparticles by flow cytometry



Panel A



Panel B



Panel C

Stage	Glycophorin A	Annexin V
Intact Non-erythroid Cells	-	+/-
Intact RBCs	+	+/-
Transitional Events	+	+
RBC Microparticles	+	+
Non-RBC Microparticles and Extracellular Vesicles	-	+/-

Panel D

Figure 1. The stages of RBC microparticle formation can be analyzed with immunochemistry. Mature, intact RBCs are anucleate, biconcave discs (Panel A), though they lose biconcavity with the progression of apoptosis and hydrolysis. The structural integrity of cells undergoing apoptosis and hydrolysis is compromised, caused in part by the depolymerization of the cytoskeleton (not shown). RBCs that have lost biconcavity are here referred to as "transitional events" — a state that occurs between RBCs and RBC microparticles (Panel B). RBC microparticles (Panel B, red dots) are small, membrane-bound vesicles released by RBCs and transitional bodies, presumably as a form of extracellular communication. Most microparticles contain bioactive molecules from their cells of origin, including proteins, lipids, and nucleic acids. The lipid bilayers of apoptotic RBCs, transitional events, and RBC microparticles isolated from stored blood products do not exhibit leaflet asymmetry of phosphatidylserine, due to the onset of apoptosis and activation of scramblases (Panel C, red phospholipid). RBCs, transition events, and microparticles isolated from stored blood products can be identified and categorized by their immunochemical profiles, when stained with anti-glycophorin A and anti-annexin V antibodies (Panel D).

Microparticles are small (50 – 1,000 nm) membrane-bound vesicles that can contain protein, DNA, or RNA, and are thought to be a form of intercellular communication.[2] Research into microparticles, like exosomes, has garnered more attention as methods for analyzing their cargoes have become more robust, and as microparticles and nanoparticles have started gaining traction as biomarkers of disease and as therapeutic delivery systems. The most commonly used method for isolating microparticles for analysis is differential ultracentrifugation, which relies on spins at several different speeds to produce a pure fraction of vesicles of the desired size, without extraneous and off-target particles.

Microparticles can be analyzed with several different methods, including nanoparticle tracking analysis, electron microscopy, and flow cytometry, with flow cytometry offering the highest throughput and least hands-on time. Flow cytometry is based on the interpretation of light-scattering patterns as individual particles pass through a beam of laser light. Larger or more complex particles cause more scatter, allowing for size discrimination. The lower-limit of size discrimination is based on the wavelength of the light used, making violet-laser flow cytometers capable of detecting smaller particles than previously possible. Nanoparticles, including exosomes, can be stained for the presence of certain proteins and phospholipids, which are also detected during their transit through the flow cell.

With the understanding that micro - and nanoparticles mediate many important biological responses, it is vital to accurately quantitate and fully characterize these particles. To demonstrate the capabilities of the CytoFLEX Flow Cytometer to resolve small particles (>150 nm), analysis of Red Blood Cell (RBC) microparticles from banked human peripheral blood was performed. Human blood samples, stored under standard blood-banking conditions for three months, were double stained with Glycophorin A (to identify RBCs and RBC-membrane vesicles) and Annexin V (to identify apoptotic cells and their vesicles); this double-labeling scheme ensured that the analyzed cells and microparticles were, in fact, of erythrocyte origin. Forward - and side-scatter analyses revealed the presence of RBC microparticles, transition events, and intact red blood cells in the stored blood sample – indications of compromised blood quality.

Materials

- Packed, non-leukoreduced RBC units (Central Blood Bank, Pittsburgh, PA) preserved with ADSOL® (Fenwal, Inc., Lake Zurich, IL)
- PE-Cy5-conjugated Glycophorin A (BD Pharmingen, San Jose, CA) Cat. # 340947
- Annexin V FITC Kit (Beckman Coulter, Brea, CA) Cat. # 55674
- CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA)

Protocol

1. Remove the units of packed RBCs from the refrigerator (1 to 6 °C).
   NOTE: Refer to detailed sample handling procedures outlined in reference 4 to avoid introducing analysis artifacts.
2. For each unit of packed RBCs, aliquot 5 µL of RBCs into a tube.
3. Add 2 µL of PE-Cy5-conujugated Glycophorin A to each of the tubes.
4. Incubate for 30 minutes at room temperature; Keep the tubes away from light.
5. Add 2 µL of FITC-Annexin V to each tube, followed by 500 µL of Annexin V Binding Buffer, per kit instructions.
6. Incubate for 30 minutes at room temperature; Keep the tubes away from light.
7. Analyze samples in triplicate by flow cytometry on the CytoFLEX Flow Cytometer*:
   - Acquire at least 100,000 events per sample (e.p.s.), at event rates not exceeding 10,000 e.p.s.
   - Set forward scatter (FSC) and side scatter (SSC) gates to log scale.
   - Refer to the gating strategy detailed in Figure 1.
8. Quantify microparticles as a percent of Glycophorin A-positive events.

*For details on the setup and correct use of the CytoFLEX Flow Cytometer, refer to the application note, Set-Up of the CytoFLEX for Extracellular Vesicle Measurement.

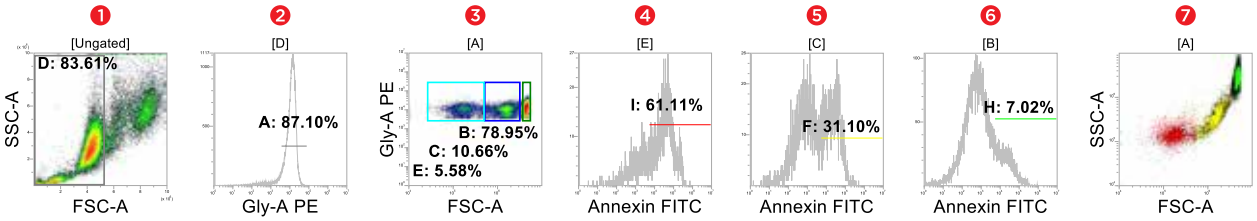


Figure 2. Gating strategy for RBC and derivative populations. Each panel (1-7, left to right) demonstrates the sequential gating strategy for analyzing RBC and derivative populations with glycophorin A. Clusters of RBCs are excluded from the gate (panel 1), and glycophorin A-dim events are excluded (panel 2). The glycophorin A-positive events in the forward-scatter channel (FSC) are then binned into low, intermediate, and high populations (panel 3). Annexin V staining is assessed in each of the three bins of glycophorin A-positive events (panel 4: low; panel 5: intermediate; panel 6: high), and panel 7 depicts the pseudocolored events from the Annexin V histograms overlaid on a dotplot of FSC X SSC, displaying RBC microparticles (red), transitional events (yellow), and intact RBCs (green). Figure 2 represents a single run.

Results and Discussion

Across the three units of packed RBCs, the majority of glycophorin-positive events demonstrated high forward scatter (~82%), suggesting the presence of relatively large particles, while a smaller group of events (~10%) demonstrated low forward scatter, consistent with smaller particles. An intermediately-sized group of particles accounted for ~5% of the events. (See Table 1, columns "Glycophorin A Positive, Low FSC" through "Glycophorin A Positive, High FSC"). The large particles are likely intact RBCs, as glycophorin A is a specific marker of erythrocyte lineage, while the small particles are likely RBC microparticles. The intermediate particles are, therefore, transitional events – meaning that they are the state that occurs as an RBC begins hemolysis and releases microparticles.

Recently published evidence supports the use of Annexin V as a marker of apoptotic microvesicles for the study of peripheral blood quality and degradation under a variety of storage conditions.[4] Annexin V, which binds with the cell-membrane component phosphatidylserine on apoptotic cells, identifies the low-FSC population as being storage-related microparticles, while the intermediate-FSC population is identified as being storage-related transitional events, and the high-FSC population is identified as being apoptotic-but-intact RBCs. For both Glycophorin A- and Annexin V-based data, the Coefficients of Variation (CVs) were acceptably low for the population of microparticles and the population of intact RBCs. (See Table 1, columns "Microparticles" through "Intact RBCs")

Table 1. Flow cytometric analysis of RBC microvesicles by CytoFLEX Flow Cytometer. Samples were run in triplicate. Inter-replicate precision is measured by CV.

RBC Sample	Number of Detected Events	Glycophorin A Positive, Low FSC	Glycophorin A Positive, Intermediate FSC	Glycophorin A Positive, High FSC	Microparticles	Transitional Events	Intact RBCs
1A	84,010	11.2%	1.0%	86.4%	63.0%	18.9%	7.6%
1B	83,365	11.7%	2.6%	83.7%	59.0%	19.6%	7.4%
1C	82,680	11.0%	4.4%	81.8%	61.9%	17.5%	7.2%
Mean	**83,352**	**11.3%**	**2.7%**	**84.0%**	**61.3%**	**18.7%**	**7.4%**
SD	**665**	**0.4%**	**1.7%**	**2.3%**	**2.1%**	**1.1%**	**0.2%**
CV	**0.8%**	**3.2%**	**64.1%**	**2.7%**	**3.4%**	**5.7%**	**2.5%**
2A	84,586	9.7%	1.8%	86.2%	55.9%	15.6%	5.2%
2B	82,371	9.2%	0.4%	88.5%	57.0%	31.0%	5.7%
2C	85,298	8.6%	1.9%	87.1%	54.8%	15.1%	4.8%
Mean	**84,085**	**9.2%**	**1.4%**	**87.3%**	**55.9%**	**20.6%**	**5.2%**
SD	**1,526**	**0.5%**	**0.9%**	**1.1%**	**1.1%**	**9.0%**	**0.4%**
CV	**1.8%**	**5.8%**	**62.6%**	**1.3%**	**2.0%**	**43.8%**	**8.4%**
3A	79,488	6.8%	9.4%	79.3%	56.8%	35.0%	7.2%
3B	73,871	5.6%	10.7%	79.0%	61.1%	31.1%	7.0%
3C	79,430	7.0%	13.7%	72.1%	54.2%	23.7%	7.1%
Mean	**77,596**	**6.4%**	**11.2%**	**76.8%**	**57.4%**	**29.9%**	**7.1%**
SD	**3,226**	**0.8%**	**2.2%**	**4.0%**	**3.5%**	**5.7%**	**0.1%**
CV	**4.2%**	**11.7%**	**19.6%**	**5.3%**	**6.1%**	**19.1%**	**1.2%**

Without centrifuging the blood sample, it is reasonable to expect a large percentage of intact RBCs in the RBC units after three months of refrigerated storage. However, the percentage of particles identified as microparticles indicates that the cells in storage were releasing exocytic microparticles, either before or on the way to hemolysis. The functional significance of these RBC-derived microparticles has been studied, and the microvesicles are implicated in poor patient prognoses.[1]

Tips for success

- This protocol does not require ultracentrifugation
- To minimize photobleaching of PE and FITC conjugates, perform all incubation steps in the dark
- Preservation of RBCs for varying amounts of time may yield more/fewer microparticles

Conclusions

Banking blood is medically necessary, and as the need for human blood products increases, the strain on the blood supply will also increase. This evidence, generated with the CytoFLEX Flow Cytometer, indicates that RBCs stored under refrigeration have a finite usable lifespan, after which the cells release microparticles which may adversely impact patients. Detecting these >150 nm microvesicles with flow cytometry is a true advance in the field, and enables deeper research into the genesis of bioactive microparticles and methods to prevent the deterioration of the banked blood supply.

Notes

The results shown here represent data generated on the Beckman Coulter CytoFLEX Flow Cytometer.
Due to differences in the performance of makes and models of flow cytometers, the authors cannot guarantee similar results with the use of other flow cytometers.

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