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International Journal of Biotechnology and Bioengineering

ISSN 2475-3432

Research Article Open Access

Biosensing of Red Blood Cell-derived Extracellular Vesicles with the advanced Bright-Field light Optical Polarization Microscopy

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Citation: Alexander E. Berezin et al. (2017), Biosensing of Red Blood Cell-derived Extracellular Vesicles with the advanced Bright-Field light Optical Polarization Microscopy. Int J Biotech & Bioeng.3:3, 61-65. DOI: 10.25141/2475-3432-2017-3.0061

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Received: March 13 , 2017; **Accepted:** March 22, 2017; **Published:** April 29, 2017

Abstract:

Background: Red blood cell (RBC)-derived extracellular vesicles (EVs) are recognized a sensitive predictive biomarker of cardiovascular risk, which allow distinguishing vulnerable population from healthy and also physiological aging from premature one. Current known methods of determination of RBC-EVs are based on several principles including flow cytometry, culture methods and various visualization techniques (surface plasmon resonance and computer tomography / magnetic resonance imaging), and various types of microscopy, i.g. high sensitive optical coherent microscopy (hs-OCM), atomic microscopy, fluorescent microscopy. However, there are some limitations and broad variabilities in cost of mentioned above methods of EV-determination.

The aim of the study was to compare a capture ability of conventional hs-OCM and advanced bright-field light optical polarization microscopy in detection and measurement of the RBC-EVs.

Methods: The study was retrospectively evolved 26 patients with established stable coronary artery disease who were examined between May 2015 and November 2016. The samples of whole blood were collected before ingestion of the meal at room temperature at the morning with powder-free gloves. We used a conventional hs-OCM and advanced bright-field light optical polarization microscopy with improved capture features. Conventional hs-OCM and advanced bright-field light optical polarization microscopy was performed with an Olympus® BH-2 microscope (Olympus, Japan). The whole sample was scanned with the Zeiss 10x objective (100x). The monochromatic laser was used to emanation of light with an appropriate wavelength. It has been identified number and relative size distribution of RBC-EVs with further analysis of morphology using original soft.

Results: hs-OCM images supported by yellow-green light allow visualizing cell-free EVs without possibilities for assay their structure and measurement of their number. In contrast, ultraviolet light-enhanced hs-OCM is able to improve capture features of RBC-EVs including their number, diameter and roughly structure. Using advanced bright-field light optical polarization microscopy associated with original soft allows distinguishing low-contrasted objects in details when we used monochromatic light with λ=370+30 nm with further math modelling.

Conclusion: the advanced bright-field light optical polarization microscopy allows detecting clinically relevant properties of EV in wide ranges and could be determined a new much promising technique, which allows assaying EV in low cost

Keywords: Extracellular Vesicles; Red Blood Cells; Measurement; Biosensing; Optical Polarization Microscopy

Introduction:

Red blood cell (RBC)-derived extracellular microvesicles (EVs) are recently recognized key regulators of cell-to-cell cooperation, blood cell function, coagulation, and probably inflammation, proliferation and tissue repair [1, 2]. Recent clinical studies have shown that the elevated circulating number of RBC-EVs has found in several cardiovascular diseases including acute coronary syndrome / acute myocardial infarction, pulmonary thromboembolism, acute and chronic heart failure, fibrillation [3-5]. Additionally, a wide range of other diseases associated with coagulopathy, thrombosis, anemia (i.e., infections, shock, respiratory distress syndrome, bleeding, vasculitis, preeclampsia / eclampsia, antiphospholipid syndrome, HELP-syndrome, malignancy, rheumatic diseases, etc.) is expressed higher circulating level of RBC-EVs due to activated secretion or increasing RBC debris [6]. Nowadays there is a large body of evidence regarding that the RBC-EVs' number could be useful circulating predictive biomarker of clinical outcomes in critical ill patients, individuals with cancer, established CV, rheumatic, autoimmune and kidney diseases [7-9].

Nowadays conventional transmitted light microscopy technique is useful and simple method to determine particle size, shape and structure [10, 11]. A highly sensitive optical coherent microscopy (hs-OCM) based on objective-type internal reflection regarding wavelength-modulation may sufficiently improve RBC-EV determination. Although hs-OCM technique has a serial limitations for data interpretation predominantly relate to use of light dose [12], this method may visualize RBC-EVs with higher accuracy and measurement limit of 40 nm [13]. To improve a capture ability of hs-OCM to detect RBC-EVs advanced bright-field light optical polarization technique might be used.

The aim of the study was to compare a capture ability of conventional hs-OM and advanced bright-field light optical polarization microscopy in detection and measurement of the RBC-EVs.

Methods

The study was retrospectively evolved 26 patients with established stable coronary artery disease (positive contrast-enhanced multispiral tomography angiography and determination of stable angina pectoris according contemporary clinical guideline [14]) who were examined between May 2015 and November 2016. All patients have given their informed written consent for participation in the study. The study was approved by the local ethics committee of State Medical University, Zaporozhye, Ukraine. The study was performed in conformity with the Declaration of Helsinki

Blood collection and storing

Blood samples were collected before ingestion of the meal at room temperature at the morning with powder-free gloves (Latex, soft-hand). We optionally used "Vacutainer sets" (BD, Europe) with 22 gauge needles and 3ml plastic syringes to collect 2ml of the whole blood in the plastic tubes with EDTA. The first 2ml of blood where discarded to prevent RBC-MC shaping due to microvascular damage. Finally we chose a 0.1ml of whole blood placed on one microscope slide with a cover slip for light microscopy examination. We used a conventional hs-OCM and advanced bright-field light optical polarization microscopy with improved capture features and original soft for further analysis of images.

Optical microscopy

Conventional hs-OCM and advanced bright-field light optical polarization microscopy was performed with an Olympus® BH-2 microscope (Olympus, Japan). The whole sample was scanned with the Zeiss 10x objective (100x). Only glass particles or micro-bubbles made the distinction difficult. In these cases we used the 40x Zeiss objective (400x) to be beyond doubt. RBC-EVs were determined as intra RBC-shaping vesicles with diameter less 400 nm. On the actual step of optical detection of the RBC-EVs we identified their number and relative size distribution, although the methods allowed determining the morphology as mean shape and ultrastructure and measure the concentration of using original soft called advanced highly dynamic resolution capture system(R).

Advanced bright-field light optical polarization microscopy

RBC-EVs could be identified by their formation in RBCs in various polarized lights. The limit of detection was 10 nm. We found an optimal reflected tight attachment that sufficiently expands scope of research through flexible combinations of polarizing light with various wavelengths and considerably simple switchover of multiple observation method. The monochromatic laser was used to emanation of light with an appropriate wavelength. All measurements were done as blinded duplicative performed by independent researchers.

Statistical Analysis

Statistical analysis of the results obtained was performed in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). The data were presented as median (Me) and 25%-75% interquartile range (IQR). To compare the main parameters of patient cohorts Mann - Whitney U-test were used. The intra assay and inter assay coefficients were calculated. A two-tailed probability value of <0.05 was considered as significant.

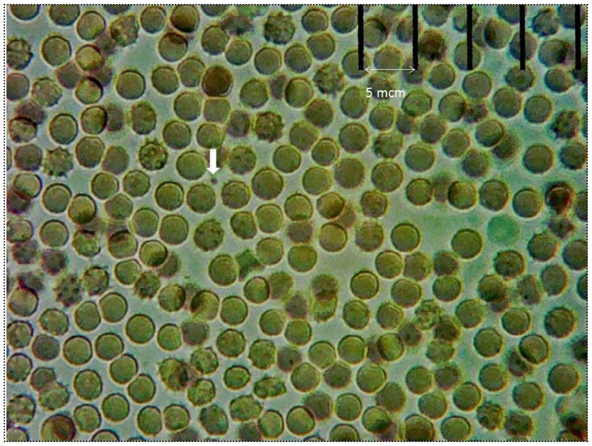

Figure 1a: Determination of cell-free EVs and EVs (arrows) at the stage of active secretion by RBCs in yellow-green (λ=560-580 nm) light using hs-OCM.

Results

Figure 1a and 1b are reported hs-OCM images of mono-layered whole blood sample received from the patients. One can see cell-free EVs and EVs at the stage of active secretion by RBCs in yellow-green (λ=560-580 nm) and ultraviolet (λ=450-470 nm) lights (Fig 1a and Fig 1b respectively). The difference between both images affects capture ability regarding determination of RBC-EVs. Indeed, hs-OCM images supported by yellow-green light allow visualizing cell-free EVs without possibilities for assay their structure and measurement of their number. In contrast, ultraviolet light-enhanced hs-OCM is able to improve capture features of RBC-EVs including their number, diameter and roughly structure. Consequently, contemporary used hs-ECM is not able to distinguished cell-free RBC-derived EVs in blood samples and does not maintain much more pretty accurate capture ability to determine RBC-EVs from debris. As a result, the prompt to calculate the number of cell-free RBC-derived EVs using a math model based on data received from hs-ECM-enhanced method may lead to increased falsely-positive results.

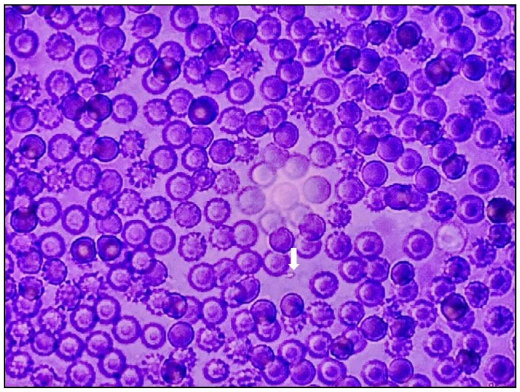

Figure 1b: Determination of cell-free EVs and EVs (arrows) at the stage of active secretion by RBCs in violet (λ=450-470 nm) light using hs-OCM.

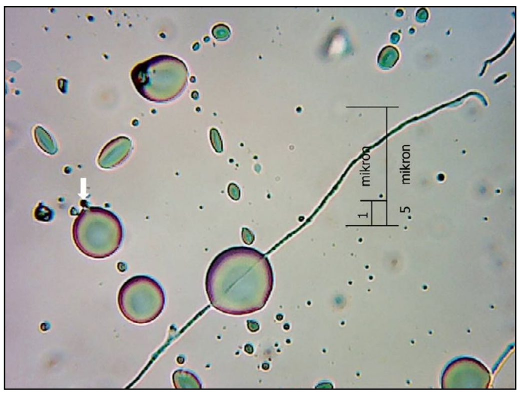

Figure 2a: Determination of cell-free EVs and EVs (arrows) using advanced bright-field light optical polarization microscopy with monochromatic ultraviolet (λ=370+30 nm)

Using advanced bright-field light optical polarization microscopy associated with original soft allows distinguishing low-contrasted objects in details when we used monochromatic light with λ=370+30 nm (Fig 2a). At the figure 2b we can see EVs with

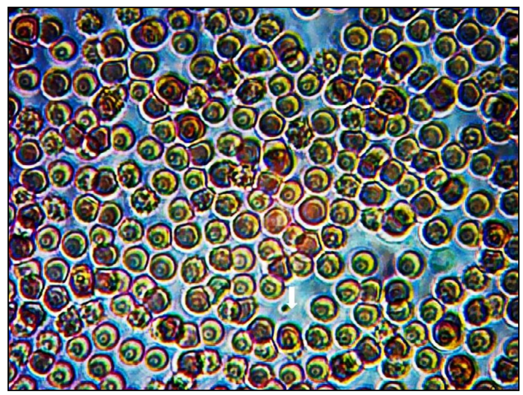

Figure 2b: Determination of cell-free EVs and EVs (arrows) using advanced bright-field light optical polarization microscopy after math modelling

Table 1: Scattering of diameter of RBC-derived EVs measured by hs-OCM and advanced bright-field light optical polarization microscopy

Features	hs-OCM		Advanced bright-field light optical polarization microscopy		P value
	Me	25%-75% IQR	Me	25%-75% IQR	
Diameter of entire RBC-derived EV population, nm	392	202 - 588	336	96 - 562	<0.012
Diameter of cell-free EV, nm	-	-	115	45 - 244	<0.001
Diameter of none cell-free EV, nm	-	-	305	149 - 537	<0.001

Abbreviation: Me, median; IQR, interquartile range diameter less 1 mcm secreting by RBCs. However, cell free RBC-EVs and cell debris could not be distinguished with the method, although the dynamic diapason of the advanced polarization microscopy was higher than this that was achieved through hs-ECM. Additionally we consequently applied ultraviolet emanation with high sensitive polarized capture through original soft to construct the image with improved contrasted features suitable for analysis of shaping, number and structure of RBC-EVs (Fig 2b).

Scattering of EV diameter measured by both methods is reported in Table 1. We found that advanced bright-field light optical polarization microscopy with original soft is able to distinguish as none-free RBC-derived EVs as well as RBC-free EVs in pretty broad ranges of diameter. Therefore the intra assay and inter assay coefficients which were determined as the standard deviation of a set of measurements were found as 7.6% and 9.5% respectively.

Discussion

In the study we first found that the advanced bright-field light optical polarization microscopy could be an alternate free-labeled optical method for quantified measured of sizes and size-related characteristics of EVs derived from RBCs. Increased resolution of new method of optical microscopy is based on interaction of polarized light with thin film of monolayer of whole blood with further mathematical analysis of image through mutual superposition each next layer over previous one. All these relate to sufficient increased capture ability and dynamic diapason extension in a way of use the same optical magnification. This is an essential advantage of advanced bright-field light optical polarization microscopy in comparison with conventional hs-OCM [15, 16]. Therefore, technically we have confirmed that the advanced bright-field light optical polarization microscopy exhibited pretty potential to accurately obtain all clinically relevant properties of single EV at a high speed, although reproducibility requires more investigations in future. Created by us mathematic model has now implemented into original soft that allow managing number and diameter of even small-sized EVs with higher sensitivity. Finally, advanced bright-field light optical polarization microscopy is a low cost method of real time detection of both types of EVs derived from RBCs (cell-free and none ell free).

These evidence may have a serious advantages regarding low depending on capture features and quality of blood sample preparation and refractive index of material that are considerable critical for conventional hs-OCM [17, 18]. It is well known that the quantity of light scattered by a single EV is proportional to the diameter of one that should optimally be at least 10 times smaller than the wavelength [19]. Therefore, the value of refractive index of prepared samples is critical to distinguish very variable in their diameter EVs [20, 21]. Indeed, recent studies have shown that the scattering intensity from EVs is periodically modulated by shifting the intensity fringes of the standing evanescent field. It has been postulated that to improve measuring contrast of scattering intensity variation during one cycle of modulation, particle sizes could be estimated easily [21, 22]. We have overcome these obstacles using original soft especially created for new method of polarized optical microscopy, which allows improving contrast of previously unrecognizable objects. Thus, we suggest that our method proposing first for easily determination of EV in pretty wide rages could be deserved further investigations.

Limitations and future directions

There is need to merge sensitivity, selectivity and reproducibility of final detection of EVs using new method of advanced bright-field light optical polarization microscopy with further comparison with other routinely used analytical methods, i.e. flow cytometry and magnetic resonance technology. However, biosensing of EVs with advanced bright-field light optical polarization microscopy requires standardization and more investigations in field of quality of measurements.

In conclusion, advanced bright-field light optical polarization microscopy allow detecting clinically relevant properties of EV in wide ranges and could be determined a new much promising technique, which allows assaying EV in low cost.

Conflict of interests

Authors declare no conflict of interest.

Acknowledgments

We thank all patients for their participation in the investigation, staff of the Regional Zaporozhye Hospital (Ukraine), and the doctors, nurses, and administrative staff in Regional Center of cardiovascular diseases (Zaporozhye, Ukraine) and Private Clinic "Vita Center" (Zaporozhye, Ukraine), general practices, and site-managed organizations that assisted with the study. We also thank the staff of the Technical Department of the Zaporozhye National Technical University (Zaporozhye, Ukraine) for preforming EV measurements and technical assistance.

Legends for figures

Abbreviations: hs-OCM, high sensitive optical coherent microscopy; EVs, extra vesicles; RBCs, red blood cells.

Notes: dimension between both nearly arranged lines on a top of the figure is 5 micrometers

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