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DARKFIELD MICROSCOPY (DFM) IN THE EVALUATION OF EXTRACELLULAR VESICLES

MICROSCOPIA DE CAMPO ESCURO (MCE) NA AVALIAÇÃO DE VESÍCULAS EXTRACELULARES

MICROSCOPÍA DE CAMPO OSCURO (DFM) EN LA EVALUACIÓN DE VESÍCULAS EXTRACELULARES

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ABSTRACT
Extracellular vesicles (EVs) are particles ranging from 30 to 5000 nm, considered mediators of intercellular communication and biomarkers of distinct types of diseases. EVs are released by most cells, including tumor cells. Therefore, research to better understand its role in the development and progression of neoplasms has been increasing every year. For such, these studies require high-tech equipment, available in highly complex research laboratories. This project sought to standardize dark-field microscopy (DFM) as a simple, effective, low-cost method to assist in research with EVs. Three samples were acquired, each one containing extracellular vesicles properly classified by size (400, 100, and 50 nm). Each one of them was analyzed by using the DFM to determine resolution capability and maximum magnification.

KEYWORDS: Cancer. Exosomes. Microscopy.

RESUMO
As vesículas extracelulares (EVs) são partículas que variam de 30 a 5000 nm, consideradas mediadoras da comunicação intercelular e biomarcadoras de diversos tipos de doenças. EVs são liberados pela maioria das células, incluindo células tumorais. Por isso, pesquisas para entender melhor seu papel no desenvolvimento e progressão das neoplasias vem aumentando a cada ano. Para tanto, esses estudos requerem equipamentos de alta tecnologia, disponíveis em laboratórios de pesquisa de alta complexidade. Este projeto buscou padronizar a microscopia de campo escuro (DFM) como um método simples, eficaz e de baixo custo para auxiliar na pesquisa com VEs. Foram adquiridas três amostras, cada uma contendo vesículas extracelulares devidamente classificadas por tamanho (400, 100 e 50 nm). Cada uma foi analisada usando o DFM para determinar a capacidade de resolução e ampliação máxima.

PALAVRAS-CHAVE: Cancro. Exossomas. Microscopia.

RESUMEN
Las vesículas extracelulares (VE) son partículas de entre 30 y 5000 nm, consideradas mediadoras de la comunicación intercelular y biomarcadores de distintos tipos de enfermedades. Las VE son liberadas por la mayoría de las células, incluidas las tumorales. Por ello, la investigación para comprender mejor su papel en el desarrollo y la progresión de las neoplasias ha ido aumentando cada año. Para ello, estos estudios requieren equipos de alta tecnología, disponibles en laboratorios de investigación de gran complejidad. Este proyecto buscó estandarizar la microscopía de campo oscuro (DFM) como un método simple, efectivo y de bajo costo para ayudar en la investigación con EVs. Se adquirieron tres muestras, cada una de ellas con vesículas extracelulares debidamente clasificadas por tamaño (400, 100 y 50 nm). Cada una de ellas fue analizada utilizando el DFM para determinar la capacidad de resolución y el aumento máximo.

PALABRAS CLAVE: Cáncer. Exosomas. Microscopía.

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JOURNAL HEALTH AND TECHNOLOGY - JHT
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1. **INTRODUCTION**

A promising study of great value for the diagnosis of oncogenetic pre-staging is the detection and classification of extracellular vesicles (EVs).

Experimental evidence indicates that intercellular communication is facilitated both by direct contact between cells and by secretion of molecules such as hormones, cytokines, and growth factors. However, a third pathway of intercellular communication has come to be studied in the last 20 years: the extracellular vesicles (EVs) [(1)].

EVs are small particles composed of a lipid bilayer, capable of transporting lipid molecules, cytosolic proteins, and RNAs inside (reviewed by XAVIER, 2018) [(13)]. Due to these characteristics, recent studies have demonstrated the relationship between EVs and several infectious and chronic pathologies such as cancer. Patients with these diseases commonly present important levels of these nanostructures in different biological fluids [(2)].

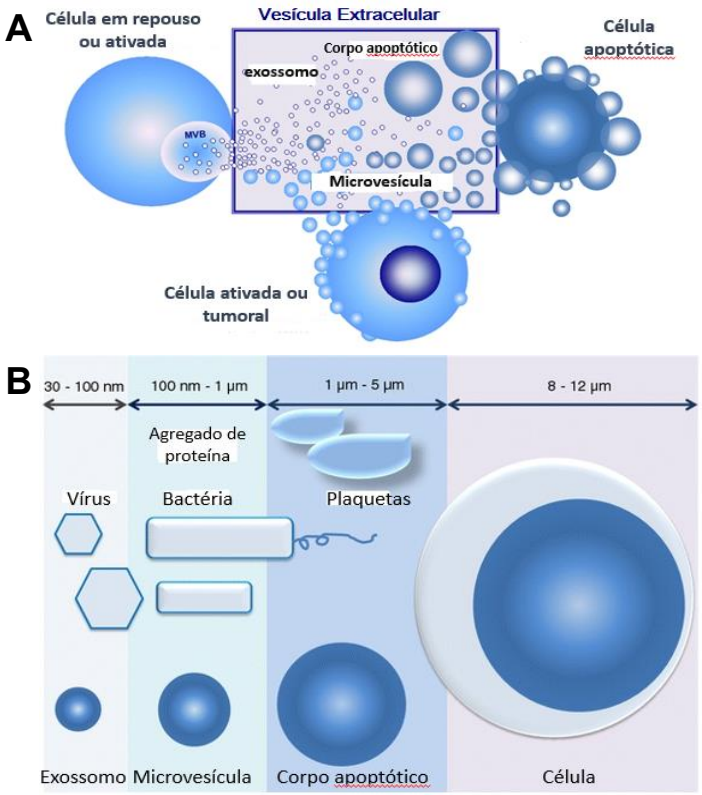
Not very recently, EVs were considered functionless cellular debris [(3)]. However, it is now known that such structures play an important role in intercellular communication. In addition, they are secreted by almost all cell types, including stem/progenitor cells [(1)].

Their biogenesis and size make EVs classified into exosomes, microvesicles, and apoptotic bodies. The exosomes correspond to the group of vesicles with a size between 30-120nm. They are synthesized from the invagination of the plasma membrane forming a primary endosome and followed by the formation of a multivesicular endosome and subsequent release of exosomes. These vesicles have several protein markers, such as fusion proteins (GTPases and Annexins), tretaspanins (CD9, CD63, CD81, CD82), cellular machinery proteins (Alix and TSG101), and other proteins such as Hsc70, HSP90, and phospholipases due to their endosomal origin. They can carry molecules such as RNAs and other proteins not involved in their genesis. On the other hand, microvesicles are structures with a size between 100 and 1000nm, originated by the plasma membrane budding, usually produced in an induced way [(3)].

1.2 Apoptotic bodies

Apoptotic bodies are structures larger than 1000nm released after cell fragmentation during the process of apoptosis. They contain cytoplasmic or nucleolar organelles [(4)].

Figure 1. Characteristics of EVs. Characterization of EVs according to (A) origin and (B) size.

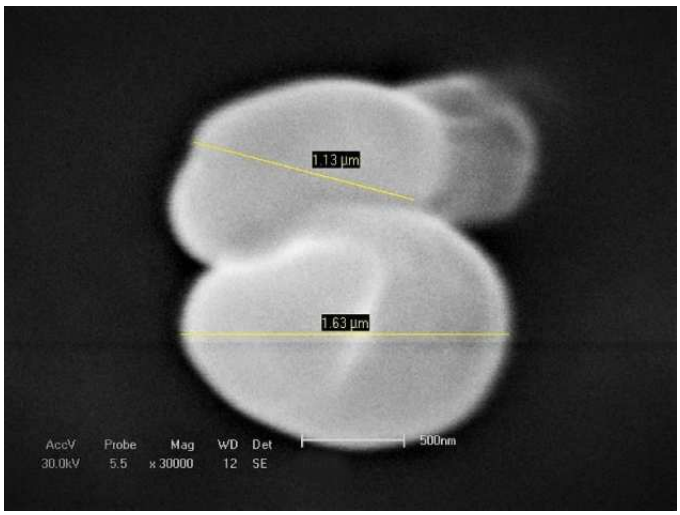


Source: Adapted from Gyorgy (2011)

The identification and classification of EVs represent a great advance in cancer research, especially in the pre-clinical stages. For this reason, important scientific publications point them out as facilitating agents in the metastatic process (reviewed by XAVIER, 2018). While a cell reaches an average size of eight micrometers, extracellular vesicles can vary on average between 120 to 1000 nanometers.

To visualize these structures, it is necessary to use a transmission electron microscope, scanning microscopy, or high-cost technological methods, such as the Cytoflex cytometer by Beckman Coulter and the NanoSight by Malvern Panalytical [(3)].

Figure 2. 30KX Scanning Microscopy with two microsomes.



Source: Author's collection

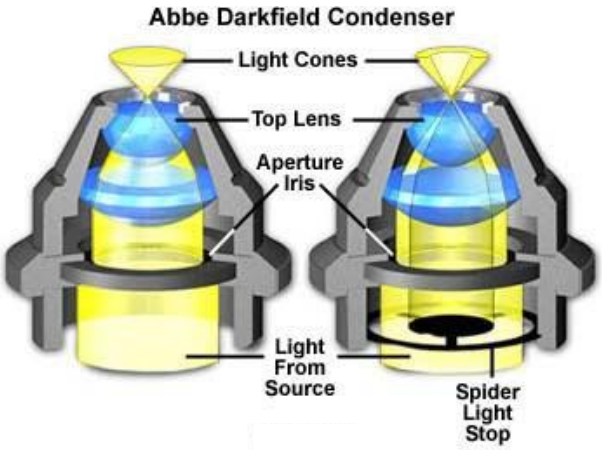
In general, such equipment is used exclusively for diagnostic purposes or for more consolidated research groups. For this reason, it is necessary to use alternative and easily accessible equipment to other research teams at university centers.

The Darkfield Microscope, like every optical microscope, is an equipment designed to produce magnified images of objects that the naked eye would have difficulty seeing. It must fulfill three basic functions: to produce a clear image of the specimen, to highlight details to the human eye, and to ensure the recording of the analyzed subject. The specimen image is enlarged when viewed through a lens. By correctly combining some of these lenses, the microscope will produce considerable image amplification values [(5)].

As for the concept of Darkfield Microscopy (DFM), it is essential to understand the special action of lenses which is ruled by principles of refraction and reflection, and geometric rules [(6)]. The Darkfield works based on the observation of the specimen from the light coming from a condenser. Inside it, more precisely in its substage, there is a stopper that does not allow the light beam to fall directly on the specimen, but in a refractive way. Thus, the light will touch the specimen making it luminous against a dark background, but only a portion of the light will pass obliquely. This light is diffracted, refracted, and reflected into the lens.

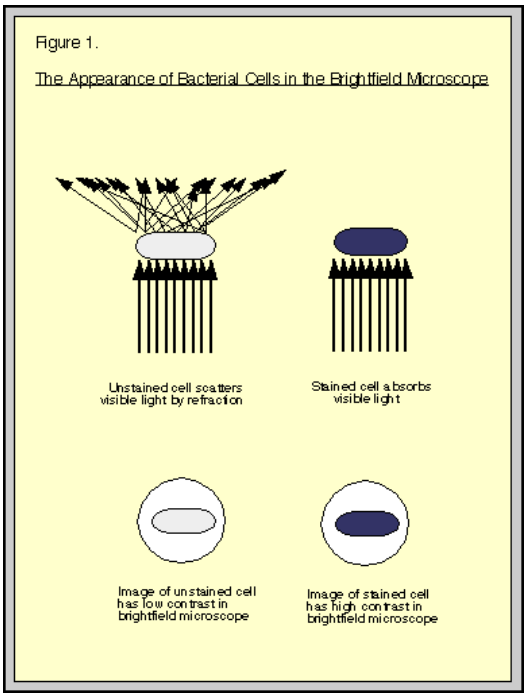
Collimated light allows the visualization of particles at nanometer scales (less than 120nm), without the use of chemical dyes and the need for high magnification. Therefore, this technique is eligible as an alternative to be tested in EVs research [(7)].

Figure 3. On the left is the Brightfield Condenser. On the right is the Darkfield Condenser. The stopper (dark circle) makes the light tangent to the specimen.



Source: Olympus BX 51 Microscope Technical Manual.

Figure 4. Collimation of light. Schematic representation of how light produces the refractive field in the specimen. Stained sample on the left. Contrasted sample photo on the right.



Source: Olympus BX 51 Microscope Technical Manual

2. OBJECTIVES

2.1 Main goal:

- ✓ Standardize the use of Darkfield Microscopy as a fast, easy, low-cost, and effective alternative method for the detection and analysis of extracellular vesicles.

2.2 Specific objectives:

- ✓ To quantify EVs;
- ✓ To determine EVs size and displacement characteristics.

3. MATERIAL AND METHODS

3.1 Previously standardized samples

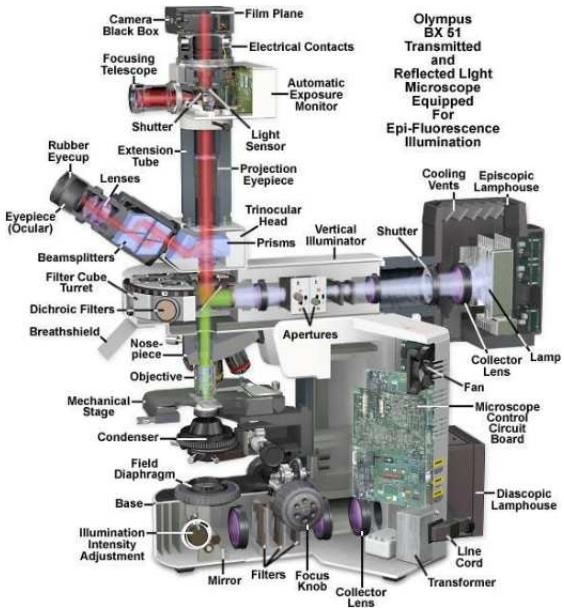
For calibration purposes and to have a comparative parameter, three samples were requested from a company. Each vial of samples contains 30 µg of lyophilized extracellular vesicles, extracted from human plasma, free of cellular debris. They were divided by sizes: 400, 100, and 50 nm. According to the instructions of the product - ref. CAT ESL-25 (Annex 1), the reconstitution of the samples was executed by adding deionized water. Afterward, they were transferred to polypropylene tubes and kept refrigerated for 2 hours.

3.2 Evaluation of the size of the vesicles through the Darkfield Microscope

The experiment used a BX 51 trinocular microscope – Olympus with the following specifications: stand and base of transmitted light BX51TF, halogen light source 100w – U-LH100-3, eyepieces 20X F.N.22, lens PLN 40X 1-U2B227, 100X UPLFLN immersion lens – N.A. 0.60 – 1.30, U-DCW oil/dry Darkfield Condenser. Digital image capture system: 7.1 MPixel macro zoom digital camera – Olympus C-7070W. 4X optical and 5X digital zoom lens = Total 20X. The images were analyzed in the public domain program - Image J.

A one µL sample of the culture was placed on a previously flamed slide. After placing the coverslip, the stabilization of the medium took thirty seconds. Then, the extracellular vesicles were observed as moving bright points on the dark background.

Figure 5. Internal view of the BX 51 microscope



Source: Olympus BX 51 Microscope Technical Manual

3.3 Statistical analysis

Statistical comparisons were managed using one-way or two-way ANOVA analysis of variance with Tukey or Bonferroni post-test. All values recorded as mean ± standard error, with a significance of 95%, considering $p<0.05$. Analysis and graphs were managed using GraphPad Version 8 Software.

4. RESULTS

The three samples (400, 100, and 50nm) were observed, totaling ten visual fields per sample. The brightness intensity of the vesicles varies according to their size. The larger the vesicle, the higher the brighter. It is, therefore, observed that the vesicle presents a refringence pattern, which promotes a light field around it, making it easily visible despite being significantly small.

In conclusion, the study demonstrates that it is possible to observe smaller-than-expected refractive particles, with a diameter that can reach fifty nanometers.

JOURNAL HEALTH AND TECHNOLOGY - JHT
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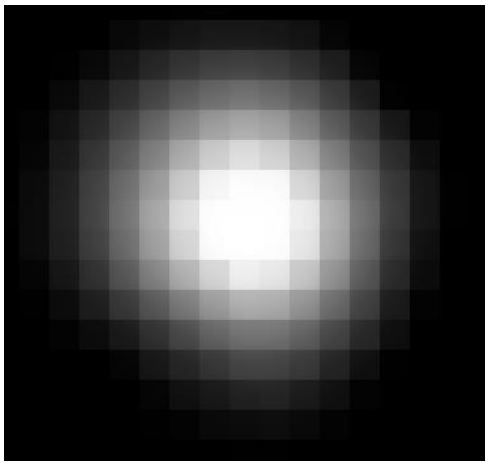
Figure 6. Extracellular vesicle of 120nm enlarged. The brightest center of the photo represents the particle itself. The halo (gray field) corresponds to 35% of its total diameter. The vesicle has an actual diameter of 78nm.



The image analysis program made it possible to create an even more specific panorama, as shown below:

Figure 7. Nanovesicles. MCE 800X



Figure 8. Processing of the image captured by the BX51 microscope camera – Olympus

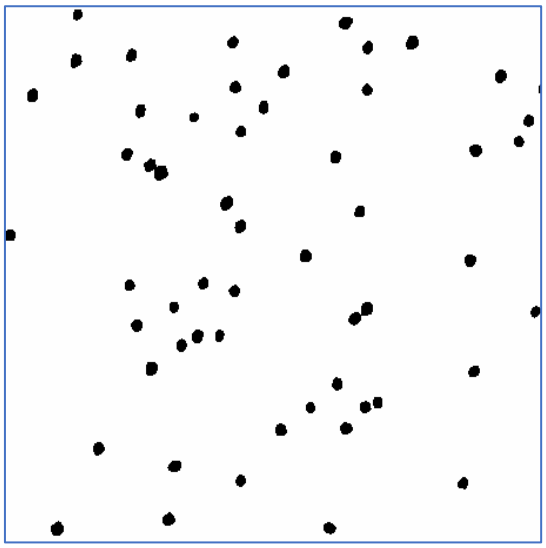
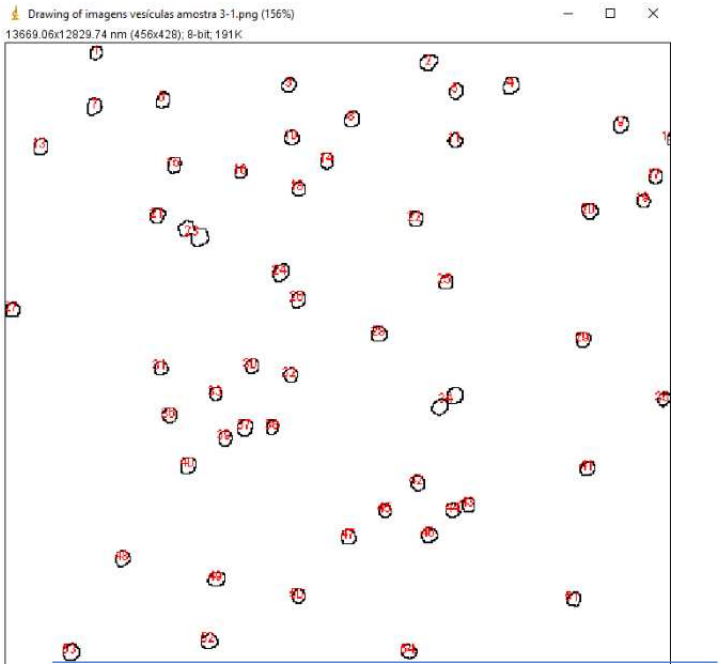


Figure 9. EV analysis and counting



The program analyzes the image and performs the counting and sizing of each of the detected particles. Using specific formulas, it is possible to calculate the sizing of the vesicles captured in the photo:

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

$$A = \pi * r^2$$

where “A” is the area and “r” is the radius.

Therefore:

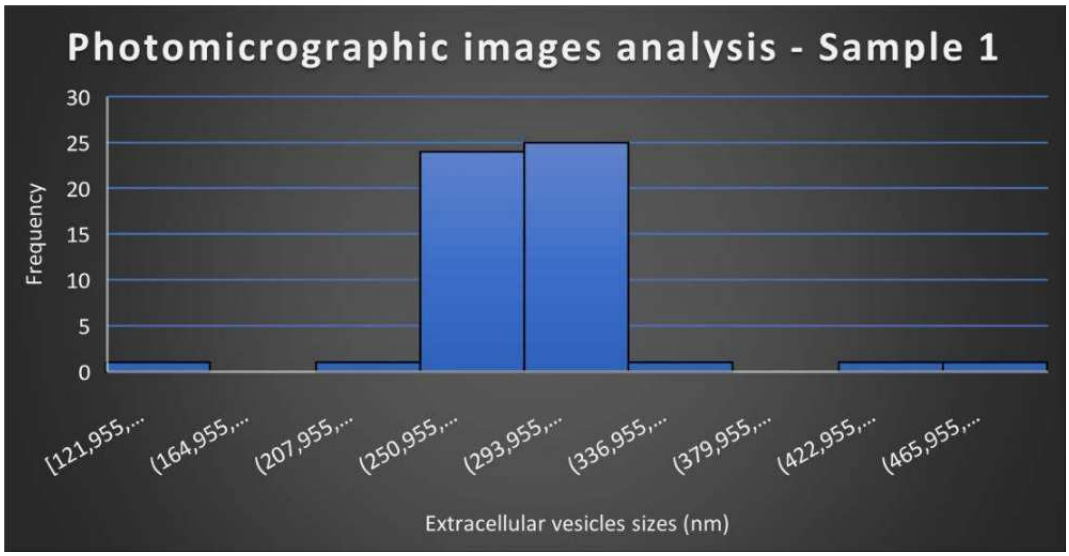
$$r = \sqrt{(A/\pi)}$$

Until reaching the diameter (d) in nanometers

$$D = 2 * r$$

The following graph shows the data collected:

Graph 1. – Sample 1: 400 nm vesicle.



The histogram shows that in a single visual field there are about 54 extracellular vesicles. The predominance in sample 1 is of extracellular vesicles measuring from 251 to 337 nanometers.

The second sample analyzed is a 100nm extracellular vesicles.

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

Figure 10. Nanovesicles – MCE 800X.

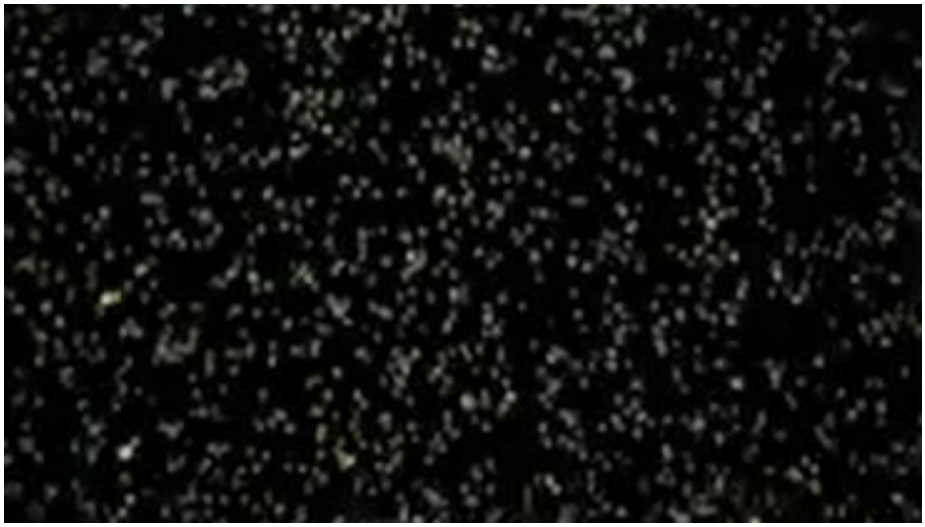
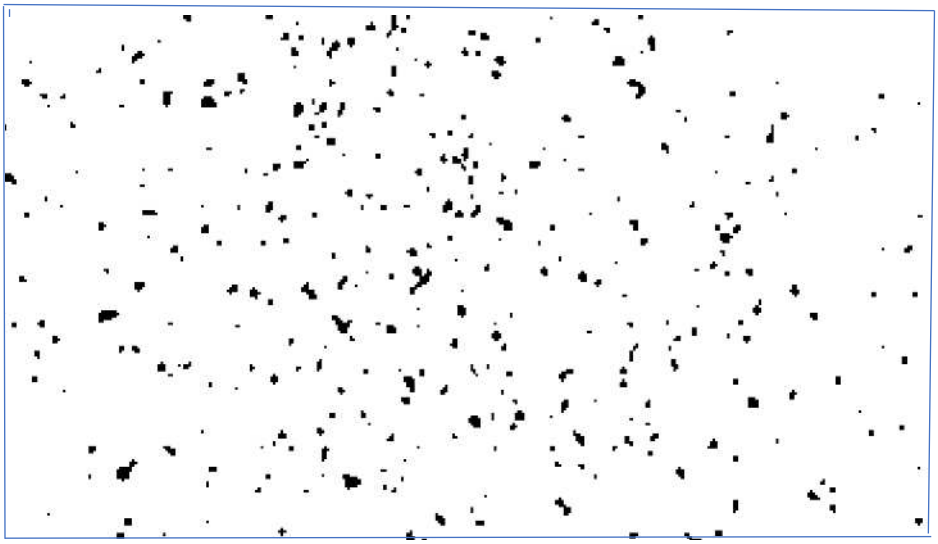
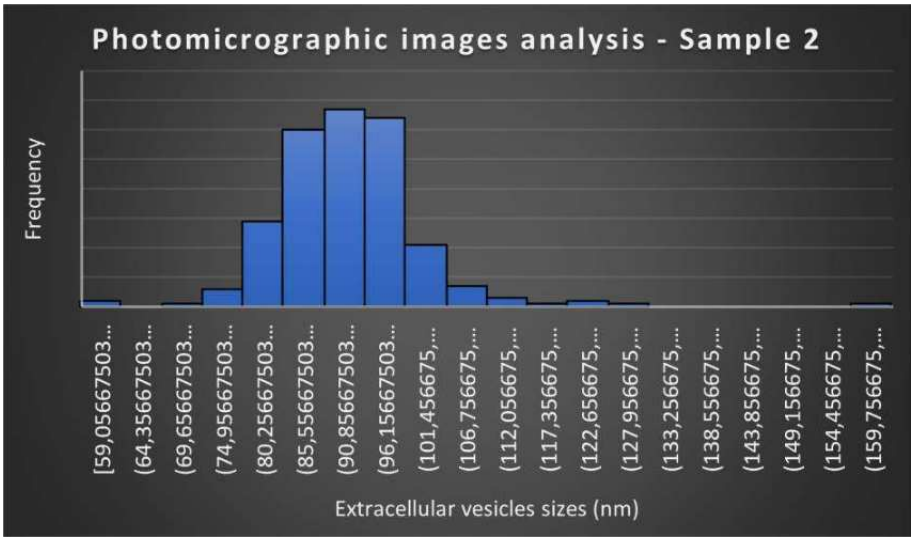


Figure 11. Processing of the image captured by the microscope camera BX51 – Olympus.



JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

Graph 2. – Sample 2: 100nm vesicles.



The third sample containing even smaller particles was analyzed to test the limits of MCE. Following the same method, it was possible to observe and record the presence of even smaller extracellular vesicles.

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Figure 12. Nanovesicles – MCE 2KX.

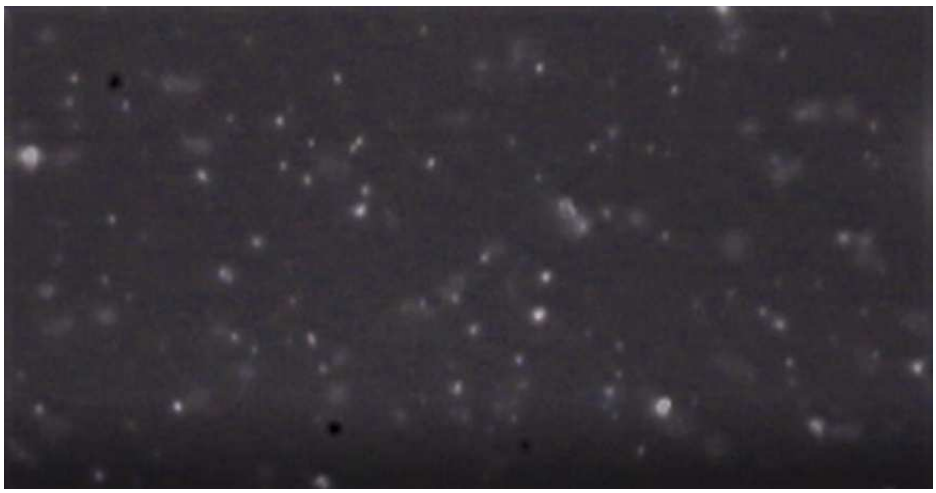
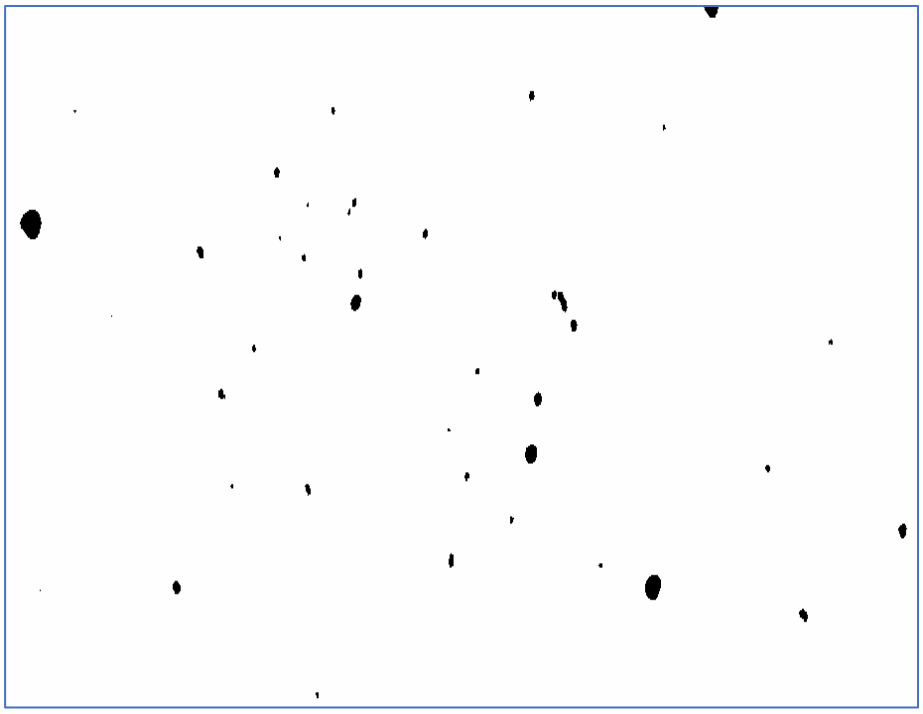
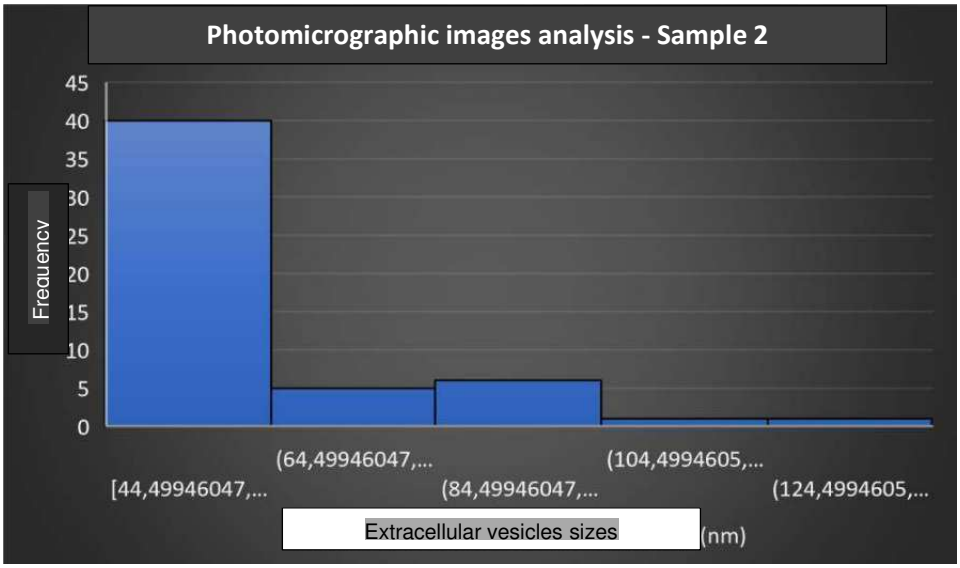


Figure 13. Processing of the image captured by the BX51 microscope camera – Olympus

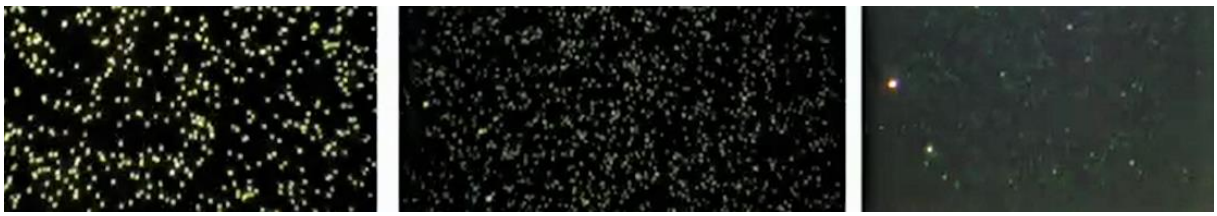


Graph 3. – Sample 3: 50nm vesicles.



It is possible to verify that in a single visual field the concentration of extracellular vesicles is fifty-three. The predominance in Sample 2 in terms of size is from 45 to 84 nanometers. However, visualization, as well as resolution of the vesicles, becomes blurred. This measurement was only possible with the image intensification feature through the image program.

Figure 14. Comparative photomicrographs of the three observed samples



200 to 400nm 800X	80 to 101nm 2KX	44 to 84nm 2KX

The first photo shows extracellular vesicles from 200 to 400nm. In the second, it is possible to see the presence of EVs that vary between 80 and 101nm. In the third photo, are vesicles from 44 to 84nm. Although they are still visible, the resolution decreases significantly. However, the image capture and brightness enhancement allow the researcher still to observe the sample.

5. CONCLUSION

The Darkfield Microscopy (DFM) technique provides the opportunity to quickly visualize extracellular vesicles (EVs), hitherto only analyzed with the use of more sophisticated equipment. Thus, DFM has proved to be a useful tool for *in vivo* visualization of nanoparticles. Its use allows the prior research and analysis of nanoparticles regarding size, displacement, and digital recording.

Another considerable aspect is image analysis. Specific software can provide an estimate of the EVs count per visual field. However, it is important to note that the formation of vesicle clusters can affect the count, although there is a feature in the program that minimizes this problem.

As for nanoparticles above 120nm, DFM can be of great help. Even smaller vesicles can be evaluated with the aid of capturing images for further analysis in programs with specific digital filters. This allows both quantitative and qualitative analysis, in addition to morphological analysis, dispersity, aggregation tendency, and even kinetic properties.

EVs are visualized by several other microscopy techniques, such as fluorescence microscopy, and other techniques, such as flow cytometry and nanosight. In recent years, cytometers of high-sensitivity flow, not yet widespread, have been infrequently used due to their high cost. On the other hand, the most widely used conventional cytometers are unable to detect particles smaller than 300nm. [8]. As a result, the particles are not visible as individual vesicles, but rather as a cluster, or they are

confused with fluorophores used to label them. Regarding the nanosight equipment, it enables accurate determination of particles ranging from 5 to 2000nm [9].

This equipment can also provide useful information about the dimensional and morphological variety of EVs, as well as specify their concentration in the medium. In addition, it determines the function of an EV according to the wide range of load inside it. To understand the heterogeneous nature of its content, it is necessary the nanosight equipment that enables the density of the charge itself to be more accurately assessed and quantified [10].

5.1 Advantage of the Darkfield Technique

The ultra-dark background provided by the condenser, both dry and in oil, provides collimated illumination that makes the extracellular vesicles glow with high contrast. The kinetics of the vesicles will serve as a parameter for the researcher familiar with the technique, concerning particles resulting from artifacts, as it can be seen in the comparative diagrams below developed from the footage taken.

Figure 15. Graph of displacement of an extracellular vesicle in 1.3 minutes

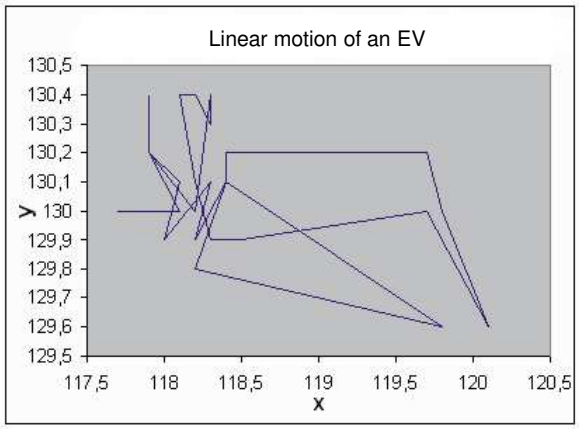
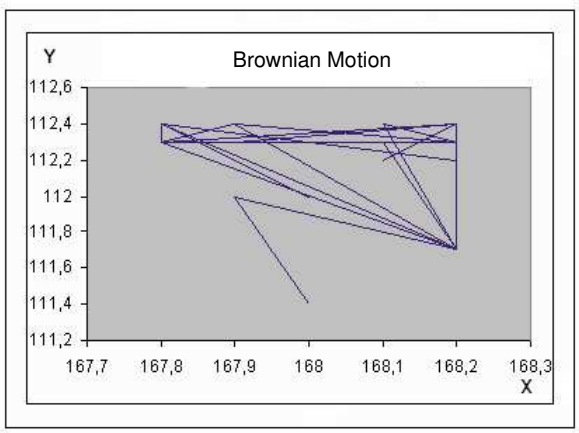


Figure 16. Graph of displacement of a pollen particle in 2 minutes



Indeed, the DFM technique has its pros and cons compared to more advanced methodologies. These allow specific and precise studies, effective in terms of molecular characterization, offering a better understanding of EVs. However, it is possible to consider the MCE as a valuable resource for a previous, fast, economical study, besides being easy to handle by the researcher. It is a viable research instrument.

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JOURNAL HEALTH AND TECHNOLOGY - JHT
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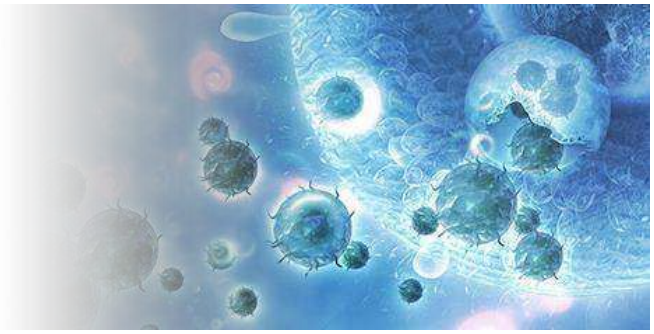
Annex 1.



Lyophilized Exosome Standard (Human Plasma)

Cat. No.: ESL-25

This product is for research use only and is not intended for diagnostic use.

Size	30 μg
Storage Conditions	4°C for up to 36 months.
Shipment Conditions	Gel Pack
Source	Lyophilized and purified exosomes available from Human Plasma
Application	Assay calibration; Control standards for exosome quantification.
Product Introduction	Lyophilized Exosomes can be used as control standards for multiple applications including FACS, WB, ELISA and as calibration standards for quantitation of exosome-derived markers from biological samples. Lyophilized exosomes can be obtained from a variety of biological sources including cell culture supernatant, human plasma, serum, urine and saliva.
Package Contents	Lyophilized exosomes standards from Human Plasma
User Supplied Reagents and Equipment	Deionized water
Note!	• Reconstitute lyophilized exosome standard by adding deionized water; • The recombinant exosome standard should be used within 2 hours after recombinant; • The remaining reconstituted standard stock solution should be aliquoted into polypropylene vials and stored at -20°C for up to a month or at -80°C for up to six months; • Avoid repeated freezing and thawing.

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