Research

Standard Operating Procedure for Interferometric Light Microscopy of Extracellular Particles within the Nanostructurome Pipeline

Romolo Anna[1], Kralj-Iglič Veronika[1,*]

1. University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia
* Correspondence: Veronika Kralj-Iglič, veronika.kralj-iglic@zf.uni-lj.si

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Abstract:
A standard operating procedure (SOP) for assessment of number density and hydrodynamic diameter of particles sized between cca 80 nm and 500 nm is presented. The aim of this SOP is to provide characterization of extracellular particles (EPs) which enables analysis of large cohorts of minimally processed samples. EPs are submicron sized particles shed by all types of cells. They are free to travel to reach distant cells and can interact with them. Therefore they present an universal mechanism of cell-to-cell communication which is a subject of studies of different mechanisms underlying physiological and pathophysiological processes.

Keywords: Bioactive compounds; Green product; Microalgae biomass; Microalgae sample preparation

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1. Definitions

ILM: Interferometric light microscopy
EPs: Extracellular particles

2. Background

EPs are present in different biological samples such as microalgae conditioned media (Adamo et al., 2021; Picciotto et al., 2021), bodily fluids (Troha et al., 2023; Berry et al., 2024) and plant tissue homogenates (Spasovski et al., 2024). Due to their small size and heterogeneous composition of samples, and the transient identity of some types of EPs, their harvesting and assessment presents a challenge. Integration of different methods is recommended by the International Society of Extracellular Particles (Welsh et al., 2024), however, new technically advanced solutions are being sought. The most commonly used method for EP harvesting involves differential centrifugation (Thery et al., 2006), optionally followed by gradient ultracentrifugation on continuous or discrete sucrose or iodixanol gradient (Iwai et al., 2016). As this technique is time consuming and is of limited capacity, alternative techniques have also been designed, e.g. ultrafiltration, dialysis, field-flow fractionation, microchip-based techniques, size exclusion chromatography, and precipitation-based methods, alone or in combination with ultra-centrifugation-based methods (Welsh et al., 2024). Immunoaffinity-based isolations for harvesting EPs with particular surface protein compositions have been developed (Beekman et al., 2019). Commercial kits have been made available. Ion exchange chromatography (Kosanović et al., 2018) is fast and cost effective capturing technique for large volumes of diluted suspensions.

However, different isolation methods were found to yield different EP preparations (Freitas et al., 2019, Tian et al., 2019, Skotland et al., 2017) reflecting the fact that the techniques applied considerably transform the samples.

As they are very small, EPs are hidden within the organisms or cell assemblies. They are observed *ex vivo* and not in their natural environment. Commonly used EP imaging methods are scanning and transmission electron microscopy, and cryogenic transmission electron microscopy (Welsh et al., 2024). Preparation requires invasive procedures such as staining, fixation or filtering. Physico-chemical techniques widely used in EP research for assessment of samples are flow cytometry, fluorescence microscopy with

analysis of Brownian motion (nanoparticle tracking analysis), light scattering, assesment of zeta potential and tunable resistive pulse sensing (Welsh et al., 2024). EPs contain proteins and genetic material (different types of RNA, in particular microRNA, and DNA), proteins, lipids and other small molecules (Hartjes et al., 2019) that are analyzed by high performance liquid chromatography-mass spectrometry-based shotgun workflows that typically lead to the identification from several hundreds to several thousands of units with interpretation depending on the data banks. Proteins in EP samples separated by gel electrophoresis are assessed also by Western Blot or specific immunosorbent assays.

Recently, a novel technological equipment Interferometric Light Microscope (ILM) was developed primarily to assess number density and size of 80 – 500 nm particles from marine water (Boccara et al., 2016). ILM was hitherto used in studies considering marine microorganisms (Boccara et al., 2016, Roose Amsaleg et al., 2017, Romolo et al., 2023), viruses (Turkki et al., 2021) and extracellular vesicles (Sabbagh et al., 2021, Romolo et al., 2022; Jeran et al., 2023; Sauset et al., 2023; Spasovski et al., 2024; Korenjak et al., 2024). The first comparative measurements of the EP samples from blood plasma, erythrocyte suspension and conditioned microalgal media, and of liposomes was reported by Romolo et al. (2022).

3. Purpose, Scope and Applicability

The purpose of the SOP is to describe the procedure for estimation of the amount and size of EPs in various biological samples. Based on the experiences gathered in recent years some tipping points regarding preparation of samples and measurement are addressed. **The scope** of the SOP is the assessment of EPs in microorganism cultures, blood products (such as blood, plasma, serum, isolates of EPs), plant cultures, apoplastic fluid, suspensions of liposomes and hybridosomes (particles artificially produced from lipid and natural sources containing EPs).

The applicability of the SOP is mainly targeted at characterization of different types of liquid biological samples (in particular – blood products, microorganism – derived samples, plant – derived samples, cell culture samples, plant – derived samples such as apoplastic liquid and tissue homogenates), including the samples that are minimally processed.

4. Health and safety warning

Biological samples may contain contagious material and measures should be taken to minimize the risk for the staff. Harvesting methods collect also other types of small particles such as viruses and potentially also yet poorly understood biologically active particles for which the safety measures are not yet known. It should be considered that the particles could be in air and that they are so small and dynamic that the acknowledged measure for protection against microbes are ineffective. Those EPs which have a lipid bilayer-based envelope are susceptible to detergents (Kralj-Iglic et al., 2000). However, some EPs are basically composed of protein and carbohydrate and are resistant to detergent or heating above 80 °C (Božič et al., 2022). It is therefore best to perform the preparation of the samples and the measurement in a room where change of the air with fresh one is available.

If the samples contain cells, also safety measures regarding these should be taken into account. Gloves and coats should be worn to prevent eventual contact of samples with skin and protective masks should be worn to avoid inhaling the material sublimating from the samples. If the samples are in the closed containers, the face should be turned aside when opening the containers. If the material is spilled on the shelf, the ground or other objects, it should be wiped with absorbing tissues and cleaned with detergent and 70% alcohol. Potentially hazardous waste should be autoclaved before disposal.

Interferometric light microscope emits flashes of blue LED light when in operation and if the shield of the sample is transparent, the operator should not look into the light or the operator should wear protective orange glasses. The microscope and the computer are charges with 220 V current.

5. Cautions

Disposal of all used material should be in accordance with applicable laws and good research and laboratory practices. Written operating instructions for the equipment used should be available to the users on site. An electronic or printed register for user reservations should be provided as well as the laboratory notebooks (log sheets) for researchers to log and keep track of the use of the instrument.

6. Personnel Qualifications / Responsibilities

Interferometric light microscope is operated by a single person. It takes about half an hour of training to learn how to measure a sample but optimal use of the equipment is achieved after measuring a larger number of samples. Anyone who uses the equipment should be trained in handling the samples and applying the SOP. Safety precautions (safety data sheets) should be reviewed prior to their use. The operator should keep equipment and desks clean. The operator must keep notes on the measurement process, preferably in paper and in digital form. The data should be properly saved and if relevant, the identity of the donors should be hided. The equipment should be regularly maintained and used as described in the manufacturer's instructions and internal guidelines. Samples should be conveniently labelled to avoid confusion.

7. Materials, Equipment and Supplies

Materials: samples, dilution medium (ultraclean water or marine water or saline or phosphate buffered saline, depending on the sample), 70% alcohol for cleaning.
Devices and other equipment used**:** ILM with computer (**Figure 1**), cover glasses from the manufacturer with the observation chamber, pipettes, pipette tips, 1.5 mL Eppendorf tubes, stand for samples, soft paper napkins for cleaning the cover glass, container for waste.
ILM is essentially composed of a light source, an observation chamber with the sample, an objective and a sensor to detect the interferometric pattern of the light. The observation chamber is created by a hole in the glass layer mounted to the cover glass. The glasses with the observation chamber are placed to the stand and moved into close contact with the glass chamber containing the immersion oil and the objective of the bright-field microscope . The light emitted from the source scatters at the particles of the sample and is then refracted by the microscope. The sensor is placed at the plane where the incident light interferes with the scattered light.
Computer Hardware and Software
Hardware: desktop computer; software: associated software, QVIR 2.6.0 (Myriade, Paris, France).

9. Step by Step Procedure

9.1. Dilution of the samples

The optimal number density range is between 5×10^8/mL to 5×10^9/mL. Some samples can be assessed undiluted. Microalgae cultures, apoplastic liquid and centrifugation supernatants can usually be assessed undiluted. If necessary, microalgae can be diluted with the medium which is used for their growth, apoplastic liquid can be diluted with liquid with which the leaves are soaked. Blood can be diluted with saline and then erythrocytes should be sedimented. Plasma and serum should be diluted with saline. Liposomes should be diluted with water. When diluting samples, the diluting liquid should be placed in the Eppendorf (or similar) tube and the sample should be added. Samples should be gently mixed by moving the pipette tip in the sample or slowly resuspending the sample. Vortexing may cause changes in the EPs.

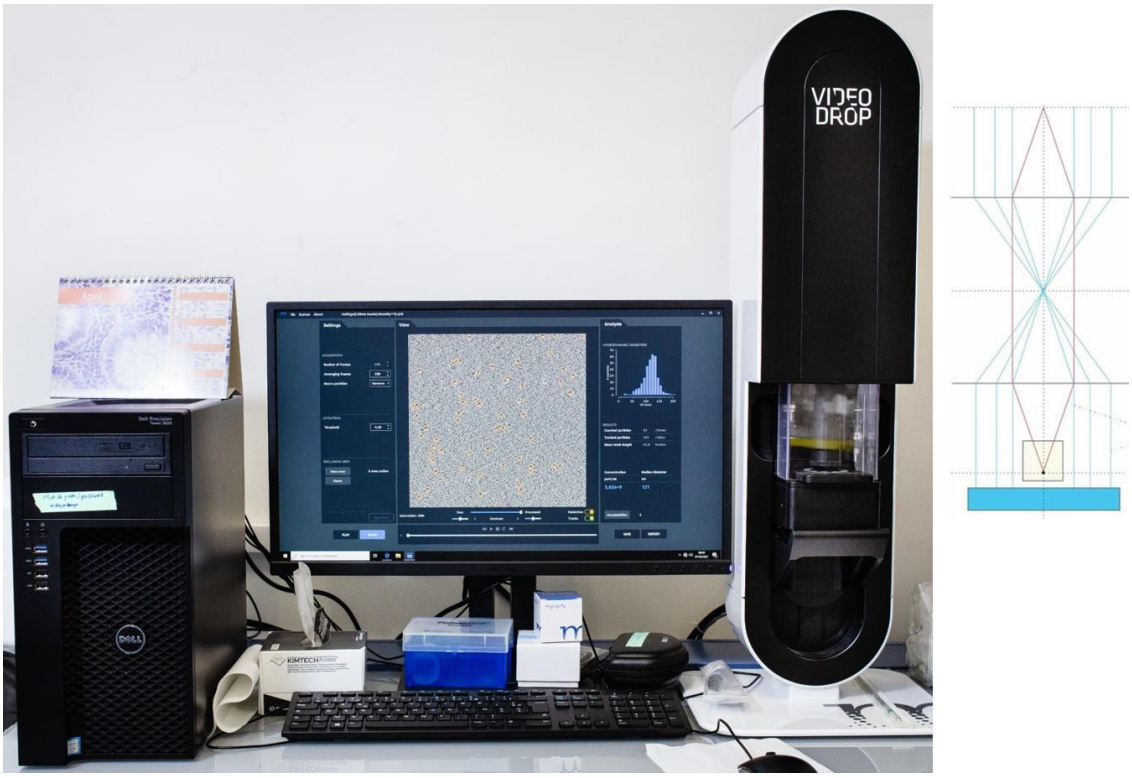

Figure 1. Interferometric light microscope Videodrop (Myriadelab, Paris, France).

9.2. Measurement of number density of EPs and their hydrodynamic diameter distribution

The instrument and the computer should be charged and the setting for the measurement should be chosen. The room temperature should be assessed and inserted to the settings. A drop of the sample (between 7 and 10 μL) is placed into the observation chamber and illuminated by 2 W of blue LED light. The light scattered on the particle is imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image is recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. Interference enhances the information in the scattered light. The contribution of the incident light is subtracted from the detected image. The obtained pattern, which includes contrasting black and white spots, is recognized as a particle, and its position in the sample is assessed. The number density of the particles is the number of detected particles within the detected volume. The typical detection volume is 15 pL. D_h is estimated by tracking the position of the imaged particle within the recorded movie. It is assumed that particles undergo Brownian motion due to collisions with surrounding particles. The motion is random, but the kinetic energies and momenta of the particles reflect the temperature of the sample. Particles with smaller masses move within a larger volume than particles with larger masses. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\,\Delta t\rangle$, while the hydrodynamic diameter was estimated by assuming that the particles were spherical and using the Stokes–Einstein relation $D_h = kT/6\pi\eta D$. Each particle that was included in the analysis is tracked and processed individually, and the respective incident light signal is subtracted from each image. The instrument enables setting of the light intensity, time of illumination, threshold of detection and size of pattern for recognition of the particle. Detection of particles and recording of movies stops when a chosen number of particles are analyzed or a chosen number of movies are taken. The number of particles tracked should be sufficient to enable relevant statistical analysis of the data.

When performing the measurements, the media (e.g. ultraclean water or saline) should be measured first to make sure that the signal is under the detection limit. Measurements of the samples should be performed in triplicate.

9.3. Data acquisition

The results are available in an outlined pdf file (**Figure 2**) with statistical analysis of the acquired data, in excel file with the data for each particle, and in the form of recorded movies.

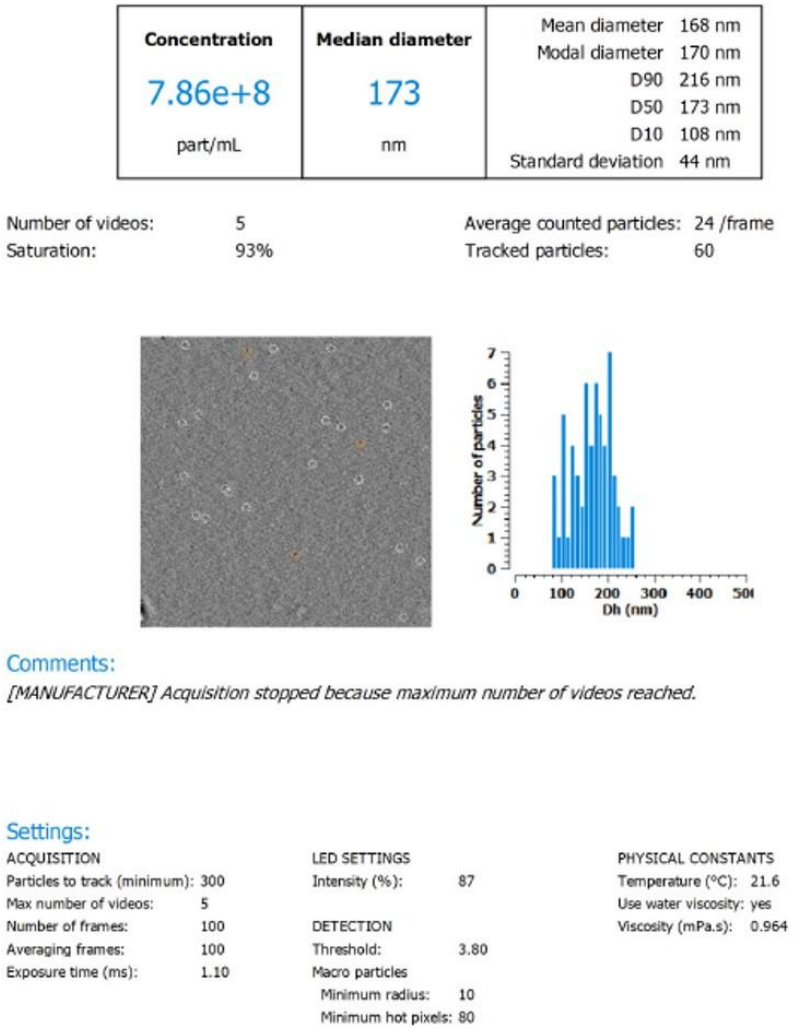

Figure 2. An example of the display of the results of measurement with ILM Videodrop (Myriadelab, Paris, France).

9.4. Troubleshooting

- Saturation of light cannot be reached. The samples may contain also particles that are smaller or larger than those detected by the instrument and these may absorb light to such extent that the measurement is not feasible. With improved dilution of the sample the saturation can be improved, however, the number density of the detected particles may decrease below the detection limit. Optimal dilution of the sample should be sought. If too many large particles are in the sample, their sedimentation can be performed before measurement.
- With dilution, particles tend to form aggregates; this is called the swarm effect (Korenjak et al., 2024). The results should be interpreted accordingly.
- If samples are too dense, their motion is hindered which is not reflected in the Einstein relation. Concentration should be optimized.

- The image is distorted. There could be crystallization of particles in the immersion oil in contact with the objective. The chamber with the immersion oil and the objective should be cleaned and fresh immersion oil should be mounted.
- The image is distrorted. The beam could be out of focus. The configuration of the microscope should be adjusted. Adjustment and maintenance of the instrument is best performed by the manufacturer.

10. Data and records management

The data are saved in the memory of at least two desktop computers and on mobile discs. The data are added to the cloud document where the results of all the methods are collected. The experimental details are documented in the lab journal.

11. Waste management

The waste includes disposed gloves and masks, Eppendorf tubes where the samples are diluted, plastic pipette tips, used paper napkins for cleaning the observation chamber and potential sample spill and remnants of the samples. Disposal material should be divided into appropriate waste fractions, according to applicable laws and good research laboratory practice. Potentially hazardous materials should be placed in special containers and delivered to the relevant acquisition units.

12. Related protocols or SOPS

This SOP includes a combination of optimized known procedures. Optimizing conditions and processing methods on the production and activity of compounds as phytohormones in microalgae can guide the development of high-quality biomass for agricultural use.

13. Quality control and quality assurance section

13.1 Instrument calibration

Instrument should be maintained and calibrated by the manufacturer. Aside from regular checkups no calibration is needed for performing measurements.

13.2 Critical processes parameters and checkpoints

Temperature should be checked and adjusted for each measurement. Locking the sample in close contact with the chamber containing the microscope objective should be performed in such way to avoid the filling of the observation chamber with the sample. To avoid mechanical disturbances of the observation chamber with the sample, the shield of the sample should be mounted before putting the sample in close contact with the chamber containing the microscope objective. The recorded movies need considerable memory of the computer and may not be necessary for every measurement. To avoid overflow of the memory the data should be transformed to an external disk and removed from the computer connected to the instrument. Finding the optimal dilution is a critical element of the determination of the number density and hydrodynamic diameter of EPs.

14. Data on procedures and samples

Data on procedures and samples are given in **Table 1**.

Table 1. Data on procedures and samples

Description of the outcome	Number density (10^9/mL), hydrodynamic diameter (nm), distribution of hydrodynamic diameter – distribution width (nm)
Time required to obtain the results	5 – 30 minutes per sample, depending on finding the optimal dilution
Volume of the sample needed	At least 20 microlitres
Estimated cost without manpower	40 EUR/sample, mostly for maintenance service
Contact person	Veronika Kralj-Iglič, kraljiglic@zf.uni-lj.si

15. Conclusions

This SOP describes laboratory procedures for assessment of number density and hydrodynamic diameter of particles sized between cca 80 and 500 nm. The skills required to perform the measurement include learning the good laboratory practices and necessary safety measures, learning how to dilute the sample and learning how to handle the instrument. These skills can be obtained by researchers, technitians and students, however, it is best that the measurements are performed under supervision of a person who is responsible for the instrument.

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Conflicts of Interest: The authors declare no conflict of interest.

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