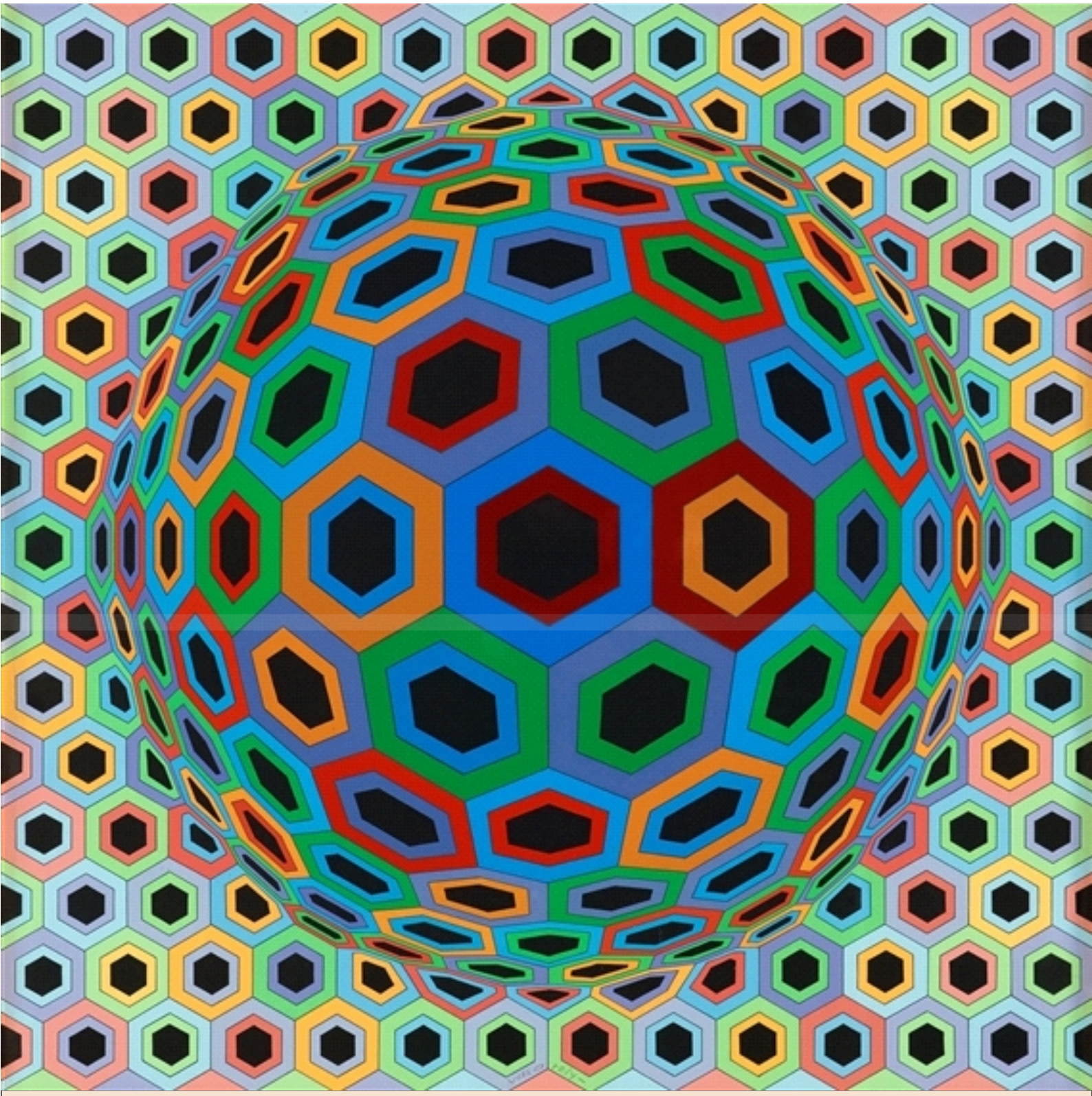

SOCRATIC LECTURES

12 TH INTERNATIONAL SYMPOSIUM LJUBLJANA, 11. 1. 2025

PEER REVIEWED PROCEEDINGS PART III - NANOSTRUCTUROME

EDITED BY VERONIKA KRALJ-IGLIČ, YELENA ISTILEULOVA AND ANNA ROMOLO
FACULTY OF HEALTH SCIENCES, UNIVERSITY OF LJUBLJANA
Z-STEAM

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12 th International Symposium, Ljubljana, January 11, 2025
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Program of the Symposium Socratic Lectures, January 11, 2025, 10:00 – 17:00 (Ljubljana time)

10.00 Welcome to participants (Veronika Kralj-Iglič, University of Ljubljana)

10.05 - 10.45 Plenary lecture: Saara Laitinen: Extracellular vesicles from blood cells and in blood. The avenue of future therapies
10.45 Break

11.00 Scientific sections

Section 1: Medicine (Chairs: A. Nemec Svete, K. Troha)

11.00 - 11.20 G. Omejec: Ultrasonographic assessment of diaphragm function
11.20 - 11.40 A. Troha: Advancements and future prospectives of platelet-rich products and extracellular vesicles in otology
11.40 - 12.00 A. Čuček: Mammary gland - anatomy, histology, pathology, and physiotherapist management post surgery
12.00 - 12.20 K. Pahovnik: A case of autosomal dominant osteopetrosis with a novel mutation in CLCN7
12.20 - 12.40 L. Dolinar: Destigmatization of erectile dysfunction
12.40 - 13.00 D. Dolinar, I. Potparič: Premature fracture of a modular femoral neck after total hip arthroplasty

Section 2 : Veterinary Medicine (Chairs: V. Erjavec, M. Šimundić)

11.00 - 11.20 L. Jarnovič: Brachicephaly in cats. A silent problem in feline health
11.20 - 11.40 B. Perc: Hematology-derived inflammatory markers in dogs with BOAC
11.40 - 12.00 I. Premrl: Foreign body removal from the canine digestive tract: findings and outcomes
12.00 - 12.20 M. Šimundić: Approach to the patient with polyuria/polydipsia
12.20 - 12.40 B. Dučić: Retained tooth roots in cats
12.40 - 13.00 M. Arko: Extracellular vesicles in bovine colostrum and milk

Section 3: Physiotherapy (Chairs: R. Vauhnik, A. Jakopič)

11.00 - 11.20 Bec: Effects of transcutaneous electrical nerve stimulation in patients with fibromyalgia syndrome
11.20 - 11.40 K. Breznik: Pain relief treatment of fresh sacral fracture for a professional snowboarder one month before participation in the Winter Olympic Games: a case report
11.40 - 12.00 A. Jakopič: Effects of hydrotherapy on functional capacity of patients after stroke
12.00 - 12.20 M. Jelen: The effects of passive loading of the knee joint after anterior cruciate ligament reconstruction on knee stability
12.20 - 12.40 E. Strle, L. Uhan: Surgical treatment of chronic post-traumatic hamstring origin pain with tendon transfer from the unfused ischial tuberosity apophysis to the ramus of the ischial bone: a case report
12.40 - 13.00 M. Zoroja: Ring shaped lateral meniscus - Raise awareness and avoid unnecessary surgery

Section 4: Green transition (Chairs: T. Griessler Bulc, M. Bavcon Kralj)

11.00 - 11.30 A. Drozd-Rzoska: Physics & Society : High Pressures for innovative pro-health foods (keynote lecture)
11.30 - 11.45 A. Kranjc Požar: Microalgal technologies: Treatment strategy for contaminants of emerging concern
11.45 - 12.00 E. Andreasidou: Uptake of contaminants of emerging concern in tomato plants irrigated with treated wastewater
12.00 - 12.15 L. Klemenčič: Harvesting of algae biomass by electrocoagulation
12.15 - 12.30 J. Danilović Luković: Impact of ionizing radiation on microalgae: Enhancing biotechnology potential

12.30 - 12.45 U. Šunta: Surface properties of algal biomass and microplastics: An exploration of point of zero charge and contact angle
12.45 - 13.00 T. Zrnec Drobnjak: Production of plant biostimulants from microalgae grown on the biogas digestat

Section 5: Nanostructurome pipeline (Chair: P. Trebše, U. Lavrenčič Štangar)

11.00 - 11.10 I. Jerman: Infrared and Raman spectroscopy
11.10 - 11.20 J. Jakše: Next generation sequencing
11.20 - 11.30 J. Hočevar: NMR Spectroscopy
11.30 - 11.40 R. Cerc Korošec: Thermoanalytical techniques
11.40 - 11.50 M. Novinec: Mass photometry for molecular mass determination
11.50 - 12.00 G. Schlosser: LC-MS lipidomics, metabolomics
12.00 - 12.10 E. Heath: LC-MS/MS methods
12.10 - 12.20 P. Hansson: SAXS and investigation of diffusion and transport of drug discovery systems in extracellular matrix models
12.20 - 12.30 V. Kralj-Iglič: Scanning electron microscopy
12.30 - 12.40 O. Vanderpoorten: Nanospacer: Nanofluidic sizing of biomolecules and EVs
12.40 - 12.50 G. Liguori: in vitro cell assays
12.50 - 13.00 S. Michelini: ELISA assays
12.50 - 13.00 All partners: Discussion on sample preparation and characterization

Section 6: FarmEVs: Different sourced extracellular vesicles and their potential (Chairs: G. Pocsfalvi, A. Di Loria)

11.00 - 11.20 G. Guerriero: Plant extracellular vesicles
11.20 - 11.40 I. Schabussova: Bacterial extracellular vesicles
11.40 - 12.00 S. Vainio: From sustainable soil to lifelong health - speculations from ecological nanointeractomics
12.00 - 12.10 R. Mammadova: Enhancing therapeutic potential: Loading strategies for plant-derived nanovesicles
12.10 - 12.20 Glamoclija: Trichinella spiralis extracellular vesicles alleviate OVA-induced allergic inflammation in BALB/c mice
12.20 - 12.30 P. Devarkanda: Bean PDNVs isolation and characterization from the dwarf organic Borlotti bean Lingua di Fuoco
12.30 - 12.40 E. Cepec: Biostimulants from algae biomass uncovered: phytohormones determination and plant growth stimulation
12.40 - 12.50 B. Korenjak: Interferometric light microscopy of diluted blood in different species

Section 7: Biophysics (Chair: A. Iglič)

11.00 - 11.20 S. Rzoska: Unique properties of high-pressure & high-temperature formed glasses: The application for new-generation batteries (Keynote lecture)
11.20 - 11.40 T. Beke Somfai: Peptide-based antibiotic supramolecules (Keynote lecture)
11.40 - 12.00 L. Mesarec: Equilibrium membrane shapes influenced by different concentrations of orientationally ordered curved rod-like membrane protein
12.00 - 12.20 M. Mussel: On spikes and sound: Debating the physical nature of action potentials
12.20 - 12.40 L. Bar: Impact of inclusions on the organization and phase behavior of lipid membrane models
12.40 - 13.00 M. Drab: Monte Carlo studies of vesicle shapes with anisotropic membrane inclusions and volume constraints

Section 8: Physics (Chair: S. Kralj)

11.00 - 11.30 A. Jelen: Microscopic patterns and complexity (Keynote lecture)
11.30 - 11.45 A.A. Sojecka: Doomsday criticality for the global society
11.45 - 12.00 T. Blazevic: Maximal entropy production
12.00 - 12.15 D. Dovnik: Fractional topological charges
12.15 - 12.30 J. Sgerm, A. Ribas, L.G. Fugina: Universality of physics of phonons
12.30 - 12.45 G. Goričan: Kibble-Zurek mechanism and applications
12.45 - 13.00 M. Župec, M. Kodrin, R. Rojko, M. Štorman: Crystal lattices in nature

Section 9: Liquid Crystals (Chairs: A. Jelen, B. Švajger)

11.00 - 11.20 T. Javornik: Importance of the critical point in thermotropic nematic liquid crystals in terms of their sensitivity
11.20 - 11.40 A. Hoelbl: Localised excitations in liquid crystals as particle analogues
11.40 - 12.00 E. Čokor: Imry-Ma patterns in confined nematic liquid crystals
12.00 - 12.20 M. Potrč: Lyotropic liquid crystalline phases
12.20 - 12.40 B. Švajger: Volume and surface phase transitions in confined nematic liquid crystals
12.40 - 13.00 M. Zid: Mode coupling and memory effects

Section 10: Education (Chair: G. Torkar)

11.00 - 11.20 G. Torkar: Environmental education for behaviour change
11.20 - 11.40 I. Devetak: Pre-service primary school teachers' understanding of biogeochemical cycling
11.40 - 12.00 L. Vinko: Environmental literacy of chemistry teachers
12.00 - 12.20 S. Beslagic: Robotics in high school
12.20 - 12.40 T. Plešnik: Undergraduate physiotherapy student perceptions of teaching and learning activities associated with clinical education
12.40 - 13.00 Ž. Rode: Pre-service teachers as citizen scientists

Section 11: Bridging Science, Health and Arts I (Chair: Y. Istileulova)

11.00 - 11.20 N. Gomes: Transformative educational strategies with artificial intelligence (Keynote lecture)
11.20 - 11.40 S. De Lasala Porta: Truth, beauty, and ethics in art, science, and health: Interdisciplinary reflections and philosophical perspectives
11.40 - 12.00 F. Banabed: Exploring health humanities from a global south perspective
12.00 - 12.20 E. Rosakebia: Balancing care and creation: The role of poetry in caregiving and emotional resilience
12.20 - 12.40 F. Dalpane: Making art as bravery: A virtue-ethics perspective
12.40 - 13.00 Y. Istileulova: Planetary health through arts: De rerum natura and Earth´s first music in cosmic harmony

Section 12: Bridging Science, Health and Arts II (Chair: V. Vidrih Perko)

11.00 - 11.20 R.S. Thomas: Combining an art therapy-trauma protocol and sensory motor art therapy to support a woman with breast cancer
11.20 - 11.40 E. Hribernik: Man on the stage of life - introduction to an Italian baroque opera
11.40 - 12.00 A.L. Mastruzzo: The performer as a multi-expressive artist in Argentina's current flute experimentation
12.00 - 12.20 A. Karboski: Vladimir Stiftar and his views on the formation of the architectural and cultural landscape of the Eupatoria resort
12.20 - 12.40 N. Paliska: Mozart iconography
12.40 - 13.00 V. Vidrih Perko: Learning - the immortal soul of humanism (Keynote lecture)

13.00 Break

14.00 Plenary lectures

14.00 - 14.30 V. Hlavackova Pospichalova: Extracellular vesicles in ovarian cancer
14.30 - 15.00 N. Gov: Magnets, Ants and Humans. The physics of collective transport by ants
15.00 - 15.30 H. Murto: Organic farming
15.30 - 16.00 E. Mihajlović: Claudio Monteverdi and so called seconda pratica or how he brings real human feelings into music

16.00 Student zone

17.00 Closing of the symposium

Satellite event: Concert at the Betteto Hall, Academy of Music, Casino Building 10.1.2025 at 18.00
Tentative Program:

Classical music:
C. Franck: Petit offertoire. Organ: Yelena Istileulova
C. Monteverdi/G.F. Busenello: Prologue from opera Coronation of Poppaea. Sopranos: Fortuna: Ronja Prapotnik, Virtu: Eva Kokot, Amore: Brina Vukovič, Harpsichord: E. Mihajlović, Organ positive: Branko Rezić
A. Aljabjev A: Nightingale. Flute: Anita Prelovšek, Piano: Elena Startseva Somun
S. Rachmaninov: Vocalise. Violin: Vasilij Meljnikov, Piano: Lara Oprešnik
C. Monteverdi/G.F. Busenello: Regina disprezzata from opera Coronation of Poppaea. Soprano: Ottavia: AlessandraTessaro, Harpsichord: E. Mihajlović, Organ positive: Branko Rezić
S. Rachmaninov: Etude Tableaux Op 33 No 3. Piano: Lara Oprešnik
G. Brun: Romance. Flute: Anita Prelovšek, Piano: Elena Startseva Somun
C. Monteverdi/G.F. Busenello: Duetto Demigella and Valetto from opera Coronation of Poppaea. Sopranos: Demigella: Nives Hadžić, Valetto: Eva Kokot, Harpsichord: E. Mihajlović, Organ positive: Branko Rezić
W.A. Mozart: Alla turca from Sonate in A major. Piano: Denis Luin
F. Chopin: Polonaise G sharp minor. Piano: Matic Bogataj

G. Ipavec/A. Čopi - Simon Gregorčič: Mountain flower: Chorus Studenec
Poetry by Ifigenia Simonović
Ifigenija Simonović is writing poetry, essays, book reviews, translating, and painting various objects for more than fifty years. She is the author of ten poetry collections, three books of essays, three books for children, and is also known as a publisher of eight books of poetry by Vitomil Zupan. In 2009, she received the Rožanec award. Between 2017 and 2021, she was the president of the Slovenian PEN Center. Her poetry is often dark, but love persistently shines through the cracks that are drawn on her path by real-time experiences.
H. Lavrenčič/Anonymus: The coque has sung: Chorus Studenec
E. Adamič/O. Župančič: Evening song: Chorus Studenec
Chorus Studenec Pivka, led by Irena Rep, is composed of experienced singers. In its 22 seasons, various genres were performed but the singers prefer to sing arrangements of folk songs. They have around 30 concerts annually, home and abroad, most importantly, the visits to Slovenian societies throughout Europe.

Contemporary music
A. von Sultanova/Titus Lucrecius: De rerum natura. Piano, Voice: Aleona von Sultanova
S Kralj: Topology. Piano: Samo Kralj
H. Mancini: Pink Panther. Trombone: Emil Somun, Piano: Elena Startseva Somun
A. von Sultanova/A. von Sultanova: For Samuel Gmelin - water, water. Piano, Voice: Aleona von Sultanova
J. Rae: Sonatine (Aquarelle, Notturne, Firedance). Flute: Anita Prelovšek, Piano: Elena Startseva Somun
A. Schnittke: Suite old style. Violin: Branko Brezavšček, Piano: Elena Startseva Somun
L. Oprešnik: Fugue. Piano: Lara Oprešnik
B. Kobal: Dic verbo. Soprano: Kaya Tokuhisa, Organ: Jana Jamšek
D. Zupanič Turković: Cantique de Baruch Spinoza from Mass in E minor. Organ: Aleona von Sultanova, Percussion: Bojan Ilievski, Piano: Lara Oprešnik, Flute: Anita Prelovšek, Voice: Veronika Kralj-Iglič

Satellite event: Recital of the organist Roberta Schmid at the Church of Assumption, Tromostovje 14.1.2025 at 20.00.
Program:
D. Buxtehude: Passacaglia in D minor BuxWV 161
J. S. Bach: Choral Das alte Jahr vergangen ist BWV 614
J. S. Bach: Choral Erbarme dich mein Gott BWV 721
A.G. Ritter: Sonate no. 2 in E minor op.19
G. Mushel: Toccata
J. Rheinberger: Passacaglia
L. Vierne: Carillon de Westminster

Editorial

12th Socratic Lectures symposium took place online on January 11, 2025. It featured five plenary lectures, 12 scientific sessions, a poster session and a newly introduced session called "The student zone".

As we received more than 40 papers, The Proceedings of the 12th Socratic Lectures is organized in three parts. Part I contains mainly papers from the medical fields, Part II contains mainly papers from the natural and social sciences and Part III contains standard operating procedures of a pipeline for assessment of extracellular particles and related publications. The pipeline was designed within the preparatory project Nanostructurome. Socratic lectures present an important meeting point for the participants of various projects and for the publication of scientific results, opinions, repositories, and any kind of documents that are useful in the management of the projects. But in the first place, the 12th Socratic lectures are a meeting point for scientists, artists and friends from many different fields. We were able to enjoy the fruit of the fields which were kindly provided to us by the participants of the events for which we remain forever thankful.

With the participation of world top scientists from the fields and integration of science in international artistic production, Socratic lectures strive to present scientific and artistic excellence to the students, involve them in the creation of science and art and push forward development of curricula within university education. We conclude with a big thanks to all the participants who in the spirit of Socrates donated their contributions and to all those who made the events possible. Kindly invited to the next Socratic lectures.

Veronika Kralj-Iglič, Anna Romolo and Yelena Istileulova

CONTENTS

TEXTS

Research

Standard Operating Procedure for Microalgae Cultivation, Harvesting and Biomass Processing

Cepec Eva[1,*], Griessler-Bulc Tjaša[1,2], Šunta Urška[1], Istenič Darja[1,2]

1. University of Ljubljana, Faculty of Health Sciences, Ljubljana, Slovenia
2. University of Ljubljana, Faculty of Civil and Geodetic Engineering, Ljubljana, Slovenia

* Correspondence: eva.cepec@zf.uni-lj.si

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Abstract:
A standard operating procedure (SOP) for the cultivation, harvesting, and processing of microalgae biomass is presented. The aim of this SOP is to optimize biomass production
in the laboratory while preserving valuable bioactive compounds through precise control of growth conditions and efficient harvesting techniques. Microalgae are increasingly recognized as a valuable source of bioactive compounds, including compounds with potential for agricultural, pharmaceutical, and environmental applications thus contributing to bioeconomy development. By following the outlined steps, high biomass yield and quality can be achieved. High biomass quality ensures that all active compounds are contained in the sample and are stable for various further treatments and chemical analyses such as for the determination of phytohormones or antimicrobial compounds when stored properly.

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

Table of contents

1. Definitions

BBM: Bold's basal medium
DO: concentration of dissolved oxygen
EC: electroconductivity
GC/F: glass microfiber filters grade GF/C
HPLC: high-performance liquid chromatography
LC-MS/MS: liquid chromatography coupled with double mass spectrometry
SOP: standard operating procedure
TSS: total suspended solids
OD: optical density

2. Background

Microalgae are a diverse group of photosynthetic microorganisms, which are capable of thriving in both freshwater and marine environments (Singh and Saxena, 2015). Due to their fast growth rates and the ability to produce a wide variety of valuable biomolecules, they have gained increasing attention across various industries, including cosmetics, food (nutritional supplements, animal feed), biofuel production, as well as in medicine and pharmaceutical industry to produce bioactive compounds (Basheer et al., 2020; Castro et al., 2023; Eze et al., 2023; Zuccaro et al., 2020) or for agricultural application (Gonçalves, 2021; Miranda et al., 2024). The cultivation of microalgae involves providing optimal conditions for their growth, including temperature, access to light, CO_2, pH, essential nutrients such as nitrogen, phosphorus, and trace elements, as well as the selection of appropriate cultivation systems (Amaro et al., 2023); namely, microalgae can be cultivated auto/hetero/mixotrophically either in closed (photo/fermentation) bioreactors or open ponds (Borowiak and Krzywonos, 2022; Muhammad et al., 2020; Tran et al., 2020).
Once microalgae reach their desired growth stage, they need to be separated from the growth medium. Common methods include centrifugation, flocculation, filtration and sedimentation. Centrifugation is highly effective and suitable for lab-scale research (Kumar et al., 2023; Singh and Patidar, 2018).

For a better understanding of the potential of microalgae to produce bioactive molecules, such as phytohormones, it is essential to standardize procedure including microalgae cultivation, harvesting and final processing steps. The procedure should ensure that all target compounds are covered and stable during the process and storage even though some compounds are sensitive to various environmental factors. SOP is therefore essential to fully uncover the bioactive potential of microalgal biomass for further applications (Thiviyanathan et al., 2024). Also, more in-depth research focused on optimizing the production of bioactive substances in selected microalgal species is needed, with an emphasis on manipulating environmental and stress factors in order to achieve the highest production of bioactive compounds (Liang et al., 2009; Magalhães et al., 2024; Mohsenpour et al., 2021). Detailed SOP for microalgae cultivation at the laboratory scale, along with harvesting and biomass processing, is presented to support advancements in this research field.

3. Purpose, Scope and Applicability

Cultivation of microalgae, particularly for biomass with high valuable compounds such as phytohormones, often necessitates optimized growth conditions, appropriate synthetic media and/or complex bioreactors (Amaro et al., 2023; La Bella et al., 2022; Menegazzo and Fonseca, 2019). The presence and the role of bioactive molecules in microalgae are still not fully understood, therefore complete understanding of the biological, chemical, and physical processes taking place during microalgae cultivation, harvesting and biomass processing, is urgently needed (Van Do et al., 2020; Senousy et al., 2023).

The purpose of the SOP is to define the key parameters and optimize the processes in order to develop an effective product from microalgae biomass. The cultivation of microalgae on laboratory scale is described, and efficient harvesting and biomass processing techniques are presented.

The scope of the SOP is to define the steps of microalgae biomass preparation to obtain bioactive compounds, starting from the cultivation to the point when samples of biomass are used for analyses/application. When developing protocols, care must be taken to maintain the bioactive properties of the biomass, as inappropriate processing techniques may lead to the degradation or loss of valuable compounds. Following the standardized procedures is crucial to ensure the effective preparation of high-quality biomass.

The applicability of the SOP is mainly targeted at the preparing of microalgae biomass for research of its bioactive potentials; however, the samples can also be further processed for other research purposes, e.g., antimicrobial activity, analysis of the main components of interest for biofuel and bioplastic production (Barsanti and Gualtieri, 2018; Van Do et al., 2021; Parmar et al., 2023).

In the framework of this SOP, the prepared samples proved to be suitable for phytohormones quantification using LC-MS/MS and biostimulatory potential screening using a commercial phytotoxicity assay to determine the direct effect of algae biomass on the germination of *Sinapis alba* (*S. alba)* seeds.

4. Health and safety warning

Microalgae biomass used in experiments is not applicable for personal use, nor approved for any other purposes, except for specific scientific research. In the laboratory, it is crucial to have a clean and organized workspace and to regularly calibrate and maintain the laboratory equipment to ensure research credibility and safe operation, respectively.

Sterile material is required for microalgae cultivation in the laboratory. Autoclaving operates under specific pressure and temperature conditions (121°C, 15 min), therefore proper training in safe operation is essential to avoid accidents. Always monitor operating parameters and follow shutdown procedures during maintenance.

The hazards and safety measures regarding the use of the centrifuge are thoroughly described in Centrifuge Safety Guidelines prepared at the University of Stanford (https://ehs.stanford.edu/reference/centrifuge-safety). Prevent leakage into the centrifuge chamber because of damaged or poorly closed tubes, additionally, rotor should be loaded in a balanced way to minimize vibrations (Kralj-Iglič et al., 2024).

Synthetic growth media used in the microalgae cultivation may contain hazardous chemicals. To minimize the risk of exposure, proper handling of the chemicals, as well as the

use of personal protective equipment such as gloves, goggles, and lab coats, is required. All personnel need to be trained in first aid and emergency procedures, including spill response and chemical handling protocols. The solvents used in sample preparation (such as ethanol) are flammable, they should therefore be stored in appropriate flammable storage cabinets.
Fine particulate matter is produced during drying. To avoid respiratory hazards, it has to be ensured that the drying is conducted in well-ventilated areas and the respiratory protection is used when handling powdered biomass. When storing biomass at the – 80 °C appropriate gloves should be used.

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 individual instruments.
Operators should be aware of the potential hazards associated with the cultivation of microorganisms and should follow established safety protocols. All chemicals in the laboratory must be kept in a separate room to which only employees have access.

6. Personnel Qualifications / Responsibilities

The staff should be trained in handling the samples and applying the SOP. Chemical handling instructions and safety precautions (safety data sheets) should be reviewed prior to their use. Staff should be qualified to perform procedures, trained to use specific equipment, such as autoclave, freeze-dryer or centrifuge, and always follow the established protocols. Staff must accurately document all process parameters, observations, and results in laboratory notebooks (log sheets) or digital systems.
Regular equipment maintenance should be conducted as per the manufacturer's instructions and internal guidelines. Repairs and adjustments must only be performed by authorized maintenance personnel with the necessary technical qualifications.
Staff must ensure that all equipment, surfaces, and tools are properly cleaned and sanitized after each use to prevent cross-contamination and comply with operational hygiene standards.
Ensure proper labelling, storage, and tracking of samples to ensure traceability and prevent sample deterioration. Reports should be submitted to supervisors.

7. Materials, Equipment and Supplies

For microalgae cultivation and harvesting: *Chlorella vulgaris* can be isolated from the environment or acquired from various sources such as culture collections, commercial suppliers, and research institutions. Controlled laboratory conditions and sterile materials are essential for axenic cultivation of microalgal culture. Use appropriate growth media, such as agar plates or liquid media, to support algal growth, and equipment as**:** Erlenmeyer flasks with stoppers facilitating gas exchange, laboratory shaker (e.g. Phoenix OS 20), appropriate room temperature (T; 20-25 °C) and light intensity (e.g. 4000 K, fluoro or LED lights; 16:8 light:dark period), centrifuge tubes, tubes' holders and sterile dH_2O.
Growth medium: Bold's Basal Medium (BBM; prepared from commercially available solid mixtures (e.g. PhytoTech Lab) or in the laboratory from primary chemicals.
Cultivation conditions are monitored using a portable multiparameter meter (e.g. Multi 3610 IDS), by measuring T, pH values, dissolved oxygen concentration (DO), and electrical conductivity (EC) in the growth medium.
Culture fitness / cell number is determined with optical microscopy (e.g. Olympus CX21) by counting in a Neubauer counting chamber, and the growth of microbes on nutrient agar is checked. Optical density at 680 nm (e.g. spectrophotometer Nanocolor, VIS, Macherey-Nagel) and Chlorophyll A concentration is measured using extraction with ethanol, as we described in Proceedings 12th Socratic Lectures 2025 Part I, and calculated according to Lichtenhaler and Buschmann (2001).

The total suspended solids (TSS) in the sample are determined by filtration using glass Whatman glass microfiber filters (GF/C) according to the standard method ISO 11923:1997 or APHA (2540 D).

Nutrients (nitrates; N, phosphates; P) are analysed spectrophotometrically according to standard methods (e.g. ISO 6878:2004 for phosphate, ISO 7150-1:1996 for ammonia, ISO 7890-3:1996 for nitrate, EN 26777:1996 for nitrite), using cuvette tests (e.g. Nanocolor, Macherey-Nagel) or by high-performance liquid chromatography (HPLC) (e.g. Agilent Technologies 1100 Series).

For biomass processing: centrifuge tubes and sterile dH_2O for washing biomass, oven for drying and freeze-dryer for lyophilization, the fridge/freezer for biomass storing.

Devices and other equipment used: autoclave, centrifuge, oven, spectrophotometer, microscope, Neubauer counting chamber, portable meter for physio-chemical parameters, cuvette tests, HPLC (e.g. Agilent Technologies 1100 Series).

8. Computer Hardware and Software

Hardware: PC; software: photo editing software e.g. ImageJ, data management and word processing software such as Microsoft Office (Word, Excel); data saving and sharing insfrastructure: hard drive or cloud-based services, communication platforms and software: e.g. Microsoft Teams.

9. Step by Step Procedure

9.1. Chlorella vulgaris cultivation in controlled laboratory conditions

Microalgae cultivation in the laboratory involves several carefully controlled steps to optimize algal growth and produce quality biomass:

- A *Chlorella vulgaris* sample obtained from AlgEn, algal technology centre Ltd. was maintained in sterile laboratory conditions in a liquid medium.
- Bold's Basal Medium (BBM) liquid and agar plates are prepared, sterilized in an autoclave and allowed to cool to room T.
- Culture is isolated from BBM liquid to the BBM agar plates: the isolates are purified by streak plating; individual colonies are then transferred from BBM with agar (15 g/L) plates into liquid BBM.
- Aseptic techniques assure maintenance of culture purity during transfer. The flasks are covered with sterile foam stoppers to prevent contamination and ensure gas exchange, and afterwards placed on an orbital shaker (160 rpm) to ensure gentle mixing and avoid sedimentation.
- This process typically takes several weeks, as it involves multiple transfer and incubation steps to ensure the purity of the isolated culture (Alam et al., 2019).

Culture growth is maintained in Erlenmeyer flasks with liquid BBM:

- Growth conditions are 16:8 light/dark cycle and an ambient temperature of 25 °C ± 2 °C with continuous mixing.
- The batch is harvested when the culture reaches the desired growth phase (Section 9.2.). Once the growth rate stabilizes, the culture enters the stationary phase (> 1.0×10^8 cells/mL), indicating that fresh inoculum should be prepared for a new batch.
- A part of the grown culture is removed for the preparation of fresh inoculum; the culture in the exponential growth phase (on the third day, when the inoculum is prepared in 100 - 250 mL BBM; OD 680 < 1, approximately 10^7 cells/mL) is harvested by centrifugation, washed, and concentrated in fresh BBM (OD 680 = 2).
- 10 % of the inoculum is transferred into sterile Erlenmeyer flasks (volume 250 - 2000 mL) containing BBM (100 – 1000 mL; added inoculum 10 - 100 mL).
- The growth of *C. vulgaris* typically stabilizes after approximately two weeks of culturing in 100 - 250 mL BBM under the specified conditions. However, the optimal time for harvesting should be determined through preliminary tests, as growth rates may vary based on specific experimental conditions; consequently, the duration of the experiment may be influenced (Section 9.6).
- In general, subculturing is performed every few weeks when the culture reaches the stationary phase to prevent aging and sustain optimal growth (Sharma et al., 2012).

- To ensure reliable and reproducible results, it is essential to maintain consistent experimental conditions and optimized biomass processing methods as described here.

9.2. Microalgae biomass harvesting

Laboratory-grown cultures are separated from the growth medium with centrifugation. Samples should be subsequently washed in sterile buffer or dH_2O before further processing by centrifuging in 50 mL tubes for 10 min, 6500 rpm, removing the supernatant and repeating process until all biomass is harvested.

For biomass washing the process is repeated 3 times:

- 25 mL of sterile dH_2O is added, vortexed 10 seconds and centrifuged (10 min, 6500 rpm).
- Supernatant is removed and concentrated biomass is prepared in buffer/fresh medium, based on target use: cultivation/analysis/application.

9.3. Biomass processing

Possible ways to process washed/unwashed biomass for further application or storage are various, including using fresh biomass, freezing and thawing, drying (in the oven at the T < 45 °C for few hours, freeze-drying for 24 h). The selection of the processing procedure affects the activity and abundance of bioactive compounds. In terms of phytohormones research, processed biomass is stored at – 80 °C till further use, but immediate use of biomass is recommended. Otherwise, storage time should be further considered.

The main steps of SOP, including microalgae cultivation, harvesting and biomass processing are presented in **Figure 1**.; The recommended analyses are described in the section below.

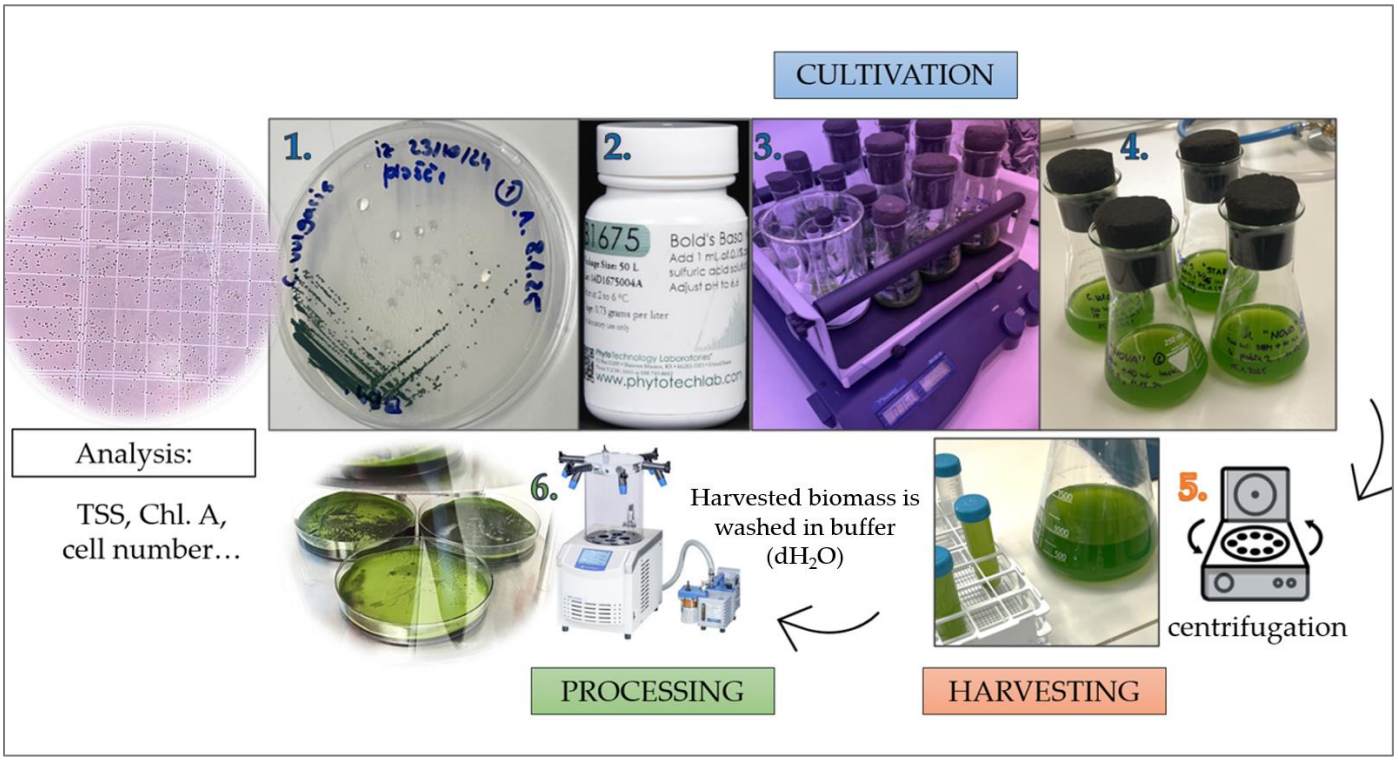

Figure 1. Cultivation of *Chlorella vulgaris* in controlled laboratory conditions; total suspended solids (TSS), chlorophyll a (Chl. A), cell counting, biomass harvesting and processing: 1. *C. vulgaris* purification on BBM + 15 g/L agar; 2. BBM medium; 3., 4. maintaining culture growth in liquid BBM; 5. harvesting; 6. processing: freeze-drying for application/analysis or storing at the – 80 °C until further use.

9.4. Recommended analysis

Cultivation conditions are daily monitored using a portable multiparameter meter (e.g. Multi 3610 IDS), by measuring T, and physicochemical parameters in the growth medium (pH, DO, EC). *Chlorella* sp. can grow in a wide pH range (4-10), but it is recommended by a supplier PhytoTech Lab to adjust the pH of BBM to 6.6. Measure initial DO, pH and EC in the medium, before and after microalgae inoculation. Parameters can vary depending on mixing, biomass density, and metabolic activity (Section 9.6.).

Periodically measure and adjust nutrient levels with controlled nutrient supplementation/diluting medium to maintain optimal levels. Namely, the appropriate NO_3 concentration at the beginning of the experiment is 250-500 mg/L, but nitrates may become limiting in late growth phases (< 5 mg/L); therefore, appropriate supplements should be added. Additionally, culture composition, OD, Chl. A, and TSS should be daily determined.

9.5. Troubleshooting

- Cultivation system not operating as usual. The problem should be defined and addressed appropriately. The natural increase in pH during microalgae cultivation can affect nutrient availability and, consequently, EC levels. When observing a decrease in EC with an increase in pH in a microalgal culture, this may indicate the precipitation of nutrients, which reduces their availability to the algae (Beltrán-Rocha et al., 2021). If key nutrients (N, P) become depleted, photosynthetic activity may drop, leading to lower oxygen production and decreased DO (Wan Hee et al., 2021). For new experiments, consider appropriate addition of nutrients.
- Variability in experimental factors; variations in flask volume, inoculum composition, or cultivation parameters such as temperature, light intensity and nutrients availability can significantly alter the growth patterns and overall performance of the culture. Therefore, controlling these factors throughout the experiment is critical for accurately monitoring growth phases and optimizing culture conditions.
- Microalgae cultures are contaminated. The culture should be discarded immediately (autoclaved before disposal). For new experiments, fresh non-contaminated culture should be used.
- Changed turbidity of (stock) solutions/reagents. The solutions/reagents should be discarded and freshly prepared.
- For better efficiency of chlorophyll extraction small volumes of microalgae culture should be used. Additionally, pellets of biomass will be more stable after centrifugation.

10. Data and records management

All experimental details should be carefully documented in the lab journal. Both raw and processed data should be securely stored in electronic format with appropriate backups. Pictures should be taken during experiments, and afterwards stored on hard drives or uploaded on cloud storage services.

11. Waste management

Disposal material used by the staff should be disposed of in appropriate waste fractions, according to applicable laws and good research laboratory practice. Chemicals should be disposed of in appropriate containers.

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

Calibration records should be securely stored and readily accessible for audits or quality assessments. If any of the instruments falls outside acceptable calibration limits, it must be adjusted or serviced before further use. Instruments should be maintained regularly.

13.2 Critical processes parameters and checkpoints

All material needed for SOP performance should be prepared ahead and sterile. Work in aseptic laboratory conditions to help prevent contamination of cultures or samples; only the intended microorganisms or materials should be involved in the experiment to maintain the integrity of the research.

The procedure from harvesting to the end of the process should be performed in the shortest time possible and follow the steps described, to obtain products with consistent quality and reduced variability. Prevent light and air exposure of biomass after drying (wrap flasks in aluminium foil), and immediately store it at – 80 °C, if not used. Ensure that there's minimal air exposure to prevent oxidation, check for properly sealed tubes. Prepared samples of biomass should be stored in small tubes, if not used at once, to avoid multiple opening-closing the tube containing entire gathered biomass. Avoid repeated freezing and thawing of samples as this can degrade or alter their integrity. Maintain detailed records of all stored samples, including storage conditions, date of storage, and any associated data.

Periodically check T conditions, instruments performance, and tube integrity to ensure samples are properly preserved. Samples should be used in the shortest time possible. Repeat chosen tests after few months of sample storage to control the quality.

14. Data on procedures and samples

Data on procedures and samples is given in **Table 2**.

Table 2. Data on procedures and samples.

Description of the yield	Processed (freeze-dried) biomass of *C. vulgaris* culture; immediate use of samples is recommended
Total volume of the culture yield	e.g., 500 mL
Total mass of the microalgae yield	0.05 g
Time required to obtain the yield	2 weeks
Estimated cost without manpower	Cost of equipment and maintenance
Contact person	Eva Cepec, eva.cepec@zf.uni-lj.si

Conclusions

This SOP describes laboratory procedures for *C. vulgaris* cultivation, harvesting and biomass processing to provide quality biomass for further analysis or application. Strict adherence to sterile conditions, proper cultivation of microalgae cultures, biomass processing and sample handling are essential to maintain the integrity of cultures, prevent contamination and obtain quality microalgae biomass. By following these critical process parameters and checkpoints, researchers can enhance the reliability of experimental outcomes and improve the overall efficiency of laboratory workflows.

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Institutional Review Board Statement: Not applicable.

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

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Scientific contribution

Preparation of Size-Controlled Unilamellar Lipid Vesicle Suspension from Lipid Powder : a Standard Operating Procedure within the Nanostructurome Methods Pipeline

Bar Laure [1,*], Lavrič Marta [2]

1. Faculty for Electrical Engineering, University of Ljubljana, Ljubljana 1000, Slovenia
2. Condensed Matter Physics Department, Jožef Stefan Institute, Ljubljana 1000, Slovenia

* Correspondence: Laure Bar; laure.bar@fe.uni-lj.si

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Abstract:
Liposomes, owing to their high biocompatibility and ease of production and manipulation, are now largely employed as nanocarriers in drug delivery and as simplified cell-membrane models in biophysics for the study of their physical, structural, and nanomechanical properties. To ensure reliable results and sample stability, liposomes are most of the time unilamellar and of a specific size range. In this proceeding paper, some widely used process steps will be combined to form a complete methodology for forming unilamellar vesicles of defined diameter.

Keywords: Lipid vesicles; Extrusion; Vesicle characterization; SUV; LUV

Table of contents

1. Definitions

SUV: Small unilamellar vesicle
LUV: Large unilamellar vesicle
MLV: Multilamellar vesicle
DLS: Dynamic light scattering
DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine

2. Background

Liposomes are spherical structures composed of one or more phospholipid bilayers surrounding an aqueous core. These artificial lipid vesicles can be classified into different categories according to the size and number of bilayers (lamellarity) constituting the vesicles. Unilamellar vesicles are characterized as small (SUV) when the diameter is below 100 nm, large (LUV) when the diameter is between 100 nm and 1000 nm, or giant (GUV) when it is larger than 1000 nm. They are usually prepared from large multilamellar vesicles (MLV) corresponding to the onion-like structures created spontaneously when phospholipids are hydrated.
Liposomes are versatile in their composition, and easy to prepare. Their high biocompatibility and low immunogenicity allow them to be widely used as nanocarriers for many active molecules in drug delivery processes (Liu et al. 2022). For the latter, they indeed enhance drug efficacy by ensuring a better control over drug distribution and release (Lombardo et al. 2022). From a more fundamental point of view, liposomes can also be used as cell-membrane models of lower complexity for studying the inherent physical, structural, and nanomechanical properties of the different vesicular structures produced (Bar et al. 2023).

3. Purpose, Scope and Applicability

For reliable and reproducible results in experiments, lipid vesicles need to reach a certain degree of uniformity in terms of size and lamellarity, which can influence the behavior, the stability, and properties of the liposomes in experimental and biological systems. For instance, unilamellar vesicles of 50 nm to 200 nm are typically used in drug delivery, for optimizing the drug encapsulation yield and the liposome circulation half-time (Nsairat et al. 2022). For experimental research, this diameter range also offers optimal stability. Techniques such as the quartz crystal microbalance with dissipation monitoring benefit from this size range, as the vesicle layer is small enough to fit within the detection range yet large enough to generate strong signals.

For preparing lipid vesicles, several procedures can be followed, firstly for the way to form liposomes (such as the thin-film hydration method, solvent injection process, or the reverse-phase evaporation method), and secondly for uniformizing them to size-controlled LUVs (such as extrusion process, or sonication) (Patil et al. 2014, Šturm et al. 2021). In this paper, a detailed step-by-step methodology for forming LUVs will be explained.

4. Health and Safety Warning

The protocol proposed here implies the use of chloroform. Chloroform can cause serious health issues if not managed properly. Direct contact with the skin or eyes can cause irritation and burns. This chemical is also suspected of causing cancer. It is necessary to familiarize with the Material Safety Data Sheet providing information on handling, storage, and emergency measures.

5. Cautions

Use chloroform always under a fume hood and make sure that you wear personal protection equipment, including gloves, glasses, and laboratory coat. Store the container in a well ventilated place and follow all the proper procedures to prevent environmental contamination. In case of accidental exposure, immediately ask for medical assistance and contact the person in charge in your institution.
To prevent degradation of lipids, protect them from exposure to light, and store them at low temperature (fridge or freezer, depending on their specific safety data sheet).

6. Personnel Qualifications / Responsibilities

People performing the steps of this protocol should be familiar with the safety rules of a chemistry laboratory, which may include or even demand additional training from the employer.

7. Materials, Equipment and Supplies

Lipids, as well as the extrusion kit are purchased by Avanti Research™. Chemicals and salts must be of high purity, to minimize the presence and potential effects of impurities.

8. Computer Hardware and Software

No computer or software are necessary for performing this procedure.

9. Step by Step Procedure for Extruded Lipid Vesicle Formation

A well-established protocol for preparing unilamellar liposomes is presented in detail below, in the form of a *to do* list and illustrated in **Figure 1**. Some of the steps are accompanied by small useful tips or recommendations.

9.1. Formation of a Lipid Dried Film

The first operation consists of the dissolution of the lipids in an organic solvent. Lipids can be either supplied diluted in chloroform or in powder form.

- For lipids diluted in chloroform, calculate the volume needed according to the solution concentration and dispense this volume in a round-bottom flask. For lipids in powder form, dispense the mass needed in the round-bottom flask and add high-purity (> 99.9 %) chloroform until complete powder dissolution.

Always manipulate chloroform with a glass syringe. Using a plastic pipette tip could lead to some sample contamination due to plastic degradation.

- Under a fume hood, slowly dry the mixture with a continuous mild flow of nitrogen or argon to form lipid films. To improve the homogeneity of the dried

lipid film, the flask can be periodically rotated during the evaporation process. If the volume is more than 2-3 ml, use a rotary evaporator for working under vacuum and earn some time.

All residual organic solvent should be completely removed. The sample can be vented or kept under a vacuum chamber overnight to ensure the elimination of the last chloroform residues.

9.2. Lipid Film Hydration

The lipid film is now composed of lipid bilayers stacked over each other. Their hydration will lead to the bilayer detachment and formation of MLVs.

- Add fresh buffer so that the lipids are at a concentration of 2 mg/ml, and hydrate the sample by immersing the flask in a temperature-controlled water bath. The temperature of the latter is kept 15°C above the main lipid phase transition temperature for having vesicles in the liquid phase. Keep hydrating for 45 min under magnetic stirring.

In order to help the dried film detachment from the glass, the buffer used for the hydration can be pre-heated to a temperature at which the lipid is in the liquid phase. After adding the warm buffer into the flask, a short sonication of 1 min can also be performed before putting the sample into the water bath.

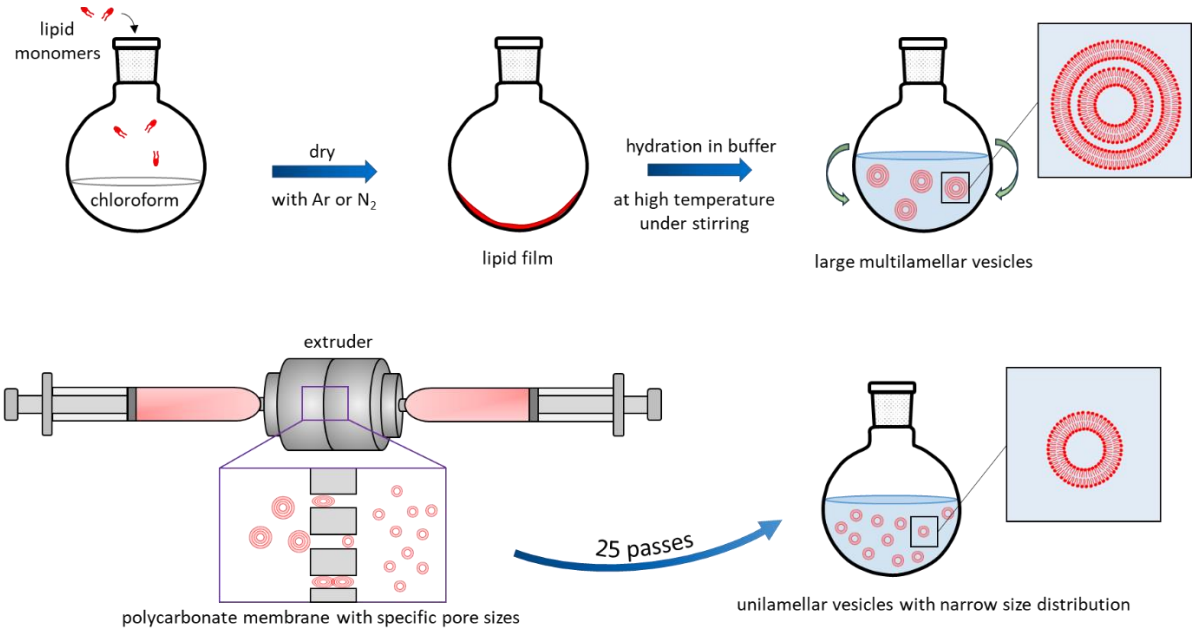

Figure 1. Scheme of the proposed protocol for forming SUVs or LUVs from lipids supplied in powder form.

9.3. MLVs Extrusion

After hydration, the MLVs solution may appear cloudy, but should be homogeneous.

- Prepare the extruder as explained in Section 10.
- Insert the lipid solution into one of the two syringes and perform a series of 25 passes.
- Dilute the extruded solution (now consisting of unilamellar vesicles) in buffer to obtain the desired working concentration.

The extrusion should be also done by keeping the lipids in the liquid phase.

10. Focus on Extrusion Material and its Preparation

Lipid vesicle extrusion (**Figure 2**) is a widely used process aiming at pressing hydrated MLV solutions through filters with specific pore sizes. This produces a uniform group of smaller vesicles (LUVs or SUVs), with their mean diameter corresponding to the pore size of the selected filters. Even though they operate in the same way, the commercial extruder sets can slightly vary in materials or features, such as the degree of automation and the regulation of temperature. The extrusion process explained here will be performed using the Avanti Research™ Mini-Extruder set. It is composed of Teflon membrane supports in which a polycarbonate membrane of specific pore size is sandwiched between two additional support filters. The solution coming from one syringe passes through this network to reach the opposite syringe. By doing this, the vesicles are broken and re-organized into smaller ones.

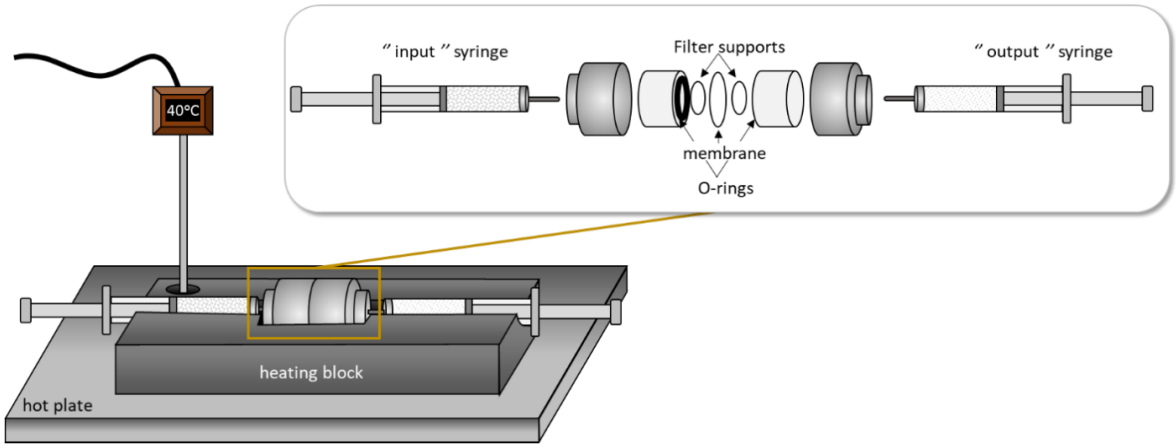

Figure 2. Scheme of an extruder setup and how it should be mounted and used for forming SUVs or LUVs.

For preparing the extruder, let's process as follows:

- Regulate the temperature of the extruder by placing the heating block and its associated thermometer on a hot plate and wait until reaching a stable temperature allowing the lipids to be in liquid phase.
- Assemble the membrane support system: use tweezers to gently handle the membranes. Filter supports can be pre-wetted with buffer before being placed, in order to reduce the dead volume.

Once the extruder is mounted, it is recommended to pass a full syringe of buffer through the setup to test if the system is well tightened and leakage-free. The buffer is then thrown away.

The extruder is now ready to be used as described in subsection 9.3.

Start the extrusion process from one syringe, and collect the solution from the syringe of the opposite side in order to retain the trapped impurities away from the suspension.

11. Data and Records Management

This section is not applicable for this SOP.

12. Related Protocols or SOPS

General instructions regarding the extrusion step can be found online on the Avanti Research website.

13. Quality Control and Quality Assurance

After extrusion, it is essential to assess the quality of the liposome suspension by checking the uniformity of the vesicles' mean diameter values and polydispersity. This can be done *via* different techniques, such as dynamic light scattering (DLS), nanoparticle tracking analysis, or transmission electron microscopy.

DLS is a non-destructive method allowing us to determine the particle size distribution by analysing the intensity variation of the light scattered within a colloidal solution, due to the Brownian motion of the particles in suspension. The Brownian motion describes the continuous collisions of particles within a suspension, which cause the particles' motion. The speed of the particles is related to their size: the smaller particles move faster than the larger ones. The Stokes-Einstein equation describing this relation highlights the influence of two parameters on the particle movement, and by consequence, the determination of the hydrodynamic diameter. These parameters are the working temperature and the viscosity of the dispersant (i.e. buffer).
An experiment can then be performed as follows:

- Switch on the DLS instrument several minutes before performing the measurement, to allow the laser light source enough time to warm up and reach a stable operating condition.
- Carefully set the parameter values needed in the software: the temperature, and the dispersant viscosity.
- Perform the measurement.

Figure 3 illustrates the size distribution of a 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) suspension before and after the extrusion in HEPES buffer. The non-extruded suspension displays two vesicle populations. The one representing 95% of the sample is characterized by a mean diameter of ~ 3.1 µm, corresponding to MLVs. Both the polydispersity index of 0.53 and the presence of a second smaller population indicate a high heterogeneity of the non-extruded suspension. On the contrary, once this solution was extruded using a membrane of 100 nm pore size, lipid vesicles attain an average diameter of 128 nm, and the polydispersity of the suspension is greatly reduced to 0.08. These data are in accordance with a homogeneous LUVs population. The final vesicle diameter is typically slightly higher than the membrane pore size, yet in the same range.

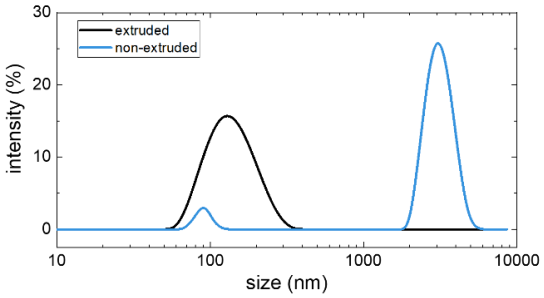

Figure 3. Size distribution plot obtained from the DLS measurement of a DMPC solution before and after extrusion with a 100 nm pore size membrane.

14. Conclusions

This paper presents a comprehensive step-by-step guide for the preparation of LUVs and SUVs utilizing widely used, standard processes. Key steps include the formation of a lipid film, the hydration of the film, and the extrusion of the resulting suspension. Due to its simplicity and high efficiency, this procedure proves exceptionally useful for applications in biochemical assays and membrane studies.

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Research

Standard Operating Procedure for Next Generation Sequencing of RNA Isolated form Extracellular Vesicles within the Nanostructurome Methods Pipeline

Kovachevikj Miona [1], Kralj-Iglič Veronika [2], Jakše Jernej[1,*], Jeseničnik Taja[1]

1. University of Ljubljana, Biotechnical Faculty, Agronomy department, Ljubljana, Slovenia
2. University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia

* Correspondence: jernej.jakse@bf.uni-lj.si

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Abstract:
The standard operating procedure (SOP) for next generation sequencing (NGS) of RNA from extracellular vesicles is presented. The SOP enables purification of small or total RNAs from isolated extracellular vesicles. Next, the SOP describes the sequencing of RNAs using NGS sequencing platform IonTorrent. In the presented protocol we define all the steps from isolation of RNA to sequencing data management, together with the cautions and checkpoints crucial for obtaining reliable high-quality sequencing reads, suitable for further analysis. NGS enables high-throughput DNA and RNA sequencing, offering accuracy, scalability, and cost-efficiency. RNA sequencing is widely used for analysing gene expression, alternative splicing, and non-coding RNAs. NGS also facilitates the study of extracellular vesicles (EVs), which play a key role in intercellular communication, especially in plant-pathogen interactions. Insights gained could inform novel disease management strategies, such as Spray-Induced Gene Silencing, for improved crop protection.

Keywords: Next generation sequencing; Extracellular vesicles; RNA-seq; Total RNA; Small RNA; IonTorrent.

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1. Definitions

NGS: next generation sequencing
EVs: extracellular vesicles
RNA-seq: sequencing of RNA molecules
SOP: standard operating procedure

2. Background

2.1. Next Generation Sequencing

Next Generation Sequencing (NGS) refers to a high-throughput DNA sequencing technology that enables the rapid and parallel sequencing of millions to billions of DNA fragments. Unlike traditional Sanger sequencing, NGS employs massively parallel processing, allowing for comprehensive genomic, transcriptomic, and epigenomic analyses with high accuracy, scalability, and cost efficiency (Goodwin et al., 2016). This technology is widely utilized in various fields, including agronomy, where NGS enables the genomic and transcriptomic analysis of crops, aiding in the identification of traits related to yield, stress resistance, and genetic diversity for improved breeding strategies. NGS facilitates the study of plant-pathogen interactions by uncovering resistance genes, defence-related small RNAs, and regulatory networks, enhancing disease resistance and crop protection efforts (Ashraf et al., 2022).

In transcriptome analysis, NGS, particularly RNA sequencing (RNA-Seq), allows for the in-depth characterization of gene expression profiles, alternative splicing events, and non-coding RNA populations, providing critical insights into cellular function, disease mechanisms, and developmental processes (Jiang et al., 2015; Krishanpal et al., 2015). In extracellular vesicle (EV) research, NGS is used to analyse the DNA and RNA cargo of EVs, their roles in intercellular communication, with emphasis on plant-pathogen interactions, where EVs play essential roles by transferring broad spectrum of biological molecules across different kingdoms (Zhang et al., 2024).

2.2. Extracellular Vesicles

Extracellular vesicles are spherical, bilayer nanovesicles, released by cells from prokaryotes, eukaryotes and archaea alike, that are primarily involved in intracellular communication (Ullah et al., 2023). Extracellular vesicles (EVs) play a crucial role in the secretory pathways of fungi, facilitating the transport of proteins, carbohydrates, lipids, different classes of RNA, nucleic acids, toxins and other macromolecules to the extracellular environment (Clos-Sanslavadore et al., 2022). Fungal pathogens, especially phytopathogens, exploit these secretory vesicles to transport a variety of effectors and secondary metabolites that aid in host manipulation and contribute to their pathogenicity (Rutter et al., 2021). On the other hand, plants secrete RNA-loaded EVs targeting pathogenicity genes, thus enhancing defence upon pathogen infection (Cheng et al., 2023). These host-pathogen inter-actions and the critical role that EVs play in them make the study of the EVs transcriptome highly coveted.

3. Purpose, Scope and Applicability

Extracellular vesicles are recently being studied as one of the key players in host-pathogen interactions, where they mediate the exchange of different biological molecules, serving as virulence/defence factors. Small RNAs are one of the classes of RNA molecules, present in the extracellular transcriptome in plant-pathogen interactions, that are transported via EVs to promote virulence in host plants upon infection with fungal pathogens. NGS RNA-seq of purified RNAs from isolated EVs and transcriptomic studies are crucial for identification of key aspects in plant-pathogen interaction. Understanding of the extracellular transcriptome is the foundation to development of novel disease management strategies, such as Spray-induced gene silencing, utilizing double-stranded RNAs. The presented SOP de-fines the key steps in NGS RNA-seq protocol, from EVs samples to bioinformatic analysis.

4. Health and Safety Warning

The SOP involves certain risks, particularly due to the use of hazardous chemicals such as phenol, chloroform, and guanidine salts for nucleic acid isolation, which are toxic, volatile, and pose risks of inhalation, skin absorption, and environmental contamination. Phenol is corrosive and causes severe chemical burns on contact. Chloroform is carcinogenic and toxic if inhaled, causes skin irritation and eye irritation.

5. Cautions

To minimize exposure, all work involving these reagents must be conducted in a properly functioning fume hood with adequate ventilation, and personnel should wear appropriate personal protective equipment, including lab coats, gloves, and safety goggles. The disposal of used chemicals must be performed according to regulative for hazardous chemicals. Additionally, the use of ultracentrifuge requires strict adherence to safety guidelines to prevent mechanical hazards, sample imbalance, and rotor failure. Users must ensure that tubes are properly balanced, rotors are securely fastened, and speed limits specified by the manufacturer are not exceeded. The centrifuge lid must remain closed during operation, and samples should be handled with caution to avoid aerosol formation and contamination. Regular maintenance and inspection of the equipment are essential to ensure safe and efficient operation.

6. Personnel Qualifications / Responsibilities

The sequencing infrastructure (Ion S5 sequencer, Ion Chef) should be used by trained personnel to avoid to prevent improper use and device deterioration. The staff who uses SOP should be trained in handling hazardous chemicals supplied with the commercial kits for nucleic acids isolation.

7. Materials, Equipment and Supplies

Materials: Monarch® Total RNA Miniprep Kit (New England Biolabs), mirVana™ miRNA Isolation Kit (Invitrogen), Ion Total RNA-Seq Kit v2 (IonTorrent, ThermoFisher), Ion 540™ Kit – Chef (IonTorrent, ThermoFisher), Agilent Small RNA kit

(Agilent), Agilent RNA 6000 Nano Kit (Agilent), Agilent RNA 6000 Pico Kit (Agilent), Agilent High Sensitivity DNA Kit (Agilent)

Devices: Agilent 2100 Bioanalyzer (Agilent), Ion Chef™ Instrument (IonTorrent, ThermoFisher), Ion GeneStudio™ S5 Prime System (IonTorrent, ThermoFisher), Qubit 4 Fluorometer (ThermoFisher)

Other equipment: microcentrifuge tubes (0.2 ml, 0.5 ml, 1.5 ml, 2 ml), micropipettes, heat block, ethanol, nuclease-free water.

8. Computer Hardware & Software

The S5 Prime system is supplied with server Dell PowerEdge T640 (2x Intel Xeon Gold 6140 Processor, 251 Gb of RAM and 25 TB of hard drive space) with Ubuntu 18.04.6 LTS operating system. Processing of the sequencing run, reporting and data availability is performed by Torent Suite software (version 5.18.1) accessible through the IP address of the computer. for automated sequencing data analysis. Torrent Suite is intuitive, browser-based interface which makes it fast and easy to plan, monitor, and view sequencing run results. Although adapters and quality trimming is performed by Torrent Suite software additional quality check can be performed by FastQC software (current version 0.12.1) (Andrews, 2010) and additional adapter and quality trimming performed by CUTADAPT (current version 5.0) (Martin, 2011).

9. Ion Torrent Semi-conductive Technology

The Ion Torrent sequencing is a next-generation sequencing technology that detects nucleotide incorporation by measuring changes in pH rather than using fluorescence-based methods. The sequencing process relies on semiconductor technology and proton detection. When a DNA polymerase incorporates a complementary nucleotide during sequencing, a hydrogen ion (H^+) is released as a byproduct. This ion release causes a slight pH change, which is detected by a semiconductor sensor. The intensity of the signal corresponds to the number of nucleotides added in a single cycle. This real-time, label-free sequencing approach enables rapid and cost-effective DNA sequencing with high throughput (ThermoFisher; https://www.thermofisher.com/si/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-technology.html).

10. Step by Step Procedure

10.1. Total RNA isolation from EVs

The Monarch Total RNA Miniprep kit is used for total RNA purification from sample of isolated EVs, according to manufacturers protocol. Briefly, the isolation of RNA is performed in two steps. Firstly, the EVs must be pelleted by centrifugation and resuspended in Lysis buffer. Next, the lysate homogenate must be loaded to the gDNA Removal Column and the flow-trough stored, as it contains RNA. The absolute ethanol is added and the suspension is loaded to the RNA Pruficiation Column. Flow-trough is discarged and the column, containing total RNA, is washed with RNA Wash buffer.

10.2. Small RNA Isolation from EVs

Enrichment of small RNAs (< 200 nt) is achieved by using the mirVana miRNA Isolation Kit, folowing manufacturers protocol. Briefly, sample of EVs is mixed with Lysis/Binding Solution to disrupt the EVs. Next, the organic extraction with Phenol:Chloroform is used to remove organic compounds. The aqueus phase is mixed with absolute ethanol and filtered trough the Filter Cartridge. Small RNAs are present in the flow-through filtrate. The filtrate is mixed with absolute ethanol and loaded to the second Filter Cartridge. After centrifugation, small RNAs are captured in the filter. The filter is washed with miRNA Wash Solution and small RNAs are eluted with nuclease-free water.

10.3. Library Preparation and Sequencing

The Ion Total RNA-Seq Kit v2 is used for the total/small RNA libraries preparation, following the manufacturer's instructions. The protocol must be performed by

authorized and trained personnel, and all essential safety guidelines must be followed as it is outlined in the user guide. The starting material for the library preparation is isolated total or small RNA. After RNA purification, the quality of the RNA must be evaluated with the Agilent 2100 Bioanalyzer to ensure that it contains the suitable fractions of small or total RNAs. If small RNAs will be sequenced, the quality and quantity of the miRNA needs to be assessed again with the Agilent 2100 Bioanalyzer. Next, appropriate input amount of RNA is to be determined for the construction of the RNA library.

Briefly, the library construction consists of hybridization and ligation, reverse transcription, purification and size selection, followed by amplification. Following the amplification, the DNA needs to be purified and size-selected by magnetic-based purification. The quality (yield and size distribution of the purified DNA) are assesed. If preparing multiple libraries, each barcoded library needst to be diluted to an equimolar concentration before mixing equal volumes to prepare a combined library.

Next, the Ion Chef Instrument is used for the amplification of the sequencing library and the sequencing. The sequencing plan and workflow needs to be created in the coresponding sequencing software.

After the planned run is created, the Ion Chef run for the preparation of the sequencing chip. The Ion 540™ Kit is used for library enrichment, chip loading and sequencing with the Ion GeneStudio S5 System, following the manufactur-er's instructions. Before starting the Ion Chef™ run, it is important to do one final review to ensure that all the settings are correct. The run needs to be monitored closely and all issues need to be addressed as they arise. After completion of the run, the data is retrieved on the correspodnig software.

10.4. Data Quality Check and Raw Data Processing

The deafult output format of the S5 prime system is UBAM format (unaligned BAM), which can be converted to standard FASTQ format inside Torrent Suite software with FileExporter plugin. TorrentSuite software reports basic QC statistics of sequencing run and barcoded samples like number of sequences, number of total bases, number of bases with quality score above 20. Standard QC software is FastQC (CITAT), which reports several standard quality related reports like basic statistics, per base sequence quality, per sequence quality scores, per base sequence content, per sequence GC content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequences and adapter content. Based o the report, decision can be made either your data has any problems of which you should be aware before doing any further analysis. The use of the software is from the bash shell like:

fastqc -t number_of_threads name_of_the_sequencing_file.fastq

The results is HTML file, which is easily viewed in any internet browser. In case additional adapter trimmin or quality trimming is required, tools like CUTADAPT can be used. Refer to manual for use, available at: https://cutadapt.readthedocs.io/en/stable/#.

11. Data Management

All sequencing data is stored in UBAM and FASTQ format on local server. The raw and processed data are archived in appropriate repositories (e.g. Sequence Read Archive - SRA).

12. Waste Management

The wase in this SOP is produced from nucleic acids isolation and sequencing protocol using commercial kits. The waste should be disposed according to manufacturers instructions and according to applicable laws. The gloves used by the staff should be disposed according to good research laboratory practices.

13. Related protocols or SOPs

The SOP specifies the use of commercially available protocols for efficient RNA isolation and sequencing, in accordance with best practices to ensure quality and accuracy of results.

14. Quality Control and Quality Assurance

14.1. Instrument Calibration

All instruments, used in the SOP, are regularly maintained and calibrated by trained service technicians and personnel.

14.2. Critical Steps and Checkpoints

The quality of RNA is essential for obtaining reliable sequencing data, as the success of the entire library preparation process hinges on the quality and integrity of the starting RNA material. Therfore, the quality of RNA must be determined before library preparation procedure. The isolated RNA must be stored at – 80 °C to prevent degradation.

Multiplexing of barcoded libryries of equimolar concentration is crucial for maximizing throughput and reducing costs, by allowing multiple samples to be sequenced in a single run, while maintaining quality and consistency. It is critical to ensure that all generated data can be attributed back to corresponding material via barcodes.

A critical step of sequencing procedure is creating the planned run, as it forms the foundation for the sequencing workflow. Selecting the correct sequencing application is vital, as it determines the entire setup based on goals, sample types and desired outputs. Accurately documenting and inputting the run details is essential for the effective analysis of the data. Proper documentation of barcodes for samples ensures that all data can be accurately linked back to corresponding libraries.

15. Data on Procedures and Samples

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

Table 1. Data on procedures and samples

	RNA isolation	Sequencing
Description of the sample	Purified EVs, stored at – 80 °C	Purified total/small RNA, stored at – 80 °C
Aliquots needed	1	1
Total volume of the sample	150 – 300 μL	10 – 100 μL isolated RNA
Estimated content needed	10^{10}/ml EVs	100 – 500 ng RNA, RIN 7
Time required to obtain results	1 day	1 week
Manpower	Highly skilled researcher	Highly skilled researcher
Estimated cost per sample without manpower	17 EUR	2500 EUR
Contact person	Jernej Jakše, jernej.jakse@bf.uni-lj.si	

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Research

Standard Operating Procedure for Quantifying Growth and Inflammatory Factors in-Cell Culture Supernatants, and Plasma via ELISA within the Nanostructurome Methods Pipeline

Michelini Sara[1], Drobne Damjana[1,*]

1. University of Ljubljana, Biotechnical faculty, Department of Biology, Ljubljana, Slovenia
* Correspondence: damjana.drobne@bf.uni-lj.si

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Abstract

Here we present the Standard operating procedure (SOP) used used to quantify growth factors and inflammatory markers in extracellular vesicles (EVs) - containing cell culture supernatants, plasma samples and using enzyme linked Immunosorbent assay (ELISA). The targets investigated in plassma and supernatant include interleukin 6 (IL-6), tumor necrosis factor α (TNFα), platelet-derived growth factor-BB (PDGF-BB), transforming growth factor β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF). These soluble mediators are crucial for cell proliferation, differentiation, and immune response regulation. They may also play a pivotal role in determining the efficacy of EV-based regenerative therapies. This SOPs was implemented within the Nanostructurome methods pipeline.

Keywords: Growth factors; ELISA; Plasma; Inflammatory factors; Supernatants.

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1. Definitions:

Ab: antibody; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CV: coefficient of variation; EGF: endothelial growth factor; ELISA: Enzyme Linked Immunosorbent Assay (ELISA); EV: extracellular vesicle; HRP: Horseradish Peroxidase, IGF-1: insulin-like growth factor 1; IL-6: Interleukin 6; O.D.: optical density; PDGF-BB: platelet-derived growth factor-BB; LLC: Large Latent Complex; PBS: phosphate buffered saline; PRP: platelet-rich plasma; RT: room temperature; TMB: 3,3, 5,5'-tetrametilbenzidine; TGF-β: transforming growth factor; TNFα: tumor necrosis factor α; VEGF: vascular endothelial growth factor

2. Background

Extracellular vesicles (EVs) are nanometer-sized vesicles secreted by virtually all cell types across a wide range of organisms, from plants to bacteria (Gill et al., 2019; Kumar et al., 2024; Peregrino et al., 2024; Bayat et al., 2021; Hu et al., 2024). In humans, EVs serve as carriers for various biomolecules, including proteins (such as cytokines, growth factors, receptors, etc.), mRNAs, miRNAs, DNA, and lipids (Kumar et al., 2024). As a result, they play a crucial role in both short- and long-range cell-to-cell communication in health and disease (Kumar et al., 2024; Mohammadipoor et al., 2023; Chang et al., 2021). The composition and quantity of EVs produced can vary depending on the stimuli a cell encounters, which in turn influences their biological effects and the cells they target. Plasma is a particularly rich source of EVs, primarily produced by hematopoietic cells, with platelets being a key contributor (Li et al., 2020; Aberro et al., 2021). Platelet-rich plasma (PRP) of autologous origin is being extensively studied in clinical dermatology because of its positive effect on skin regeneration and more applications are now being considered. The positive effect of PRP can be linked to the presence of different active molecules in platelets; but also, to the high concentration of biologically active EVs. Depending on the protocol used for plasma preparation, the concentration and type of EVs might vary, and thus the efficacy of the preparation. This is applicable also to cell-derived supernatant samples.

3. Purpose, Scope and Applicability

The purpose of this SOP is to describe the procedures for quantifying soluble mediators—such as IL-6, TNFα, PDGF-BB, TGF-β, EGF, IGF-1, and VEGF—in EV-containing plasma and cell-culture supernatants, using commercially available ELISA kits from R&D and Peprotech. This protocol was implemented within the Nanostructurome methods pipeline.

4. Health & Safety Warning

The hazards and safety measures regarding working with blood-derived products are described in Guidance on Working Safely with Human Blood and Plasma (https://www.sgul.ac.uk/about/our-professional-services/safety-health-and-environment/documents/guidance-on-working-safely-with-human-blood-or-plasma.pdf.) and must be carefully followed by the staff which should always wear suitable DPI during the procedure to reduce risk.

5. Cautions

The disposal of consumables should be in accordance with applicable laws and good research and laboratory practices. All non-disposable equipment should be disinfected at the beginning and end of the procedure. Adequate aeration of the room should be provided. An electronic or paper register for user reservations of the spectrophotometer should be provided. Operating procedures of the spectrophotometer, safety cabinet, pipettes and vacuum pump should be available for the users.

6. Personnel Qualifications / Responsibilities

When testing plasma samples, the staff should be informed regarding the hazards connected to testing these samples. The staff should undergo training on how to handle blood-derived samples. Usually, staff members with work experience in a biological/cell laboratory such as re-searchers are qualified enough to test these samples.

7. Materials, Equipment and Supplies

7.1 Samples:

Plasma samples must be transported to the laboratory at room temperature and processed immediately. If immediate processing is not possible, samples should be stored at -80*C and used immediately after tawing. Multiple freezing and thawing steps must be avoided. The samples should be tested as soon as possible after thawing. The same principles apply to cell-culture supernatants.

7.2 Other Equipment, Materials and Consumables:

Consumables usually provided within the ELISA kits or ELISA accessory kits: 96-well plate, standards, blocking buffer, sample diluent concentrate, assay buffer concentrate, capture and biotinylated detection antibodies, streptavidin or avidin – HRP, wash buffer, TMB or ABTS (chromogen), stop solution, adhesive plate covers.

Extra equipment and consumables: reagent reservoirs, PBS, spectrophotometer, multichannel and single-channel pipettes, tips, safety cabinet, waste disposal bins, protective gloves, goggles, lab coats, glass bottles to dilute diluents and buffer concentrates, - 80*C freezer and fridge for sample and material storage, plastic tubes, ddH_2O and sterile milliQ water to dilute reagents, vacuum pump with container to collect liquid waste.

8. Computer Hardware & Software

Hardware: PC, plate reader. Software: Microsoft Excel, GraphPad Prism, online data analysis resources such as assayfit pro. Data saving/sharing: Cloud and/or Drive documents.

9. Step by Step Procedure

The procedure is highly dependent on the type of kit used and the specific analyte/sample being investigated. These steps are always thoroughly detailed in the kit protocol.

For example, for measuring the analytes in EVs-containing plasma (cytokines and growth factors), we use Peprotech and R&D kits. Below, we provide a general step-by-step procedure; for more detailed instructions, please refer to the respective kit protocol.

9.1 Reconsituting and Storage of the Reagents:

- Reconstitute capture Ab and detection Ab reconstituted in the most appropriated solvent and store the aliquots at -20*C.
- Reconstitute standards in the most appropriate solvent and store the aliquots at -20*C.
- Dilute streptavidin-HRP or avidin-HRP in the most appropriate solvent and store the aliquots at 2-8˚C or at -20*C respectively.

9.2 Plate Coating with Capture Ab and Blocking:

- Dilute capture Ab in the most appropriated solvent to the concentration specified in the kit and add it to each well.
- Cover the plate with adhesive covers and incubate overnight at RT.
- Wash the plate 4x with the wash buffer and add the blocking buffer to each well. This step will reduce nonspecific binding.
- Incubate for at least 1 hour at RT and then repeat the washing steps.

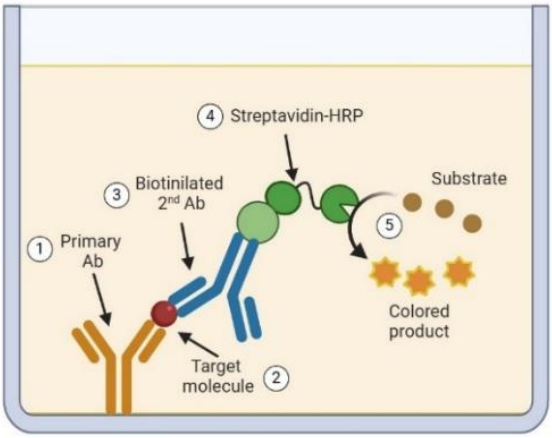

Figure 1: steps of a sandwich ELISA. 1) Plate coating with capture, 2) Incubation with the sample containing the target molecule, 3) addition of biotinylated detection Ab, 4) Incubation with the streptavidin-horseradish peroxidase, 5) colorimetric reaction induction by addition of the HRP substrate (e.g. TMB). Data are then acquired with a spectrophotometer after stopping the reaction (if necessary) using HCl. Image created with Biorender.com

9.3 Standard and Sample Preparation:

- Unfreeze and vortex the samples. Depending on the analyte under investigation, the sample should be diluted differently, For, example, the analytes mentioned in section 3 are diluted as shown in **Table 1**.

Table 1: table showing the most appropriated sample dilution factors for a given analyte.

Target	Dilution Factor	KIT
IL-6	Pure	ELISA TMB Peprotech
TNF-α	Pure	ELISA TMB Peprotech
VEGF	Pure	ELISA TMB Peprotech
EGF	1:2	ELISA ABTS Peprotech
PDGF-BB	1:50	ELISA ABTS Peprotech
IGF-1	1:50	ELISA TMB R&D
TGF-β1	Free, pure/ Total - 1:80	ELISA TMB R&D

- The dilution factors were determined by performing pilot experiments. *NOTE*: TGF-β1 can be found in the plasma both in an "active" (or free) form or in an "inactive" form which is bound to proteins of the Large Latent Complex (LLC) (11). The inactive form cannot be measured by the ELISA unless the sample is exposed to 1N HCI to allow LLC denaturation and protein release. After neutralization with 1N NaOH the sample can be added to the plate and measured. This process will allow to detect the total amount of TGF-β1 present in the sample (active + inactive).
- Add undiluted or diluted samples in the plate at least in duplicates.
- Unfreeze the standard aliquots and prepare 8 serial dilutions from the highest (concentration is kit dependent) to zero in the most appropriated solvent. Add the standard to the plate at least in duplicates.
- Incubate the plate at RT for 2 h.

9.4 Analyte Detection and Colorimetric Reaction:

- Wash the plate 4 times.
- Prepare the detection Ab by diluting the stock solution in diluent to the concentration specified within the kit. Add 100µl per well.
- Keep the plate at RT for 2 h then wash again 4 times.
- Prepare the Streptavidin-HRP or Avidin-HRP Conjugate by diluting the stock in The concentration specified in the kit and aliquot it in each well and incubate 30 min at RT.
- Wash the plate 4 times. Then add TMB (for plates incubated with streptavidin-HRP) or ABTS (to plates incubated with Avidin-HRP) to each well and allow the colorimetric reaction to develop at RT.
- For TMB kits wait at least 20 min, then add the stop solution and read absorbance at 450nm with wavelength correction set at 620nm.
- For ABTS kits instead measure the plate over time at 405nm with wavelength correction at 650nm. Save the data when the O.D of the highest standard in the curve reaches 1.4 units.

9.5 Data Acquisition

Save the excel file containing the results provided by the spectrophotometer. Copy the obtained O.D. values in the favorite analysis software. For example, use the online ELISA assay results calculator: assayfit pro. In assayfit pro insert the plate layout, the samples dilution factors and the standard curve fit input values. Analyze the data using a four-parameter logistic (4-PL) curve-fit. Ensue that the R2 value of the curve fir is > 0.99. Analyze and plot the obtained concentration data with GraphPad Prism.

9.6 Troubleshooting

High background:

- No/not enough washing steps.
- No/incorrect/insufficient plate blocking.
- Longer incubation times.
- Cross-reactivity / contaminated buffer / sample cross-contamination.

The signal is weak or absent:

- Sample not enough concentrated. Consider using a kit with a lower detection range.
- Incorrect storage, preparation or dilution of the components.
- Expired or omitted reagents.
- Enzyme inhibitor present or the assay is performed at too low temperatures.
- The plate reader is not set to read the correct wavelength.

The signal is too high:

- Incorrect dilution/preparation of reagents.
- Not enough washing steps.
- Too long incubation of the reagents (e.g. HRP)

Edge effects:

- Different temperature across plate.
- Evaporation – use adhesive plates covers.

Poor standard curves:

- Pipetting errors or incorrect calculations/pipetting technique.
- Reduced capture Ab binding: use a proper plate.
- Wells not properly aspirated or poorly mixed reagents.
- High coefficient of variation (CV) among sample replicates:
- Pipetting errors
- Contamination of plates or reagents.
- Different temperature across plate or evaporation – use adhesive plates covers.

10. Data and Records Management

All experimental details must be recorded in detail in the lab book. Both raw and processed data must be stored electronically both in the staff computers, in the cloud and in a physical backup for 10 years after generation.

11. Waste Management

Gloves, liquid waste, plates, tubes, tips and all plasticware which was in contact with the samples must be disposed of in biological sanitary waste, in compliance with applicable laws and proper research laboratory practices.

12. Related Protocols or SOPs

Not applicable.

13. Quality Control and Quality Assurance

Usually linearity range, linearity, lower limit of detection, precision, recovery and specificity tests are routinely performed by the kit producer and results are described within the data sheet of the specific kit. Some of these parameters change depending on the kit lot number and thus must be always carefully consulted before assay performance and purchase of the kit. To ensure the generation of quality data during the assay is important to: 1) run standard curves on every plate, 2) run samples/standards in duplicates/triplicates. The coefficient of variation (CV) of the samples/standard should be ≤ 20%, 3) include background controls and (if possible) positive controls, 4) Analyze the data using a 4-parameter curve fit, and 5) ensure that the O.D. values of the samples fall in the linear range of the standard curve. This will ensure the correct quantification of the analyte of interest. To allow this, consider sample dilution.

It is important to note however that human – derived samples composition might strongly differ because of donor-to-donor variance. To obtain reliable results with O.D. values that are in the linear range, is important to either: 1) perform a detailed literature research to determine the expected range of analyte concentration in healthy/non-healthy plasma in order to define (if necessary) the most suitable dilution factor of the samples, 2) if point one is not possible, perform a pre-test of the sample at different concentrations in order to define the dilution factor.

13.1. Instrument Calibration

Periodically calibrate the spectrophotometer using the appropriate calibration microplate. Calibration must be performed by a laboratory technician or by trained personnel

13.2. Critical Processes Parameters and Checkpoints

The temperature and incubation time of samples and capture/detection antibodies are critical during the assay performance. Additionally, sample / reagent storage and preparation can strongly influence the results.

14. Data on procedures and samples

Data on procedures and samples are given in **Table 2**.

Table 2. Data on procedures and samples

Description of the sample	Liquid. If not tested immediately, must be stored frozen at -80°C. Repeated freeze-thaw cycles mush be avoided.
Aliquots needed	Depends on the number of analytes to be investigated. If IL-6, TNFα, PDGF-BB, TGF-β, EGF, IGF-1, and VEGF are under investigation: 3 aliquots with at least 1ml, 200 µl and 400 µl are needed.
Total volume of the sample	300 µl per type of analyte (if tested in triplicates and undiluted).
Time required to obtain results	2 days
Manpower	Skilled researcher
Estimated cost per kit without manpower	Kit price: 300-600 euro each.
Contact person	Damjana Drobne, damjana.drobne@bf.uni-lj.si

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Conflicts of Interest:

The authors declare no conflict of interest.

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Research

Standard Operating Procedure for Testing Plant Biostimulants from Air Dried Microalgae with Germination Tests within the Nanostructurome Pipeline

Zrnec Drobnjak Tanja[1,*], Resman Lara[2], Žitko Vid[1], Schwarzmann Ana[1], Mihelič Rok[1]

1. University of Ljubljana, Biotecnical Faculty, Agronomy Department
2. Chamber of Agriculture and Forestry of Slovenia – Murska Sobota

* Correspondence: tanja.zrnecdrobnjak@bf.uni-lj.si

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Abstract:
A standard operating procedure (SOP) for testing plant biostimulants from air dried microalgae with germination tests is given. Microalgae-based biostimulants offer a sustainable approach to enhancing plant growth and resilience in response to environmental stressors such as drought and soil degradation. This study presents a standardized protocol for evaluating microalgae-based biostimulants through germination assays. The protocol encompasses sample preparation, seed germination tests, and statistical data analysis to assess biostimulant effectiveness or phytotoxicity. Experimental procedures involve preparing biostimulant solutions from dried microalgae biomass, testing their effects on seed germination and root elongation, and analyzing germination indices. ImageJ software is used for root length measurements, with data subjected to statistical validation. Standard laboratory quality control measures and data management should be followed. This protocol provides a reliable method for assessing the agricultural potential of microalgae-derived biostimulants, facilitating their further development and application in sustainable farming practices.

Keywords: Sustainable agriculture; Microalgae; Plant biostimulant; Germination test

1. Definitions

Biostimulant: A natural substance or microorganism applied to plants or soil to improve nutrient uptake, growth, and resilience to environmental stress.
Microalgae: Microscopic algae, such as Scenedesmus sp., used in this context for biostimulant production.
Biogas Digestate: A nutrient-rich by-product of anaerobic digestion of organic materials from biogas plant.
Germination Index (GI): A measure of seed germination performance and early root growth.
Germination Test: An experimental setup used to assess seed viability, root development, and the effectiveness of growth-promoting treatments.
PPE: personal protective equipment
DM: dry matter

2. Background

Microalgae-based biostimulants present sustainable solutions to contemporary agricultural challenges, including drought, irregular rainfall, and soil degradation (Miranda et al., 2024). These biostimulants enhance seed germination, root development, and nutrient utilization, thereby improving plant resilience to environmental stress (Bulgari et al., 2014; Van Oosten et al., 2017). The efficacy of microalgae as biostimulants is influenced by their growing conditions, with nutrient availability and environmental stresses (Khalid et al., 2024) playing crucial roles in their biochemical composition and effectiveness (Petkov et al., 2009; Alling et al., 2023).
Cultivating microalgae on liquid digestate exemplifies circular economy principles by converting waste into valuable agricultural inputs (Barzee et al., 2022). The liquid fraction of the digestate, obtained after mechanical separation of solid components, is rich in nitrogen, phosphorus, potassium, calcium, sodium, and trace elements. This nutrient-rich medium is cost-effective for cultivating microalgae such as *Scenedesmus* sp. (Resman et al., 2021).

The digestate provides essential nutrients for microalgae growth, supporting the production of secondary metabolites, including amino acids, polysaccharides and other bioactive compounds that are critical for their biostimulatory properties (Hossain Sani et al., 2024). This SOP outlines the testing process for the effectiveness of plant biostimulants using germination tests and data analysis to evaluate their efficacy.

3. Purpose, Scope and Applicability

The purpose of this SOP is to provide a standardized protocol for testing the efficacy of plant biostimulants. This ensures consistent and reliable evaluation of biostimulant performance through germination tests.

This SOP applies to biostimulants produced from microalgae. It encompasses the entire testing process, including sample preparation, germination tests, data collection, and analysis. It is used to determine optimal treatments, concentrations and potential phytotoxicity of developed microalgae biostimulants for further tests on agricultural plants.

It is intended for application in research laboratories, quality control departments, and agricultural product development settings. It is designed for researchers, technicians, and other personnel involved in the development and testing of biostimulants.

4. Health and Safety Warning

When handling biogas digestate (used for algae growth media in our experiments) and microalgae samples, it is crucial to wear appropriate personal protective equipment (PPE), including gloves, lab coats, and safety goggles. This precaution helps prevent skin contact, ingestion, or exposure to possible harmful substances. Proper laboratory ventilation is necessary, especially during material drying. When processing with chemicals e.g. solvents, extractants utilizing fume hoods or local exhaust systems can minimize the inhalation of potentially harmful gasses or particles. Regular inspection and maintenance of ventilation equipment are required to ensure effective operation and safeguard the health of laboratory personnel. Operating centrifuges, microfiltration units, and drying cabinets in accordance with the manufacturer's instructions and laboratory safety guidelines is imperative. Regular inspection, maintenance, and calibration of all equipment help prevent accidents or damage. Training personnel in the safe operation of laboratory equipment and emergency procedures further enhances laboratory safety. Exercise caution when handling chemicals like sodium hypochlorite for seed sterilization and use chemical-resistant gloves and work in well-ventilated areas or fume hoods. It is important to ensure that personnel are aware of the potential hazards of each chemical, including risks of burns, inhalation, and environmental impacts.

5. Cautions

Dispose of biostimulant solutions, germination materials, and other waste must be according to laboratory biohazard protocols. Segregate waste, use designated biohazard containers, and follow safe transport and disposal procedures. Regularly reviewing and updating waste disposal protocols to comply with regulations is essential for maintaining a safe and compliant laboratory environment. After use sterilize reusable glass equipment in an autoclave and disinfect growing plates with 70% ethanol after washing them. Microalgae biomass produced in experiments is strictly for scientific research and is not approved for personal use or any other unauthorized purposes. Ensuring that all personnel understand this restriction is crucial for maintaining the integrity and safety of the research.

6. Personnel Qualifications / Responsibilities

Personnel involved in the testing of plant biostimulants must have a background in laboratory techniques, including experience in handling biological materials and conducting germination tests. They should be familiar with the operation of laboratory equipment such as ventilated areas or fume hoods, autoclave, centrifuges, microfiltration units, and drying cabinets. All safety protocols must be followed, including the use of PPE, proper handling of chemicals, and disposal of waste. Regular safety audits and training personnel in emergency procedures must be conducted. Ensuring that all personnel are thoroughly trained in first aid, emergency procedures, spill response, and safe chemical handling is

vital. Additionally, insufficient training can compromise the accuracy and reliability of experimental results, leading to data integrity issues and potentially invalidating research findings. Laboratory technicians are responsible for preparing samples, conducting germination tests, and recording data accurately. They must ensure that all procedures are followed according to the SOP and maintain a clean and organized workspace. Supervisors oversee the entire testing process, ensuring adherence to the SOP and verifying the integrity of the data collected. They are responsible for training personnel, maintaining equipment, and addressing any issues that arise during the testing process. Training all personnel in the correct use of PPE and emphasizing its importance during sample handling and processing is essential for maintaining a safe laboratory environment. Statistical analysis of the experimental results must be accurate and reliable of the data and provide detailed report on the findings.

7. Materials, Equipment and Supplies

The testing of plant biostimulants derived from algae biomass requires a comprehensive array of materials, equipment, and supplies to ensure accurate and reliable results. Essential materials include microalgae biostimulant samples, which are derived from microalgae species such as *Scenedesmus* sp., at different formulations, testing kits such as Phytotoxkit liquid samples and test plant seeds. These biostimulants are prepared in various concentrations to test their efficacy on plant growth. Various types of seeds can be used in the germination tests specified by the experiment. The choice of seeds can depend on the specific objectives of the study and the plants relevance to agricultural practices. Seeds that are too big for germination test plates used in this SOP are unsuitable for this type of germination tests (e.g. avocado or other stone fruits). Deionized water is used for preparing biostimulant suspensions and for rinsing seeds, as it is free from ions and contaminants that could affect the results of the germination tests. Deionised water is also used in control group.

Germination tests are customized after Zhao et al. (1992) and Maunuksela et al. (2012). Phytotoxkit liquid samples (MicroBioTests Inc.) test plates were used to assess the phytotoxicity of the biostimulants. The Phytotoxkit is a standardized bioassay that measures the effects of substances on seed germination and early plant growth and can be used for toxicity testing, helping to determine the potential toxic effects of the biostimulants on plant growth and ensuring that they are safe for agricultural use. The ISO 18763:2016 soil quality standard outlines the methods for determining the toxic effects of pollutants on germination and early growth of higher plants. Adhering to this standard ensures that the testing procedures are consistent and reliable. Sterile testing plates are used to conduct germination tests. Sterility is crucial to prevent contamination that could affect the results. They provide a controlled environment for observing seed germination and root growth. Filter paper is placed on testing plates to support seed germination. Pipettes or micropipettes are used for accurate measurement and application of biostimulant suspensions. Precision in measuring and applying solutions is essential for obtaining reliable and reproducible results. Measuring cylinders are used for preparing and measuring biostimulant suspensions, ensuring that the correct volumes of solutions are prepared for the experiments. Sterile forceps are used for handling seeds and samples to prevent contamination. Gloves and laboratory safety equipment are essential for personal protection and maintaining a safe laboratory environment. An incubator or controlled temperature environment is used for maintaining consistent conditions during germination tests. The incubator provides a controlled environment with precise temperature and light conditions, ensuring that the germination tests are conducted under optimal conditions. In practice, a temperature-controlled room is also acceptable (25±5°C).

8. Computer Hardware and Software

Needed hardware is a personal computer with installed software, such as photo editing software e.g. ImageJ for analysing root length and germination data, data management and word processing software such as Microsoft Office (Word, Excel). Used data saving and sharing infrastructure can be any hard drive or cloud-based services and communication platforms. For statistical analysis, the suitable programme is needed e.g. in R language (R Commander).

9. Step by Step Procedure for Testing Plant Biostimulants from Microalgae

9.1 Sample Preparation

- Prepare obtained microalgae biomass. It can be used as fresh green biomass or dried at 35°C±2 to air-dryness and subsequently ground into fine powder (fraction size passes 0.2 mm mesh).
- Microalgae biostimulant solution preparation depends on the chosen treatments for germination tests. Mix the powder in deionized water to prepare suspensions with at least three different concentrations such as 1 g DM/L, 5 g DM/L, and 10 g DM/L and control with only dH_2O (this is crucial for statistical evaluation of the results).
- Suspension volume depends on number of repetitions. For one germination test plate (Phytotoxkit liquid samples, MicroBioTests Inc.; Figure 1) 20 mL of biostimulant suspension is needed to fully saturate the absorbing material (foam sheet or absorbing paper towels) of the test plate.

9.2 Germination Test Setup

- Use seeds of selected plant species to account for the plant species variability in sensitivity, the assays are performed with the seeds of three plant species: one monocotyl (proso millet - *Panicum milliaceum*) and two dicotyls (e.g., garden cress – *Lepidium sativum*, cabbage - *Brassica Oleracea* var. *capitata*).
- It is recommended to sterilize seeds by soaking in 1% sodium hypochlorite (NaOCl) solution for 5 minutes, followed by thorough rinsing with deionized water. This depends on plant species. Note: check for possible adverse effect of NaOCl on seeds germination, e.g. garden cress can be susceptible. If NaOCl is not used the chance of mould development on seeds is higher, therefore use more replicates.
- For preparation of growing medium, foam sheet and black filter paper are placed on test plates. Instead of foam sheet folded paper towel can be also used (3 paper towels folded in thirds and then in half so it fits test plate).
- First foam sheet or paper towel is saturated with the first half (10 mL) of the prepared suspension. After that the black filter paper is placed on top.
- Distribute 5-10 seeds (depending on seed size; more seeds when smaller) evenly on each half of test plate on black filter paper. Seeds must be offset towards the top of test plate.
- The remaining 10 mL of suspension per replicate is carefully evenly applied with pipette on black filter paper with seeds.
- Prepare at least three replicates for each treatment concentration and the control.
- Place the prepared test plates for incubation in a growth chamber or incubator vertically at 25±5°C for at least three days (time depending on plant species). It can be placed in dark or under dimmed even lighting. Monitor daily.

Preparation of biostimulant suspensions for germination tests and two germination test plates with treated cabbage seeds are shown in **Figure 1.**

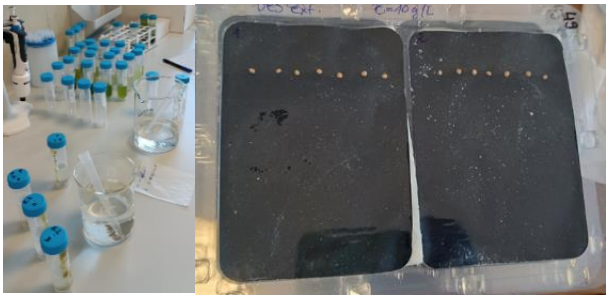

Figure 1. Preparation of different microalgae biostimulant suspensions for germination tests (left) and two germination test plates with cabbage seeds treated with 10 g DM microalgae biostimulant/L (right).

9.3 Observations and Data Collection

- After seeds germinate (in compliance with ISO 18763:2016 root length at least 1 mm) and when the root sprout shoots (approximately 3 days, depending on plant species and temperature), take the digital photograph with the measure (ruler) next to it.
- Measure the length of roots (in compliance with ISO 18763:2016 from seed to root tip) and shoots (in compliance with ISO 18763:2016 from seed to tip) using the ImageJ software or similar tools. Measure is determined with the help of ruler next to the taken photograph of the germinated test plate.
- In ImageJ software determine scale with the help of the ruler on the photograph (new scale for each photograph). This is crucial if you do not have a fixed stand for the camera (if the digital photograph was taken with the camera on the stand, you can determine one scale for all photographs).
- Count number of successfully germinated seeds.
-
- Calculate Germination Index (GI):

$$\text{GI}\,[\%] = \frac{(\text{no. germinated seeds} * \text{root lenght})}{(\text{no. germinates seeds in control} * \text{root lenght in control})}\ \text{x}\ 100 \qquad (1)$$

- Log all germination percentages, root lengths, and GI values in a laboratory notebook or spreadsheet.

9.4 Data Analysis

- Analyse the calculated GI using suitable software for statistical evaluation for determining significant differences among biostimulants at 95 % confidence. Compare treated samples against controls using appropriate statistical tests (e.g., t-tests).
- The efficacy of the biostimulant is determined on observed improvements in GI compared to control.

9.5 Quality Control

- Conduct the tests at least in triplicates to ensure consistency and reliability of results.

9.6 Troubleshooting

- Seeds may fail to germinate because of poor seed quality or dormancy, incorrect incubation conditions (temperature, light, or moisture), contamination in the germination medium. Therefore the seeds must be fresh, tested for germination prior to experiments (FAO and ISTA, 2023). Incubation must be verified and conditions adjusted to match species requirements. Preparation of germination materials must be in sterile enviroment.
- Uneven germination across treatments may be because of variability in suspension application or filter paper saturation or uneven distribution of seeds. Even saturation of filter paper before placing seeds must be ensured. Seeds must be placed evenly and maintain uniform spacing.
- Contamination (e.g., mold growth) may result from inadequate sterilization of seeds, tools, or containers, or from high humidity and prolonged incubation periods. Ensure thorough sterilization of all materials and equipment. If mold is detected the test is not valid and should be repeated.
- Inconsistent root growth measurements may be caused by errors in image capture or analysis, or variations in seedling orientation during measurement. Calibrate imaging equipment and use consistent lighting. Align seedlings and make shure the roots are not crossed before capturing images. Validate measurements in ImageJ manually if needed.
- Phytotoxicity may arise from excessive biostimulant concentrations or impurities in the microalgae biomass. Reduce the concentration of the biostimulant ssuspension.
- Double-check of all recorded data (photograps) before ImageJ analyses and calculations; since some photographs can be blured or light reflection may be visible which all distort results.

- Poor ventilation may result from inefficient systems or malfunctioning fume hoods. Inspect and clean ventilation systems regularly.

10. Data and Records Management

All raw data, observations, and measurements, including germination percentages, root lengths, and Germination Index (GI) calculations, must be systematically recorded in a laboratory notebook or digital spreadsheet. Ensure experimental conditions, such as incubation temperature, light cycles (in hours) and light intensity (bright light, dimed light, in darkness), and biostimulant concentrations, are documented clearly to support reproducibility. Digital data, including images and analysis outputs, must be securely stored on systems with regular backups, using standardized file naming conventions (e.g., experiment name, date, treatment identifier). Apply version control to digital documents to maintain a traceable record of changes, and export image analysis results in formats compatible with statistical software such as R. Validate data regularly by cross-referencing entries with original observations or images to ensure accuracy. Restrict access to raw and processed data to authorized personnel and share data securely using encrypted methods or password-protected files. Archive finalized datasets and reports in designated repositories with metadata descriptions that include study objectives, methods, and data collection dates. All data management activities must comply with institutional policies, legal requirements, and ethical standards while safeguarding confidentiality and intellectual property rights.

11. Waste Management

Dispose of all biostimulant suspensions, germination materials, and other waste according to local biohazard disposal protocols to prevent environmental contamination and health hazards.

12. Related Protocols or SOPs

This SOP includes a combination of optimized known procedures. Related protocols and SOPs include the SOP for Microalgae Cultivation, Harvesting and Biomass Processing, which outlines the procedures for cultivating microalgae in laboratory conditions, harvesting methods such as sedimentation or microfiltration, and drying processes. The SOP for Procedure for Interferometric Light Microscopy of Extracellular Particles can be used to analyze microalgae biomass as microalge exude extracelular compounds.

13. Quality Control and Quality Assurance Section

13.1 Instrument calibration

Instruments calibration (laboratory scale, thermostat of drying cabinet) regularly according to the manufacturer's specifications is essential to maintain the accuracy and reliability of results. Calibration schedules should be documented and strictly followed, with a record of all calibration activities maintained for quality assurance purposes. Any deviations in equipment performance must be identified and corrected promptly to avoid inaccuracies in measurements.

For imaging systems, use a calibration scale (e.g. millimeter graph paper) or ruler to verify accurate measurements in software such as ImageJ. 13.2 Critical processes parameters and checkpoints

Critical process parameters and checkpoints are essential to maintaining the quality and reliability of the testing process. During microalgae biostimulant preparation, it is vital to confirm the exact concentrations of suspensions (e.g., 1 g DM/L, 5 g DM/L) and ensure uniform mixing to avoid variability. Seed sterilization must be conducted using appropriate sterilizing agents, such as sodium hypochlorite, with thorough rinsing to prevent residual chemicals from interfering with germination. In the germination test setup, filter papers must be evenly saturated with the correct volume of biostimulant solution or control water (e.g., 20 mL per test plate), and seeds must be distributed uniformly on test plates to minimize bias. Incubation conditions, including temperature (25±5°C) and light intensity of environment. Light intensity is generally not critical for seed germination in most

plant species. Many seeds germinate best in darkness, while some require light to trigger germination. Light preferences depend on a plant species germination demands (e.g. garden cress, *Lepidium sativum,* is somewhat sensitive to light during germination. While it does not require complete darkness, light can enhance its germination rate compared to total darkness. However, it can still germinate under a wide range of light conditions as long as moisture and temperature are optimal).

Data collection requires careful validation of root length measurements using calibrated imaging systems, with recorded values double-checked for accuracy. Calculations of the Germination Index (GI) should be double-checked to ensure consistency and precision. Adherence to these parameters and checkpoints ensures the accuracy, reproducibility, and credibility of the testing process.

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 microalgae sample	Dried microalgae biomass
Needed dry microalgae	10 g
Number of plant seeds	120 (10 per grow plate)
Minimal volume of the suspensions (dH_2O) needed	20 mL/grow plate
Time required to obtain results	4 to 5 days (3 days germination + 1 day image analyses)
Manpower	Technician
Minimal estimated cost without manpower	25 €/treatment (min. 3 replications + control)
Contact person	tanja.zrnecdrobnjak@bf.uni-lj.si

15. Conclusions

This SOP describes laboratory procedures for testing the germination stimulation potential of microalgae-based biostimulants.

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Research

Standard Operating Procedure for Trace Analysis of Organic Contaminants by Liquid Chromatography Coupled to Mass Spectrometry (LC-MS), a Part of Nanostructurome Methods Pipeline

Heath Ester[1,2,*], Andreasidou Eirini[1,2], Heath David[1]

1. Jožef Stefan Institute, Ljubljana, Slovenia
2. International Postgraduate School Jožef Stefan, Ljubljana, Slovenia

* Correspondence: Private Parts; ester.heath@ijs.si

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Abstract:
This Standard Operating Procedure (SOP) provides guidelines for the trace analysis of organic contaminants in different matrices using Liquid Chromatography coupled to Mass Spectrometry (LC-MS). It covers key steps such as sample collection, preparation, and extraction, followed by chromatographic separation and mass spectrometric analysis. The SOP ensures accurate quantification and identification of contaminants by employing calibrated standards, internal standards, and quality control measures. The procedure emphasizes adherence to safety protocols, instrument maintenance, and troubleshooting for consistent, reliable results, meeting both regulatory and quality assurance standards for analytical performance. This SOP is a part of Nanostructurome methods pipeline.

Keywords: Liquid chromatography; Mass spectrometry; Extraction; Organic contaminant; Trace analysis

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1. Purpose

This Standard Operating Procedure (SOP) outlines the steps to be followed for the trace analysis of organic contaminants in various matrices, such as water, soil, food, and biological samples, using liquid chromatography coupled to mass spectrometry (LC-MS). The procedure ensures the accurate and reproducible quantification and identification of contaminants at trace levels (**Figure 1**). LC-MS/MS is employed due to its high sensitivity and selectivity, with the ability to detect a broad range of compounds in complex matrices. This SOP was developed for the purpose of Nanostructurome methods pipeline.

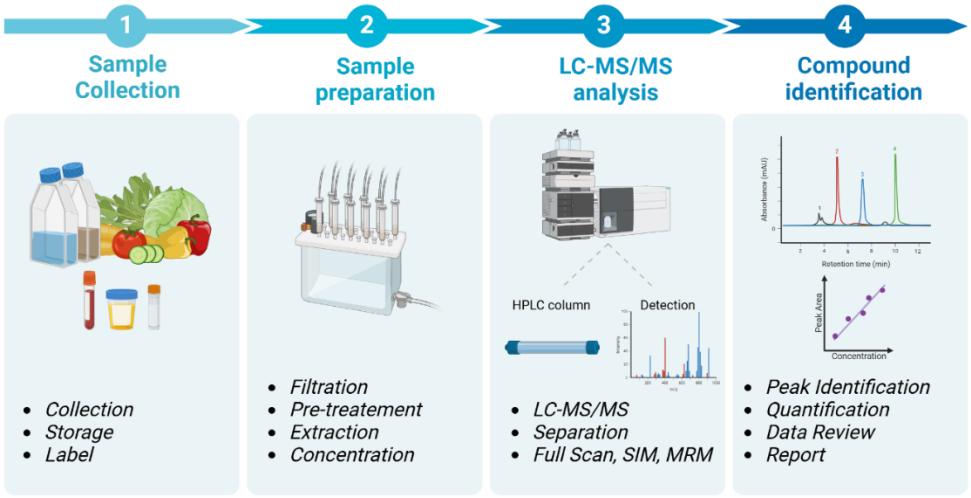

Figure 1. Steps of SOP for LC-MS/MS analysis.

2. Scope

This SOP is applicable to the analysis of organic contaminants, such as pesticides, pharmaceuticals, and industrial chemicals in liquid and solid samples. It includes sample preparation, LC-MS analysis, and data interpretation. Applicable sample types include:

- Water samples: surface water, groundwater, wastewater, drinking water
- Solid matrices: soil, sediments, biosolids
- Food and agricultural products: Fruits, vegetables, grains
- Biological samples: plasma, urine

3. Involved Personnel

Involved Personnel include the following:

- Analyst: Responsible for performing the analysis, following this SOP, ensuring instrument calibration and documenting all relevant data.
- Quality Control (QC) Officer: Responsible for ensuring compliance with quality standards, reviewing results, validating calibration and method performance, and identifying any errors that require additional actions.
- Laboratory Supervisor: Responsible for overall procedure adherence, including laboratory safety measurements, regulatory guidelines, equipment maintenance, and troubleshooting.

4. Materials, Equipment and Supplies

The SOP requires the following:

- Liquid Chromatograph (e.g., HPLC, UPLC) equipped with a suitable column (e.g., C18, reverse phase).
- (Hybrid) Mass Spectrometer (e.g., QTrap: Quadrupole Ion Trap, Orbitrap, tQ: triple Quadrupole or ToF: Time of Flight) with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) source.
- Solvents: HPLC-grade methanol, acetonitrile and water. Additives or buffers such as 0.1% formic acid, ammonium acetate, ammonium formate or ammonium fluoride may be used to enhance separation efficiency and ionization.
- Standards: Certified Reference Materials (CRMs) for target organic contaminants prepared as individual solutions after weighing and dissolving a proper amount of each compound in a specific solvent.
- Internal Standards: Stable isotope-labelled compounds or structurally similar analogues for quantification to correct for matrix effects and variability in ionization.
- Consumables: Sample vials (e.g., glass vials with PTFE-lined caps), syringes, and filters (e.g., 0.22 or 0.45 μm, PTFE or nylon filters), solid-phase extraction (SPE) cartridges (if applicable for sample clean-up), pipettes and volumetric flasks (for precise solvent and standard preparation).
- Calibration standards: Prepare at different concentrations of target analytes to generate calibration curves for quantification.

5. Procedure for Sample Collection and Storage, Preparation and Analysis

5.1. Sample Collection and Storage

5.1.1. Collect samples in clean, contaminant-free containers. Containers can be glass or plastic (High-Density Polyethylene and Polypropylene) according to target analyte polarity to prevent adsorption.

5.1.2. Transport samples in temperature-controlled conditions, ensuring minimal exposure to light and contamination.

5.1.3. Store samples according to matrix requirements:

- Water samples: Refrigerate at 4°C and analyze within 7 days (unless preserved).
- Biological samples: Freeze at -20°C or lower to prevent degradation.
- Soil and sediment samples: Store at 4°C and dry, if necessary, before extraction.

5.1.4. Label and document all samples, including sample type, location, date and time, storage conditions and other relevant metadata.

5.1.5. Use of appropriate preservatives if required.

5.2. Sample Preparation

5.2.1. Spiking with Internal Standard: Spike a known quantity of internal standard (stable isotope-labelled compounds or analogues) to the sample. This step helps to monitor the extraction efficiency and ensures accurate quantification.

5.2.2. Filtration: Filter liquid samples through a 0.45 μm membrane to remove particulate matter.

5.2.3. Sample Pre-treatment: Deconjugation for biological samples: For biological samples such as urine or plasma, deconjugation may be necessary to release conjugated analytes (e.g., glucuronides or sulphates) through enzymatic or acid hydrolysis to ensure the target analytes are in their free forms for accurate quantification. For water and other aqueous samples, adjust the pH to the desired pH (acidic or basic) using suitable reagents (e.g., HCl for acidic conditions and NaOH for basic conditions). Adjusting the pH can improve extraction efficiency, stability, and recovery of specific analytes, as some compounds are more stable or extractable under specific pH conditions. For aqueous samples, the addition of preservatives such as EDTA or a chelating agent is optional to prevent the formation of complexes with metallic ions.
5.2.4. Extraction (if applicable): For solid samples, extract contaminants using appropriate solvents (e.g., acetonitrile, methanol). Extraction techniques may include sonication, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) or microwave-assisted extraction, depending on the sample type and target analytes. For aqueous samples, consider solid phase extraction (SPE) or direct dilution.
5.2.5. Concentration: Evaporate solvent under gentle nitrogen flow or a rotary evaporator to concentrate the sample to an appropriate volume.
5.2.6. Reconstitution: Reconstitute the extracted sample in an appropriate solvent (e.g., 50:50 mixture of water and acetonitrile) prior to analysis

5.3. Instrument Setup

5.3.1. LC System: Set the appropriate chromatographic conditions (flow rate, column temperature, mobile phase composition) based on the chemical properties of target analytes.

- Choose an LC column that is suitable for the target analytes and is equilibrated adequately with the mobile phase prior to sample injection to ensure reproducible results.
- Ensure pressure and temperature are within expected/optimal ranges to prevent column damage or degradation of compounds during the analysis.

5.3.2. Mass Spectrometer:

- Source Temperature: Set the optimal source temperature to achieve stable ionization and maximize sensitivity for the selected ionization mode.
- Collision Energy: Adjust collision energy to ensure optimal fragmentation of target analytes.
- MS Scan Range: Configure the mass spectrometer scan range (e.g., m/z 100-1000) to capture the full range of relevant ions.
- Ensure proper tuning and calibration of the mass spectrometer using calibration standards to improve accuracy and reproducibility.

5.3.3. Data Acquisition Method:

- Full Scan: Detection of a broad range of ions, mainly for identifying unknown compounds.
- SIM (Selected Ion Monitoring): focus on specific ions that correspond to target analytes.
- MRM (Multiple Reaction Monitoring): Monitoring of precursor and product ion of each analyte (Figure 2).
- Scheduled Acquisition: Mass spectrometer switches between analytes at specific times based on different retention times (U.S. EPA, 2009).

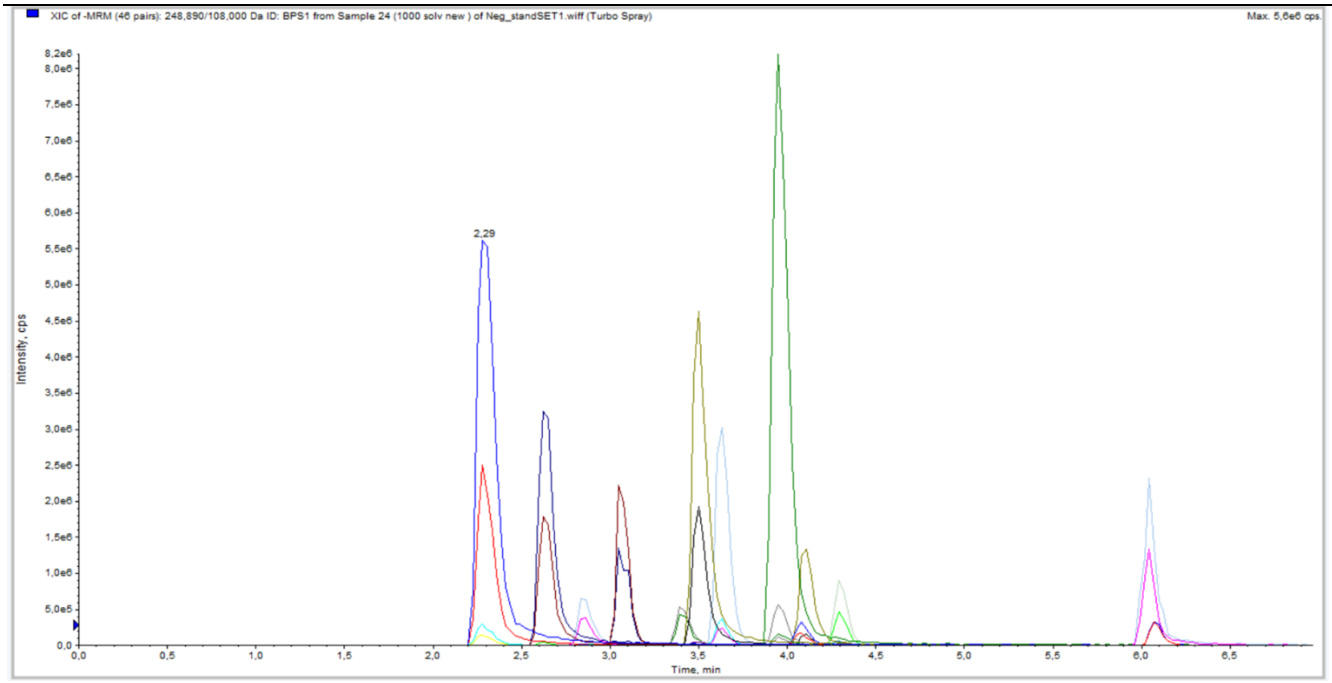

Figure 2. Example of Extracted Ion Chromatogram (XIC) following MRM monitoring for 14 organic contaminants and respective internal standards.

5.4. Calibration and Quality Control

5.4.1. Calibration Curve:

- Prepare a set of calibration standards with known concentrations of target organic contaminants.
- Standards should cover the expected concentration range for the analytes of interest, typically covering low, mid, and high concentrations.
- Generate a calibration curve for each analyte using the area ratio, peak area or height vs. concentration.
- Ensure that the lowest concentration selected is near the limit of quantification (LOQ) and the highest concentration is near expected levels within the sample.

5.4.2. Quality Control of Samples:

- Analyze at least five blank samples (or pseudo-blank with low concentration of analytes) to check for contamination or background noise.
- Analyze one low-level standard and one high-level standard to ensure the sensitivity of the analysis and the expected range, respectively.
- Analyze solvent or blank samples, especially following high-concentration samples, to evaluate the carry-over effect and ensure that analytes from previous samples are not contaminating subsequent injections.
- Analyze a replicate QC sample for each batch of samples, which includes using a known concentration of target analytes in solvent or matrix.
- Ensure that the recovery of the analytes is within acceptable limits (e.g., 80%-120%) (Magnusson and Örnemark, 2014; European Commission, 2021, 2023).

5.5. Sample Analysis

5.5.1. Inject the prepared sample into the LC-MS system, ensuring the use of appropriate injection volume following optimization (2-20 μL).

5.5.2. Monitor the chromatographic separation of analytes. Ensure proper retention times and resolution.

5.5.3. Collect MS data in the chosen mode (Full scan, SIM, or MRM).

5.5.4. Analyze the data for peak identification, quantification, and confirmation of the analytes.

5.6. Data Processing and Analysis

5.6.1. Peak Identification:

- Identify the analyte peaks by comparing retention times and mass spectra with those of the calibration standards and known reference materials. As part of the identification criteria of compounds for MS/MS techniques, signal-to-noise ratio should be higher than 3, or in case of noise absence, a signal should be present in at least five subsequent scans. The minimum number of ions is set at 2 product ions of typical MS/MS systems. Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap, while the ion ratio from sample extracts should be within ± 30 % (relative) of the average of calibration standards from the same sequence (Pihlström NFA et al., 2017).

5.6.2. Quantification:

- Use the calibration curve to calculate the concentration of each analyte in the sample, adjusting for sample dilution or concentration steps performed during preparation.

5.6.3. Data Review:

- Verify the accuracy of the results by ensuring that the internal standard response is within acceptable limits.
- Check the performance of the calibration curve through a correlation coefficient (r^2) of > 0.99 to confirm good linearity and reliable quantification.

5.6.4. Reporting:

- Record the results in the laboratory information management system (LIMS) or report them manually.
- Include details such as sample ID, analyte concentrations, method parameters, and QC results.

6. Safety and Waste Management

- Follow laboratory safety protocols when handling solvents and reagents, especially those that are hazardous or toxic. For specific safety information on each chemical, refer to Safety Data Sheets.
- Dispose of waste solvents and contaminated materials in accordance with institutional and local regulations.
- Wear appropriate personal protective equipment (PPE), including gloves, lab coat, and eye protection.
- Use fume hood when handling hazardous solvents to minimize inhalation exposure.

7. Troubleshooting and Maintenance

7.1. Instrument Issues:

- Pressure issues: If the system pressure increases or fluctuates abnormally, which may result to retention time drift, check for clogged filters, column blockages, or air bubbles in the mobile phase lines. Leaks in the LC system can lead to pressure fluctuations, so connections and fittings need to be checked for tightness.
- Ionization issues: If the ion source is not functioning optimally, check for contamination or deposition of sample residue. Ensure the ion source temperature

and voltage settings are optimized for the target analytes. For electrospray ionization (ESI), ensure the spray needle is not clogged and that the sheath gas and nebulizer gas flow rates are appropriate.

7.2. Data Issues:

- If chromatographic peaks are not well resolved, adjust the column, mobile phase composition, or flow rate. Ensure that the column temperature is stable and within the recommended range for optimal separation.
- If there are issues with sensitivity or signal-to-noise ratio, optimize the MS parameters (e.g., ion source voltage).

8. 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 sample	Aqueous sample, frozen
Aliquots needed	1
Total volume of the sample	LQ: 100-250 Ml, SOLID: 1-10g (depending on matrix/expected concentration of analyte)
Estimated content needed	
Time required to obtain results	1 month (target analysis), 6 month (suspect/non-target)
Manpower	Highly skilled researcher
Estimated cost per sample without manpower	Depends on matrix and type of analysis
Contact person	Ester Heath, ester.heath@ijs.si

9. Conclusions

In conclusion, this SOP outlines a comprehensive and systematic approach for the trace analysis of organic contaminants in environment, food or health related matrices using LC-MS, ensuring reliable and accurate results. By following the procedures for sample preparation, instrument calibration, and quality control, analysts can achieve precise quantification and identification of contaminants. Adhering to safety protocols and maintaining equipment ensures consistent performance, making this SOP an essential tool for conducting high-quality analytical work in environmental, food, and pharmaceutical testing. Moreover, this SOP ensures that the process for trace analysis of organic contaminants by LC-MS is conducted in a controlled, reproducible manner, meeting quality assurance and regulatory requirements.

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

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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.

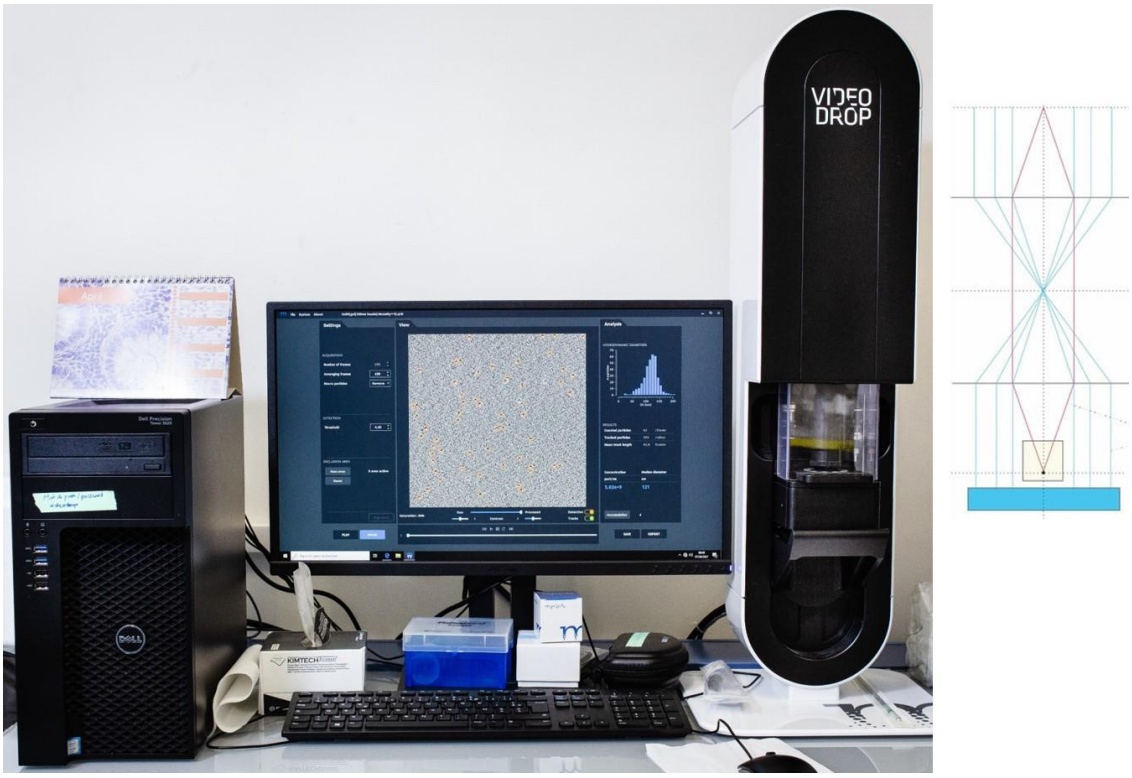

Figure 1. Interferometric light microscope Videodrop (Myriadelab, Paris, France).

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.

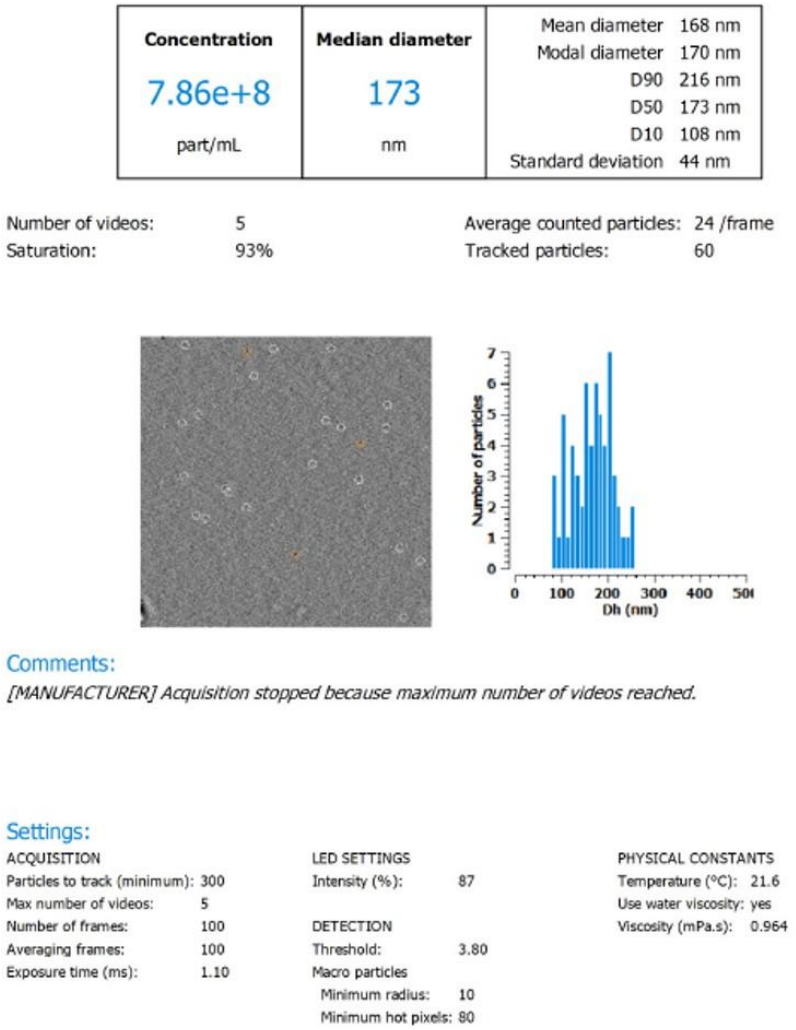

Figure 2. An example of the display of the results of measurement with ILM Videodrop (Myriadelab, Paris, France).

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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Research

Microalgae as a Source of Extracellular Vesicles: Laboratory Cultivation within the Nanostructurome Methods Pipeline

Tanović Marija [1±], Santrač Isidora [1±], Dimitrijević Milena [1], Ćurić Valentina [2], Kovačević Snežana [1], Stanić Marina [1], Danilović Luković Jelena [1*]

1. University of Belgrade, Institute for Multidisciplinary Research, Belgrade, Serbia
2. University of Belgrade, Faculty of Biology, Belgrade, Serbia

* Correspondence: reginadelphy@yahoo.com

± Both authors have contributed equally to this work

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Abstract:
Extracellular vesicles (EVs) are cell-derived, membrane-bound structures that play vital roles in intercellular communication, biomolecule transport, and various physiological processes. Recent studies have shown that, in microalgal cells, EVs act as key facilitators of intercellular communication via transfer of bioactive compounds to convey regulatory signals and instructions. In aquatic ecosystems, this is most evident in the influence EVs have over community structure, trophic interactions, and cell fate. Microalgae-derived EVs have promising applications in biotechnology, environmental monitoring, and sustainable bioengineering. However, for large-scale production of these microalgae-derived EVs to be feasible and cost-effective, several challenges are yet to be solved, including development of standardized isolation methods, further characterization of EVs biochemical composition, as well as a more comprehensive understanding of their function. Cultivating microalgae in a controlled laboratory setting, with standardized protocols for culture maintenance, is crucial in order to address and solve these challenges, efficiently and reliably.

Keywords: Microalgae; Extracellular vesicles; Laboratory cultivation; Good laboratory practice

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1. Definitions

EVs: Extracellular vesicles

2. Background

Extracellular vesicles (EVs) are membrane-bound structures released by cells into the extracellular space. They play crucial roles in intercellular communication, biomolecule transport, and various physiological and pathological processes (Petrovčíková et al., 2018). EVs carry a diverse array of bioactive compounds, including lipid mediators, proteins, and nucleic acids, which can modify the recipient cell's phenotype (Record et al., 2018). Microalgae, as sustainable sources of bioactive compounds, are also known to produce EVs (Picciotto et al., 2021). EVs-mediated intercellular communication in aquatic ecosystems can influence community structure and trophic-level interactions, and modulate cell fate, morphology, and susceptibility to viruses (Schatz & Vardi, 2018). In recent years, the sustainability, scalability, and renewability of the EVs derived from microalgae were investigated and appeared promising compared to EVs from other sources (Paterna et al, 2022). For example, EVs from microalgae *Tetraselmis chuii* are taken up by human and *Caenorhabditis elegans* cells, recognized and internalized via energy-dependent mechanisms, localizing in the cytoplasm of specific cells and persisting for several days (Picciotto et al., 2021).

Microalgae-derived EVs can be used as nanocarriers for bioactive compounds in health supplements, cosmetics, and food ingredients, and have potential in therapeutic applications due to their ability to deliver drugs or other therapeutic molecules (Picciotto et al., 2021). Up to this point, the existing literature on this topic remains relatively limited, and further research is needed to fully confirm and explore the potential of EVs in these applications. For cost-effective production and utilization of microalgae-derived EVs to be attainable, there are a few recognized challenges that have yet to be overcome:

a) Standardized isolation methods - Developing robust and standardized methods for isolating and purifying microalgae EVs is crucial for reproducible research and applications.
b) Characterizing EVs content - Thoroughly characterizing the composition of microalgae EVs is needed to understand their functions and potential applications. Unlike mammalian exosomes, where there are many known proteins that are used as biomarkers for the presence of these unique EVs [http://www.exocarta.org/], to date,

there are no known candidates to serve as biomarkers for EVs in aquatic environments

c) Understanding EVs' functions - Further research is needed to elucidate the specific roles of microalgal EVs in both microalgal physiology and potential interactions with other organisms.

Overall, microalgae represent a promising source of EVs with potential applications in various fields. Continued research focusing on overcoming the current challenges will unlock the full potential of these vesicles.

In this review, the cultivation methods and proper maintenance of microalgae cultures will be exemplified using several common species (*Chlorella sorokiniana, Chlamydomonas reinhardtii, Chlamidomonas acidophila, Haematococcus pluvialis*).

3. Purpose, Scope, and Applicability

In recent years, the interest for the concept of employing and optimizing microalgal cultivation techniques for various biotechnological and industrial applications, has been steadily growing. One of the key research angles has been the cultivation of microalgae as a means for large-scale production of EVs (Picciotto et al., 2021). The advantage behind the utilization of microalgae for these purposes lies, at least in part, in their ability to thrive in various unfavorable environments. This has biotechnological implications as an opportunity to exploit underutilized resources such as industrial byproducts, wastewater and acid mine drainage ponds (Liberti et al, 2024). Different microalgae strains can be screened and selected for their EV production capabilities. Cultivation in laboratory settings is needed to provide a controlled environment for optimization of microalgal growth conditions (Paterna et al., 2022), as well as allow for development of scalable approaches to EV production and isolation (Picciotto et al., 2021). For example, modifying culture conditions, such as media composition or growing microalgae in co-culture with bacteria, can enhance the culture growth rate or EVs production (Martin et al., 2021). Research in controlled laboratory conditions can also enhance our understanding of the EV-mediated communication in aquatic environments, as a major challenge in the development of EV-based environmental monitoring (Schatz & Vardi, 2018).

4. Health and Safety Warning

Before disposal, sterilization of previously used cultures is performed by autoclaving (125 °C, 60 minutes), to ensure that the risk of contamination is kept to a minimum.

5. Cautions

There is no risk regarding the use of hazardous chemicals in microalgae culture maintenance. Chemicals used for culture media comprise of inorganic salts that should be disposed of according to regulations for disposal of non-hazardous chemicals. To minimize the risk of potential contamination spreading from personnel or the laboratory environment to the microalgae cultures (and vice versa), all work surrounding microalgal culture must be performed in a properly functioning laminar flow hood. All personnel should follow sterile technique procedures when working with microalgae, and should wear protective gear such as lab coats, protective gloves, and goggles, in accordance with the experiments conducted. Microbiological waste generated during the handling of microalgae cultures should be managed according to strict safety and disposal guidelines. It should be treated with appropriate care to avoid contamination and the spread of pathogens. Key steps include:

a) segregation, microbiological waste should be separated from general waste and placed in designated biohazard containers;

b) disinfection, disinfectants or sterilization methods (such as autoclaving) should be used to decontaminate waste before disposal;

d) disposal, dispose of the waste following local environmental and health regulations, often through licensed waste disposal services for biohazardous materials.

Handling microalgal cultures involves the use of glass laboratory equipment (e.g., flasks), requiring careful handling to prevent potential injuries, including cuts. The use of centri-

fuges requires following safety guidelines to prevent mechanical issues and sample contamination. Regular equipment maintenance must be performed to ensure safe and efficient operation.

6. Personnel Qualifications / Responsibilities

Staff handling microalgae cultures should have qualifications in microbiology and/or biotechnology, with training in laboratory safety, biohazard management, and proper handling and disposal of biological materials (Brand et al, 2013).

7. Materials, Equipment, and Supplies

a) Materials:

Nutrients - Inorganic salts, vitamins for medium preparation

Culture vessels (glass or plastic bottles, Erlenmeyer flasks, or tubes for closed systems)

b) Equipment:

Phytotron - chamber for controlled growth conditions of plants and algae, equiped with a light source – LED lamps or fluorescent lamps with an appropriate light spectrum (usually 400–700 nm, PAR spectrum) and temperature controll mechanism.

Orbital shakers - provide continuous circular motion to the liquid culture vessels to maintain cells in suspension, enhance gas exchange and ensure uniform nutrient distribution. They are placed inside the fitotron.

Optical microscope – to monitor cell morphology, track growth parameters such as cell number and viability. Should be equiped with a mountable microscope camera for imaging.

Temperature regulation - incubator with shaking platform for temperature control, thermostatic water bath, phytotron with temperature control

Sterilization equipment – autoclave, sterile filters (0.2 μm), and UV lamps to prevent contamination

Aeration and mixing – aerators or magnetic stirrers to enrich the culture with CO_2 and prevent sedimentation

Centrifuges or membrane filtration systems – for biomass separation

Monitoring equipment (pH sensors, CO_2 dosers)

c) Other equipment: microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2 ml), micropipettes, micropipette tips, sterile deionized water

8. Computer Hardware & Software

Optika PROView, a professional image analysis software for light microscope (Optika, Italy). As part of the essay for assessing cell viability, after obtaining images on the light microscope, the analysis is performed using the Image J software (NIH, USA).

9. Overview of the microalgae cultivation and maintanence

9.1. Media preparation

Cultivation of microalgae in the appropriate cultivation medium is of key importance because it provides all the necessary nutrients and optimal conditions for their growth and development. Microalgae require specific nutrients, such as nitrogen, phosphorus, iron and trace elements, which enable maximum growth and productivity. Maintaining proper pH and salinity is important, as improper conditions can slow growth or even cause cell death. A properly formulated medium also prevents the growth of competing microorganisms, such as bacteria and fungi, which can contaminate the culture and reduce its yield. Many established recipes exist for preparing microalgae culture media, such as CCAP procedure (Culture Collection of Algae and Protozoa, 2022; https://www.ccap.ac.uk/) and Andersen's Algal Culturing Techniques (Andersen, 2005). These recipes specify the amounts of nutrients, trace metals, and vitamins needed for optimal growth. To prepare the medium, the individual components must be accurately weighed and mixed. All prepared media must be sterilized. Commercial microalgae culture media are also available from various suppliers (e.g.,

https://www.variconaqua.com/). These media provide convenience and consistency as they are pre-mixed and quality-controlled. However, they may be more costly and are typically restricted to commercially available strains. It's worth noting that some studies have explored the use of agricultural fertilizers as a cost-effective alternative for large-scale microalgae production (Novoveská et al, 2023).

Culture maintenance can be carried out through the use of liquid medium or agar plates, according to current needs. The difference between the two types of cultivation medium is reflected in their composition and application. Liquid medium is most often used because it allows for even distribution of nutrients and better absorption of light, which is crucial for photosynthesis. Microalgae in a liquid medium grow in suspension, which enables continuous mixing and enrichment of the medium with gases such as CO_2. On the other hand, a solid medium, usually prepared with the addition of agar (w/v 1.5%), is used for the isolation and preservation of pure cultures of microalgae (Sánchez-Bayo et al, 2020). On the surface of agar plates, microalgae form colonies, which facilitates the recognition and selection of specific morphologies. This type of medium is not suitable for mass production, but is useful in laboratory research and culture preservation.

9.2. Contamination Prevention

Sterilization of media and equipment is essential to prevent contamination. Autoclaves are generally used for this. Axenic (pure) cultures are often desired, and aseptic techniques are practiced during inoculation and sub-culturing to prevent contamination from other microorganisms (Vu et, 2018). Media should not be stored for more than two weeks and stock solutions for media preparation should be prepared in small quantities. Particular care should taken with phosphates and NaCl, as they are the highly susceptible to contamination and mold growth. This recommendation is based on laboratory experience.

9.3. Cultivation conditions

Although the conditions vary between species in certain subtleties (detailed examples for some common species will be provided as a supplement), maintenance and cultivation of microalgae in laboratory conditions require the following:

Culture vessels/devices: Various containers used for microalgae cultivation: a) flasks, laboratory-scale, with aeration or agitation, b) suitable rubber stoppers ili silicone stoppers, or cotton plugs , c) glass tubes or petri dishes, for strain maintanance on agar media and d) phytotron, a controlled environment chamber for precise growth conditions, d) laminar flow hood, for work with microalgae provides a sterile environment by directing filtered air in a smooth, uniform flow to prevent contamination during cultivation and handling.

Lighting: Controlled light intensity and spectrum are crucial for the optimal growth of microalgae. Different species may require specific light conditions, such as a particular intensity (measured in lux or μmol photons m^{-2}/s^{-1}) and wavelength range (spectrum), to ensure efficient photosynthesis. Artificial lighting systems like LEDs or fluorescent lamps are commonly used to provide consistent light conditions in laboratory settings. The growth of microalgae is significantly influenced by light quality, with blue light (450-495 nm) being the most efficient for photosynthesis, while red light (620-750 nm) and white light also play important roles in biomass production (Sánchez-Bayo et al., 2020).

Shaking: The shaking of microalgae in the lab involves agitating the culture medium to ensure uniform distribution of nutrients, gases, and light while preventing sedimentation of the microalgae. This is typically achieved through mechanical stirrers, air bubbling, or rotary shakers. Proper mixing ensures that microalgae receive adequate nutrients and exposure to light, which are essential for their growth and productivity. For most commonly cultivated species, the average velocity ranges from 50 to 120 rpm.

Aeration: Aeration of microalgae in the lab refers to the process of introducing air or oxygen into the culture medium to provide the necessary oxygen for algal growth and maintain proper gas exchange. This is typically done using air pumps, sterile filters, or bubbling systems to ensure that the microalgae receive sufficient oxygen and carbon dioxide, promoting optimal growth conditions.

Monitoring: Equipment to track pH, temperature, and cell density is essential for ensuring optimal growth conditions for microalgae. pH can be monitored using a pH probe and adjusted if necessary. Temperature is monitored with a thermometer or temperature sensor (typically between 20°C to 25°C, depending on the species). Cell density is established using a spectrophotometer or cell count chambers (e.g., Sedgewick-Rafter, Neubaer, etc.; see Andersen et al, 2005). Common viability assays are also recommended for this purpose, and they include live/dead staining (FDA-PI) and membrane integrity tests (Evans Blue) (Elder et al, 2021). Regular monitoring allows for adjustments to the culture conditions as needed, ensuring healthy and productive microalgal growth.
Harvesting (if needed): Centrifuges or filtration systems are used to collect microalgae from the culture medium. Centrifugation separates cells from the medium based on their density, while filtration removes the algal cells using mesh or membrane filters.

9.4. Growth curve parameters

Monitoring optical density (OD), biomass, viability, and cell count are common methods for characterizing microalgae growth (Humphrey et al., 2019; Ling et al., 2021). These parameters provide a comprehensive understanding of the growth curve.
OD is a quick and easy way to estimate the cell density in a culture (Humphrey et al., 2019) (Nielsen & Hansen, 2019). It measures the opacity of the culture, which increases as the number of cells increases. To avoid interference from chlorophyll absorption, it is more accurate to estimate the cell density of photosynthetic microorganisms using a wavelength outside the pigment absorption range, such as 750 nm.
Biomass is a direct measure of the total mass of cells in a culture. It provides a more accurate representation of the amount of biological material produced during growth than OD alone (Bellinger, 1974).
Viability indicates the proportion of live cells in a culture. It is important for understanding the overall health and productivity of a culture. A decline in viability can indicate stress or nutrient limitation.
Cell count provides a direct measure of the number of cells in a culture (Sarrafzadeh et al., 2015) and is useful for determining the growth rate and generation time of a culture. Still, it can be time-consuming, especially when done manually. Also, cell numbers alone may not accurately reflect the biomass of the population if the cells differ in size (Bellinger, 1974).
The growth of a microalgae culture occurs in five phases: lag phase, exponential phase, declining relative growth phase, stationary phase, and lysis/death phase (Price & Farag, 2013). The two of them are the key phases of microalgae culture growth. The exponential phase is characterized by rapid cell division and a high metabolic rate. During this phase, microalgae are actively growing and multiplying, leading to a steep increase in the biomass.
The stationary phase is characterized by the growth rate slowing down significantly as resources become limited or waste products accumulate (Zhao et al., 2018). The number of new cells produced is roughly equal to the number of cells dying, resulting in a stable population size. The metabolism of the cells also slows down as they adapt to the less-favorable conditions. The duration of these phases and the overall growth time can vary depending on the microalgae species, culture conditions (e.g., temperature, light, nutrients), and the specific goals of the cultivation (Krzemińska et al., 2013; Bernard & Lu, 2022). While 15-20 days is a reasonable average, some species may grow faster or slower (Ra et al., 2016).

9.5. Culture maintenance

The transfer of the microalgae culture into a fresh medium is carried out under a laminar flow hood under sterile conditions. Before starting, the hood is irradiated with UV-C light for 1 hour to minimize the risk of biological contamination. Vessels for culture maintenance are sterilized at 160ºC for 3 hours (dry sterilization), while freshly prepared media, deionized water, lids for Erlenmeyer flasks, and pipette tips are subjected to wet sterilization (114ºC, 25 minutes) and consequential drying in the case of stoppers. All items, including vessels containing old cultures need to be sprayed with 70% ethanol

before being placed in the laminar hood. When working in the laminar hood, it is mandatory to wear a clean coat, gloves disinfected with 70% ethanol, and ensure the ventilation system is operating. After completing the inoculation, the work surface is wiped with 70% ethanol, and the hood is again exposed to UV-C radiation.

The culture is directly transferred from one liquid medium to another. Still, in the case of some species such as *C. acidophila*, a specific volume of the old culture is firstly centrifuged at 5000×g for 5 minutes, the pellet is then washed with deionized water (dH_2O), centrifuged again and resuspended to the initial volume. Further, it is filtered through standard filter paper to remove potential fungi and bacteria. The filtrate is inoculated into a fresh medium.

The transfer from agar to liquid medium follows a similar procedure as for a liquid ones. It is directly applied on the cooled, previously sterilized agar in a Petri dish or test tube, or firtsly washed without filtration in case of *C.acidophila*. The transfer is performed using a sterile loop, previously sterilized on the flame of flame burner. It is essential that the agar is firmly set and well-cooled before use.

These procedures are repeated continuously, depending on the species-specific time (Supplement, Table 2), to prevent contamination and maintain the culture in a mature state. To reduce the growth of present fungi and bacteria, the cultures of these microalgae are treated with specific antimycotics and antibiotics every two to three months, or as needed.

10. Data management

Irrelevant.

11. Waste management

The waste should be disposed of according to the manufacturer's instructions and according to applicable laws. The gloves used by the staff should be disposed of according to good laboratory practices.

12. Related Protocols or SOPs

Supplement – examples of some common microalgae species cultivation and maintanence conditions.

13. Quality Control and Quality Assurance

Instrument maintanence

All instruments are regularly maintained and calibrated by trained service technicians and personnel.

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

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Supplement

Chlorella sorokininana is a green freshwater mixed algae species from the *Chlorophyta* division. The strain of this microalga, CCAP 211/8K, was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK. The optimal medium for maintaining this culture is 3N-BBM + V, whose initial pH is ~ 7.5. The medium is prepared according to the CCAP procedure (https://www.ccap.ac.uk/wp-content/uploads/MR_3N_BBM_V.pdf). The culture is maintained on liquid and solid medium (1.5% w/v agar in medium). These microalgae are grown at 22ºC in a phytotron on orbital shakers (120 rpm) with a continuous photon flux density of 120 μmol m–2s–1. The early stationary phase in case of *C. sorokiniana* is reached after 20 days of growth.

Hematococcus pluvialis is a freshwater microalga species in the *Chlorophyta* division. The culture is usually grown in a liquid medium 3N-BBM + V, with an initial pH of ~7.5. The microalga is grown at 25ºC (room temperature), without shaking, under a 16:8 light-dark cycle and photon flux density of 50-80 μmol m–2s–1. Cultures are maintained only in a liquid medium. The growth curve is determined by monitoring the following parameters: optical density, biomass, viability, and cell count. The growth period for *H. pluvialis* is approximately 30 days, after which the culture needs to be transferred to a fresh medium. This alga is characterized by the complex life cycle consisting of four distinct phases/cell types. Microzooids are small motile green cells, <10 μm, and macrozooids are green, ovoid cells with a prominent gelatinous extracellular matrix and two flagella, ranging from 10 to 20 μm in diameter. These two types are dominant in the exponential phase. Palmella are green, spherical cells (average diameter 20-40 μm) and typically appear during the 'green' phase of culture growth. Hematocysts are red, spherical cysts (30-60 μm) and represent a metabolically dormant cell type characteristic of the 'red' phase of culture growth, which is triggered by the depletion of essential resources or specific stress conditions. The palmella stage contains the red pigment astaxanthin, which is widely exploited in the food and cosmetics industries.

Chlamydomonas reinhardtii is a green unicellular alga with an approximate diameter of 10 μm, moving using two flagella. It is used as a model organism in cell and molecular biology research. This alga is an extensively studied biological model organism, appreciated for its ease of cultivation and highly tractable genetics, which allows precise manipulation for research purposes. The cells of *Chlamydomonas reinhardtii* are mostly spherical but can also have an ellipsoidal shape, with a very thin cell wall. The optimal medium for maintaining this culture is TAP (TRIS-acetate-phosphate) (CCAP, https://www.ccap.ac.uk/wp-content/uploads/MR_TAP.pdf). The culture is maintained in both liquid and solid media (1.5% w/v agar in medium). This microalga is cultivated at 22ºC in a phytotron, on orbital shakers (120 rpm), under a continuous photon flux density of 120 μmol $m^{-2}s^{-1}$. The growth period is approximately 28 days, after which the culture needs to be transferred to a fresh medium.

Chlamydomonas acidophila 136 and 137 are green extremophilic microalgae. The strains of these microalgae, CCAP11/136 and CCAP11/137, were obtained from the Collection of Algae and Protozoa (CCAP), UK. *Chlamydomonas acidophila* PM01 is a green extremophilic microalga species isolated from acidic mining lakes. This strain was collected from Parys Mountain, Anglesey, North Wales, UK.

Maintenance is carried out in MAM medium at pH 3 (Table 1). The medium is prepared following the recipe from Olaveson & Stokes (1989), with certain modifications. The cultures are maintained in a liquid medium.These microalgae are cultivated at 22ºC, in a phytotron, on orbital shakers (120 rpm), with a continuous photon flux density of 120 μmol m–2s–1. The growth curve is determined by monitoring the following parameters: optical density, biomass, viability, and cell count. The growth period of these microalgae is approximately 20 days, after which the culture must be transferred to a fresh medium.

Table 1. MAM medium recipe

Component	50 ml	100 ml	800 ml	400 ml
$(NH_4)2SO_4$	2.5 g	5 g	8 ml	4 ml
$CaCl_2 \cdot 2H_2O$	0.05 g	0.1 g	8 ml	4 ml
$MgSO_4 \cdot 7H_2O$	2.5 g	5 g	8 ml	4 ml
KH_2PO_4	1.5 g	3 g	8 ml	4 ml
NaCl	0.15 g	0.3 g	8 ml	4 ml
$Na_2EDTA \cdot 2H_2O$	1 g	2 g	0.4 ml	0.2 ml
Trace Metals	See below	0.8 ml	0.4 ml	
$FeSO_4 \cdot 7H_2O + H_2SO_4$	0.249 g + 0.05 ml	0.498 g + 0.1 ml	0.8 ml	0.4 ml
Vitamin B12	1 mg/ml	1 mg/ml	0.8 ml	0.4 ml
Trace Metals Mix (1 ml)				
Component	Amount			
H_3BO_3	2.86 g			
$MnCl_2 \cdot 4H_2O$	1.81 g			
$ZnSO_4 \cdot 7H_2O$	0.222 g			
$Na_2MoO_4 \cdot 2H_2O$	0.390 g			
$CuSO4 \cdot 5H_2O$	0.079 g			
$Co(NO_3)2 \cdot 6H_2O$	0.0494 g			

Table 2. Overview of the time points for reaching of the early stationary phase of growth and transfer to media in days for each of microalgae species

Species	Time to early stationary phase (days)	Transfer point to fresh medium (days)
Chlorella sorokiniana	20	30
Chlamydomonas reinhardtii	28	40
Chlamydomonas acidophila, strains 136 and 137	15	27
Chlamydomonas acidophila, strain PM01	15	20
Haematococcus pluvialis	27	30

Research Article

Standard Operating Procedure for Wound Healing Cell Migration Assay within the Nanostructurome Pipeline

D'Antonio Concetta [1], Mantile Francesca [1], Liguori Giovanna L[1,*]

1. Institute of Genetics and Biophysics (IGB), National Research Council of Italy, Naples, Italy.
* Correspondence: Giovanna L Liguori; giovanna.liguori@igb.cnr.it

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Abstract

Standardization of scientific procedures is a key issue in making research reproducible, cost-effective, shareable and easily accessible for technology transfer and data reuse. Here we present a standard operating procedure (SOP) for setting-up, running and analyzing a cellular wound healing assay. Wound healing assays allow the ability of different substances, such as conditioned medium, natural extracts, natural and synthetic compounds, drugs, extracellular vesicles, and synthetic nanoparticles to influence the ability of cells to migrate to be evaluated. Different cell types can be tested, including both normal and tumor cell lines. The output of the assay is to determine the effect of the tested sample on the migratory ability of specific cells, compared to untreated cells, by measuring the percentage of wound closure over time. These data are relevant in all the biological contexts in which cell migration occurs, including morphogenetic events during embryonic development, tissue repair and regeneration, tumor infiltration and invasiveness, and metastasis.

The SOP provides a step-by-step description of the procedure, lists the materials and equipment required, and identifies safety measures, instrument cautions and critical process parameters that must be controlled to ensure the quality of the output. It is therefore a valuable tool for staff training, competence transfer, quality assurance and management of the process, as well as facilitating technology transfer.

Keywords: Cell migration; Wound healing assay; Wound closure rate; Tumor invasion and metastasis, Tissue repair and regeneration; Standard operating procedure

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1. Definitions

EVs: extracellular vesicles
FBS: fetal bovine serum
N: number
NT: non treated
PBS: phosphate-buffered saline
PF : paraformaldehyde
qPMO: quality and project management OpenLab
SOP: standard operating procedure
T (T_0,T_1,T_2): time
T: treated
V_f: final volume
WA: wound area
WH: wound healing

2. Background

Cell migration is a key biological process for embryo development and adult life as well as an integral mechanism of many pathological processes, including tissue repair, tumor cell infiltration, invasiveness and metastasis. Cell migration depends on complex and integrated relations with the surrounding cells as well as the extracellular space. External biochemical cues that can induce cell migratory behaviour include soluble factors, chemoattractants and extracellular vesicles released by surrounding cells and present in the extracellular milieu. These stimuli have to be internalized in order to induce the cytoskeletal remodelling and adhesion rearrangements capable of conferring cell polarization and migratory behaviour (Merino-Casallo et al., 2022; Liguori and Kralj-Iglič, 2023)

Among the experimental assays available to assess cell migration is the wound healing assay, which is based on the formation of a wound on a confluent layer of cells and the subsequent monitoring of the healing process as cells migrate into the wound to close it. This assay has been used in several studies to determine the effect of different treatments or samples, including growth factors, drugs, natural products and extracellular vesicles, on the ability of different types of cells to migrate (Tong and Wang, 2017; Mantile et al., 2022; Alqarni et al., 2025). This assay is capable of assessing the ability of different samples/substances to induce or inhibit cell migration. Substances capable of inducing cell migration may be of interest in tissue and wound repair, whereas substances capable of inhibiting cell migration may be relevant in the context of tumour therapy to reduce the invasiveness and spread of cancer cells.
Several factors can influence the performance of the assay and the reproducibility of the results, including cell culture density, wounding method, the use or not of antiproliferative drugs, and the amount of sample to be tested. Therefore, a Standard Operating Procedure (SOP) describing the method and the critical steps to be controlled is useful for setting up, running and analysing a cellular wound healing assay.

3. Purpose, Scope and Applicability

The **purpose** of this SOP is to describe a procedure to determine the effect of a specific sample or treatment on the cell migratory behaviour over time.
The **scope** of the SOP is to describe how to perform and analyse a wound healing assay, mainly using silicone cell culture inserts with a defined cell-free gap (Ibidi) to increase the reproducibility of the wound and the reliability of the results.
The SOP is **applicable** to all types of adherent cells, especially those involved in tissue repair or tumour dissemination, and to all types of extracellular substances (natural compounds, synthetic peptides and drugs, chemokines, growth factors, extracellular vesicles, synthetic nanoparticles), purified or not, that can be diluted in the cell-conditioned medium.

4. Health & Safety Warning

All recommended measures for working with cell cultures should be followed. All experimental procedures should be carried out under a vertical laminar flow hood, wearing gloves and coat. All surfaces should be cleaned with 70% alcohol before and after use. Cell culture material should be discarded with biological waste soon after experiments. For further details, please refer to the guidelines for working in cell culture laboratories (Lacerra et al., 2013) identified by the quality and project management OpenLab (qPMO) network of the National Research Council of Italy (Bongiovanni et al., 2015), according to a quality-based model for life science research guideline (Digilio et al., 2016).

5. Cautions

All measures recommended for working with cell cultures must be followed to avoid cell culture contamination, as previously described (Lacerra et al., 2013). For imaging at different times, if the microscope used is equipped with a top incubator, it is possible to leave the cell samples in the top incubator throughout the experiment. If not, the cell samples are normally grown in the cell incubator and then transferred to the microscope at fixed times in clean, closed containers for image acquisition. In the latter case, the image acquisition step must be as fast as possible so as not to disturb the cell cultures. The image coordinates must be determined very carefully in order to monitor the same field of view over time and thus ensure the reliability of the results. All surfaces must be cleaned with 70% alcohol before and after use.

6. Personnel Qualifications / Responsibilities

The personnel responsible for the assay must be trained to work in both the cell culture and microscopy facilities. Personnel must know the basic rules for working with cells under a laminar flow hood, how to use an inverted microscope, perform live image acquisition, and analyse images and data using specific software (e.g. ImageJ, Excel, Prism, Power Points, Adobe Photoshop).
The cell culture and microscopy facility managers, together with the technical staff, ensure the performance of the facilities and equipment involved and are responsible for identifying and resolving any malfunctions, taking also advantage of external specialized technical support.
The Principal Investigator in charge of the project supervises the design, execution and analysis of experiments.
The other users of the facilities are responsible for respecting the operating rules and their reservation times so as not to interfere with the other experiments.

7. Materials, Equipment and Supplies

The following facilities and equipment are required to carry out the wound healing process:
- a cell culture facility equipped with a laminar flow hood that allows work to be carried out under sterile conditions; an incubator that maintains constant temperature, O_2 and CO_2 parameters; a bench-top centrifuge to collect cells from the cultures; an inverted phase contrast microscope to observe and count the cells;
- a microscopy facility with an inverted light microscope equipped with a motorized stage (e.g. DMI6000 microscope); automated image acquisition software that allows specific positions to be registered for time-lapse analysis, preferably with a top incubator to ensure that optimal growth conditions and sterility are maintained during the experiments.

The experiments will also require the following supplies:
1. sterile cell culture plates and/or flasks, strippettes and pipette tips;
2. pipettes and electronic pipettor;
3. a Burker chamber for cell counting;
4. 24-well cell culture plates;
5. removable 2-well silicone culture inserts to create a cell-free gap for migration assays (Ibidi, cat.no. 8209);
6. sterile tweezers.

and the following reagents:
1. Growth medium specific for the cell line used, complete with antibiotics, and specific supplements;
2. PBS1X for cell washing;
3. Mitomycin C to block cell proliferation;
4. Trypan Blue 0.4% for live cell counting.

8. Computer Hardware & Software

The microscope has to be equipped with a PC, a camera and a software for automated image acquisition. Specifically, we used the Leica DMI6000 inverted light microscope and Leica LAS AF software. Different software is required for image analysis, panel construction and data analysis (Image J, Adobe Photoshop, Power Point, Microsoft Excel and GraphPad Prism).

9. Step by Step Procedure

Scheme of the wound healing procedure is shown in **Figure 1.**

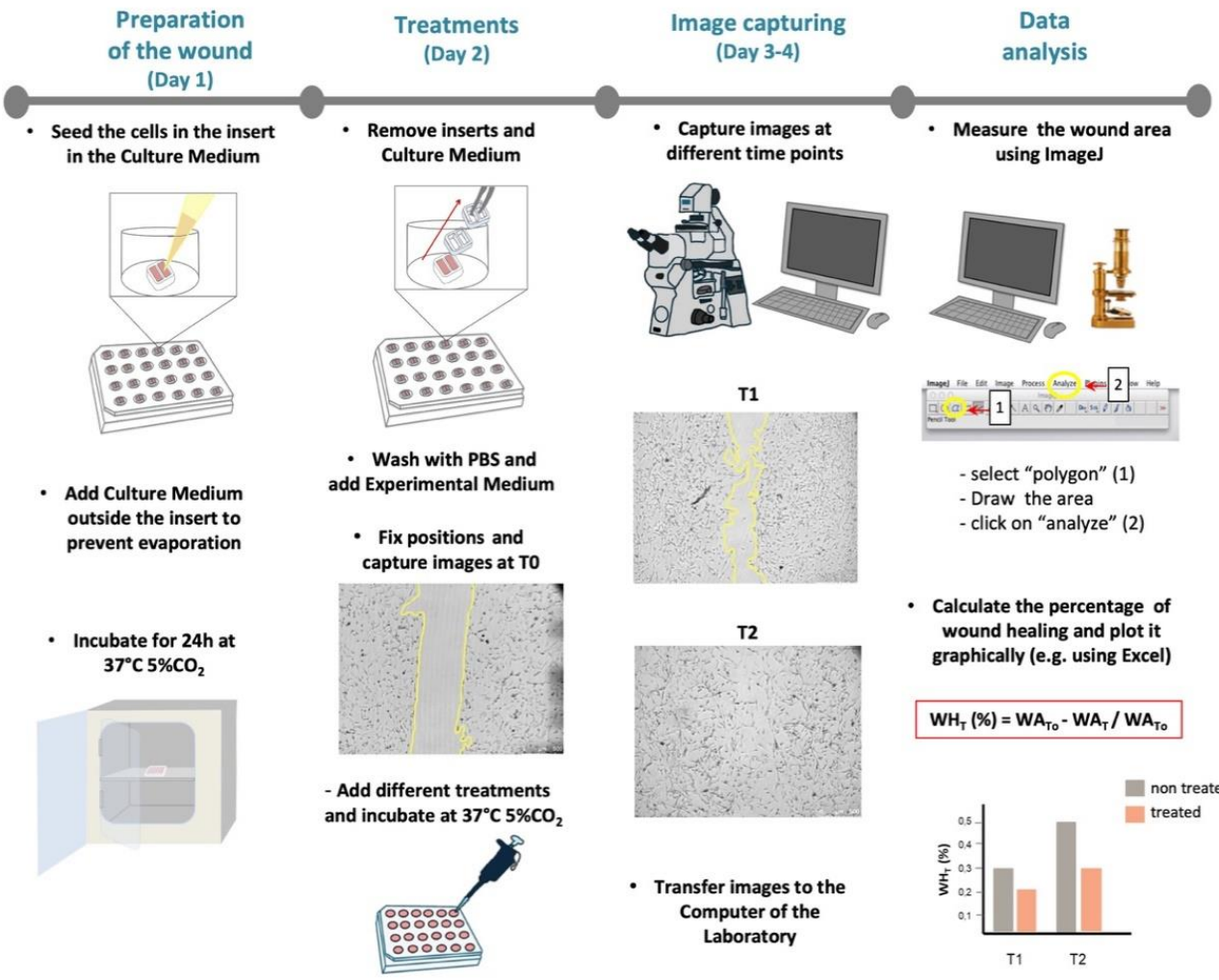

Figure 1. Scheme of the key step in the wound healing procedure.

9.1 Preparation of the cells and samples to test

9.1.1 Cell cultures

Cells are grown under their specific growth conditions (culture medium, supplement(s), T, CO_2 and/or O_2 atmosphere), which may vary from one cell line to another. It is recommended to use cell cultures at low passages, which have not been grown in culture for a long time and which have been tested for the absence of mycoplasma, whose contamination could alter the metabolism and behaviour of the cells and affect the reproducibility of the results. It is recommended that each assay is repeated three times using different batches of cells but at comparable passages.

9.1.2 Samples to test

The sample to be tested should be carefully prepared to reduce the risk of cross contamination, accurately quantified, aliquoted and stored at the optimum temperature to maintain its characteristics and to avoid repeated freezing and thawing which may affect the integrity and performance of the samples. It would be advisable to repeat each experiment three times using different batches of samples and testing different sample concentrations to measure a dose-response curve.

EVs are emerging as key particles capable of influencing cell migration (Sung et al., 2021) during both tumorigenesis (Mantile et al., 2020; Liguori and Kralj-Iglič, 2023) and wound repair (Narauskaitė et al., 2021; Li et al., 2025). In our case study, we determined the ability of EVs isolated from teratocarcinoma cells to affect cell migration of glioblastoma cells (Mantile et al., 2022).

9.2 Execution of the assay

Detailed description of the assay is given in **Table 1.**

Table 1. Detailed description of the wound healing assay procedure.

Day 1_Preparation of the Wound	
Step	Description
1	Calculate the number of cells required for the wound healing experiment: TOT N cells = N cells/well x N treatments x N replicates for each treatment The **Number (N) of cells** to be seeded in each well must be sufficent to form a confluent or subconfluent cell monolayer 24 hours after seeding. Therefore, it should be defined separately for each cell type. For U87 glioblastoma cells we used 15000 cells per well.
2	Detach cells with Trypsin 0.25% (incubation time and temperature depend on the specific cell line) or other methods if specifically recommended. Wash with PBS1X, and collect cells in Culture Medium in a 15ml Falcon tube. A single 100mm cell culture dish at 80% confluence should be sufficient.
2	Centrifuge at 1200 rpm for 5-8 minutes, preferably with gentle acceleration/deceleration, to collect cells. Discard the supernatant and resuspend the cells in 0.5-1 ml of Culture Medium.
3	Count the cells using a Burker chamber, making a cell dilution of 1:5 or 1:10 in a vital dye such as Tripan Blue 0.4%.
4	Transfer the amount of cells required for the entire experiment to another 15 ml Falcon tube.
Optional	Dilute in Culture Medium, resuspend well with Stripettes and count cells again with Burker chamber (1:5 dilution) to ensure that the desired number of cells are present.
5	Add Culture Medium to obtain the required V_f **V_f = 70ul (V for half insert) x 2 x N wells**
6	Under a laminar flow hood, open the box of inserts and, using sterile tweezers, place them in the centre of the well in a 24-well plate. Ensure that the Ibidi insert is firmly attached to the plate and does not move.
7	Always resuspend the cells just before aliquoting them and add 70 ul of cell culture to each side of the insert (140 ul for each well.)
8	Incubate at 37°C and 5%CO_2 approximately 1 hour to allow the cells to adhere to the multiwell plate.
9	Add 500 ul of Culture Medium to each well, outside the insert, to prevent evaporation. Incubate at 37°C 5%CO_2 for at least 24 hours to reach the required confluence.

Day 2_Treatments	
Step	Description
1	Check the cell density under the microscope to ensure that a monolayer is visible, then remove the medium outside the insert.
2	Using sterile tweezers, remove inserts from the wells by gently pulling on one corner. Check under the microscope that the monolayer is still attached and that the wound is homogenous.
3	Wash the wells with PBS 1X and add 550 ul of Experimental Medium containing a reduced concentration of FBS and an antiproliferative agent (e.g. mitomycin C) to block cell proliferation. The concentration of Mitomycin C and FBS to be used in the Experimental Medium should be determined separately for each cell type. For U87 cells we used 2% FBS instead of 10% in the Culture Medium and 2 ug/ml of Mitomycin C to block cell proliferation.
4	Place the multiwell under the inverted light motorized microscope (e.g. DM6000, Leica). Fix the coordinates of the positions in the culture cell wound and take the images for the T0 point (2 images for each well, at 5X magnification).

5	Under the hood, add 50 ul of the appropriate treatments (NB. For non-treated samples, add 50 ul of the Experimental Medium). It is recommended to test different concentrations and each concentration at least in duplicates, preferably in triplicates. For our test with extracellular vesicles we use a range of concentration between 2 and 20 ug/ml (2-5-10-20 ug/ml).
6	Incubate at 37°C and 5%CO_2 in the top stage incubator (if the microscope is equipped with one) or return the well to the cell culture incubator.
7	At fixed times (e.g. T1=8h, T2=24h, T3=40h), take new images at the same positions as previously fixed.
Optional	At the end of the experiment collect the medium and store it at -20°C, while the cells are fixed in PFA 4%, then stored at -20°C in PBS/Glycerol 1:1 for further analysis.
8	Transfer images from the microscope PC to your own laboratory computer for futher storage and data analysis (e.g. using Image J for wound area quantification, Adobe Photoshop and Microsoft Power Point for image processing, Microsoft Excel and/or GraphPad Prism for data analysis and graphing) .

9.3 Data acquisition and calculation

Using ImageJ software, the area of the wound can be measured in the images at different times. Two main parameters can then be calculated and plotted:

1) the Relative Wound Healing at different time shows the percentage of healing compared to initial wound area (**Figure 2**). It is defined as

$$WH_T\ (\%) = (WA_{T0} - WA_T)/\ WA_{T0}\ , \qquad (1)$$

where WH_T (%) is the percentage of wound healing at a given time t, WA_{T0} is the area of the wound at time t0; WA_T is the area of the wound at a given time t.

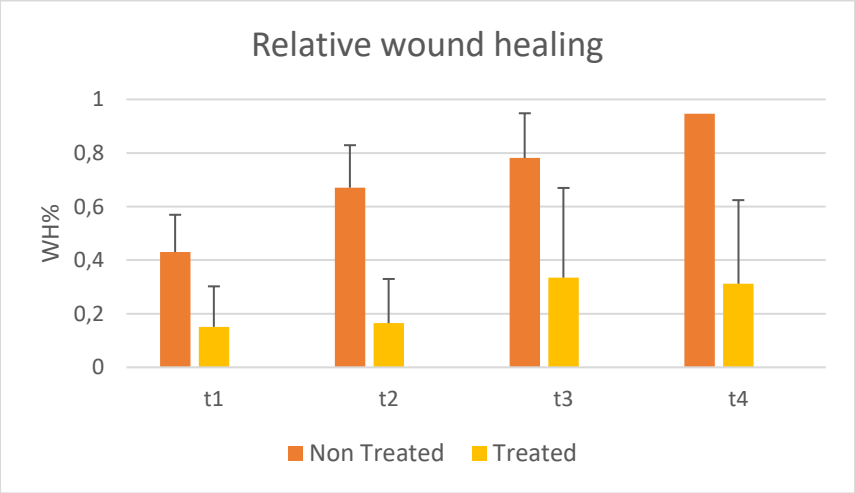

Figure 2. Example of the graphical output of the relative wound healing in dependence on time.

2) the Absolute Rate of Gap Closure (in um² or pixels over time) as the slope of the line obtained by reporting the area of the wound covered by cells (ordinate) at different times (t1, t2, t3, t4) (abscise) (**Figure 3**), before reaching the saturation phase.

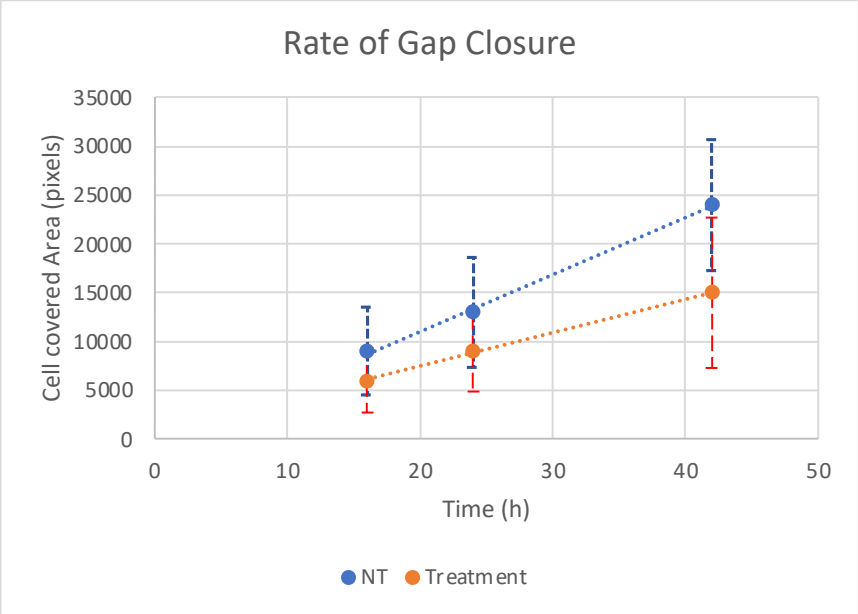

Figure 3. Example of the graphical output of the Cell covered area in dependence on time.

9.4 Troubleshooting

Envisaged problems and possible solutions are given in **Table 2.**

Table 2. Envisaged problems and possible solutions

Step	Problem	Possible reason	Solution
Step1, Day2	Cells density too low or too high	• Inaccurate cell counting • Poor cell resuspension	• Count cells twice • Pipette cells again just before plating cells into the inserts
Step1, Day2	Evaporation of the medium	Multi-well plate not closed properly	Check that the well plate is closed
Steps2 and 3, Day2	Cells detach from the plate	• Rough removal of culture inserts • Strong PBS1X washes	Be gentle and check the integrity of the cell monolayer and of the wound after insert removal
Steps6 and 7, Day2	Cells continue to proliferate	• Suboptimal concentration of FBS and/or Mitomycin C • Mitomycin C has lost its activity	• Test different concentrations • Check expiry date and/or change batch
Step7, Day2	The image field of the wound at different times does not match	Problem with position fixing at the microscope	Check that the microscope and software are working properly before starting the experiment
Step7, Day2	The technical replicates are very heterogeneous	Heterogeneity in plating cells, in aliquoting treatments, and/or in removing inserts	Prepare more technical replicates, to exclude those with technical problems
Step9, Day2	Treatment has no effect or is toxic	Insufficient concentration of treatment or presence of contaminants	• Increase or reduce the amount of treatment. A dose-response curve would be helpful. • Try different batches

10. Data and Records Management

All output files are temporarily stored on the PC connected to the microscope and then transferred to the server and to the laboratory computer(s) for further analysis. At the end of the project, the data are stored on external hard drives.
The protocols used are stored in electronic and paper registers. Any stable modification to the protocol is adequately documentd and included in the update version of the SOP.
The experimental plan and the results are recorded in the Laboratory Notebook and in the electronic files of the experimenter and are presented periodically to the supervisor, which also keeps a copy of the files on his/her computer.

11. Waste Management

At the end of the experiment, all materials used will be disposed of as liquid or solid biological waste.

12. Related Protocols or SOPS

For more informations on the use of culture inserts in wound-healing assays please visit https://ibidi.com/img/cms/downloads/ag/FL_AG_033_Wound_Healing_150dpi.pdf

13. Quality Control and Quality Assurance

13.1 Instrument Maintenance

Laminar flow hoods, incubators and microscopes are regularly inspected by internal technical services. In the event of a malfunction, users inform the facility managers, who investigate the problem and, if necessary, contact external technical support.

13.2 Critical Process Parameters and Checkpoints

A list of critical processes parameters and checkpoints is given in **Table 3**.

Table 3. A list of critical process parameters and checkpoints.

Critical Process Parameter	Checkpoint
Cell density	Check under the microscope that the cells have reached the adequate confluence before starting the experiment. If necessary, leave the cells in the incubator for a longer period of time
Wound area	Check that all the wounds at T0 are regular and measure approximately 500um, as foreseen for using Ibidi culture inserts. It is recommended to prepare more wells than necessary, so to choose the best ones for the experiment
Cell proliferation	Check that the cell density does not increase during the experiment, indicating that cell proliferation is occurring and contributing to the wound healing, thus confounding the results. In this case, repeat the experiment and check that the conditions used (serum and/or antimitotic drug concentration) are adequate. Check also the expiry date of the drug
Amount of Sample	Test different amounts of sample to obtain a dose-response curve
Reproducibility of technical replicates	Check the standard deviation and/or mean error among technical replicates. If there are visible problems in some replicates (e.g. cell density too low or too high; heterogeneity of initial wounds), consider discarding the corresponding data. In any case, the experiment must be repeated. Consider checking the quantity and quality of the cell and treatment batches used and/or using different batches
Reproducibility of biological replicates	Check the standard deviation or mean error between biological replicates. Check the quality of the cell culture batches (presence of mycoplasma, vitality, cell metabolism) and treatment batches (quantity and quality of preparation) and discard batches with problems. Repeat the experiment

14. Data on procedures and samples

Data on procedures and samples are given in **Table 4**.

Table 4. Data on procedures and samples

Description of the outcome	Relative wound healing and Absolute rate of gap closure as a measure of the effect of the treatment on cell migration
Time required to obtain the results	1 week
Amount of the sample needed	pg-ng for purified molecules ug for heterogenous samples
Estimated cost without manpower	10 EUR for each single test
Contact person	Giovanna L. Liguori IGB, CNR, Naples giovanna.liguori@igb.cnr.it

15. Conclusions

Optimization and standardization of the procedures and multivariable cellular assays are fundamental to increase the performace of the experiments, the reliability and reproducibility of the results, and to minimize the time and resources required, as the scientific community is increasingly aware and committed (Mancinelli et al., 2015, 2021; Hollmann et al., 2020, 2022). Based on our personal experimental practice, the current literature and the manufacturers' guidelines, we present a SOP describing the fundamental steps to perform a wound healing assay using silicone cell culture inserts with a defined cell-free gap. This method is more reproducible than the scratch method using the yellow tip to create the wound, and, unlike the scratch, does not risk to cause cell damage. The SOP focuses on the materials and equipment required, the critical parameters to control in order to obtain reliable and reproducible results, as well as the possible causes of failures and relative solutions. The SOP also includes safety measures, cautions and the required competence of personnel. It is worth noting that the wound healing assay can be used as a preclinical in vitro assay to test potential cell migration agonists or antagonists as possible candidates for use in wound repair or anti-tumor therapy, respectively. The SOP, therefore, is an essential tool for training staff, transferring competence, managing processes, ensuring the quality of the output, and ultimately facilitating technology transfer.

The assay can be used with purified compounds as well as more complex and heterogeneous samples such as cell conditioned medium, EVs or synthetic nanoparticles. The nature of the sample to be tested and the method used to prepare and/or purify and quantify it can also affect the reproducibility of the results. Therefore, all input variables and upstream processes must be also carefully checked in order to analyse the data correctly. This is particularly true in the context of EV research due to the high heterogeneity of both the methods used and the different batches and preparations obtained, which requires a large number of chemico-physical, biochemical and functional parameters to be checked, as well as the implementation of tools for quality management and control (Reiner et al., 2017; Ayers et al., 2019; Nieuwland et al., 2020; Liguori and Kisslinger, 2021, 2022; Loria et al., 2023; Shekari et al., 2023; Welsh et al., 2023).

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Research

Standard Operating Procedure for Determination of Lignin Structure with NMR Spectroscopy Within the Nanostructurome Methods Pipeline

Hočevar Jan[1], Prinčič Griša Grigorij [1], Iskra Jernej[1,*]

1. Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia
* Correspondence: Jernej.Iskra@fkkt.uni-lj.si

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Abstract:
Standard operating procedure (SOP) for the characterization of Organosolv lignin from wood using nuclear magnetic resonance (NMR) spectroscopy is presented. The aim of this procedure within the Nanostructurome methods pipeline is to determine the structural composition of lignin, including the relative proportions of syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units, as well as the distribution of interunit linkages such as β-O-4, β-β, and β-5 bonds. The extracted lignin was analyzed using 2D heteronuclear single quantum coherence (HSQC) NMR spectroscopy. This method enables precise structural elucidation of signals from aromatic and aliphatic regions. The mathematical model quantifies the abundance of aromatic units and interunit linkages, allowing for comparison of lignin fractions. Proper laboratory practices, including chemical handling, equipment maintenance, and data management, are outlined to ensure the reliability and reproducibility of results. Health and safety measures are emphasized, particularly regarding the disposal of byproducts such as black liquor and solvent waste. The SOP serves as a valuable guideline for lignin structural analysis, facilitating its application in biorefinery processes, material development, and bioengineering.

Keywords: Biomass; Lignin; NMR spectroscopy; 2D HSQC; Structure determination; Nanostructurome

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1. Definitions

NMR: nuclear magnetic resonance
HSQC: heteronuclear single quantum coherence spectroscopy
SOP: standard operating procedure
S-units: syringyl units
G-units: guaiacyl units
H-units: *p*-hydroxyphenyl units

2. Background

Due to the increasing awareness of environmental issues, significant attention is being directed towards obtaining substances from natural renewable sources, among which plant biomass is the only renewable source of organic carbon (Foong et al., (2020); Yoo et al., (2020); Lobato-Peralta et al., (2021)). The most common form of biomass is lignocellulosic biomass, which represents the most extensive and promising renewable carbon source on Earth, with an annual production of 181.5 billion tons (Dahmen et al., (2019)). The characterization of lignin is essential for determining its structure and properties. This, in turn, defines its final applicability and facilitates the development of general methods for its depolymerization and valorisation (Sun, (2020)).

Lignin is composed of cross-linked phenylpropane subunits: *p*-coumaryl (4-hydroxycinnamyl), coniferyl (3-methoxy-4-hydroxycinnamyl), and sinapyl alcohol (**Figure 1**) (Chio et al. (2019)). The proportion of individual monomeric subunits (H, G, and S) in lignocellulosic biomass depends on the type of plant biomass, its age, the plant part from which lignin is extracted, and the fractionation method used. Lignin with a high number of ether bonds, such as β-O-4, and fewer condensed C–C bonds, represents an excellent source for conversion into aromatic molecules. On the other hand, lignin with a high content of hydroxyl groups is suitable for polymer preparation (Sun, (2020)).

The most precise and useful method for identifying lignin structure is nuclear magnetic resonance spectroscopy (NMR), specifically two-dimensional heteronuclear single quantum coherence (2D HSQC) spectroscopy (**Figure 1**). This technique enables the rapid and straightforward determination of S, G, and H monomeric subunits and their linkages. Data from the aromatic region allow the determination of the ratio between the monomeric sub-

units of the entire lignin, while data from the aliphatic region provide insight into the proportion of C–C and C–O linkages. Using NMR spectroscopy, a quick structural analysis of lignin can be obtained, which is crucial for its further applications. Based on the ratio of S, G, and H monomeric subunits, the preferential conversion of a specific lignin into different products can be predicted (Wen et al., (2013); Lu et al., (2017); Mansfield et al., (2012)).

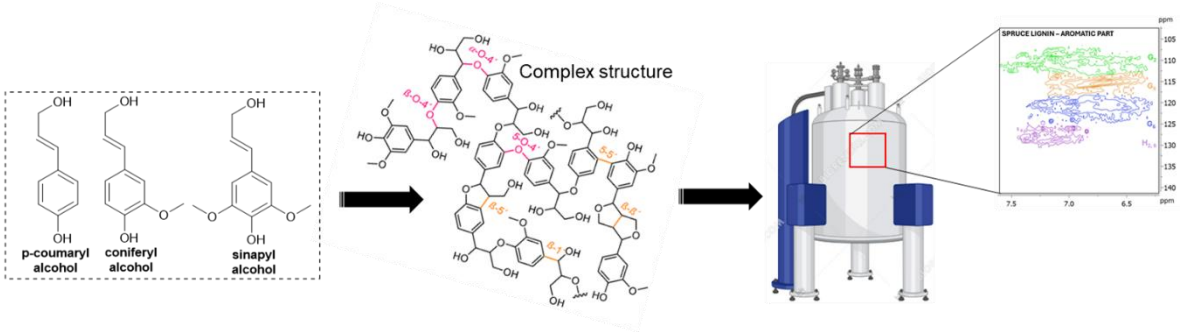

Figure 1. Left: simple propylene subunits that lignin structure is composed of after polymerization. Middle: complex structure of lignin macromolecule with specific bonds colored. Right: NMR instrument and 2D HSQC NMR spectrum of lignin. Different aromatic regions are in colors.

3. Purpose, Scope and Applicability

There are many examples of lignin valorization; however, characterization of lignin is urgent and important research prior to lignin valorization. When considering the utilization of lignin as a promising feedstock, there are some key aspects regarding the characterization of lignin to bear in mind. The under-utilization of industrial lignins is mainly due to their complicated, heterogeneous, destructive, and condensed structures. Simultaneously, different biorefinery processes also produce increasing lignin streams with various structural characteristics and properties. Various fractionation processes cause condensation of lignin, and this poses many difficulties for lignin valorization. Particularly depolymerization of condensed lignin leads to low yields of monomers. Therefore, other than developing viable valorization methods for different sources of lignin, structural characterization of lignin fractions could open the way towards new fractionation and valorization methods. In other words, the different sources of lignin complicate the development and optimization of new processes for their value-added applications (Sun, (2020); Orella et al., (2019)).

The purpose of this SOP is to focus on characterization of kraft lignin and determination of its structure

The scope of this SOP is to determine the structure of lignin derived from spruce wood and produced with craft method.

The applicability of this SOP is broad across various scientific fields, as lignin structural characterization plays a crucial role in multiple applications. In bioengineering, understanding lignin's composition and monomeric ratios enables genetic modifications to improve plant digestibility and industrial processing efficiency. In materials science, lignin with a high content of hydroxyl groups is essential for developing lignin-based polymers, while lignin rich in β-O-4 linkages is valuable for catalytic depolymerization into aromatic chemicals. In environmental and energy research, structural insights aid in optimizing biorefinery processes for sustainable biofuel production. Furthermore, advancements in lignin analysis support the emerging field of lignin nanoparticles, expanding applications in nanotechnology and biomaterials.

4. Health and Safety Warning

Lignin extraction and purification processes pose several health and environmental risks that require careful management. The disposal of black liquor, a byproduct of lignin extraction, generates high concentrations of persistent organic pollutants (POPs) as effluent, which harm aquatic flora and fauna. Adverse effects can include respiratory stress, mixed oxygenase activity, toxicity and mutagenicity, liver damage, or genotoxicity. They can also

cause health hazards such as diarrhea, vomiting, headaches, nausea, and eye irritation in children and employees (Mandal et al., (2023)).
Additionally, certain extraction methods, like the sulfite process, yield lignin products with high sulfur content and co-extract hemicellulose, complicating purification efforts. The lack of selectivity in these processes can lead to lignin with high ash and carbohydrate content (Saadan et al., (2024)).
Moreover, the use of strong acids in some extraction methods poses risks of unwanted side reactions and alterations in the biopolymer structure, which can affect the quality and functionality of the extracted lignin (Karlsson and Lawoko, (2023)).
Therefore, it is crucial to carefully consider and mitigate these risks when selecting and optimizing lignin extraction and purification methods to ensure safety and maintain the integrity of the lignin product.

5. Cautions

All chemicals used in the lignin extraction and purification process should be handled in accordance with applicable safety regulations and good laboratory practices. Proper storage conditions, including temperature control and segregation of incompatible substances, must be maintained to prevent hazardous reactions. Chemical waste should be disposed of following institutional guidelines and environmental regulations. The NMR spectrometer should be operated following manufacturer guidelines and institutional safety protocols. Regular maintenance, including cryogen refilling and probe calibration, must be performed to ensure optimal performance.

6. Personnel Qualifications / Responsibilities

Handling with chemicals should be performed by trained professionals. Operation with delicate NMR equipment should be performed by trained professionals.

7. Materials, Equipment and Supplies

Materials: Spruce biomass was milled to 0.25 mm particles. The following chemicals were used: ethyl acetate (Sigma-Aldrich), hydrochloric acid (Sigma-Aldrich), acetone (Sigma-Aldrich), and deuterated dimethyl sulfoxide (DMSO-d6, Eurisotop).
Devices: Retsch ZM200 mill, Bruker Avance Ultrashield 600 Plus NMR spectrometer.
Other equipment: NMR tubes for preparing lignin solutions in d6-DMSO.

8. Computer Hardware and Software

Hardware: Office PC.
Software: Microsoft Excel, Bruker TopSpin 4.1.1, MestReNova.
Saving and sharing: Cloud and/or Drive documents.

9. Mathematical Model of Lignin 2D HSQC NMR Calculations

In this SOP we follow the general course from previously published articles (Zijlstra et al., (2019)).
The amount of **all aromatic units (T.A.)** in lignin is defined as

$$T.A. = S_{unit} + G_{unit} + H_{unit}, \quad (1)$$

where S_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the S and S' units, G_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the G_2 and G_5 and G_6 units, H_{unit} is the sum of the integrals in the 2D HSQC NMR spectrum for the H and H' units (**Figure 2**).

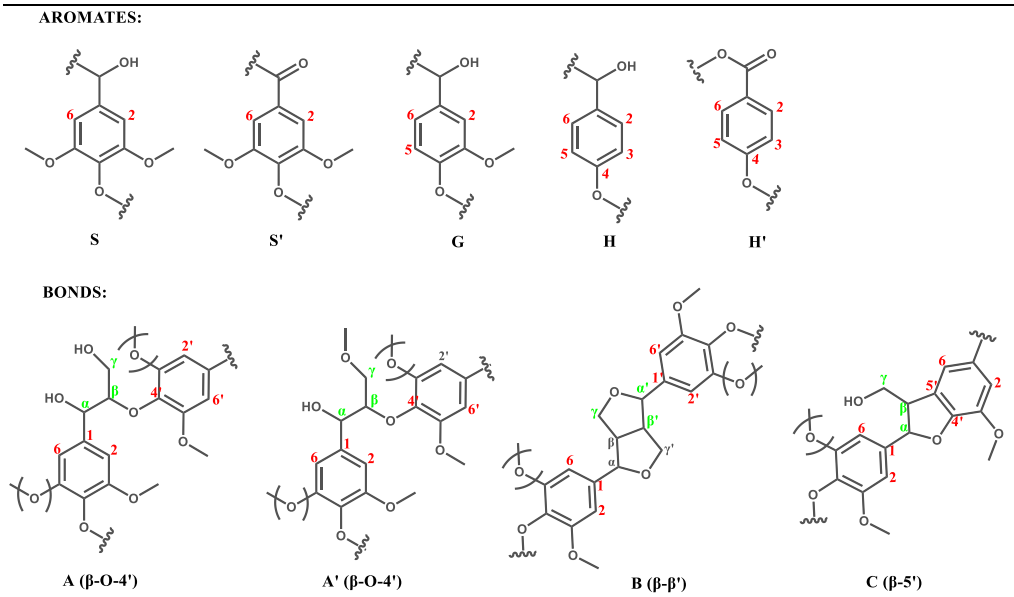

Figure 2: Schematic representation of the aromatic units and the bonds between them in the structure of lignin. H atoms used in integration are in red, bonds in green.

Since there are two S-unit regions in the 2D HSQC NMR spectrum, the following applies

$$S_{unit} = (S_{2/6} + S'_{2/6}) / 2, \quad (2)$$

where $S_{2/6}$ is the area integral of the $S_{2/6}$ unit in the 2D HSQC NMR spectrum and $S'_{2/6}$ is the area integral of the $S'_{2/6}$ unit in the 2D HSQC NMR spectrum.

Since there are two H-unit regions in the 2D HSQC NMR spectrum, the following applies

$$H_{unit} = (H_{2/6} + H'_{2/6}) / 2, \quad (3)$$

where $H_{2/6}$ is the area integral of the $H_{2/6}$ unit in the 2D HSQC NMR spectrum and $H'_{2/6}$ is the area integral of the $H'_{2/6}$ unit in the 2D HSQC NMR spectrum.

Since there are three G-unit regions in the 2D HSQC NMR spectrum, the following applies

$$G_{unit} = (G_2 + G_5 + G_6) / 3, \quad (4)$$

where G_2 is the area integral of the G_2 unit in the 2D HSQC NMR spectrum, G_5 is the area integral of the G_5 unit in the 2D HSQC NMR spectrum, G_6 is the area integral of the G_6 unit in the 2D HSQC NMR spectrum.

Since the G units in the 2D HSQC NMR spectrum partially overlap with the H units, the latter have to be subtracted from the G units to obtain

$$G_{unit} = (G_2 + G_5 + G_6 - H_{2/6} - H'_{2/6}) / 3, \quad (5)$$

When the Eq.(2), Eq.(3) and Eq.(5) are included in Eq.(1), we get

$$T.A. = (S_{2/6} + S'_{2/6}) / 2 + (G_2 + G_5 + G_6 - H_{2/6} - H'_{2/6}) / 3 + (H_{2/6} + H'_{2/6}) / 2 \quad (6)$$

The number of all bonds between monomeric units in lignin structure is defined as

$$\sum bonds = (A_\alpha(bonds) + B_\alpha(bonds) + C_\alpha(bonds)) * 100 / T.A., \quad (7)$$

where A_α(bonds) is the sum of the integrals in the 2D HSQC NMR spectrum from A_α and A'_α bonds, B_α(bonds) is the integral in the 2D HSQC NMR spectrum from the B_α bonds, C_α(bonds) is the integral in the 2D HSQC NMR spectrum from the C_α bonds.

Since there are two A_α(bonds) regions in the 2D HSQC NMR spectrum, the following applies

$$A_\alpha(bonds) = (A_\alpha + A'_\alpha) / 2, \quad (8)$$

where A_α is the integral in the 2D HSQC NMR spectrum for A_α bonds and A'_α is the integral in the 2D HSQC NMR spectrum for A'_α bonds.

When the Eq.(8) is included in Eq.(7), we get

$$\sum bonds = ((A_\alpha + A'_\alpha) / 2 + B_\alpha(bonds) + C_\alpha(bonds)) * 100 / T.A.$$

10. Step by Step Procedure

10.1. Isolation of lignin

Lignin is isolated using ACE high-pressure tubes at 120 °C. Ground spruce biomass (5 g) is placed into the tube, followed by the addition of 50 mL of ethyl acetate and 1 mL of 37% hydrochloric acid. The mixture is stirred at 120 °C for 1 hour.
After the reaction, the residual biomass is filtered off, and the solvent is evaporated under reduced pressure using a rotary evaporator. The resulting concentrated residue is dissolved in 10 mL of acetone, and 100 mL of water is slowly added to induce lignin precipitation. The precipitated lignin is collected by filtration, washed with water, and air-dried overnight.

10.2. Sample preparation for NMR analysis

All lignin samples are prepared following the same procedure. A total of 40 mg of dry, pre-isolated lignin is dissolved in 0.5 mL of deuterated DMSO (d-DMSO). The solution is then filtered and transferred into an NMR tube for analysis.

10.3. NMR analysis

2D HSQC NMR spectra are recorded at 25 °C using a Bruker Avance III 500 spectrometer. The following acquisition parameters are used: F2 range = 10 to 0 ppm, F1 range = 158 to -8 ppm, number of scans (ns) = 24, dummy scans (ds) = 16, number of increments (ni) = 256, relaxation delay (d1) = 1.47 s, CNST[2] = 145, and pulse program = hsqcetgpsi2. The spectra are processed using Bruker TopSpin 4.1.1 and MestReNova software.

10.4. Determination of lignin structure

Integrate the signals in the aromatic and aliphatic region that correspond to the three different aromatic units and bonds between them (**Table 1**).
The aromatic region is used as a reference standard, and the fraction of bonds between aromatic subunits is expressed as the number of occurrences per 100 aromatic units.

Percentage of aromatic units:
T.A. (total aromatics) is calculated by using Eq.(6).
S_{unit} is calculated by using Eq.(2).
H_{unit} is calculated by using Eq.(3).
G_{unit} is calculated by using Eq.(5).

Percentage of S-units = S_{unit} / T.A. × 100%
Percentage of G-units = G_{unit} / T.A. × 100%
Percentage of H-units = H_{unit} /T.A. × 100%

Percentage of bonds between monomers:
T.A. (total aromatics) is calculated by using Eq.(6).
A_{α}(bonds) is calculated by using Eq.(8).

Percentage of β-O-4′ bonds = A_{α}(bonds) / T. A. × 100%
Percentage of β-β′ bonds = B_{α}(bonds) / T. A. × 100%
Percentage of β-5′ bonds = C_{α}(bonds) / T. A. × 100%

10.5. Data acquisition

Table 1. The area of each sub-unit (S, G, H, I and -OCH_3) and the linkages between (A, B, C) in the 2D HSQC NMR spectrum (Yuan et al., (2011)).

Mark	δ_H/δ_C [ppm]	Assignation
$S_{2,6}$	[6.3-7.0/101.5-108.0]	C_2-H_2 and C_6-H_6 in syringyl units (S)
$S'_{2,6}$	[7.2-7.4/105.0-109.0]	C_2-H_2 and C_6-H_6 in oxidised (C=O) syringyl units (S')
G_2	[6.7-7.2/108.5-113.0]	C_2-H_2 in guaiacyl units (G)
G_5	[6.38-7.15/113.2-117.5]	C_5-H_5 in guaiacyl units (G)
G_6	[6.5-7.0/117.5-123.0]	C_6-H_6 in guaiacyl units (G)
$H_{2,6}$	[7.1-7.29/126.5-131.0]	C_2-H_2 and C_6-H_6 in *p*-hydroxyphenyl units (H)
$H'_{2,6}$	[7.5-7.75/127.3-131.0]	C_2-H_2 and C_6-H_6 in *p*-hydroxybenzoate units (H)
I_{α}	[6.44-6.54/128.1-128.5]	C_{α}-H_{α} in *p*-hydroxycinnamyl alcohol
$I_{ß}$	[6.25-6.45/128.1-128.5]	$C_{ß}$-$H_{ß}$ in *p*-hydroxycinnamyl alcohol
I_{γ}	[4.0-4.10/61.4]	C_{γ}-H_{γ} in *p*-hydroxycinnamyl alcohol
-OCH_3	3.7/55.6	C-H in methoxy groups
A_{α}	[4.6-5.0/70.0-74.0]	C_{α}-H_{α} in ß-0-4' units (A)
A'_{α}	[4.50-4.85/79.0-83.0]	C_{α}-H_{α} in ß-0-4' units (A)
$A_{ß}$, $A'_{ß}$	[4.0-4.45/80.0-86.0]	$C_{ß}$-$H_{ß}$ in ß-0-4' units (A)
A_{γ}, A'_{γ}	[3.1-4.1/83.5-87.0]	C_{γ}-H_{γ} in ß-0-4' units (A)
B_{α}	[4.5-4.8/83.5-87.0]	C_{α}—H_{α} in ß-ß' resinol units (B)
$B_{ß}$	[2.96-3.2/52.0-55.5]	$C_{ß}$-$H_{ß}$ in ß-ß' resinol units (B)
B_{γ}	[3.7-3.94/69.5-73.5]; [4.1-4.2/69.5-72.5]	C_{γ}-H_{γ} in ß-ß' resinol units (B)
C_{α}	[5.35-5.65/85.5-89.0]	C_{α}—H_{α} in phenylcoumaran units (C)
$C_{ß}$	[3.36-3.58/52.0-53.7]	$C_{ß}$-$H_{ß}$ in phenylcoumaran units (C)
C_{γ}	[3.5-3.9/61.5-64.5]	C_{γ}-H_{γ} in phenylcoumaran units (C)

11. Data and Records Management

All the experimental details are recorded within the lab journal carefully. All raw as well as treated data is stored in electronic form with physical backup for a minimum of 10 years after data generation.

12. Waste Management

In this SOP, ethyl acetate is collected during the vacuum evaporation process and is either recycled or disposed of as a waste organic solvent. The first filtration process generates a

solid residue composed of cellulose and hemicellulose, which is repurposed for further reactions. In the second filtration step, where lignin is obtained as the solid residue, the remaining aqueous phase is classified and collected as waste aqueous solvents.

13. Related Protocols or SOPS

There are many reported protocols for determination of lignin structure (reviewed for example by Lupoi et al., (2014), Jiang et al., (2018), Karlsson et al., (2023), Lui et al., (2024)).

14. Quality Control and Quality Assurance Section

14.1. Instrument Calibration

Calibrating a Nuclear Magnetic Resonance (NMR) spectrometer is essential to ensure accurate and reproducible results. The calibration process involves several key steps, including frequency calibration, field homogeneity adjustments, and shimming optimization (Andris et al., 2021).

14.2. Critical Processes Parameters and Checkpoints

Number of Scans (NS) and Relaxation Delays (D1) are critical parameters. The former has a major impact on the signal-to-noise ratio, while the latter must be suitable for quantitative analysis, which is what lignin structure determination is.

15. Conclusions

NMR spectroscopy is a powerful analytical tool that enables precise structural elucidation, quantitative analysis, and dynamic studies of molecular systems. The implementation of standardized methodologies, rigorous experimental protocols, and continuous technological advancements enhances the accuracy, reproducibility, and efficiency of NMR-based investigations. To ensure optimal performance and reliability, best practices and standard operating procedures should be regularly reviewed and updated in alignment with scientific progress and evolving research demands. Moreover, integrating NMR spectroscopy within a comprehensive quality management system tailored to scientific exploration can further improve data integrity while preserving the flexibility necessary for innovative research.

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Data Availability Statement: Data available at request.

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Research

Thermal Analysis of Supported Thin Films within the Nanostructurome Pipeline

Cerc Korošec Romana[1,*] and Lavrenčič Štangar Urška[1]

[1] University of Ljubljana, Faculty of Chemistry and Chemical Technology, Večna pot 113, SI-1000 Ljubljana, Slovenia
* Correspondence: romana.cerc-korosec@fkkt.uni-lj.si

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Abstract:
A comparison of the results obtained with thermoanalytical techniques can show considerable differences between thin film samples and the corresponding xerogels/powders. One reason for this is that the substrate material itself influences the properties and morphology of the deposited films. It therefore makes sense to carry out measurements for thin film samples that have been deposited on a substrate. Thermogravimetric and differential scanning calorimetry measurements on thin films are possible even if the mass of the thin film on a massive substrate is very small. With the described approach, it is shown that each chemical system behaves differently depending on the type of precursor and deposition method, but nevertheless the results of the thin film measurements are helpful in optimising the properties of the final films for a specific application.

Keywords: Thermal analysis; Thin films; Supported films; Thermogravimetry; Differential scanning calorimetry; Nickel oxide

1. Introduction

The applicability of different types of thin films is becoming increasingly important both in science and in many areas of modern technology (Leskelä et al.,1993). They change the chemical and/or physical properties of the substrate and can be used for various purposes, e.g. as surface protection against corrosion, wear and abrasion, as optically active coatings or as coatings for certain chemical applications (Niinistö, 1999), including photocatalytic coatings (Šuligoj et al., 2022). The preparation of thin films involves several steps, from the synthesis of precursors to the final application. The characterization of the prepared films as well as the understanding of the processes by which they are prepared are important for the optimization of the preparation process.

After the film has been deposited on the substrate using one of the chemical or physical deposition techniques, thermal treatment is required. During heating, sufficient adhesion of the film to the substrate is ensured; in addition, certain chemical reactions can occur, during which the structure suitable for the application is formed. For the latter, thermoanalytical techniques allow the determination of the temperature window within which it is essential to monitor the development of the structure using *ex-situ* diffraction and/or spectroscopy techniques.

Thermoanalytical measurements on thin films (thickness below 1 μm) deposited on a planar substrate are a demanding procedure and routine measurements are not yet common. The reason for this is that in thermogravimetry (TG) the mass changes are in the range of buoyancy and aerodynamic effect, while in differential scanning calorimetry (DSC) the heat released or consumed by the sample diffuses into a more massive substrate, so the thermal changes are often below the detection limit (Niinistö, 1999; Cerc Korošec and Bukovec, 2006). Therefore, measurements are performed on the corresponding xerogel powders instead. There are several reasons why thermally induced effects occur at different temperatures for both types of chemically equivalent samples (Przyłuski et al., 1981; Cerc Korošec et al., 2008). Since explanation of the properties of thin films based on TG and DSC measurement results of the xerogels could lead to misinterpretations, it is important that the measurements are performed for thin films themselves.

In this contribution, a protocol for TG and DSC measurements of thin films deposited on planar substrates is briefly presented.

2. Materials and Methods

2.1. Preparation of Thin Films for Thermoanalytical Measurements

Thin film can be deposited from the sol (colloidal solution) by dip or spin coating. Various substrate materials can be used: microscope cover glass **(Figure 1A),** thin platinum foil (thickness 0.025 mm), aluminium foil **(Figure 1B)** (Cerc Korošec et al., 2003). The substrate must be inert (must not react with the thin film) and must withstand the high temperature. For example, microscope cover glass is not a suitable substrate for temperatures above 650 °C because it undergoes a glass transition; in this case, Pt foil can be used. After drying, the microscope glass covered with a thin film is placed between two clean sheets of paper and pressed so that the glass broke into small pieces **(Figure 1C),** which could be placed in a 150 μL Pt crucible **(Figure 1D).**

For DSC measurements, two circles large enough to cover the thermocouples can be cut out of the Pt foil mentioned above; the uncoated circle is used as a reference, while the other is covered with the sample. The underside of this circle is cleaned, dried and placed directly on the sample side of thermocouples (the thin film is deposited to the top side of this circle).

2.2. Instrumental

Measurements were performed on a Mettler Toledo TG/SDTA 851e Instrument and DC analysis on a Metter Toledo 20 Cell; all in dynamic air atmosphere and with a heating rate 5 K/min.

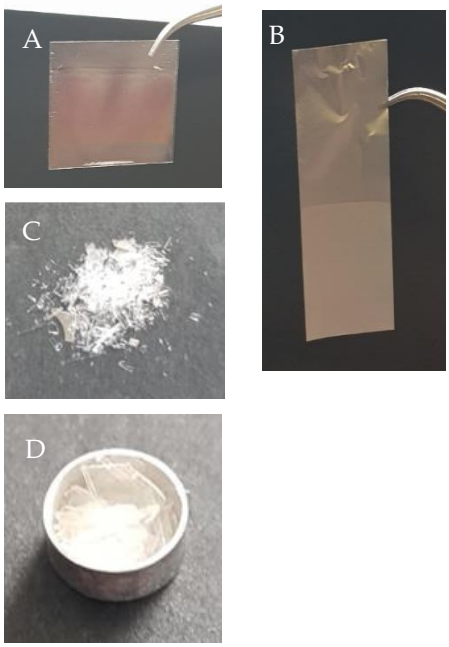

Figure 1. Thin film, prepared by sol-gel method and deposited on a microscope-cover glass by dip-coating – A, on aluminium foil – B; broken pieces of microscope cover glasses with deposited film – C, and pieces of the latter in 150 μL Pt crucibles – D.

3. Results

Figure 2A shows a comparison of the dynamic TG curves of thin film and corresponding xerogel. The thin film was prepared by alternately dipping deposition from $NiSO_4$ and a LiOH solution, while the xerogel was formed by mixing the two solutions and drying the slurry at room temperature. Onset temperature at which NiO forms differs by more than 100 degrees (220 °C for the thin film and 325 °C for the xerogel sample). The reason for this is the formation of carbonate anions in the thin film structure (they are formed during the reaction between atmospheric CO_2 and the alkaline surface of the thin film), while sulfate anions are present in the xerogel samples (they originate from the precursor) (Cerc Korošec et al., 2008). The reason for the large difference in thermal stability is therefore different anionic species; sulfate anions stabilize the primary hydroxide structure that forms at room temperature. From **Figure 2B** it is evident that the temperature difference for NiO formation in sol-gel films and xerogels is only 30 degrees. In this case, the reason most likely lies in the smaller aggregates of colloidal particles in the thin film sample, which have a higher surface energy. **Figure 2C** represents the results of the DSC measurement of the thin film described above **(Figure 2B);** when the film was applied to aluminium foil, cut into small pieces and placed in a crucible covered with a pierced lid, a) curve was obtained. If the film was applied to a Pt circle and placed directly on the thermocouples (as described in Materials and Methods), a different result was obtained, which is shown in curve b). The reason for this difference lies in the fact that in an open system the gasses formed during thermal decomposition can easily leave the system. Therefore, the initial temperature is lower (265 °C) compared to a partially closed system in which an equilibrium reaction A(s) ↔ B(s) + C(g) raises it (295 °C), according to Le Chatelier's principle.

The results of thermal treatment optimization, based on TG measurements of thin films, are for NiO sol-gel films for electrochromic applications summarized in (Cerc Korošec and Bukovec, 2006).

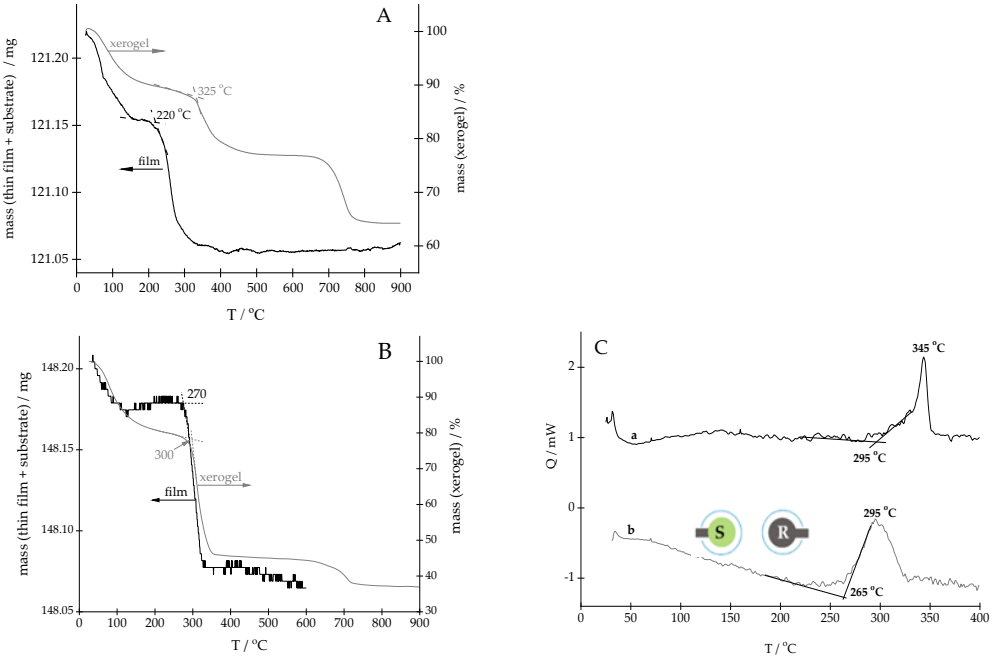

Figure 2. Comparison of TG measurements between supported thin films and corresponding powders for $Ni(OH)_2$ samples, prepared by alternately dipping deposition – A, sol-gel prepared thin films and xerogels – B, and DSC curves of the films, prepared by the sol-gel method, for two different measurement approaches – C.

4. 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 sample	Sol (colloidal solution) in glass container or solid sample.
Aliquots needed	1
Total volume of the sample	5 mL (liquid) or 10 mg (solid sample)
Time required to obtain results	2 weeks
Manpower	Highly skilled researcher (thin films analysis) or researcher (solid sample)
Estimated cost per sample without manpower	250 EUR (thin films analysis) or 150 EUR (solid sample)
Contact person	Romana Cerc Korošec, romana.cerc-korosec@fkkt.uni-lj.si

5. Discussion

In some cases, the thermal analysis of thin films can be carried out in the usual way as for a xerogel/powder sample: I) when the thin film exists as a free-standing film, e.g. thin metal films or thin films of polymers; II) when the film is deposited on a powdered substrate, e.g. mica or ceramic support, and III) when the thickness of the film exceeds 1 μm, in which case it can be scraped off the substrate. However, the results obtained for deposited thin film samples and the corresponding xerogels/powders may differ significantly. For this reason, and also if the film is very thin (in this case it is impossible to obtain a sufficient mass of the film by removing it from the substrate), it is possible to obtain reliable results from TG and DSC measurements of supported films.

6. Conclusions

Thermal analysis of thin films deposited on planar substrates requires a special approach, but the results are useful for understanding the processes that take place during thermal treatment, even on a larger scale for industrial applications.

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

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Reflection

The Golden Ratio of Creativity. Unity in Diversity: The Mathematics of the Soul and the Art of Truth

Oprešnik Lara[1,2,*]

1. University of Ljubljana, Academy of Music, Ljubljana, Slovenia
2. University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenija

* Correspondence: opresnik9@windowslive.com

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Abstract:

We reflect on the profound relationship between art and mathematics, focusing on their mutual search for truth. Art, often perceived as an emotional and subjective expression, delves into deep societal and personal truths, while mathematics serves as the logical language through which we understand the universe's fundamental principles. We argue that these two fields are not opposing, but complementary in their quest to uncover meaning and order. By balancing emotion and reason, both art and science contribute to the discovery of universal truths. As an example, we discuss Bach's Kunst der Fuge (The Art of Fugue), where the mathematical structure and artistic expression harmonize. Also we examine how the golden ratio and the Fibonacci sequence, both present in nature and art, represent harmony and guide the creation of aesthetically pleasing works. The connection between art, mathematics, and nature reveals that the search for balance is essential in understanding the world and seeking deeper truths.

Keywords: Art; Mathematics; Golden ratio; Fibonacci sequence, Bach, Universal understanding

1. Introduction

Defining art and clinging to a single explanation of its existence or understanding of its purpose seems meaningless. The number of perspectives and roles that art plays in society probably corresponds to the number of human experiences. According to the Slovene Dictionary (SSKJ), art is defined as an activity "whose purpose is the creation and formation of works of aesthetic value." However, besides creating aesthetic value, art can also be a means of expressing the full spectrum of emotions that humans are capable of experiencing and feeling. Art can be an escape, a detachment from reality, a journey into fantasy, imagination, and dreams… It can hold up a mirror to society and address current issues, or it can accompany the individual on a spiritual journey toward transcendence – a search for higher truth and the essence of humanity. In contrast to this deeper perspective, art can also be expressed through play, emerging from spontaneity and curiosity, thereby creating a space not so much for answers as for questions and new perspectives. As we often hear in life: the purpose of art may simply be art itself. Yet, often – especially within society (which, in our opinion, could not survive without art) – it also carries a connecting element: a universal language that crosses borders, time, and linguistic barriers. It is the language of the universe; at its core, it touches the darkest and brightest particles of our souls, and its power resonates all the way into the infinity of the cosmos. This is where a surprisingly deep connection with mathematics arises – not as the opposite of art, but as its silent ally in the search for universal order. Italian physicist, astronomer, mathematician, and philosopher Galileo Galilei believed that mathematics was the language in which the universe was written, and that only through it could we understand its order and laws (Galilei,1623). In our opinion, mathematics is the language of patterns and structures, a way of modeling and understanding reality. Mathematics too seeks the truth – but it does so through proof.

2. Emotion and Reason:

2.1. A Philosophical Exploration

In a superficial, simplified comparison, today we would associate art with emotion, and mathematics with reason. These two opposing poles have sparked philosophical debates since the time of the ancient thinkers of Western tradition. The roots of these discussions can be traced back to ancient philosophy, where Plato placed reason above art. Reason – that is, structure and form – he described as eternal and perfect, while art, which was of secondary importance to him, was merely an imitation of the real world (Badiou 2016). He indicated that art could only progress if guided by a didactic principle – that is, mathematics. In contrast, Nietzsche, the 19th-century German philosopher, proposed that the human connection to truth was most directly expressed through art, not mathematics. He criticized rationalism and idealism by stating that life, emotions, and instincts were more important than "cold reason." (Badiou, 2016). He attributed an existential role to art indicating that it helps us to survive and makes suffering meaningful (Badiou, 2016). My own view on the role of art and science (mathematics) is close to my reflection on the legacy of Aristotle. It seems that Aristotle understood both, art and science, as explorations of reality – but each in its own way. We may understand that for him, mathematics was not separate from sensory experience, but rather a logical language that could also be beautiful and harmonious: human beings learn and explore reality through experience, observation, and systematic explanation; mathematics, therefore, coexists with art. (Badiou, 2016).

2.2. The Coherence of Structure and Creativity

The connection between reason and emotion, often projected onto the relationship between mathematics and art – or between the intuitive, sensory creator and the logical thinker – is, in its duality, rather narrow. We think that masterful art does not exist without rational structure – architecture, composition, form, even poetry, all involve order, logic, structure, and rules. Conversely, mathematics also seem to require aesthetics, imagination, and an experimental moment. Many mathematicians speak of the beauty of equations, the elegance of proofs, and even moments of experimentation, curiosity, and creativity (Penrose, 2004). Both fields carry with them ratio and emotio – and we suggest

that it is in the intersection and right balance of both, where the greatest masterpieces have the potential to be created. Why is this so? We believe that this is in accordance with nature, which is our greatest teacher, and that the greatest artistic and scientific works are born in the perfect balance and ratio between emotion and reason. The concept of duality continuously teaches us through nature, from which everything we know, do, and live today was born. Nature also teaches us that we cannot define the existence of something without its opposite – therefore, the balance between two concepts is all the more important. We believe that opposites, such as light and darkness, life and death, joy and sorrow, cannot be properly defined without recognizing their counterpart. Nature teaches us that everything exists in a balance of opposites, which together form a complete understanding of the world. Without darkness, there is no light; without death, there is no life; without sorrow, there is no joy. This dialectical nature of opposites is fundamental to understanding our existence and encourages us to seek balance. As Hegel presented in his *Science of Logic*, opposites do not exist independently but continuously define and complement each other through their interaction. Similarly, Heraclitus taught that everything is in constant flux, where opposites cannot be considered in isolation, as they form a dynamic balance that enables life as we know it. We think balance is what nature strives for – and with it, humanity. Balance brings peace, which is often the goal. We believe, however, that the greatest masterpieces are born when we push the extremes – when we move beyond our limits, stepping outside our comfort zone. But these are only moments in the trajectory of life, which, in its overall average, tends toward balance. Structure and emotion, then, complement each other and create the highest form of interdisciplinarity, which creates space for mastery.

3. The Convergence of Art and Mathematics

3.1. Bach's Kunst der Fuge

If art and mathematics, or science – or more broadly, emotion and reason – are not separate worlds, but merely different expressions of the same search for truth, the question arises: what is within the common denominator of the intersection of these two fields? In which human creations do structure and sensibility, order and imagination, proof and abstraction intertwine? The answer may lie precisely in that which is most natural to humans: in rhythm, in patterns, in harmony, in analysis – perhaps in music, mathematically one of the most precise forms of art. Since I myself come from a musical background, I will begin by touching upon this subject. As an example of mastery or the intersection of mathematics and art, I will present the work *Kunst der Fuge* (The Art of Fugue) by Johann Sebastian Bach – one of the greatest composers of all time, a source of inspiration for numerous generations of artists, and undoubtedly a symbol of creative synthesis between strict structure, mathematical logic, and deeply spiritual artistic expression. Bach is often referred to as the greatest master of the musical form of the fugue. In his lifetime, he wrote more than a hundred fugues, both as part of larger works and as independent compositions. But what exactly is a fugue as a musical form? It is an instrumental, orchestral, or choral polyphonic composition in which the main theme appears successively in all voices. A fugue is an extraordinarily strict and structured form that uses specific rules for how the theme develops in different voices – for example, in retrograde form, inversion, augmentation, diminution, crab progression, in various tonalities and modulations, through contrapuntal, rhythmic, melodic, and harmonic changes. *Kunst der Fuge* is one of Bach's most complex, intellectually remarkable, and symbolically rich works, often cited as the pinnacle of his polyphonic art. Undoubtedly, it is his most mathematical, analytical, and philosophical creation. The entire work, lasting approximately an hour and a half, consists of fourteen fugues, each derived from a single musical theme, developed and broken down through various contrapuntal techniques. It is designed with extraordinary mathematical precision; each fugue follows a logic that can be analyzed almost like a mathematical proof. Yet despite the strictness of the rules, the listener or performer, when in contact with the music, feels depth, peace, transcendence – a sensation that could perhaps be defined as a spiritual experience or even a form of meditative state, of prayer. Bach's *Kunst der Fuge* is one of those rare masterpieces where art and science not only meet but align in perfection. It represents a bridge between two seemingly opposing

yet actually complementary worlds – and for this reason, it has become the subject of study not only by musicians but also by mathematicians, philosophers, and theologians.

3.2. *The Golden Ratio: A Universal Principle in Art and Nature*

Musical compositions, in general – and not just in the fugue form – require a great deal of structure. The communicative power of music is most effectively expressed when supported by substantial knowledge of the rules of harmony, melody, music form analysis, music history, dramaturgical arcs, and contextual placement in time. Alongside this, it also requires contemplation of its purpose, its idea, and its expression. This holds true for composers as well as performers, who, through their own development and process of musical expression and the shaping of their artistic personalities, touch upon the most diverse extremes and possibilities, within the space, time, and period, as well as within their own compass of individual paths. As in life and the universe, so too in art and science – meanings are often layered, and interpretations, answers, and paths may be multiple rather than singular. The interplay between mathematics and art thus reveals itself on many levels, including within visual art.

One of the most well-known examples of this connection is the Golden Ratio – a proportion found both in nature and in numerous artistic masterpieces. Often regarded as the most perfect compositional law in nature, the golden ratio appeals to the human eye as innately beautiful, as it embodies a harmony between linear precision and apparent irregularity or imperfection. Mathematically, it refers to a relationship in which the ratio of the smaller part to the larger is the same as the ratio of the larger part to the whole. This proportion approximates 1.618:1, and the point dividing a whole according to this ratio is known as the golden point. It can be expressed with the equation: A : B = (A + B) : A. The Golden Ratio has been known since the time of the ancient Greeks, but it experienced a profound revival during the Renaissance (World History Edu, n.d.). Artists, architects, and sculptors of that period deliberately employed it as a tool to achieve visual balance and aesthetic harmony. Leonardo da Vinci—Italian Renaissance architect, inventor, scientist, engineer, sculptor, and painter—is often linked to the golden ratio, particularly in works like the *Vitruvian Man* and the *Mona Lisa*. While many scholars and art historians speculate that elements of the golden ratio are present in these compositions, it remains uncertain whether Leonardo intentionally applied this principle, and such interpretations are still subject to debate (World History Edu, n.d.). In this way, the golden ratio functions as an unwritten law of universal order, flowing seamlessly through nature and art—as though inscribed in the very fabric of existence. This recurring tendency toward balance, symmetry, and formal coherence appears to be inherently inscribed in the human psyche—precisely because it is inscribed in nature itself.

3.3. *Fibonacci's Sequence: The Blueprint of Nature's Order*

Closely tied to the golden ratio is the Fibonacci sequence—a mathematical series of numbers in which each number is the sum of the two preceding it (1, 1, 2, 3, 5, 8, 13, 21 …). As the values increase, the ratio between consecutive numbers increasingly approximates the golden mean, or 1.618. This link between numerical order and natural harmony is not merely a theoretical curiosity—it has numerous practical applications in art, architecture, and the natural world. Fibonacci sequences and the golden ratio provide artists and architects with tools to create works that convey not only aesthetic appeal but also inner balance and order. From ancient structures to modern architecture, proportions based on these principles often evoke a sense of coherence and natural beauty. A classic example could be *St. Peter's Basilica* in the Vatican, where some scholars suggest that numerous elements may follow proportions aligned with the Fibonacci sequence (World History Edu, n.d.). Traces of these two fascinations can also be found in the *Parthenon* in Athens, where architectural proportions and relationships in structures such as columns and windows appear consistent with the golden ratio (Finch, 2017). In sculpture – for example – some scholars argue that Michelangelo used elements aligned with mathematical principles, including the golden ratio, when creating works like the *Sistine Chapel* (Huntley, 1970). The architectural and artistic work he designed for the chapel's ceiling contains compositions where the ratios between individual paintings and body segments appear to

reflect this mathematical ideal. Some interpretations of Michelangelo's *David* suggest that its proportions may reflect an intuitive sense of mathematical harmony, often associated with the golden ratio – a concept long admired for its aesthetic appeal (World History Edu, n.d.).

3.4. The Sound of Numbers: Mathematical Beauty in Music

The Fibonacci sequence and the golden ratio are also present in music. In many of Mozart's works—such as sonatas and symphonies—musical structures follow rhythmic and harmonic patterns that align with Fibonacci numbers (BBC, 2023.). It is said that numerous phrases and sections in his compositions end after a specific number of bars corresponding to consecutive Fibonacci values. A similar principle can be observed in the works of French Impressionist Claude Debussy, whose rhythmic cycles and harmonic progressions often convey not only aesthetic sensibility but also a deeper coherence that can be linked to the golden ratio and Fibonacci sequence (Frey, 2015). We believe there are quite a few more of these examples in music.

3.5. The Mathematical Language of Nature

The fascination with the golden ratio and Fibonacci sequence is also found in natural structures such as flowers, leaves, shells, and even galaxies – and while humans may not be consciously aware of it, we often experience it as aesthetically pleasing. A few examples: the spiral shape of snail shells follows a mathematical structure based on golden ratios. The family tree of bees intriguingly reflects the Fibonacci sequence. A male bee (drone) is born from an unfertilized egg and thus has only one parent – the queen. The queen, however, has two parents – a drone and another queen. If we continue tracing the lineage backwards, we see that the number of ancestors in each generation follows the Fibonacci pattern: 1 (drone), 2 (queen), 3, 5, 8, 13 … and so on (The Math Learning Centrum, 2024). In the plant kingdom, this ratio is frequently observed: daisies, chrysanthemums, and primroses often have 34, 55, or even 89 petals, numbers that are consecutive Fibonacci values. Sunflower seeds are arranged in spiral patterns, typically 34 in one direction and 21 in the other—again, two consecutive Fibonacci numbers (Liu, 2024). This arrangement is not only visually appealing but also biologically optimal, facilitating the densest possible packing of seeds. A similar pattern is evident in the arrangement of leaves on a stem or branch—known as phyllotaxis—which often reflects this mathematical order (Liu, 2024). In the human body, proportions approximating the golden ratio are frequently found: distances between joints, limb lengths, or facial features. Although not every body is perfectly proportioned, idealized representations, such as Leonardo da Vinci's *Vitruvian Man*, are based on ratios close to this ideal (Liu, 2024). This may explain why the human form appears so naturally harmonious.

5. Conclusion: Echoes of Unity: A Path to Universal Understanding

There are likely as many examples of the indispensable reciprocity between mathematics (science), art, and nature as there are ways to connect creative imagination with the logical-mathematical component, all with the common aim of shaping, understanding, and making sense of the world in which we live. The intersections between mathematics and art reveal numerous shared foundations. We may view them as the ones seeking order, structure, and essence – in time, space, and existence. Both transcend functionality and open up as expressions of an inner necessity. They explore form, harmony, contrast, and experiment – and most importantly, both create space for the new: new theories, new artistic forms, fresh thought processes, and unpredictable paths of understanding. It is at this intersection that ideas are born which push the boundaries of the known – and in doing so, also push us forward.

If we resist structure and order within art, or if we deny freedom, exploration, and creativity in mathematics or other scientific fields, we are, in fact, resisting nature itself.

And if we resist nature, we resist humanity, human life, and ultimately, existence itself. I envisage that being is a shared consciousness from which we all emerge and in which the truth is hidden. This truth, in its purest form, is a space where there are no longer divisions between "I" and "you"; there, we are all one. And it is from this state of oneness that the greatest masterpieces are born: in art, science, architecture, sport, and beyond... wherever a person surpasses oneself and touches something greater. Sometimes, we can feel the essence of life inside the Sagrada Familia in Barcelona, other times while listening to Bach's Mass in B minor. If something within us moves, if for a moment we better understand the universe, the human experience, or if it encourages us to look into the darker parts of our soul that we haven't dared approach until now, then it has fulfilled its purpose. In the end, we are left with a single, long or surprisingly short, trajectory of life, to which each of us may - or may not - add our own mark. The choice is ours. In reality, everything belongs to the same fabric – tone, number, colour, breath, human, question, answer. And this awareness may be the first step toward understanding what it means to exist. For when we recognize that we are not separate from creation, but part of it, then art, science, and life finally meet in the same breath.

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Research

Standardization of Procedures: the Nanostructurome Pipeline

Liguori Giovanna L [1,*] and Kralj-Iglič Veronika [2,*]

1. Institute of Genetics and Biophysics (IGB), National Research Council of Italy, Naples, Italy.
2. University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia

* Correspondence: giovanna.liguori@igb.cnr.it ; veronika.kralj-iglic@zf.uni-lj.si

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Abstract

Large scale ecological features are driven by the interactions between organisms at all life scales, in particular the microorganisms living in the air, soil, and water. If we want to understand how an ecosystem will respond to natural and human-induced changes, we need to understand the state of the unseen majority – the microorganisms. Understanding of a system means characterising the function of its components and their interactions. In the presently acknowledged view, an ecosystem is considered as an organization of molecules (genes, mRNAs, proteins, and metabolites) into complex molecular pathways (such as gluconeogenesis and tricarboxylic acid cycle). It is thought that interacting molecules form functional modules (such as groups of molecules involved in the same biological process), which then drive larger scale biological processes. Supported by the omic-level high-throughput data acquisition and collections, comprehensive models of the interactions among biomolecules are sought. Since the discovery of DNA molecule as a powerful carrier of the information, solutions at the molecular level have been in focus so far. But for understanding of some essential cellular processes downscaling from micro to molecular level is too big to overcome in a single step. Apparently, the substance (and with it the information) is exchanged between cells within nano-sized extracellular particles (EPs) and as this level is at the core of interaction between the microorganisms, it could be the lever to shift the state of the ecosystem. By creating a pipeline of standardized complementary methods for characterization of samples containing EPs we are approaching the largely unknown landscape of a "nanostructurome" that will in the future become increasingly filled with knowledge on sub-micron cellular structures that convey matter and information between cells.

Keywords: Interactome; Extracellular particles; Standardization; Reproducibility, reliability; Standard operating procedures; Nanostructurome; Pipeline

1. Introduction

Interacting molecules form functional modules which drive large scale biological processes. Interactome studies, then, give us the tools to prepare for and confront the significant challenges that society is grappling with, such as disease prevention and cure, hunger relief, population growth, and climate change. Researchers are seeking comprehensive models of the interactions among biomolecules, with omic-technology providing support.The problem of different interactive relationships between genes considering their unique intrinsic properties and defects is incorporated in cellular interactomes. Narrowing the focus, a metagenomics approach was developed to study the plasmidome (the overall plasmid content in a given environment) which indicate that gene mobility between different phyla is still fairly common in microbially rich environments. The interactome network of protein–protein interactions aims to capture the molecular interactions that underlie organismal complexity. However, the basic principles of how such interactome networks respond to environmental unpredictability and change during evolution are largely unknown. Nanoparticles and nanotechnology offer a fascinating and evolving field of study with many practical applications. At the same time, due to their extremely small size, the study of nanoparticles requires a multidisciplinary platform that integrates data from different sophisticated and complementary technologies.

The progress of technology generates a huge array of data, which poses new challenges in quality control, reproducibility, management and sharing of the experimental results. Optimized and standardized procedures are essential for generating, validating, integrating, analyzing, storing and reusing the wide variety of data available as well as to increase research performance and reduce related cost and time (Liguori and Kisslinger, 2021; Hollmann et al., 2022). Optimization of multivariable processes can be achieved by varying one specific factor at a time, as in the traditional OFAT method, or implementing statistical approaches and quality management tools, such as Design of Experiments (DoE), for a more powerful analysis. DoE has been used in many different fields to identify the effect of simultaneous variations in variable process factors, the interaction between the factors and ultimately the optimal setting that maximizes process output. (Mancinelli et al., 2015, 2021; Xu et al., 2020; Schade and Middendorf, 2021; Rampado and Peer, 2023). Following optimization, information on the optimal factor combination(s) to use, the critical process parameters to monitor, as well as the required equipment and professional competence needed can be formalized in quality assurance tools such as guidelines and standard operating procedures or SOPs (Digilio et al., 2016; Hollmann et al., 2020; Liguori and Kisslinger, 2022). These tools are fundamental to guarantee uniformity in the performance of a specific function or process, which is the basis for data reproducibility.

2. Standard Operating Procedures

Based on the existing literature on the definition of SOPs (Hattemer-Apostel, 2001; Gough and Hamrell, 2010; Akyar, 2012; Hollmann et al., 2020) and our direct experience in dealing with standardization issues in multicentric national and international consortia (Bongiovanni et al., 2015; Liguori and Kisslinger, 2021), we here summarise the main characteristics and purposes of a SOP.

SOPs are detailed step-by-step written instructions for performing a procedure to achieve a predetermined specification and quality end result. In addition, SOPs also contain key information on the materials, equipment and software/hardware required, health and safety warnings, cautions and instructions for the management of records, data and waste, as well as the roles, competencies and responsibilities of all personnel involved. This information, which nevertheless has a major impact on the performance

of the procedure, is sometimes left out, whereas SOPs provide an overview of all the key aspects involved in the process and thus allow it to be kept under control.

The purpose of SOPs is to ensure that all operators perform tasks in the same way, which is a necessary condition for obtaining the expected output from the process. Standardized procedures guide workers and reduce the possibility of missed steps or other errors that affect the performance of the process and the quality of the results. SOPs are obviously useful in meeting compliance requirements in regulatory environments where a Quality Management System (QMS) and/or Good Manufacturer Practice (GMP) is in place (e.g. manufacturing and biotechnology industries). However, SOPs are just as useful in non-regulated research to work in a consistent and efficient manner, improve resource management, mitigate health and safety risks, and ultimately support overall research performance and technology transfer. Moreover, SOPs are useful for training new personnel and ensuring appropriate transfer of skills and knowledge, which is particularly relevant in research environments, where staff turnover is typically high. In the context of multicentre projects and consortia, especially the one developing cutting-edge technologies to address current scientific challenges, the identification and sharing of agreed SOPs is essential for data reproducibility, analysis and integration.

3. The Importance of Micro and Nanostructures in Pursuing the One Health Principle

The health maintenance faces many challenges. Societal need for effective disease prevention, treatment, and rehabilitation has been recognized by scientists from diverse fields such as physics, chemistry, biology, and interdisciplinary sciences who are striving to unravel the underlying mechanisms at the cellular level. It is acknowledged that when living cells are exposed to stressful conditions, the secreted cellular substance provides valuable information for other cells for coping with destabilizing stress events. Secreted small (nano-sized) extracellular particles EPs (e.g. extracellular vesicles, lipoproteins and antibody complexes) can transport cargo to neighboring or distant cells (Herman et al., 2021, Lenzini et al., 2020) and thereby mediate and trigger inter- and intracellular communication. It was found that this communication is relevant to biofilm formation, antibiotic resistance, and toxin delivery (Woith et al., 2019). Entering in the process as active players, scientists envisage nanoparticulate platforms as potential carriers of beneficial substances such as vaccines, regenerative material and therapeutics for various diseases (Armingol et al., 2021, Jin et al., 2021, Combarnous et al., 2020, Fais et al., 2016). Nanoparticles are very small particles, generally in the range of 1 to 100 nanometres, that have special physical and chemical properties compared to bulk materials due to their minute size. Nanoparticles, are used in a wide range of applications, including electronics, energy, environmentl applications and medicine, where they play a key role in therapeutic drug-delivery (Joudeh and Linke, 2022). Nanovesicles enclosed by artificial membranes (liposomes) have been considered as substance carriers already for decades. Recently, research has focused also on development of cell-based drug carriers that better mimic the complexity of the natural membrane, however, their survival, migration, function, and toxicity were found to be of concern (Hermann et al., 2021). In addition to lipid nanoparticles and liposomes, extracellular vesicles (EVs) produced by cells have emerged as promising natural nanovectors for drug delivery in several pathological contexts, including cancer, due to their high biocompatibility, safety, targeting capabilities and ability to cross body barriers (Mantile et al., 2020; Herrmann et al., 2021). Finally, bioinspired nanoparticles, which combine the advantages of both synthetic and natural nanoparticles, have also

been produced to enhance drug delivery performance (Liu et al., 2023; Tripathi et al., 2023).

Nowadays, it is of great interest to find solutions for promotion of health and search for alternative bioinspired materials that are abundant, easily accessible and fulfill environmental friendliness and sustainability. Considering ocean as a vast reservoir of living organisms, the research includes the possibilities provided by marine microalgae. Microalgae are the primary source in the food chain and by performing photosynthesis, they also contribute to the formulation of the atmosphere. Besides being acknowledged for their important ecological role, they are being considered for various human uses (Balsanti and Gualitieri 2006, Rosales-Mendoza 2016).

Microalgal EPs (so called nanoalgalosomes) (Picciotto et al., 2021, Adamo et al., 2021) were isolated, imaged by electronic microscopy and characterized according to the guidelines of the International Society for Extracellular Vesicles (ISEV) (Thery et al., 2018). They were found non-toxic and able to transmit the substance to eukaryotic cells (Adamo et al., 2021). As the study (Adamo et al., 2021) was the first systematic aproach to microalgal EPs, further research is necessary to consider also other microalgae species that could be subjected to different mechanisms of formation of SCPs and yield higher quality and quantity EPs (Božič et al., 2022). Preliminary results indicate that the mechanisms of EP formation can be profoundly different in different microalgae species.

Plants provide the core basis for life on Earth and they are the single most important pillar of human nutrition, but healthy plants are not something that we can take for granted. Plants, which make up 80 percent of the food we eat, and produce 98 percent of the oxygen we breathe, are under constant and increasing threat from pests and diseases. Numerous plant pathogens, including bacteria, fungi, and nematodes, are responsible for many plant diseases, which reduce the yield and quality of agricultural production worldwide every year (Fisher et al., 2012; Savary et al., 2019).

The global plant biotechnology market is projected to expand from usd 51.73 billion in 2025 to reach usd 76.79 billion by 2030 (AGI 9348, 2025). Demand for genetically modified seeds is growing as for the needs in India and China which drives the growth of the plant biotechnology market and also induced also a huge shift in terms of innovative products and patent registrations (AGI 9248, 2025). Global players are investing in R&D to manufacture products that are economical and suitable for use with fertilizers and pesticides. The top plant biotechnology market companies are BASF SE (Germany), Corteva Agroscience (USA), Bayer AG (Germany) and Syngenta AG (Switzerland) (AGI 9348, 2025). The outlined factors for growth of the plant biotechnology market are growing demand for high value crops and use of plant growth regulators to combat climatic changes. Protecting plant health can help end hunger, reduce poverty, protect the environment, and boost economic development (UN news, 2019).

Exploring the interaction between plants and microorganisms is conducive to plant disease control and agricultural production. Namely, plants and pathogens secrete multitudes of molecules into the extracellular environment for cross-border communication, which is crucial to plant defense and pathogen virulence (Delaunois et al., 2014; Toruno et al., 2016). As key resources for life on the planet (energy, nutrients, water, soil) are becoming ever more subject to climate and ecological pressures, European Commission has specified nature based solutions as likely means for understanding and managing the urban ecosystem's equilibrium.

Biostimulants are substances with growth-promoting activities on plants, mostly related to nutrient uptake and stress tolerance. We consider EPs as a major way to achieve communication between plants and microorganisms to enhance crop resilience,

productivity and quality. EPs can be up-taken by other cells and therefore mediate intercellular interaction by transferring proteins, lipids, nucleic acids and other molecules to neighbouring or distant cells. By changing the functioning of the recipient cells, EPs play an important role in many physiological and pathophysiological processes (Welsh et al., 2024). Communities of microorganisms managed or functionally enhanced through a greater understanding of EPs communication have several additional extraordinary advantages, key among them being scalability and cost. While urban farming, and bioremediation often require large-scale investment and equipment, EPs are cheaper and easier to scale, including to the individual household level, allowing easier adaptation of local communities and neighbourhoods to climate change system and global changes soon to be impacting urban areas, especially in less-well developed economic areas. One can distinguish between macro and micro remediation methods and contexts, and also along the social-ecological, natural and technical axes differentiating resilience management at city and household scales, while all scales are governed by the mechanisms at the cellular level. Since EP field is a relatively new rapidly developing field the key mechanisms are not yet fully understood and the methods for EP harvesting and characterization and extraction of the compounds are not yet being fully explored. Furthermore, interpretation of the results on new substances in connection with other parameters of the medium urgently need improvements and clarifications.

4. The Nanostructurome

Nanostructurome contains knowledge on nanostructures, in this particular case those that are relevant in living systems. It focuses on investigation how matter and information are transported at the cellular level in the form of EPs or EVs whose size is in the nanometre range. There are many challenges in harvesting, characterization and application of EPs and presently, it is recommended that the samples are assessed by orthogonal methods (Welsh et al., 2024). Part III of the 12th Proceedings of the Socratic Lectures is devoted to the efforts to assemble and coordinate processing of samples containing EPs to develop a standardized pipeline and an integrated platform for the physico-chemical, molecular and functional study of EPs. The Part III of the 12th Proceedings of the Socratic Lectures presents the first 9 standardized procedures developed inside the Nanostructurome pipeline, spanning from biomass production (Cepec et al., Danilović Luković et al), biophysical analysis (Romolo and Kralj-Iglič), physico-chemical analysis (Heath et al, Bar and Lavrič, Hočevar et al) to molecular and functional analysis (Kovačevskij et al, Michelini et al, D'Antonio et al). Cepec et al. and Danilović Luković et al address biomass production and processing, using microalgae as natural source of EPs. Other SOPs are dedicated to analytical methods such as Interferometric Light Microscopy (Romolo and Kralj Iglic, 2025), Liquid Chromatography coupled to Mass Spectrometry (Heath et al, 2025), NMR spectroscopy (Hocevar et al., 2025) Thermal Analysis (Bar and Lavrič, 2025; Cerc and Lavrencic, 2025) and Next Generation Sequencing (Kovachevikj et al., 2025). Finally, two SOPs focus on biological aspects, such as the quantification of growth and inflammatory factors in extracellular medium (Michelini et al., 2025) and a wound-healing functional assay to determine the effect of nanoparticles on cell migration (D'Antonio et al., 2025). All SOPs are written according to specific guidelines and have the same structure and content. The present SOPs together with those in progress will identify a standardized procedural pipeline for the production, physicochemical, molecular and functional analysis of both unpurified and purified EPs.

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