NANOANALYTICS OF EXTRACELLULAR VESICLES – Standards for isolation, characterization and reporting

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NCI NANO-WORKING GROUP
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Our mission is to leverage public and private investment to promote nanoscience research at the interfaces between disciplines, to translate discoveries into knowledge-driven commercial enterprises, and to educate the next generation of scientists and engineers.
Scanning Probe Microscopy
Nano & Pico Characterization Laboratory

At-a-Glance

- Provides state-of-the-art SPM imaging tools
- Nanoscale imaging and spectroscopy in nearly any environment
- In-house instrument and method development
- Quantitative tools for nanomechanical analysis
- Biomaterial and live cell characterization

From atoms → molecules → materials → cells → organisms
Scanning Probe Microscopy
Nano & Pico Characterization Laboratory

PeakForce Tapping™ mode provides automatic parameter optimization, high-speed scanning and quantitative nanomechanical mapping.
Electron Microscopy
Electron Imaging Center for NanoMachines

At-a-Glance

- Leader in the field of cryoEM
- Provides state-of-the-art Electron Imaging Tools
- Develops cutting-edge technology of cryoEM reconstruction
- Study virus structures and infection processes; important sub-cellular complexes; engineered nanostructures and devices

CryoEM

- Electron Source
- Complexes embedded in vitrified buffer
- Magnetic lens

Image Analysis

- 2D projection
- Determination of orientation/center
- 3D model
- 3D data merging
- Serial Slices

CryoET

- Electron Source
- Tilt
- Magnetic lens

Image Analysis

- Tilt Series
- 3D model
- 3D Fourier transformation
Fluorescence Imaging
Advanced Light Microscopy/Spectroscopy Laboratory
and Macro-Scale Imaging Laboratory

At-a-Glance

- Fluorescence imaging at all length scales
- Consultation, service and training
- Dissemination and teaching
- Collaborative research and development
- Academic and industrial partnerships
- First super-resolution STED microscopy in the US (sub-70 nm resolution)
- Macromolecules, cellular dynamics and nano-scale characterization of bio-materials
- 10 controlled-environment optical rooms

Fluorescence imaging at all length scales: from single-molecule detection to in vivo small animal imaging
The CNSI Ecosystem

Team Science Platform
- Health, energy, environment, and information technology research focus
- Seed and support collaborative teams with expertise that spans disciplines

Education Platform
- Entrepreneurship training and educational outreach programs
- Workforce development programs for grads and postdocs

Technology Platforms
- 6 Core Centers provide and develop leading-edge technology
- Nanofabrication, characterization and high-throughput screening

Entrepreneurship Platform
- 2,800 sq. ft. incubator for CNSI start-ups, 1,000 sq. ft. co-working space
- Currently incubating 16 start-ups; VC fund under development

DISCOVERY NANOSCIENCE NANOSYSTEMS NANOTECHNOLOGY

DEVELOPMENT START-UP VENTURES LICENSING & TRANSFER INDUSTRY ALLIANCES

The CNSI Ecosystem
OUTLINE

• Brief introduction to EVs and major applications

• Correlative techniques - High resolution imaging and characterization of EVs using EM, AFM and Force spectroscopy

• Current challenges- lack of gold standards for isolation and characterization-
  Minimal Requirements- ISEV position papers

• Reporting requirements and transparency of results: EV-TRACK

• Characterization needs for nanoscale and heterogeneous EVs- Biology meets nanoanalytics. Example from European Union METVES

• Convergence with ISA_TAB_NANO for Nanotechnology data sharing standards

• Collaborative opportunities at CNSI/UCLA, comments, ideas, critique
EXTRACELLULAR VESICLES:

- [http://www.the-scientist.com](http://www.the-scientist.com)
EXOSOME

- Pre-metastatic niche
- Immune response inhibition
  + Biomarker
  + Immune response stimulation
- Disease transmission
- Misfolding protein delivery
  + Diagnostic tool
- Spreading of infections
  + Immune stimulation (vaccine)
  + Biomarker
- Their increase inhibits the immune response against the fetus
  + Biomarker
- It promotes the utero-placental angiogenesis
- It favors the maternal tolerance to the fetus

Front. Immunol., 2015
Brief introduction to EVs

EXTRACELLULAR VESICLES:
Naturally occurring

Biological NANO-particles (30-100nm)
EVs: CHARACTERIZATION NEEDS

Isolation

Characterization
- Adhesion, buoyancy, charge, size, shape, concentration, monodispersity, refractive index, stiffness
- Membrane proteins

Applications
- Biomarker discovery
- Exosome engineering
- Drug delivery

A nano-jigsaw puzzle

Khatun et al. Nanomedicine (2016)
Correlative microscopic techniques

EXTRACELLULAR VESICLES “SIZE” IN PERSPECTIVE

- Different scales provide different *types* of information
- Combine techniques to obtain comprehensive structure/dynamic understanding
- Correlative microscopy aims to overcome inherent limitations of different microscopy techniques
Electron Microscopic “Cup shaped” Structure of Exosomes


Palanisamy V. et al. PLOS ONE 2010
AFM topography and Phase images of human saliva derived exosomes
The structure of exosomes - FESEM

Single isolated vesicles as round bulging structures and inter-vesicular connections
Mechanical properties of exosomes

Increase in exosome size under increasing AFM imaging forces (white arrows show scan direction)
AFM based immunolabeling of exosomes extracted from human saliva

Exosome

Primary Ab (antiCD63)

sAb coated gold nanoparticles

260nm
Force spectroscopy to quantitatively map Exosome surface receptor density
Cancer exosome populations significantly increased in saliva and display irregular morphologies, increased vesicle size, and higher inter-vesicular aggregation.

The presence of increased exosome counts and irregular morphology in cancer saliva samples was observed irrespective of whether they solely received chemotherapy, surgery or both.
Release of exosomes from multi-vesicular bodies (MVs) seen in oral cancer patient salivary exosomes
Peak force AFM of exosomes motivated by our earlier FESEM observation.
Results were confirmed by imaging samples obtained from two independent and commonly used isolations, with and without sucrose gradient purification.
Cross section heights: EV and filaments

Vertical height (nm) vs Lateral distance (nm)
Immune Affinity (IA) isolation method shows greater roughness of EVs.
Thanks also to FEI for FESEM - Michael Schmidt & Cliff Mathisen
Laurent Bentolila Advanced Microscopy Lab, CNSI, UCLA
Extracellular vesicles are amazing **BIO**functional **NANO** particles. Nano at its best (or worst)
The need for nanoscale characterization of Extracellular vesicles

Standardization of sample collection, isolation and analysis methods in extracellular vesicle research

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Discussions of the round table are supplemented with a review of published materials and our experience. Controversies and outstanding questions are identified that may inform future research and funding priorities. While we emphasize the need for standardization of specimen handling, appropriate normative controls, and isolation and analysis techniques to facilitate comparison of results, we also recognize that continual development and evaluation of techniques will be necessary as new knowledge is amassed. On many points, consensus has not yet been achieved and must be built through the reporting of well-controlled experiments.
Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper

ABSTRACT
The release of RNA-containing extracellular vesicles (EV) into the extracellular milieu has been demonstrated in a multitude of different in vitro cell systems and in a variety of body fluids. RNA-containing EV are in the limelight for their capacity to communicate genetically encoded messages to other cells, their suitability as candidate biomarkers for diseases, and their use as therapeutic agents. Although EV-RNA has attracted enormous interest from basic researchers, clinicians, and industry, we currently have limited knowledge on which mechanisms drive and regulate RNA incorporation into EV and on how RNA-encoded messages affect signalling processes in EV-targeted cells. Moreover, EV-RNA research faces various technical challenges, such as standardisation of EV isolation methods, optimisation of methodologies to isolate and characterise minute quantities of RNA found in EV, and development of approaches to demonstrate functional transfer of EV-RNA in vivo. These topics were discussed at the 2015 EV-RNA workshop of the International Society for Extracellular Vesicles. This position paper was written by the participants of the workshop not only to give an overview of the current state of knowledge in the field, but also to clarify that our incomplete knowledge – of the nature of EV(-RNA)s and of how to effectively and reliably study them – currently prohibits the implementation of gold standards in EV-RNA research. In addition, this paper creates awareness of possibilities and limitations of currently used strategies to investigate EV-RNA and calls for caution in interpretation of the obtained data.
EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research


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Implementation of EV-TRACK knowledgebase

Flowchart demonstrating four different data flows available to registered EV-TRACK users
Percentage of experiments that adhere to EV metric parameters for various bio-fluids

- **EV-enriched proteins**: Lysate preparation, Antibody specifics, UC specifics, EV density, Density gradient, Electron microscopy images
- **Non EV-enriched proteins**: Qualitative and quantitative particle analysis

Legend:
- Red: Cell culture supernatant
- Blue: Blood plasma
- Green: Serum
- Yellow: Urine
- Orange: Mammalian, other
- Grey: Nonmammalian
Metrological characterisation of micro-vesicles from body fluids

Welcome to the website of European Metrology Research Programme (EMRP) Joint Research Project (JRP) HLT02: Metrological characterisation of micro-vesicles from body fluids as non-invasive diagnostic biomarkers (METVES).

Human blood contains numerous cell-derived microvesicles (left). METVES combines state-of-the-art clinical and biochemical knowledge with advanced metrological techniques (center, right) to quantify - for the first time - clinically relevant properties of microvesicles as novel biomarkers of disease, thereby enabling earlier detection of common diseases, improving healthcare, and reducing the costs of health care.
Optimal properties for biological reference material for EV studies
Biological reference materials for extracellular vesicle studies

CURRENT CHARACTERIZATION TECHNIQUES AND REFERENCE STANDARDS FOR EVs
EXTRACELLULAR VESICLES

Small in size, big in action

EV Core - an analytical technology platform to drive discovery of EV-derived biomarkers and therapeutic targets

Enhanced production: 2D vs 3D cell cultures

Standardization: Development of reference materials

Isolation
- EV sub-populations
- Affinity purification
- Novel methods
- Small sample optimization (biobanks)

Purity of the EV preps: Identification and removal of contaminants

Improved EV characterization
- Sample optimization for omics
- Flow cytometry of multiple EV-markers
- Omics comparisons

High throughput: analysis of clinical samples
Flowchart showing the relation between Work Packages (WPs) of Joint Research Project (JRP) HLT02. Red arrows depict the transport of samples, whereas green arrows depict the knowledge flow between WPs.
Conclusions

• EVs are emerging as novel class of disease biomarkers and drug delivery agents

• To utilize this potential, standardization of isolation and extensive physiochemical and biological characterization of EVs at the single vesicle and population levels; and reporting of results for different applications is a priority

• Search for biological reference material for EVs

• Convergence of biology and nanoanalytics is needed

• Exploring ISA_TAB_NANO
Exploring ISA_TAB_NANO for EV data sharing

**EV CHARACTERIZATION AND FUNCTIONAL DATA**

Microscopy data: AFM, EM, STED
Particle tracking/scattering: NTA, DLS
Flow cytometry/ NanoFACS
Functional Assays
Proteomic/genomic/lipidiomic
Western blot
Thank you!

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