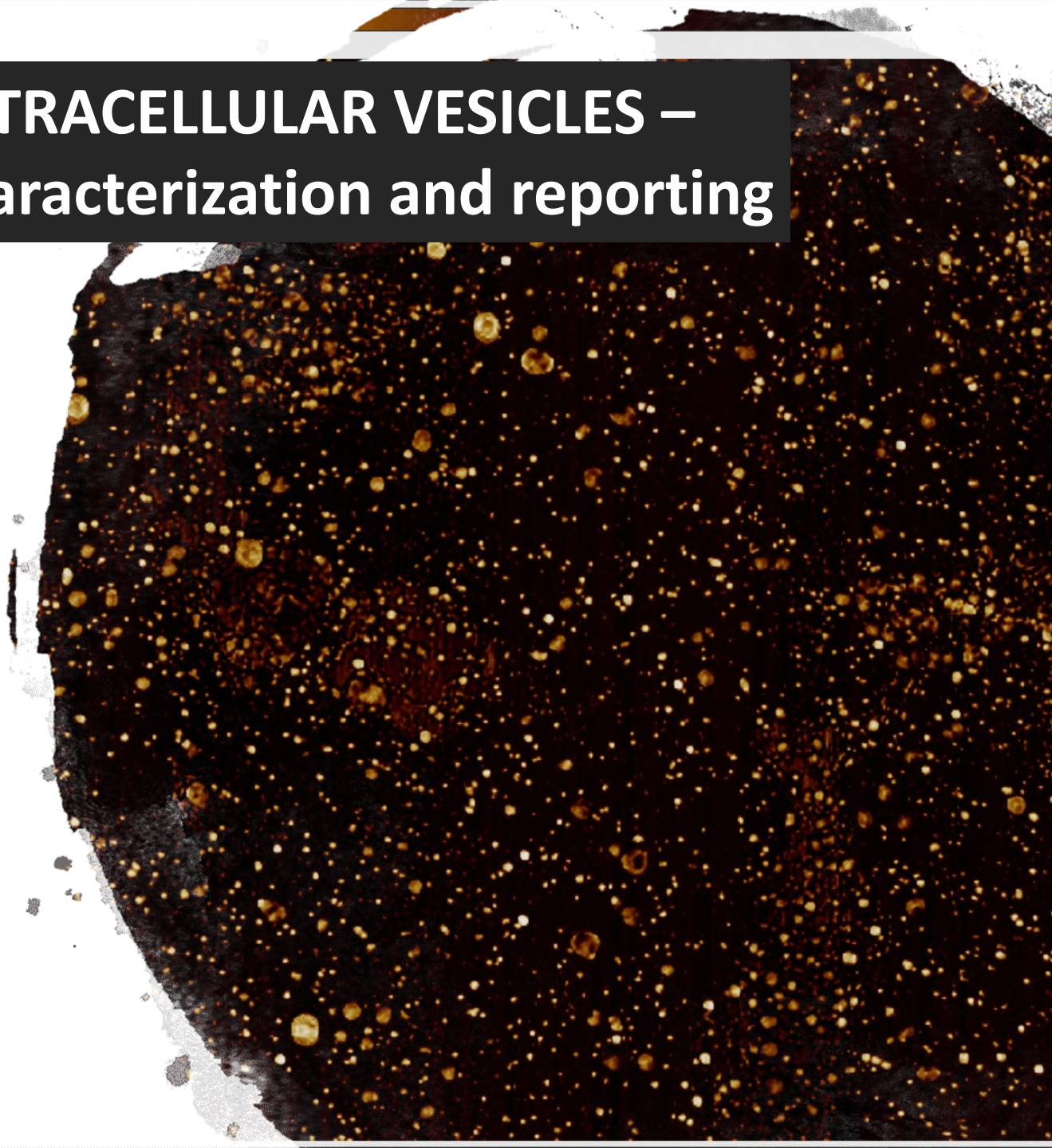
NANOANALYTICS OF EXTRACELLULAR VESICLES – Standards for isolation, characterization and reporting



Shivani Sharma

California NANOSystems Institute

University of California, Los Angeles

NCI NANO-WORKING GROUP
May 25, 2017

California NanoSystems Institute

CNSI at UCLA

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.

Advanced Light Microscopy / Spectroscopy Lab

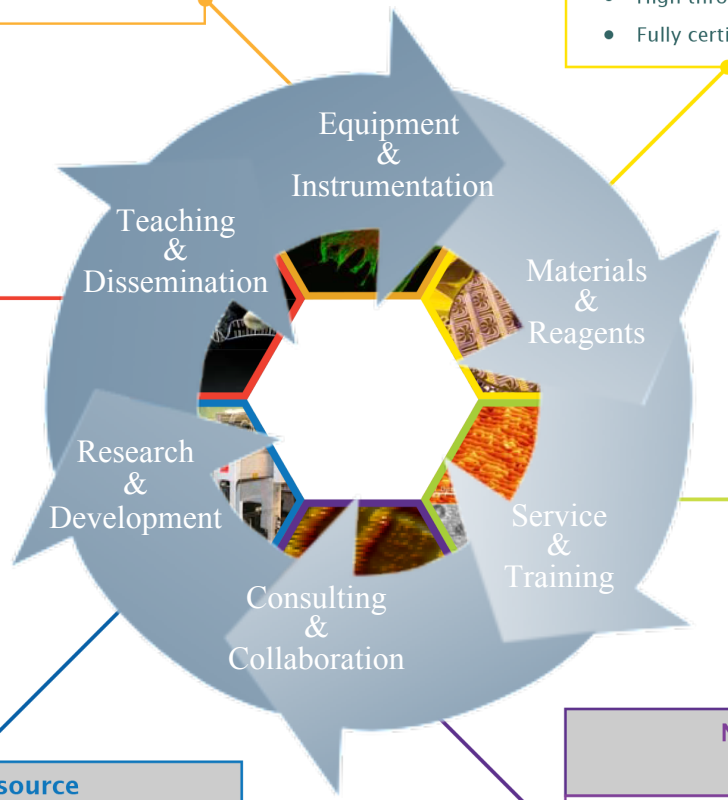
http://alms.cnsi.ucla.edu

- Confocal & multi-photon fluorescence microscopy
- Super-resolution nanoscale imaging
- Advanced techniques: FCS, FLIM, FRET, FRAP, TCSPC
- Light-sheet 3D microscopy
- Pre-clinical small animal imaging

Integrated Systems NanoFabrication Cleanroom

www.isnc.cnsi.ucla.edu

- 10,000 sq.ft. of fully-equipped cleanroom space for nanoscale fabrication and characterization
- Electron beam lithography with sub-10 nm resolution
- High-throughput optical lithography with 300-nm resolution
- Fully certified biosuites (BSL-1 and BSL-2)



Electron Imaging Center for NanoMachines

www.eicn.ucla.edu

- A Leader in the field of cryoEM
- Study virus structures and infection processes; sub-cellular complexes; engineered nanostructures and devices
- Provides state-of-the-art electron imaging tools
- Develops cutting-edge technology for cryoEM reconstruction

Integrated NanoMaterials Lab

http://inml.cnsi.ucla.edu

- Semiconductor nanomaterials synthesis & characterization facility
- III-As/Sb and III-N MBE epitaxial growth foundry services
- High quality alloys for electronic / photonic devices
- Integration of dissimilar nanomaterials for novel applications

Molecular Screening Shared Resource

www.mssr.ucla.edu

- High Throughput Screening and Drug Discovery
- All plater reader based readouts supported
- High Content Screening (confocal and epifluorescence)
- Functional Genomics (CRISPR, cDNA, shRNA and siRNA)
- FACS sorting for C.Elegans, cell spheroids and tumeroids

Nano & Pico Characterization Lab

http://nanopicolab.cnsi.ucla.edu

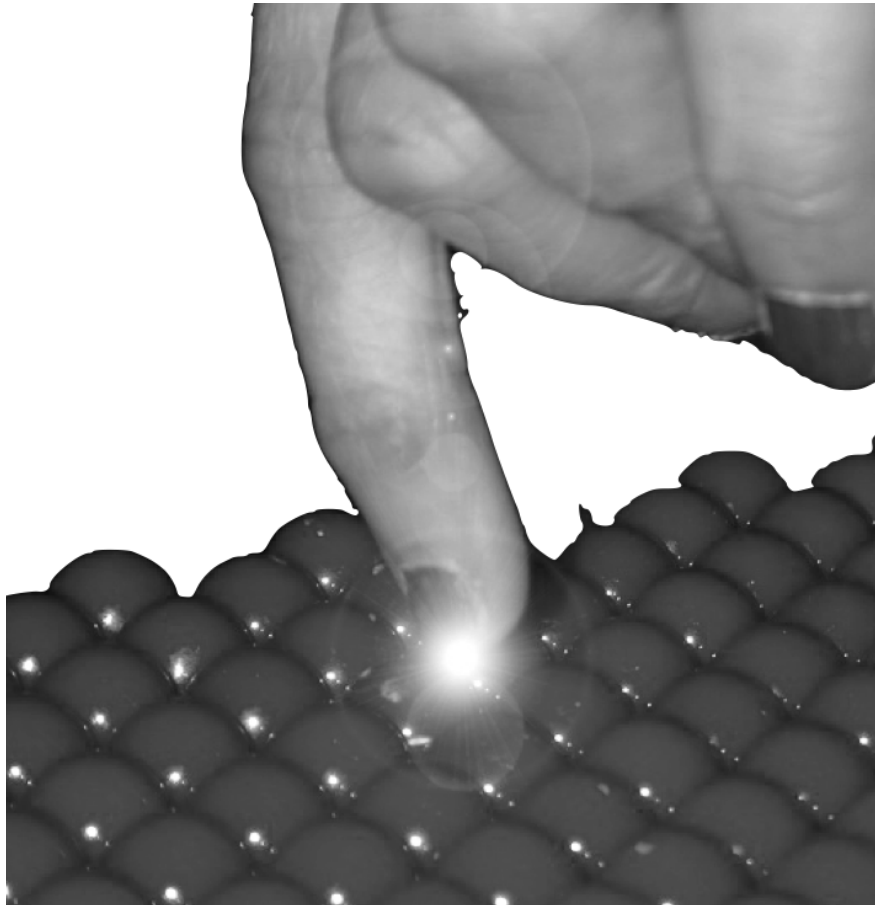
- State-of-the-art instrumentation for scanning probe microscopy
- Quantitative methods for electrical, magnetic, and mechanical analysis
- Biomaterials and live cell characterization with nanoscale precision
- AFM & STM Imaging and spectroscopy in nearly any environment

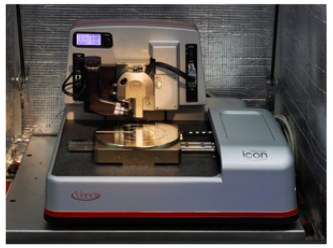
Scanning Probe Microscopy

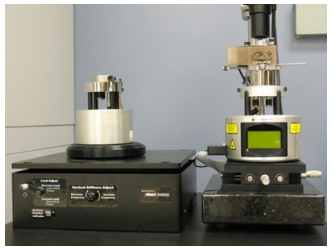
Nano & Pico Characterization Laboratory

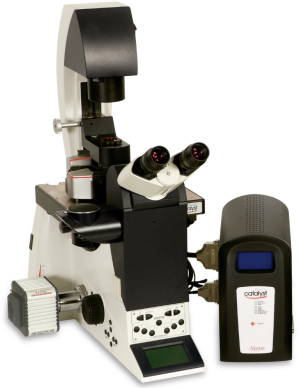
At-a-Glance

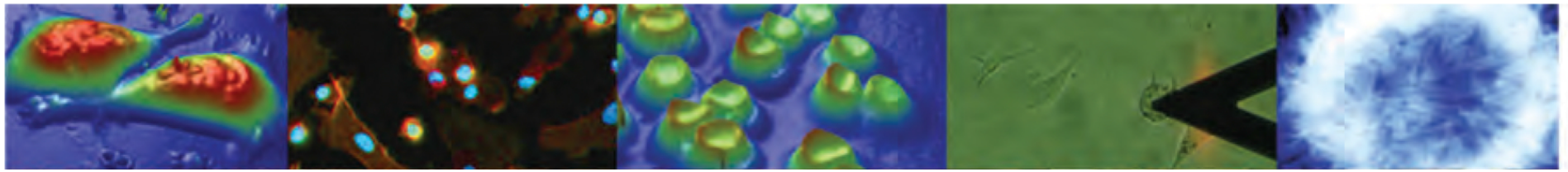
- Provides state-of-the-art SPM imaging tools
- Nanoscale imaging and spectroscopy in nearly any environment
- In-house instrument and method development
- Quantitative tools for nanomechanical analysis
- Biomaterial and live cell characterization











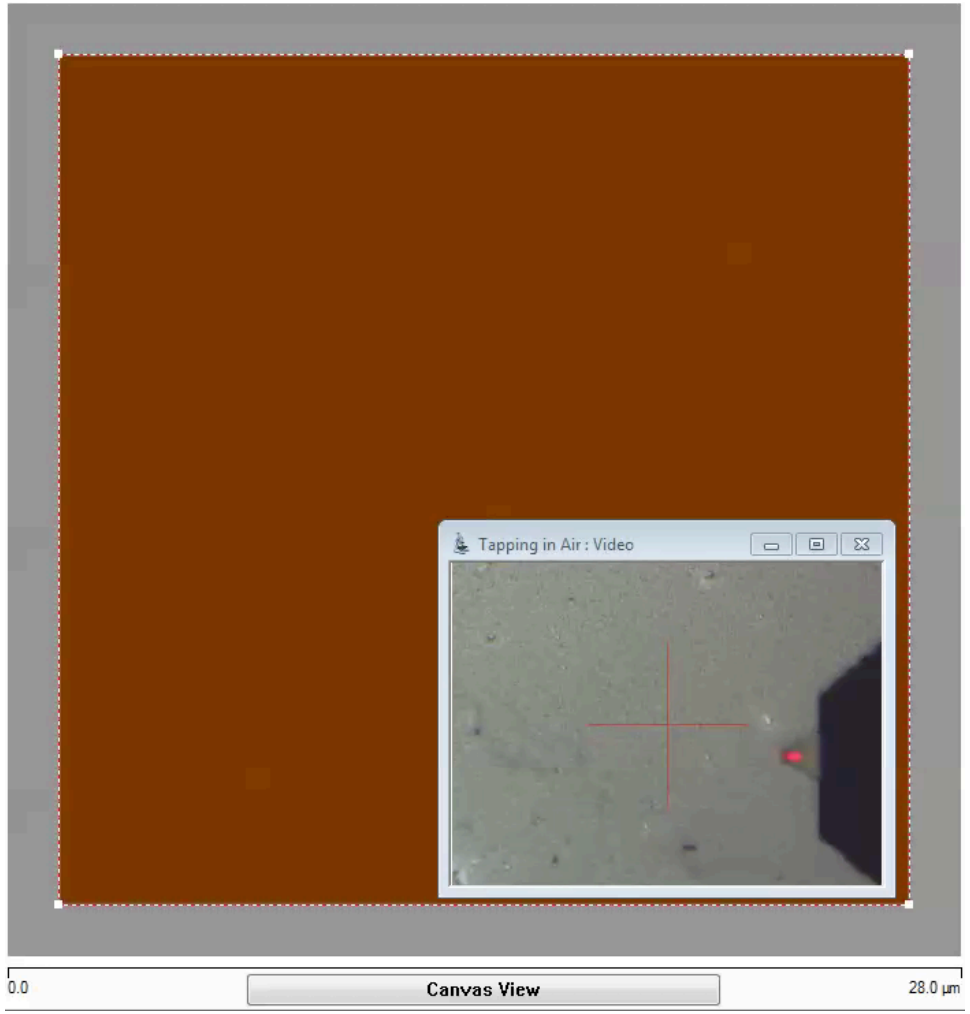
From atoms ⟷ molecules ⟷ materials ⟷ cells ⟷ organisms

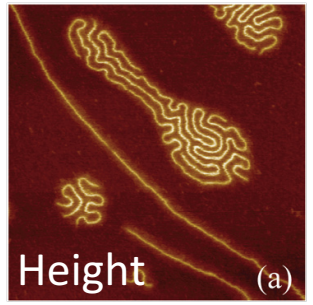
Scanning Probe Microscopy

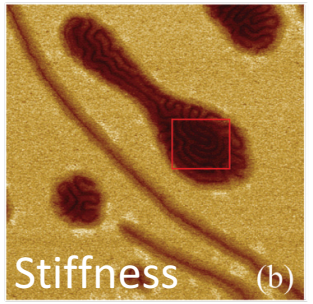
Nano & Pico Characterization Laboratory

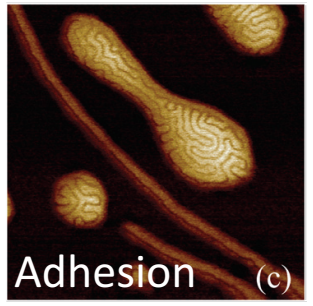
At-a-Glance

- 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









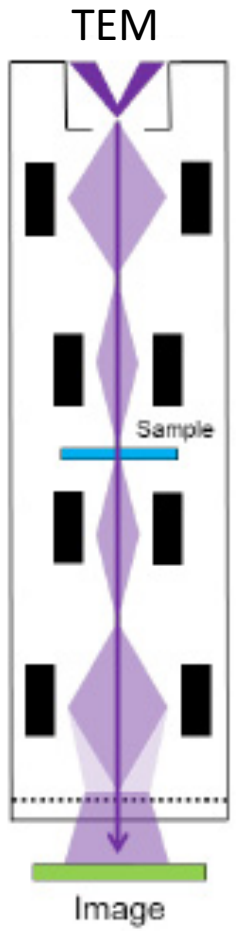
PeakForce Tapping™ mode provides automatic parameter optimization, high-speed scanning and quantitative nanomechanical mapping

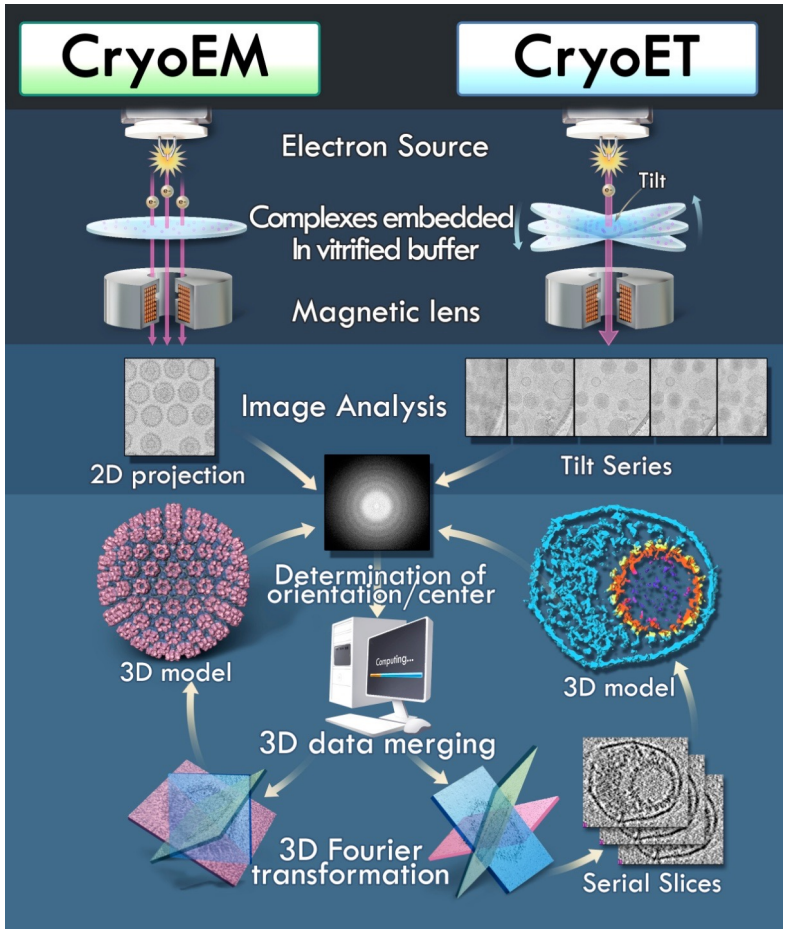
Electron Microscopy

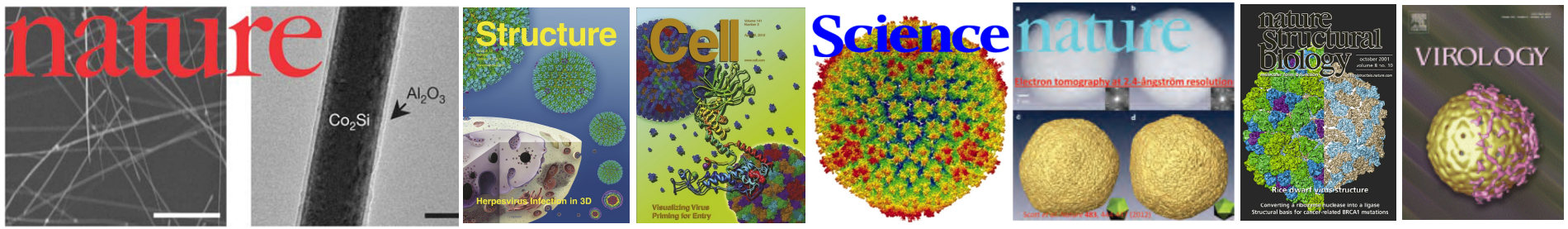
Electron Imaging Center for NanoMachines

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







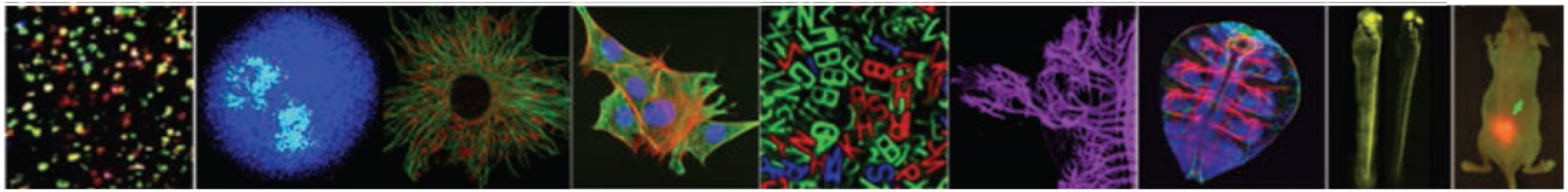
Fluorescence Imaging

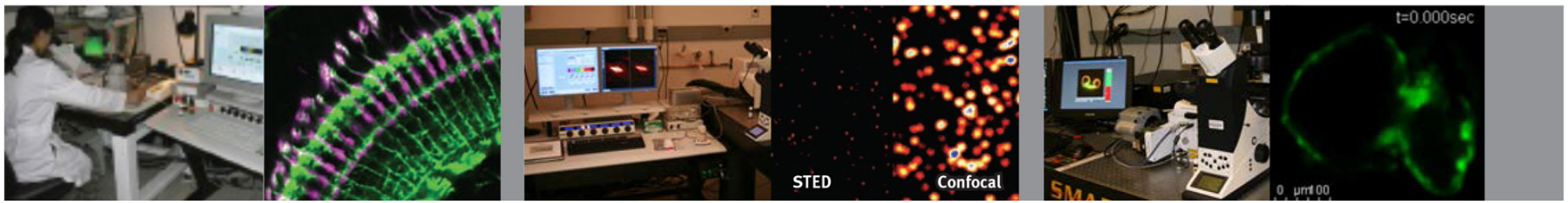
Advanced Light Microscopy/Spectroscopy Laboratory and Macro-Scale Imaging Laboratory

At-a-Glance

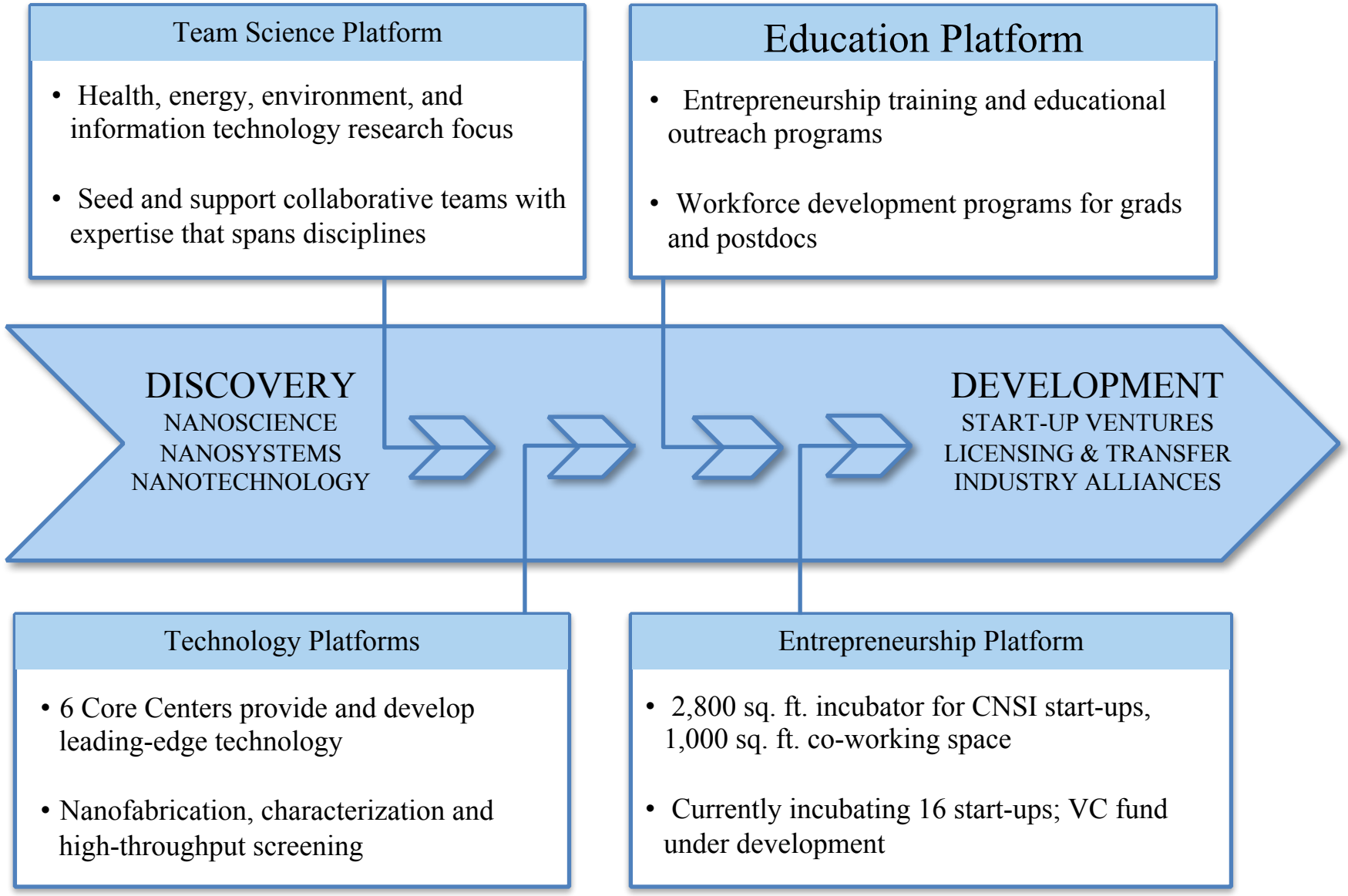
- Fluorescence imaging at all length scales
- Consultation, service and training
- Dissemination and teaching
- Collaborative research and development
- Academic and industrial partnerships
- First super-resolution STED microscopy in the US (sub-70 nm resolution)
- Macromolecules, cellular dynamics and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

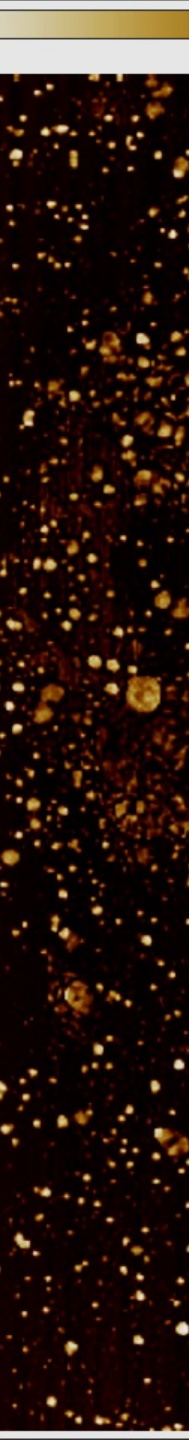
Fluorescence imaging at all length scales: from single-molecule detection to *in vivo* small animal imaging





The CNSI Ecosystem



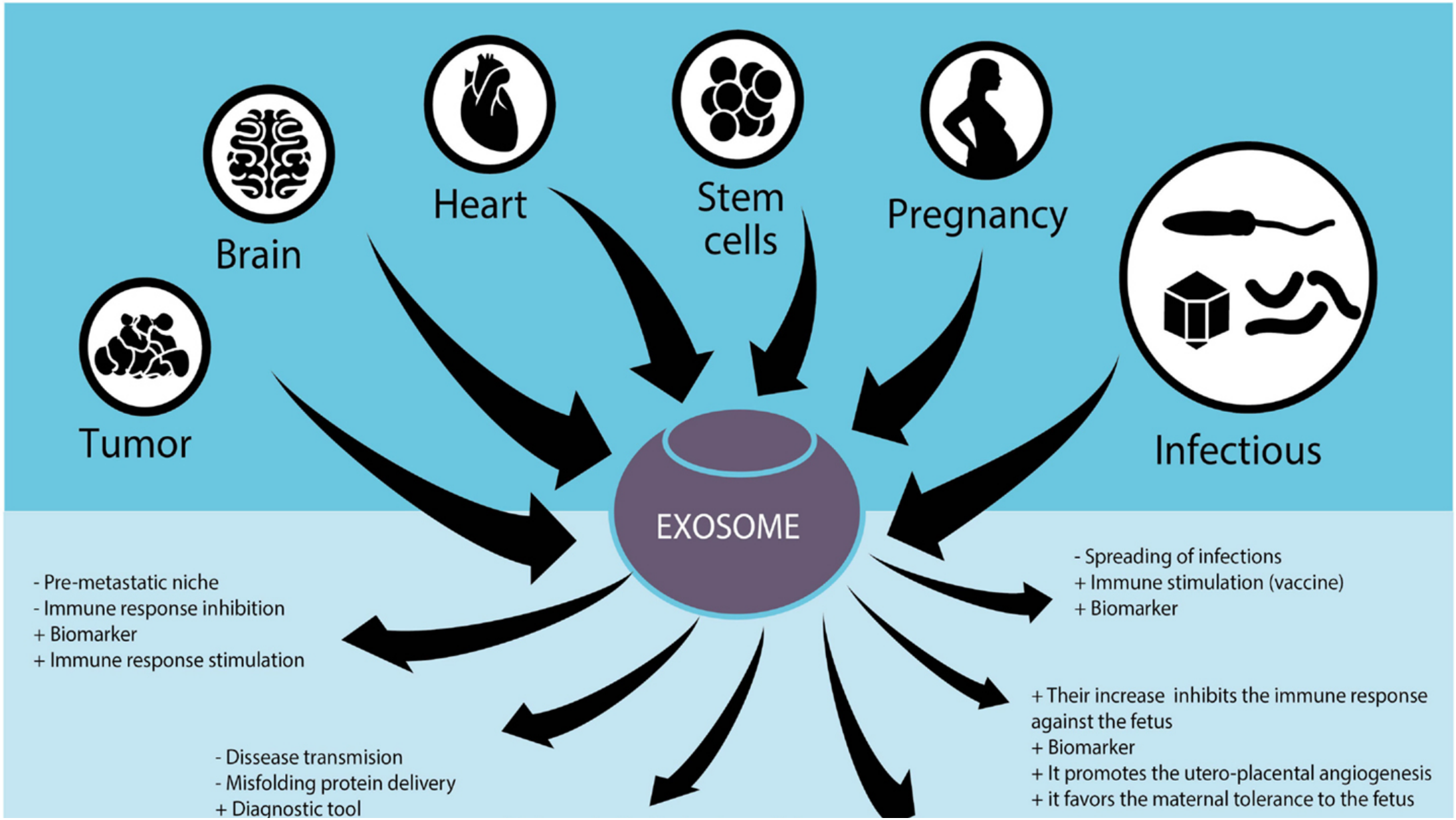


OUTLINE

- 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 ?articles.view/articleNo/30793/title/Exosome-Explosion/*



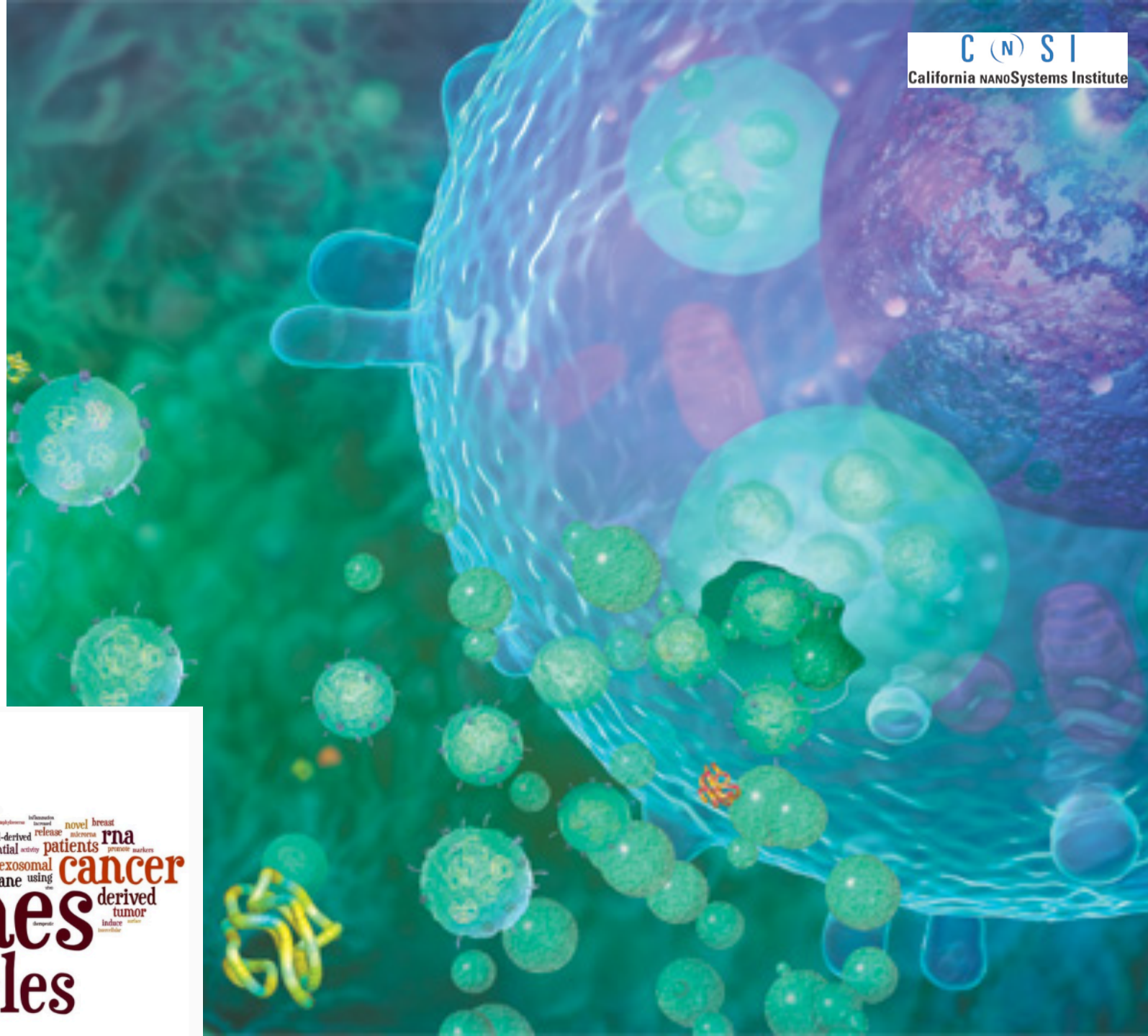
Front. Immunol., 2015

Brief introduction to EVs

EXTRACELLULAR VESICLES:

Naturally occurring

Biological ***NANO-*** *particles (30-100nm)*



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California NANOSystems Institute

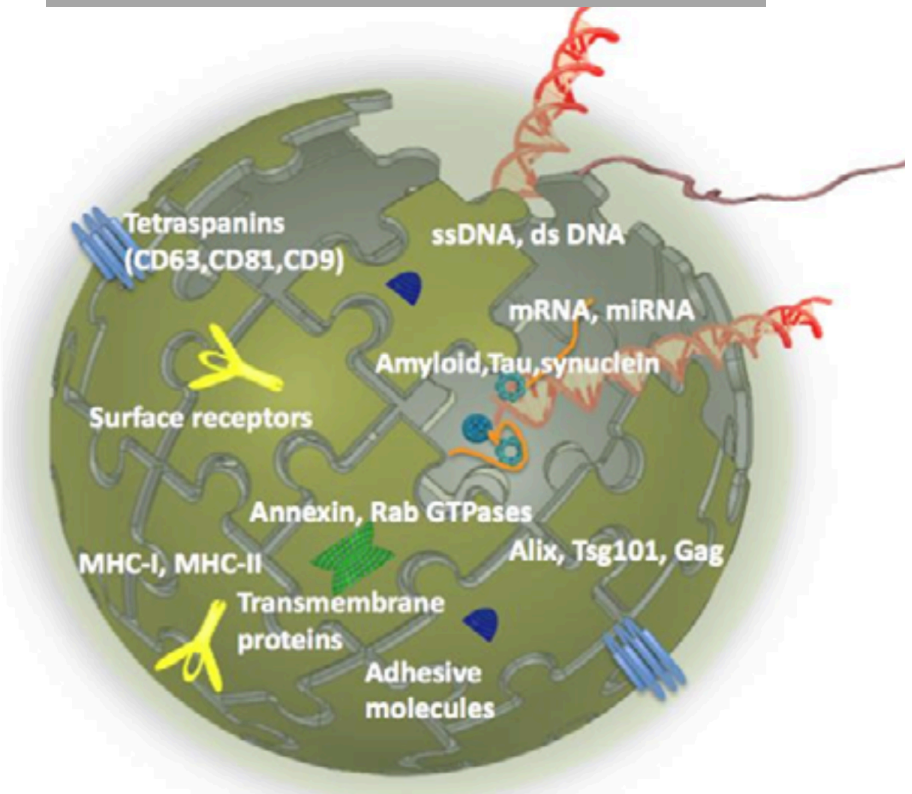
EVs: CHARACTERIZATION NEEDS

Isolation → **Characterization**

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

→ Applications

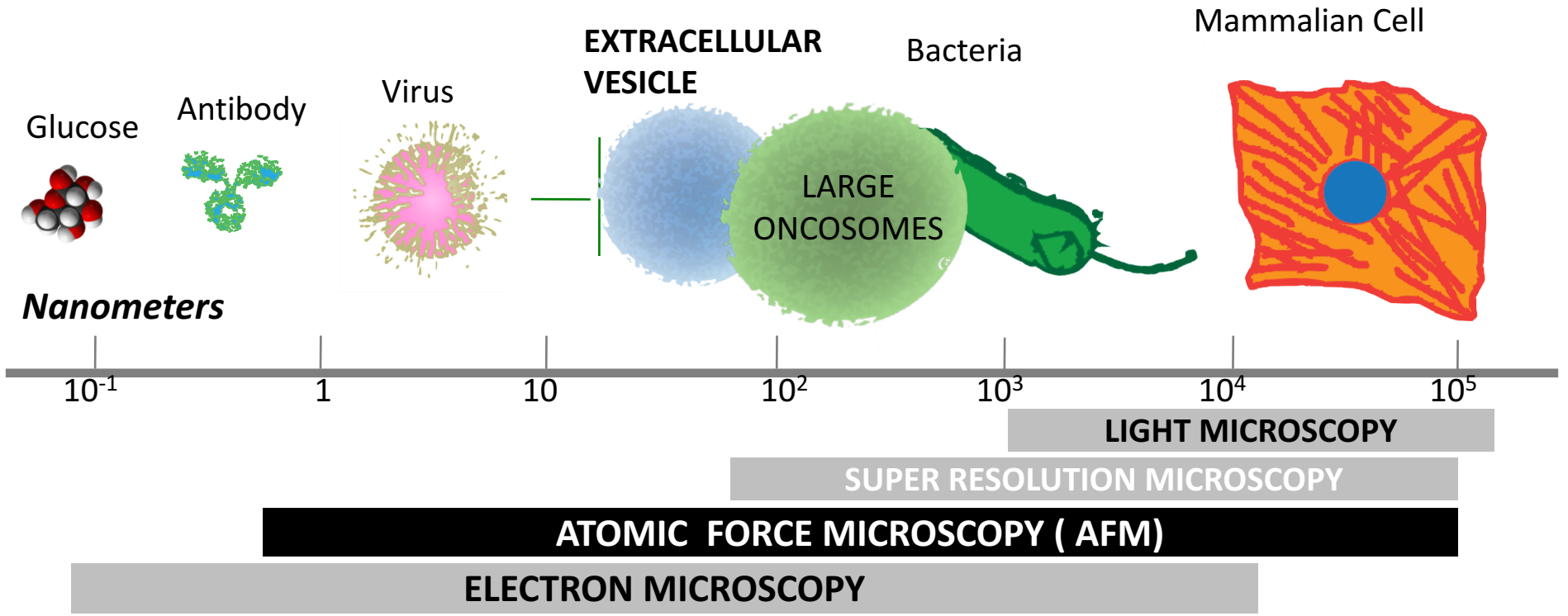
- Biomarker discovery
- Exosome engineering
- Drug delivery



A nano-jigsaw puzzle

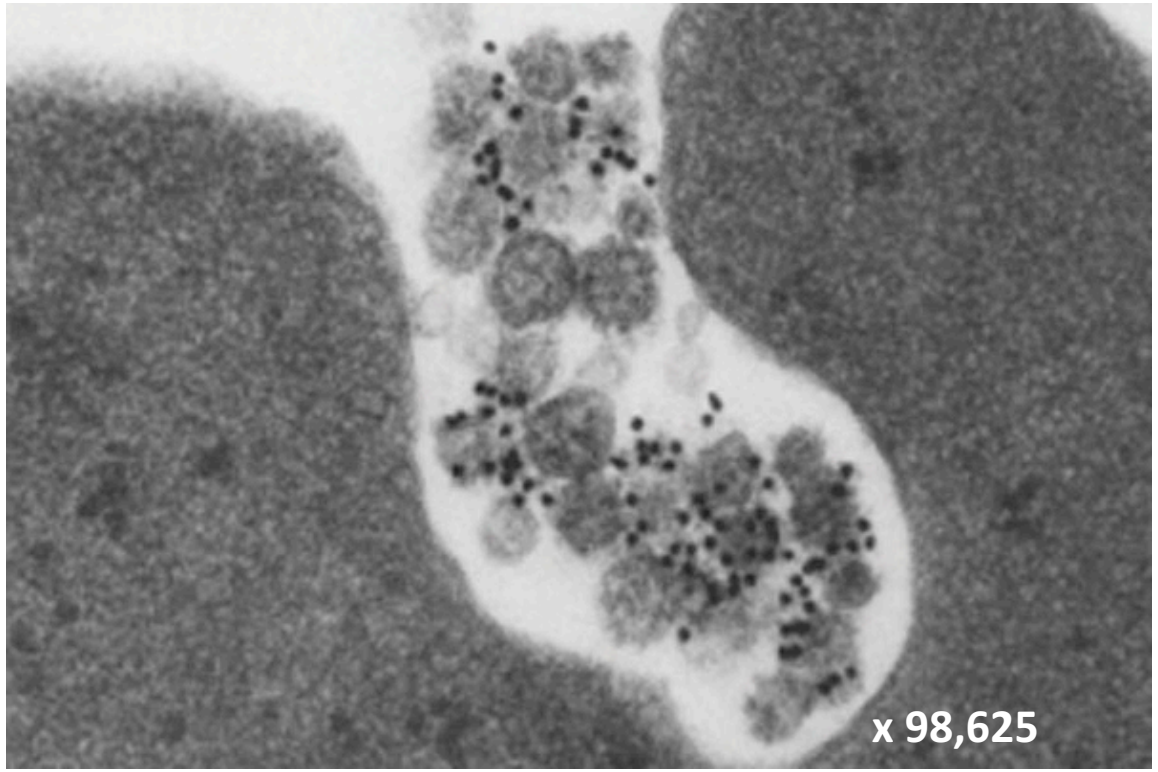
Khatun et al. Nanomedicine (2016)
Sharma et al. J Nanomed Nanotechnol (2015)

EXTRACELLULAR VESICLES "SIZE" IN PERSPECTIVE

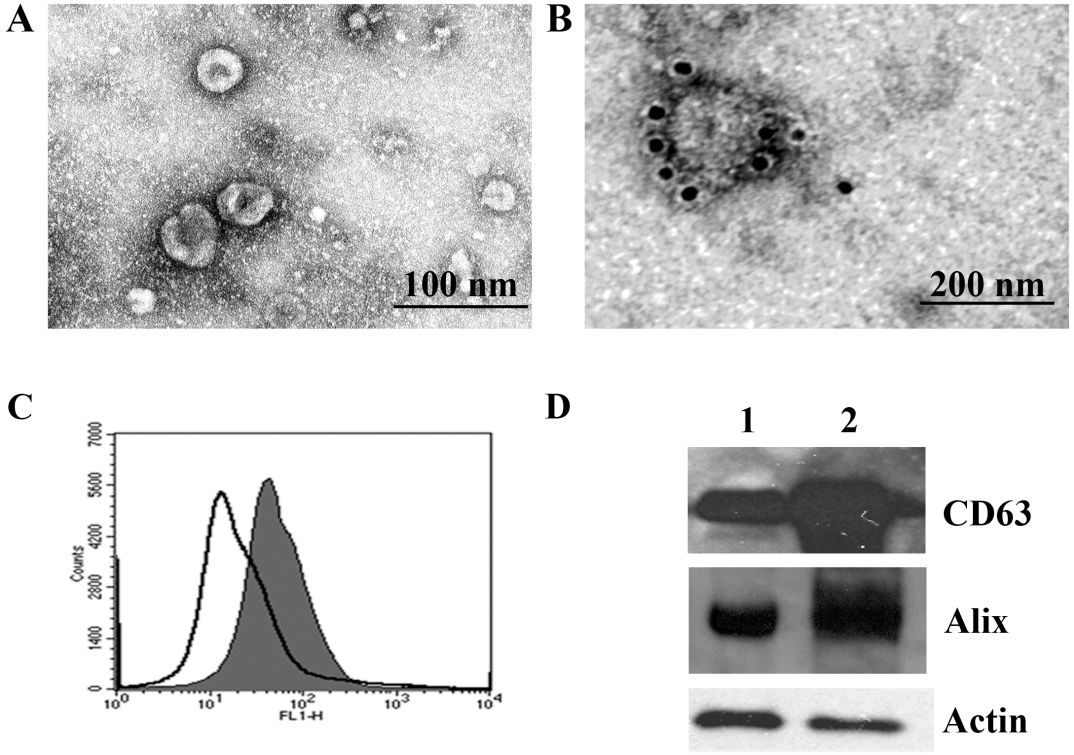


- Different scales provide different *types* of information
- Combine techniques to obtain comprehensive structure/dynamic understanding
- Correlative microscopy aims to overcome inherent limitations of different microscopy techniques

Electron Microscopic “Cup shaped” Structure of Exosomes



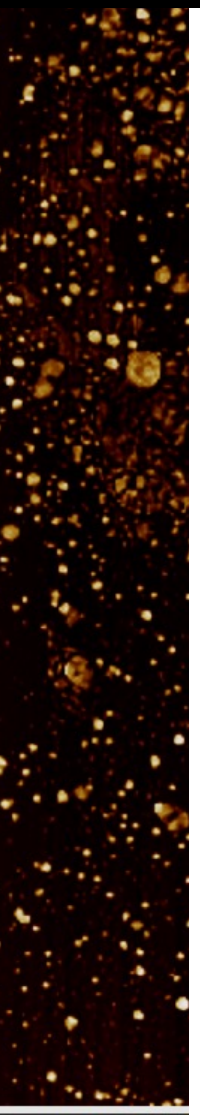
BT Pan et al. J Cell Biol. 1985 Sep;101(3):942-8



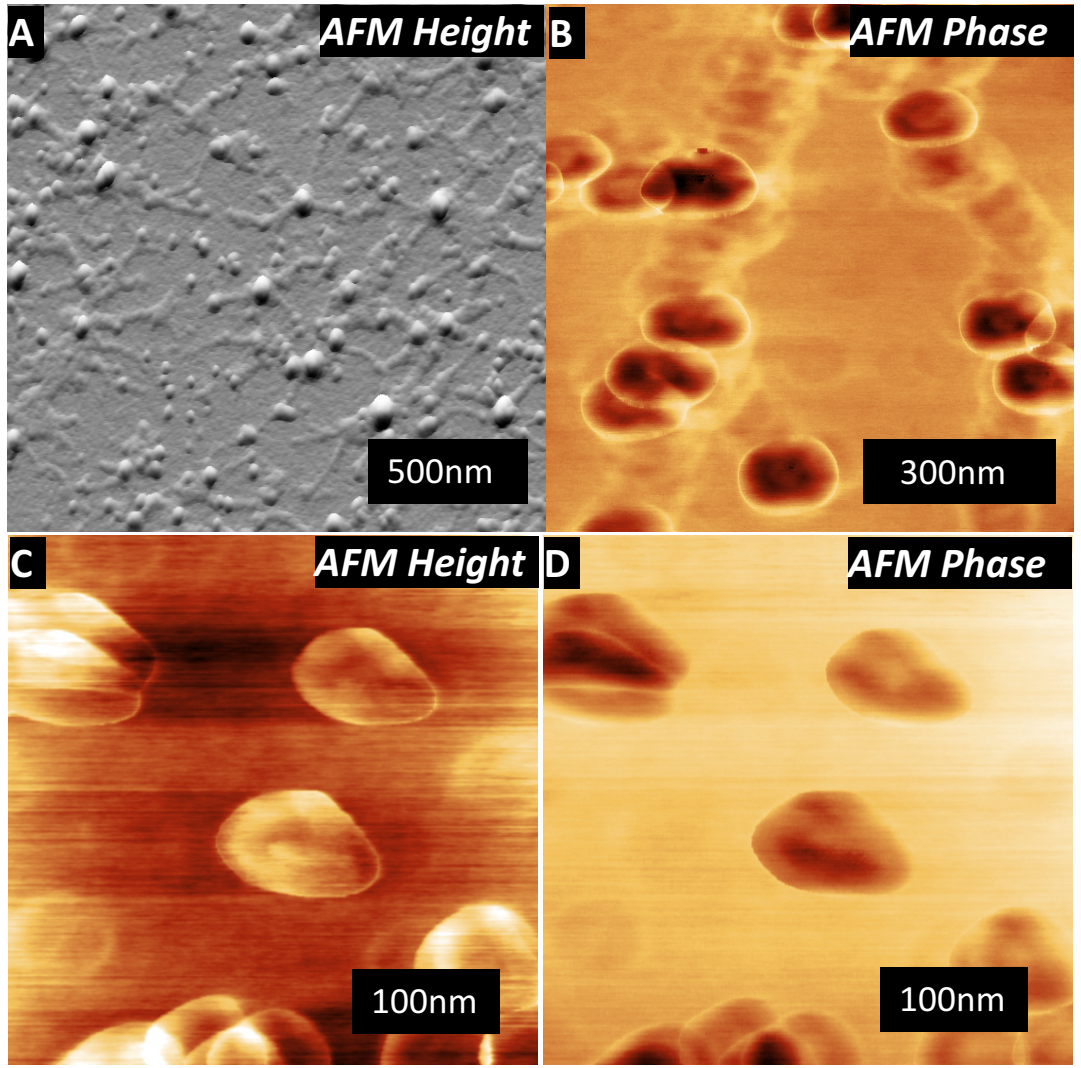
Palanisamy V. et al. PLOS ONE 2010

Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy

Sharma S. *et al*. ACS Nano 2010

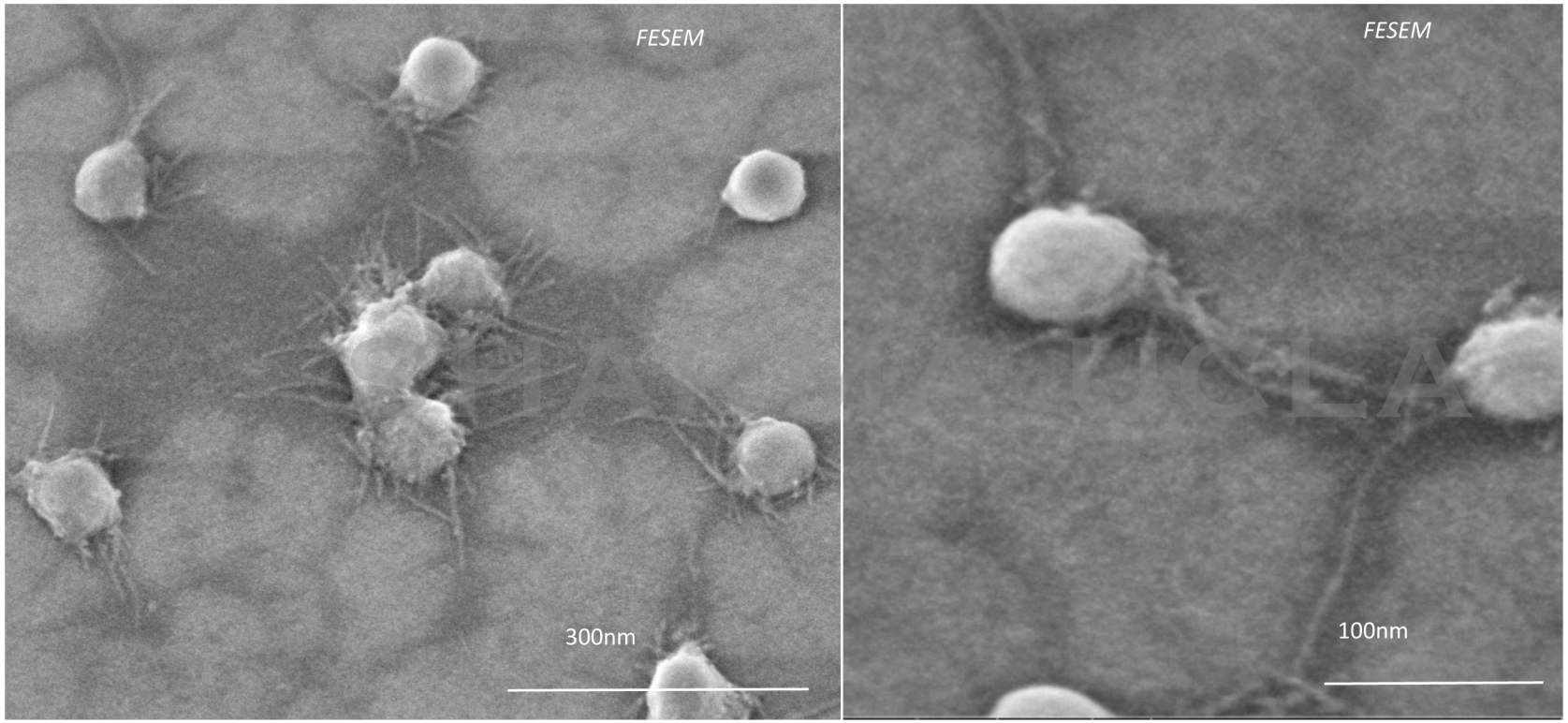


AFM topography and Phase images of human saliva derived exosomes



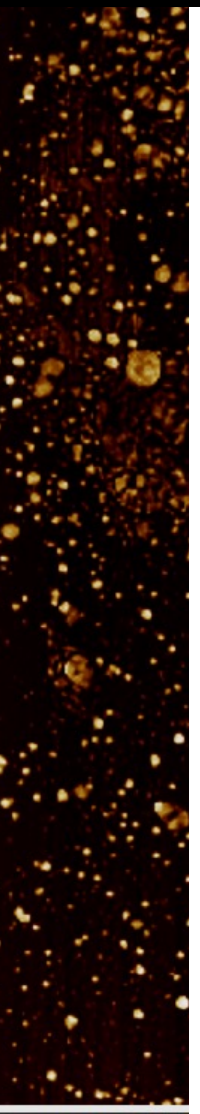
Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy

The structure of exosomes- FESEM



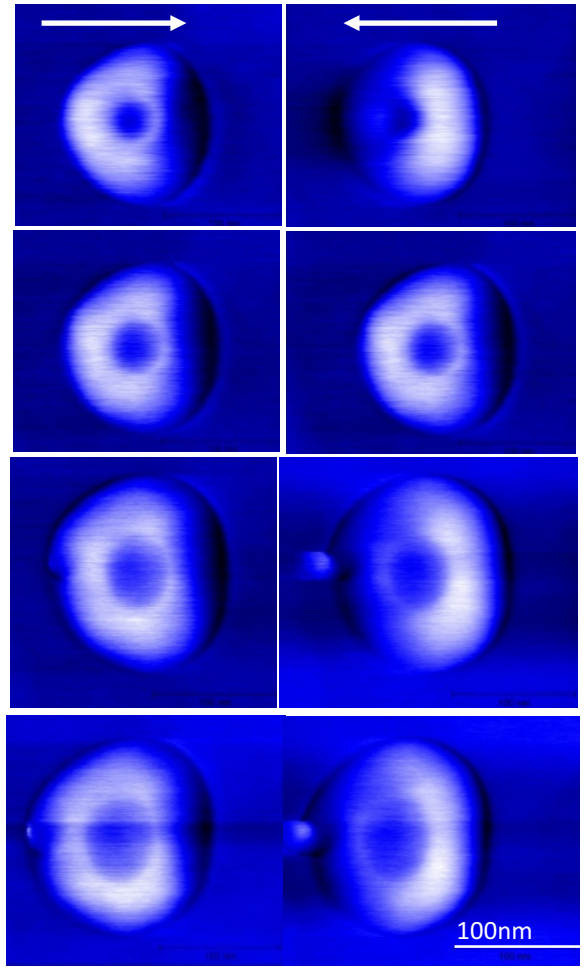
Single isolated vesicles as round bulging structures and inter-vesicular connections

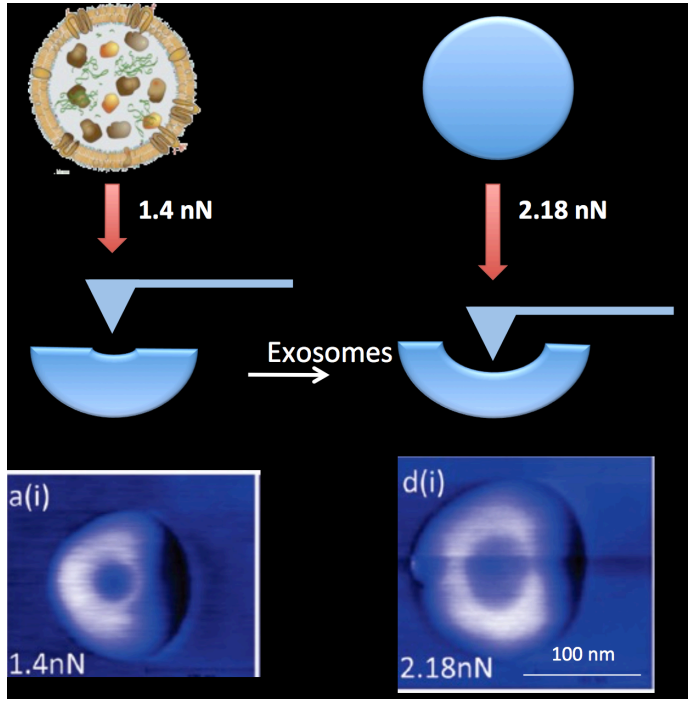
Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy



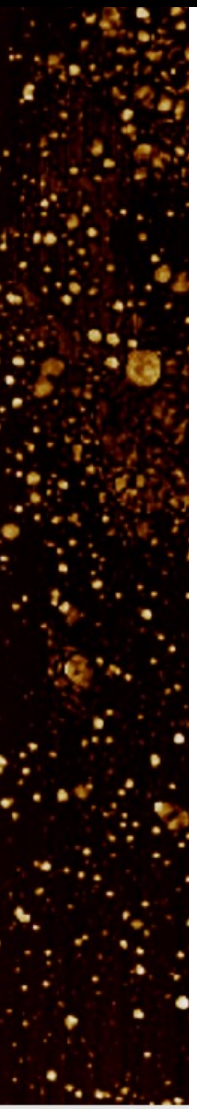
Mechanical properties of exosomes

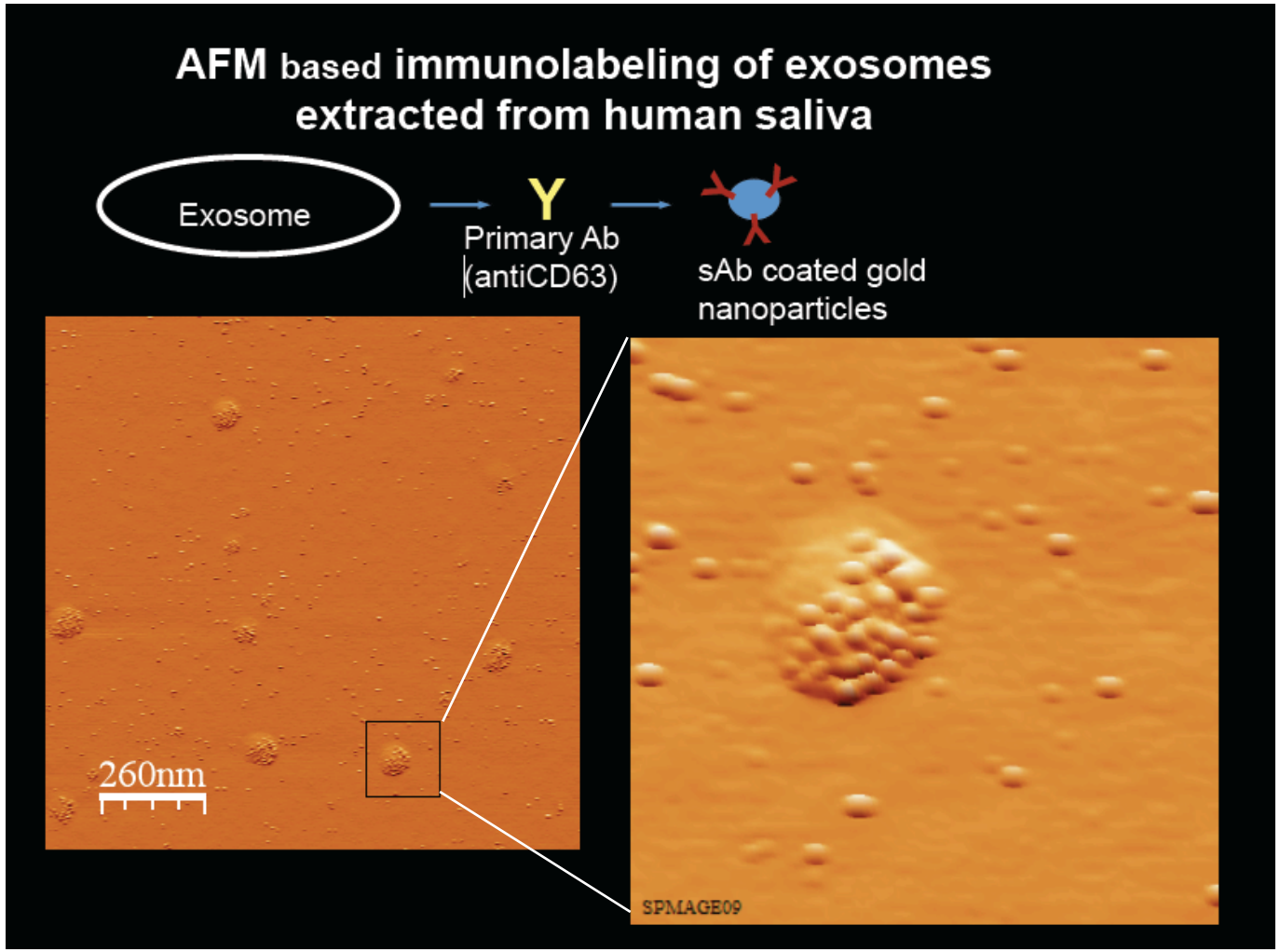
Increase in exosome size under increasing AFM imaging forces (white arrows show scan direction)





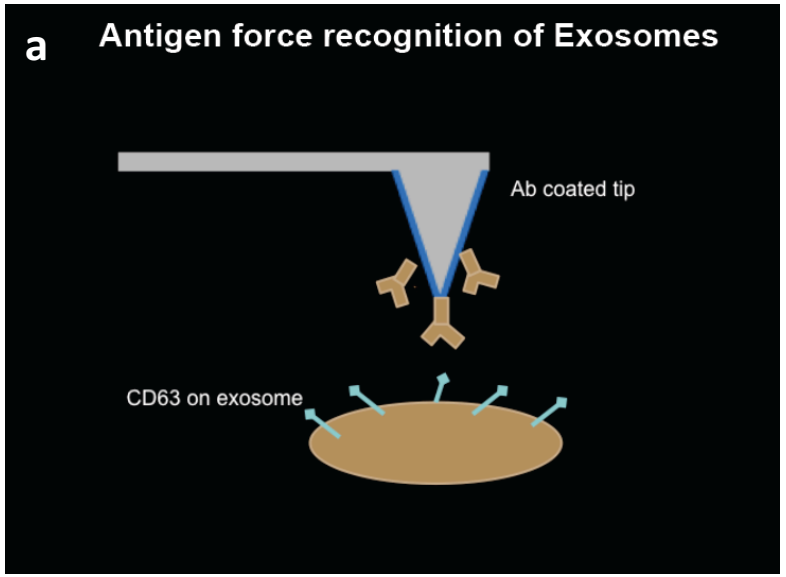
Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy

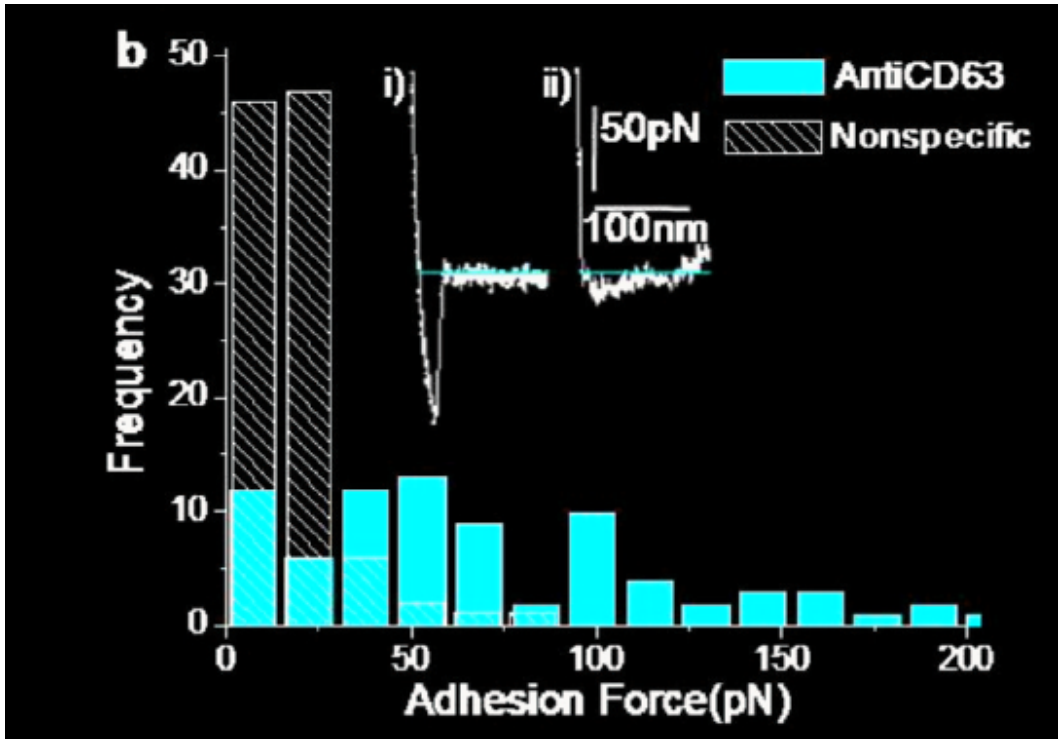




Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy

Force spectroscopy to quantitatively map Exosome surface receptor density

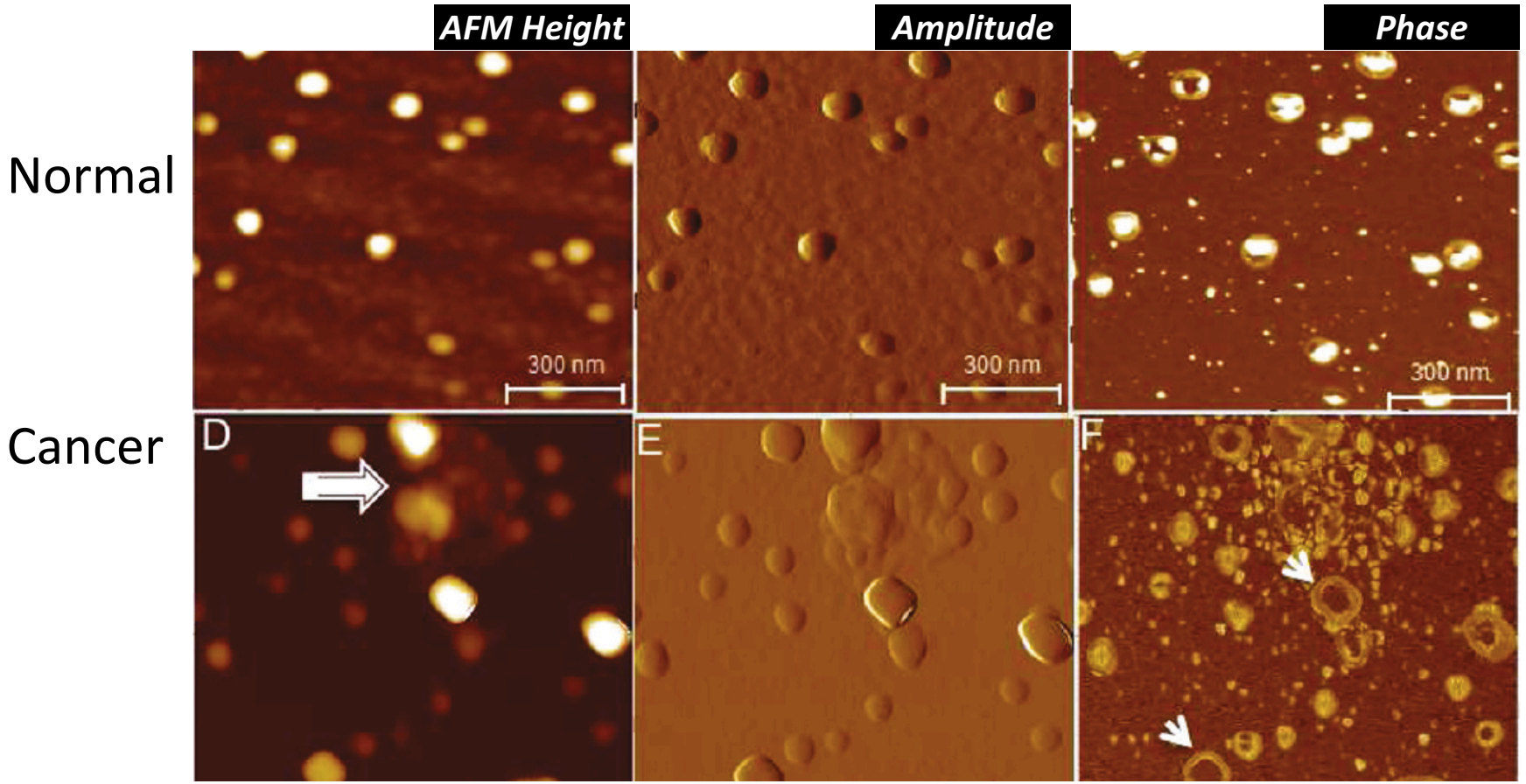


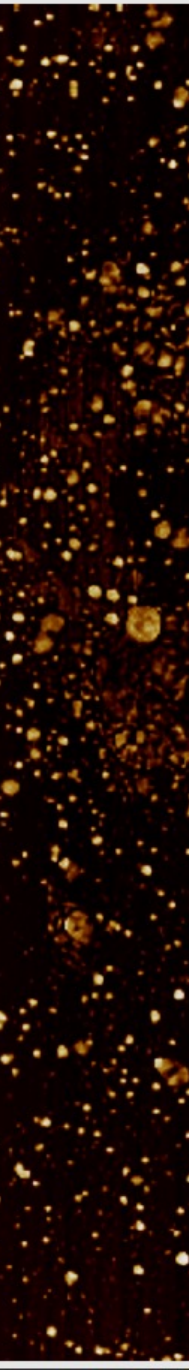


Quantitative Nanostructural and Single-Molecule Force Spectroscopy Biomolecular Analysis of Human-Saliva-Derived Exosomes

Sharma S. *et al*. Langmuir 2011

Cancer exosome populations significantly increased in saliva and display irregular morphologies, increased vesicle size, and higher inter-vesicular aggregation





AFM analysis of exosomes

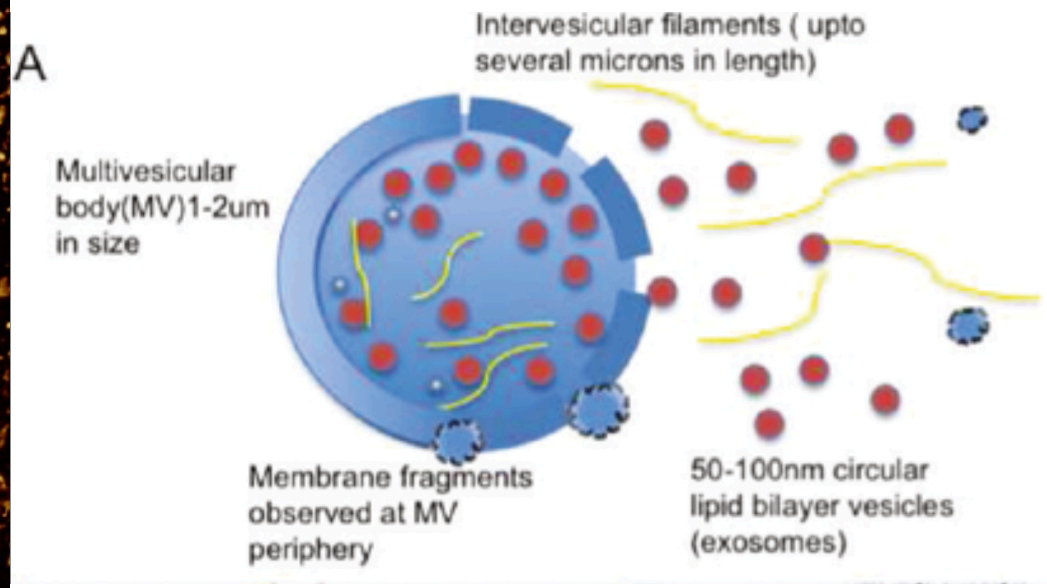
Table 1. Patient History and Histopathology versus Saliva Exosome Characteristics

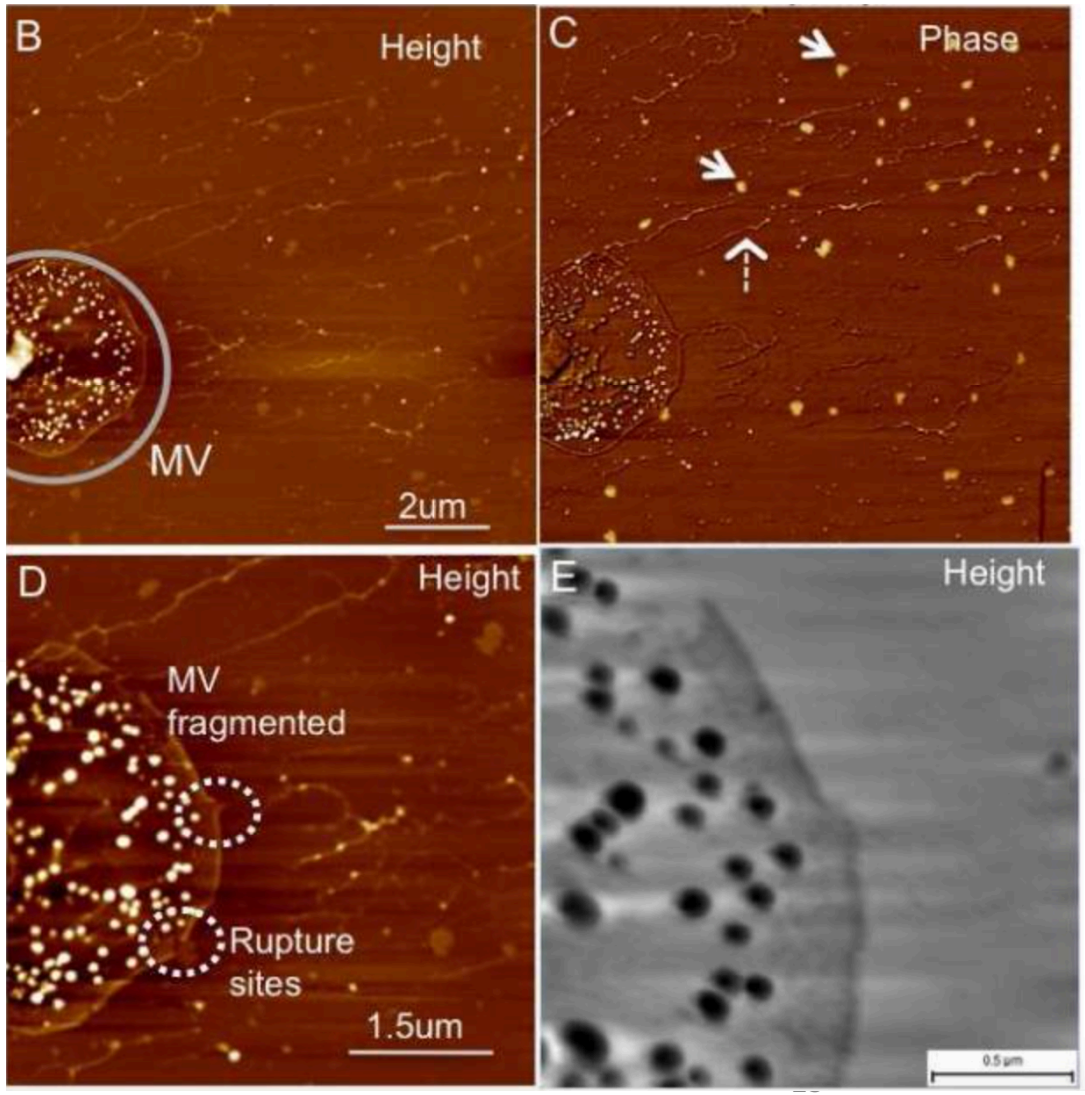
case no.	stage- histological grade[a]	size (nm)[b]	counts/64 μm^2	vesicle morphology	MVs[e]	treatment
1	4a	77.0 ± 3.7	345	IR[c]	++	chemotherapy
2	4a	138.6 ± 6.8	342	IR	–	chemotherapy
3	4a	102.7 ± 6.7	414	IR	+	chemotherapy + surgery
4	1	92.5 ± 4.6	242	IR	–	surgery
5	4a	NA[f]	Na	NA	NA	chemotherapy
6	2	80.9 ± 6.1	268	IR	–	surgery
7	normal	62.3 ± 3.4	128	R[d]	–	none
8	normal	67.4 ± 2.6	137	R	–	none
9	normal	66.4 ± 3.6	96	R	–	none
10	normal	67.4 ± 2.9	193	R	–	none
11	normal	71.6 ± 1.7	126	R	–	none

[a] Grades 1–4 and a–c indicates less advanced to more advanced stages and types of cancer. [b] Mean ± SEM. [c] Irregular. [d] Regular circular. [e] Multivesicular bodies. [f] Not used for analysis because of sample serum contamination. Normal and cancer samples studied consisted of sex (males) and age (between 54 and 75 years) matched.

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

Quantitative Nanostructural and Single-Molecule Force Spectroscopy Biomolecular Analysis of Human-Saliva-Derived Exosomes





Release of exosomes from multi-vesicular bodies (MVs) seen in oral cancer patient salivary exosomes

JOURNAL OF THE ROYAL SOCIETY Interface

rsif.royalsocietypublishing.org

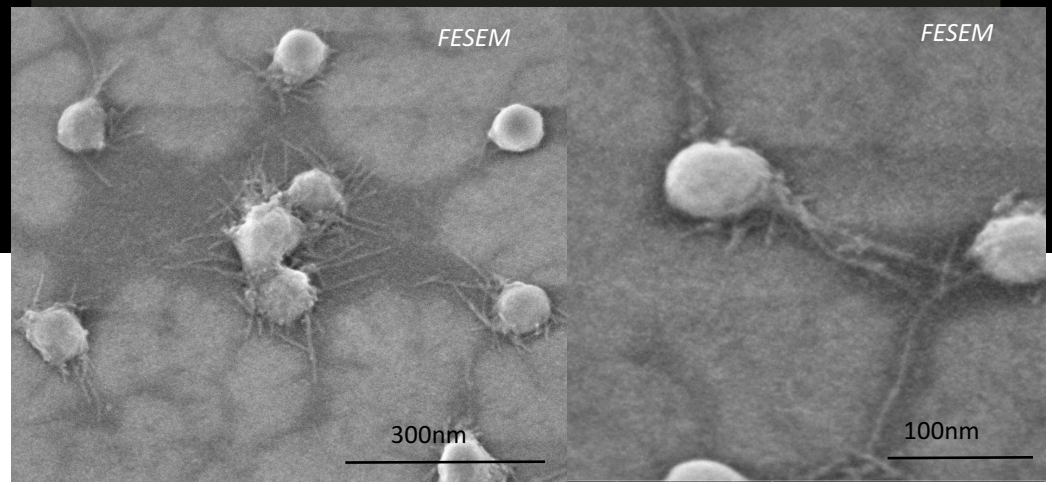
Research

Cite this article: Sharma S, Das K, Woo J-R, Gimzewski JK. 2014 Nanofilaments on glioblastoma exosomes revealed by peak force microscopy. *J. R. Soc. Interface* **11**: 20131150. http://dx.doi.org/10.1098/rsif.2013.1150

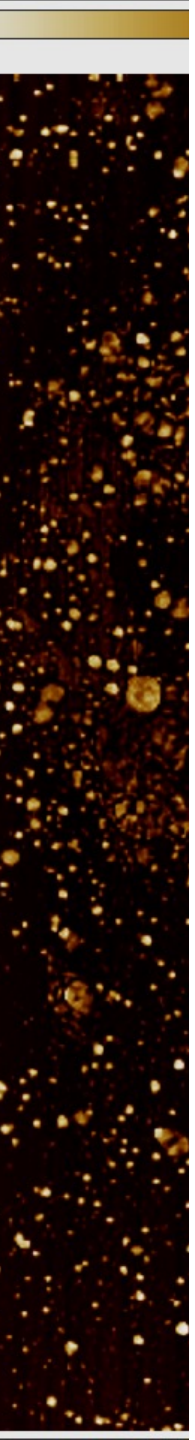
Nanofilaments on glioblastoma exosomes revealed by peak force microscopy

Shivani Sharma[1,2], Kingshuk Das[3], JungReem Woo[1] and James K. Gimzewski[1,2,4,5,6]

[1]Department of Chemistry and Biochemistry, [2]California NanoSystems Institute, [3]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, and [4]Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA 90095, USA
[5]International Center for Materials Nanoarchitectonics Satellite (MANA), National Institute for Materials Science (NIMS), Tsukuba, Japan
[6]Centre for Nanoscience and Quantum Information, University of Bristol, Bristol, UK

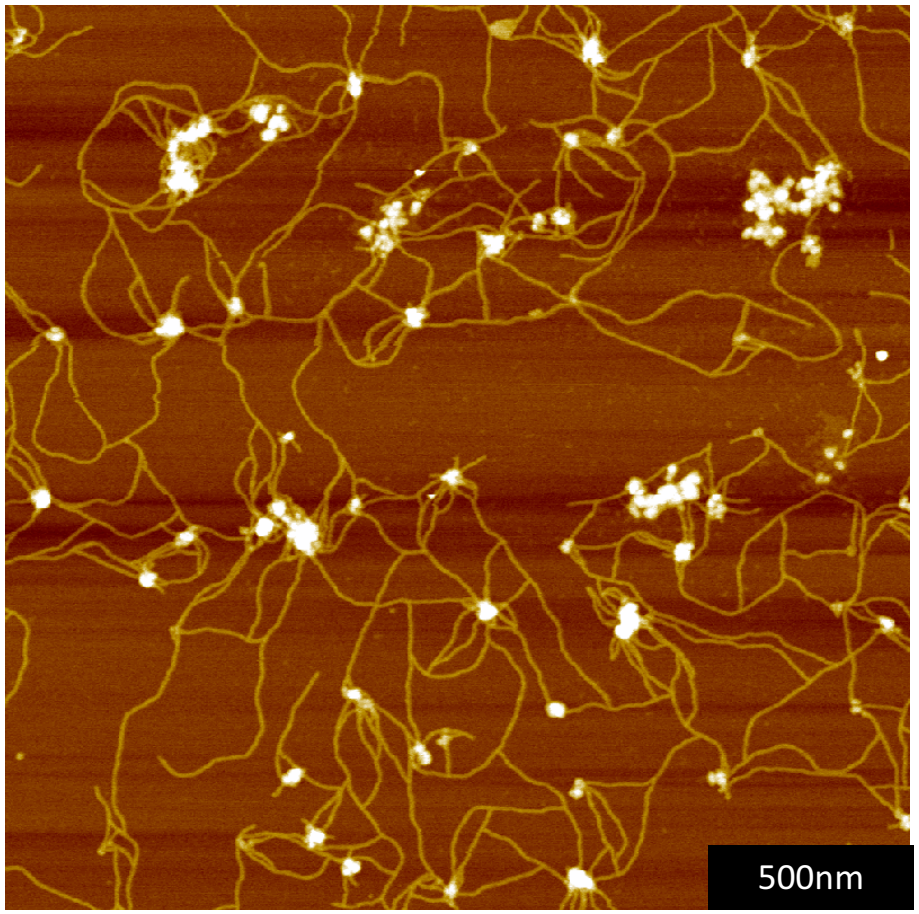


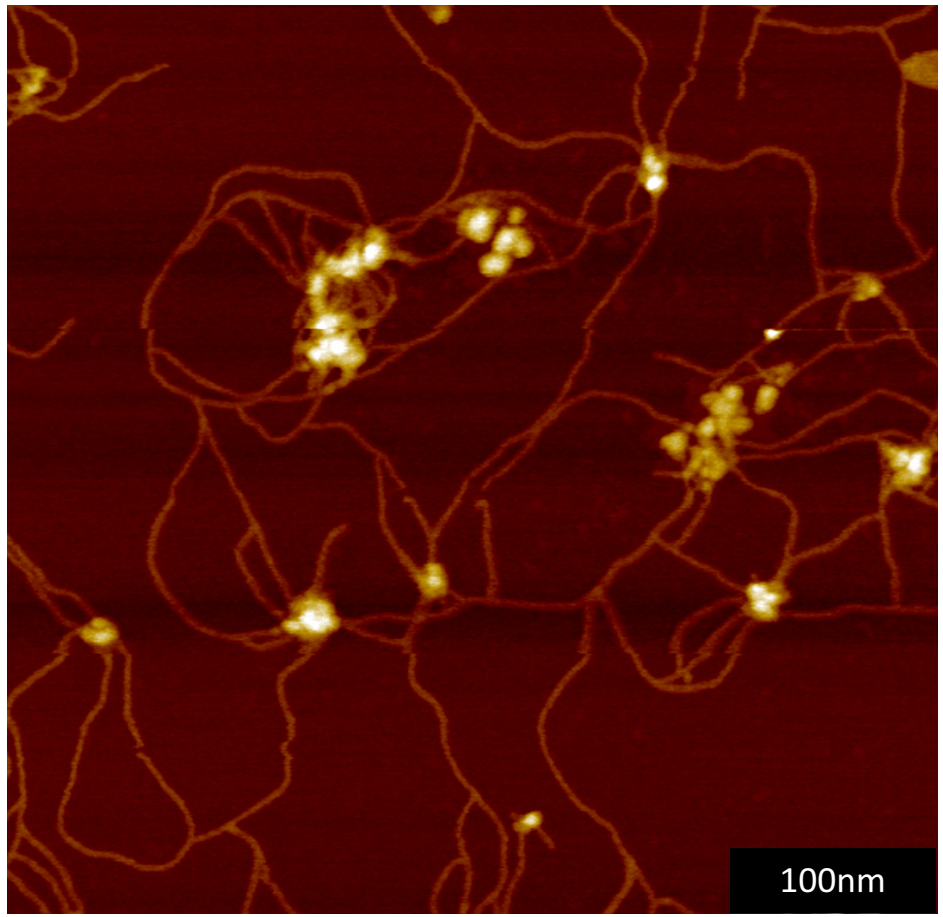
Peak force AFM of exosomes motivated by our earlier FESEM observation



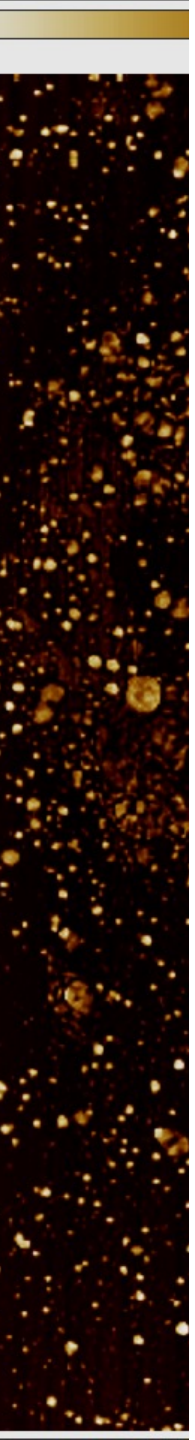
Sharma S. *et al*. J.R. Interface 2014

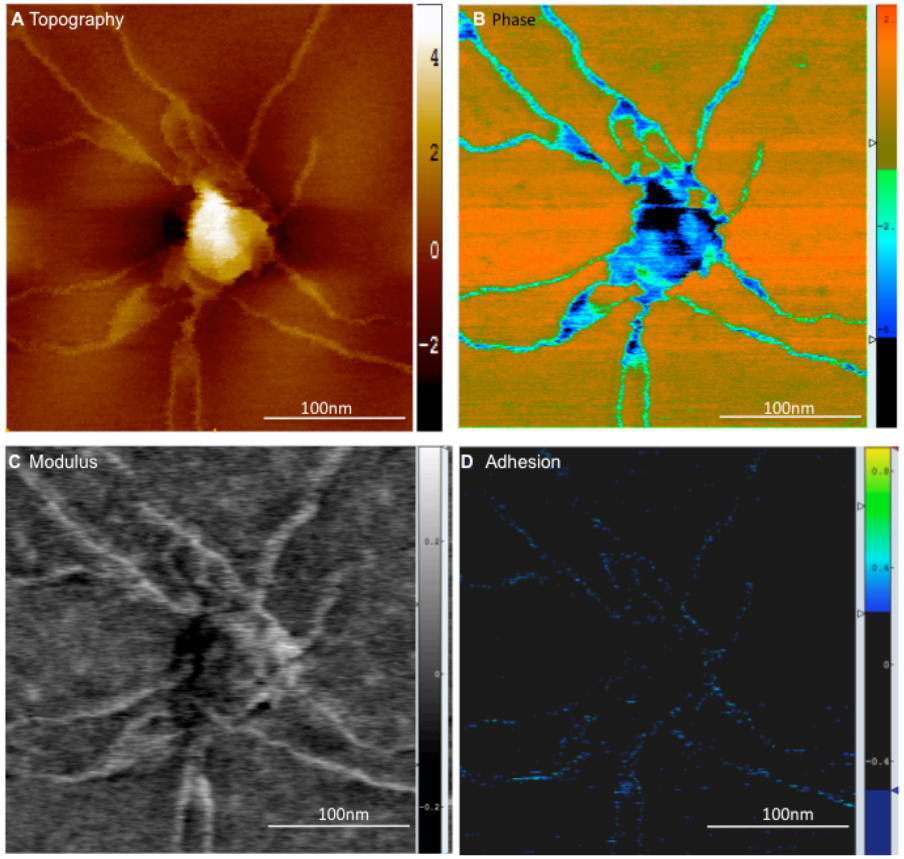
PeakForce imaging of U87 exosomes

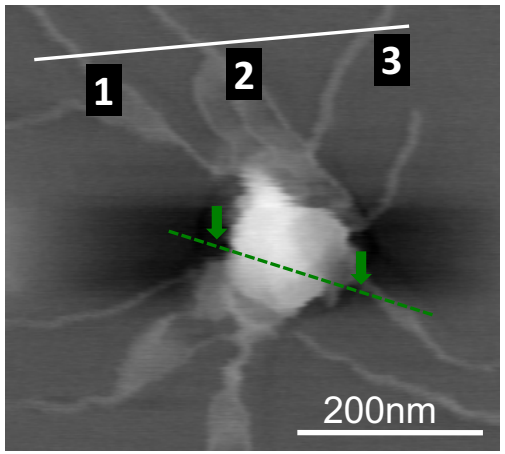




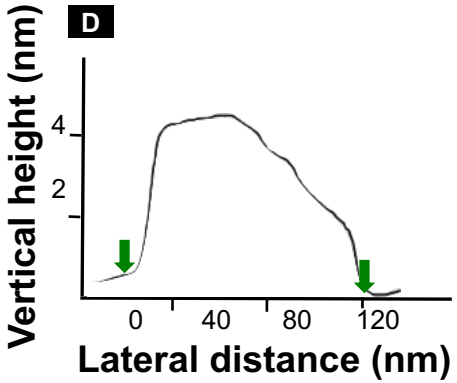
Results were confirmed by imaging samples obtained from two independent and commonly used isolations, with and without sucrose gradient purification.

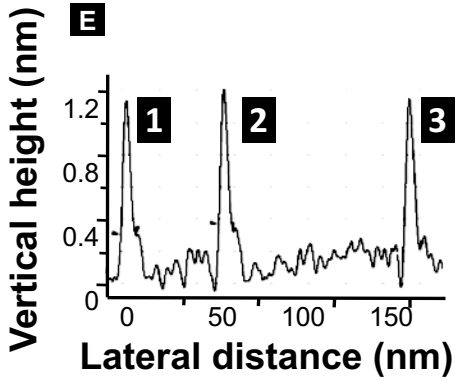






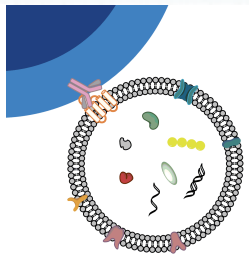
Cross section heights: EV and filaments



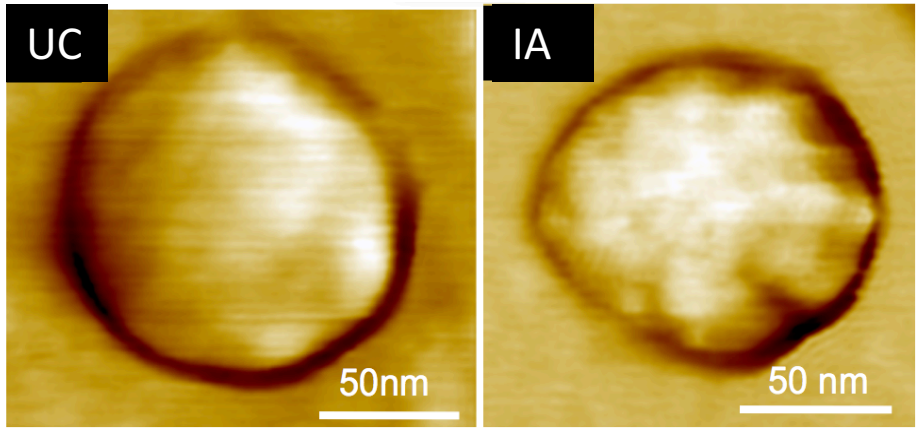


The Role of Isolation Methods on a Nanoscale Surface Structure and Its Effect on the Size of Exosomes

JOURNAL OF CIRCULATING BIOMARKERS

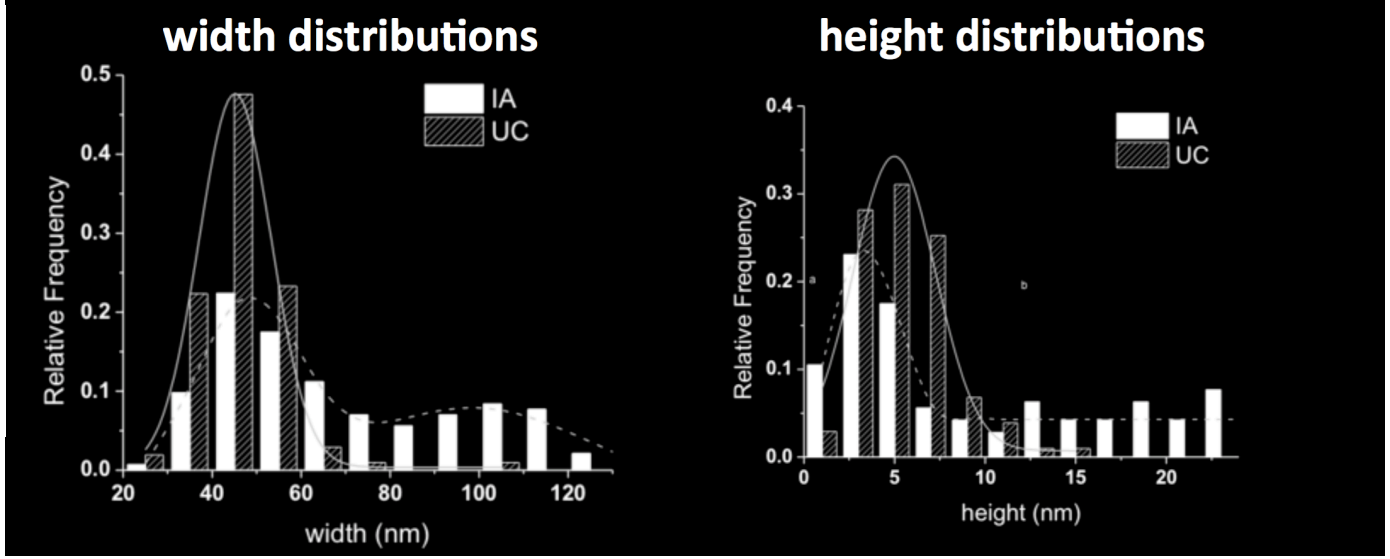


Woo J. *et al.* 2016



Immune Affinity (IA) isolation method shows greater roughness of EVs

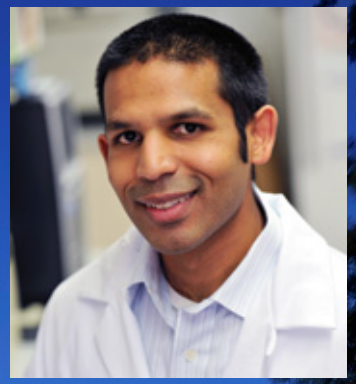
The size distributions of exosomes depending on isolation methods




James Gimzewski


Jung-Reem Woo


Shivani Sharma


Kingshuk Das

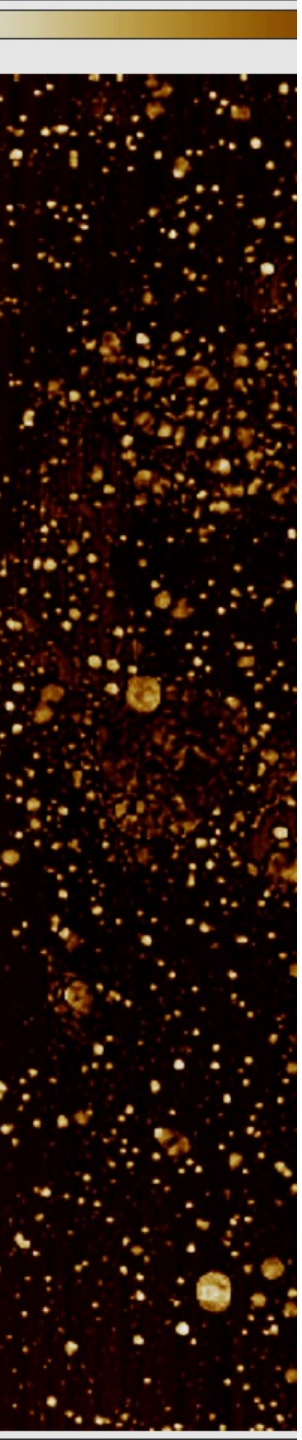

Haider Rasool


David T W Wong DMD


V Palanisamy


Michael LeClaire

Thanks also to FEI for FESEM -Michael Schmidt & Cliff Mathisen
Laurent Bentolila Advanced Microscopy Lab, CNSI, UCLA



Extracellular vesicles are amazing BIOfunctional NANOparticles

Nano at its best (or worst)

Current challenges-

JOURNAL OF EXTRACELLULAR VESICLES

The need for nanoscale characterization of Extracellular vesicles

AN ISEV POSITION PAPER

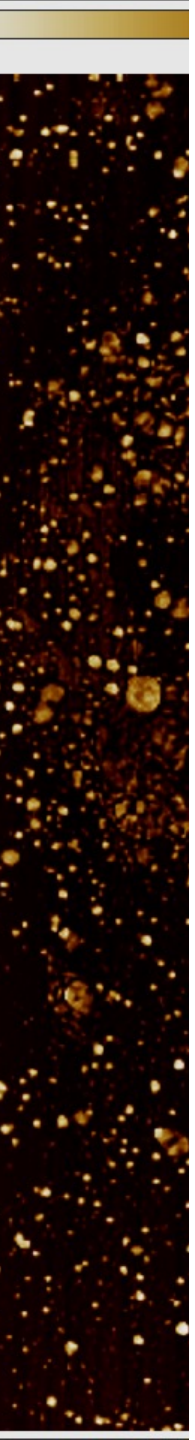
Standardization of sample collection, isolation and analysis methods in extracellular vesicle research

Kenneth W. Witwer[1]*, Edit I. Buzás[2], Lynne T. Bemis[3], Adriana Bora[4], Cecilia Lässer[5], Jan Lötvall[5], Esther N. Nolte-'t Hoen[6], Melissa G. Piper[7], Sarada Sivaraman[8], Johan Skog[9], Clotilde Théry[10,11], Marca H. Wauben[6] and Fred Hochberg[8]

xtracellular vesicles (EV) has highlighted
e and therapeutic targets. These findings
ogy, prompting expanded interest in the
derstanding of EV subtypes, biogenesis,
ues that can be harnessed to address the
orkshop of the International Society for
s part of the "ISEV Research Seminar:
, 6 round-table discussions were held to
V, purification and analysis of associated
ntervention. This article arises from the

discussion of EV isolation and analysis at that meeting. The conclusions 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.

ISEV
INTERNATIONAL SOCIETY FOR EXTRACELLULAR VESICLES



JOURNAL OF EXTRACELLULAR VESICLES, 2017
VOL. 6, 1286095
http://dx.doi.org/10.1080/20013078.2017.1286095

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ORIGINAL RESEARCH ARTICLE

OPEN ACCESS Check for updates

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.

Reporting requirements and transparency of results: EV-TRACK

nature methods
Techniques for life scientists and chemists

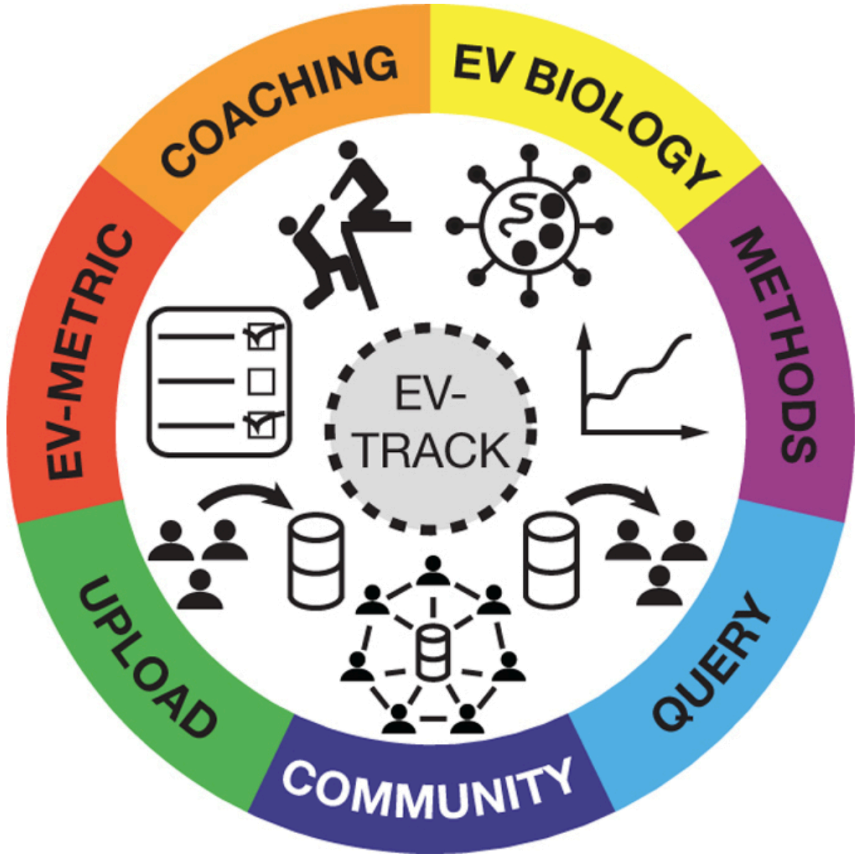
NATURE METHODS | COMMENTARY

EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

EV-TRACK Consortium, Jan Van Deun, Pieter Mestdagh, Patrizia Agostinis, Özden Akay, Sushma Anand, Jasper Anckaert, Zoraida Andreu Martinez, Tine Baetens, Els Beghein, Laurence Bertier, Geert Berx, Janneke Boere, Stephanie Boukouris, Michel Bremer, Dominik Buschmann, James B Byrd, Clara Casert, Lesley Cheng, Anna Cmoch, Delphine Daveloose, Eva De Smedt, Seyma Demirsoy, Victoria Depoorter, Bert Dhondt + *et al.*

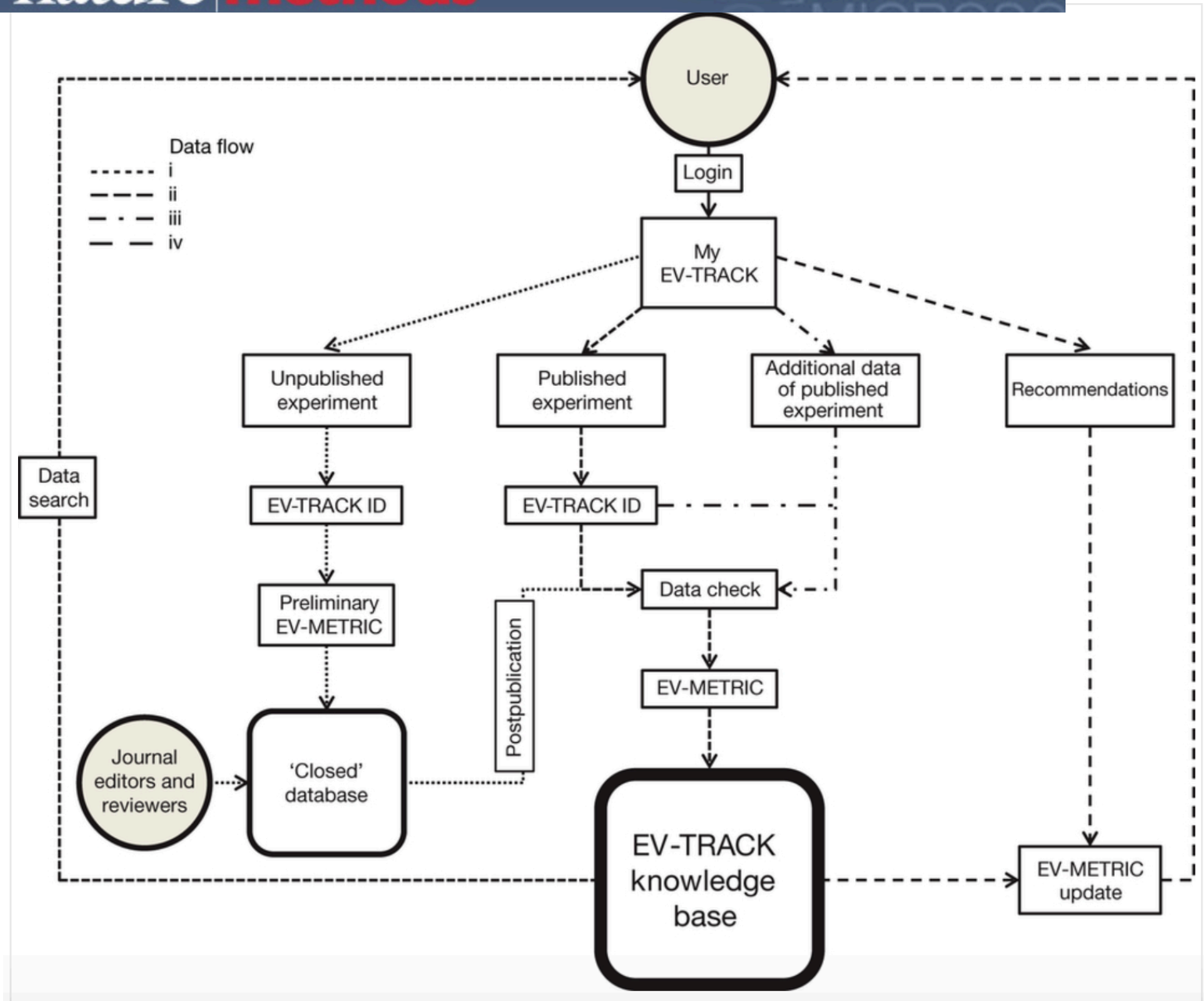
Affiliations | Contributions | Corresponding author

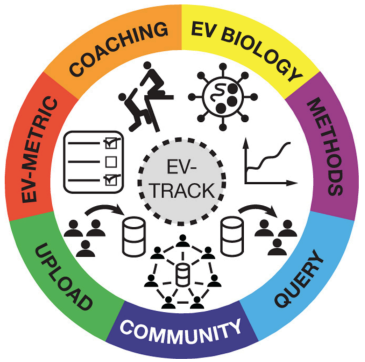
Nature Methods **14**, 228–232 (2017) | doi:10.1038/nmeth.4185
Published online 28 February 2017



nature methods

Implementation of EV-TRACK knowledgebase

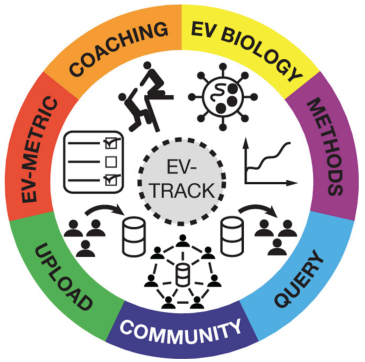




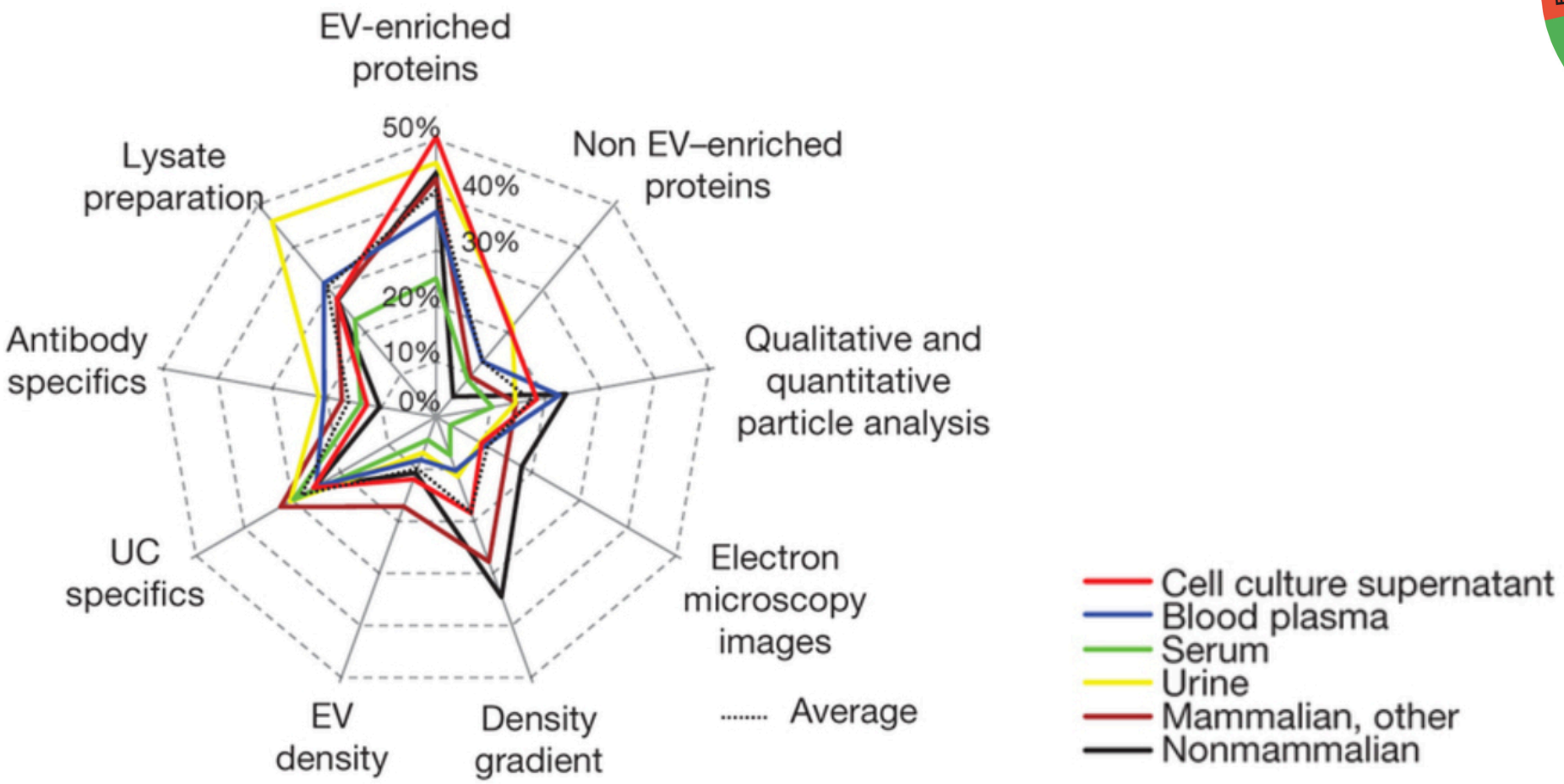
Flowchart demonstrating four different data flows available to registered EV-TRACK users

nature methods

EV metric parameters



Percentage of experiments that adhere to EV metric parameters for various bio-fluids



Biology meets Nanoanalytics

METVES

METROLOGICAL CHARACTERISATION
OF MICRO-VESICLES FROM BODY FLUIDS
AS NON-INVASIVE DIAGNOSTIC BIOMARKERS

EURAMET
European Association of National Metrology Institutes

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- News
- Project summary
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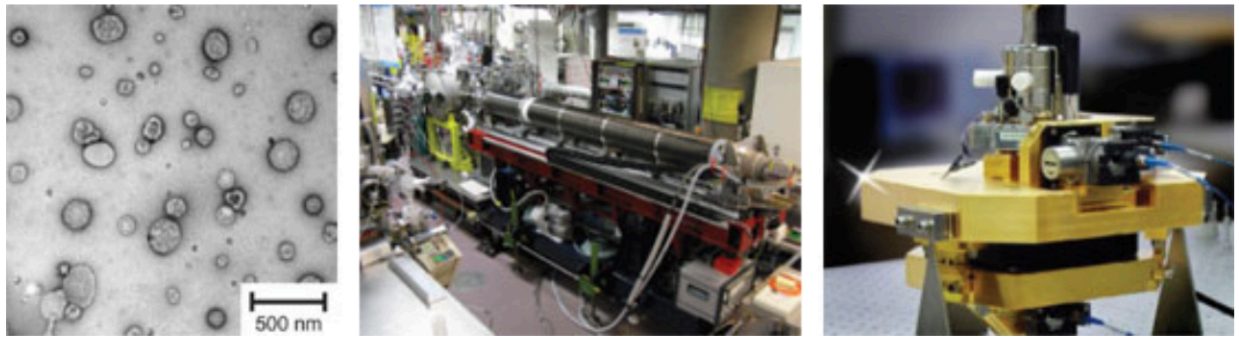
Metrological characterisation of micro-vesicles from body fluids

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.

Biological reference materials for extracellular vesicle studies

Contents lists available at ScienceDirect

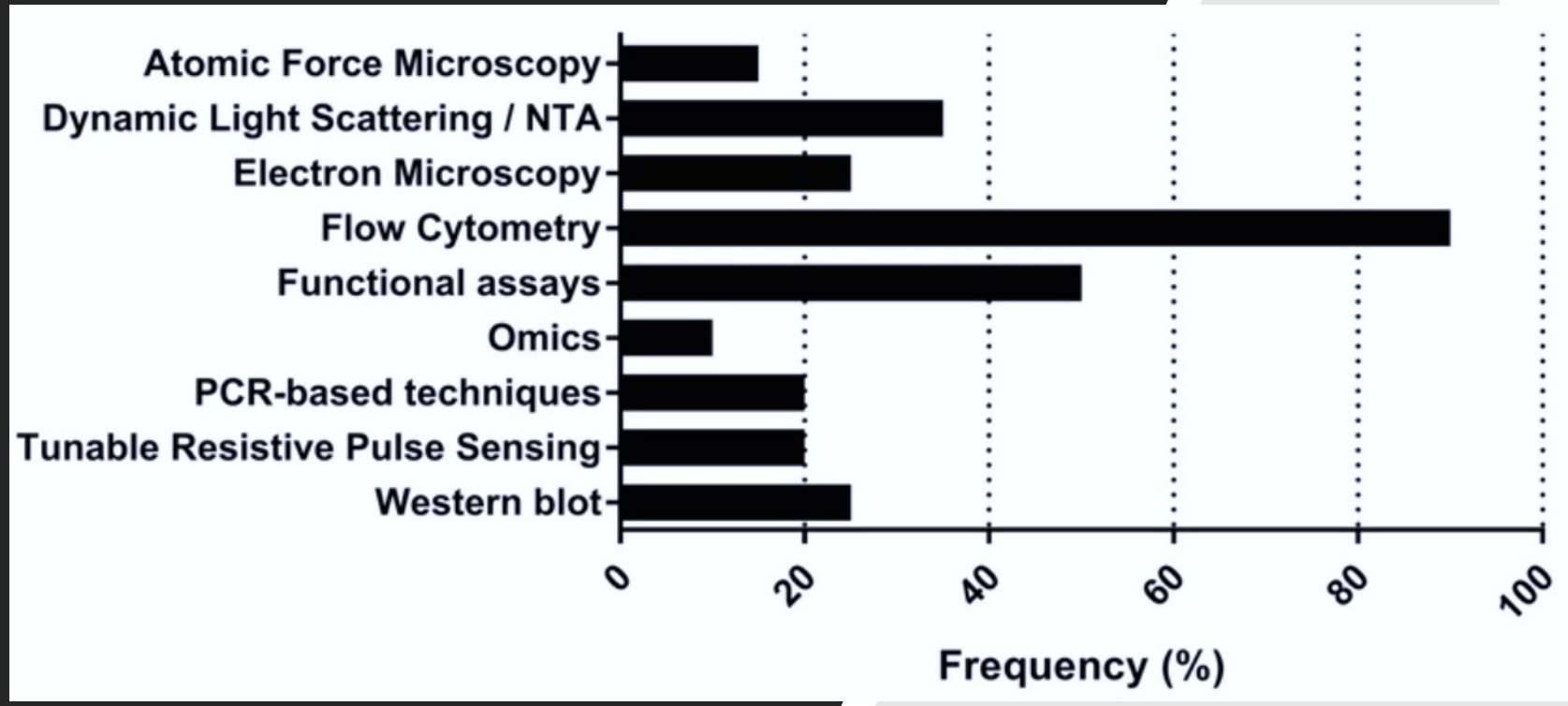
European Journal of Pharmaceutical Sciences

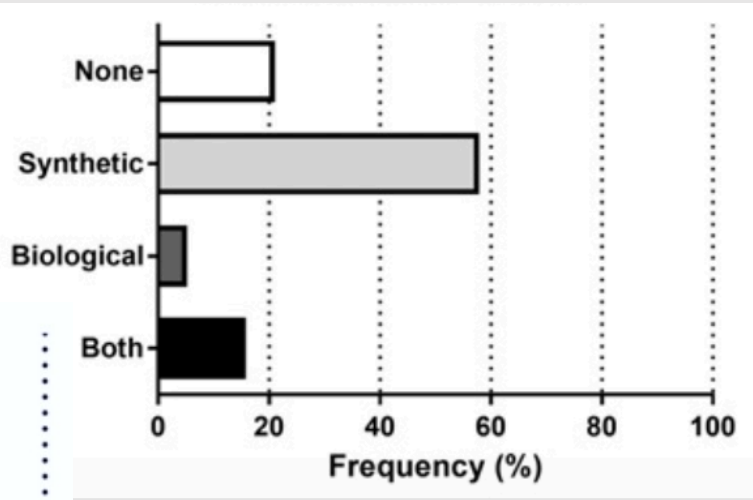
journal homepage: www.elsevier.com/locate/ejps

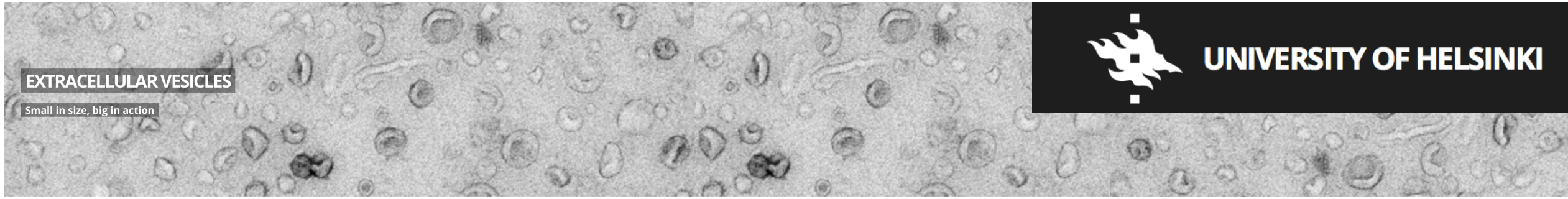
OPTIMAL PROPERTIES FOR BIOLOGICAL REFERENCE MATERIAL FOR EV STUDIES

Biological reference materials for extracellular vesicle studies

CURRENT CHARACTERIZATION TECHNIQUES AND REFERENCE STANDARDS FOR EVs







UNIVERSITY OF HELSINKI

Enhanced production: 2D vs 3D cell cultures

Standardization: Development of reference materials

Isolation

EV sub-populations

Affinity purification

Novel methods

Small sample optimization (biobanks)

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

Improved EV characterization

sample optimization for omics

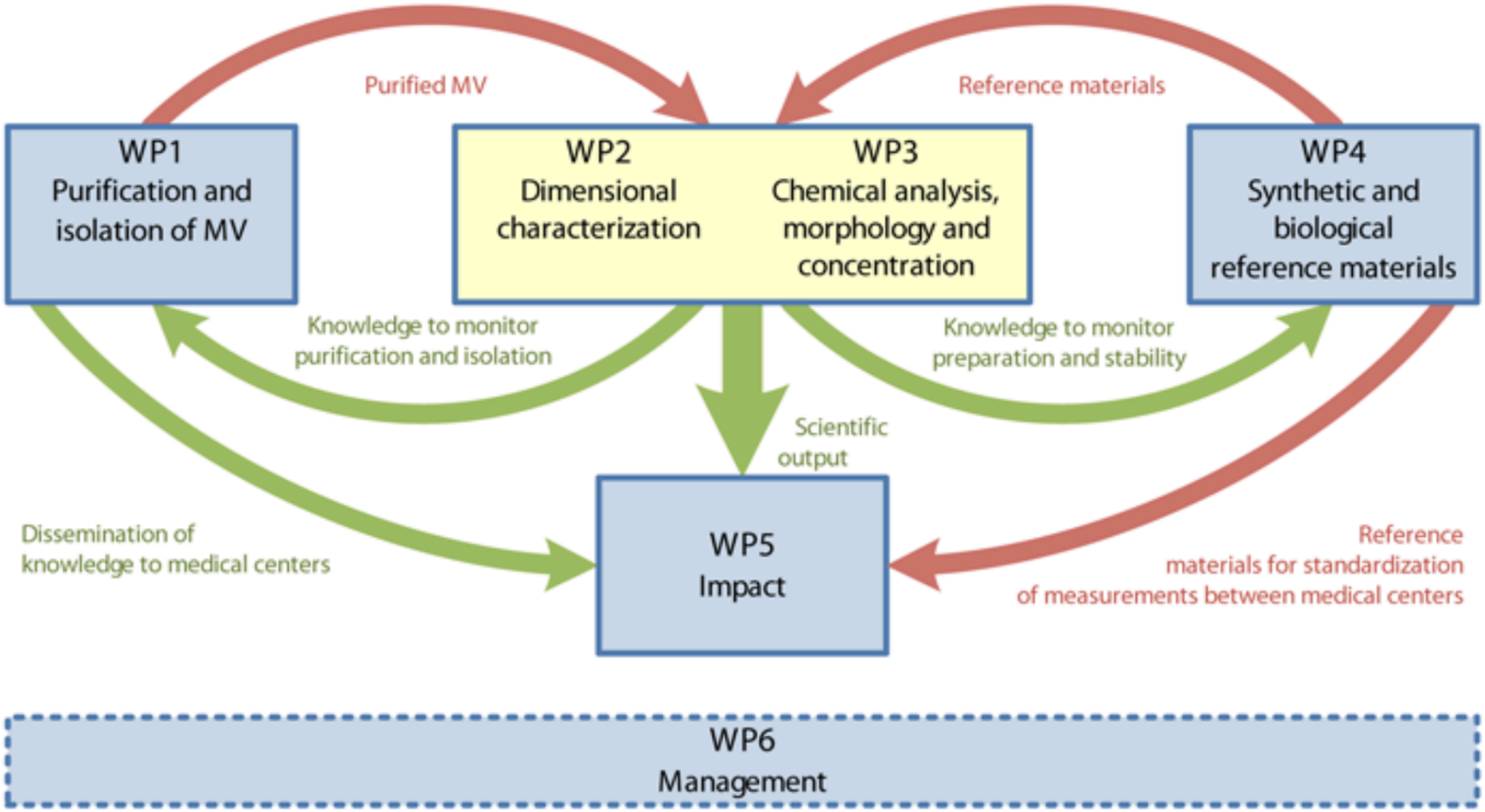
flow cytometry of multiple EV-markers

Omics comparisons

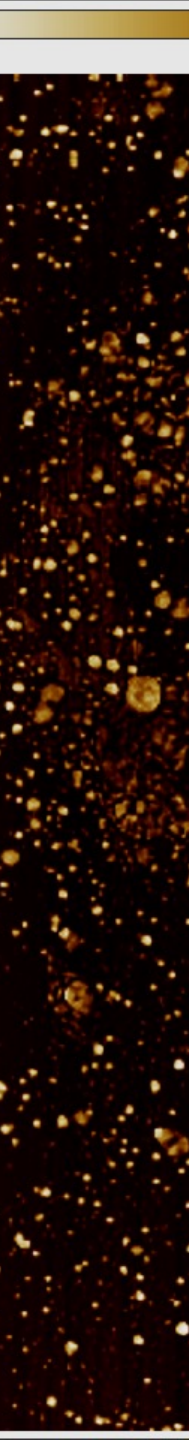
Purity of the EV preps: Identification and removal of contaminants

High throughput: analysis of clinicial samples

Metves.eu



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!

Shivani Sharma, PhD
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California NanoSystems Institute, UCLA
sharmas@cnsi.ucla.edu