

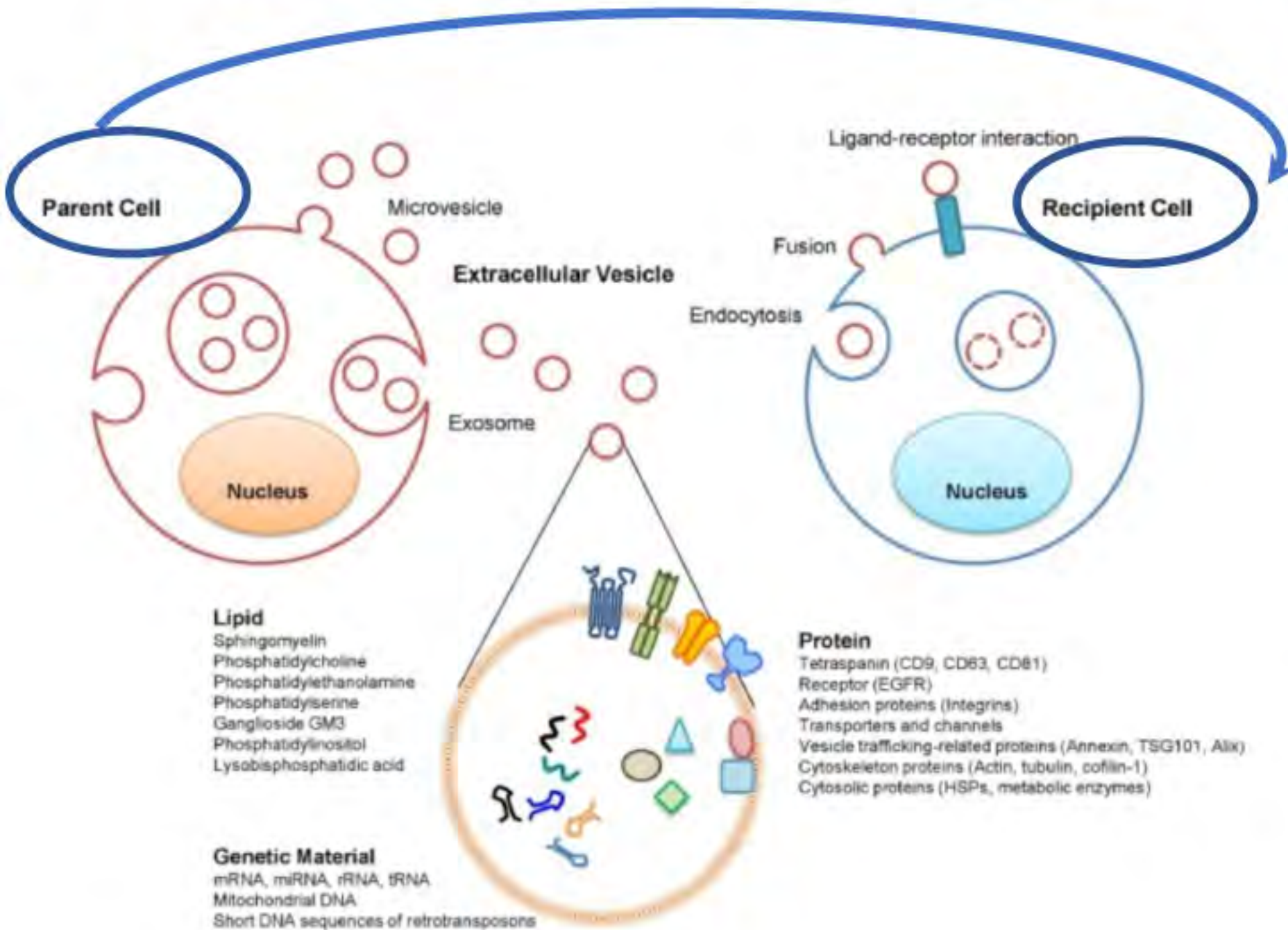


Istituto di Chimica del Riconoscimento Molecolare
Consiglio Nazionale delle Ricerche

Microarray based platforms for extracellular vesicles imaging and molecular characterization

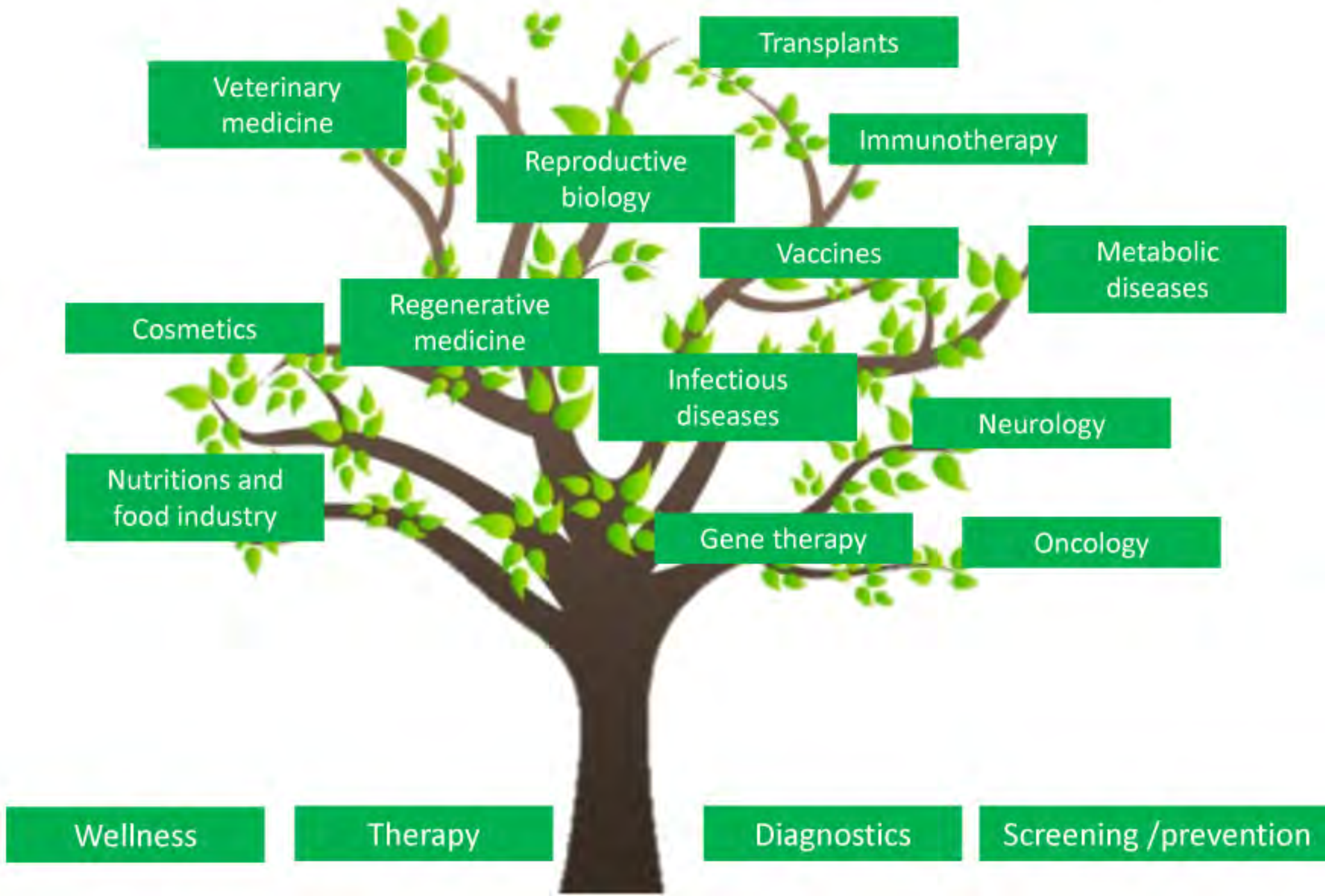


Extracellular Vesicles (EVs): intercellular communication

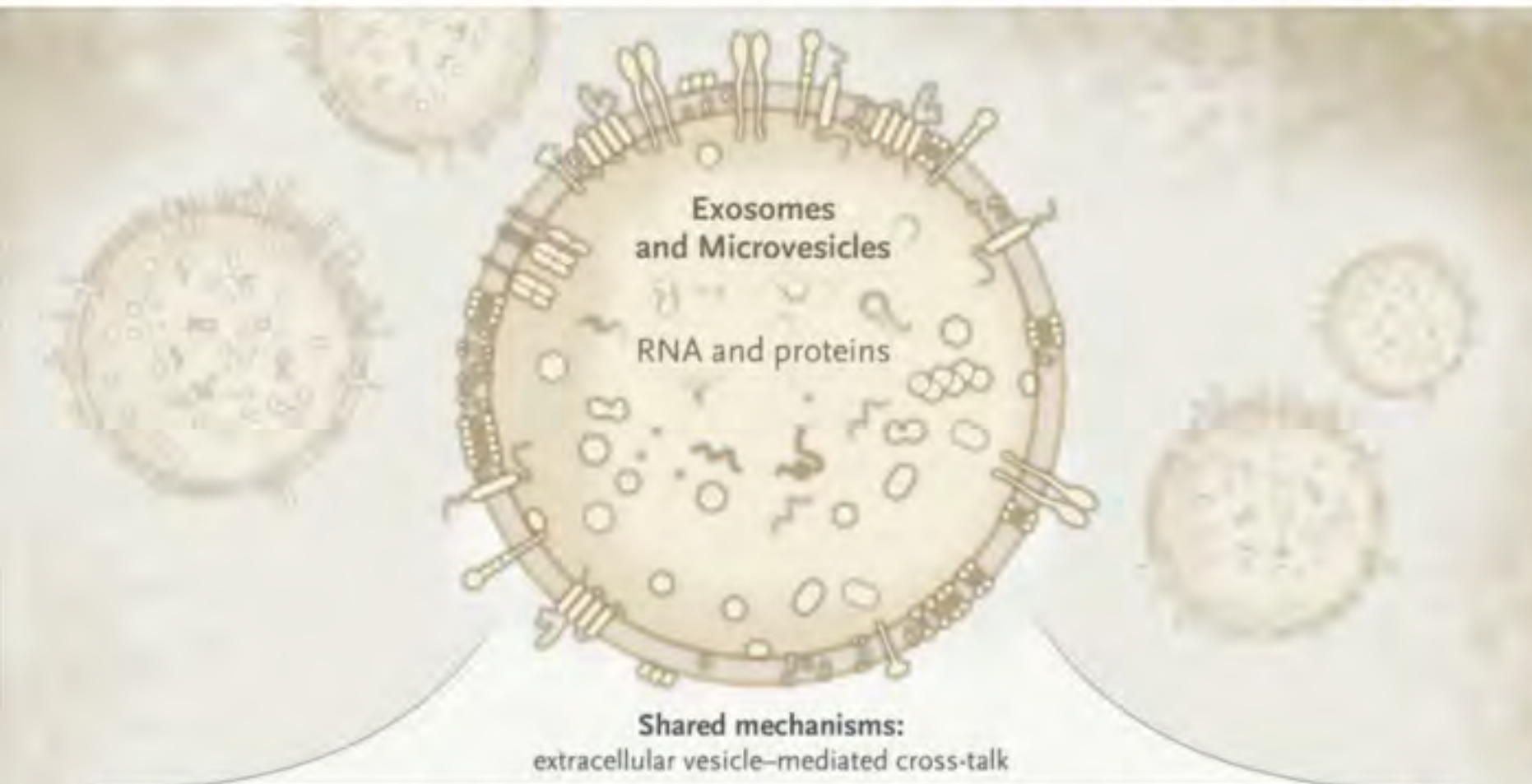


- Contain a large amount of bioactive molecules (packed and protected in extracellular environment)
- Endowed with extraordinary target specificity (via receptor mediated association)
- Precise temporal control of cell to cell signalling
- Secreted in any biological fluid
- Able to cross any biological barrier
- The list of normal and pathological processes they are involved in is continuously growing

EV research: one root different directions

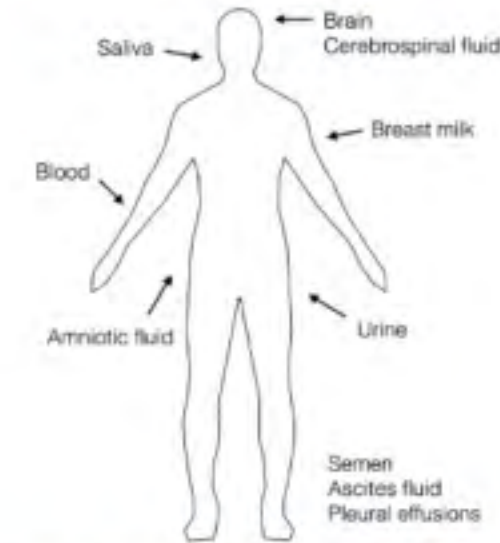


EVs as circulating biomarkers



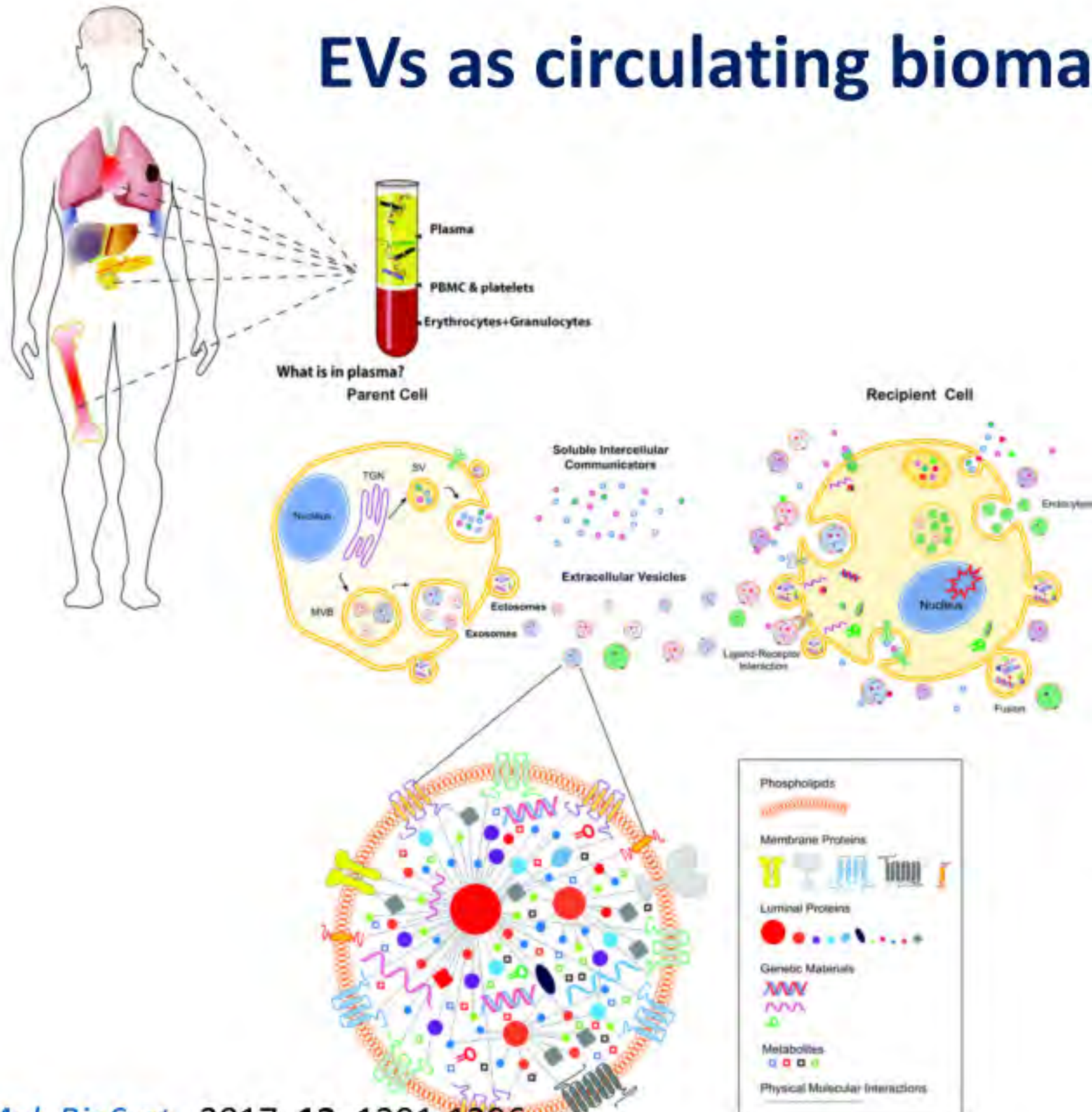
Aspects of disease affected by the function and contents of extracellular vesicles			
Cancer	Cardiometabolic disease	Neurologic disease	Infectious disease
<ul style="list-style-type: none"> • Chemotherapy resistance • Oncogenesis • Tumor immunity • Metastatic disease 	<ul style="list-style-type: none"> • Cardiomyocyte size • Cardiovascular risk factors and prognosis • Potentiation or attenuation of cardiac hypertrophy • Allograft rejection • Metabolic syndrome 	<ul style="list-style-type: none"> • Neurodegenerative diseases • Trauma • Stroke 	<ul style="list-style-type: none"> • Immune surveillance • Response to therapy • Early detection • Tracking of disease activity

- Circulate in peripheral blood and in different biological fluids



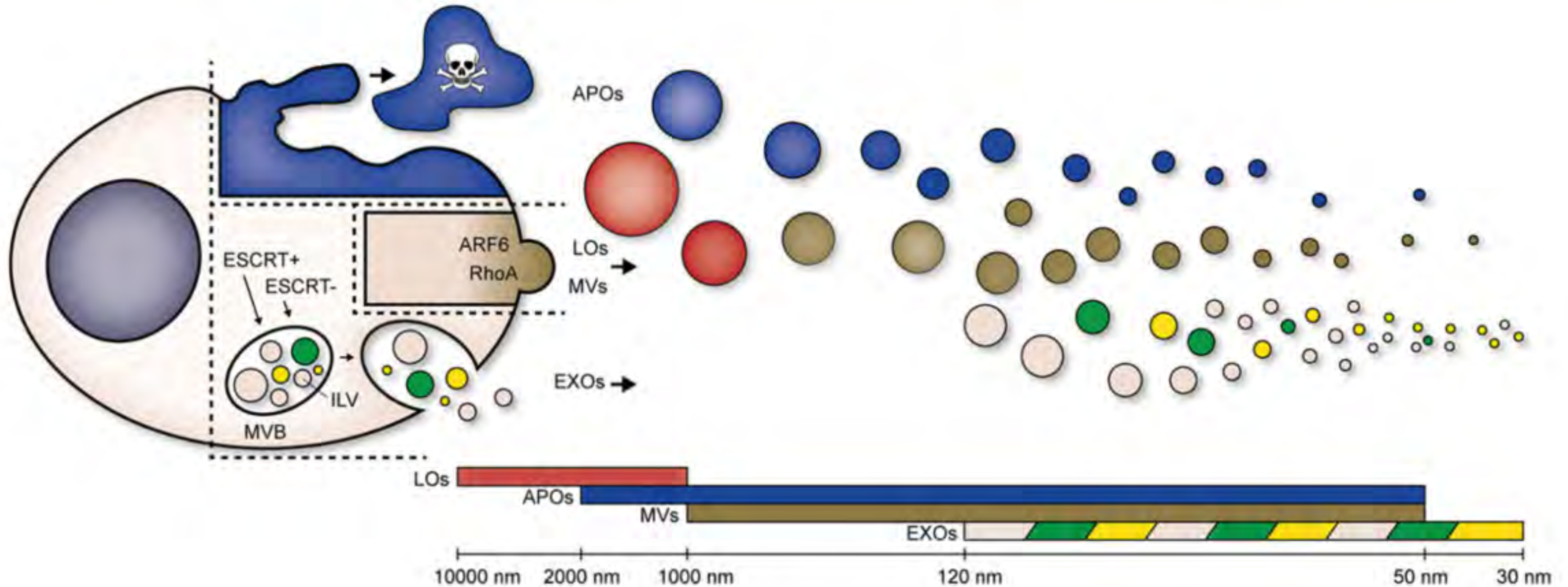
- As concentrators of biomarkers:
 - ✓ reflect the protein and genomic content of parent cells
 - ✓ enable secretion of otherwise non-soluble proteins
 - ✓ enrich low abundant molecular species
 - ✓ can be selectively isolated

EVs as circulating biomarkers: the challenges



- Complexity of information to decode
- Heterogeneity in origin, size, cargo and displayed molecules
- Sensitivity needed for EVs subpopulations
- Small size
- Low refractive index
- Disparate EV handling and analysis protocols

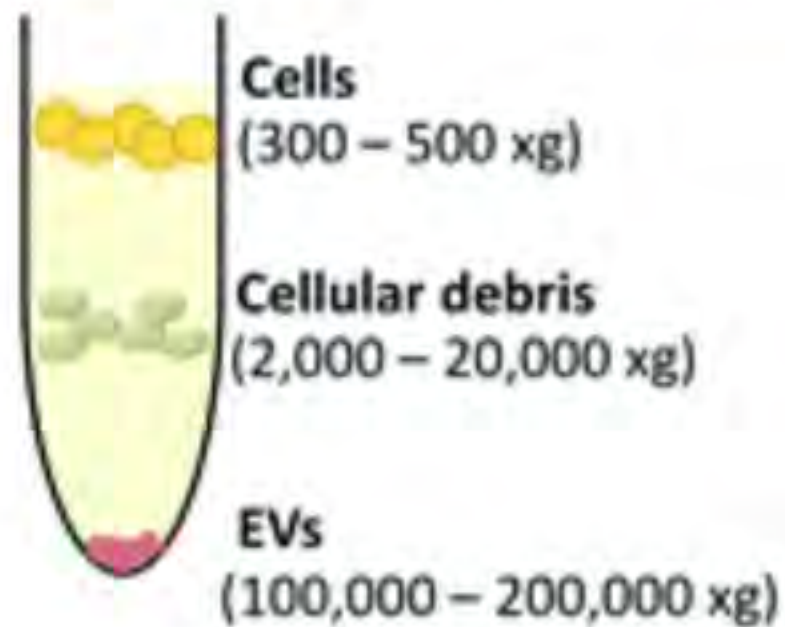
Extracellular Vesicles (EV): an heterogeneous population



EV separation and enrichment methods

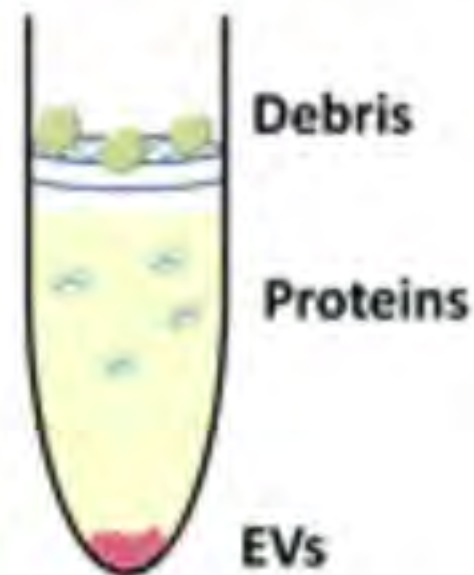
Ultracentrifugation, sucrose gradient techniques, and precipitation reagents

Density



Filtration through membranes filters
SEC: size exclusion chromatography

Size

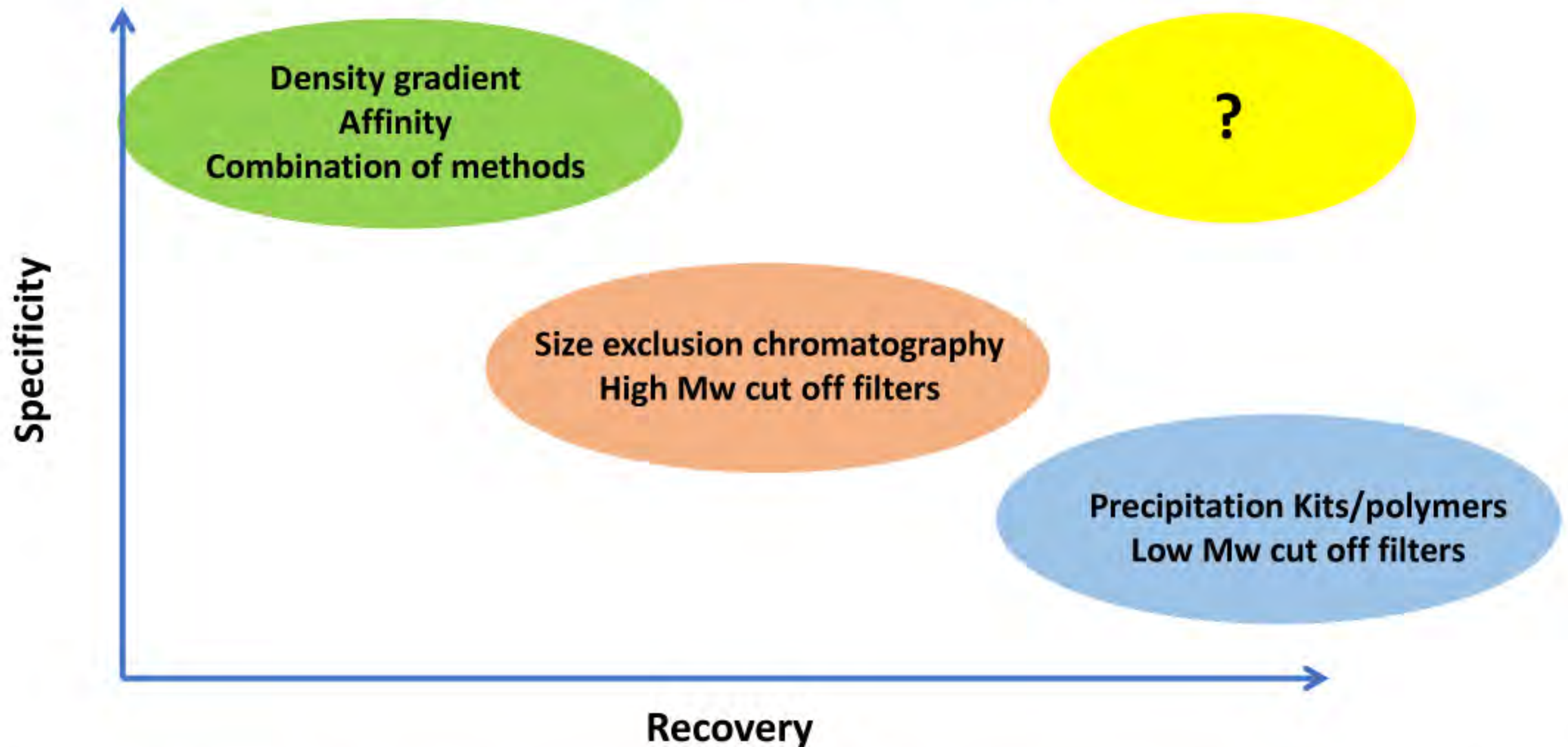


Affinity-based separation

Surface markers

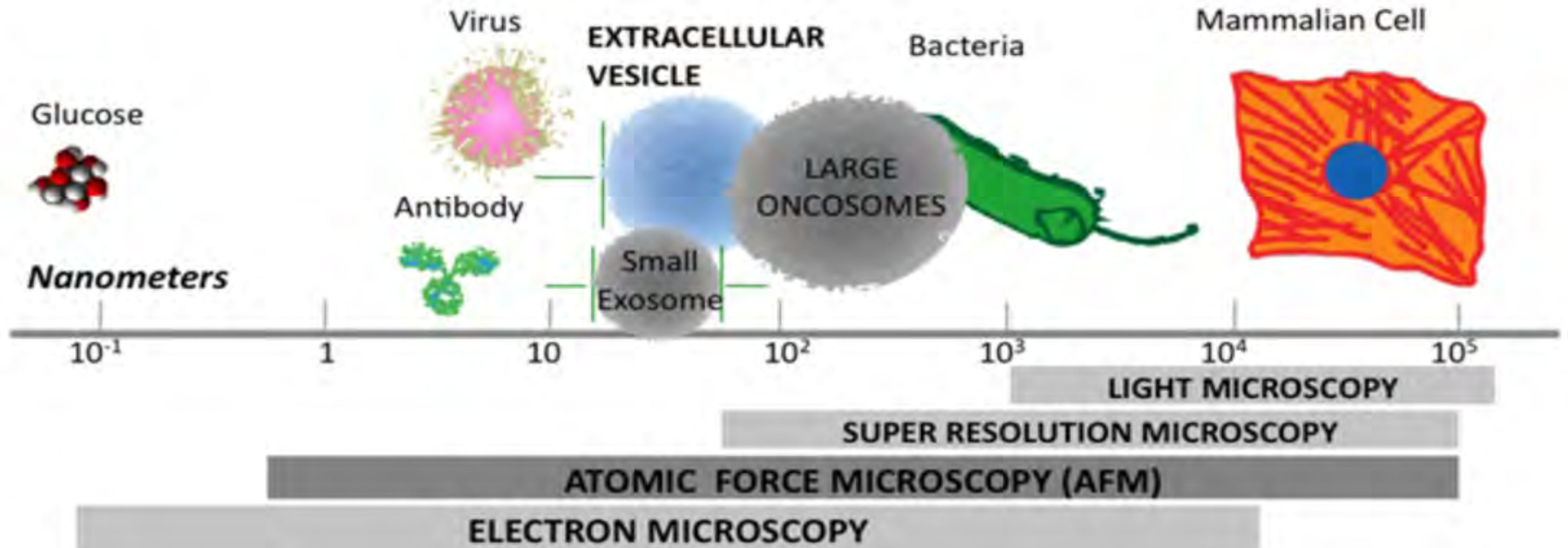


EV separation and enrichment methods



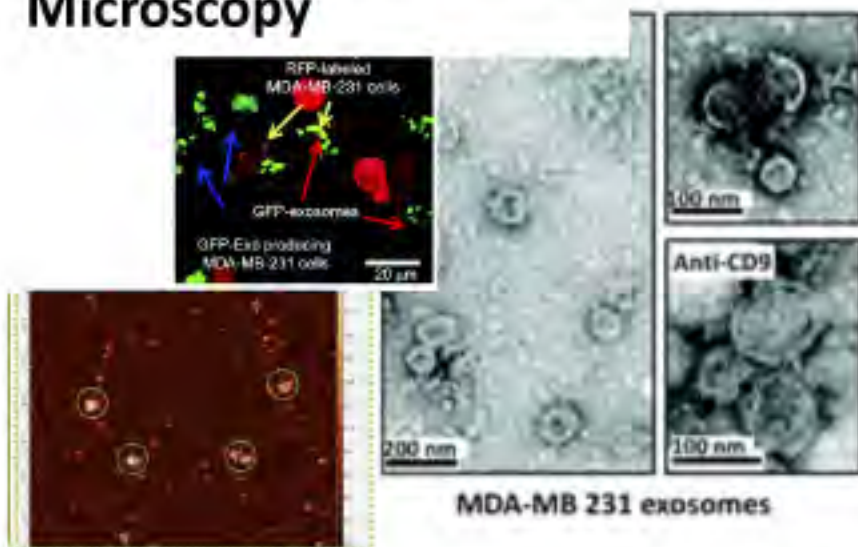
Absolute purification, or complete isolation of EVs from other entities is an unrealistic goal... MISEV 2018

Sensing/detecting EVs: a challenging task

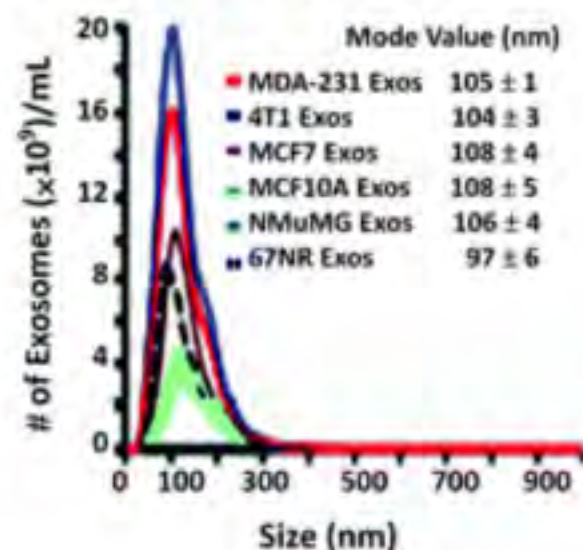


Physical and molecular characterization of EVs

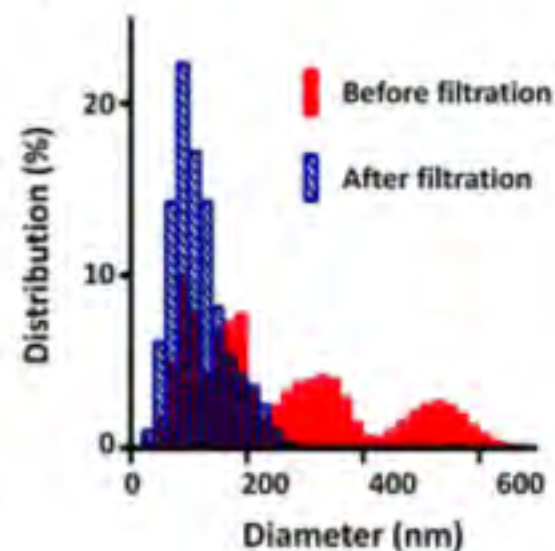
Electron/Atomic Force Microscopy



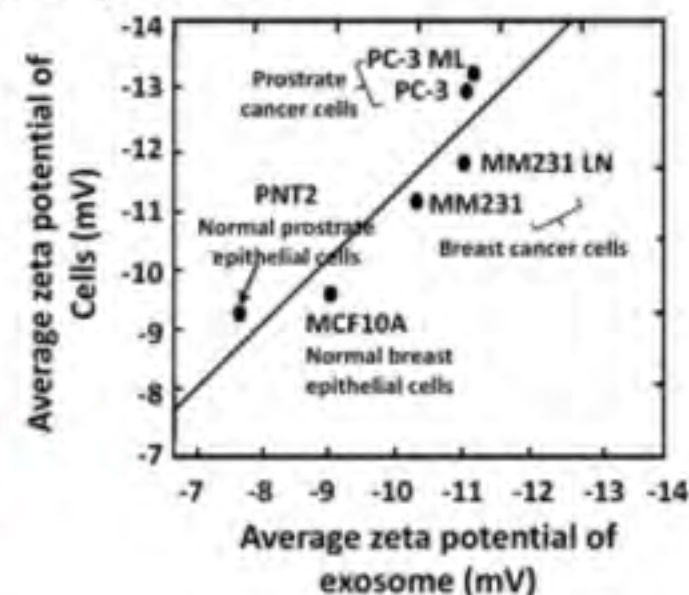
NTA-Nanosight



DLS

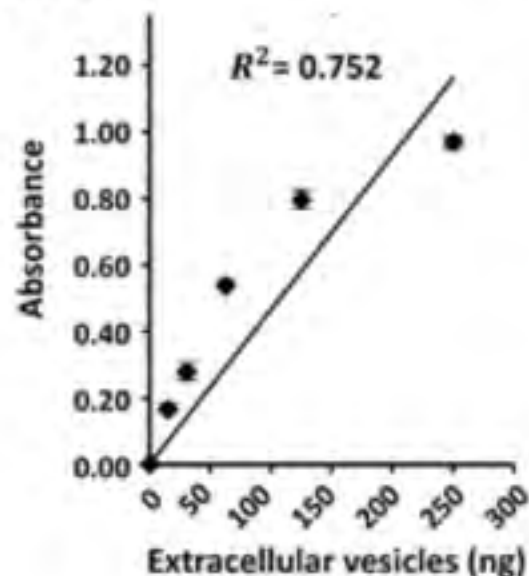


Zeta potential

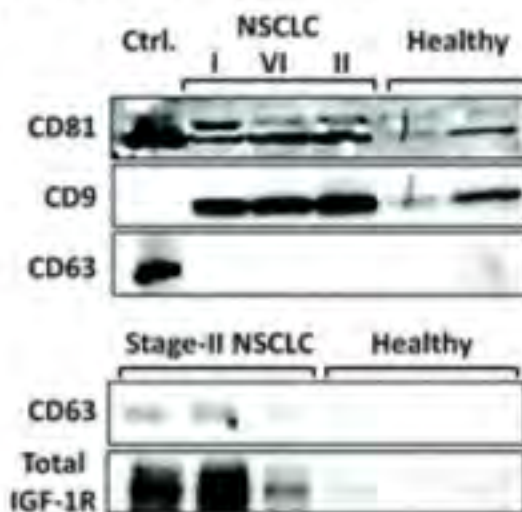


Surface markers and protein analysis of EVs

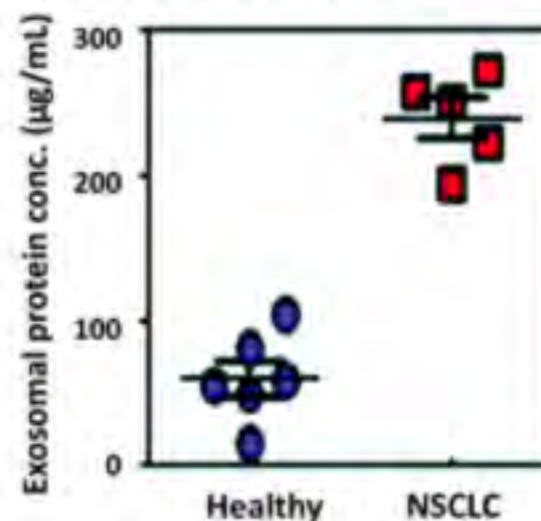
ELISA



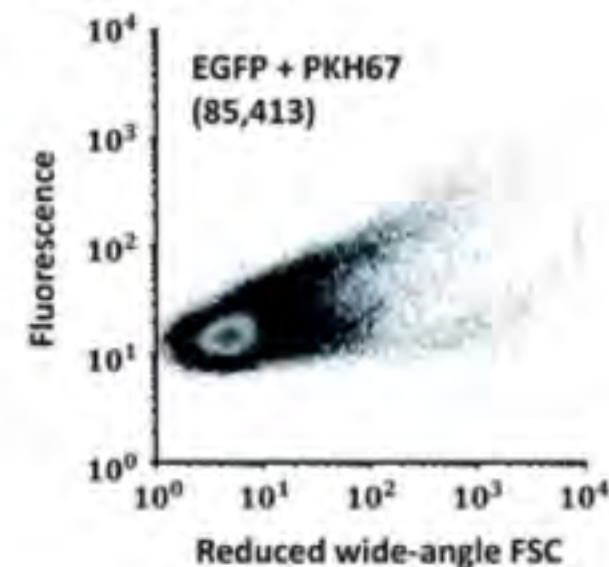
Western blotting



Tot. protein content

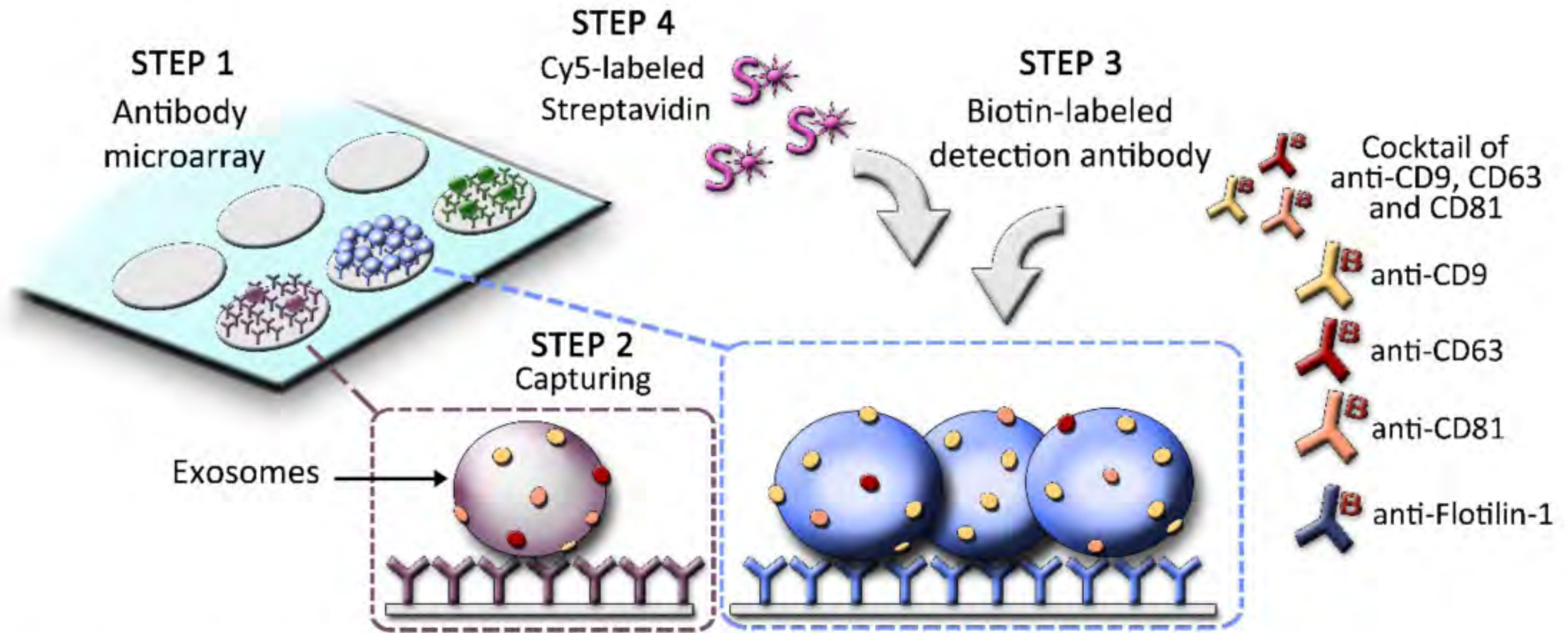


Flow Cytometry



Modified from *Analyst*, 2016, **141**, 371-381

EV microarrays





Integrated nanoparticle isolation and detection system for
complete on-chip analysis of exosomes

H2020-FETOPEN-2016-2017 – Grant agreement n° 766466

<http://www.indexproject.eu>



The Trustees of Boston University (BU)

Hansabiomed Life Sciences OU

Region Nordjylland (AALBORG University)



Institut Curie

Fluigent SA

Consiglio Nazionale delle Ricerche (CNR)

- 1. Develop a novel EV sensor to detect and classify individual biological nanoparticles as small as 30nm**
- 2. Develop a novel microfluidic device for efficient magnetic isolation of nanoparticles**
- 3. Integrate isolation and EV sensor modules within a prototype instrument to demonstrate complete on-chip EV analysis**
- 4. Demonstrate the application of the system with clinical samples for lung cancer**

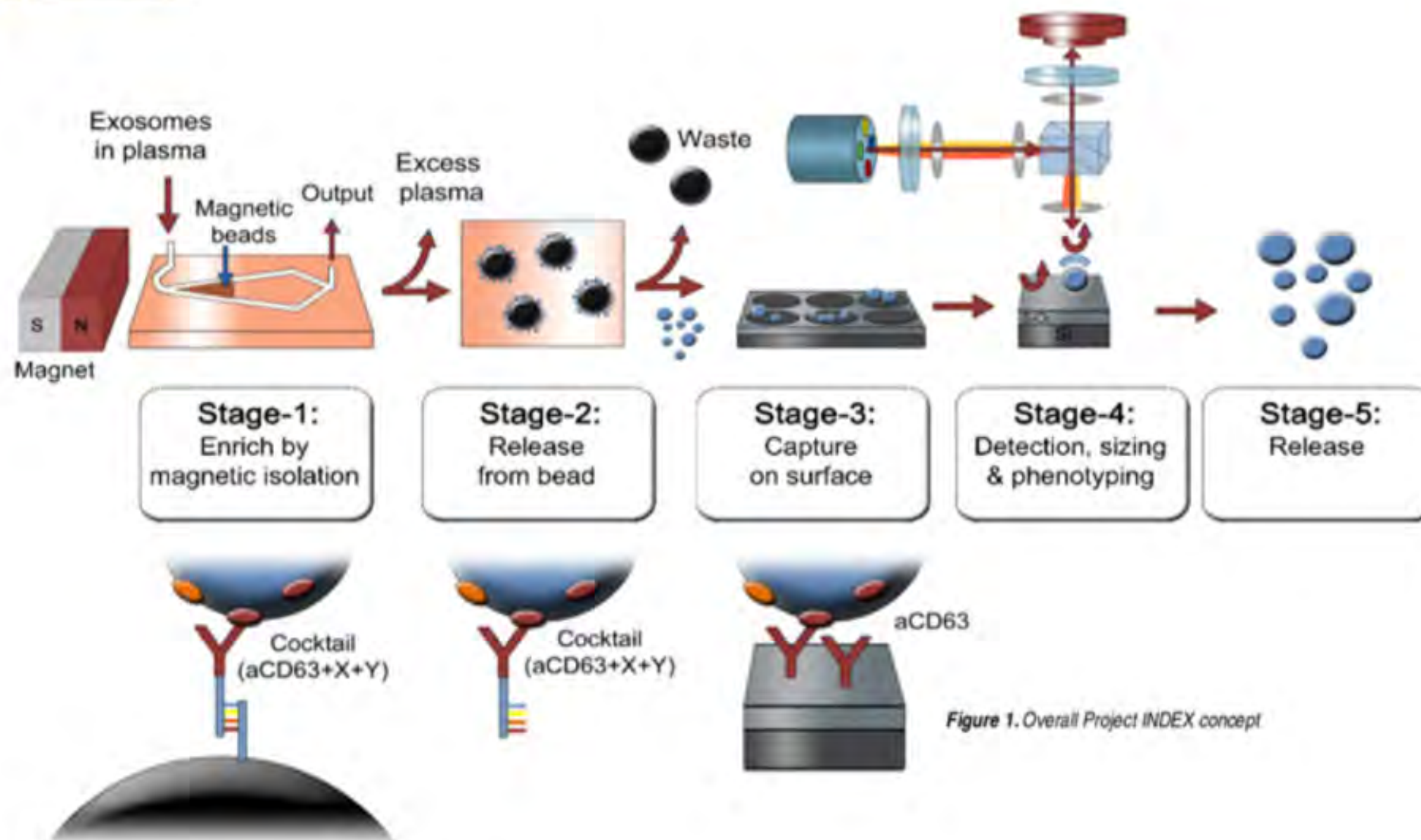
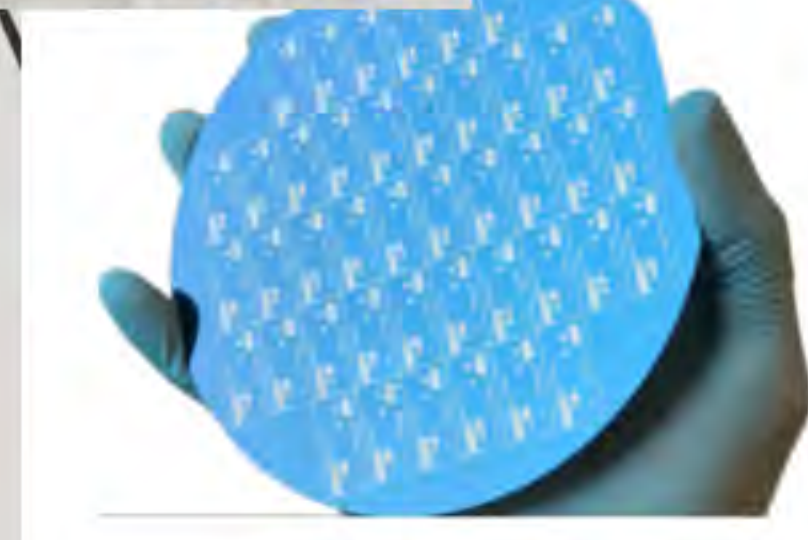
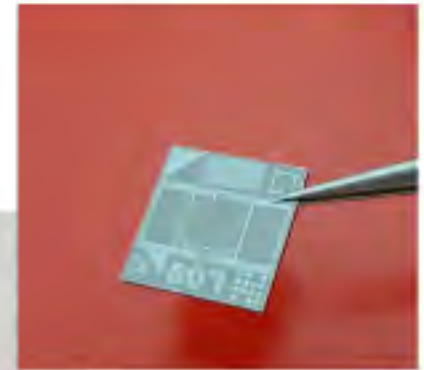
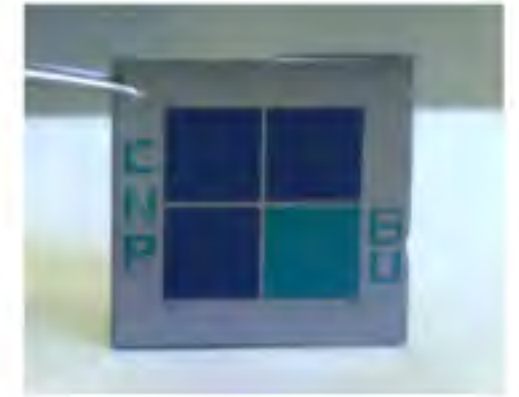


Figure 1. Overall Project INDEX concept

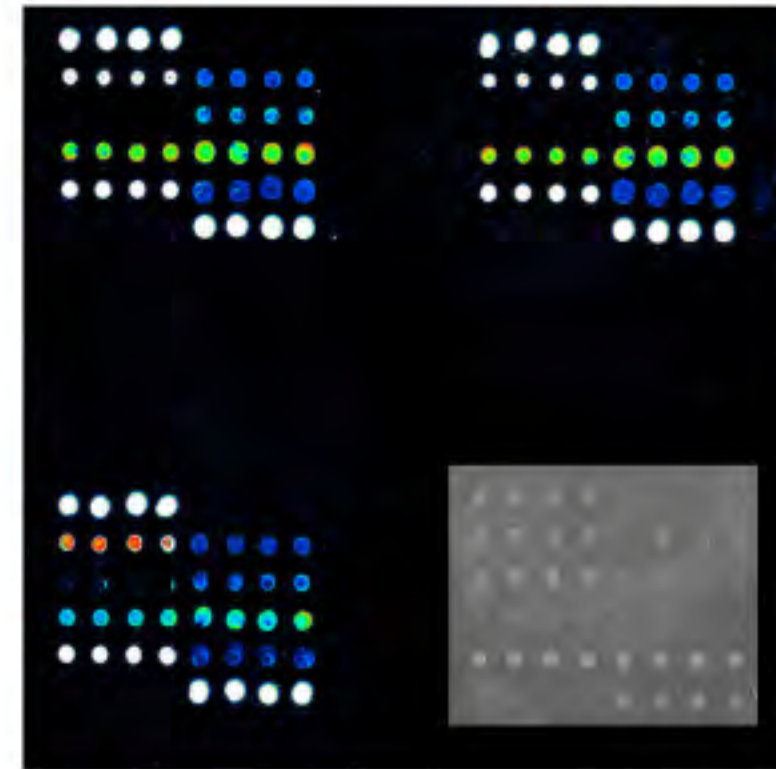
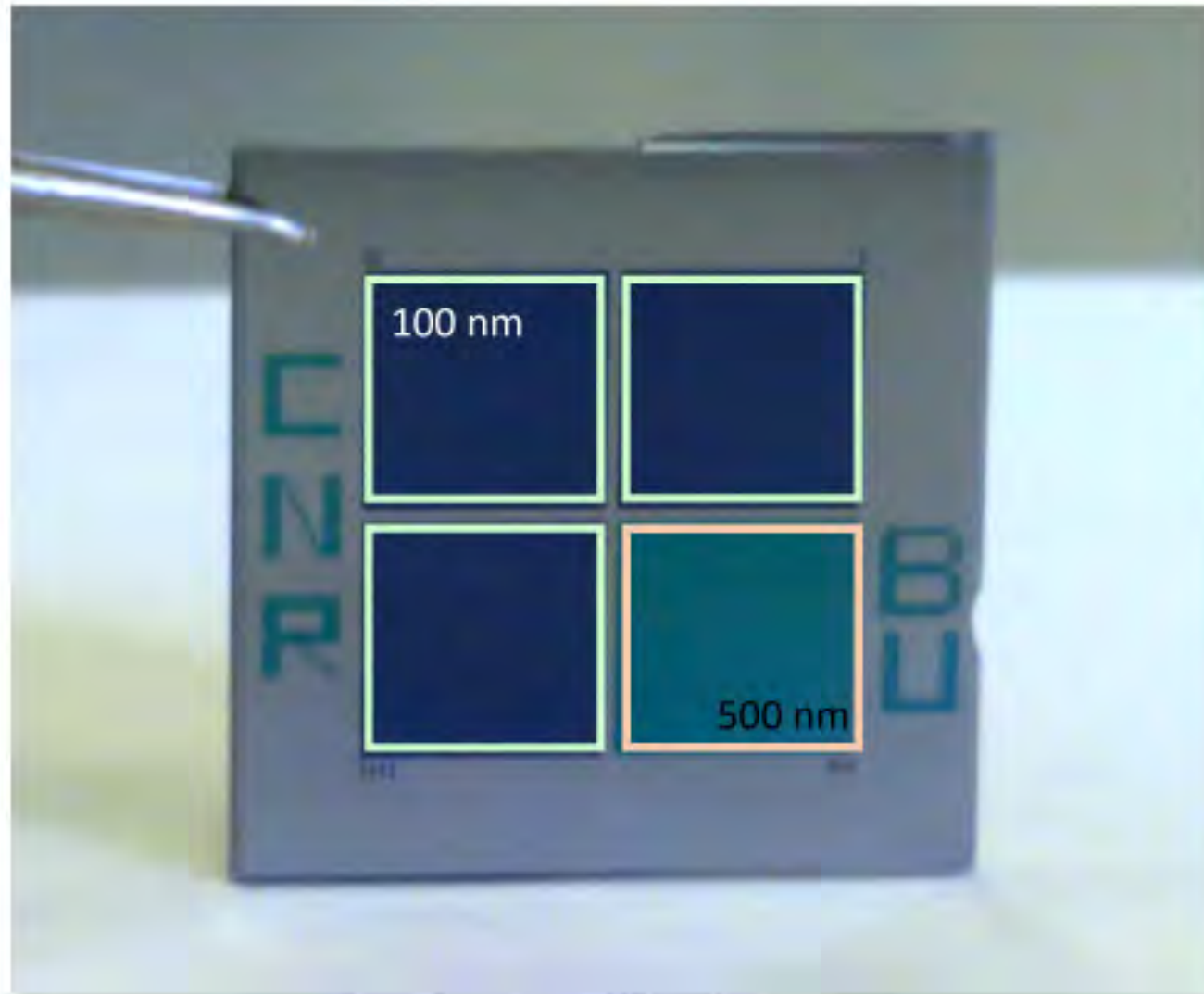
The microarray platform at ICRM-CNR

Support: silicon slide + thermal silicon oxide 100/500nm thickness

- High purity
- Flat and smooth surface
- Potential use as sensor element in integrated in microelectronic devices
- Scalable/manufacturable
- Fluorescence signal enhancement
- Interferometric (Label free) detection
 - Mass quantitation
 - Enhanced particle scattering



The microarray platform at ICRM-CNR

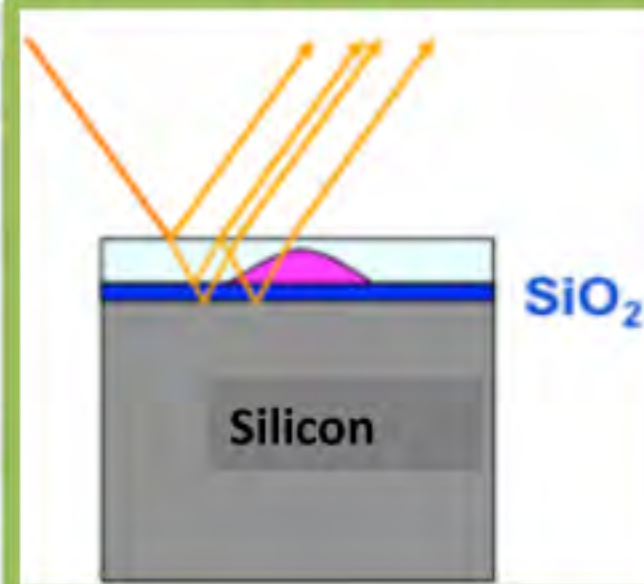
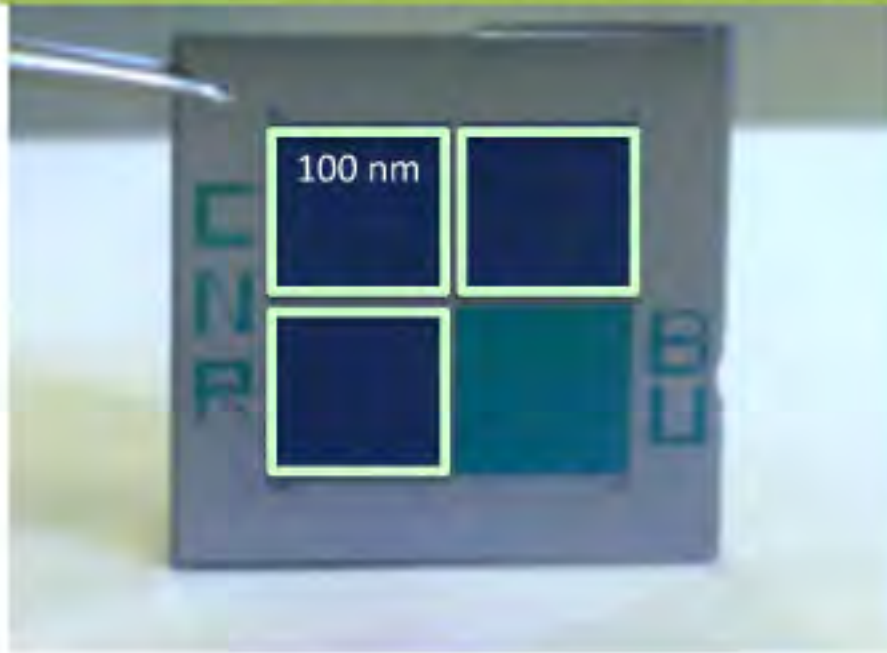


Collaboration
with Prof. Selim
Unlu's group

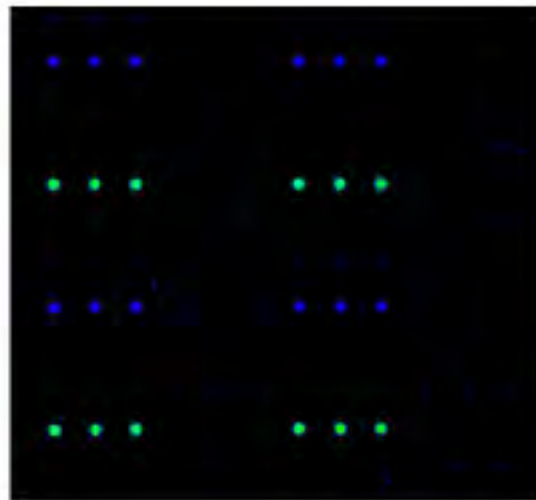
- ✓ Each quarter of the chip is spotted with the same protein array within the same spotting session
- ✓ Within a single experiment the chip allows imaging of the quality of the spotted array and detection of fluorescence of the bioassay

The microarray platform at ICRM-CNR

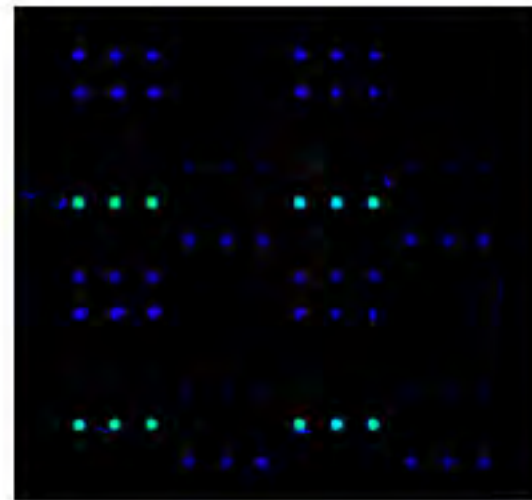
Constructive optical interference: Enhanced Fluorescence detection



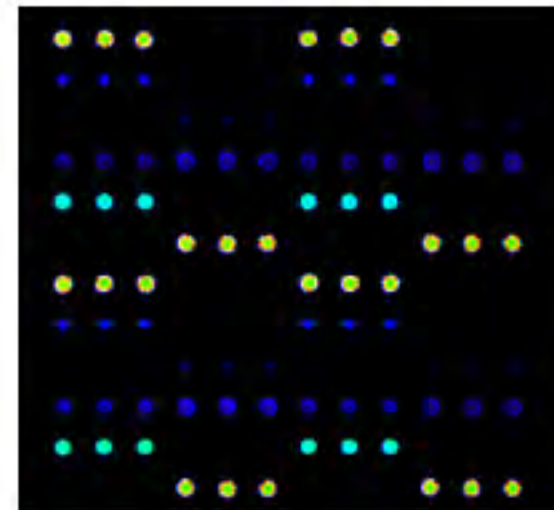
Optimized 100 nm SiO_2 act as interference layer and reflects, towards the detector, the light that would be otherwise absorbed by the substrate
Enhancement of fluorescence up to 7 times



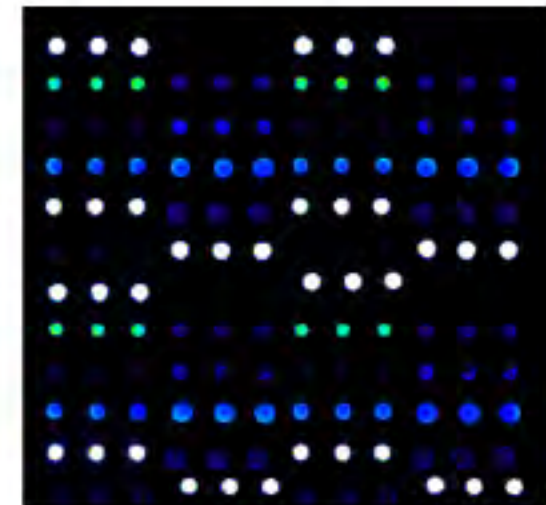
SuperEpoxy 2



MirrorEpoxy
substrates



Copoly (DMA-NAS-MAPS)
coated glass

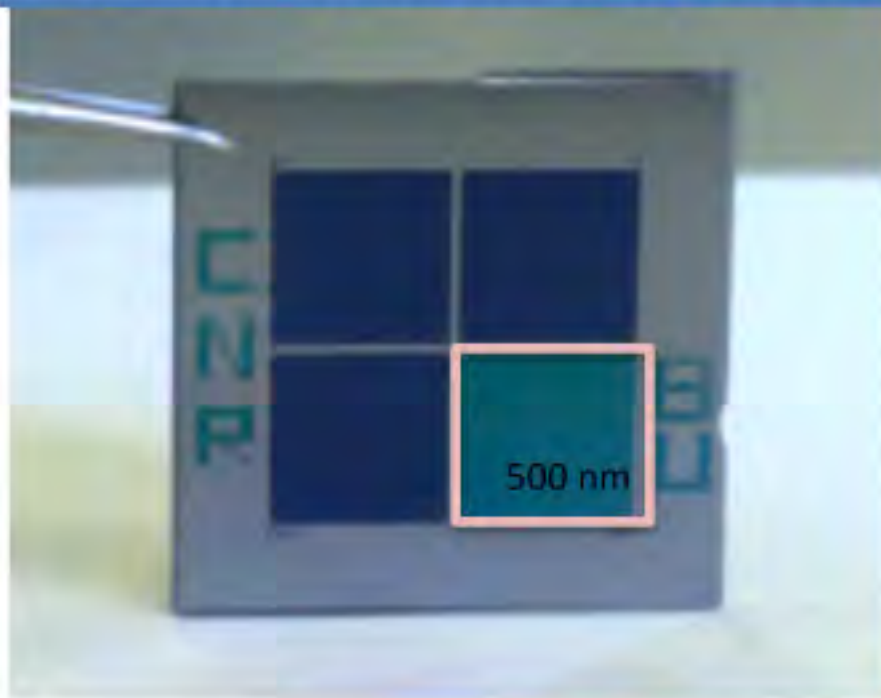


Copoly (DMA-NAS-MAPS)
coated silicon

Collaboration
with Prof. Selim
Unlu's group

The microarray platform at ICRM-CNR

Label-free detection by LED based Interferometric Reflectance Imaging Sensor (IRIS)

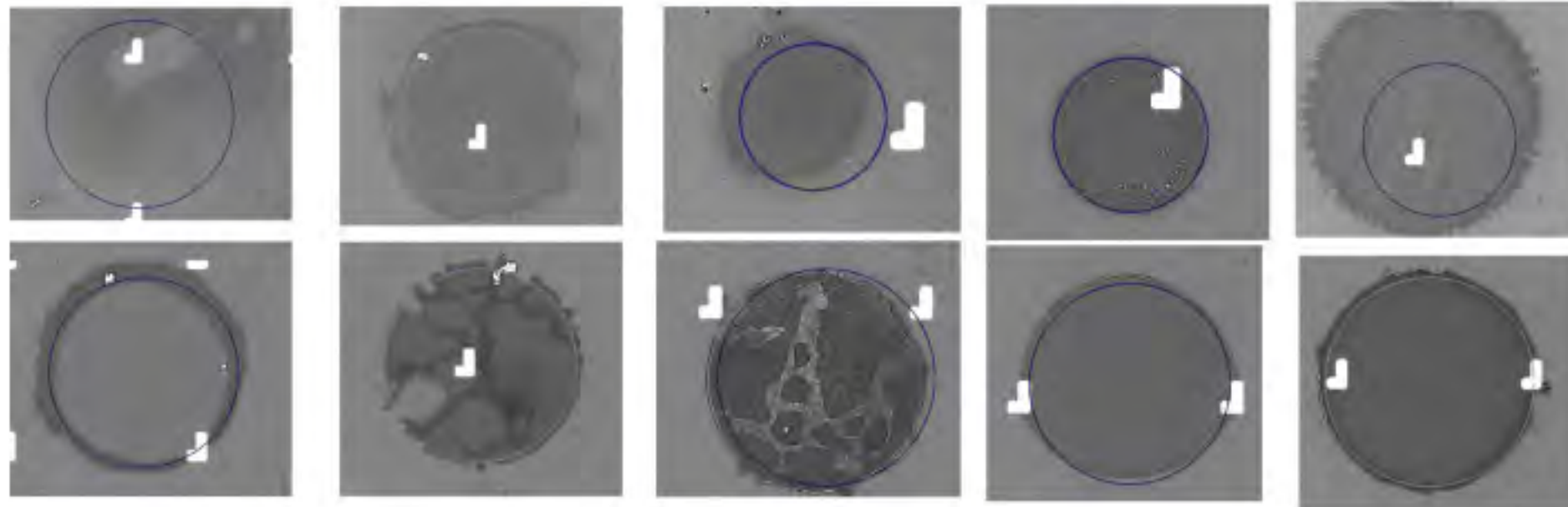


Monitoring of the shift of reflectivity resulting from surface accumulation of biological mass

Precise quantification of immobilized probe

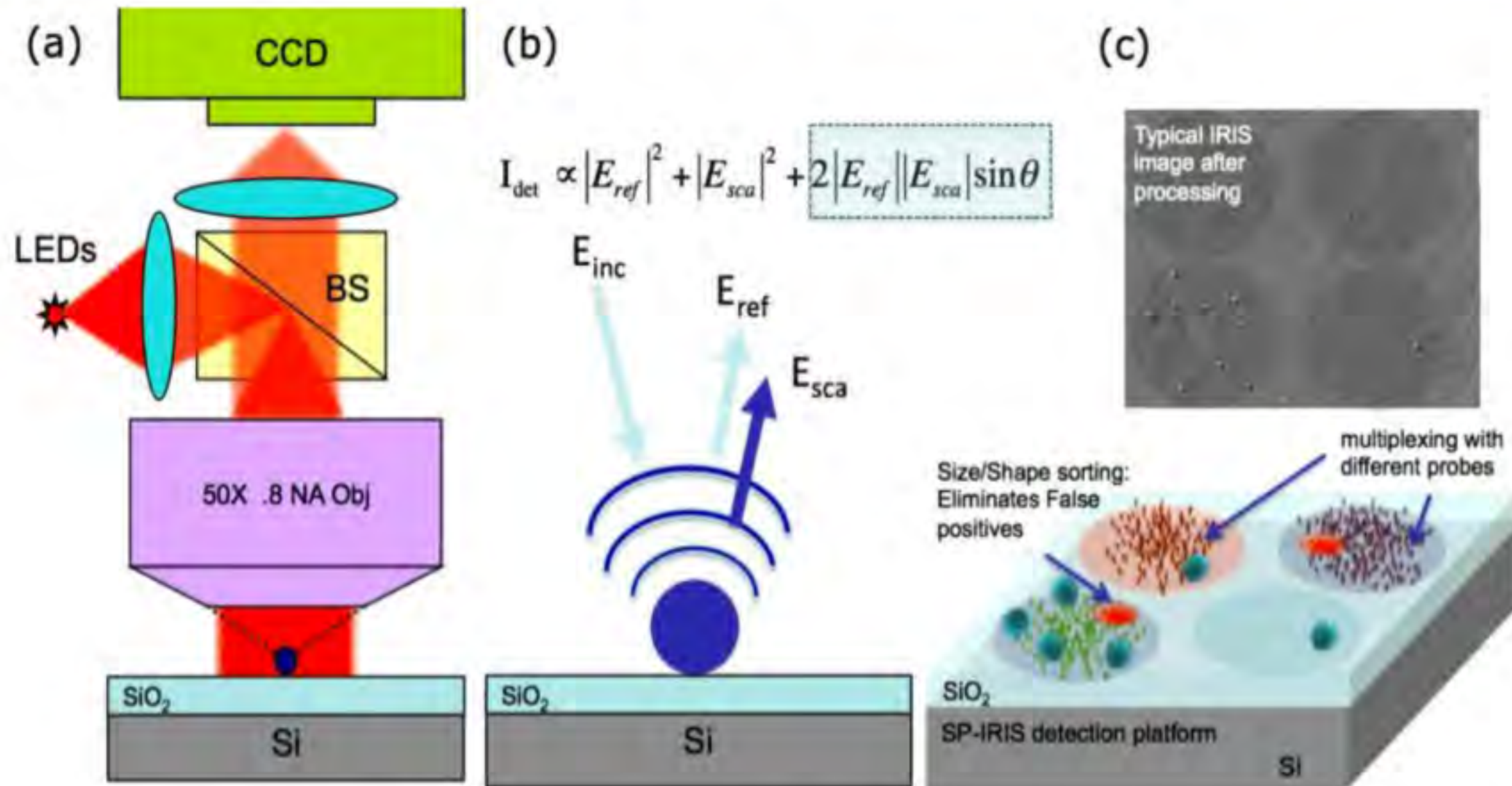
SiO₂ on Si reflector 8

Imaging of spotted ligands: Morphology, Homogeneity, Binding yields



Collaboration
with Prof. Selim
Unlu's group

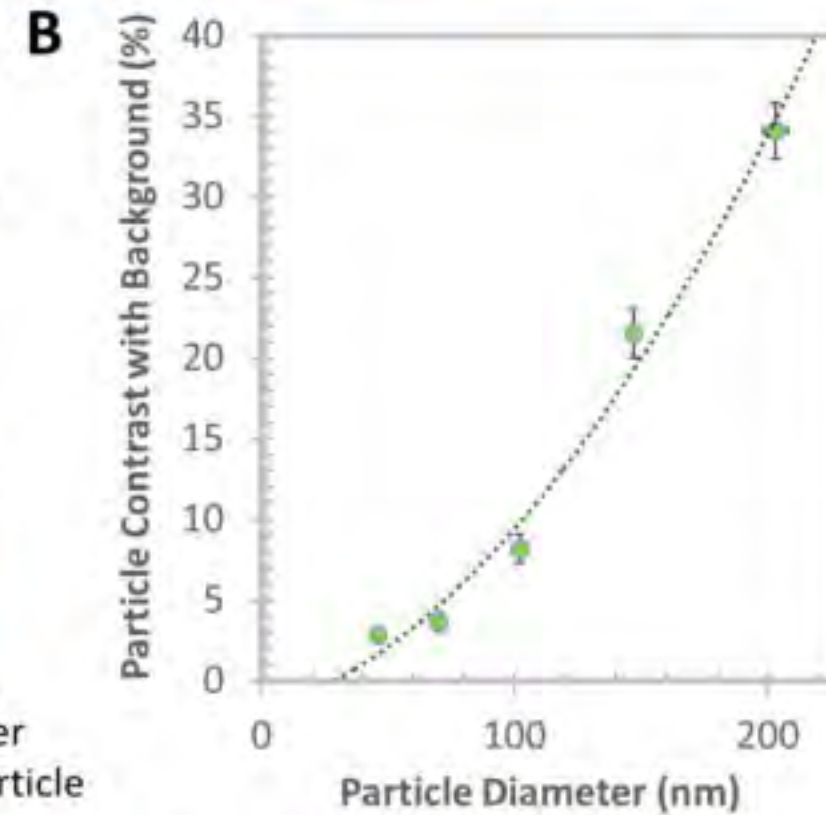
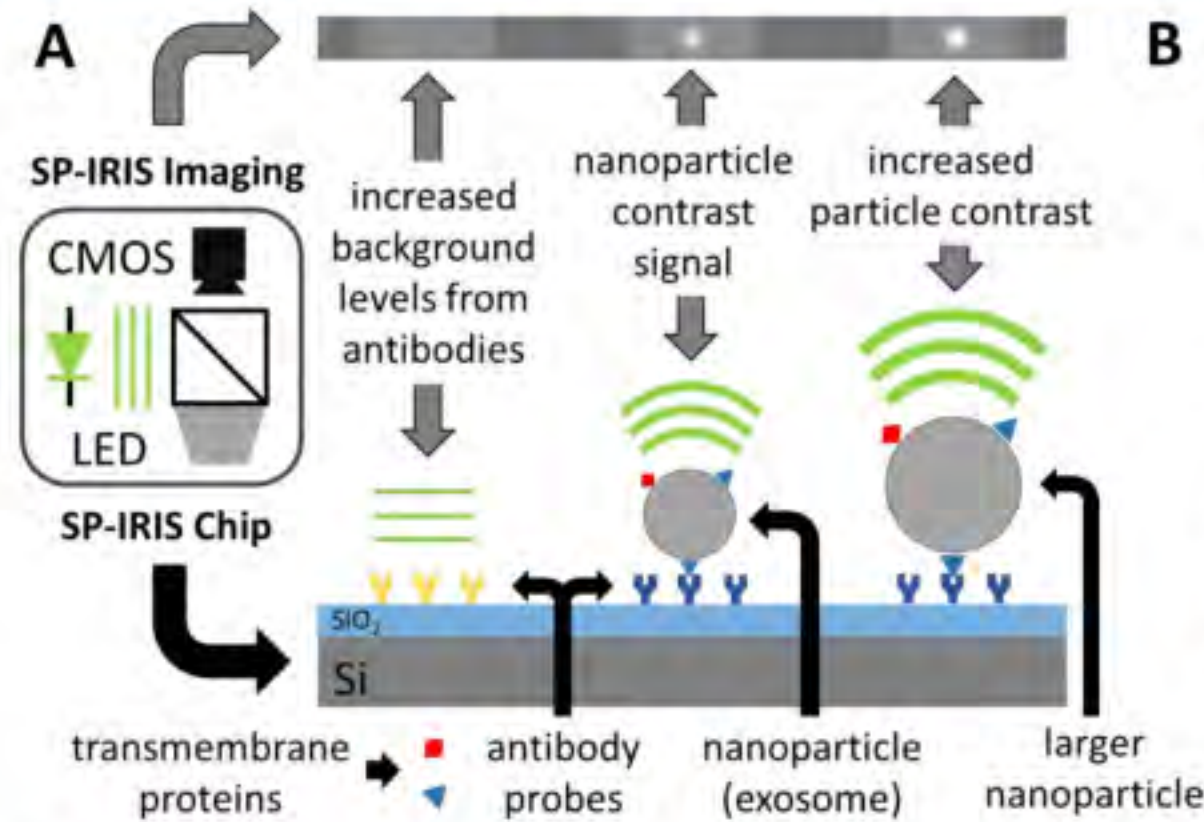
Single Particle (SP) Imaging (SP-IRIS)



Prof. Selim Unlu's
group

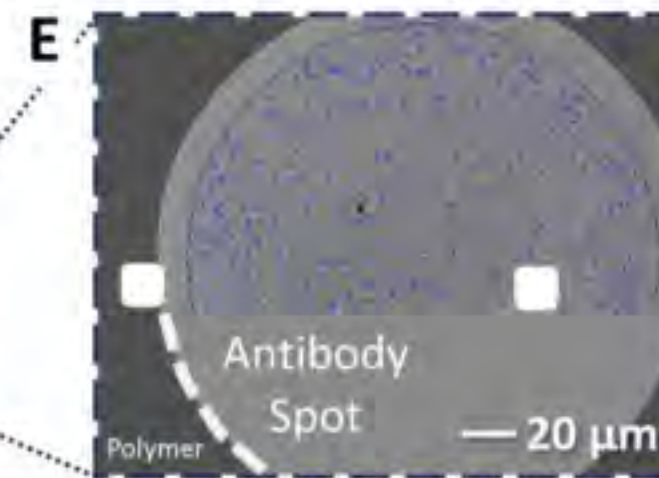
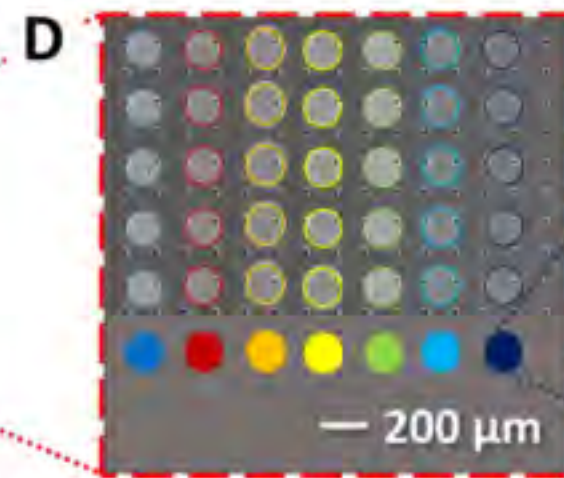
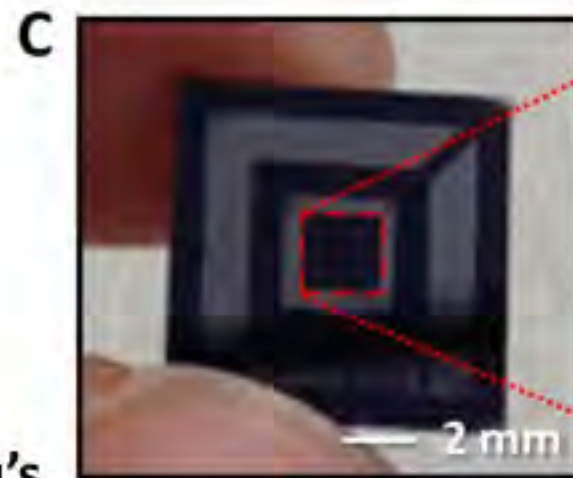
A visible LED provides illumination and reflection image is captured on a CCD camera. The key to improved visibility of nanoparticles is the enhanced contrast in the scattering signal that is generated using a layered substrate

Single Particle (SP) Imaging (SP-IRIS)



SP-IRIS detection principle:

- 1) Monochromatic LED light illuminates the sensor surface
- 2) Nanoparticle scattering signature is enhanced interferometrically
- 3) And is captured on a CMOS camera.

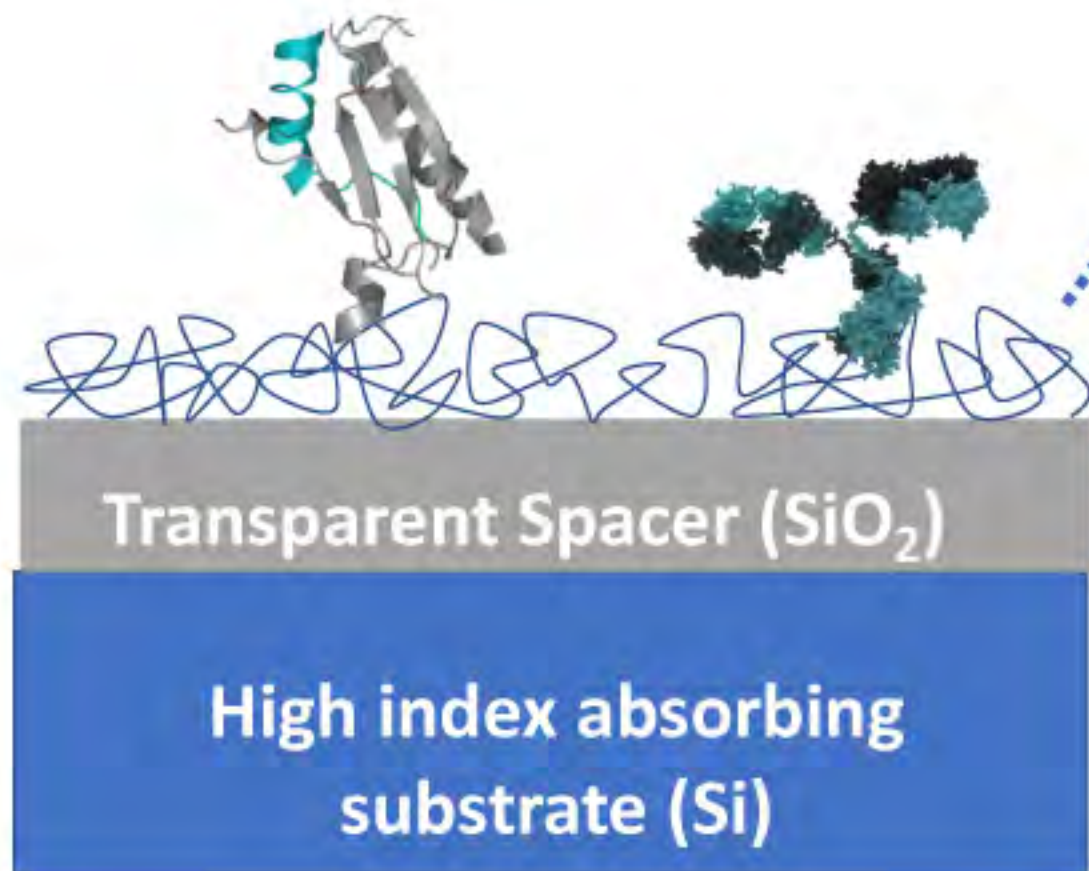


Microarray for EVs:

- Phenotyping
- Counting
- Sizing

Prof. Selim Unlu's group

The key to silicon based multimodal analysis: Surface functionalization

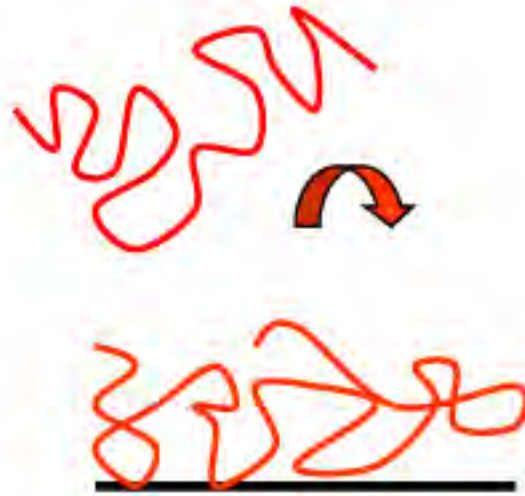


- Functional groups for immobilization of molecules of interest
- The surface is inherently inert and resists non-specific adsorption
- Binding strong enough to retain the protein on the surface, but also sufficiently non intrusive to have minimal effect on its 3D structure
- Retention of optical properties

Surface functionalization: polymeric coatings

Physisorption

Pre-synthesized polymers physisorbed and/or linked on substrate surface

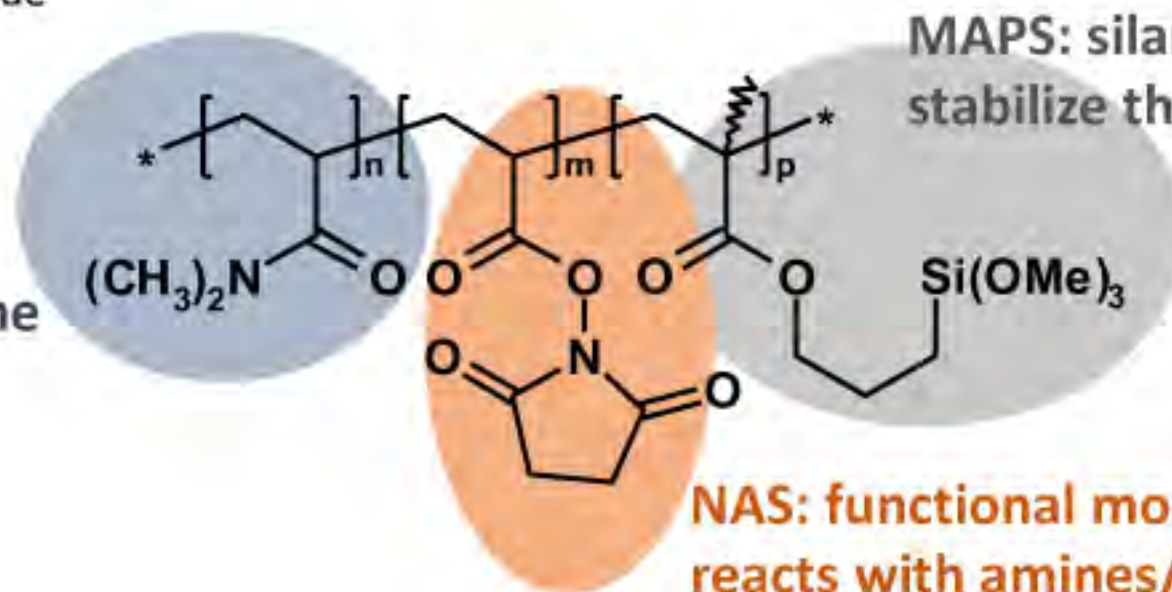


- Robust and user friendly coating protocol
- Glass, silicon, NC, plastics, gold...

Copoly(DMA-NAS-MAPS)

a ter-copolymer based on N,N-dimethylacrylamide (DMA),
N-acryloyloxysuccinimide (NAS) and
3-(trimethoxysilyl)propyl-methacrylate (MAPS)

DMA: polymer backbone
self-adsorb onto SiO_2
surfaces

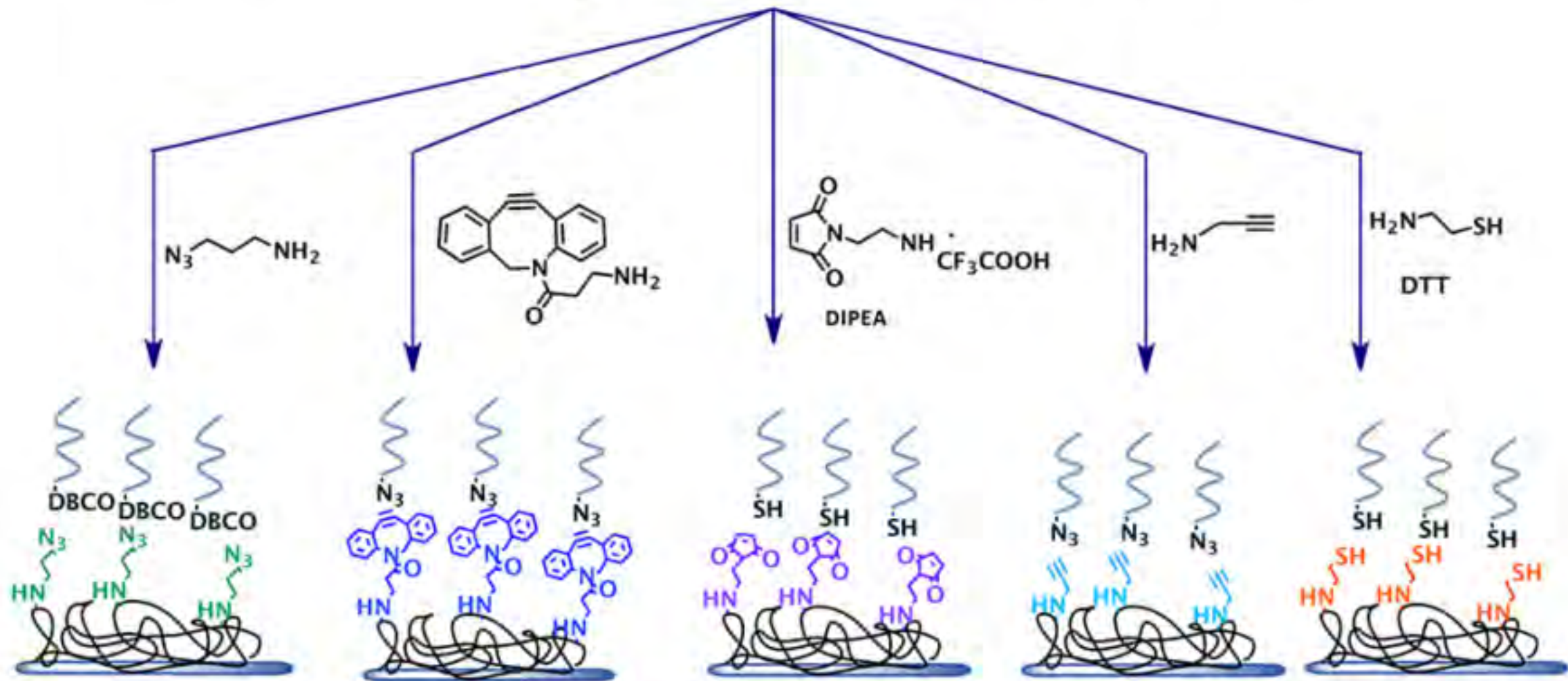
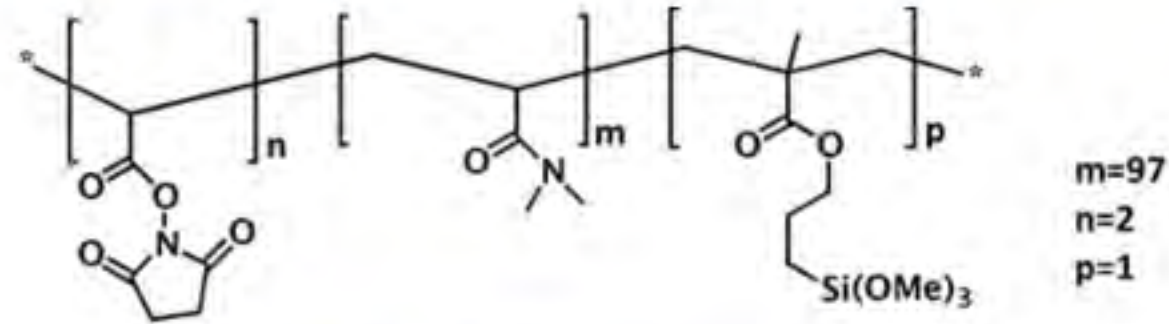


MAPS: silanating monomer
stabilize the coating

NAS: functional monomer
reacts with amines/nucleophiles

"Clickable" polymeric coatings

Parent polymer
Copoly(DMA-NAS-MAPS)



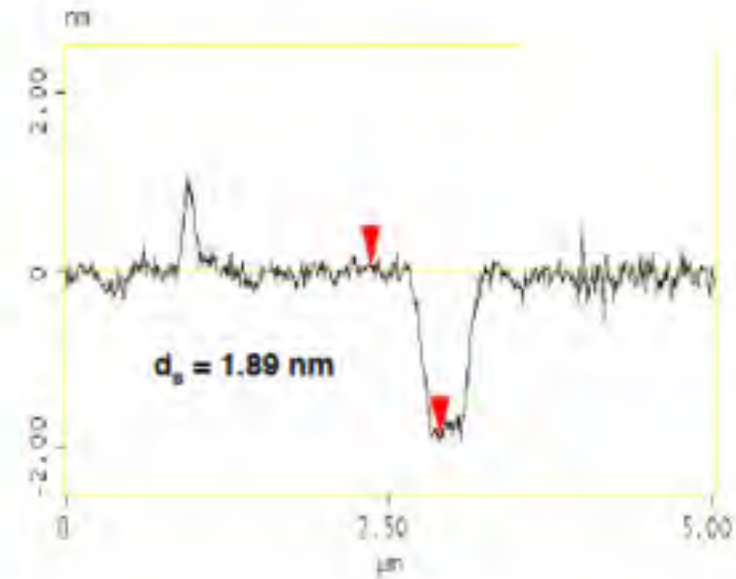
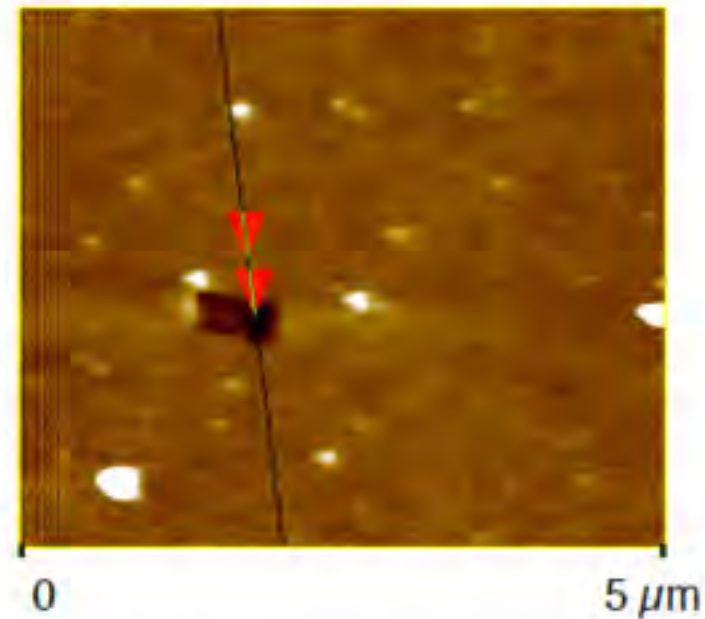
Clickable polymeric coatings for microarray surfaces

Surface functionalization: polymeric coatings

Roughness and Thickness of polymeric coating by AFM



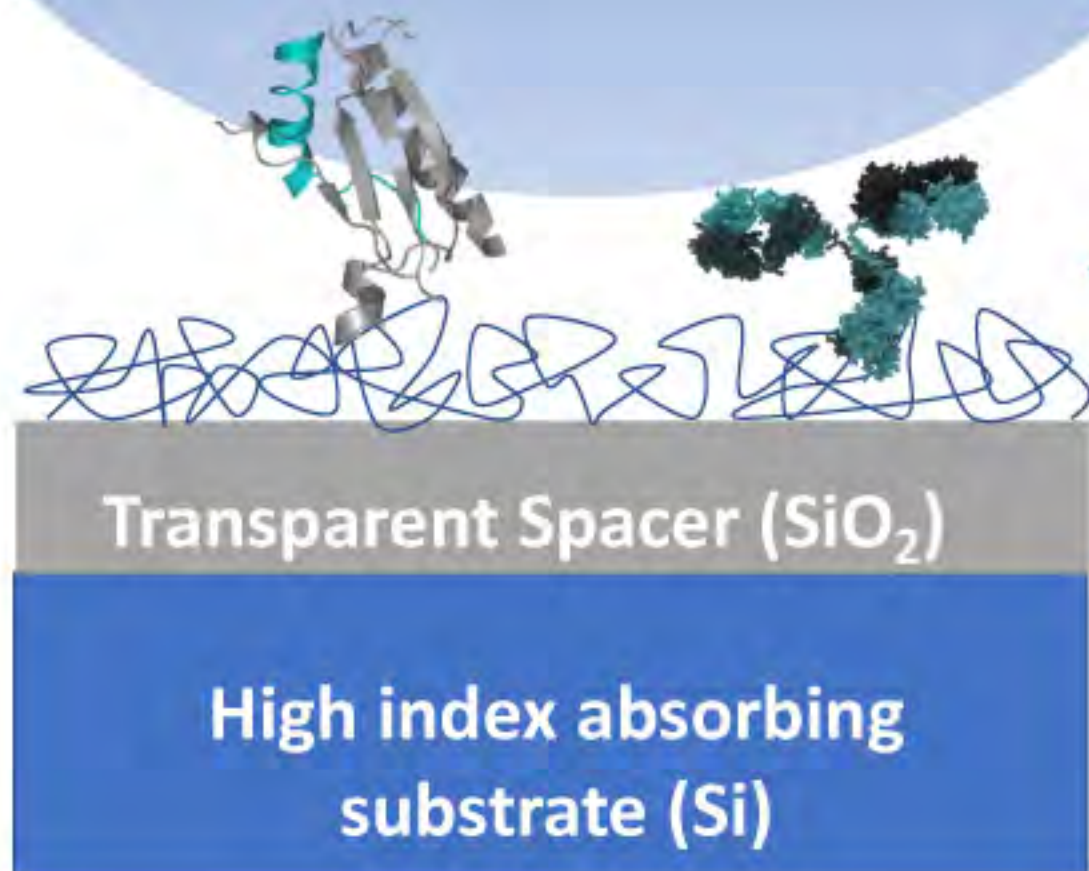
$R_q = 0.097 \text{ nm}$



Characterization of the polymeric coating on silicon by Dual Polarization Interferometry using AnaLight® Bio2000 (Farfield)

	RI	Thickness (nm)	Mass (ng/mm ²)	Density (g/cm ³)
Copoly(DMA-MAPS-NAS)	1.36 ± 0.05	15.73 ± 1.4	2.15 ± 0.04	0.15 ± 0.02

Silicon based microarray platform for EVs



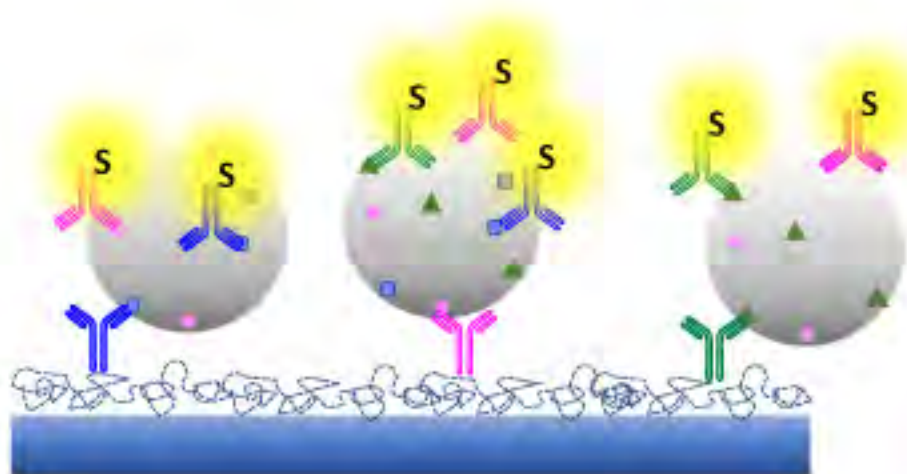
➤ Special layered silicon chips for multimodal analysis:

- Fluorescence
- Spot imaging
- Single particle detection

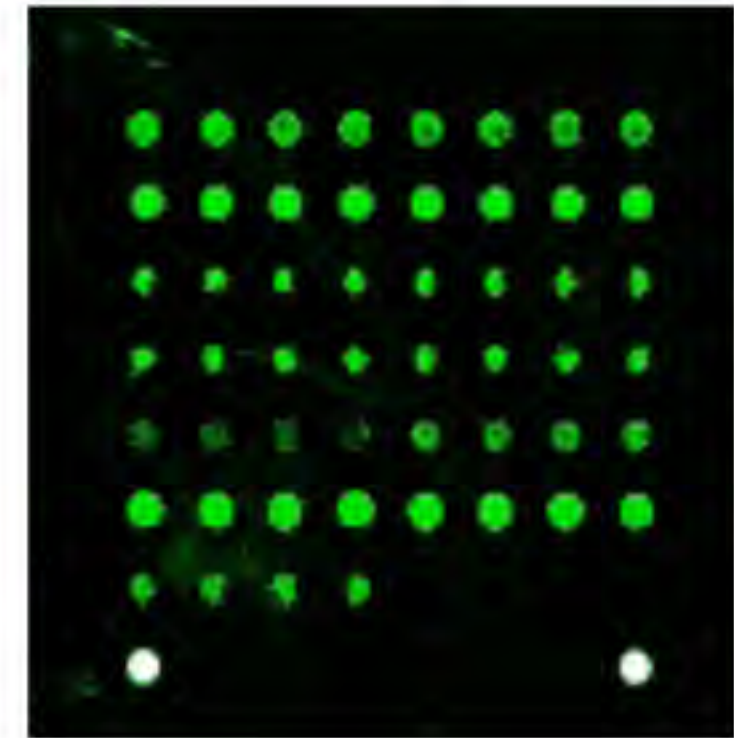
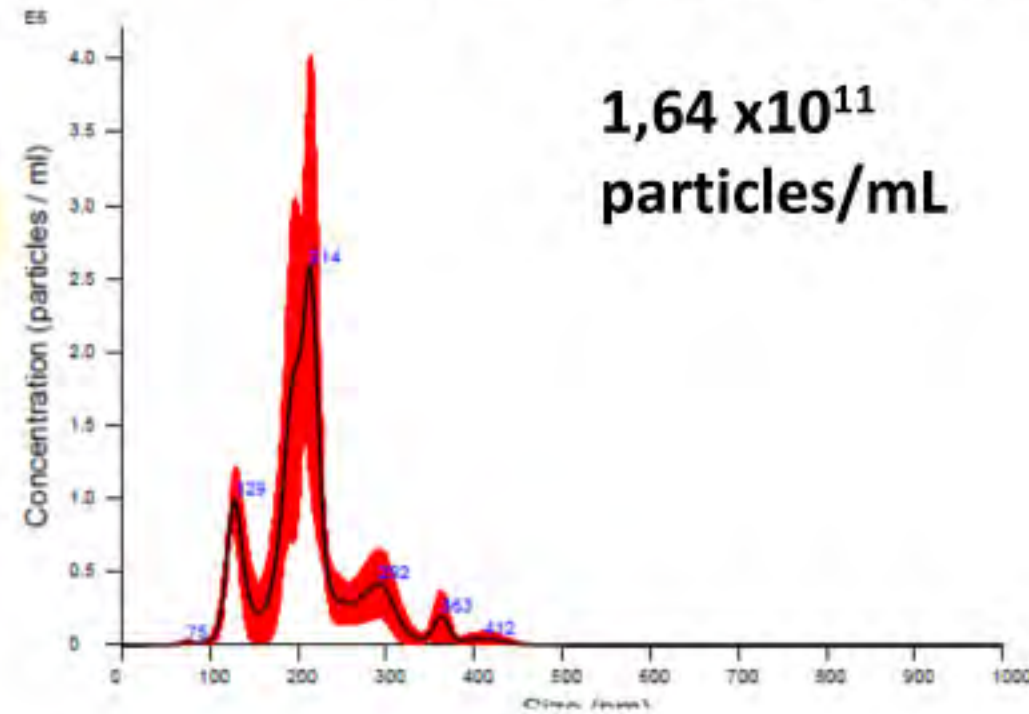
➤ A polymeric coating provides functional groups for covalent binding, suppression of non-specific interactions

➤ Retention of optical properties

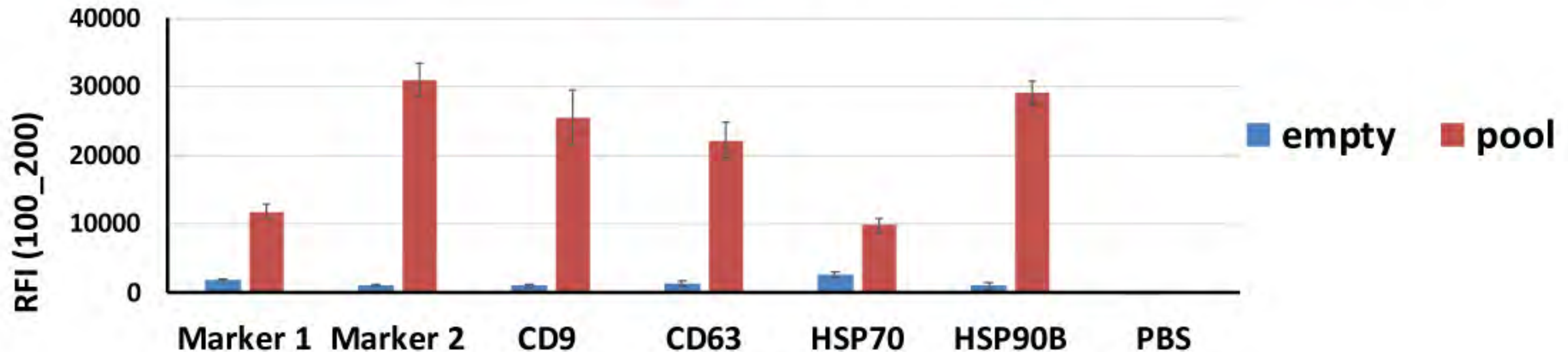
Fluorescence EV arrays on antibody microarrays



Pool of serum samples

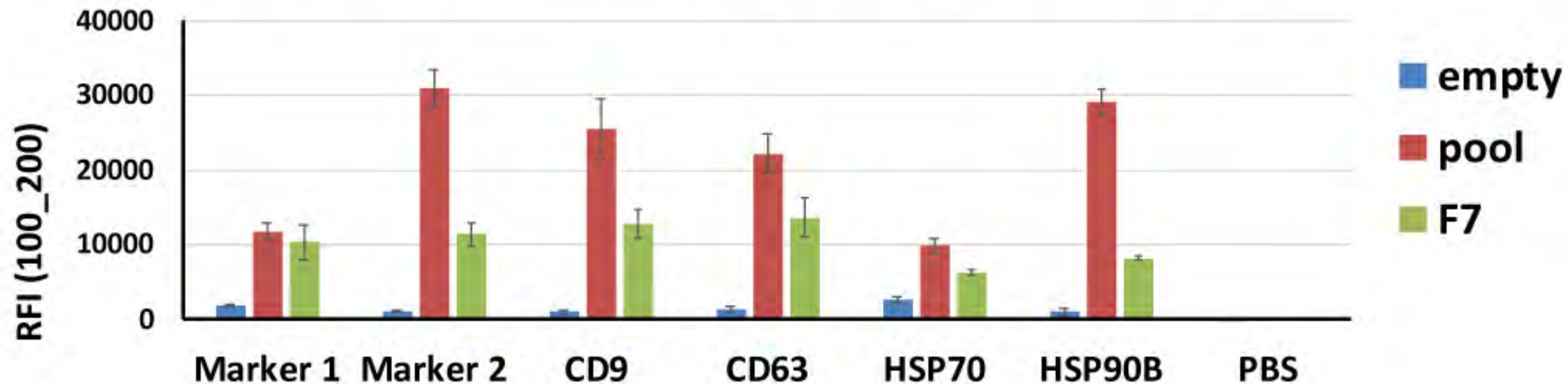
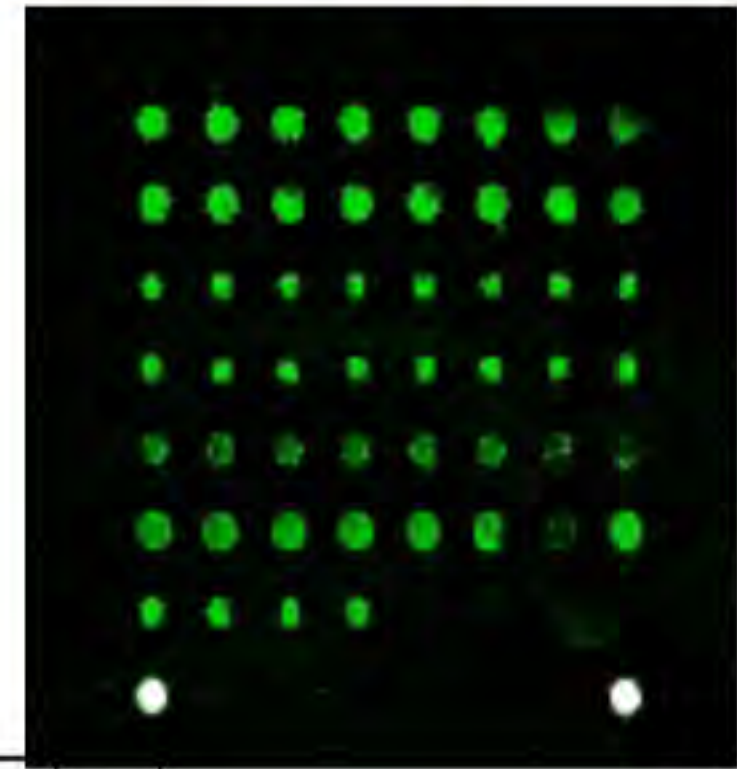
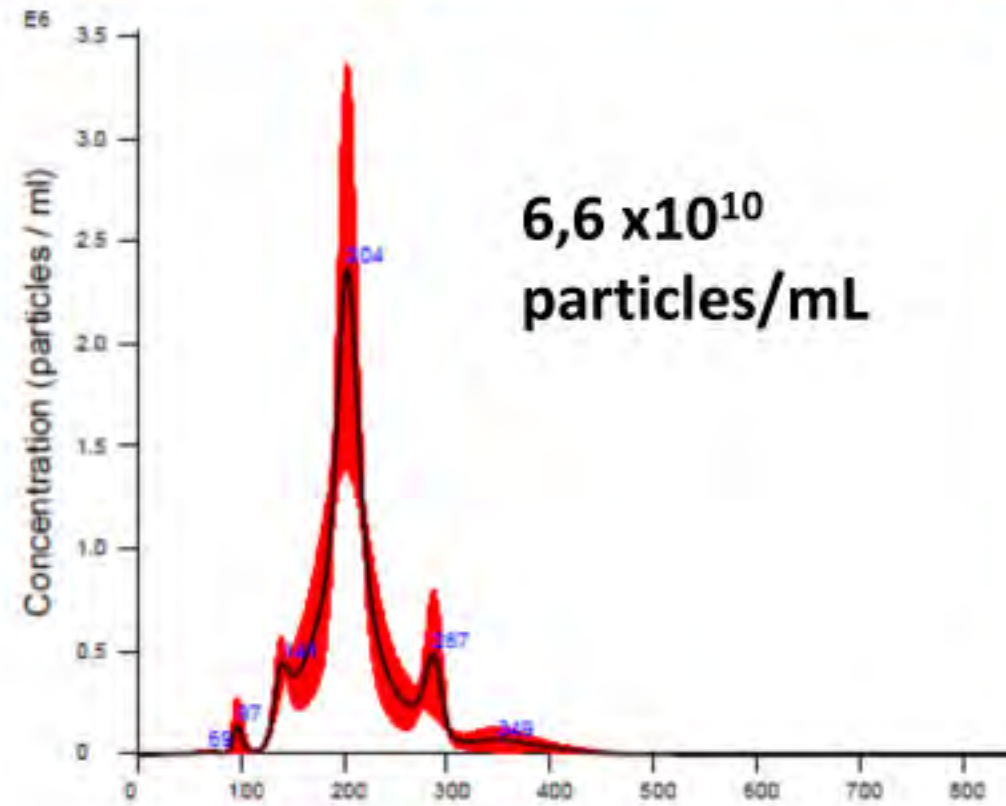
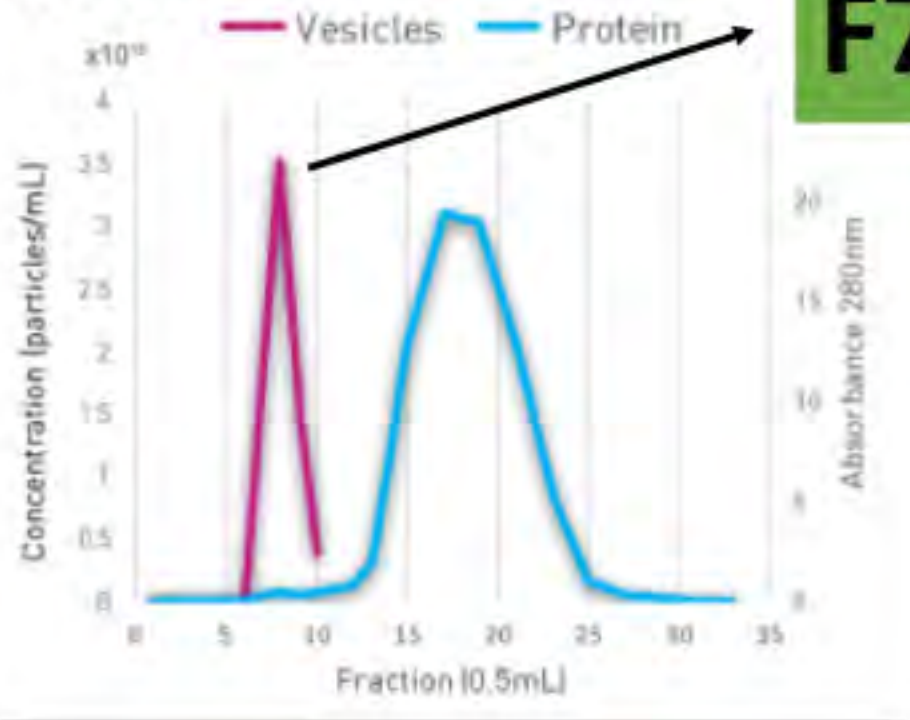


Marker 1
Marker 2
CD 9
CD 63
HSP70
HSP90 β



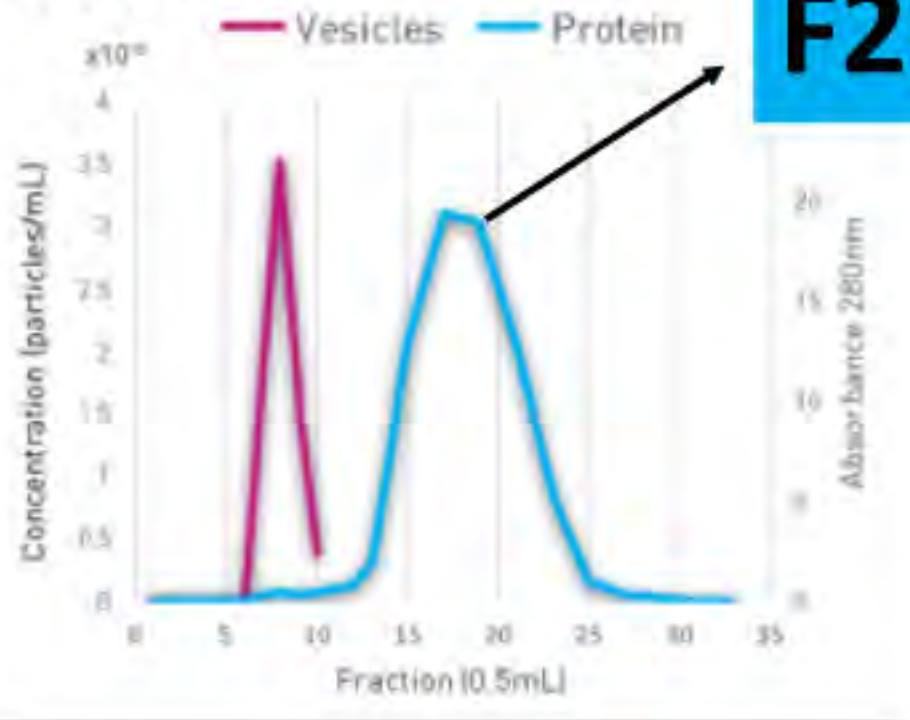
Fluorescence EV arrays on antibody microarrays

IZON SEC

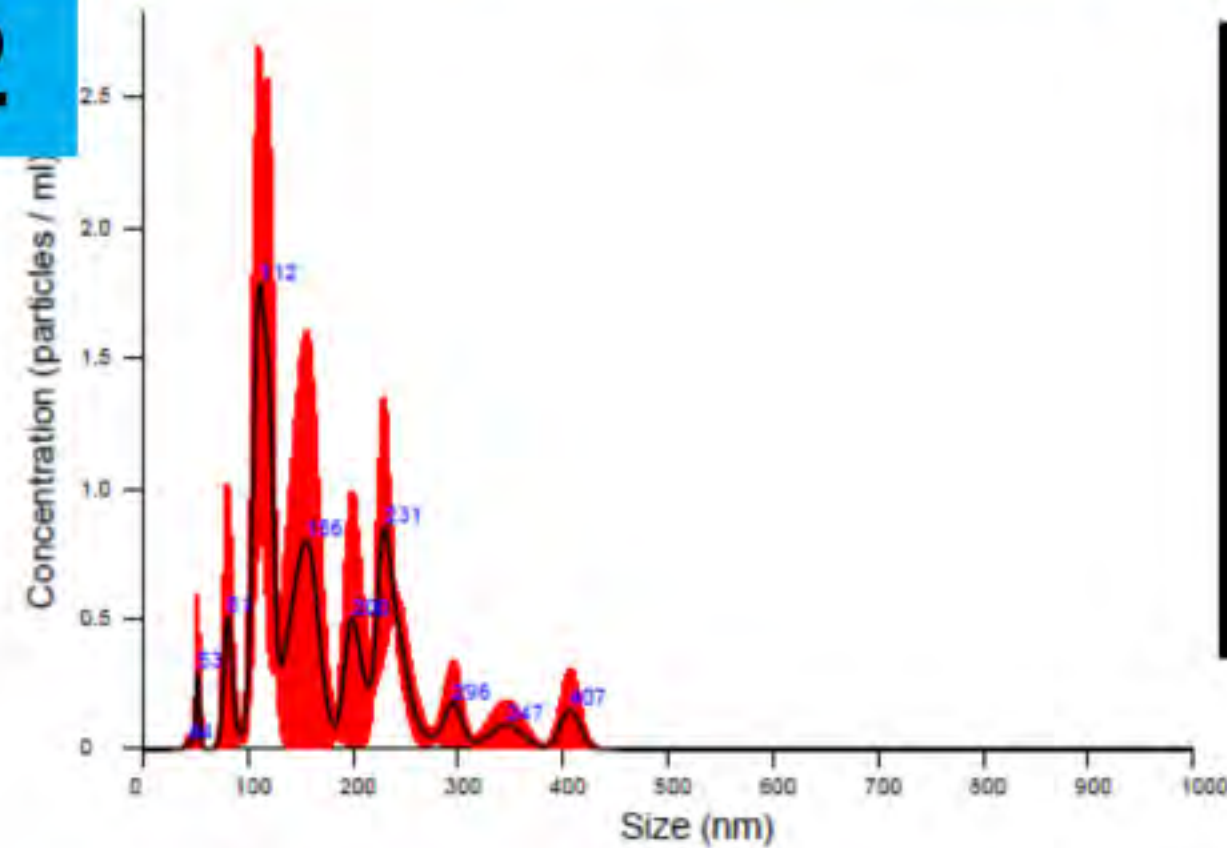


Fluorescence EV arrays on antibody microarrays

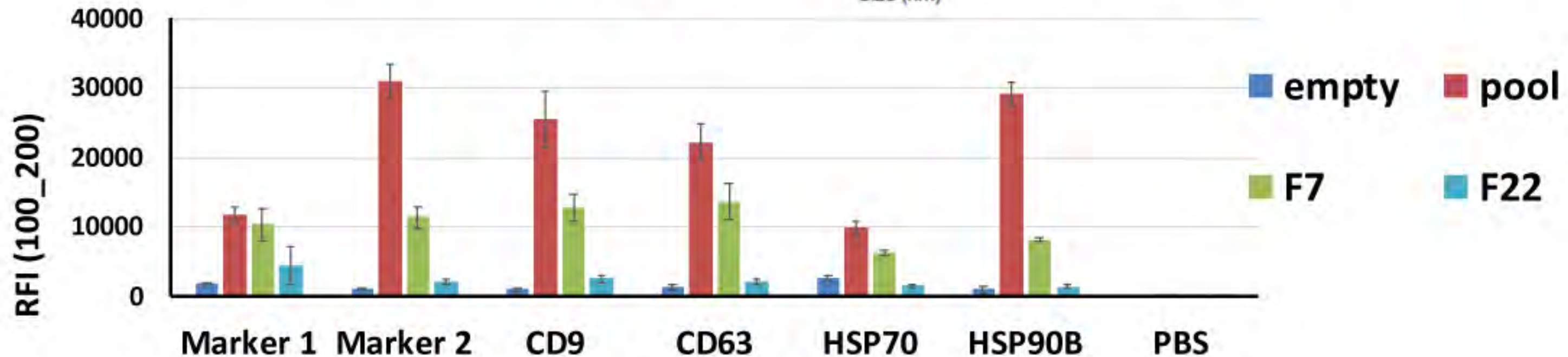
IZON SEC



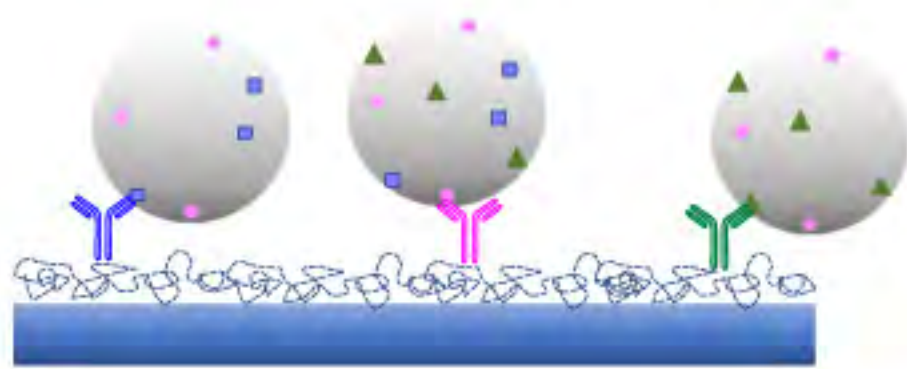
F22



Marker 1
Marker 2
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CD 63
HSP70
HSP90 β

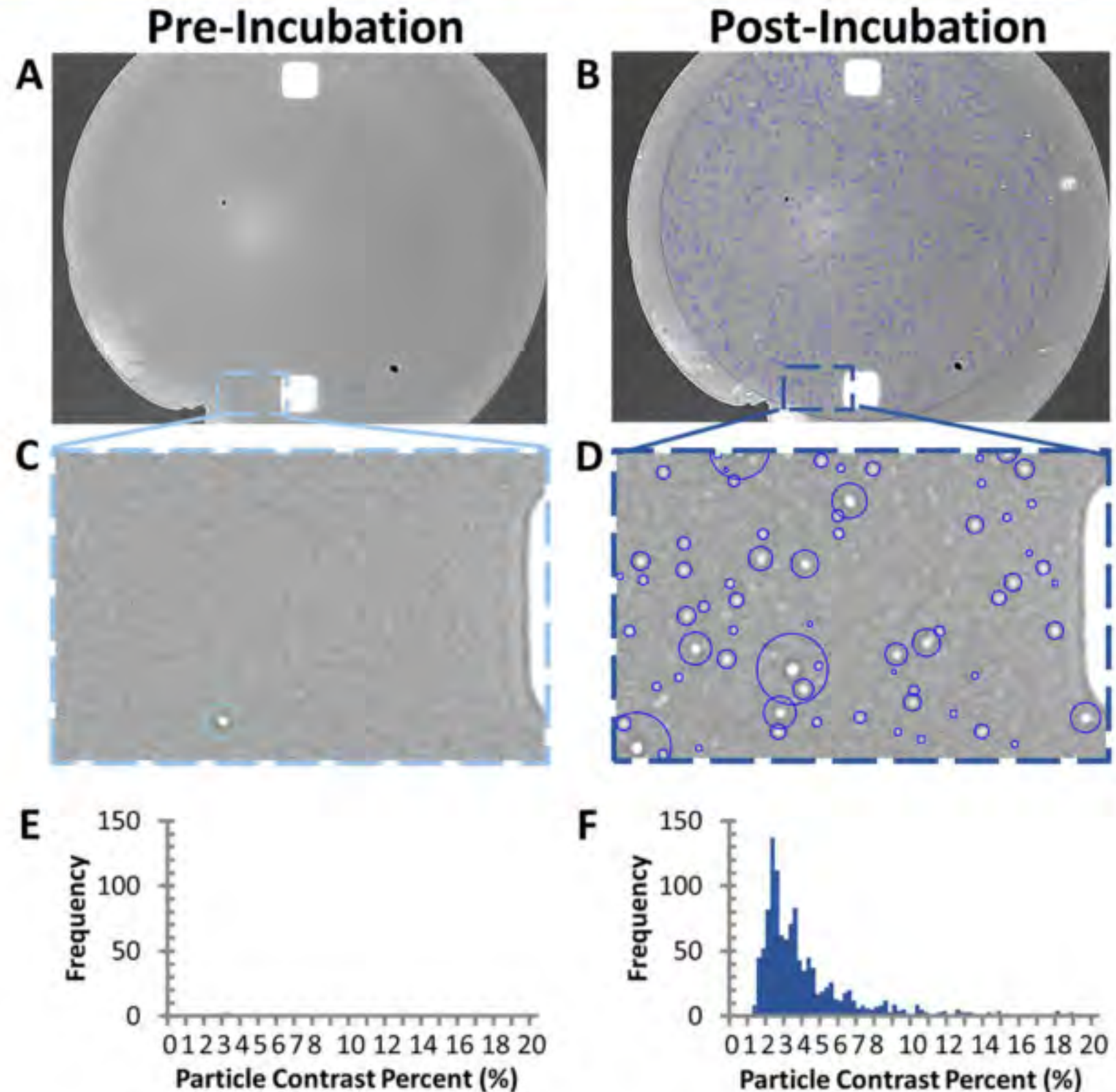


Single-EV counting and phenotyping

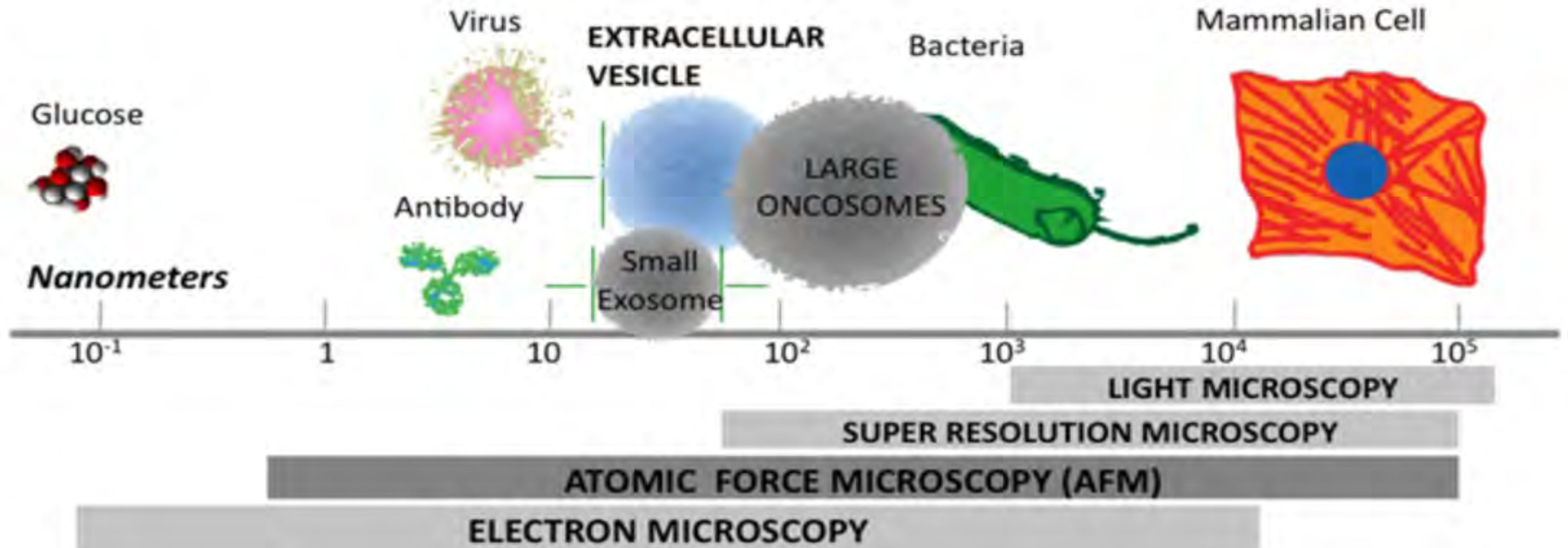


Exosome capture, digital counting, and relative sizing.

Anti-CD81 capture probe image acquired before and after incubation with purified Human Embryonic Kidney 293 (HEK293) cells derived exosomes.

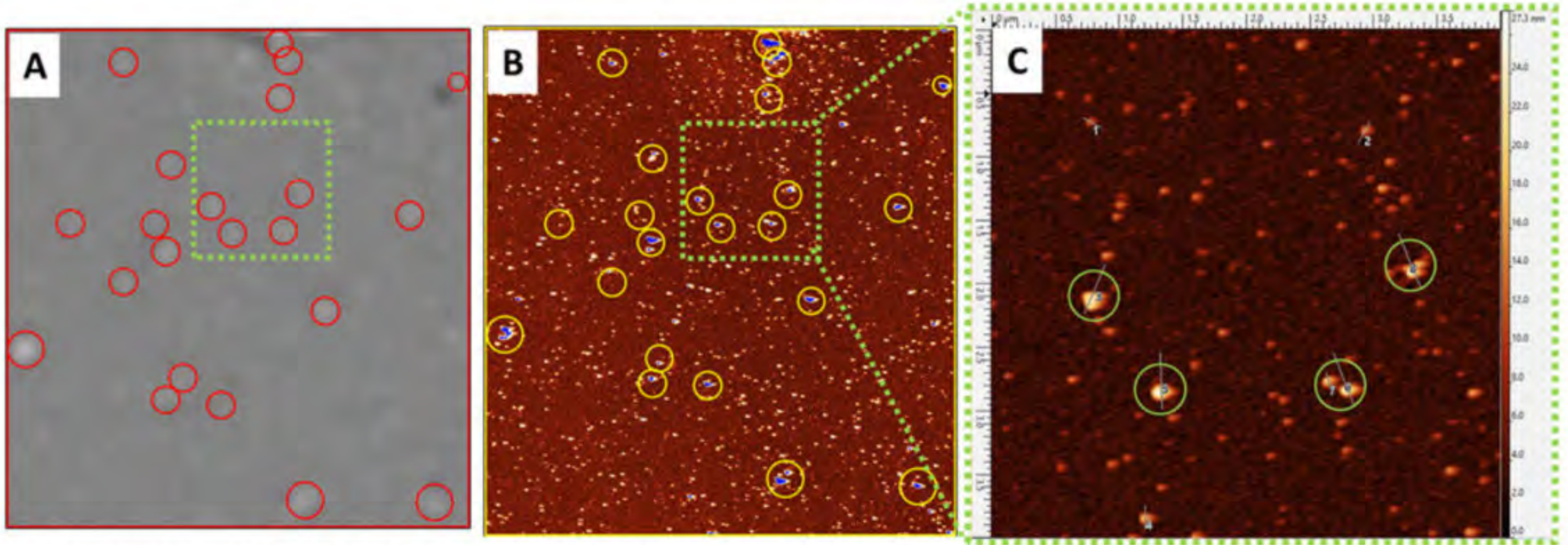


Sensing/detecting EVs: a challenging task



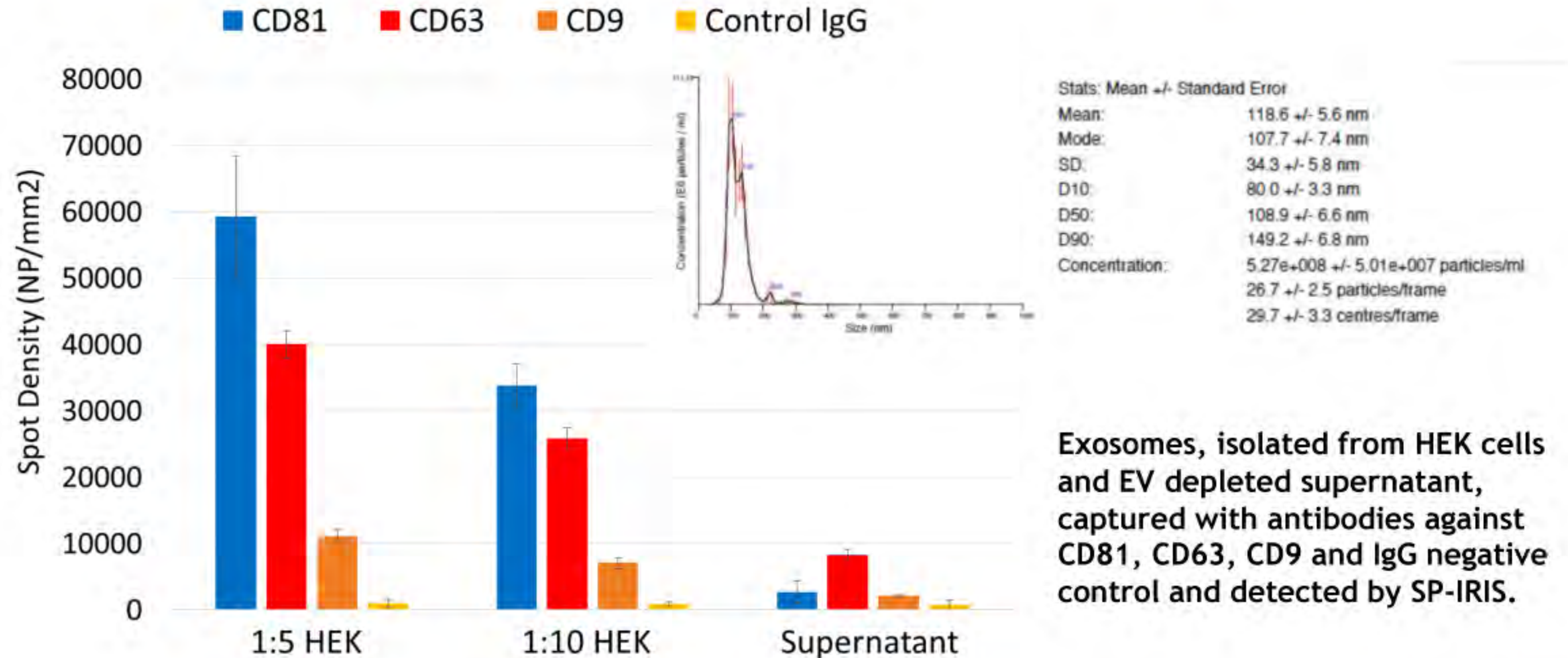
Single-EV counting and phenotyping

Validation by Atomic Force Microscopy

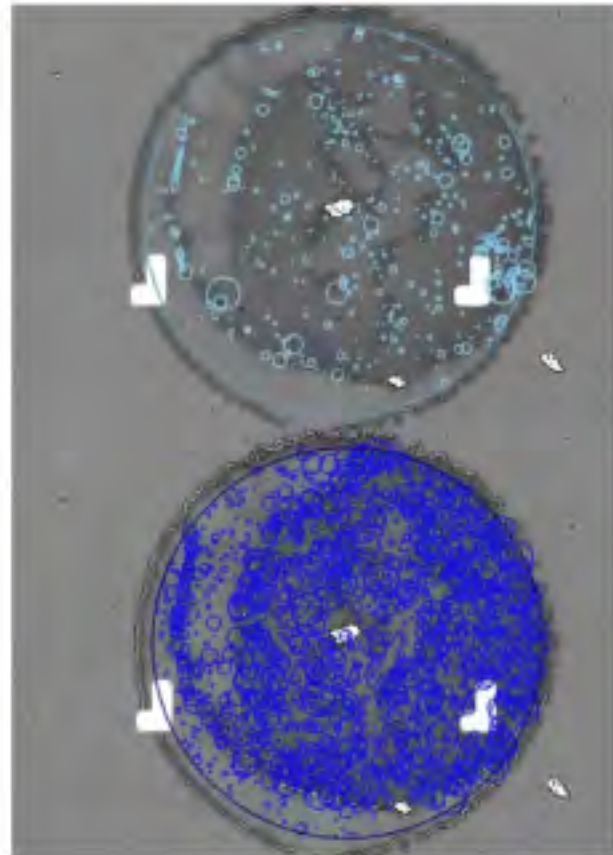


Exosomes purified from HEK cell line, captured with anti- CD81 antibody on silicon chip and detected by SP-IRIS (A) and AFM (B, C).

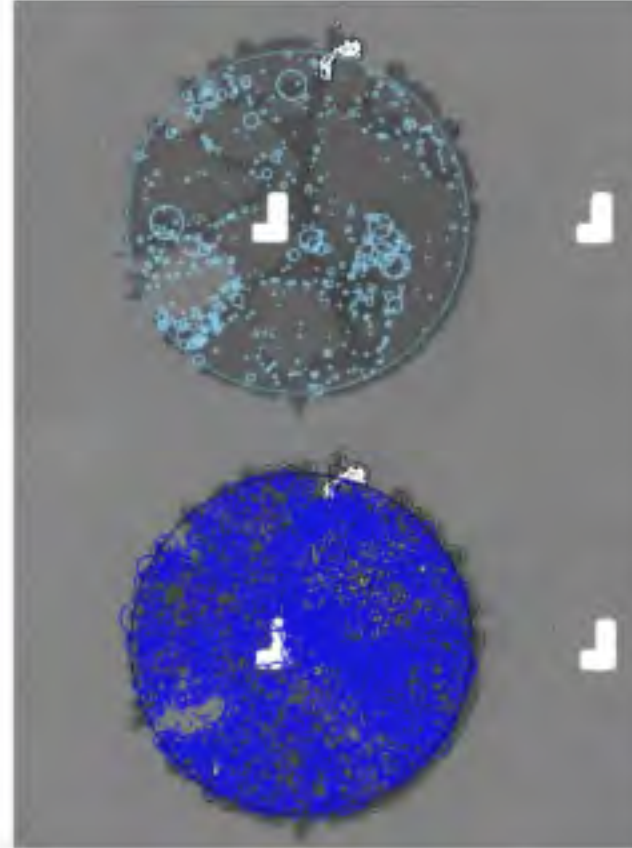
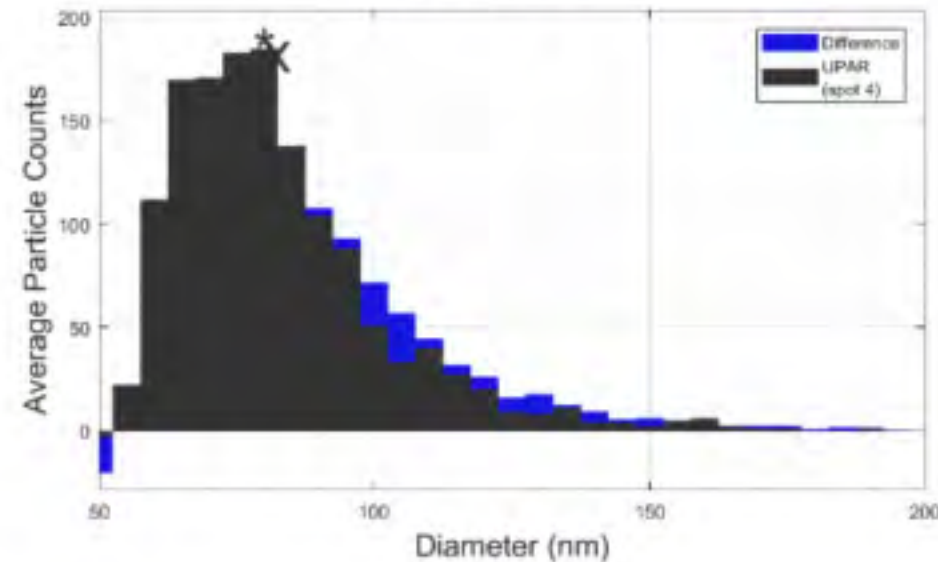
Single-EV counting and phenotyping



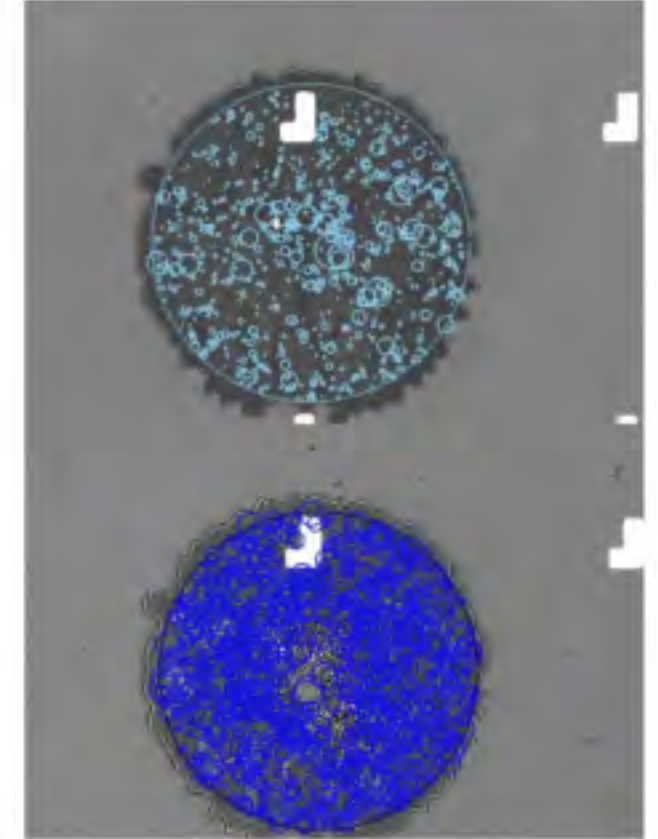
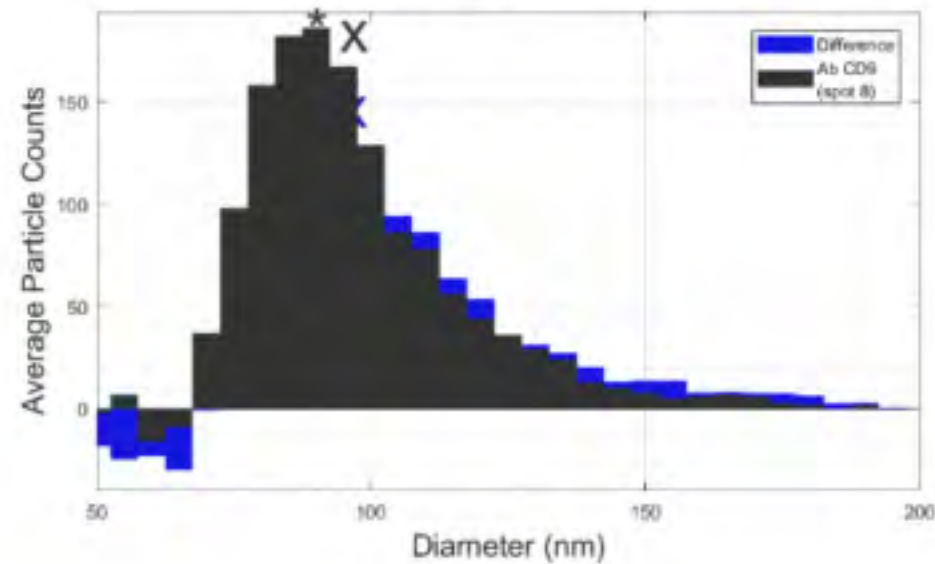
Single-EV counting and phenotyping



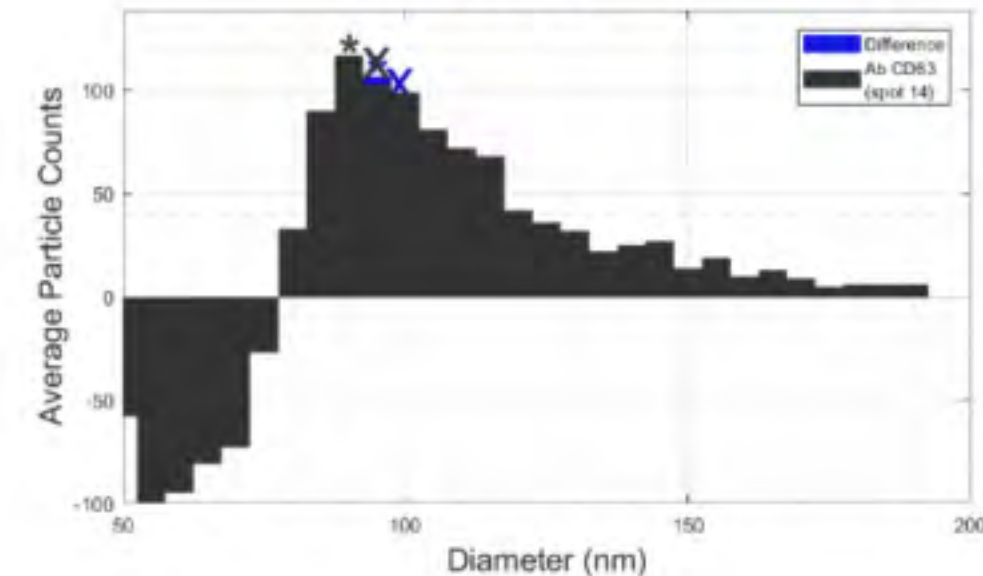
Marker 1



CD 9



CD 63



Conclusions

True requirement for EV based diagnostics: streamlined isolation and sensitive quantification of associated molecules, taking into considerations EV complexity and heterogeneity

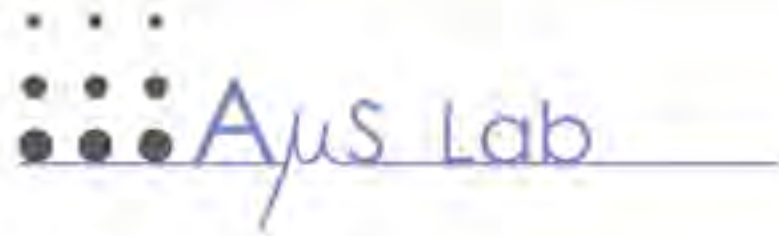
A Si/SiO₂ platform enable MULTIMODAL microarray assays:

- 1. Quantification/quality control of immobilized ligands (label free)**
- 2. High sensitivity due to enhanced fluorescence by constructive optical interference**
- 3. Single particle counting by enhanced particle scattering**

Surface properties are controlled by polymeric coatings

The combination of these technologies allows counting, sizing and phenotyping of extracellular vesicles in a high-throughput manner

Acknowledgements



Marcella Chiari
Francesco Damin
Laura Sola
Alessandro Romanato
Tea Odinolfi
Dario Brambilla
Lucia Ferraro
Elisa Chiodi



Selim M. Unlu
Natasa Zarovni



Riccardo Vago
Silvia Galbiati



H2020-FETOPEN-2016-2017 – Grant agreement n° 766466
<http://www.indexproject.eu>





THANK YOU

