Introduction : Methods of characterization of extracellular vesicles / exosomes

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Workshop « Vésicules Extracellulaires / Exosomes & Cancer » - Cancéropole-GSO

5 Décembre 2017 - Toulouse

PLAN

I- Question de nomenclature :

Extracellular Vesicles, Exosomes, ...

II- Imaging and quantification of Extracellular Vesicles

by immuno-cryo-TEM and flow cytometry

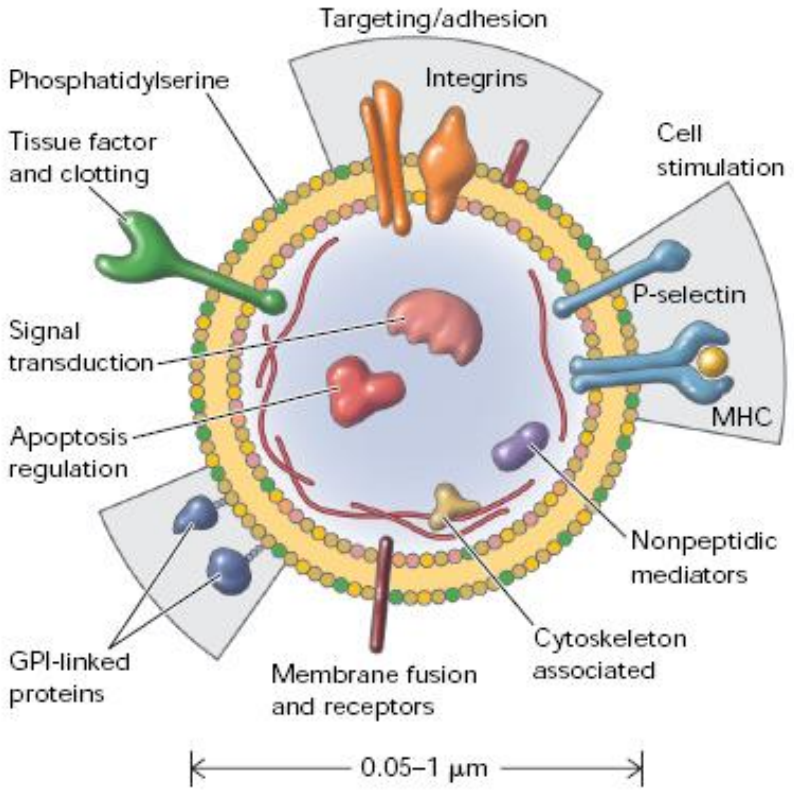
Extracellular Vesicles, Microparticles, Microvesicles, Exosomes

• Cells release membrane vesicles in the extracellular milieu upon activation

• EVs are found in body fluids: plasma, urine, CSF, .. and in cell culture supernatants

• EVs present surface receptors and contain elements (RNAs, miRNAs ..) allowing to identify their cell of origin

• EVs participate in a multitude of processes in health and disease



Hugel et al 2005 *Physiology*

• EVs present bio-medical applications, as disease biomarkers, therapeutic agents, drug delivery systems, vaccines, …

Un peu d'histoire …

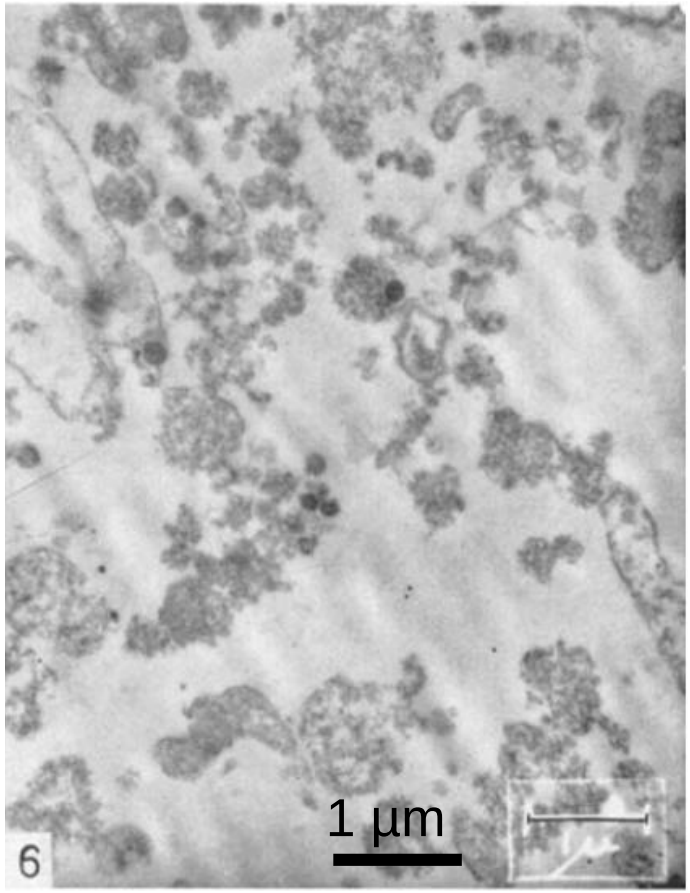
Plasma contains minute particulate material, called "platelet dust", responsible for coagulant activity.

Peter Wolf *Brit. J. Haemat. 1967, 13:269-288*

The purpose of the present communication is to provide evidence for the occurrence in normal plasma, serum and fractions derived therefrom of coagulant material in minute particulate form, sedimentable by high-speed centrifugation and originating from platelets, but distinguishable from intact platelets. It is suggested that this material, hereafter referred to as 'platelet-dust', is responsible for the phenomena referred to above. (*coagulant activity*)

à l'origine des termes

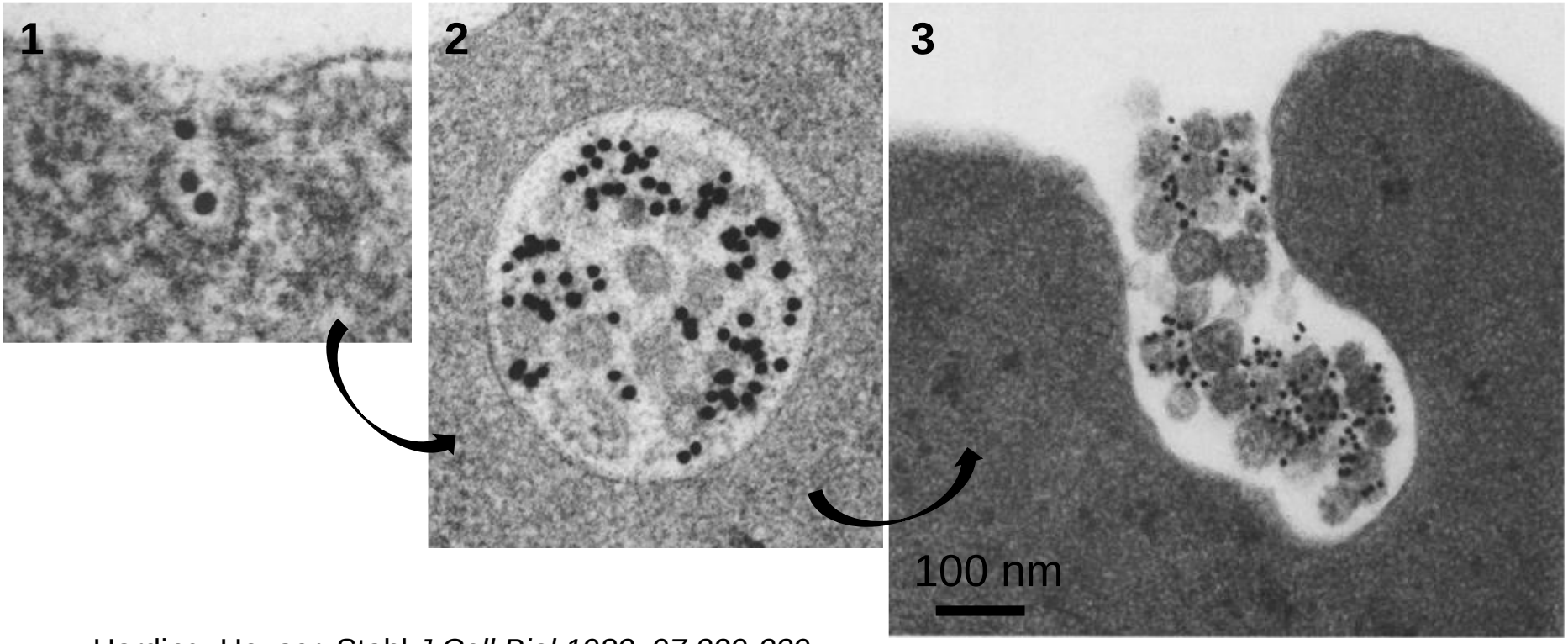
Microparticles, Microvesicles



Platelet dust from serum

"… for the most part, the material consists of agglutinates of small particles"

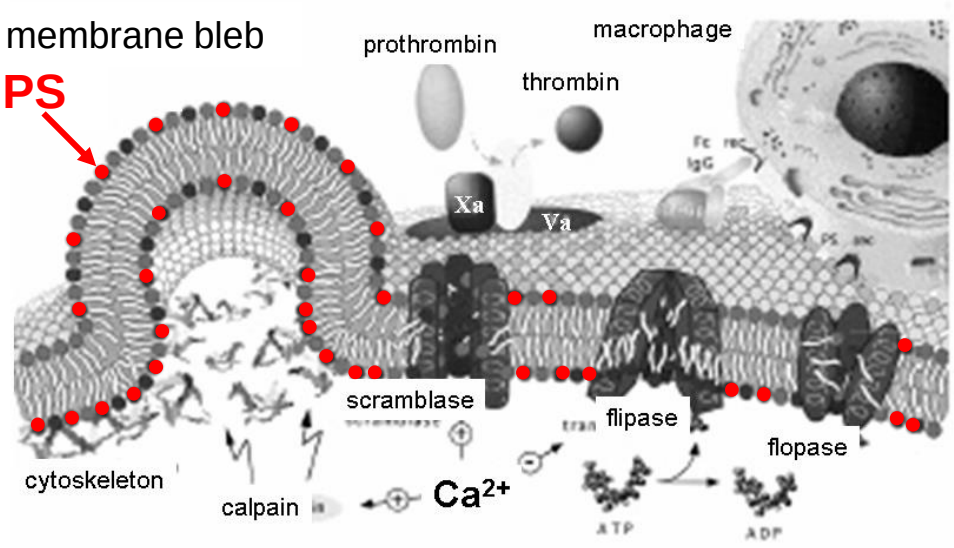
The formation of **exosomes**: a functional mechanism for shedding membrane proteins during maturation of reticulocytes into erythrocytes



Harding, Heuser, Stahl *J Cell Biol 1983, 97:329-339*

Johnstone, Pan et al. *J Cell Biol 1985, 101:942-948*

Mechanisms of formation of extracellular vesicles

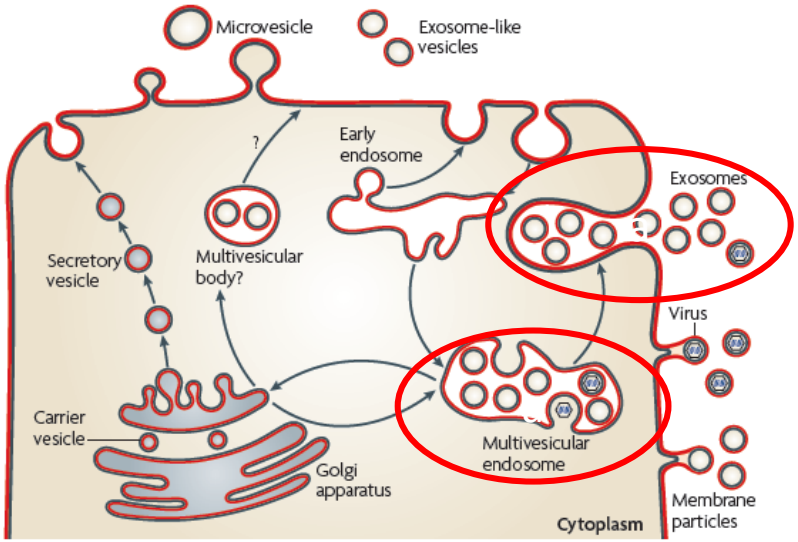


Zwaal & Schroit 1997, *Blood* 89 (4):1121-1132

Microparticles, Microvesicles

(100 nm - 1 µm) form at the cell plasma membrane:

- entrance of Ca^{2+} & increase of $[Ca^{2+}_i]$
- Ca^{2+}-dependent regulation of enzymes
- loss of membrane phospholipid asymmetry
- exposure of phosphatidylserine (**PS**) on the outer membrane leaflet
- blebbing and shedding of microparticles

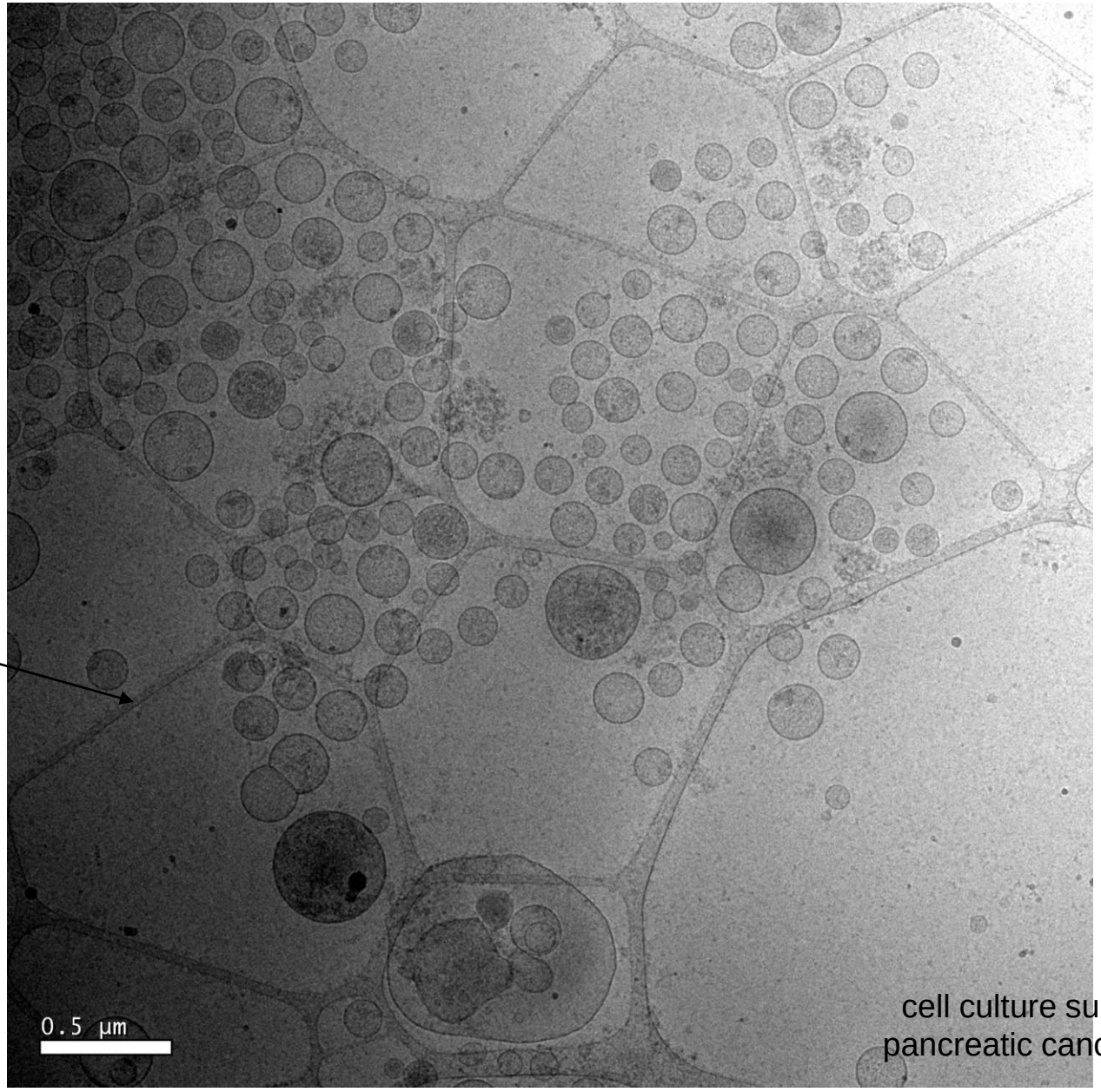


Théry et al. *Nat. Rev. Immunology* 2009, 9:581-593

Exosomes (50 - 100 nm)

- form in multi-vesicular bodies (MVBs)
- are released in the extracellular milieu after fusion of MVBs with the cell plasma membrane
- rich in tetraspanins (CD63, CD81, CD9)

Sur quels critères distinguer les vésicules issues de la membrane plasmique de celles issues des MVBs, … ?



cell culture supernatant
pancreatic cancer cell line

1- La taille ?

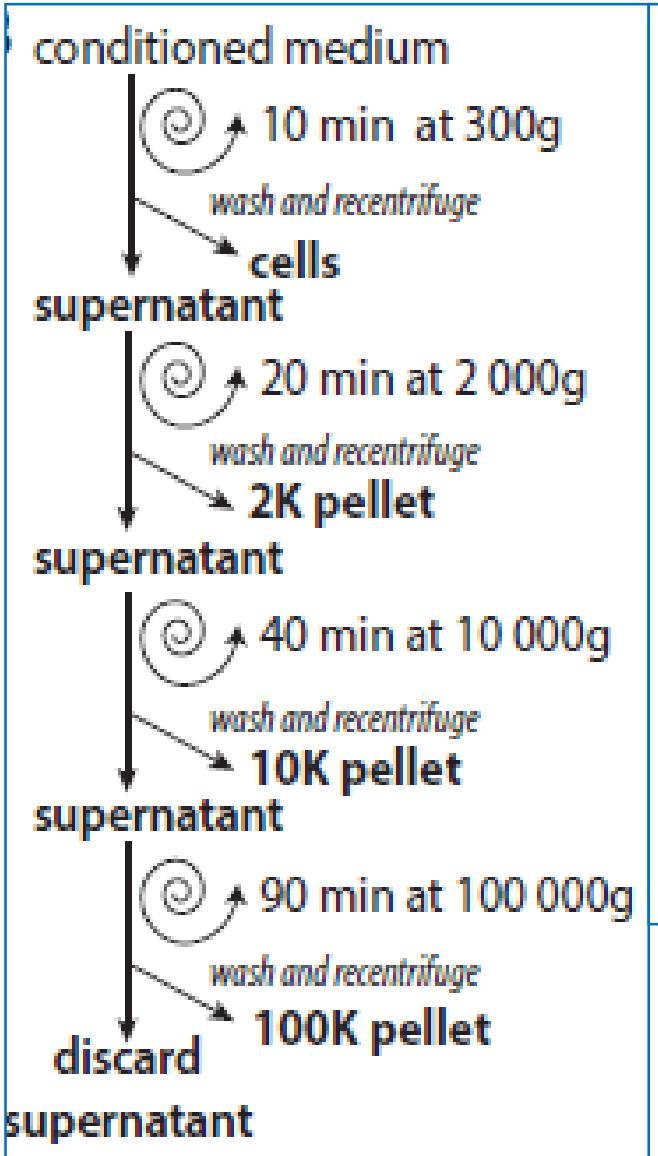
- Exosomes : 30 nm à 100-150 nm
- Toutes les vésicules < 150 nm sont-elles des exosomes ?

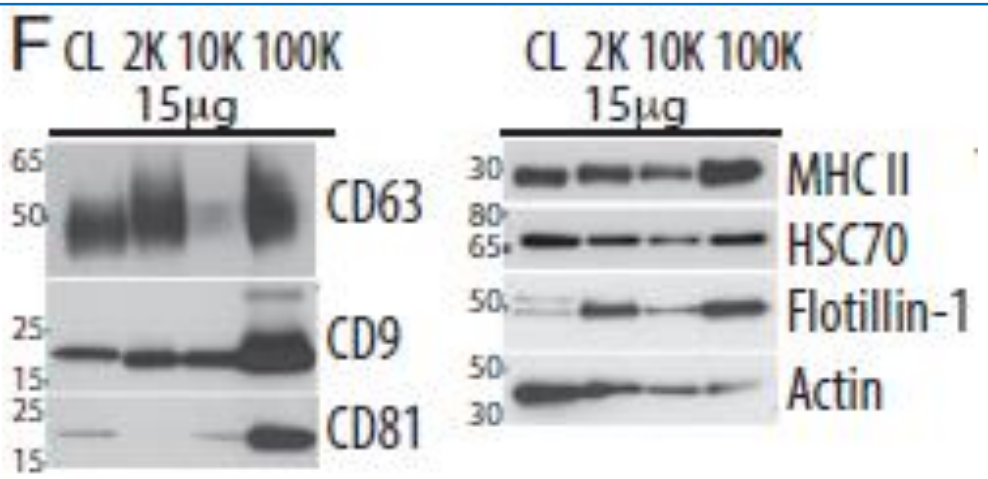
2- La présence de marqueurs de surface ?

- Toutes les vésicules issues de la membrane plasmique exposent-elles de la PS ?
- Quels sont les marqueurs spécifiques des exosomes ?
  - tetraspanins: CD63, CD81, CD9
  - MHC-I, MHC-II, flotillin, HSC-70, …

Heterogeneity of EVs isolated from a single cell type (human dendritic cells)

(Kowal et al., PNAS 2016)





"…. As expected for sEV-markers, tetraspanins (CD63, CD9, CD81) are enriched in 100K, but CD9 and CD63 are also abundant in the 10K and 2K pellets respectively.

Other putative sEVs markers (MHC II, HSC70, flotillin-1, actin) are ubiquitously present in the three pellets." ….

Recommandation de l'ISEV (2012)

Use the term "Extracellular Vesicle" … when there is no proof of the vesicle origin (plasma membrane, MVB, other …)

Cette recommendation est loin d'être suivie de manière rigoureuse,

entre autre par les compagnies qui commercialisent des "exosome isolation kits"

Box 1 Nomenclature issue

Here, we use the term 'exosomes' as defined by Rose Johnstone in 1987 [7], and not the more general use for any vesicles released by cells [79]. Currently, the use of the term 'exosomes' for MVB-derived EVs is generally accepted in the field, although the variety of EVs secreted by cells and difficulties in proving the actual origin of EVs led to a less strict usage: either for any small EVs (of 50–100 nm diameter by transmission electron microscopy), or for EVs recovered after 100 000 × *g* ultracentrifugation. As proposed recently by S Gould and G Raposo [80••], given the lack of perfect demonstration of EVs' endosomal origin, we can only suggest that researchers clearly specify their interpretation of whatever term they use for the EVs they analyse.

Kowal, Tkach & Théry Curr. Opin. Cell Biol. 2014, 29

Methods of EV characterization

***Single EV detection methods**:*

- Flow cytometry
- Nanoparticle Tracking Analysis (NTA)
- Electron microscopy
- Resistive Pulse Sensing (tRPS, qNano)
- Atomic Force Microscopy

***Bulk methods**:*

- Western blotting
- "Omic" methods: mass spectrometry, RT-qPCR
- Functional assays: measurement of procoagulant activity, …
- Surface-sensitive methods: SPR, SPFS, ..
- …

Methodological Guidelines to Study Extracellular Vesicles
Coumans et al., *Circulation Research* 2017, 120:1632-1648

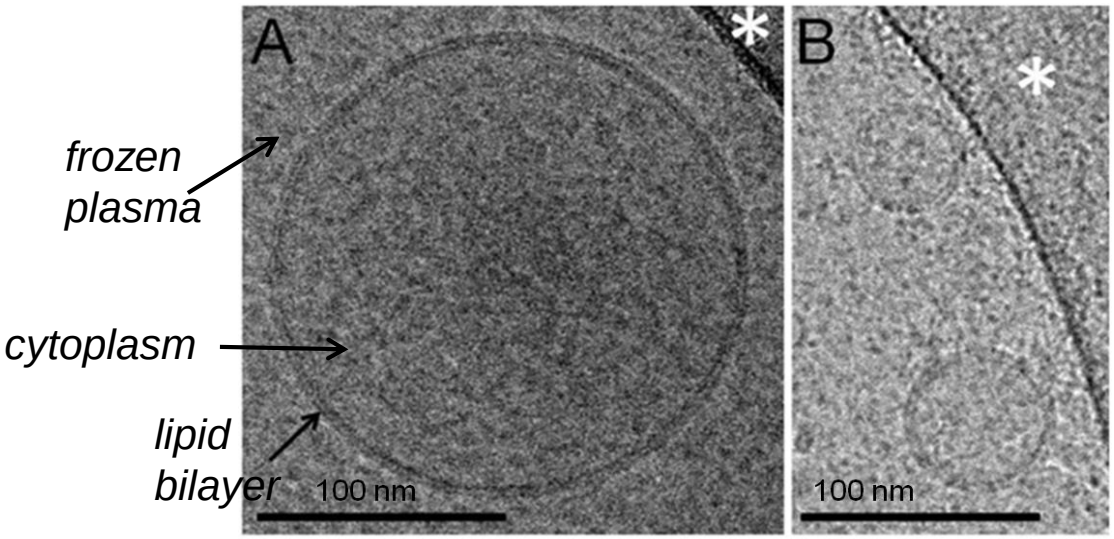
II- Imaging and quantification of Extracellular Vesicles,

by cryo-transmission electron microscopy (TEM),

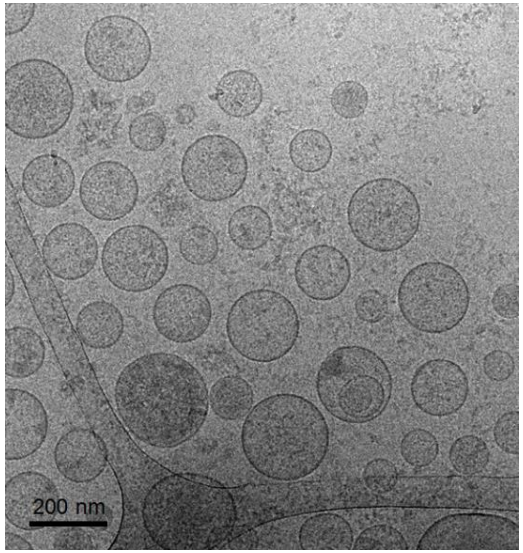
immuno-gold labelling and flow cytometry

1- Cryo-TEM, the gold-standard method for imaging EVs

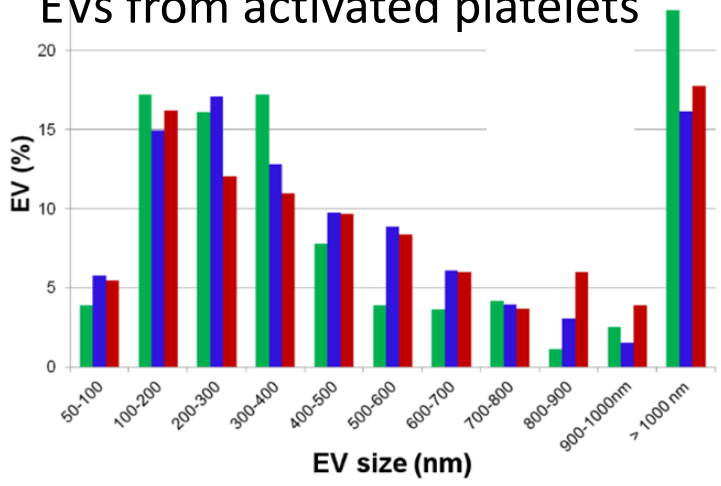
EVs in pure plasma



EVs from BxPC3 cells



EVs from activated platelets



For most/all body fluids

- EVs range in size from 30-50 nm to over 1 µm
- the majority of EVs measures from 50 to 500 nm

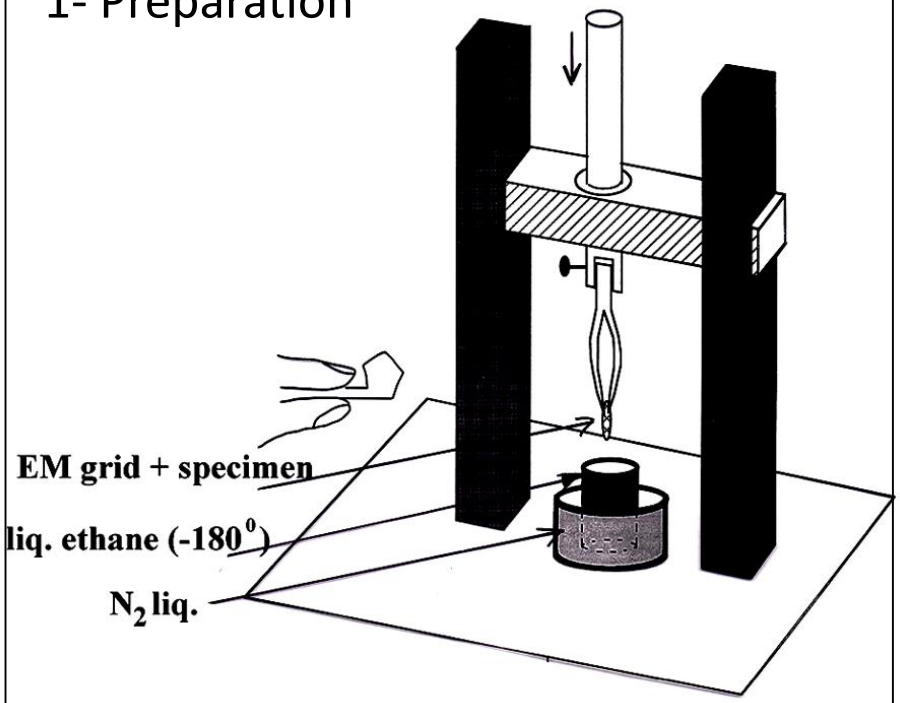
Arraud et al., *J. Thrombosis & Haemostasis*, 2014, 12: 614
Brisson et al., *Platelets* 2017 28:263

Principle of Cryo-TEM :

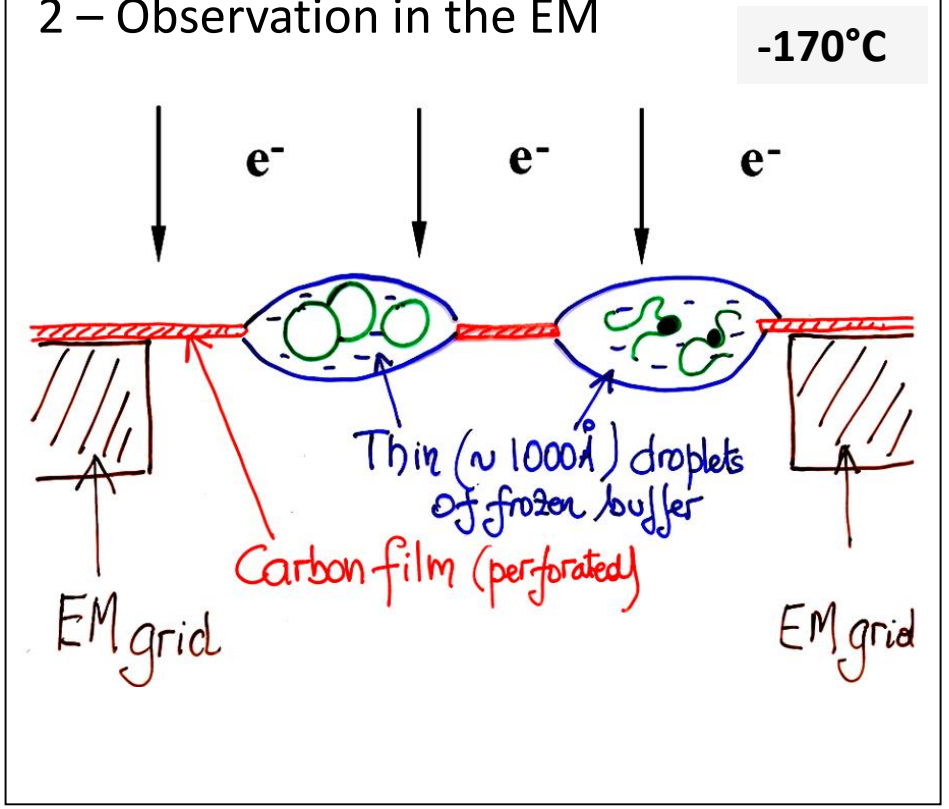
1- a sample is quickly frozen by plunging in a cryogen,

2- then is observed at low T in the microscope

1- Preparation



2 – Observation in the EM



- cryo-EM is the least invasive EM method
- samples are not dried, not fixed, not stained, not thin-sectioned
- samples are observed in their native hydrated state

The Nobel Prize in Chemistry 2017

© Nobel Media. Ill. N. Elmehed

Jacques Dubochet

Prize share: 1/3

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Joachim Frank

Prize share: 1/3

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Richard Henderson

Prize share: 1/3

The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank and Richard Henderson *"for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution"*.

The standardization of EV preparation methods is a major challenge in EV research

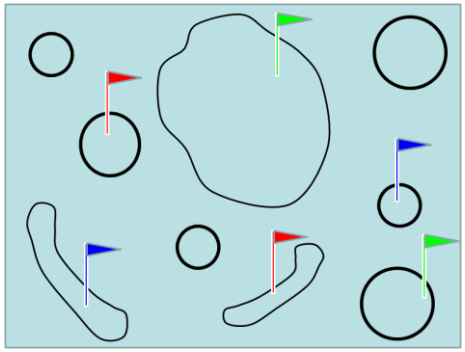
*Platelet-Free Plasma (PFP)

- blood collected over citrate

- 2 x (2,500g x 15 min, 25°C)

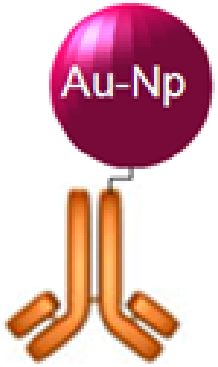
(Lacroix et al. *J. Thromb. Haemost. 2012 10,437-446)*

2- Cryo-TEM combined with *immuno*-gold labelling allows phenotyping EVs

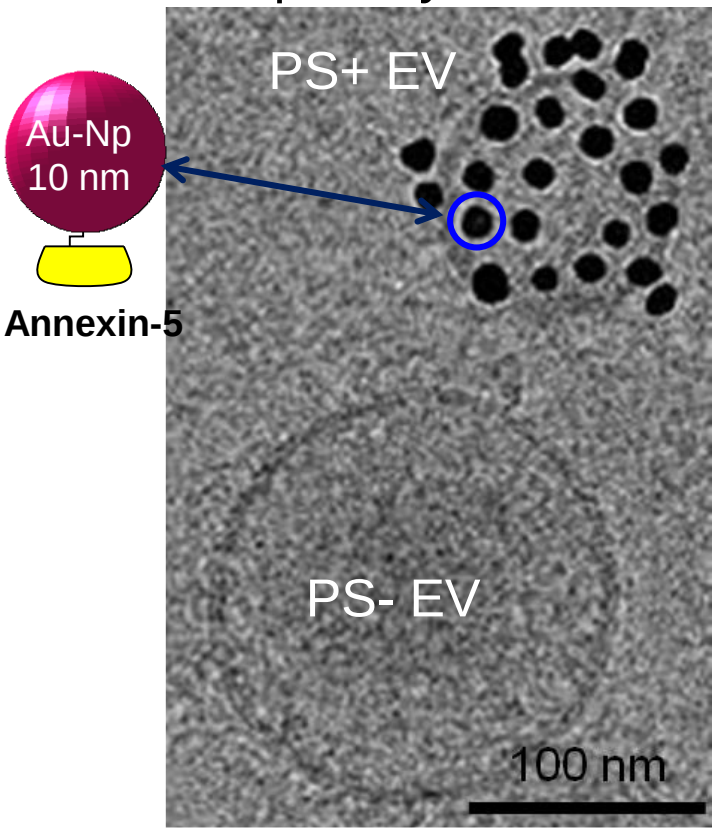


Identification of EV phenotype

tool : protein-conjugated gold nanoparticles



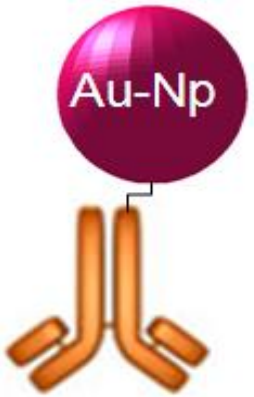
Phosphatidylserine+ EV



- ***A minority of EVs bind Anx5/expose PS in plasma …. & in most/all body fluids***

- *in contrast with the classical theory of EV formation at plasma membranes*

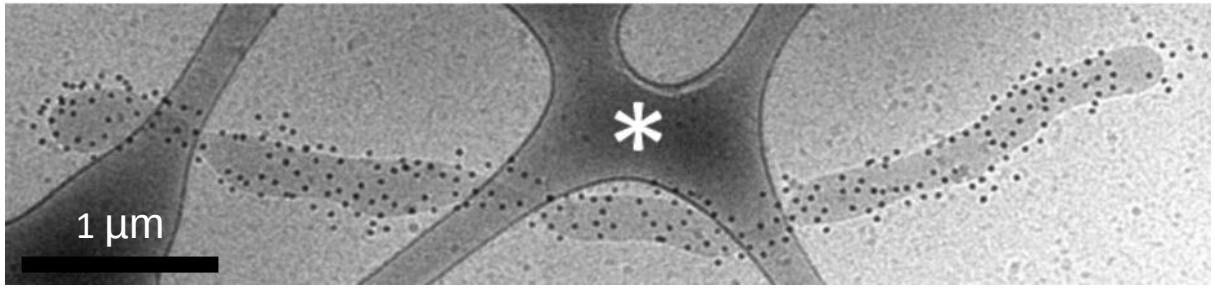
Phenotyping plasma EVs by Immuno-Cryo-TEM



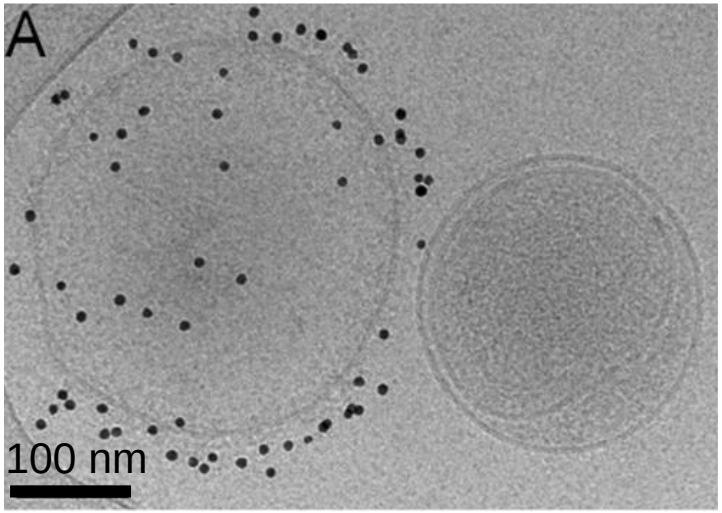
cell-specific IgG

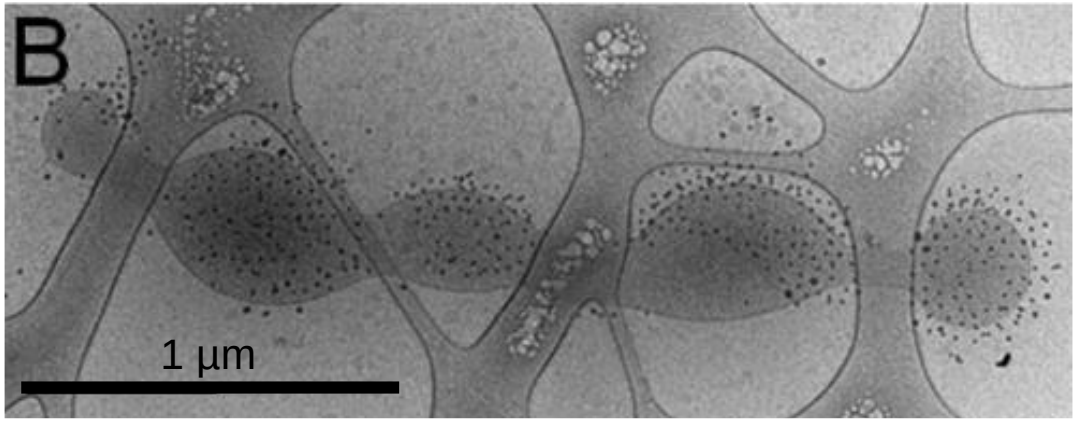
Erythrocyte-derived EVs

CD235a-Ab gold particles

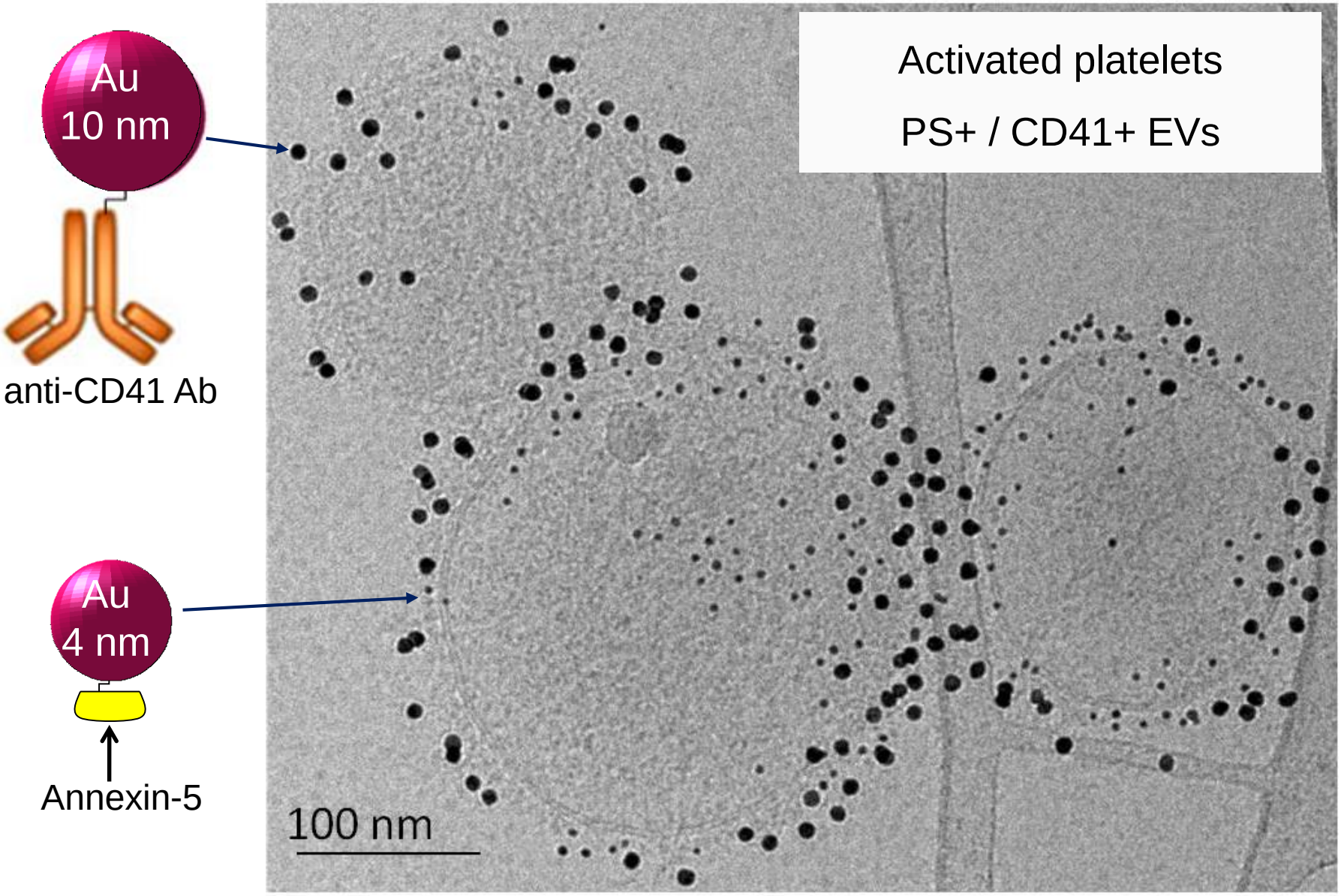


Platelet-derived EVs - CD41-Ab gold particles

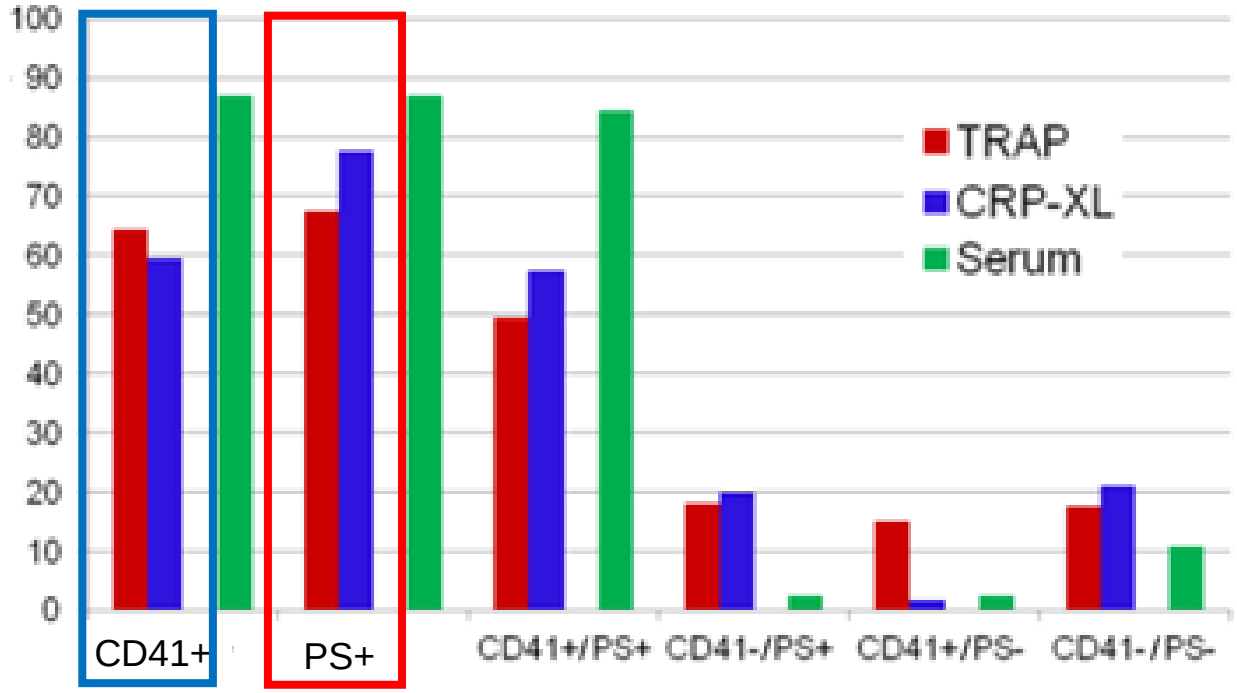




Study of EVs released by activated platelets



Distribution of EV phenotypes in activated platelets



- ~ 25 % of EVs from activated platelets do not expose PS

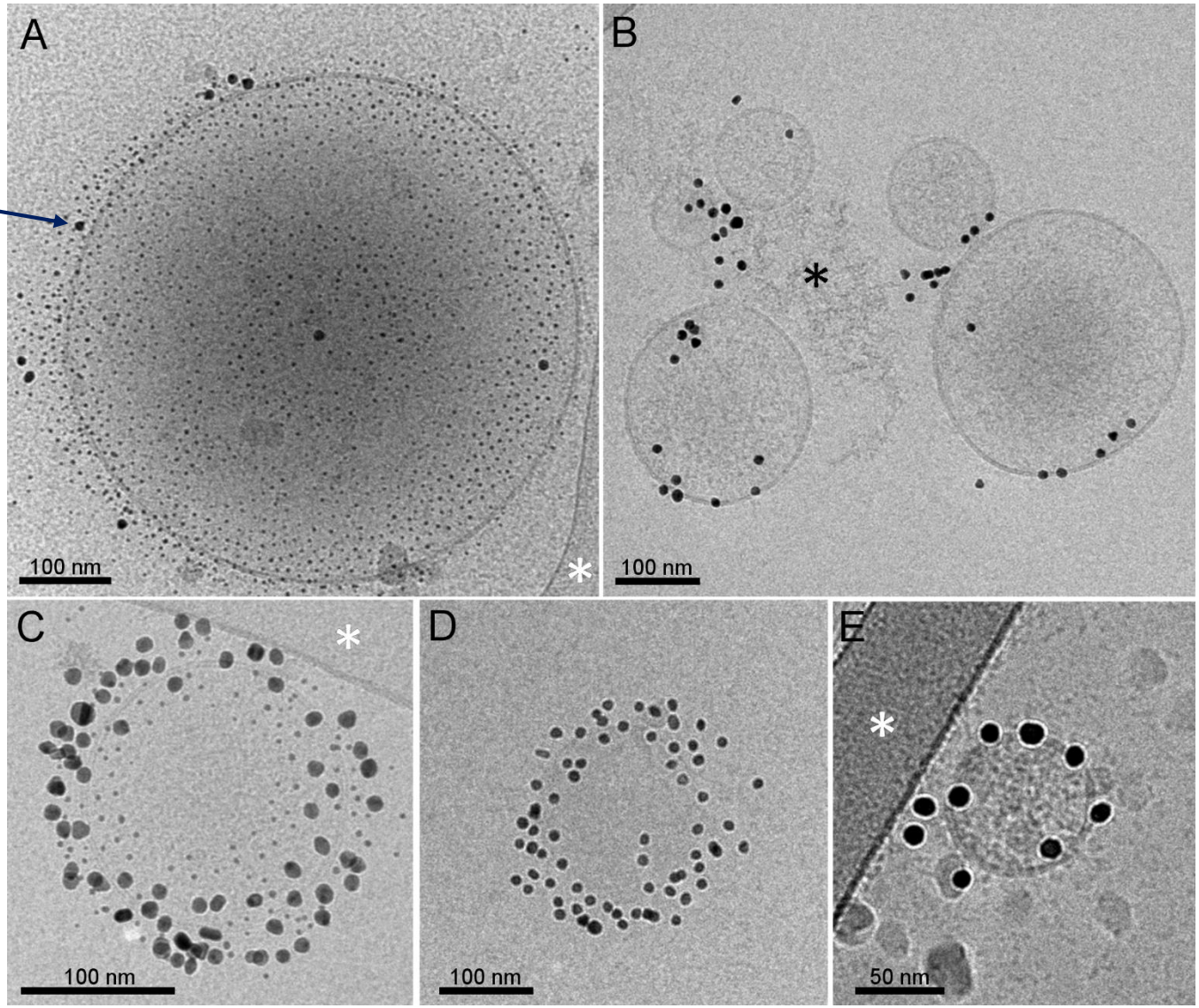
- ~ 40 % of EVs from activated platelets do not expose CD41

- How do they form ? Do they have a function ?

Brisson et al., *Platelets* 2017 28:263-271

Gallery of CD63-exposing EVs in activated platelets

anti-CD63 mAb

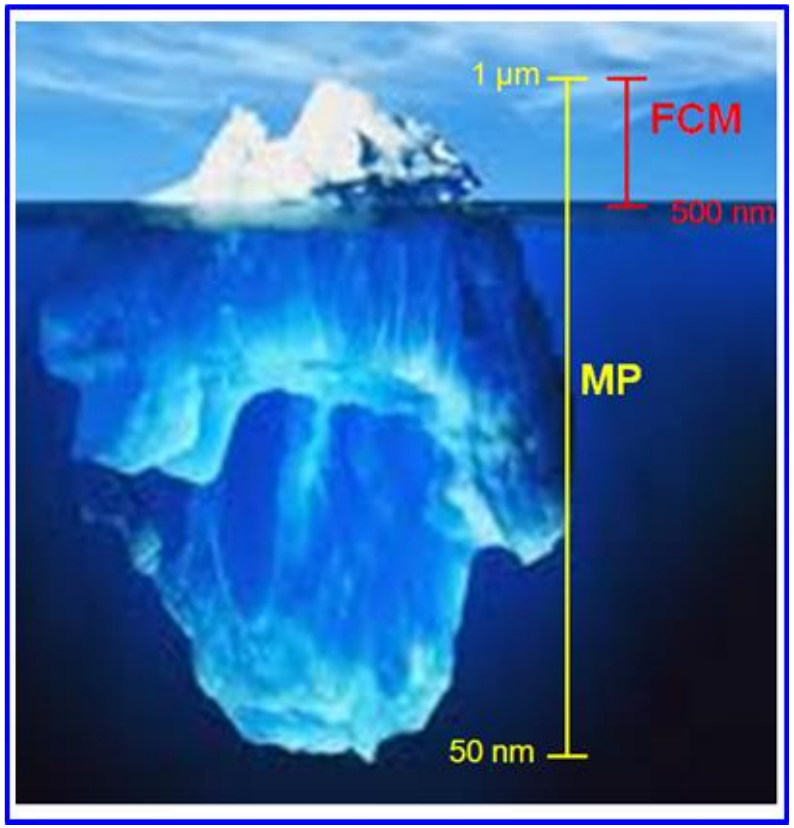


1- Most/All EVs expose CD63, whatever their size

2- The smallest EVs -*the exosomes (?)*- present a higher density of CD63

3- EV quantification

Flow cytometry

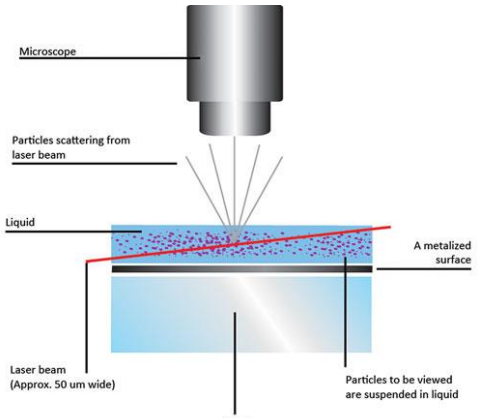


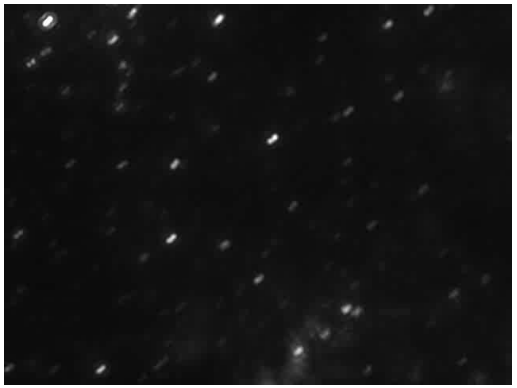
Flow cytometers are able to detect polymer particles of ~200 nm

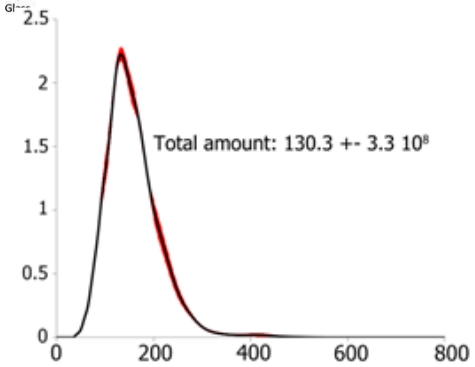
but detect only

"the peak of EVs' iceberg"

Nanoparticle Tracking Analysis







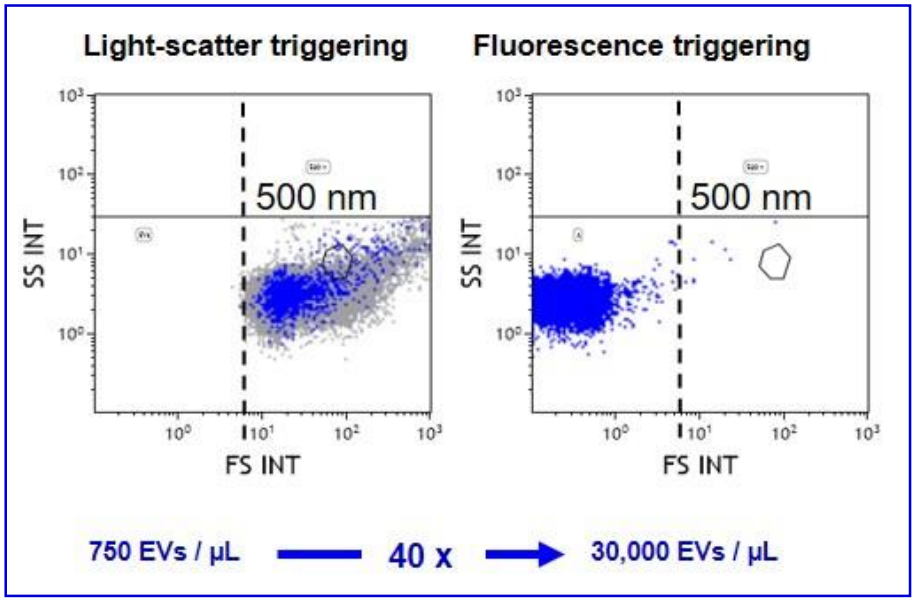
NTA detects particles down to ~20 nm

but detects also non EV material,

e.g. lipoproteins

van Deun et al., J. Extracell. Ves.2014

EV quantification by Flow Cytometry



Arraud et al., *J. Thromb. Haemost.* 2015 13:237

Catalog of EVs in healthy plasma

EV concentrations in plasma (/ µL)	
Anx5+ / CD41+	10 000
Anx5+ / CD41-	25 000
CD41+ / Anx5+	12 000
CD41+ / Anx5-	10 000
Anx5+ / CD235a+	1 000
Anx5+ / CD235a-	35 000
CD235a+ / Anx5+	1 500
CD235a+ / Anx5-	10 000

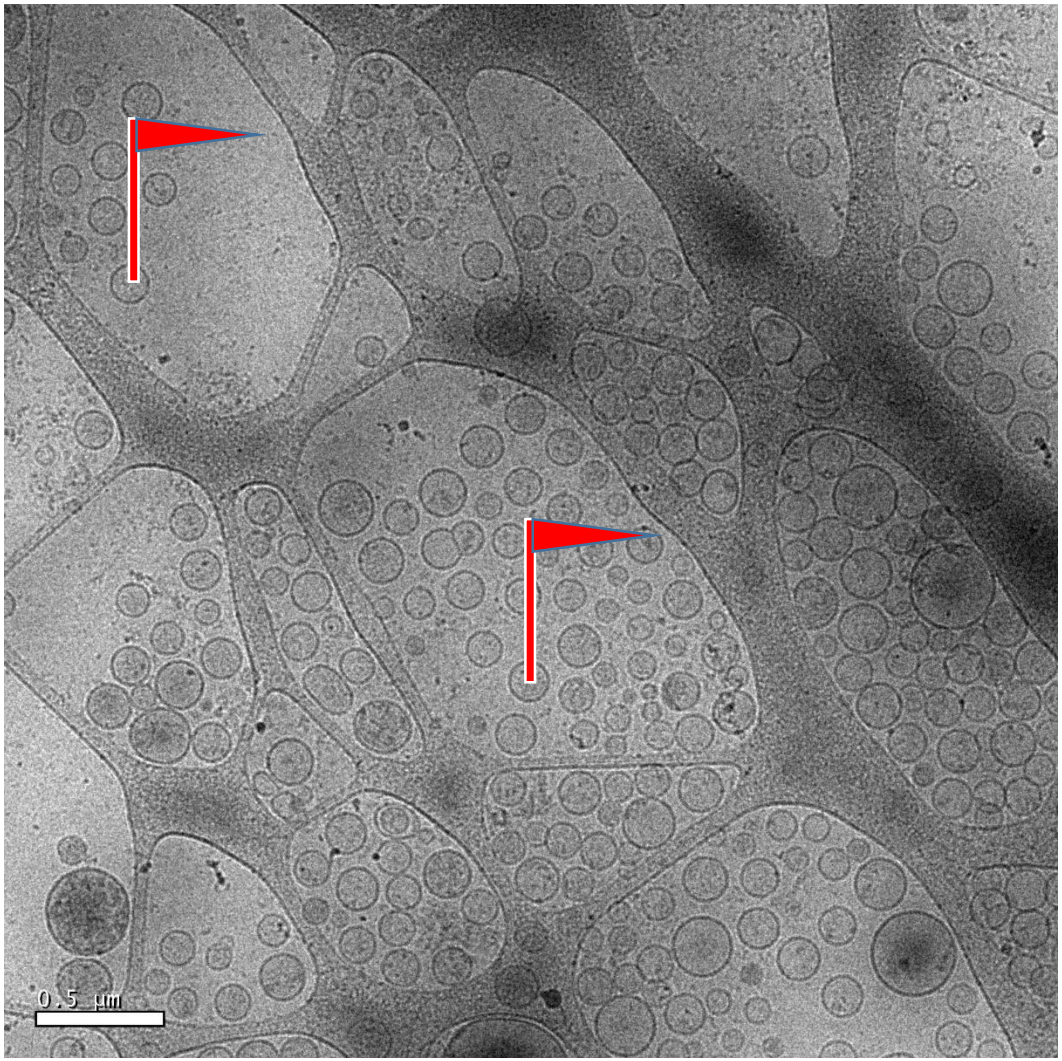
N. Arraud, C. Gounou, D. Turpin, A.R. Brisson, ***Cytometry A*** 2016 89(2):184-95

Fluorescence triggering : a general strategy for enumerating and phenotyping EVs by flow cytometry

"Best paper of the year in Cytometry-A 2016 (ISAC-2017)"

4- Characterization of EVs in diseases

Can Immuno-Cryo-EM help identifying disease-specific EV signatures ?



Conditioned medium from pancreatic cancer BxPC3 cells

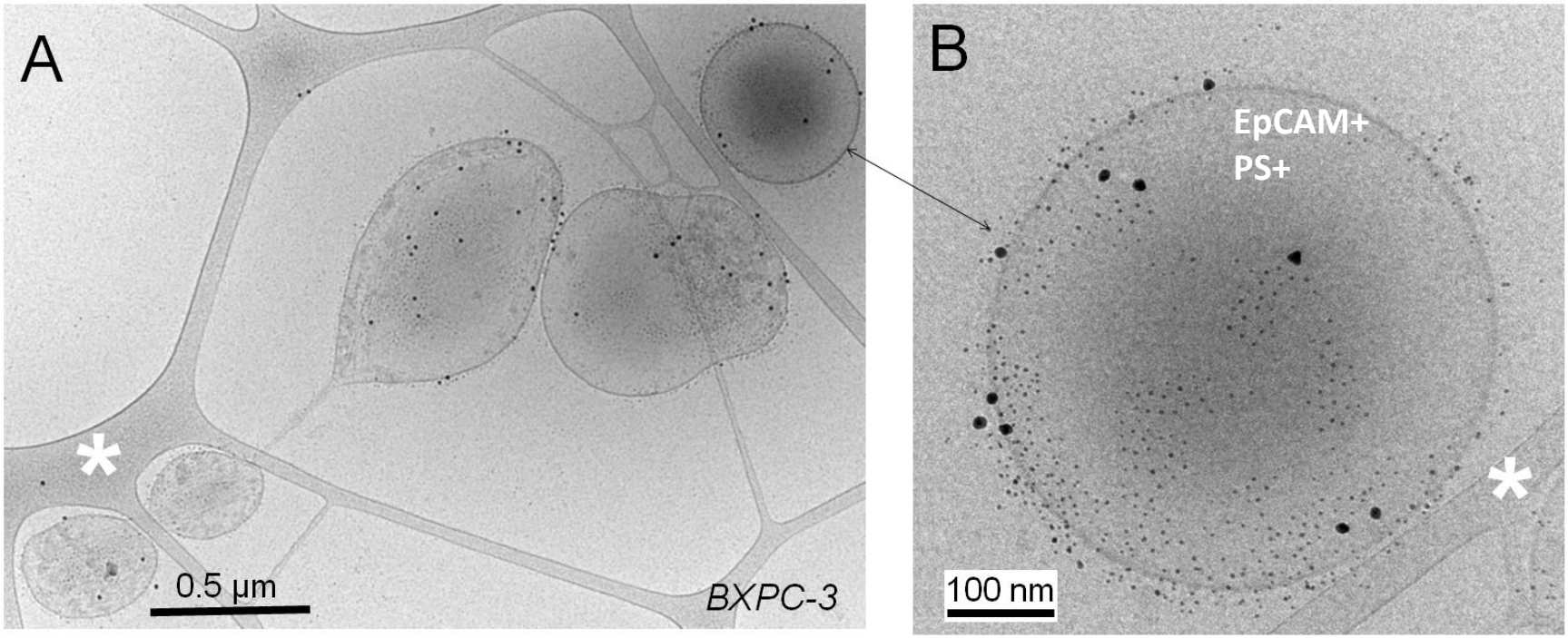
Characterization of EVs in Pancreas Cancer

Sandrine Dabernat, Etienne Buscail (UMR-INSERM 1035, Bordeaux Univ., CHU-Bordeaux),
Guido David, Pascale Zimmermann (Leuven Univ.; Inst. Paoli-Calmette, Marseille)

- EpCAM
- Glypican-1
- *phosphatidylserine*

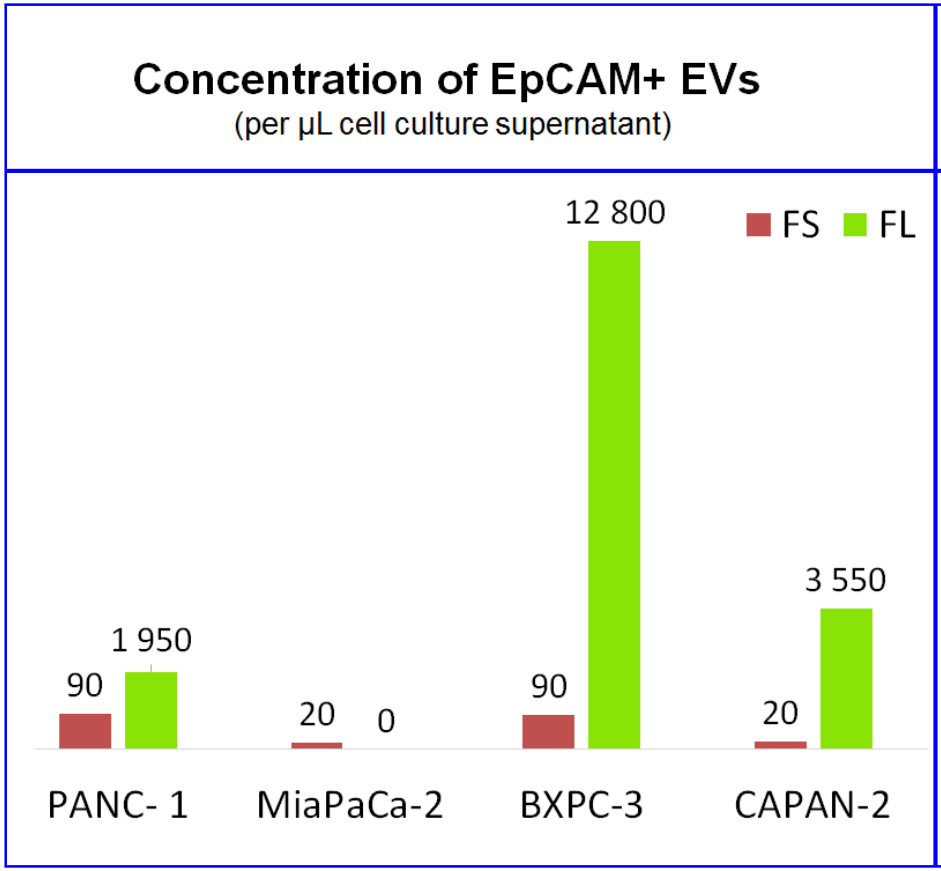
- PDAC cell lines : CAPAN-2, BXPC-3, PANC-1 MiaPaCa-2
- Plasma (platelet-free plasma) from pancreas cancer patients

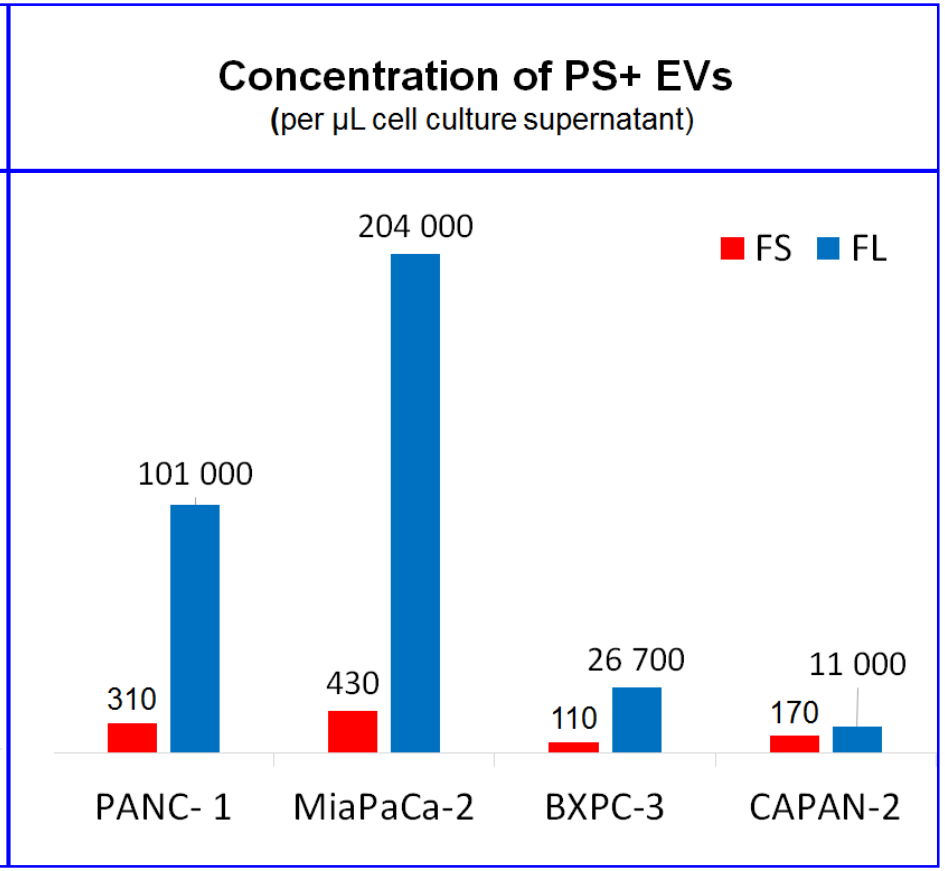
Heterogeneity of EVs in size, phenotypes, …



- cell culture supernatant
- 5,000g x 10 min

(AB, unpublished)





FS detects only large EVs, size > ~ 800 nm
FL detects small & large EVs, size > 100-200 nm

- **EpCAM+ EVs**: BXPC-3 > CAPAN-2 > PANC-1 >> MiaPaCa-2 (~ fibroblasts),

in keeping with the epithelial phenotype of BXPC-3 and CAPAN-2.

- **Double-positive EpCAM+/PS+ EVs**: BXPC-3 (70%); CAPAN-2 (50%).

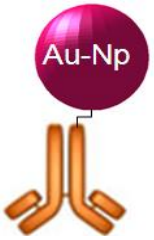
In progress: Immuno-magnetic isolation of EpCAM+ EVs *(AB, unpublished)*

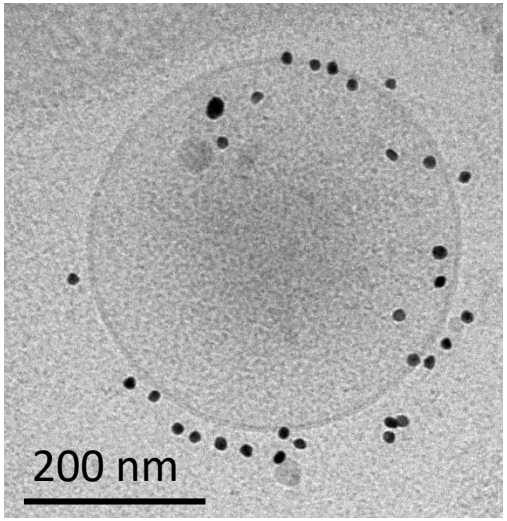
Characterization of EVs in Ovarian Cancer

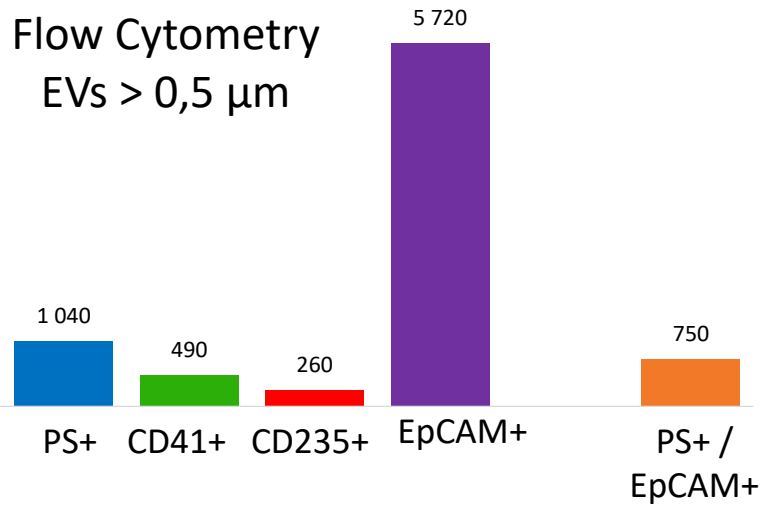
Coll. with Dr. V. Catros (CRB-Rennes), Pr. V. Lavoué INSERM-U-1242, CHU-Rennes)

Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and **EpCAM.** S. Runz et al., Gynecologic Oncology 2007, 107:563–571

EpCAM+ EVs are found in 100% ascites from ovarian cancer patients







(AB, unpublished)

4- Conclusion

- Cryo-EM allows imaging EVs in pure, unprocessed, body fluids and suspensions, giving information on EV morphology, size, heterogeneity .. phenotype, purity

- Immuno-cryo-EM and flow cytometry are highly complementary, providing quantitative description of the main EV populations

- ***Recommendations*** :
- Apply immuno-cryo-EM as a quality control assay for:
  - Ab validation
  - EV/exosome purification methods
  - EV/exosome production for therapeutic applications, providing objective evaluation of the reproducibility, presence of contaminants, concentration