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Extracellular Vesicle Isolation Efficiency: Method Comparison using Multispectral Nanoparticle Tracking Analysis

November 16, 2021

HORIBA Webinar

Particle Characterization Series

COLUMBIA | MAILMAN SCHOOL OF PUBLIC HEALTH

Overview

- Background: EVs, ALS, Research Questions
- Standardized NTA Protocol for Reproducibility
- Comparing CNS-EV Isolation Methods
- Conclusions and Future Directions

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- Standardized NTA Protocol for Reproducibility
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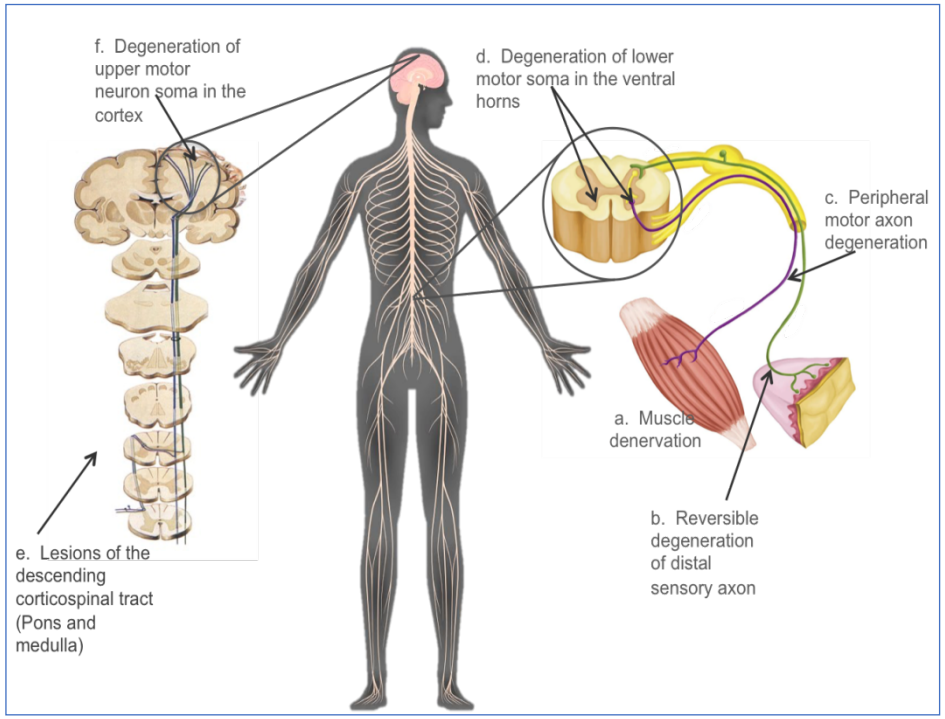
ALS: A Motor Neuron Disease with No Reliable Biomarkers of Progression

- Characterized by progressive muscle weakness, atrophy, and paralysis
- Age of onset 50-60 years
- Fatal within 1-5 years of onset of symptoms
- No cure
- ***No validated, reliable biomarker of disease progression***

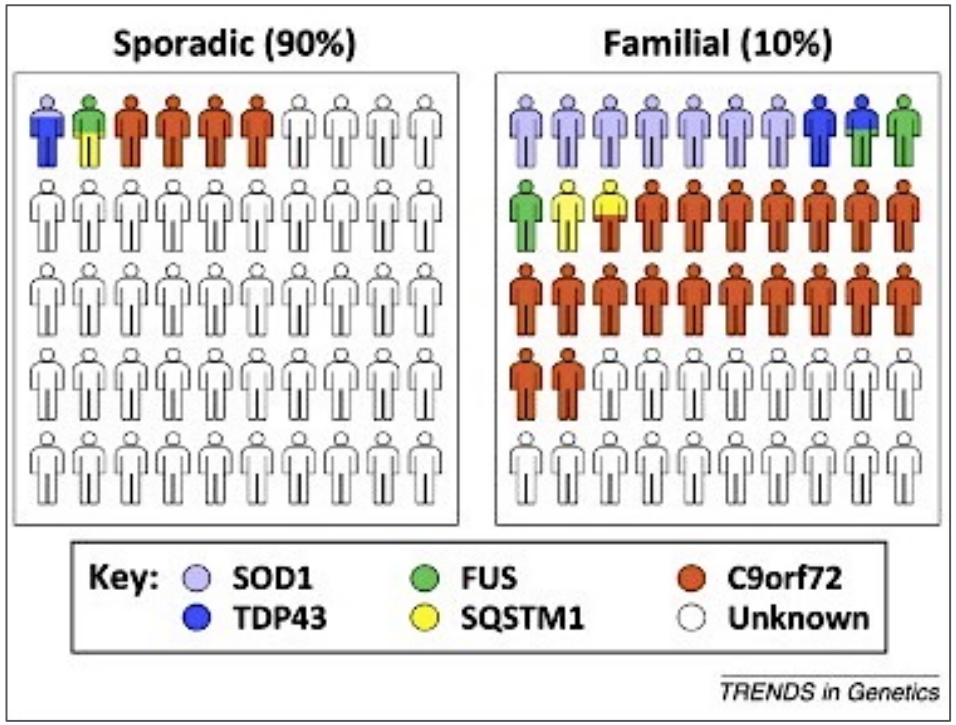
ALS: Amyotrophic Lateral Sclerosis



Merwin, Obis, Nuñez, Re. *Archives of Toxicology*, 2017

The Mysterious Etiology of ALS Points to an Environmental Origin

- 90% of cases sporadic
- Causative genetic mutations explain:
  - 68% of familial ALS
  - 11% of sporadic ALS

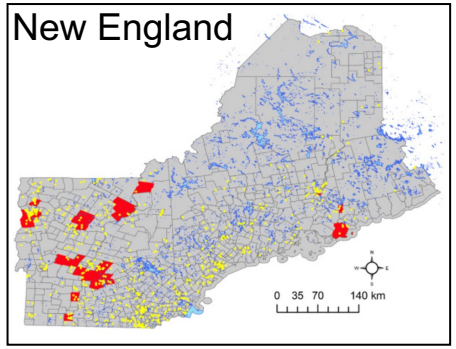


The Mysterious Etiology of ALS Points to an Environmental Origin

- ALS twin registry studies:
  - Disease discordant in ≥ 90% of monozygotic twins!

Graham et al., 1997;
Al-Chalabi et al., 2010

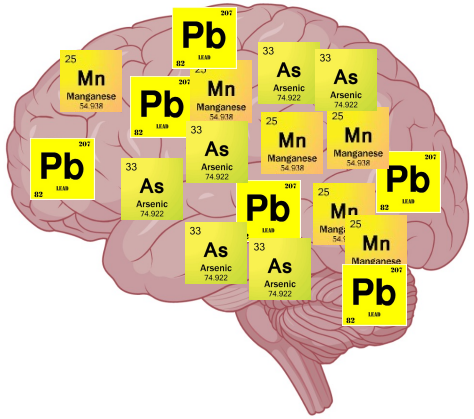
- ALS geographical clusters



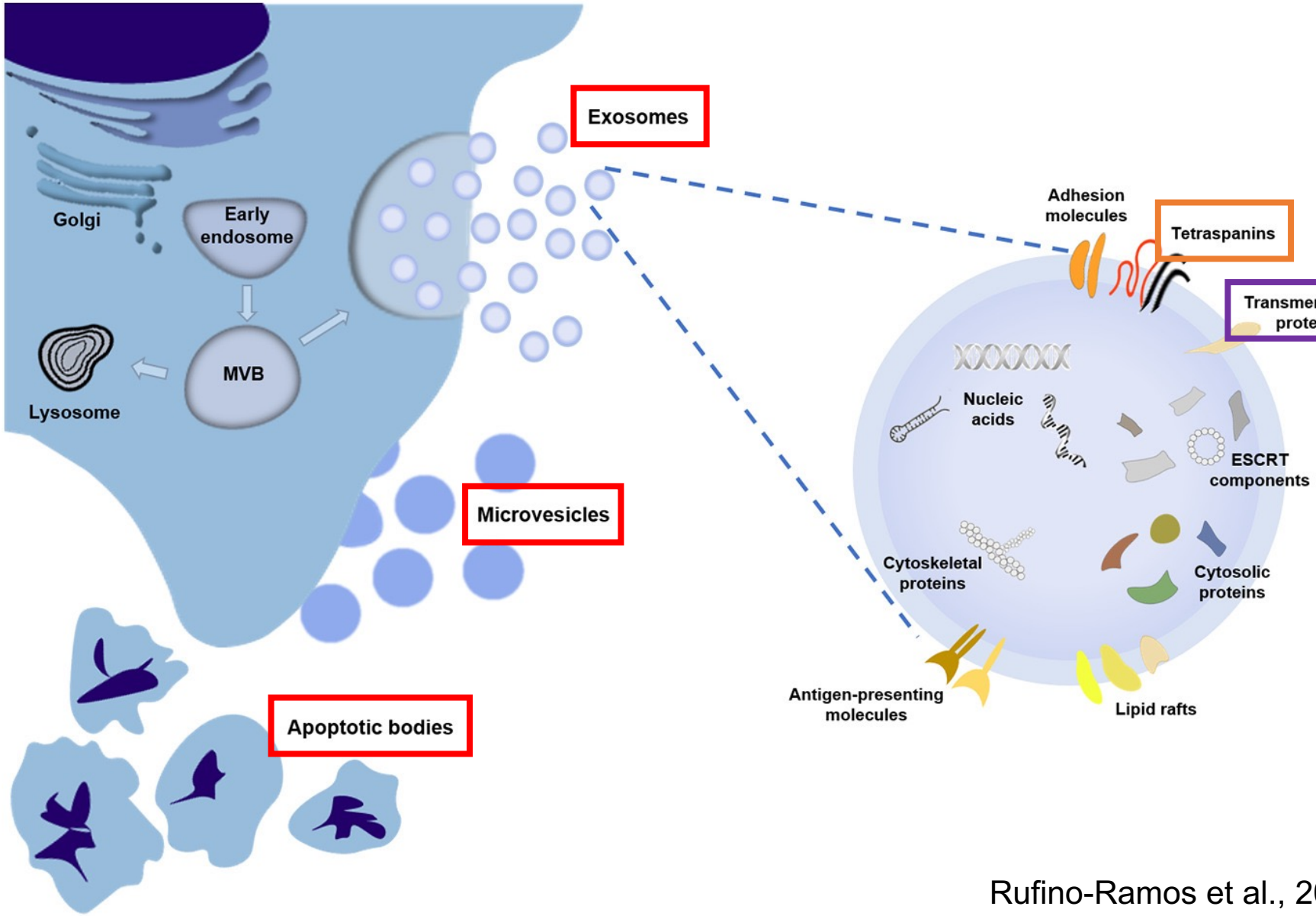
Torbick N. et al. Int J Health Geogr. 2014
Plato C. et al., Am J Epidemiol 2003

ALS Research is Hampered by Lack of Biomarkers of CNS Toxicant Exposure

- Predominantly sporadic condition (no family history) with unknown etiology
- Metal exposure has been linked to ALS risk and progression
- ***No accurate and non-invasive biomarkers of central nervous system (CNS) metal burden***
- Circulating **extracellular vesicles (EVs)** can address this urgent research gap



No access to brain metal load



"Hallmarks of EVs"

CD9

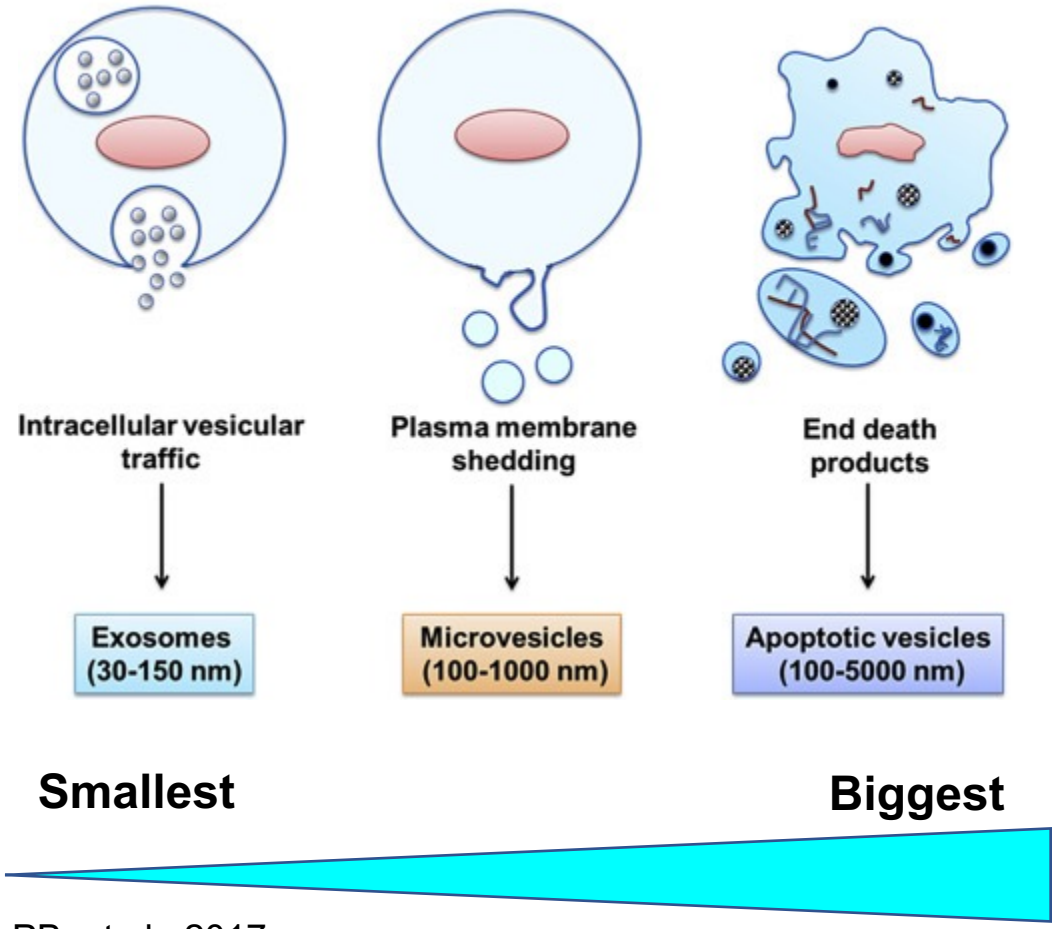
CD63

CD81

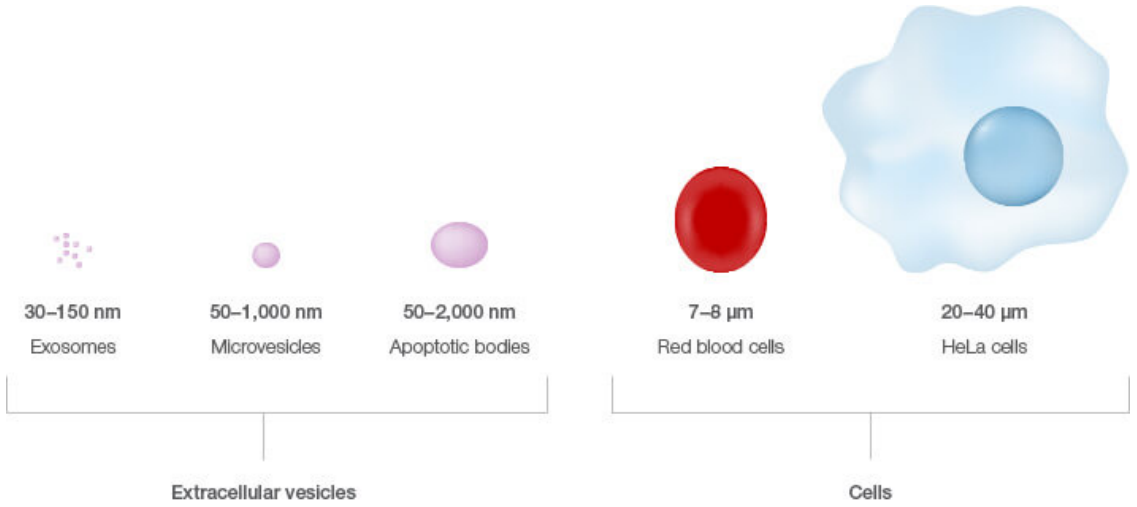
Cell-type specific

Rufino-Ramos et al., 2017, *J Control Release*

CNS EVs: New Biomarker Opportunities for Environmental Exposures and ALS



Devhare PB, et al., 2017

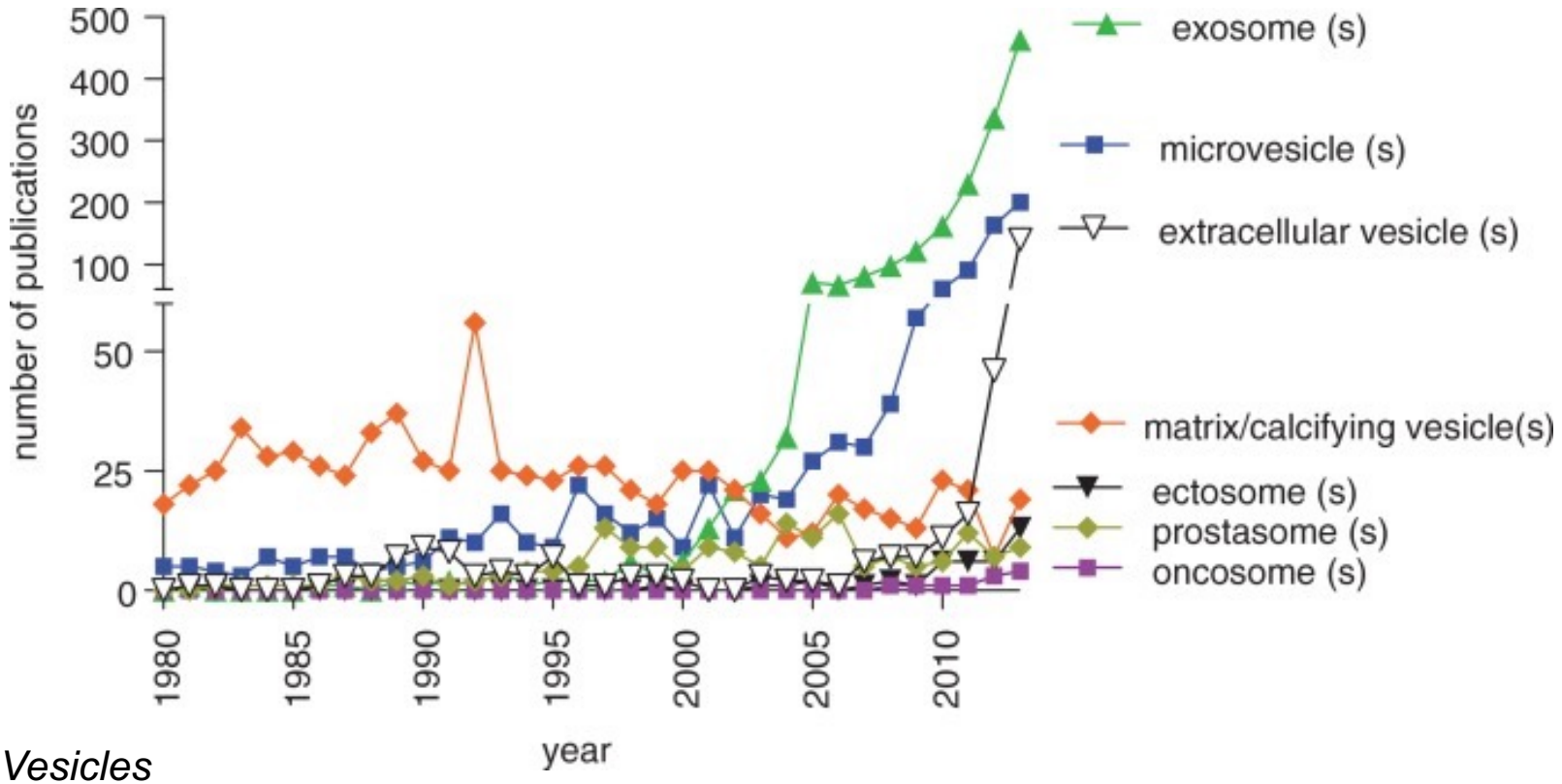


Barnette A, Bio-Rad Laboratories, Inc., 2018

EVs Found in Various Biofluids

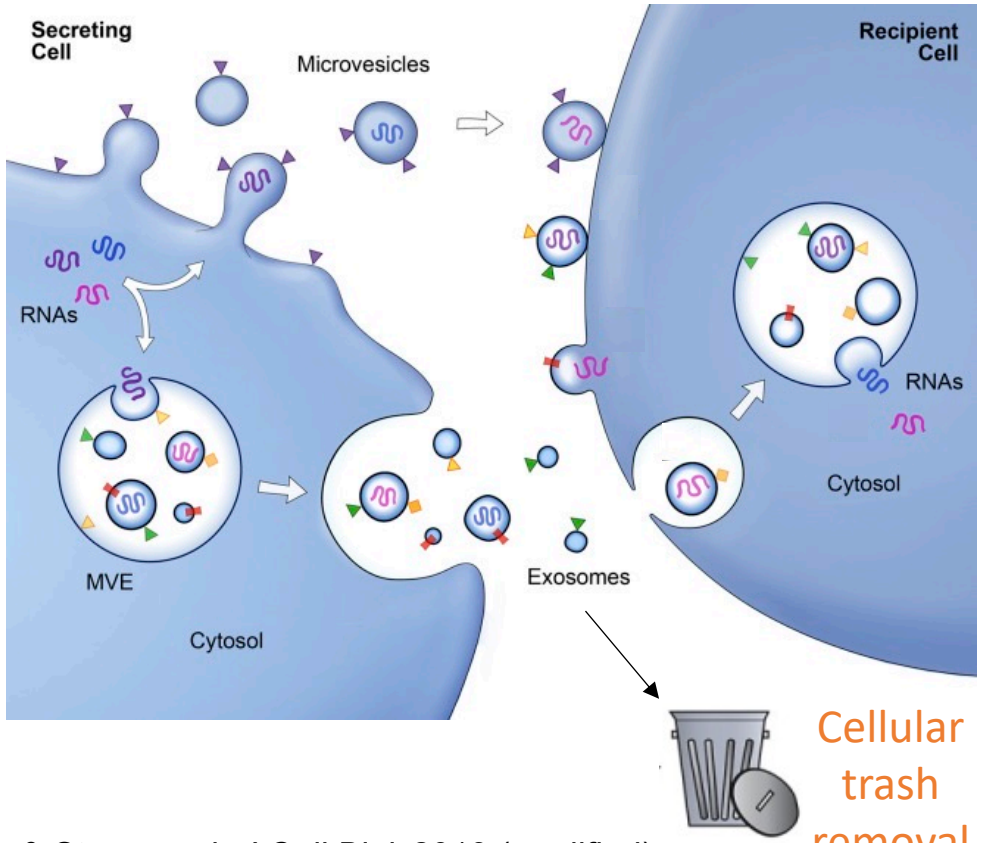
INTERNATIONAL SOCIETY *for* EXTRACELLULAR VESICLES

"Extracellular Vesicle"



Lötvall et al., 2014, *J Extracell Vesicles*

EVs Play Important Roles in the Cellular Disposal System



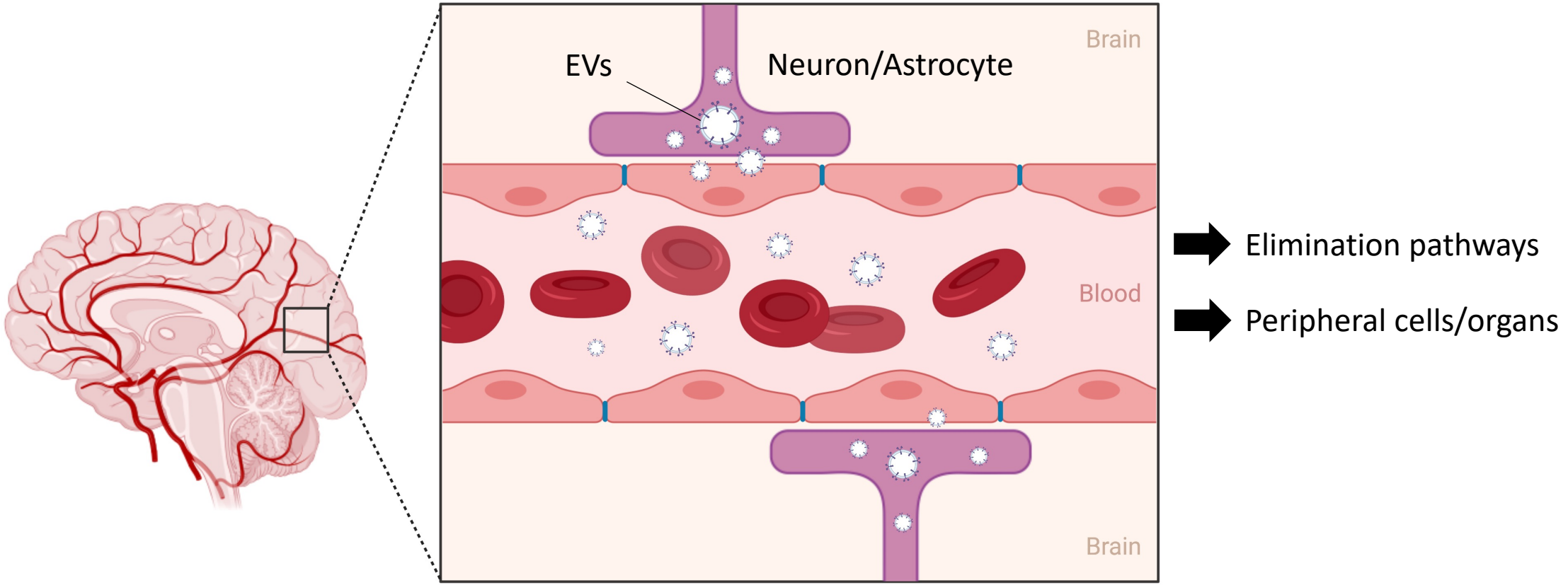
Raposo & Stoorvogel, *J Cell Biol*, 2013 (modified)

- Regulation of gene expression
- Activation of signaling
- Distribution of catalytic activity
- **Disposal system**

Why Study EVs Derived from the Central Nervous System (CNS)?

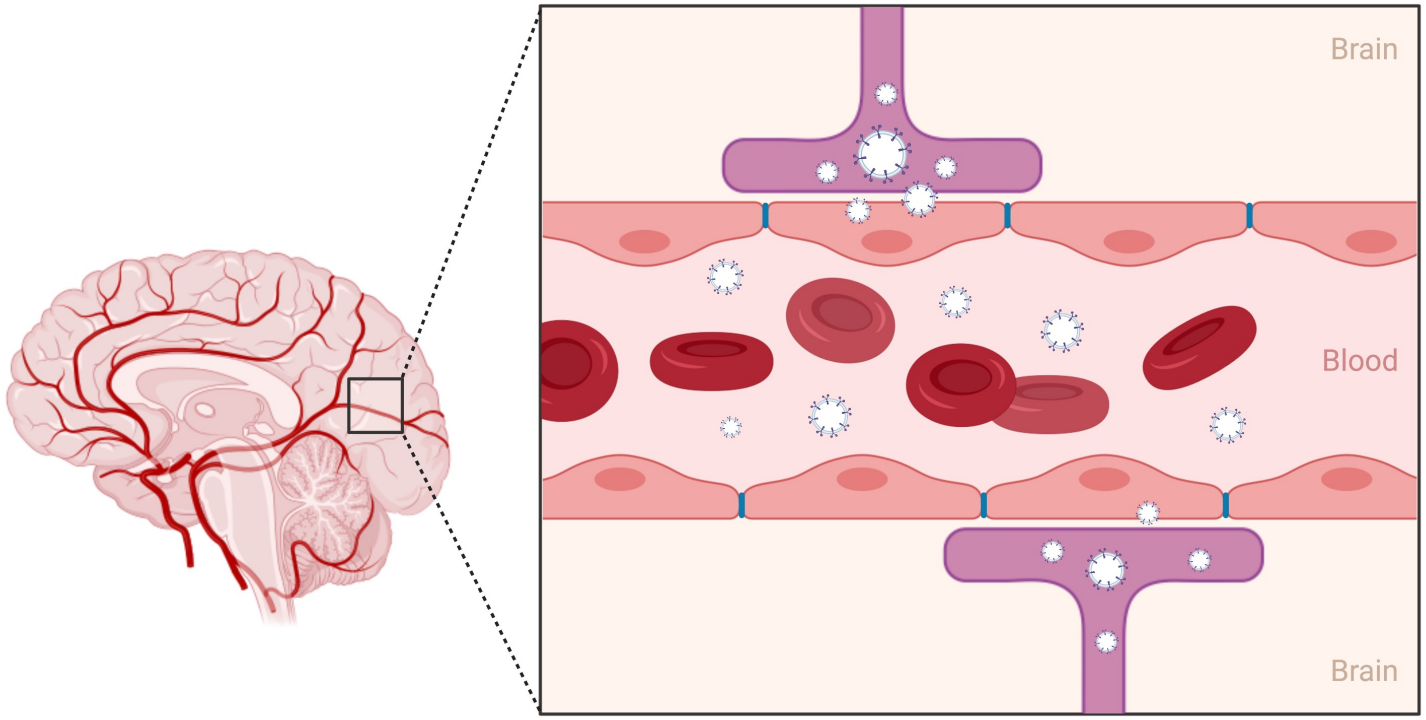
- CNS biomarker studies previously limited by:
  - Inability to perform brain biopsies on the living
  - Postmortem tissue providing information after degenerative processes have occurred

CNS EVs Can Cross the Blood-Brain Barrier and Enter the Circulation

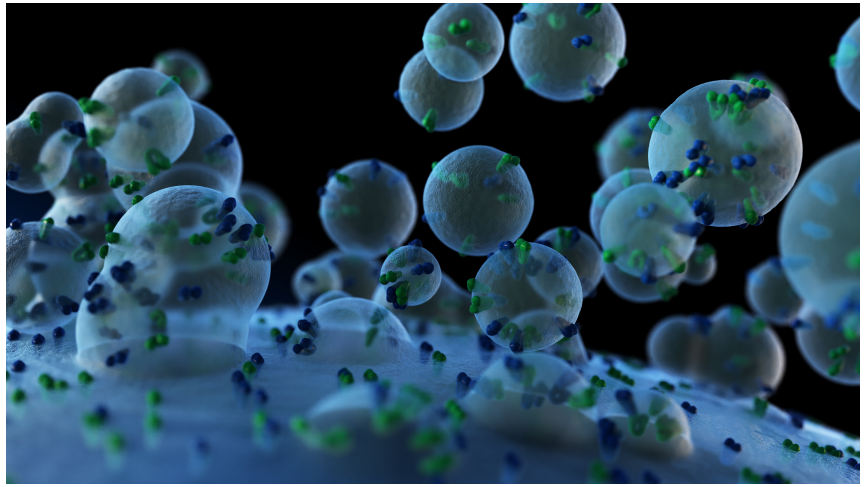


Created with BioRender.com

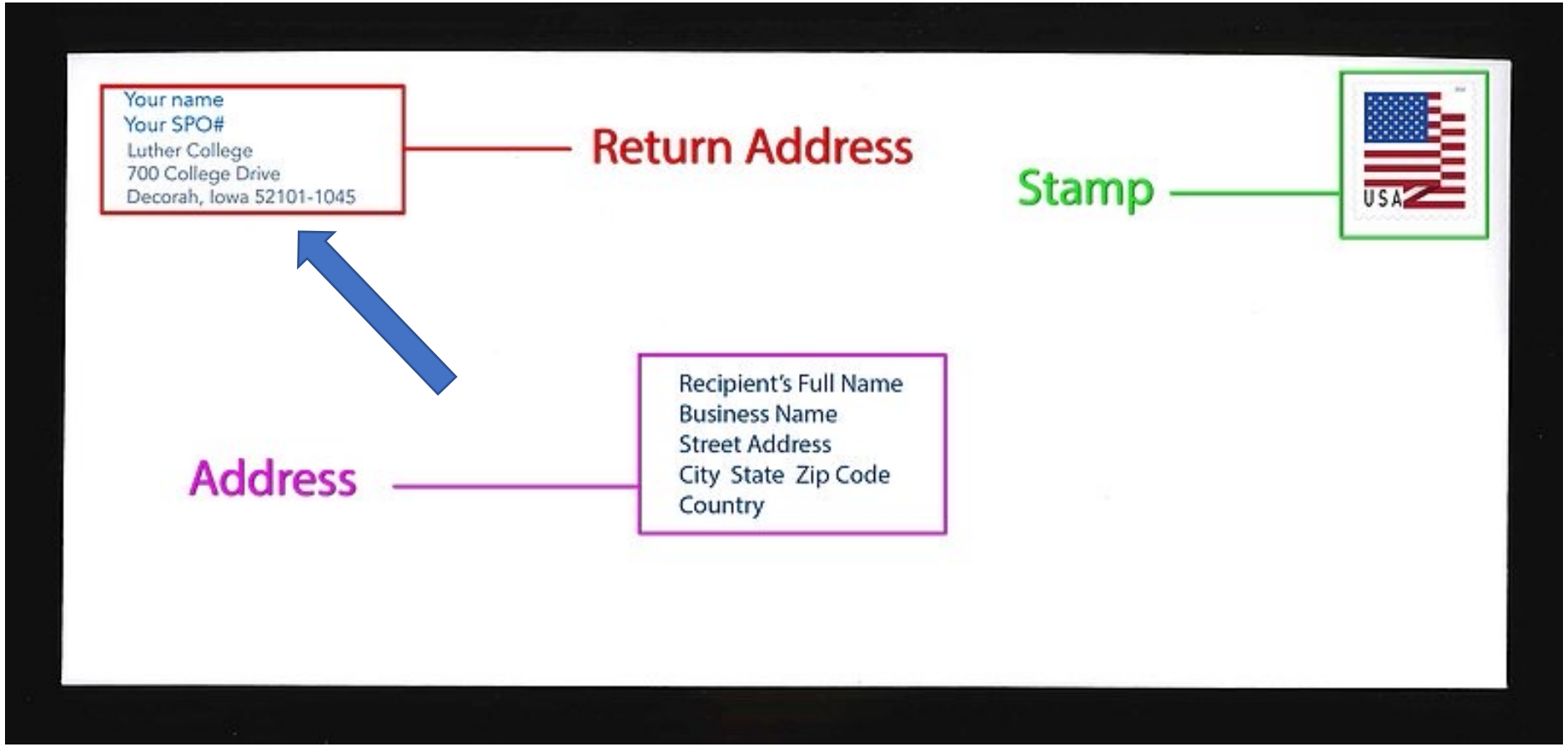
CNS EVs Can Be Isolated from Blood



Membrane memory



Membrane surface markers analogous to return address on envelope



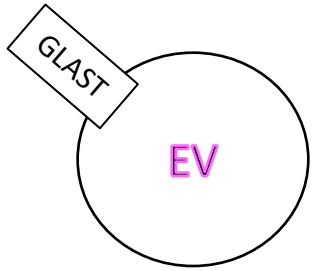
CNS Cell-Type Specific Proteins

CNS Cell Types

Astrocytes

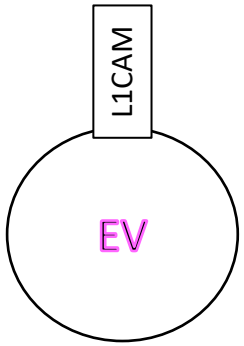
"Return Addresses" for CNS

- Glutamate aspartate transporter **(GLAST)**



Neurons

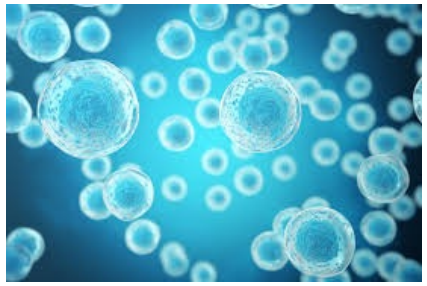
- L1 cell adhesion molecule **(L1CAM)**



EVs and Metals: EVs Loaded with Metal Metabolism Proteins

- EVs contain many proteins involved in metal metabolism

Can CNS cells also use EV production as a mechanism of metal homeostasis?

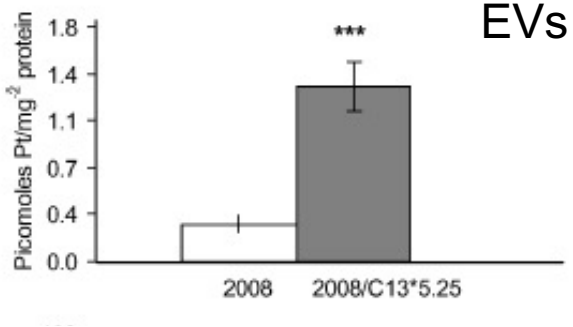


The secret life of extracellular vesicles in metal homeostasis and neurodegeneration

Shayne A. Bellingham*†, Belinda Guo*† and Andrew F. Hill*†‡[1]

Biol. Cell (2015) 107, 380-418

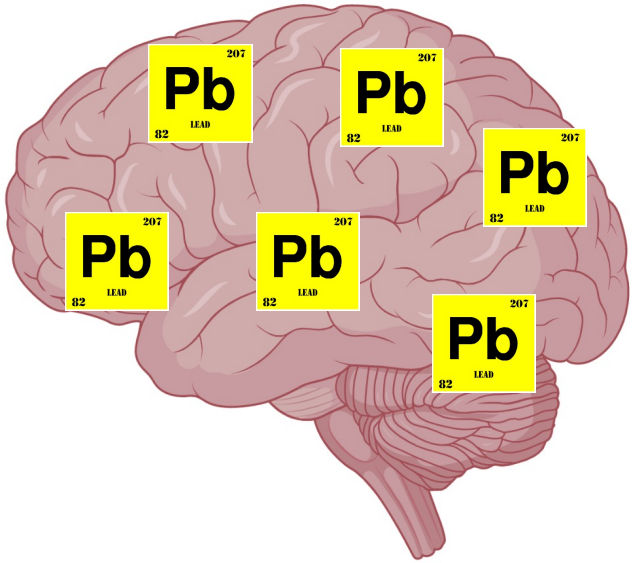
Cisplatin-resistant tumors extrude Pt metal via increased EV release



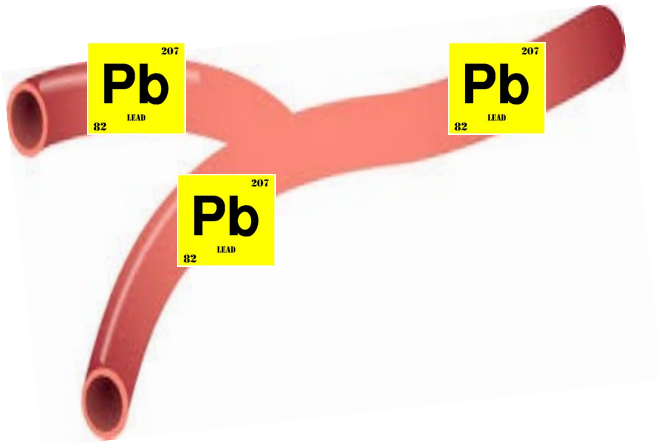
Safaei, R., Larson, B.J., Cheng, T.C., Gibson, M.A., Otani, S., Naerdemann, W. and Howell, S.B. (2005) Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol. Cancer Ther. **4**, 1595–1604

Could patients who are better metal extruders have slower disease progression?

High Pb in ALS patient CNS



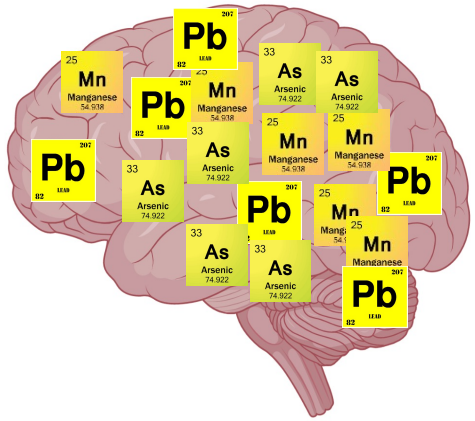
Higher Pb in blood = slowly progressing ALS



CNS-EV Metal Levels as a Biomarker of CNS Metal Burden and ALS Progression

- Question 1: **Could circulating CNS-EV metal load serve as biomarker of actual CNS metal burden?**

- Question 2: Could circulating CNS-EV metal load serve as biomarker of ALS progression?



Develop and validate a reliable, reproducible method to isolate CNS-EVs and measure their metal content

Research Question:

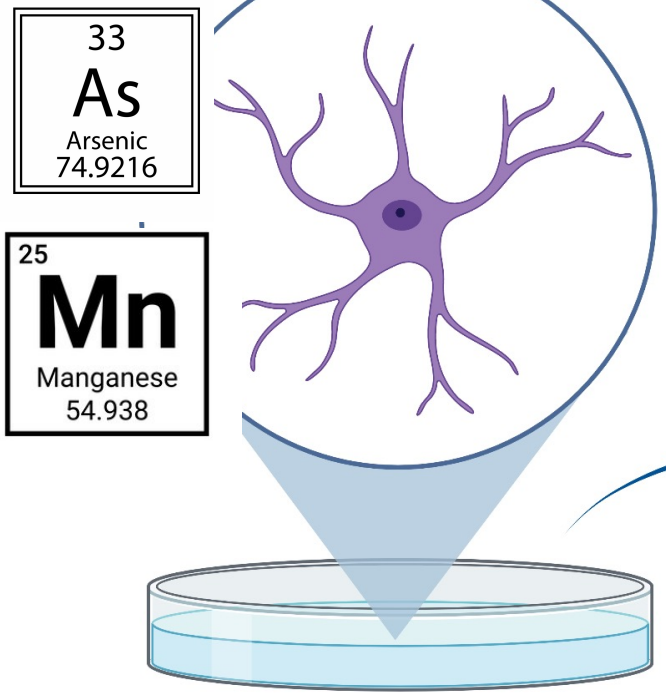
Are metals extruded in EVs from CNS cells?

Objective:

Measure metal content in EVs released from astrocytes treated with As or Mn *in vitro.*

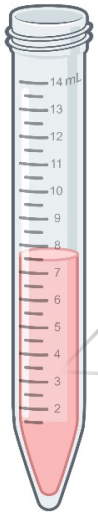
Methods: *in vitro* pilot

Primary cultures

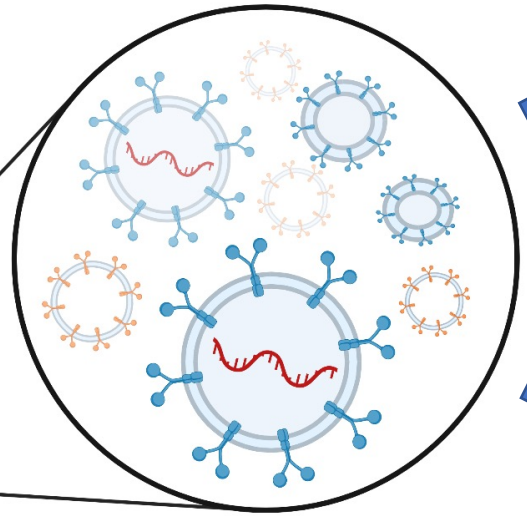


2.5 µM

3 or 7 days



Culture media



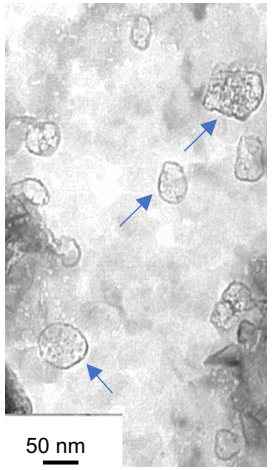
Culture Media EVs

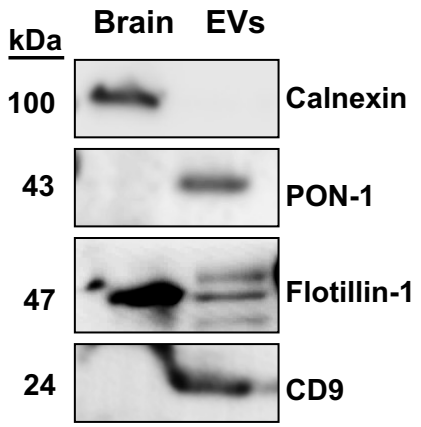
Western Blot (EV quantity)

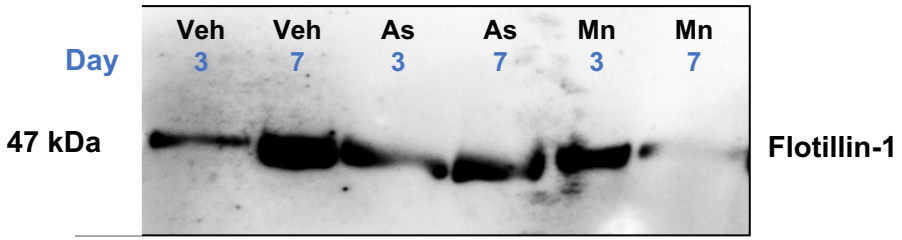
ICP-MS (metal levels)

CNS Cells Can Extrude Excess Metals via EVs

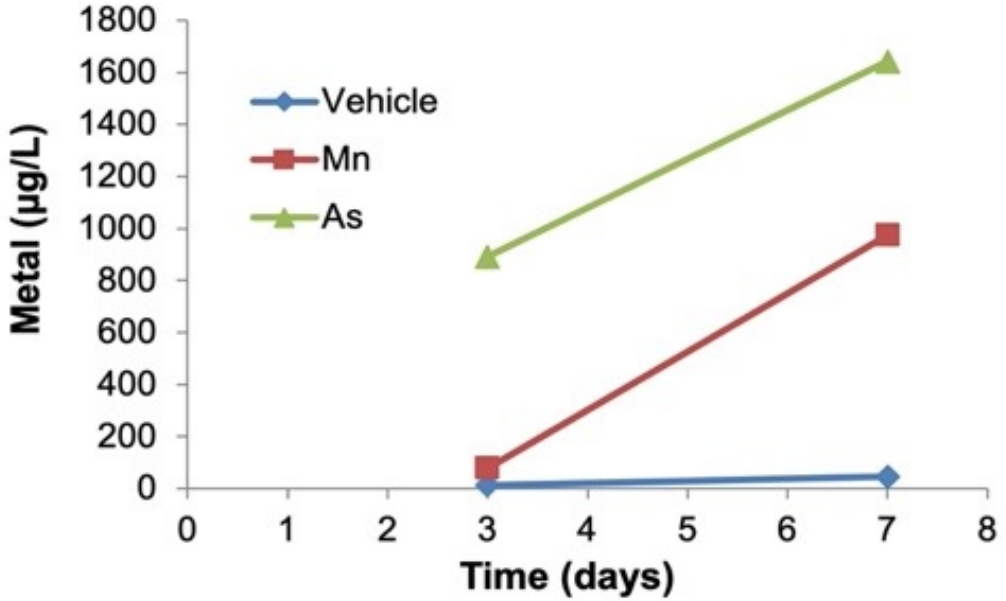
TEM Image
92000X magnification







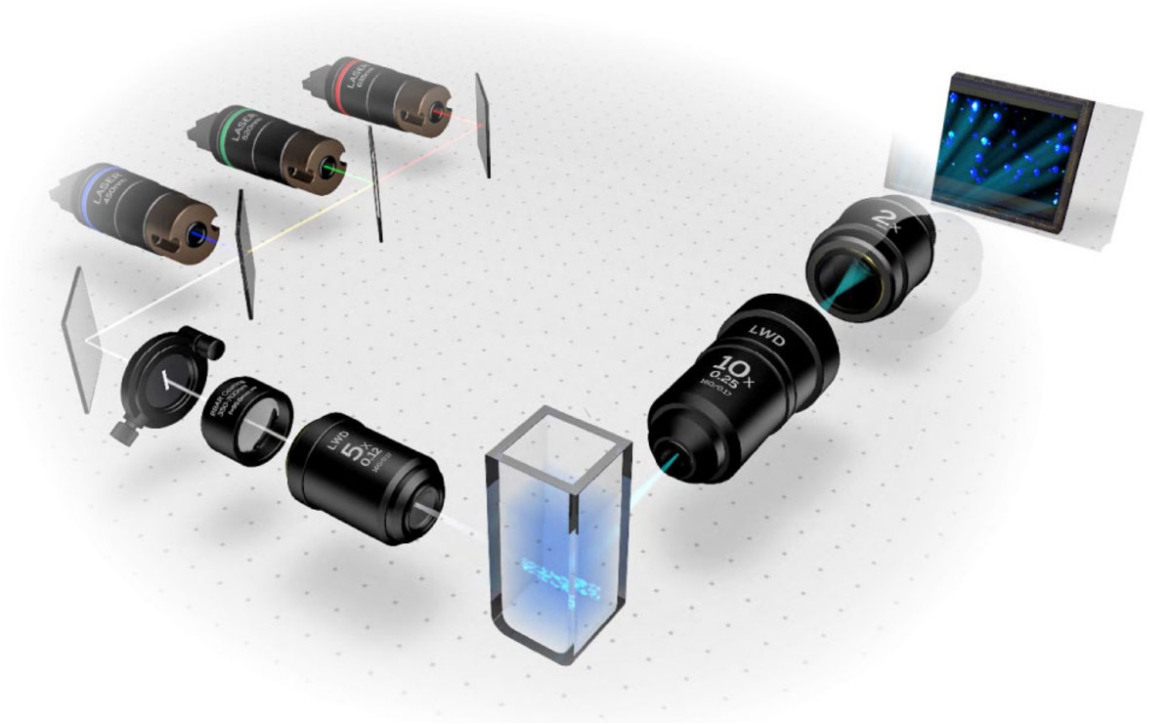
Arsenic (As) and manganese (Mn) accumulate in astrocyte EVs



Overview

- Background: EVs, ALS, Research Questions
- **Standardized NTA Protocol for Reproducibility**
- Comparing CNS-EV Isolation Methods
- Conclusions and Future Directions

Sizing, Counting, and Phenotyping of EVs: Nanoparticle Tracking Analysis (NTA)



horiba.com

Standardized protocols needed for reproducibility in EV research

- Characterization of EVs needed for interpretation of results and comparison of results across labs

J Extracell Vesicles. 2014; 3: 10.3402/jev.v3.26913.
Published online 2014 Dec 22. doi: 10.3402/jev.v3.26913
PMCID: PMC4275645
PMID: 25536934

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles

Jan Lötvall,[✉1] Andrew F. Hill,[2] Fred Hochberg,[3] Edit I. Buzás,[4] Dolores Di Vizio,[5] Christopher Gardiner,[6] Yong Song Gho,[7] Igor V. Kurochkin,[8] Suresh Mathivanan,[9] Peter Quesenberry,[10] Susmita Sahoo,[11] Hidetoshi Tahara,[12] Marca H. Wauben,[13] Kenneth W. Witwer,[14] and Clotilde Théry[15]

J Extracell Vesicles. 2018; 7(1): 1535750.
Published online 2018 Nov 23. doi: 10.1080/20013078.2018.1535750
PMCID: PMC6322352
PMID: 30637094

Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines

Standardized protocols needed for reproducibility in EV research

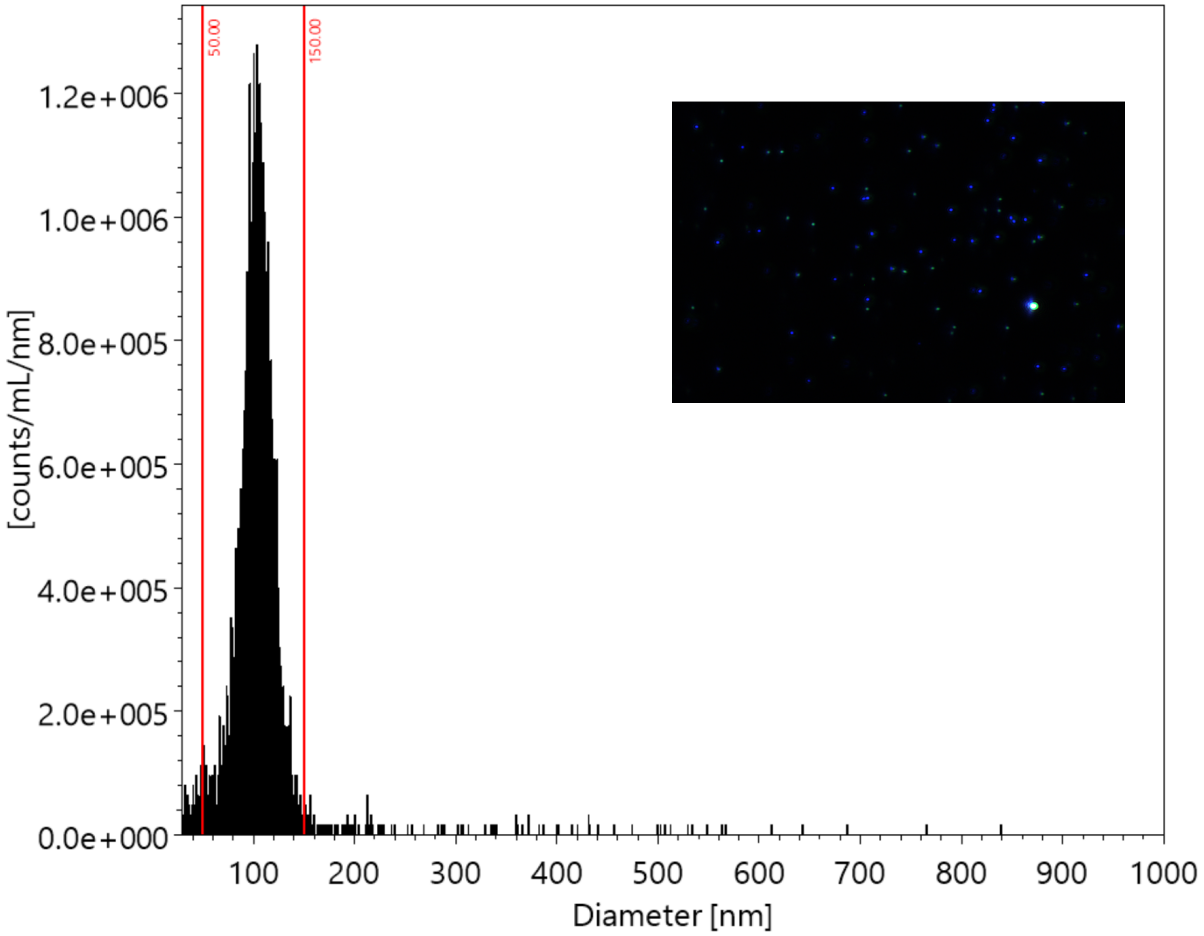
- Characterization of EVs needed for interpretation of results and comparison of results across labs
- Published optimized protocol for EVs in *J Vis Exp* (doi: 10.3791/62447)
- Demonstrated effect of altering various parameters, e.g., laser powers

Standardized protocols needed for reproducibility in EV research

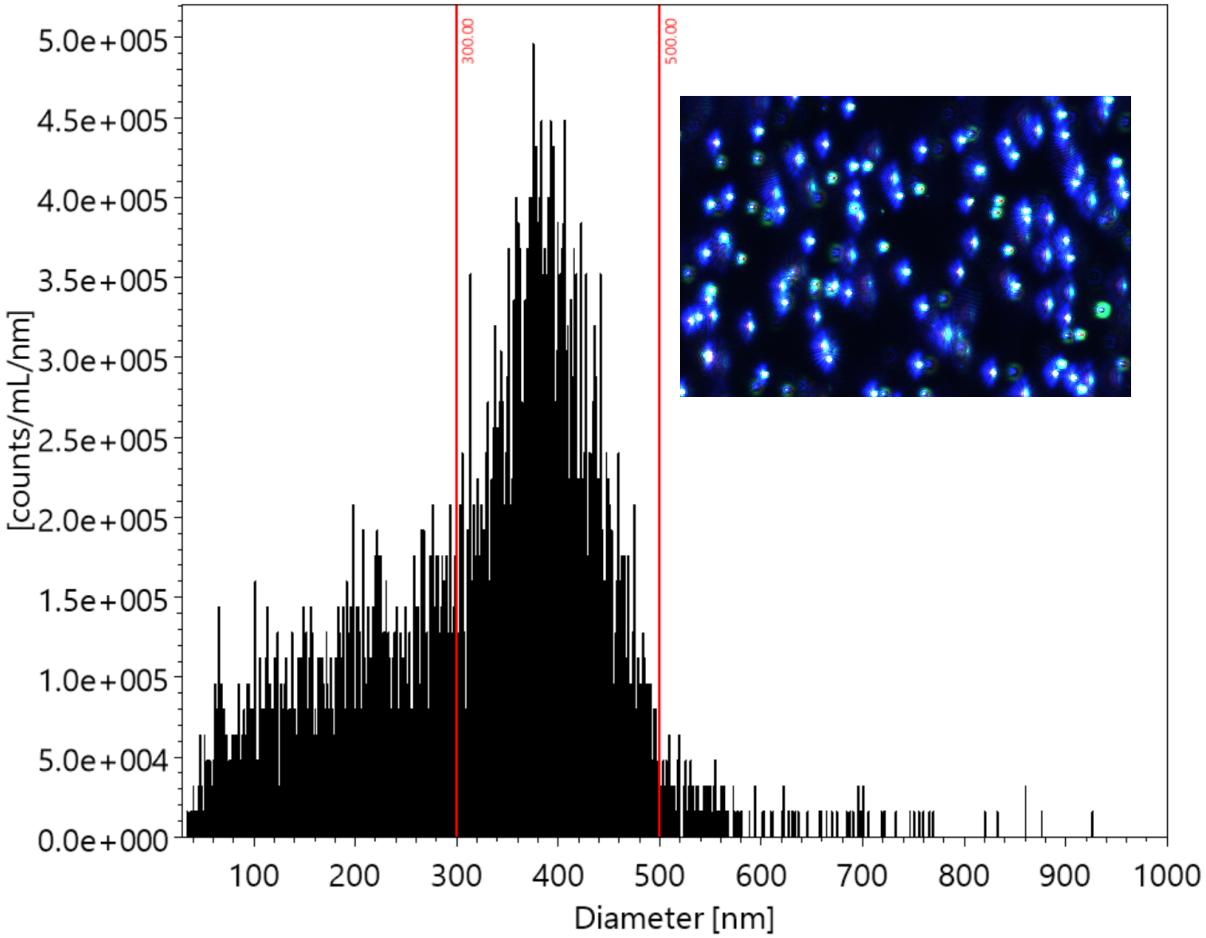
Gain [dB]	Blue Laser [mW]	Green Laser [mW]	Red Laser [mW]	Total Concentration, particles/mL (raw)	Average size [nm]*	Standard deviation of size / CV	Modal size	D10 / D50 / D90
18	70	12	8	5.90E+07	133	89 / 0.66	67	66.57 / 131.08 / 322.70
24	70	12	8	1.00E+08	118	73 / 0.61	64	60.21 / 117.93 / 257.08
30	210	12	8	1.70E+08	105	57 / 0.54	80	59.83 / 104.74 / 206.07
30	70	12	8	1.10E+08	122	75 / 0.61	74	73.17 / 123.09 / 278.70
30	210	0	0	1.00E+08	116	70 / 0.6	82	71.33 / 115.63 / 257.65
30	70	0	0	7.00E+07	126	79 / 0.63	82	74.81 / 125.16 / 284.23
30	0	12	0	2.80E+07	169	106 / 0.63	100	98.28 / 163.39 / 392.50
30	0	24	0	4.40E+07	148	95 / 0.64	88	84.24 / 147.69 / 354.15
30	0	0	8	1.50E+07	175	129 / 0.74	62	8.81 / 15.32 / 108.93
30	0	0	16	9.20E+06	246	147 / 0.6	13	8.55 / 14.93 / 136.75

Size distributions of monodisperse polystyrene bead standards

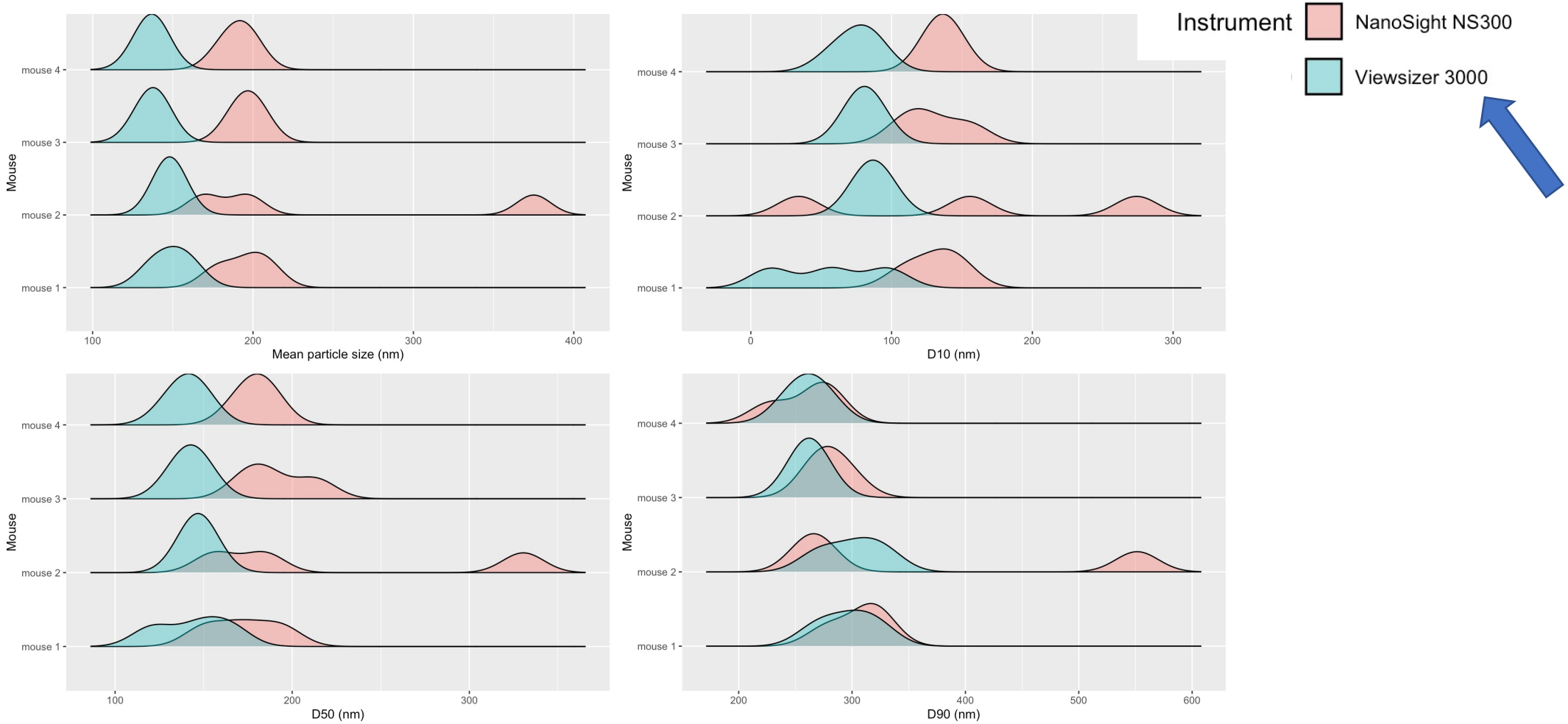
100 nm polystyrene bead standard



400 nm polystyrene bead standard



Particle size measurements across multiple dilutions: ViewSizer 3000 vs. NanoSight NS300



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Published online 2021 Mar 28. doi: 10.3791/62447

PMCID: PMC8243380
NIHMSID: NIHMS1705513
PMID: 33843938

Nanoparticle Tracking Analysis for the Quantification and Size Determination of Extracellular Vesicles

Nicole Comfort,[1] Kunheng Cai,[2] Tessa R. Bloomquist,[1] Madeleine D. Strait,[1] Anthony W. Ferrante, Jr.,[2] and Andrea A. Baccarelli[1]

Pros/Cons of ViewSizer for NTA in EV Research

Pros

- Doesn't require expertise to perform/not technically challenging
- Not as time-consuming as TEM, little hands-on time
- Multispectral illumination allows visualization over wider range of sizes in polydisperse samples like EV isolate
- Sample prepared in cuvette (viewed in native state, EV recovery possible)
- Fluorescent NTA possible

Cons

- Reduced sensitivity for small particles (< 50 nm, but LOD will be sample-dependent)
- Overestimates EV sizes
- Will measure all particles, not just EVs
- Little work on standardization of protocols
- Minimum 300-350 µL volume (400-500 µL recommended)
- Bleaching in fluorescent NTA common

Considerations for reproducible NTA in EV research

- Use of hollow organosilica beads encouraged (light-scattering properties similar to that of EVs)
  - Otherwise, silica nanoparticles preferred over polystyrene standards
- Perform NTA on multiple dilutions of the same samples
  - Particle concentrations should scale proportionally with the dilution factor but not reported size measures
- Researchers should explicitly state ViewSizer settings used to generate data for transparency and reproducibility
- Recommended to always characterize EVs using orthogonal method(s)

Applications of NTA in EV research

- Applications of NTA in EV research:
  - Particle size and count (characterization)
  - Particle number: assessing EV secretion under different conditions
  - Particle number: normalization factor
  - Particle number: efficiency of isolation, used for comparison of methods

Overview

- Background: EVs, ALS, Research Questions
- Standardized NTA Protocol for Reproducibility
- **Comparing CNS-EV Isolation Methods**
- Conclusions and Future Directions

Research Question:

Can metal levels measured in circulating CNS EVs predict CNS metal load in ALS patients?

Objective:

First, compare methods to isolate CNS-EVs from peripheral blood.

CNS-EV Isolation Method: Characterization, Validation, Adaptation

Our Constraints:

1. ALS human samples are rare, difficult to obtain → **limited in quantity**
2. Both neurons and glial cells have a critical role in ALS and astrocytes are instrumental in metal homeostasis → **EVs enriched from a unique cell type may not give a full picture**
3. For a large study (with thousands of samples), **cost per EV isolation needs to be realistic**
4. Total time/amount of work per EV isolation needs to be feasible
5. Normalization factors used for CNS-EV isolation efficiency in other studies were **not validated in the context of ALS**
6. Our method needed to be adapted to be compatible with trace metal measurement by ICP-MS → **as metal-free as possible**

Comparing 1-step vs. 2-step CNS-EV Isolation Protocols

Starting from
0.5 mL of Plasma

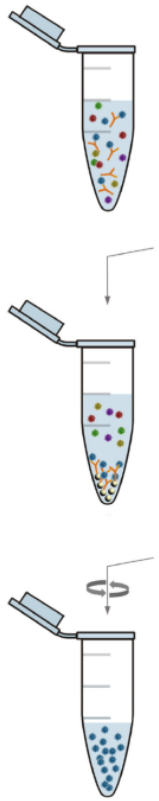
2-Step Isolation
(Mustapic et al., 2017)

Step 1. Isolate Total EVs by ExoQuick

Step 2. GLAST EV IP or L1CAM EV IP

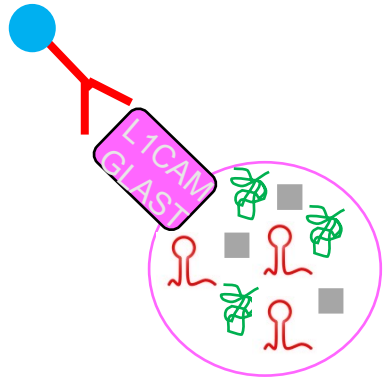
Step 1. Total EV Isolation

Step 2. Immunoprecipitation (IP)



1-Step Isolation
Direct IP
(Shi et al., 2017)

Step 1. Direct IP
GLAST/L1CAM EVs IP



Comparing 1-step vs. 2-step CNS-EV Isolation Protocols

2-Step Isolation

Pros:

- Total EV step decreases Ab quantity needed
- Total EV step decreases pulldown of unwanted soluble proteins
- More published studies to compare data with

Direct Isolation

Pros:

- Fewer steps, less reagent, less potential metal contamination
- Faster, less labor, more adapted to population studies

Comparing 1-step vs. 2-step CNS-EV Isolation Protocols

2-Step Isolation

Cons:

- More steps, more reagents, more potential metal contamination
- More labor- and time-intensive
- Most studies re-used the method with minimal re-validation

Direct Isolation

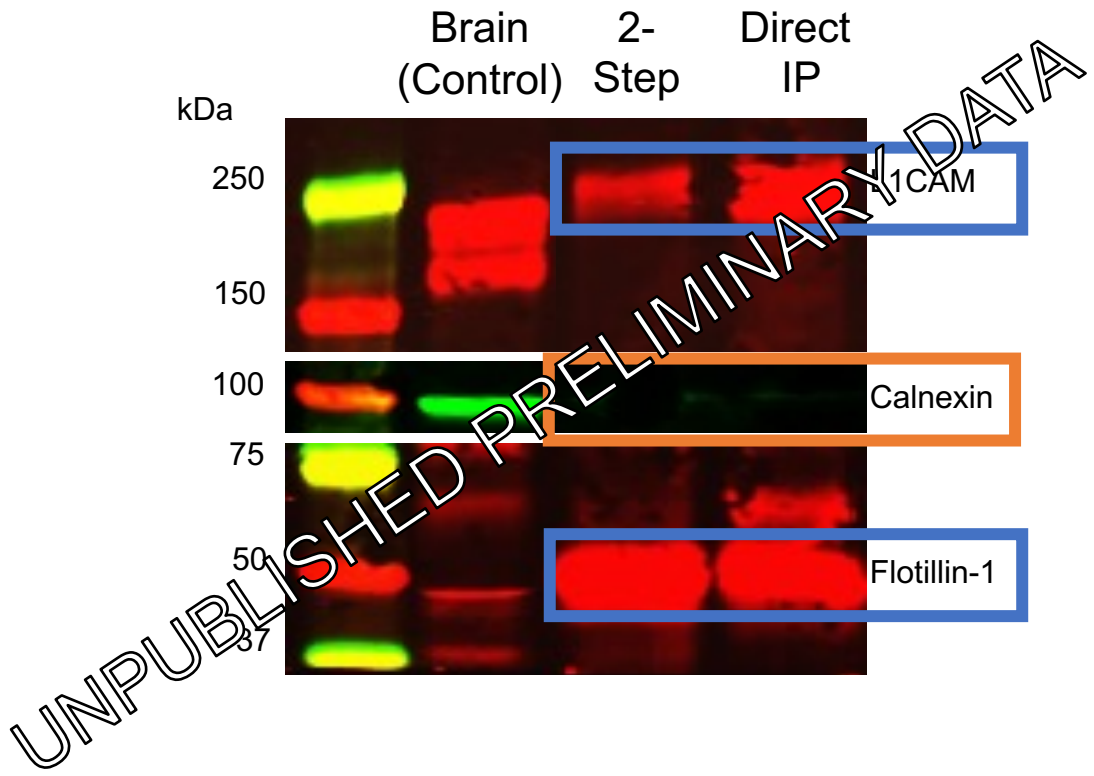
Cons:

- More Ab needed
- Potential pulldown of non-EV soluble proteins
- Published by only one group
- Uses magnetic beads! Needed to adapt to Streptavidin-Agarose resin

Evaluating and validating CNS-EV isolation methods

- Characterization of EVs (TEM, Western Blot, NTA) isolated using antibodies for:
  - GLAST (astrocyte)
  - L1CAM (neuron)
- Isolated from both whole blood and plasma samples
- EVs isolated using both 2-step and direct IP methods

L1CAM/GLAST-EV IP Validation: Characterization of L1CAM EVs



Enrichment of EV markers

Absence of non-EV markers

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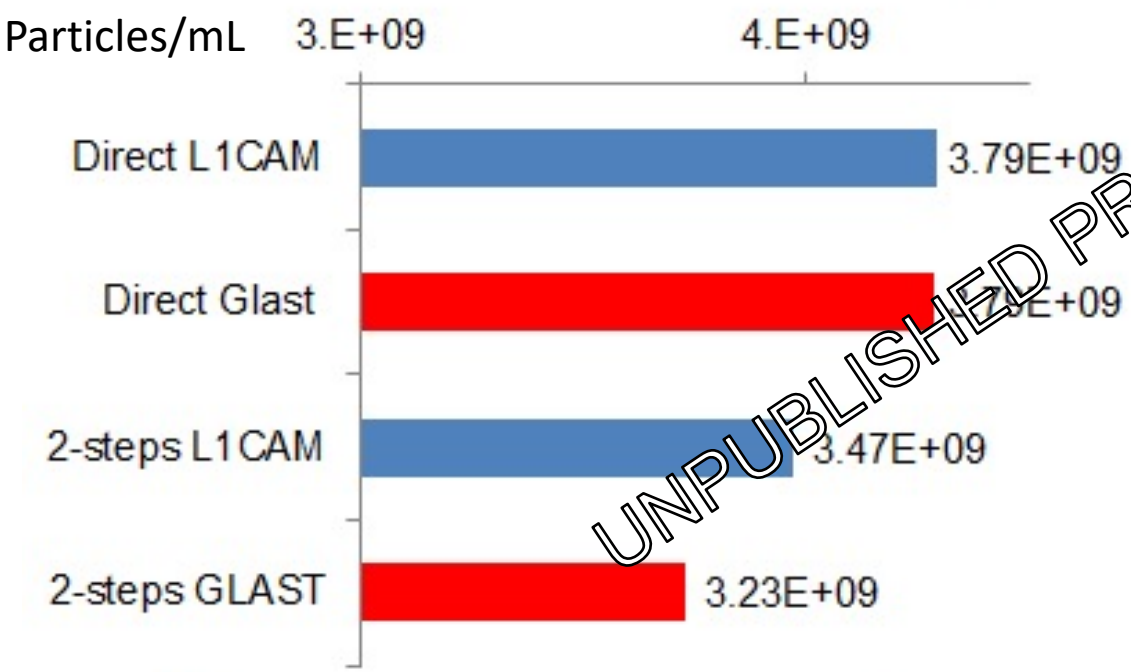
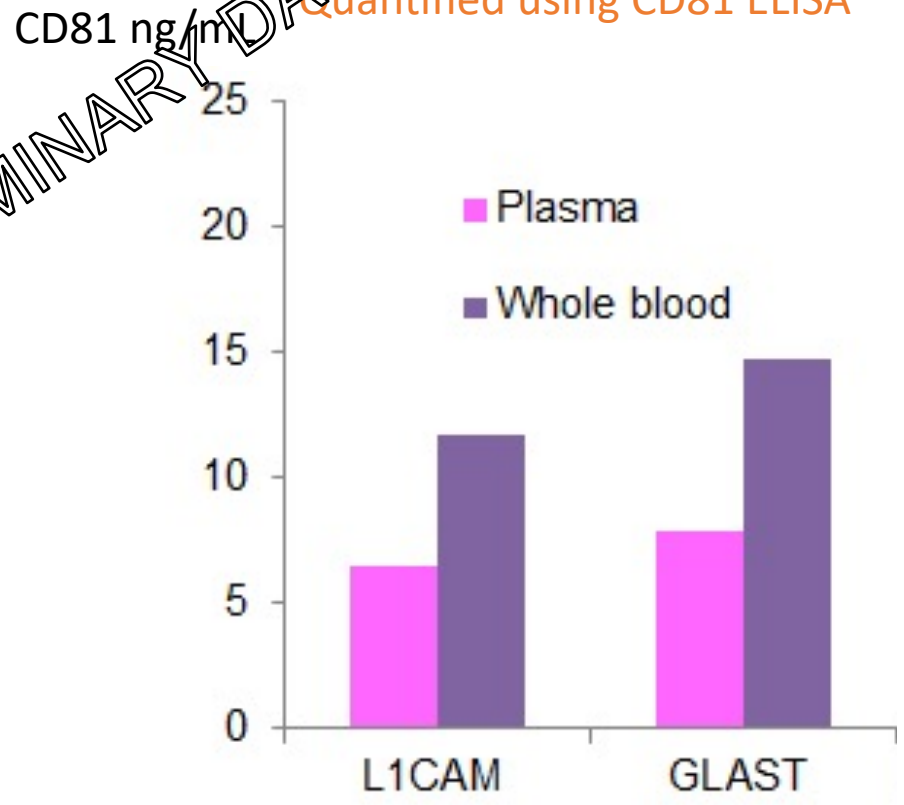
L1CAM/GLAST-EV IP Evaluation

Concentrations of CNS-EVs/mL
Quantified using Fluorocet Assay

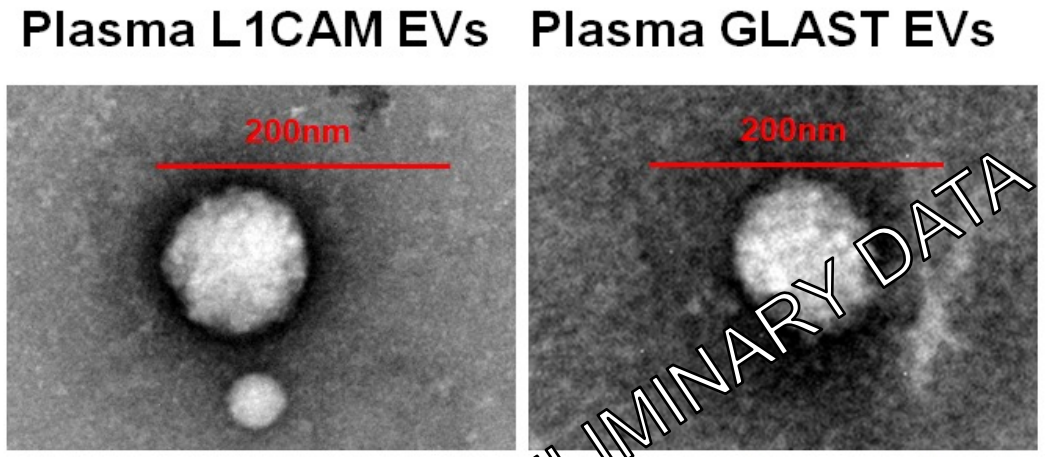


Levels of CD81 in CNS EVs
Quantified using CD81 ELISA

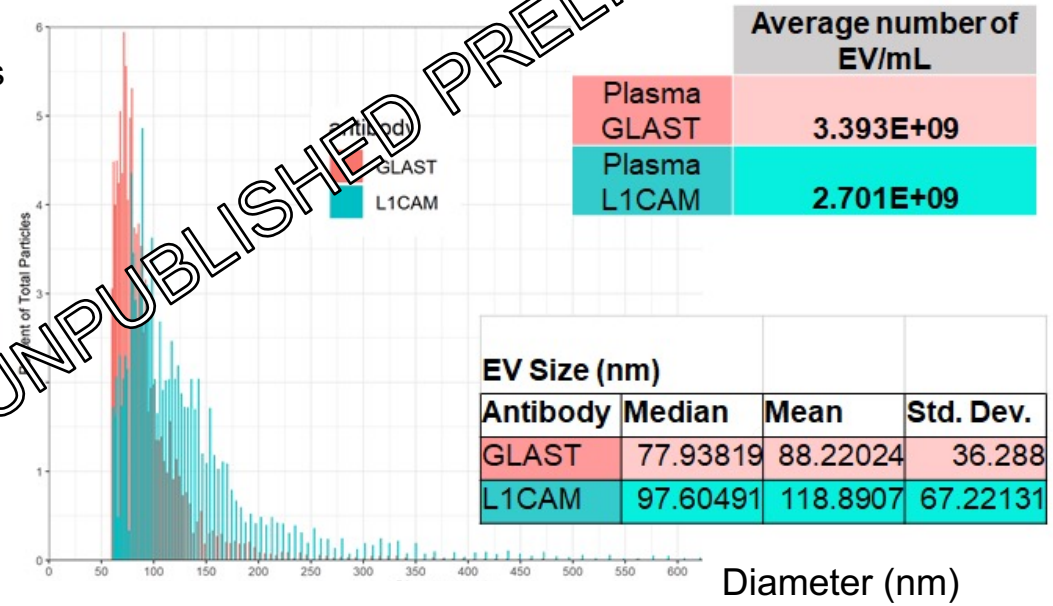


Characterization of GLAST and L1CAM EVs:

GLAST antibody more efficient at CNS-EV isolation than L1CAM antibody



Transmission Electron Microscopy (TEM)



	Average number of EV/mL
Plasma GLAST	3.393E+09
Plasma L1CAM	2.701E+09

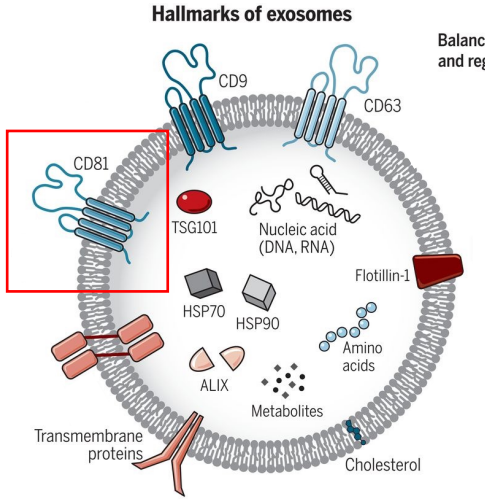
EV Size (nm)

Antibody	Median	Mean	Std. Dev.
GLAST	77.93819	88.22024	36.288
L1CAM	97.60491	118.8907	67.22131

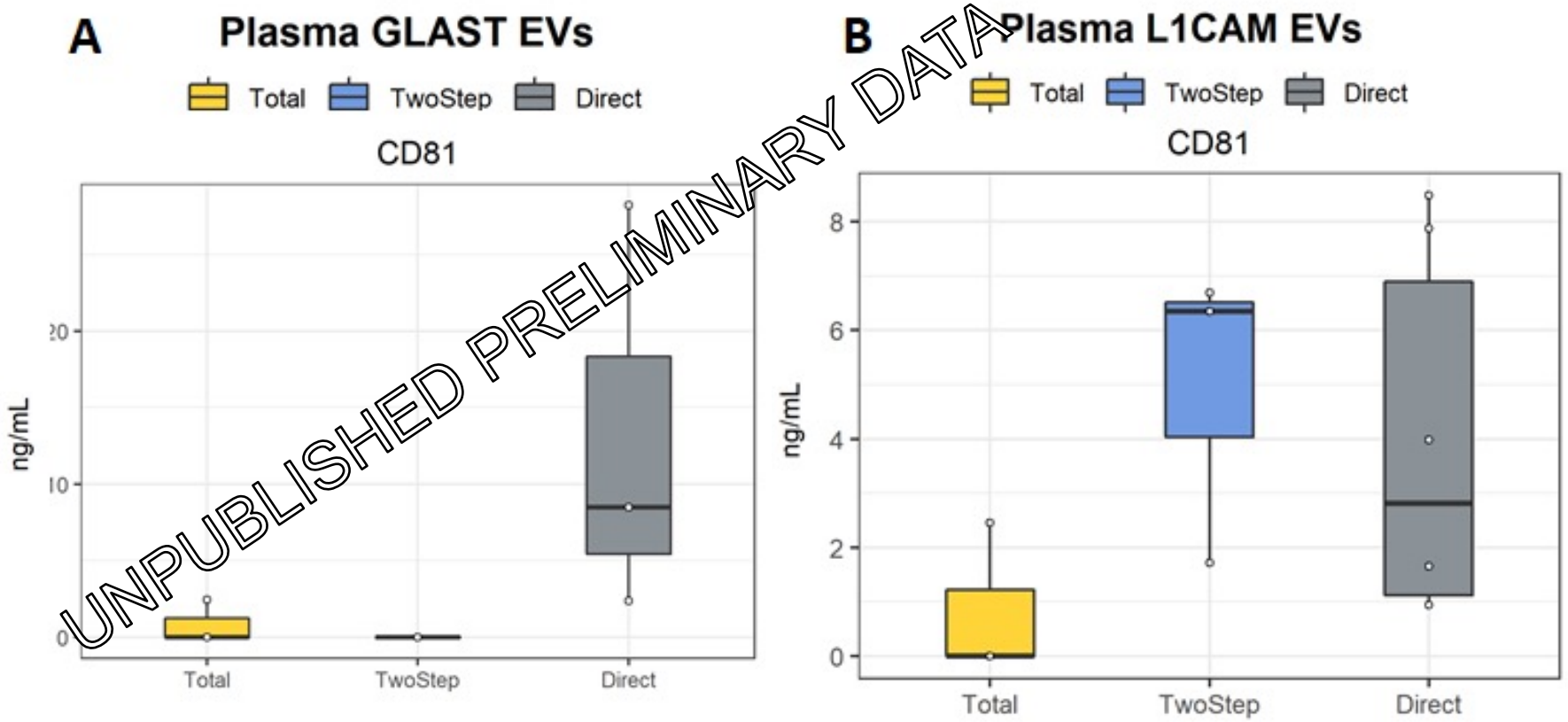
Nanoparticle Tracking Analysis (NTA)

- EVs in expected size range
- GLAST isolated greater number of EVs
- Concentration agrees with previous quantitation using Fluorocet assay

Efficiency of 2-Step vs. 1-Step CNS-EV Isolation Method: Assessed by CD81 ELISA



Kalluri & LeBleu, *Science*, 2020



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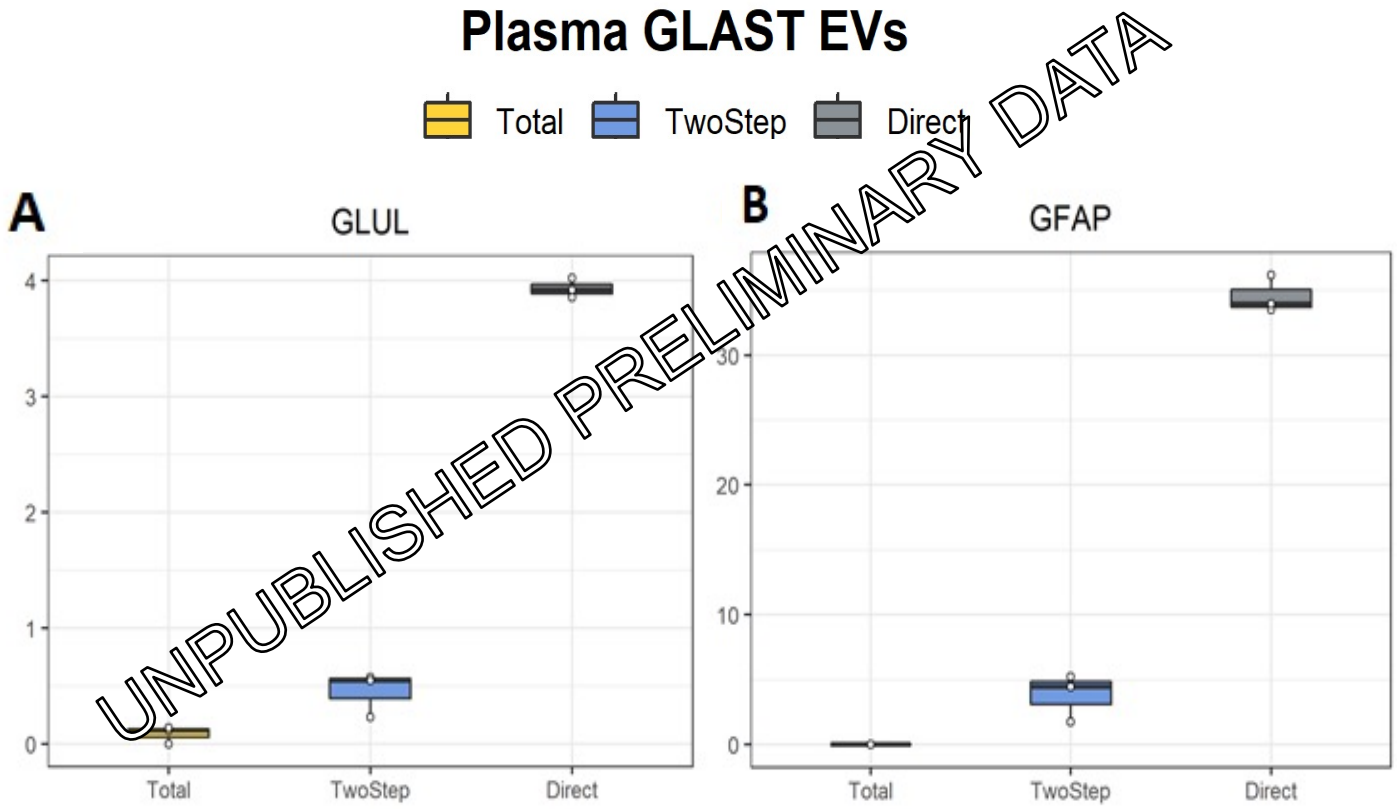
Direct IP more efficient than 2-step in isolating GLAST EVs

Efficiency of 2-Step vs. 1-Step GLAST-EV Isolation Method: Enrichment in Astroglial Markers in GLAST EVs



Legend:
GLUL – glutamine synthetase
GFAP – glial fibrillary acidic protein

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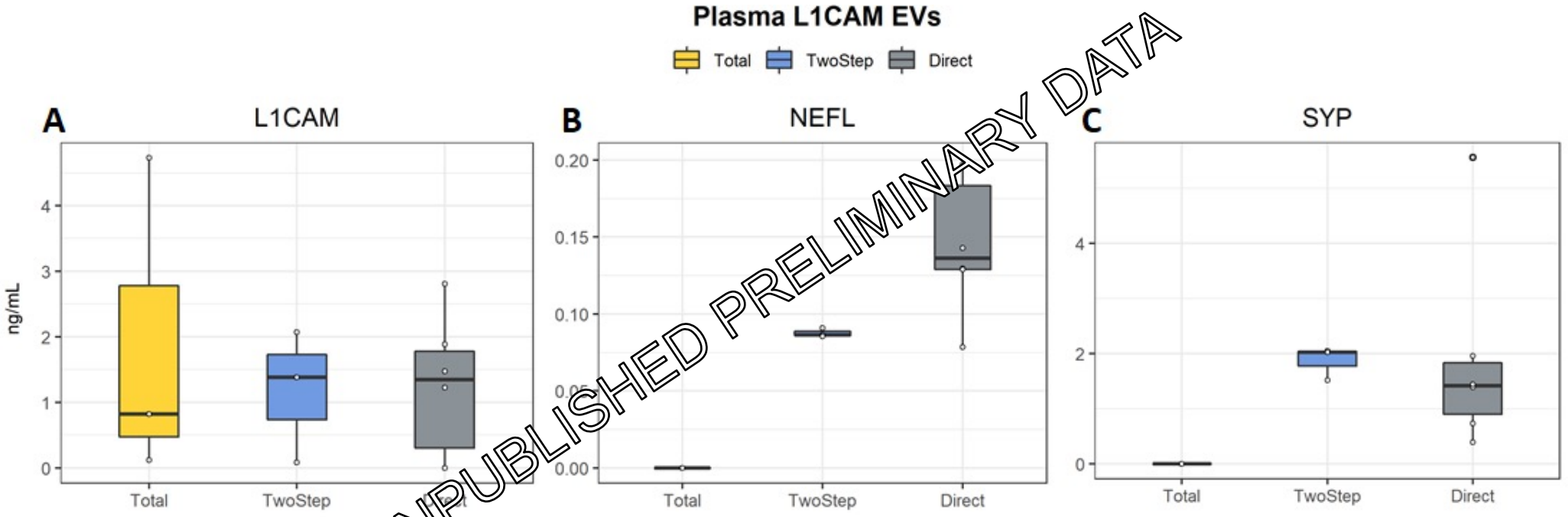
Direct IP more efficient than 2-step method in enriching other astrocyte markers

Efficiency of 2-Step vs. 1-Step L1CAM-EV Isolation Method: Enrichment in Neuronal Markers in L1CAM EVs



Unexpectedly, we found that L1CAM was not significantly enriched by either method in L1CAM-EVs as compared to the Total EV fraction

Legend:
NEFL – neurofilament
SYP – synaptophysin

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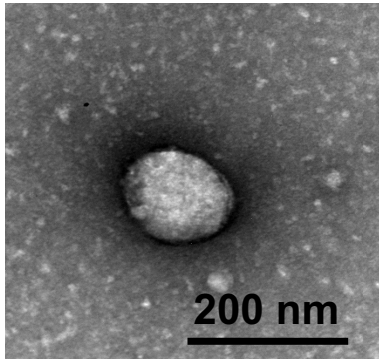
Direct IP vs. 2-step Method

- Found direct IP method equally or more efficient in purifying CNS-EVs, determined by:
  - TEM, NTA, Fluorocet Assay, ELISA

- Enrichment Ratios (measured by ELISA)

	GLAST				L1CAM		
	Direct/Total		Direct/TwoStep		Direct/Total		Direct/TwoStep
Marker	Plasma	Whole Blood	Plasma	Whole Blood	Plasma	Whole Blood	Plasma
CD81	15.95	5.20	11[illegible].05	514.81	4.87	0.96	0.81
GLAST	3.95	**25.37**	0.44	25.37			
GFAP	156.04	**2[illegible]1.14**	9.10	11.40	122.37	157.93	5.21
GLUL	46.34	**668.34**	8.70	10.10	14.93	151.20	1.51
L1CAM					0.65	8.67	1.04
NEFL	9.63	28.05	1.74	3.16	13.26	23.68	1.67
SYP	152.36	331.72	17.65	15.95	43.28	55.98	1.02



TEM (100,000x) of GLAST-EVs.

Conclusions

- Astrocytes exposed to neurotoxic metals can extrude excess metals through homeostatic mechanism involving EV release
- Direct IP more efficient and reliable overall than 2-step isolation
- Which cell surface marker to target? Confirmed high reliability of GLAST antibody (greater than L1CAM)
- NTA can be used for size distribution/particle count comparing samples in EV research, but researchers should measure multiple dilutions and report all parameters

Future Directions

- Repeat experiments with increased sample size
- Exploring contactin-2 (CNTN2) as more specific neuronal marker (in contrast with L1CAM)
  - https://www.proteinatlas.org/ENSG00000184144-CNTN2/brain
- Can metal levels measured in circulating CNS EVs predict CNS metal load in ALS patients?

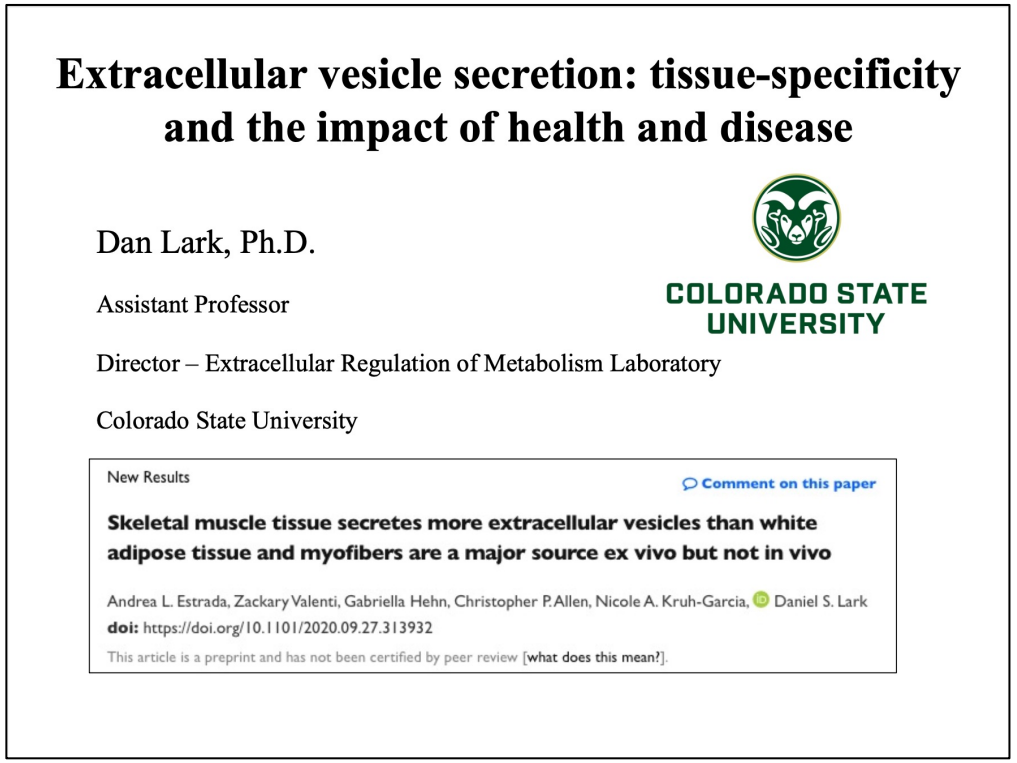
  Measure metal levels in blood CNS-EVs and matched brain/spinal cord samples

  Normalize metal levels by IP efficiency using particle count and/or CD81 levels
- Are differences in CNS-derived EV metal levels associated with ALS progression?

  Use linear mixed models adjusted for potential confounders
- Differences between GLAST-EV metal levels among ALS cases vs controls?

Check out the other webinars in this particle series for more on EVs!

Excellent background and overview



Another discussion and application of tissue-specific EVs

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Thank You