Establishing a Unique Analytic Platform for Extracellular Vesicle-Based Brain Injury Studies: An MTBI² Presentation

MTBI² Uniformed Services University

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Abstract

Background: The recent advancement in discovery of blood-based biomarkers for diagnosing brain injury, encephalopathy, and post-injury symptoms has emerged from use of state-of-the-art technology that has been developed and refined over the past decade. However, low-abundance biomarkers existing in peripheral blood at the beginning of and/or throughout the disease course frequently fall out of low detection limits of most advanced biomarker analysis technologies. Further, the specificity of the emerging biomarkers for identifying brain traits still needs validation. Extracellular vesicles (EV), which carry specific biological signatures from their sites of origin and protect their contents from degradation by proteases or ribonucleases, can traverse the blood–brain barrier (BBB) and be isolated from peripheral biofluids, and become a promising new avenue for brain injury biomarker discovery and assessment tool development (Alberro 2021, Yáñez-Mó 2015, Guedes 2020, Puffer 2020).

Services: The MTBI² Bioanalysis Division (BA) establishes EV characterization platform by combining nanoparticle tracking analysis, fluorescence colocalization, and zeta potential techniques to quantify size, concentration, and cell origin specificity of EVs on ZetaView® PMX-430 QUATT. The BA also provides multiple downstream ultra-sensitive biomarker analysis services, which facilitate biomarker assessments after EV characterization and enrichment. The combined biomarker analysis expertise possessed in MTBI² is essential for biomarker studies in cell-derived EVs, which brings unique support for TBI and the brain health research community.

Background

Although the cargo of circulating **brain cell–derived EVs (bEVs)** may contain biomarkers specific to the status of their original cells, the biggest challenge for this burgeoning field is that there is no accepted and standardized methodology to isolate bEVs. For instance, to date, the L1 cell adhesion molecule (L1CAM, also known as CD171) has been most frequently used to obtain materials of putative CNS neuronal origin for molecular profile discovery and validation associated with neurodegenerative diseases and TBI. However, a study has reported that interpretation of the L1CAM-associated results is complicated, because neuron surface marker L1CAM is not solely expressed in neurons and in the CNS. This criticism reiterates the urgent need for a precise strategy to establish a reliable and standardized methodology for bEV isolation and biomarker studies, Figure 1.

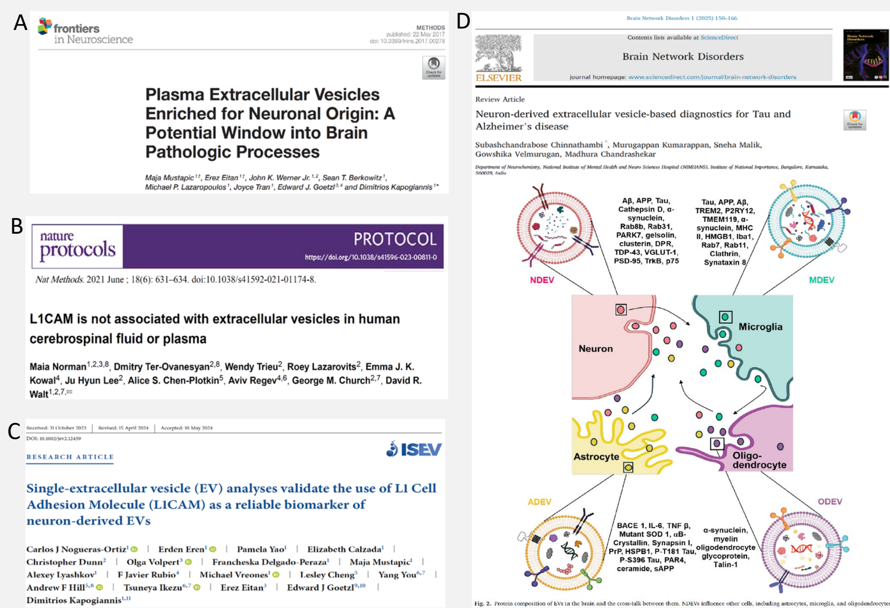

Figure 1. Research progress overview in bEV characterization

Methods

We apply **Nanoparticle Tracking Analysis (NTA)** technology and **fluorescence colocalization** measurement features to perform cell-specific EV characterization and biomarker measurement. The ZetaView allows real-time visualization of nano-sized biological nanoparticles ranged from 40 nm–800 nm. NTA is part of the "Minimal information for studies of extracellular vesicles" (Welse, 2024). Double or multiple fluorescence co-staining with cell-specific markers and exosome-specific markers, such as CD9, CD63, and CD81, can be applied to validate the origins of EVs by using fluorescence co-localization features. We also provide ultra-sensitive **proteomics and transcriptomics analysis platforms** to ensure robust molecular studies in EVs. Comprehensive **pathway and network analysis** for potential EV–derived biomarkers can also be achieved.

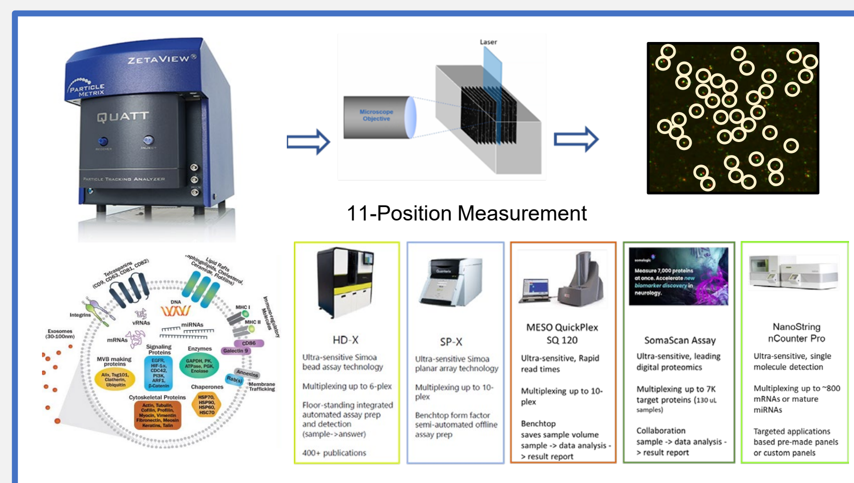

Figure 2. Analysis platforms accessible in the MTBI² Bioanalysis Division. (Upper panel) The ZetaView® PMX-430 QUATT model offers a nanosized particle assessment platform for cell-specific EV characterization. (Bottom panel) A schematic diagram of identified composition of small size EV, exosome. Our biomarker analysis platforms ensure ultra-sensitive downstream applications specifically suitable for molecular analyses in EVs.

Results: Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis

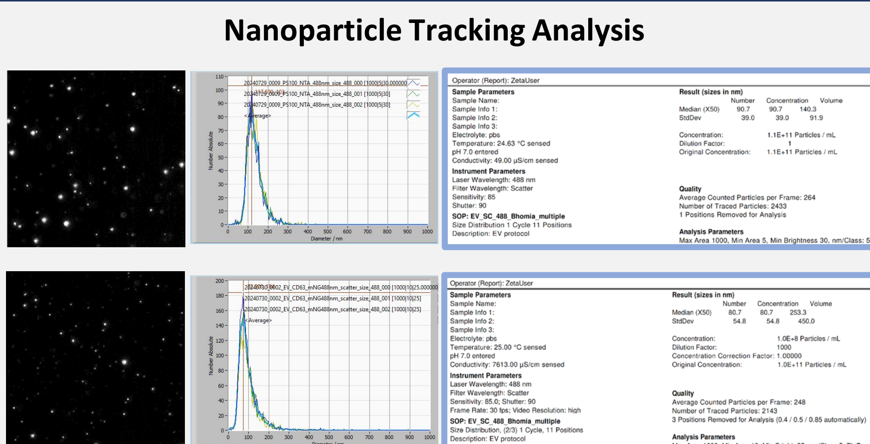

Figure 3. NTA analysis. (Upper panel) The representative bright field scatter image and measurements of sizes and concentrations of 100 nm polystyrene beads (PS100) were acquired by using the NTA technology. PS100 beads had a mode peaked at 90.70 µm, and estimated original concentration was around 1.1E+11 particles/mL. The data were averaged from 3 acquisitions of 11-pos measurements from the same sample, with a total of ~7,500 active events measured and evaluated. (Lower panel) The representative bright field scatter image and measurements of sizes and concentrations of human blood–derived EVs were acquired by using the NTA technology. EV particles had a mode peaked at 80.70 µm, and estimated original concentration was around 1.0E+11 particles/mL. The data were averaged from 3 acquisitions of 11-pos measurements from the same sample, with a total of ~ 6,000 active events measured and evaluated.

Results: Fluorescence Colocalization (F-NTA)

The detection of fluorescence (e.g., vesicles labeled with fluorescently tagged antibodies) allows bio-specific nanoparticle characterization with **fluorescence-based NTA (F-NTA)** with 405, 488, 520, or 640 nm laser. **Colocalization NTA (C-NTA)** enables the simultaneous distinction and analysis of various particle types in heterogeneous samples. This feature offers a deeper understanding of the sample's composition, correlations, and dynamics.

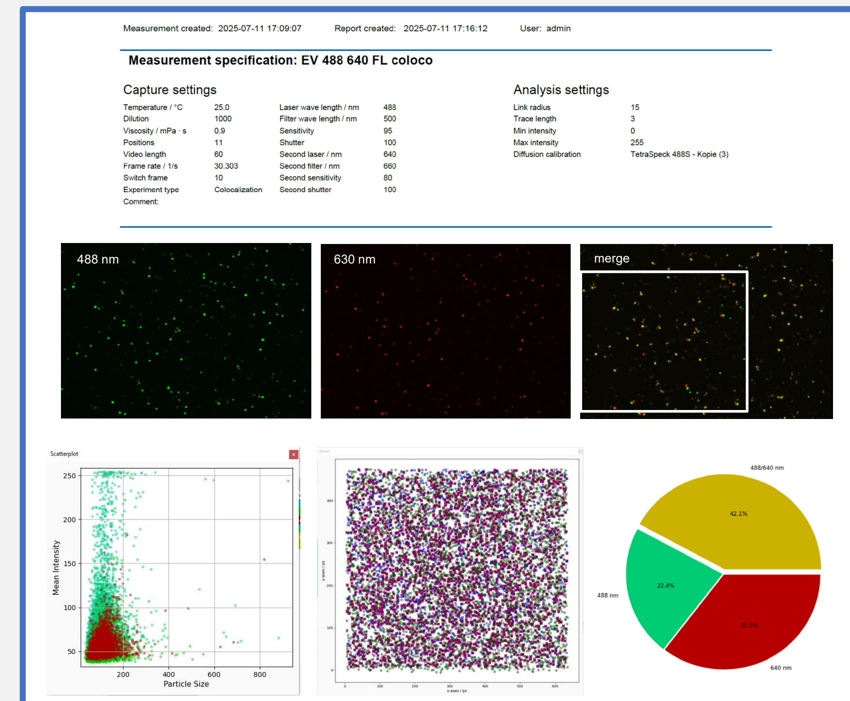

Figure 4. F-NTA and C-NTA are powerful features for cell-specific EV characterization. (Upper panel) A representative Measurement Specification for a colocalization NTA analysis. To identify EV phenotypes in the sample, the particles were multi-labeled with anti-CD9, CD63, and CD81 in green (captured with 488 nm laser), and a cell-specific surface marker in far-red (captured with 640 nm laser). (Middle panel) Representative images captured in the setting. (Bottom panel) Left. Scatter plot of the fluorescence labeled particles. Middle and right. Colocalization analysis of the stained particles.

Results: Zeta -potential (ZP)

Zeta potential (ZP) is a popular method to measure the surface potential of EVs, while used as an indicator of surface charge and colloidal stability influenced by surface chemistry, bioconjugation, and theoretical model applied (Midekessa, 2020; Rasmussen 2020). ZP can also function as a powerful characterization tool to unravel the complexities of EVs especially in applied aspects, such as for theragnostic purposes.

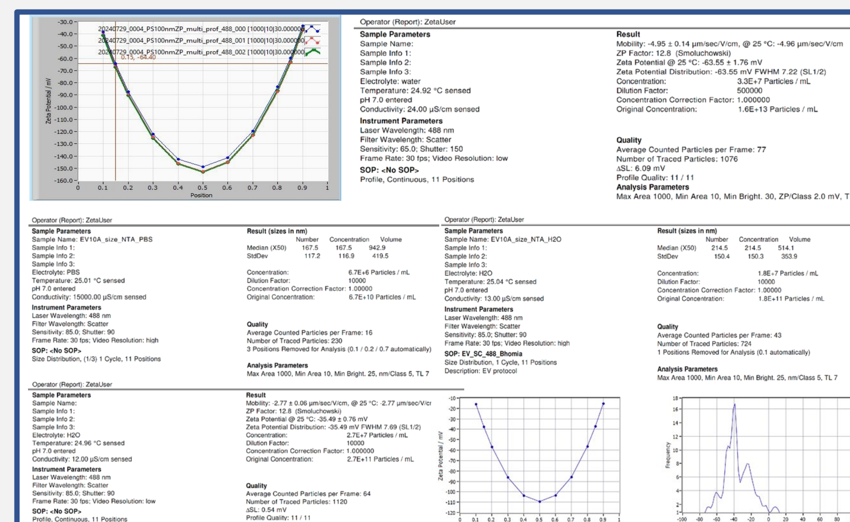

Figure 5. Zeta-potential (ZP) analysis. (Upper panel) Zeta-potential measurements of PS100 beads at all measuring positions were displayed in parabolas, and details were shown in the result table. (Middle panel) NTA measurements of human blood–derived EVs in PBS buffer vs. those in water. The EVs in water displayed an increased median of EV size vs. those in PBS buffer. The concentrations of EVs in PBS and water were also measured. Results indicated that buffer variation may affect not only EV sizes but also EV concentrations. (Lower panel) Representative measurement results of ZP measurements, ZP parabola, and frequencies of ZP distribution in an EV sample.

Summary

We have established the EV characterization platforms with NTA technology, fluorescence and colocalization, and zeta-potential measurement features to quantify size, concentration, and cellular origin specificity of EVs. We also provide downstream protein and transcriptomics analysis services for comprehensive molecular analyses for TBI and brain health–associated biomarker discovery.

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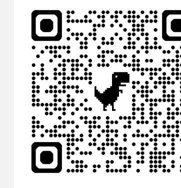

Reference

Alberro A, et. al. Extracellular Vesicles in Blood: Sources, Effects, and Applications. *Int J Mol Sci*. 2021 Jul 29;22(15):8163. doi: 10.3390/ijms22158163. PMID: 34360924; PMCID: PMC8347110.

Yáñez-Mó M, et. al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015 May 14;4:27066. doi: 10.3402/jev.v4.27066. PMID: 25979354; PMCID: PMC4433489.

Guedes VA, et. al. Extracellular Vesicle Proteins and MicroRNAs as Biomarkers for Traumatic Brain Injury. *Front Neurol*. 2020 Jul 16;11:663. doi: 10.3389/fneur.2020.00663. PMID: 32765398; PMCID: PMC7378746.

Puffer RC, Cumba Garcia LM, Himes BT, Jung MY, Meyer FB, Okonkwo DO, Parney IF. Plasma extracellular vesicles as a source of biomarkers in traumatic brain injury. *J Neurosurg*. 2020 Jul 24;134(6):1921-1928. doi: 10.3171/2020.4.JNS20305. PMID: 32707544.

Mustapic M, etl al. Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes. *Front Neurosci*. 2017 May 22;11:278. doi: 10.3389/fnins.2017.00278. PMID: 28588440; PMCID: PMC5439289.

Norman, M., et al. L1CAM is not associated with extracellular vesicles in human cerebrospinal fluid or plasma. *Nat Methods*. 18, 631–634 (2021). doi: 10.1038/s41592-021-01174-8

Nogueras-Ortiz CJ, et. al. Single-extracellular vesicle (EV) analyses validate the use of L1 Cell Adhesion Molecule (L1CAM) as a reliable biomarker of neuron-derived EVs. *J Extracell Vesicles*. 2024 Jun;13(6):e12459. doi: 10.1002/jev2.12459. PMID: 38868956; PMCID: PMC11170079.

Chinnathambi S, Kumarappan M, Malik S, Velmurugan G, Chandrashekar M. Neuron-derived extracellular vesicle-based diagnostics for Tau and Alzheimer's disease. *Brain Network Disorders*. Volume 1 (3), Sep 2025, Pages 150-166.

Welsh JA, et. al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles*. 2024 Feb;13(2):e12404. doi: 10.1002/jev2.12404. Erratum in: J Extracell Vesicles. 2024 May;13(5):e12451. doi: 10.1002/jev2.12451. PMID: 38326288; PMCID: PMC10850029.

Midekessa G, et. al. Zeta Potential of Extracellular Vesicles: Toward Understanding the Attributes that Determine Colloidal Stability. *ACS Omega*. 2020 Jun 30;5(27):16701-16710. doi: 10.1021/acsomega.0c01582. PMID: 32685837; PMCID: PMC7364712.

Rasmussen MK, Pedersen JN, Marie R. Size and surface charge characterization of nanoparticles with a salt gradient. *Nat Commun*. 2020 May 11;11(1):2337. doi: 10.1038/s41467-020-15889-3. PMID: 32393750; PMCID: PMC7214416.