ADNI Alzheimer's Disease Neuroimaging Initiative

Quantification of Extracellular Vesicles Carrying L1CAM, Amyloid beta 42, or Amyloid beta 40 in Human Plasma

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Summary

To assess the diagnostic potential of blood extracellular vesicles (EVs) likely derived from the central nervous system (CNS) or carrying known Alzheimer disease (AD) markers, the amounts of L1CAM-, amyloid beta 1-42 (Aβ42)-, and amyloid beta 1-40 (Aβ40)-positive EVs in blood plasma were quantified using an Apogee nanoscale flow cytometry platform and our newly developed labelling protocol. Briefly, plasma was ultracentrifuged and stored at -80°C before use. L1CAM, Aβ40, and Aβ42 antibodies were coupled to fluorescent dye using Zenon Antibody Labeling Kits. Ultracentrifuged (EV-enriched) plasma samples were thawed and then incubated with dye coupled antibodies overnight at 4°C prior to being diluted with PBS and read on an APOGEE Micro-PLUS flow cytometer. The levels of L1CAM-, Aβ42-, and Aβ40-positive EV subpopulations were acquired, and the proportions of these subpopulations among all detected plasma EVs are reported.

Method (Assay Protocol)

Materials & Instruments

1. Zenon IgG labeling kits (Invitrogen/Life Technologies),
   Including Component A (Dye) and Component B (IgG Block):
   Zenon™ Alexa Fluor™ 405 Mouse IgG1 Labeling Kit (Z25013) for L1CAM
   Zenon™ Alexa Fluor™ 647 Mouse IgG1 Labeling Kit (Z25008) for Aβ40
   Zenon™ Alexa Fluor™ 647 Rabbit IgG Labeling Kit（Z25308）for Aβ42
2. 2% bovine serum albumin (BSA): dissolve 2 g BSA heat shock fraction (pH 7, ≥98%; Sigma-Aldrich, A9647) in 100 ml PBS, pH7.4; 0.22 μm membrane filtered and stored at 4°C before use.

3. 10× Protease Inhibitor Cocktail (PIC): PIC powder (Sigma-Aldrich, cat# P2714), resuspended in 10 ml de-ionized and 0.22 µm membrane filtered water and stored at -20°C before use.
4. Mouse anti-L1CAM antibody [UJ127] - BSA and Azide free (Abcam, ab80832)
5. Mouse anti-β-Amyloid, 1-40 (Aβ40) antibody (Biolegend, 805401)
6. Rabbit anti-β-Amyloid (Aβ42) recombinant monoclonal antibody (Thermo Fisher Scientific, 700254)
7. 4% paraformaldehyde (PFA): 4% PFA Phosphate Buffer Solution (FUJIFILM Wako Pure Chemical Corporation, US), 0.22 µm membrane filtered
8. 0.22 µm membrane filters: Medical Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, sterilized by gamma irradiation (Millipore Sigma, SLGPM33RS)
9. 1.5-ml ultracentrifugation tubes (Beckman Coulter Life Sciences, 357448)
10. 1.5-ml microcentrifuge tubes (Fisher Scientific, 05-408-129)
11. Micro-PLUS nanoscale flow cytometer (Apogee Flow Systems, UK)
12. Optima TLX Ultracentrifuge with TLA55 rotor (Beckman Coulter Life Sciences, Indianapolis, IN)

Sample Ultracentrifugation (EV collection)

1) Thaw plasma samples in a 37°C water bath for 60 seconds, spin down samples once thawed. (12 samples are thawed at a time).
2) Measure plasma sample volume and add 1/10 volume 10× PIC.
3) Centrifuge 3000 × *g* for 30 minutes at 4°C.
4) Transfer supernatant to a new 1.5-ml microcentrifuge tube.
5) Centrifuge 10,000 × *g* for 30 minutes at 4°C.
6) Transfer 100 µl of centrifuged plasma to ultracentrifugation tube.
7) Add 1000 µl 0.22 µm filtered PBS to dilute the plasma sample.
8) ultracentrifuge 100,000 × *g* for 1 hour at 4°C.
9) Discard the supernatant, leaving a residual volume of around 100 µl to avoid disrupting the pellet.
10) Add 200 µl 0.22 µm filtered PBS, pipetting up and down 200 times to resuspend the pellet.

11) Add 900 µl 0.22 µm filtered PBS. Gently vortex and balance samples tubes (within ±1.0 mg) for centrifugation with filtered PBS if needed.
12) Ultracentrifuge 100,000 × *g* for 1 hour at 4°C.
13) Discard the supernatant, leaving a residual volume of around 50 µl.
14) Add 200 µl 0.22 µm filtered PBS, resuspend the pellet by pipetting up and down 200 times.
15) Aliquot the ultracentrifuged sample (~40 µl/aliquot) and store at -80°C.

Antibody Labelling Note: The antibody species and isotype must be matched to the correct Zenon IgG labelling kit. Furthermore, when multiple markers are being assessed, the Zenon IgG labelling kit wavelengths cannot overlap between pairs of markers.

Sample Labeling

1) Thaw ultracentrifuged plasma samples at room temperature.
2) Add 10 µl ultracentrifuged plasma to a 1.5-ml tube.
3) Add 10 µl 2% BSA and mix well by pipetting.
4) Incubate for 60 minutes at room temperature (around 25°C) to block non-specific labeling.
5) Dilute the blocked sample with 10 µl 0.22 µm filtered PBS.
6) Prepare 1 µg of antibody per 10 samples (minimum 1 µg of antibody per reaction) in PBS.
7) Vortex Zenon Component A (dye) for at least 30 seconds. Add 5 µl of Component A per 1 µg antibody to the antibody solution, vortex well and spin down, and incubate for 20 minutes at room temperature (25°C). Protect from light.
8) Vortex Zenon Component B (IgG Block), dilute 1:10 in 0.22 µm filtered PBS (for example, 2 µl component B into 18 µl 0.22 µm filtered PBS). Add 3 µl of diluted Component B per 5 µl of Component A to the mixture from step 7 and incubate for 10 min at room temperature. Protect from light.
9) Dilute the Zenon complex (step 8) by adding sufficient filtered PBS to result in a final volume of 50 µl per 1 µg of antibody (e.g., 1 µg antibody + 5 µk Component A + 3 µl diluted Component B + 41 µl filtered PBS = 50 µl total volume)

10) Add 5 µl diluted Zenon complex labeled anti-L1CAM, anti-Aβ40, or anti-Aβ42 to the thawed ultracentrifuged plasma sample. Mix well by pipetting and incubate overnight at 4°C. Protect from light. Equivalent antibody amount per sample is 0.1 µg.

Sample Post fixation

Add 20 µl of 0.22 µm membrane filtered 4% PFA to the labeled sample and incubate for 20 minutes at room temperature. Protect from light.

Sample Reading on Apogee

Dilute the sample to 200 µl with 0.22 µm membrane filtered PBS and read on Apogee instrument.

APOGEE settings

Apogee settings may require optimization between instruments. Specifically, the laser power, PMT, and thresholds may require adjustment. Additionally, positive event histograms should be applied to distinct populations of particles appearing above unlabeled particle signal. Settings used are described below:

Sample control:

- 100 µl of Zenon labelled, diluted ultracentrifuged plasma sample was injected through the APOGEE instrument at a flow rate of 1.50 µl/min for approximately 60 sec per sample.
- 2 flush cycles were applied between samples.
- Sheath pressure was set at 150mbar.

Laser Power, Gain, PMT, and Thresholds :

- All lasers were set at 75mW power.
- All wavelength gain was set to 1.0.
- PMT was set as shown in the following table:

Wavelength	Voltage
405-LALS	350
405-Blu	450
638-Red	500

- Thresholds were set as shown in the following table:

Wavelength	Threshold
405-LALS	17
405-Blu	30
638-Red	30

Histograms:

- Histogram wavelengths must match Zenon dye wavelengths and colors (e.g., 405-Blu for a 405 wavelength Zenon IgG dye kit, 488-Grn for a 488 wavelength Zenon IgG dye kit).
- 405-LALS is used to measure all events and is utilized as the x-axis of histograms measuring single positive events (e.g. 405-Blu y-axis and 405-LALS x-axis for a 405 wavelength Zenon IgG dye kit, 488-Grn y-axis and 405-LALS x-axis for a 488 wavelength Zenon IgG dye kit).
- L1CAM was labelled and measured under 405-Blu, Aβ40 under 638-Red, and Aβ42 under 638-Red.
- Histogram ROIs should be applied to delineate particle populations that are above unlabeled particle signal and recognized as positive events by APOGEE (**Figure 1**).

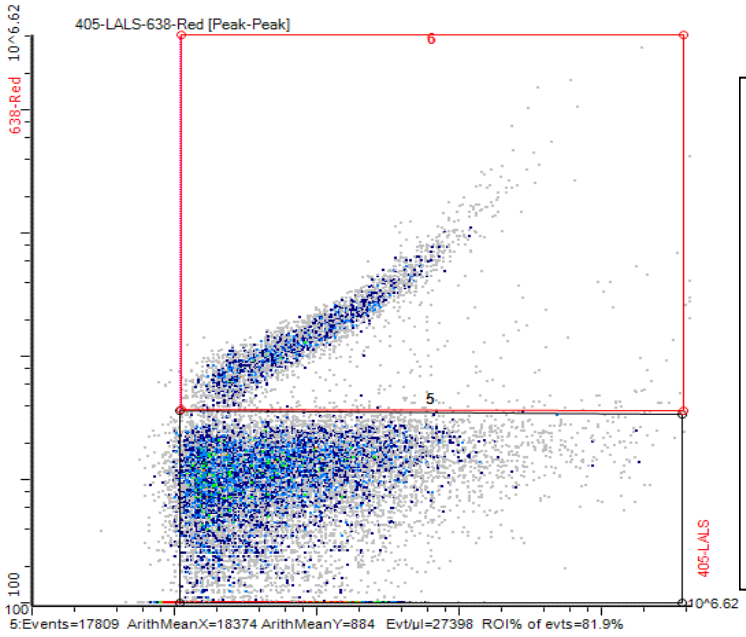

Figure 1 A representative Apogee histogram. Positive particle population is distinctly separated and above the unlabeled particle population. In this example, ROI 6 (red) encompasses all positive particles, while ROI 5 (black) contains unlabeled particles.

- Events within positive ROIs (L1CAM+, Aβ42+, or Aβ40+ EVs) and the total event concentrations (all detected EVs) are acquired. Data is reported as the proportion (%) of positive particles (EVs) among all detected plasma particles (EVs) in the dataset.

Assay Validation and Reproducibility

The APOGEE flow cytometery protocol for measuring L1CAM-, Aβ 42-, and Aβ 40-positive EVs in ultracentrifuged plasma samples was validated through the assessment of EV enriched and EV depleted plasma samples. Plasma was ultracentrifuged with the supernatant (**UC depleted**) and pellet (**EV enriched**) saved prior to application of the above protocol. EV enriched pellet fractions of plasma showed a robust increase of positive events when compared to the EV depleted supernatant after both fractions were incubated with the same antibody/dye complex (L1CAM, Aβ42, or Aβ40) (see **Figure 2** next page). Further, when isotype-matched normal IgG was applied to the dye labelling protocol in place of L1CAM, Aβ42, and Aβ40 antibodies, positive events in the EV enriched fraction were diminished and close to levels seen with no antibody addition (blank) (**Figure 2**).

Intra-day assay reproducibility was assessed by duplicate measures of four distinct reference plasma samples over five days. Intra-day %CVs of 8.6%, 8.9%, and 2.4% were obtained for L1CAM, Aβ42, and Aβ40, respectively. Inter-day assay reproducibility was assessed by a mean of daily means model across five days, resulting in an overall inter-day %CV of 10% or lower across all three markers. In our experimental cohort, inter-day %CVs of 9.4%, 9.4%, and 3.8% were obtained for L1CAM, Aβ42, and Aβ40, respectively, as determined by reference plasma control samples. Day-to-day correction/normalization was determined to be unnecessary.

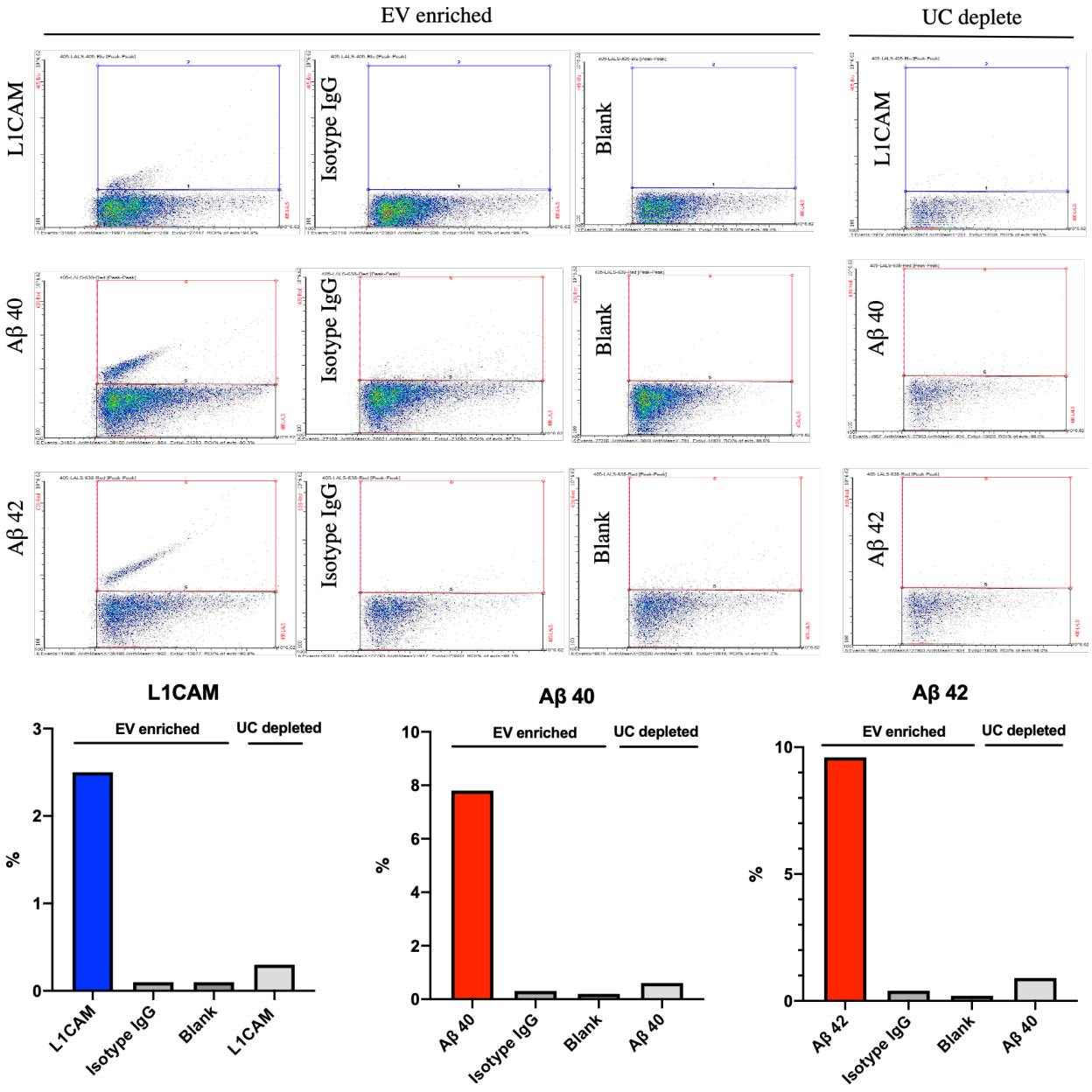

Figure 2 Representative histograms comparing L1CAM, Aβ42, and Aβ40 positive events to their respective isotype-matched normal IgG, without the addition of antibody during dye labelling, and L1CAM, Aβ42, and Aβ40 positive events in ultracentrifuged plasma supernatant.

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Version Information

This is the first (1.0) version of this document.

Dataset Information

This methods document applies to the following dataset(s) available from the ADNI repository:

Dataset Name	Date Submitted
Plasma_EV_Apogee_data_UW	August 4, 2020

References

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