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Amyloid-β Plaques in Clinical Alzheimer’s Disease Brain Incorporate Stable Isotope Tracer In Vivo and Exhibit Nanoscale Heterogeneity

Norelle C. Wildburger1*, Frank Gyngard2, Christelle Guillermier3,4,5, Bruce W. Patterson6, Donald Elbert1, Kwasi G. Mawuenyega1, Theresa Schneider1, Karen Green8, Robyn Roth9,10, Robert E. Schmidt8, Nigel J. Cairns1,8,11,12, Tammie L. S. Benzienger11,13,14, Matthew L. Steinhauser3,4,5 and Randall J. Bateman1,11,12*

1Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States, 2Department of Physics, Washington University in St. Louis, St. Louis, MO, United States, 3Nanomaging Center, Division of Genetics, Brigham and Women’s Hospital, Cambridge, MA, United States, 4Department of Neurology, Brown Medical School, Providence, RI, United States, 5Department of Medicine, Washington University School of Medicine, St. Louis, MO, United States, 6Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, United States, 7Department of Neurology, The University of Texas at Austin Dell Medical School, Austin, TX, United States, 8Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO, United States, 9Washington University Center for Cellular Imaging, Washington University School of Medicine, St. Louis, MO, United States, 10Knight Alzheimer’s Disease Research Center, Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States, 11Hope Center for Neurological Disorders, Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States, 12Department of Radiology, Washington University School of Medicine, St. Louis, MO, United States, 13Department of Neurosurgery, Washington University School of Medicine, St. Louis, MO, United States

Alzheimer’s disease (AD) is a neurodegenerative disorder with clinical manifestations of progressive memory decline and loss of executive function and language. AD affects an estimated 5.3 million Americans alone and is the most common form of age-related dementia with a rapidly growing prevalence among the aging population—those 65 years of age or older. AD is characterized by accumulation of aggregated amyloid-beta (Aβ) in the brain, which leads to one of the pathological hallmarks of AD—Aβ plaques. As a result, Aβ plaques have been extensively studied after being first described over a century ago. Advances in brain imaging and quantitative measures of Aβ in biological fluids have yielded insight into the time course of plaque development decades before and after AD symptom onset. However, despite the fundamental role of Aβ plaques in AD, in vivo measures of individual plaque growth, growth distribution, and dynamics are still lacking. To address this question, we combined stable isotope labeling kinetics (SILK) and nanoscale secondary ion mass spectrometry (NanoSIMS) imaging in an approach termed SILK–SIMS to resolve plaque dynamics in three human AD brains. In human AD brain, plaques exhibit incorporation of a stable isotope tracer. Tracer enrichment was highly variable between plaques and the spatial distribution asymmetric with both quiescent and active nanometer sub-regions of tracer incorporation. These data reveal that Aβ plaques are dynamic structures with deposition rates over days indicating a highly active process. Here, we report the first, direct quantitative measures of in vivo deposition into...
INTRODUCTION

Alzheimer’s disease (AD) is a devastating neurodegenerative disorder characterized by progressive cognitive and functional decline and is the most common form of age-related dementia (1, 2). The incidence of AD-related death and disability is rapidly growing in persons 65 years of age or older whose risk of developing the disease doubles every 5 years (3); currently, an estimated 5.3 million Americans are affected (4). Amyloid-beta (Aβ), a 38–43 amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein, is implicated as a pathogenic species in AD (5–10). Abnormal accumulation and aggregation of Aβ in the cerebral extracellular space result in one of the pathological hallmarks of AD—amyloid plaques. As a result, the onset and rate of amyloidosis has been intensively studied over the past several decades. Although older individuals may have extensive Aβ deposition without clinical signs of dementia (11–14), there is a strong association of amyloid plaques with AD dementia. Longitudinal measures of amyloid pathology have shown increases in amyloid deposition 15–20 years prior to dementia onset (15) in early onset, familial AD and predict future cognitive decline (16–20).

As amyloid pathology increases in vivo, a concomitant decline in cerebrospinal fluid (CSF) Aβ42 occurs (21). More recent studies in the production and clearance of Aβ in CSF (22) demonstrate that the clearance of Aβ42 is decreased in AD. Intriguingly, with the onset of amyloidosis, only the Aβ42 proteoform (23, 24) demonstrated faster turnover kinetics attributed to rapid deposition into plaques—for ~50% of all Aβ42 produced (22). Further, there was a positive correlation between increased Aβ42 turnover kinetics and rate of amyloidosis measured by PET (22). In plasma, the altered Aβ42 turnover kinetics and lower Aβ42/40 ratio with amyloidosis is also present albeit to a lesser extent (25). These data suggest that alterations in Aβ concentrations and kinetics are strongly associated with amyloidosis reflecting deposition of Aβ into plaques.

Longitudinal PET studies demonstrate that amyloid plaque pathology increases slowly and plateau with the clinical presentation of dementia (15, 20, 26–29). The slow accumulation of amyloid tracer binding over decades before clinical presentation of dementia has been suggestive of stability in plaque accumulation. The plateau of amyloid tracer binding may indicate that amyloid pathology reaches dynamic equilibrium or quiescence globally at the clinical stage of dementia. Similarly, several animal model studies of plaques have demonstrated that plaques grow, typically at earlier ages (30–32), to a stable size (32–34), but that plaques may be in equilibrium with their environment if continual deposition is assumed (33).

However, despite the relevance of amyloid plaques in AD, measures of in vivo growth and distribution of human plaques are lacking due to analytical challenges and limitations. Previous human in vivo studies (22, 25, 35) relied on CSF and plasma, which are indirect measures of the brain compartment and do not directly measure plaques. PET imaging of human brain is an average measure of amyloid pathology over ~1 cm, which does not measure individual amyloid plaques, and amyloid tracers bind only a subset of aggregated Aβ (36). Interpretation of PET results is limited because amyloid binding agents measure binding sites and not necessarily the true amount of amyloid plaques. Fluorescence-based assessments using multi-photon imaging of over-expression models, which do not fully replicate the human disease (37–39), encounter limitations resulting from specificity of dyes, decreased sensitivity due to tissue autofluorescence, and the inability to distinguish new versus previously existing Aβ or other proteins found deposited into plaques (40). As individual plaque dynamics have not been directly measured in humans, we therefore sought to measure the in vivo incorporation of protein into amyloid plaques to resolve whether plaques are stable or dynamic structures in the human AD brain.

Stable isotope labeling kinetics (SILK) was developed to determine protein production and clearance by administering a stable isotope labeled precursor in vivo and sampling during and after labeling (41). The development of SILK allowed quantitation of protein turnover in humans, providing a direct readout of changes underlying pathophysiology of disease and pharmacodynamics of therapeutics (22, 24, 42). However, SILK performed from CSF or plasma does not convey localization of labeled biomolecules in tissue. Nanoscale secondary ion mass spectrometry (NanoSIMS) allows both imaging and measurement of stable isotopes at high spatial resolution (50–100 nm or <1 μm) (43, 44). While NanoSIMS has been applied to the study of AD (45, 46), it has not been used in combination with SILK for the measurement of in vivo protein translation in cells or deposition into plaques in normal or diseased brain. Here, we coupled SILK to NanoSIMS in a method termed SILK–SIMS to directly measure and image the distribution and rate of protein deposition (a proxy for growth taking into account area) into individual plaques at the nanometer level (44, 47–49). Our aim was to measure the stability or growth of individual amyloid plaques in AD animal models and in the human brain.

Initial SILK–SIMS experiments in a mouse model of AD demonstrated in vivo detection of the stable isotope tracer 13C6-leucine in native Aβ plaques. Further, plaques incorporated more of the tracer via protein deposition compared to protein translation in the surrounding brain parenchyma, but less than neurons. In human AD brain, we found 13C enrichment in multiple diffuse and dense-core plaques in both the frontal lobe and precuneus. 13C enrichment was highly variable and asymmetric between plaques with both quiescent and active nanometer sub-regions.
of tracer incorporation. These data reveal that human Aβ plaques are dynamic structures and suggest that they have growth or turnover rates that can be directly quantified in AD brain. Our results provide the first, direct measures of in vivo plaque dynamics in human AD brain. We predict that SILK–SIMS will provide a precise understanding of protein deposition into amyloid plaques in human AD brain and other CNS disturbances characterized by protein aggregation.

MATERIALS AND METHODS

Cell Culture

B-cell hybridoma line (produced in-house by Holtzman Lab, Washington University) was grown for 5 days in leucine-free media that was supplemented with either 13C6-leucine or 13C6-leucine at 26 mg/L and mixed at the appropriate percentage of heavy isotope-containing media with 2% FBS. Cells were harvested and spun at 5 mL per 5 min at room temperature (RT). Cell pellets were resuspended in 4°C Ringers wash solution for 5 min, spun, and then fixed with 4% paraformaldehyde in 100 mM NaCl, 30 mM HEPES, 2 mM CaCl$_2$, pH 7.2 (NaHCa) for 2 h. This was followed by three rinses of NaHCa at RT and overnight incubation at 4°C in NaHCa. Centrifugation was used throughout the following steps in order to re-concentrate the cells to a pellet. The following morning, pellets were placed into ddH$_2$O and then infiltrated with LR White Embedding Media (Catalog #14383, EMS, Hatfield, PA) using the manufacturer’s published protocol with minor modification. Partial dehydration was accomplished by using 20% EtOH 15 min, 40% EtOH 15 min, 50% EtOH 15 min, 70% EtOH 15 min, 85% EtOH 10 min, followed by 1 h in a 2:1 LR White to 85% EtOH. Sections of LR White embedded samples were cut on a Leica UC7 Ultramicrotome using a diamond knife. 200 nm and 400 nm sections were picked up with a perfect loop, placed on top of a polished silicon (Si) wafer (Catalog #534, University Wafer Inc., South Boston, MA, USA), and let air dry on a 35°C hot plate.

APP/PS1 Plasma Leucine

Mouse whole blood was spun at 1,000 × g for 10 min, and the plasma (supernatant) was removed. Plasma proteins were precipitated with ice-cold acetone followed by de-lipidation with hexane, and the aqueous fraction was dried in vacuo (51). 1:1N-Methyl-N-tert-butyl(dimethyl)silyl trifluoroacetamide/acetonitrile was added, and samples were incubated at 70°C for 30 min. Duplicate 1 µL injections were made into an Agilent 5973 MSD mass spectrometer using a 30 µm × 0.25 mm DB-5MS column (Agilent Technologies). Electron impact ionization and selected ion monitoring were used to measure endogenous unlabeled leucine at m/z 200 (molecular ion minus C-1 as CO$_2$-tert-butyl(dimethyl)silyl; CO2-tBDMS), and 13C6-leucine (tracer) was measured at m/z 205 as an m + 5 ion. The tracer-to-tracee ratio (TTR) was taken as the m + 5/m + 0 peak area ratio of the biological sample minus the m + 5/m + 0 ratio of a natural abundance leucine sample. The molar fraction of labeled leucine was calculated as: MFL = TTR/(1 + TTR).

Human Tissue

Clinically and neuropathologically well-characterized human brain tissue samples were obtained from the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (Knight ADRC), Washington University School of Medicine, Saint Louis, Missouri. At the time of death, written informed consent in accordance with the Declaration of Helsinki was obtained from the next-of-kin in accordance with the protocol approved by the Washington University Human Studies Committee and the General Clinical Research Center Advisory Committee. Cognitive status at expiration was determined using a validated retrospective post-mortem interview with an informant to establish the clinical dementia rating (CDR) (52). We used frozen tissue from the frontal lobe (Brodmann areas 8/9) of P2 with mild AD dementia (CDR 1, age at death = 88 years; post-mortem interval = 15 h; post-labeling interval (delta) = 8 days; Table 1). The right cerebral hemisphere was coronally sliced at 1 cm intervals and snap frozen by contact with pre-cooled Teflon®-coated aluminum plates, and temperature equilibrated by immersion into liquid nitrogen vapor in a cryo-vessel. Following freezing, tissues were placed in Ziploc® storage bags and stored in freezer at −80°C. Participants 2 and 3 were given the stable isotope tracer 13C6-leucine as part of previous SILK studies (22, 35). These AD participants had made previous arrangements for brain donation to Washington University.
Formalin-fixed tissue from the frontal lobe, including the cortical ribbon, of Pt1 (negative control, no labeling), Pt2 (delta = 8 days), and Pt3 (delta = 4.5 years) were post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, en bloc stained with 3% aqueous uranyl acetate for 1 h, dehydrated in graded ethanols, and embedded in PolyBed 812 catalog #08792-1 (Polysciences, Hatfield, PA, USA). Blocks were polymerized at 80°C for 72 h. Tissue blocks were sectioned using a diamond ultrathin section knife on a Reichert Ultra-Cut E ultramicrotome at 300–500 nm. Sections were transferred to a single polished Si wafer for SILK–SIMS analysis. Serial adjacent sections were placed on glass microscope slides for toluidine blue staining for light microscopy. The precuneus of Pt2 was prepared and embedded in LR White as described above for animal tissue along with the precuneus of Pt3, which was serially sectioned with the second set (precuneus), Pt2 precuneus samples (embedded in PolyBed812) and Pt1 frontal lobe samples (embedded in LR White). Toluidine blue stained sections were imaged with a Hamamatsu NanoZoomer 2.0-HT System. Imaging was done to guide feature identification and location for electron microscopy.

**Electron Microscopy**

Images of the tissue and reference points were taken with a field emission scanning electron microscope (FE-SEM; Quanta™ 3D FEG, FEI, Hillsboro, OR, USA) in order to document plaque locations and provide an absolute coordinate system for the tissue. In-house coordinate transformation software was used to translate tissue regions-of-interest (ROIs) and reference points found in the FE-SEM to the NanoSIMS instrument stage coordinate plane for relocation of the same ROIs. Additional sections were cut at 70–90 nm for transmission electron microscopy (JEOL JEM-1400Plus) to image selected plaques to define ultrastructure. Anti-Aβ antibody 82E1 (1:50; Aβ N-terminal epitope (1-16); IBL-America, Minneapolis, MN, USA) was used with goat anti-mouse secondary antibody conjugated to 10 nm gold particles (1:15).

**Magnetic Resonance Imaging (MRI) and PET-PiB Imaging**

Participants were labeled with the radiotracer N-methyl-2-(4-methylaminophenyl)-6-hydroxybenzothiazole (Pittsburgh compound B, PiB) for human brain PET imaging of amyloid deposition. PiB was prepared as previously described (53). PET and MRI were performed as previously described (54).

**NanoSIMS**

Data were acquired on either a Cameca NanoSIMS 50 at Washington University (cells and human tissue) or NanoSIMS 50 L at Brigham and Women's Hospital (mouse tissue). Images of the B-cell hybridoma used to calculate the 13C6-leucine standard curve were acquired with a 50 µm primary beam raster for 15 min at 1 ms/px and 67.5 s/plane (dwell time) for a total of 10 planes (i.e., cycles) per mass (256 × 256 px). APP/PS1 mouse brain tissue was acquired at a 17–60 µm raster for 11–87 min at 2 ms/px and 131 s/plane (dwell time) for a total of 5–40 cycles (i.e., planes) per mass (256 × 256 px). For all APP/PS1 measurements, the non-labeled cells (0%) were measured prior to the SILK–SIMS data acquisition. The unlabeled cells and the natural abundance of carbon-13 (55) were used for quantitation of normalized ratios for mouse plaques and neuron. Plaque ROI outlines were drawn based on plaque morphology and areas around the plaque defined such they do not overlap or intersect with the predefined plaque ROIs.

The human tissue was analyzed in three sets. In the first set (frontal lobe), Pt1 (negative control) and Pt2 frontal lobe were embedded in PolyBed 812 and pre-sputtered using the D1-1 aperture for 10 min followed by data acquisition at a raster size between 35 and 50 µm raster with a D1-2 aperture. Data acquisition was 4 h at 5 ms/px and 327.68 s/plane (dwell time) for a total of 40 planes (i.e., cycles) per mass (256 × 256 px). In the second set (precuneus), Pt2 precuneus samples (embedded in LR White; Figures S9 and S11 in Supplementary Material) were pre-sputtered at 30 µm raster with a D1-1 aperture for 10 min followed by data acquisition at 25 µm raster with the D1-2 aperture. Data acquisition was 2.5 h at 5 ms/px and 327.68 s/plane (dwell time) for a total of 25 planes (i.e., cycles) per mass (256 × 256 px). Pt1, also embedded in LR White, was used as the negative control in this experimental set. Finally, unlabeled AD tissue (Pt1; in Supplementary Material) embedded in PolyBed812 was acquired with a 45 µm raster for 11.6 h at 4 ms/px and 1,048,576 s/plane (dwell time) for a total of 40 planes (i.e., cycles) per mass (512 × 512 px). Pt3’s (in Supplementary Material) brain tissue was acquired with a 55 µm raster for 18 h at 5 ms/px and 1,310 s/plane (dwell time) for a total of 40 planes (i.e., cycles) per mass (512 × 512 px). For all measurements, the non-labeled material (Pt1) was measured prior to the SILK–SIMS data acquisition. In this way, the negative control data were acquired first before

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**Table 1**

| Participant | Amyloid status PET-PiB | CDR* | AD dementia | MMSE* | Gender | Age | ∆ Between labeling and DOE (days) |
|-------------|------------------------|------|-------------|--------|--------|----|----------------------------------|
| 1           |           |      |            |        |        |    |                                 |
| 2           | pos       | 1    | Mild       | 22     | M      | 88 | 1,150 and 8Δ                   |
| 3           | pos       | 1    | Mild       | 11     | M      | 88 | 1,648                           |

Clinical autopsy provided a diagnosis of Alzheimer’s disease (AD) for all participants. CDR, clinical dementia rating; MMSE, mini mental state exam; DOE, date of expiration.

*This participant was not part of the SILK studies.

This participant was in two SILK studies and thus has two time lapses.
SILK data acquisition, and the electron multipliers were no longer adjusted after negative control data were acquired.

**NanoSIMS Data Analysis**

Each analysis was performed in 24-h blocks with measurements on a SiC standard of known isotopic composition to assess instrument stability followed by measurements on an unlabeled control prior to SILK–SIMS data acquisition. Raw image data were imported into the L’Image image processing software (Larry Wittler, Carnegie Institution of Washington) to produce quantitative ratio images of heavy and light isotopes and determine where isotopic anomalies were located. Images were automatically segmented into 10 × 10 pixel ROIs using L’image, and the heavy/light isotopic ratios were calculated from the summed ion counts within each ROI. The fractional uncertainty, \( f \), of each ROI ratio is defined as:

\[
f = \sqrt{\frac{\sigma_{\text{Std}}}{R_{\text{Std}}}^2 + \frac{\sigma_{\text{ROI}}}{R_{\text{ROI}}}^2}
\]

where \( R_{\text{Std}} \) is the average ratio of repeated measurements on unlabeled tissue, and \( R_{\text{ROI}} \) is the ratio calculated from summing the counts of every pixel contained within the individually defined ROI. This uncertainty represents the entire experimental precision and accuracy, including: counting statistics, matrix effects, systematic error, instrumental tuning, and differences between standards and samples. From this uncertainty, the amount, significance, and location of heavy isotopic labeling can be quantitatively determined. Those ROIs with heavy/light isotope ratios greater than or equal to \( \mu + 2\sigma \) value of the unlabeled sample were analyzed by a one-sample \( t \)-test. Two SDs from the mean (\( 2\sigma \)) was chosen to ensure high confidence in \( \sigma \) unlabel sample were analyzed by a one-sample from the mean (\( 2\sigma \)) was chosen to ensure high confidence in

\[
\text{Retention time alignment was based on the 15N internal standard, and digested overnight with 0.25 ng/µL Lys-N (Catalog #100965-1, Seikagaku Biobusiness Corp., Tokyo, Japan) at 4°C.}
\]

**Mass Spectrometry**

Samples were resuspended in 1% FA/10% ACN (v/v) with 20 nM BSA digest (Catalog #1863078, Pierce, Rockford, IL, USA). Samples were analyzed in triplicate by nanoLC-MS/MS on an LTQ-Orbitrap Fusion (Thermo Fisher Scientific) in positive ion mode. Separations were performed using an online NanoAcquity UPLC (Waters, Milford, MA, USA) using an ACQUITY UPLC HSS T3 (360 µm OD × 75 µm ID) column packed with 10 cm C\(_{18}\) (1.8 µm, 100 Å, Waters) at 300 nL/min and heated to 65°C. Mobile phases were 0.1% FA in water (A) and 0.1% FA in ACN (B). Samples were eluted from the column with the gradient starting at 12% B, which was ramped to 32% B over 10 min and further increased to 90% B over 5 min and held for 1 min, before re-equilibration to 12% B over 2 min. Total run time, including column equilibration, sample loading, and analysis, was 30 min. The mass spectrometer was operated in targeted MS2 mode. MS2 spectra were acquired in the Orbitrap (30,000 at \( m/z \) 400) in centroid mode using XCalibur, version 4.0 (Thermo Fisher Scientific). Ion injection times for the targeted MS2 scans for labeled and unlabeled samples, respectively (in ms) were: A\(\beta\) mid-domain (54, 1080), A\(\beta\)40 (54, 540), and A\(\beta\)42 (54, 1080). The Orbitrap automatic gain control targets were set to \( 5 \times 10^7 \) for all proteoforms except A\(\beta\)42, which was set to \( 1 \times 10^8 \). The targeted precursor ions were sequentially isolated in the quadrupole and fragmented in the Orbitrap using HCD (isolation width 1.6 Da, normalized collision energy 25%, activation Q 0.250, and activation time 10 ms). The general mass spectrometric conditions were as follows: spray voltage 2.2 kV, 60% S-lens, and ion transfer tube temperature 275°C.

**Mass Spectrometry Data Analysis**

Data (.raw files) were imported into a Skyline template containing the Lys-N C-terminal peptides of A\(\beta\)38, 40, 42, and the mid-domain. Retention time alignment was based on the \( ^{13} \text{N} \) internal standard. The sum of all transitions (\( b \) ions monitored for each parent peptide) for unlabeled and \( ^{13}\text{C}_{6}\)-leucine labeled A\(\beta\) peptides were exported from Skyline to Excel. The ratios of labeled/unlabeled of each replicate (triplicate injections) for Pt2 sample were taken followed by isotopic background subtraction of the mean ratio of an unlabeled participant to give the TTR minus background for each replicate injection. Next, the background subtracted TTRs were used to calculate the mean and SD (i.e., the mean of the ratio ratios) of enrichment for each A\(\beta\) peptide.

**Data Availability Statement**

NanoSIMS image files (.im) and L’Image saved session (.sav) files are available upon request from corresponding authors. Excel data analysis files for human NanoSIMS images can be found in the Supplementary Material.
RESULTS

Quantification of $^{13}$C and In Vivo Labeling

To quantify carbon isotopes in biological material, we measured carbon as negative ions of $^{12}$C, $^{13}$C, $^{12}$C$^{14}$N, and $^{13}$C$^{14}$N. Using a B-cell hybridoma grown in leucine-free media supplemented with increasing percentages of $^{13}$C$_6$-leucine, we characterized the signal response for $^{13}$C enrichment. Figure 1 demonstrates the linearity of response of SILK–SIMS measurements to increasing isotopic enrichment of $^{13}$C$_6$-leucine in culture measured as the ratio of $^{13}$C$^{14}$N/$^{12}$C$^{14}$N, which produced improved image quality and more accurate quantification (Figure S1–3 in Supplementary Material). To determine whether $^{13}$C$_6$-leucine enrichment could be detected in vivo in native Aβ plaques, two individual APP/PS1 mice (3.5 months of age) were administered $^{13}$C$_6$-leucine tracer for 10 and 5 weeks (Figure 2A). The mouse labeled for 10 weeks (Figure 2B) reached a $^{13}$C$^{14}$N/$^{12}$C$^{14}$N ratio of 2.4% ± 0.03% in plaque (expected unlabeled at 1.1% due to $^{13}$C natural abundance) compared to brain parenchyma (2.2% ± 0.03%) in both Area 1 and Area 2 (Figure 2B; Figure S4 in Supplementary Material). A plaque from the APP/PS1 mouse labeled for 5 weeks, followed by a 5-week washout, remained enriched at 1.6% ± 0.02% relative to natural abundance (Figure 2C), and compared to adjacent areas (1.4% ± 0.02%). Despite being labeled for half the amount of time with an additional 5 weeks of tracer washout, the plaque enrichment was approximately two-thirds of that measured in the 10-week labeled animal (1.6% ± 0.02% vs. 2.4% ± 0.03%). The enrichment of plasma leucine measurements taken at the time of collection was fourfold lower (72 vs. 16% isotopically labeled leucine in the 10-week vs. 5-week labeled mouse, Table 1). A neuron incorporated substantially more tracer than the surrounding parenchyma (neuron = 3.3% ± 0.05% vs. area 1 = 2.4% ± 0.03% vs. area 2 = 2.4% ± 0.03%) and the plaque (Figure S5 in Supplementary Material) in the 10-week labeled mouse. These results demonstrate that $^{13}$C$_6$-leucine incorporation into Aβ plaques and other features could be measured and is localized to protein deposition (i.e., plaques) and protein translation (i.e., cells).

In Vivo Labeling and Quantification of Human AD Plaques

The APP/PS1 mouse model (50) is an over-expression model of APP mutations typically found in early onset, familial disease, which accounts for <1% of AD cases (58) and does not replicate all aspects of the human disease or human amyloid-plaques (37–39). Further, despite intensive study of plaque dynamics in animal models (30–34), little is known about individual plaque dynamics in human AD brain. The demonstrated feasibility of measuring $^{13}$C$_6$-leucine incorporation in native Aβ plaques from mouse brain (Figure 2) prompted us to investigate post-mortem tissue from previous SILK study participants (22, 35). Plaques from unlabeled AD participant tissue (Table 2; Figure S6 in Supplementary Material) were similar to the natural abundance of $^{13}$C as expected. In a previous SILK participant (Pt3) (22), who had a post-labeling interval of 4.5 years (Figure S7 in Supplementary Material), we were also unable to detect $^{13}$C enrichment in the examined areas. In contrast, post-mortem tissue from Pt2, who was labeled (35) and passed away 8 days later due to an unrelated cause, had significant $^{13}$C enrichment in amyloid plaques (Figure 3). Four diffuse plaques in the

![Figure 1](image-url)
Figure 2 | Stable isotope incorporation within plaques of APP/PS1 mouse model of Alzheimer’s disease. (A) Labeling scheme to determine the feasibility of detecting in vivo incorporation of stable isotope $^{13}$C in native Aβ plaques. (B,C) From left to right, $^{12}$C$^{14}$N ion map, $^{13}$C$^{14}$N ion map, $^{13}$C$^{14}$N/$^{12}$C$^{14}$N ion map, and normalized $^{13}$C$^{14}$N/$^{12}$C$^{14}$N ratios of 10-week and 5-week labeled APP/PS1 mice, respectively (35 × 35 μm images; see, Figure S4 in Supplementary Material, for region-of-interest (ROI) outlines based on plaque morphology and areas around the plaque defined such they do not overlap with plaque ROIs). Error bars indicate ± SD in quadrature of the SD of the average ratios measured for non-labeled cells (0%) and the Poisson errors of the feature itself (Equation 5 in Supplementary Material). Dashed horizontal line represents natural abundance of $^{13}$C (1.1%). Scale bar, 2 μm.

Table 2 | Plasma leucine in labeled APP/PS1 mice.

|              | $^{12}$C$_6$-Leucine peak area | $^{13}$C$_6$-Leucine peak area | $^{13}$C/$^{12}$C | TTR | Mol fraction labeled | Average Mol fraction labeled |
|--------------|-------------------------------|-------------------------------|-------------------|-----|---------------------|-----------------------------|
| 10 week      | 4,579                         | 11,793                        | 258%              | 258%| 72%                 | 72%                         |
|              | 4,601                         | 11,845                        | 257%              | 257%| 72%                 | 72%                         |
| 5 week       | 14,438                        | 2,878                         | 20%               | 20% | 16%                 | 16%                         |
|              | 14,256                        | 2,724                         | 19%               | 19% | 16%                 | 16%                         |

Each sample was injected twice with excellent reproducibility.
TTR, tracer-to-tracee ratio.

Frontal lobe displayed puncta of $^{13}$C labeling intercalated in the periphery and fibrillar interior (Figure 3C). Notably, not all plaques demonstrated equivalent amounts of $^{13}$C incorporation or the same number of enriched regions (max $^{13}$C = 1.26, 1.34, 1.49, and 1.20%, for plaques 1–4, respectively). Among plaques with an equivalent number of enriched regions (e.g., plaques 2 and 3) $^{13}$C enrichment varied, suggesting differing rates of $^{13}$C incorporation among “dynamic” plaques (Figures 3C,D). Additionally, the number of areas with significant enrichment drastically differed, indicating that plaque incorporation of $^{13}$C is highly asymmetric and can be constrained to specific regions or sub-regions; in the case of plaque 4, it is confined to a 3.0 μm$^2$ area (Figures 3C,D; Figure S8 in Supplementary Material). To confirm this result was not restricted to a single brain region, we examined plaques in the precuneus (Figure S9 in Supplementary Material). Of the two dense-core plaques imaged by SILK–SIMS and verified by immunoelectron microscopy (Figure S10 in Supplementary Material), only plaque 5 had $^{13}$C enrichment within the plaque, the peri-plaque region (Figures S9A–F in Supplementary Material), and up to 50 μm away (Figure S11 in Supplementary Material). In contrast, plaque 6 had a low number of enriched regions, which appear part of the surrounding brain parenchyma (Figures S9G–L in Supplementary Material) rather than the plaque itself, suggesting a quiescent plaque. To
provide orthogonal validation of the presence of $^{13}$C$_6$-leucine Aβ proteoforms (23) in Pt2 brain, we used targeted nLC-MS/MS. In Aβ from insoluble aggregates (57), we quantified $^{13}$C$_6$-leucine labeled Aβx-40, 42, and mid-domain peptides, which were enriched at relative abundances of 0.112, 0.022, and 0.053% above natural abundance, respectively, while Aβx-38 was not detected as expected (Figure 4).

**DISCUSSION**

In the current study, we utilized brain tissue from participants enrolled in previous SILK studies (22, 35) for NanoSIMS imaging. During the *in vivo* stable isotope labeling procedure (41) newly synthesized proteins are labeled simultaneously. As a result, many different features (e.g., plaques, parenchyma, and neurons) and...
suggesting no net change (20, 26–29). Level and plaque nanometer sub-region level, despite PET studies can be at both dynamic equilibrium and quiescence within a single participant who had a post-labeling interval of eight days. Yet, to the best of our knowledge, this represents the first report of imaging of individual human amyloid plaques in vivo dynamics at the nanometer scale not appreciable by previous methods.

**Human Aβ Plaques Are Dynamic Structures**

The incorporation of $^{13}$C$_6$-leucine into plaques (i.e., protein deposition) in a participant with clinical stage AD dementia suggests that plaques can be dynamic with activity over days even in later stages of the disease. Notably, we were able to demonstrate dynamic activity in both diffuse and dense-core plaques in contrast to previous animal model studies (30–34), which were restricted to imaging dense-core plaques due to the use of fluorescent plaque binding reagents Thioflavin S and Methoxy-04. The activity of individual plaques can be highly variable as demonstrated by varying isotope enrichment, suggesting differing rates of protein incorporation even among individual plaques in the same brain region. A single plaque could simultaneously demonstrate nanometer scale regions of dynamic incorporation of the tracer as well as quiescence—highly asymmetric tracer incorporation.

Plaque asymmetry was previously suggested (33) in a mouse model of AD but was attributed to asymmetrical clearance. The quiescence regions and sub-regions of individual plaques could be interpreted as either asymmetric clearance or differential nanometer scale deposition. However, not all plaques incorporated the tracer indicating a lack of protein deposition during the same timeframe. Together, these results demonstrate that plaques can be at both dynamic equilibrium and quiescence within a single participant even with dementia, at both the individual plaque level and plaque nanometer sub-region level, despite PET studies suggesting no net change (20, 26–29).

**CONCLUSION AND OUTLOOK**

The application of SILK–SIMS to future studies will allow estimates of the rate and distribution of plaque deposition at the nanometer level across human AD cohorts. The results will provide a better understanding of the rate of AD pathophysiology and may have a significant impact on the development of therapeutic strategies; for example, drugs targeting amyloid plaque growth and resorption through a variety of mechanisms. However, SILK–SIMS, unlike PET imaging, is currently limited to post-mortem tissue. Biopsies are an alternative but are invasive and only feasible with surgery to relieve other conditions such as normal pressure hydrocephalus. Nonetheless, SILK–SIMS may also be applied to measuring other proteinaceous deposits such as Lewy bodies (alpha-synuclein), tau, and prion protein), or MALDI imaging (60, 61) may be used in parallel to obtain molecular identities of plaque constituents in situ, though at micrometer resolution.

**ETHICS STATEMENT**

All animal procedures were conducted in accordance with the animal protocol approved by the Washington University Animal Studies Committee and are consistent with the National Institutes of Health (NIH) guidelines for the care and use of animals. Clinically and neuropathologically well-characterized human brain tissue samples were obtained from the Charles F. and Joanne Knight Alzheimer’s Disease Research Center.
(Knight ADRC), Washington University School of Medicine, Saint Louis, Missouri. At the time of death, written informed consent in accordance with the Declaration of Helsinki was obtained from the next-of-kin in accordance with the protocol approved by the Washington University Human Studies Committee and the General Clinical Research Center Advisory Committee.

**AUTHOR CONTRIBUTIONS**

NW, DE, FG, and RB designed the experiments. NW, FG, and CG operated the NanoSIMS instrument for SILK–SIMS data acquisition. NW performed all animal experiments and biochemical fractionation of SILK AD tissue. BP contributed APP/PS1 and human plasma leucine measurements, data analysis, and critical discussions regarding relevance of NanoSIMS to Aβ peptide SILK studies. KM assisted with mass spectrometry data acquisition and analysis. TS performed sample digestion and preparation for mass spectrometry analysis. KG and RR performed histology and EM sample preparation. RS provided unlabeled tissue, supervised the EM preparation, and confirmed plaque pathology. NC provided SILK tissue and advised about plaque location in tissue and sample processing. TB provided PET-PiB and MRI images and analysis. MS and RB supervised analyses and provided critical feedback at all junctures. NW made the figures. NW, FG, and RB wrote the manuscript. All authors approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00169/full#supplementary-material.
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Conflict of Interest Statement: RB and Dr. Holtzman (Chair of the Department of Neurology) and Washington University in St. Louis have equity ownership interest in C2N Diagnostics and may receive royalty income based on technology licensed by Washington University to C2N Diagnostics. In addition, RB and Dr. Holtzman receive income from C2N Diagnostics for serving on the Scientific Advisory Board. Washington University, with RB and Dr. Holtzman as co-inventors, has also submitted the U.S. non-provisional patent application “Methods for measuring the metabolism of CNS derived biomolecules in vivo,” serial #12/267,974. Washington University, with NW, RB, FG, and DE, has also submitted the U.S. provisional patent application “Methods and systems for measuring, detecting, and determining the location of a biomolecule or drug,” #62523811.

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