Capturing protein production and clearance in neurodegeneration with SILK

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Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD) are characterized by protein dysregulation, misfolding and accumulation in the central nervous system. The stable isotope labeling kinetics (SILK) technique labels proteins using a naturally occurring heavy isotope, thereby allowing for rates of production and clearance to be determined *in vivo*. Over the past decade SILK has been used to determine the normal turnover of key pathogenic proteins amyloid-β (Aβ), tau, and superoxide dismutase 1 (SOD1) in cerebrospinal fluid, and to identify several factors that alter production and/or clearance rate, including age, sleep, and disease-causing genetic mutations. Aβ turnover has also been successfully measured in blood and within brain tissue. SILK has potential to elucidate a range of neurodegenerative disease mechanisms including neuroinflammation and synaptic dysfunction, and to demonstrate target engagement of novel disease modifying therapies.

Introduction

The accumulation of misfolded proteins in the CNS is a common mechanism underpinning neurodegenerative diseases. Understanding rates and mechanisms of production and clearance for specific proteins will be key to understanding how such a state of disequilibrium occurs.

Stable isotope labeling kinetics (SILK) is a technique used to measure protein synthesis and clearance *in vivo* and *in vitro*. Stable isotopes have been used to interrogate human physiology since their discovery in the 1920s[1]. However, only relatively recently has the technique been employed to probe central nervous system (CNS) physiology and *patho*physiology. Stable isotopes are naturally occurring chemical elements (e.g., carbon-13, nitrogen-15 or hydrogen-2) that possess additional neutrons in their atomic nuclei, which make them fractionally heavier but otherwise non-radioactive and chemically identical to the natural element. Stable isotope labeled amino acids can be administered to humans intravenously or orally, effectively "labeling" newly translated proteins. These proteins are typically immunoprecipitated, digested using an enzyme (e.g. Trypsin, Lys-N, or AspN) and then quantified using targeted mass spectrometry (MS). Isotope-labeled peptides can be distinguished from their unlabeled counterparts using MS because of their mass difference, which quantifies the labeled to unlabeled peptide ratio, and reflects the rate of amino acid incorporation into a protein. This provides protein kinetic measures of synthesis, release into body fluids or tissue, and clearance. Further, SILK has the potential to provide more immediate *in vivo* evidence of target engagement in clinical trials, advancing therapeutic discovery.

Here we discuss the principles of SILK, summarize the key SILK studies, and discuss utility across different biological models.

(FIG 1 here)

Principles of SILK

SILK has been used in both preclinical models systems, including induced pluripotent stem cell, cell culture, and rodents, as well as in humans to measure protein synthesis and clearance rates (Figure 1). The SILK method combines administration of essential amino acids (indispensable amino acids that cannot be synthesized by the body) enriched with naturally occurring stable (heavy) isotopes with the quantification of incorporation of these amino acids into new protein synthesis over time. Leucine labeled with $^{13}C_6$ is the most widely used amino acid in SILK studies to date. After amino acid administration is stopped, the decline in the quantity of the stable isotope labeled amino acid in the protein target is quantified over time representing the protein clearance or degradation rate. Most *in vitro* and *in vivo* biological systems cannot discriminate $^{13}C_6$- from $^{12}C_6$-labeled amino acid (as is the case for the different nitrogen isotopes ^{15}N and ^{14}N), so incorporation of [$^{13}C_6$] leucine into newly synthesized proteins follows identical pathways and kinetics as endogenous $^{12}C_6$-leucine[2]. When [$^{13}C_6$] leucine is administered, each leucine in the target protein may incorporate: 1) a [$^{13}C_6$] heavy or 2) a [$^{12}C_6$] light form. MS can then be applied to samples (tissues, fluids) subsequently collected to identify and quantify the presence of these two forms, quantifying the heavy-to-light ratios of the protein or peptides. Kinetic models of the system may then be used to estimate *in vivo* production, aggregation, transport, and degradation rates.

By way of example, amyloid beta (Aβ) is produced primarily by neurons in the brain, followed by secretion into the cerebrospinal fluid (CSF)[3]. Aβ peptides in the CNS are continually produced, degraded or cleared during the labeling experiment. Aβ clearance from the CNS may occur via interstitial/CSF fluid (ISF) transport and resorption[4], transport across the blood brain barrier[5], reuptake by brain tissue, *in situ* proteolysis[6] and glymphatic clearance[7]. Irreversible deposition into amyloid plaques removes Aβ from the ISF, but not from the CNS. Through serial sampling of blood and CSF, SILK can be used to investigate the

processes of protein turnover, which is the sum total of the underlying mechanisms for target protein accumulation, aggregation, and deficit. When a stable isotope labeled amino acid (tracer) is introduced, the isotopic enrichment (abundance) of heavy Aβ peptides increases over time as unlabeled peptides are removed and replaced by newly synthesized, isotope-labeled peptides (Supplementary videos 1,2,3). After tracer administration is discontinued, the stable isotope labeled amino acid enrichment in the target protein or peptide returns (declines) to natural abundance, which is negligible. Changes in the isotopic enrichment of Aβ can be measured via immunoprecipitation and MS allowing for the calculation of protein synthesis and clearance rates from ratio of labeled:unlabeled Aβ (Figure 1). SILK can be used to measure the kinetics of a variety of neuronally derived proteins; as rates of protein synthesis and differ, specific labeling protocols need to be established for each protein of interest (i.e. tau, SOD1, alpha-synuclein).

Compartmental models can provide a mathematical framework describing the flow of tracer from site of administration through protein synthesis to a final sampled product (e.g. Aβ in CSF) (Supplementary video 3, FIG. 2). The model's structure and rate constants affect the shape and magnitude of the predicted enrichment time course and adjusted to optimize the fit to the observed SILK data. The compartmental model typically identifies a fractional turnover rate (FTR) for the whole system, which is the fraction of a rate-limiting compartment irreversibly removed per unit time. Mass flux rates along a pathway are calculated as the product of compartment mass times the rate constant. Specifically, if compartment 3 in FIG. 2 represents CSF Aβ, then the flux (*i.e.*, production rate) of Aβ through CSF as ng/mL/hr (which equals the rate of appearance of Aβ into and disappearance of Aβ from CSF at steady state) is the product of the CSF concentration (ng/mL) x k_{03} (pools/hr). The labeling data is transformed by the model into a set of parameters, that may be used to determine the effect of factors such as age[8], sex, apolipoprotein E4 (*APOE4)*[9] , ADAD mutation[10], drug[11], sleep[12], and amyloid status[8] on Aβ kinetics.

Application of SILK to key pathogenic proteins

In this section we summarise the major findings from SILK experiments to date, focusing on the proteins aggregates seen in neurodegenerative diseases.

Aβ SILK

In order to measure Aβ CNS kinetics, a translational protocol in humans was developed to label and track Aβ over 36-hr during and after a 9-h intravenous infusion of [U-$^{13}C_6$] leucine[8,13,14]. This protocol was then applied to studies of autosomal dominant AD (ADAD)[10] and in sporadic AD (SAD) and normal controls[8,] to measure turnover of Aβx–38, Aβx-40 and Aβx-42. A highly consistent finding in <u>*both*</u> ADAD and SAD is that the turnover or clearance of soluble Aβx-42 is specifically increased when amyloidosis is present, whereas Aβx-38 and Aβx-40 FTR were unaltered. Furthermore, taken as a ratio, Aβ42/Aβ40 turnover rate was positively correlated with amyloid plaque load as measured by Pittsburgh compound B (PiB) positron emission tomography (PET) (r=0.45) and with amyloid plaque growth rate (r=0.75), suggesting that faster Aβ42 turnover represents increased deposition of Aβ42 relative to Aβ40 into amyloid plaques[8,10]. In addition to altered clearance with plaques, the Aβ42/Aβ40 production rate ratio was higher in *PSEN* mutation carriers compared to non-carriers, suggesting that Aβ42 overproduction also contributes to ADAD. As a potential mechanism of age-related risk of amyloid accumulation, there was a dramatic slowing of the turnover rate of all Aβ peptides with age (2.5-fold longer half-life over five decades of age)[8]. The physiological Aβ kinetics enabled testing mechanisms of action for proposed disease modifying therapeutics[15]. For example, gamma secretase and BACE inhibitors resulted in a dose-dependent reduction in the accumulation of newly synthesized Aβ[11,16] without altering clearance kinetics[11].

FIG. 3 here

When applied to measure blood plasma concentrations of Aβ, SILK in both SAD and control participants demonstrated that Aβ turns over ~3x faster in plasma than CSF[17]. Plasma Aβ42 and Aβ40 kinetics are virtually identical in amyloid negative subjects, whereas in individuals with significant brain Aβ accumulation (amyloid positive) Aβ42 fractional turnover is faster, the same pattern observed in CSF albeit at a smaller magnitude. These findings of plasma Aβ SILK led to the discovery that plasma Aβ42/40 concentration ratios are highly specific for brain amyloid plaques and CSF Aβ[17,18].

Effects of sleep on Aβ kinetics

It was shown in mouse and human studies that brain interstitial fluid (ISF) and CSF Aβ concentration oscillates with the sleep-wake cycle, respectively[19,20]. Since Aβ deposition in the brain is concentration-dependent, prolonged wakefulness due to sleep disturbances may increase risk of AD by an Aβ-mediated mechanism[21]. To determine the kinetics underlying this Aβ oscillation, a recent SILK study found that CSF Aβ concentration increased ~30% during sleep deprivation but the Aβ SILK curves were similar to normal sleeping controls[12,21]. In the SILK model, changes in production rate are directly proportional to concentration while the shape of the SILK curves are most sensitive to changes in clearance (Supplementary Videos 1 and 2). This suggests that Aβ production is the mechanism for sleep-mediated changes in Aβ concentration.

Aβ SILK and human brain tissue

The faster soluble CSF Aβ42 clearance rate in amyloid positive subjects suggests incorporation into amyloid plaques[8]. Combined with SILK, nanoscale secondary ion MS (NanoSIMS) was used to image, at the nanometer scale, heavy isotope ([U-$^{13}C_6$] leucine) incorporation in post-mortem brain sections. (FIG.

4), allowing for quantification of protein deposition that occurred *in vivo* into AD brain amyloid plaques[22] from patient post-mortem samples (8 days to 4 years after labeling). The findings confirm that plaques incorporate isotopically labelled Aβ42 and other proteins over the course of several days, indicating that plaques are dynamic and that protein/peptide sequestration is a highly active process. This promising result from a small number of participants has led to the accelerated launch of a hospice study to measure plaque dynamics in human brains. This technique, called SILK-SIMS, has large potential to quantify the dynamic evolution of other proteins in a range of different neurodegenerative conditions.

ApoE SILK

SILK has been applied to plasma and CSF apolipoprotein E (apoE) protein turnover to understand the biological risk of apoE proteotypes for AD[9,23-25]. ApoE isoforms have different plasma turnover rates (apoE4 is two-fold faster than apoE3 which is two-fold faster than apoE2) but apoE isoforms have similar turnover rates in CSF[25]. In transgenic mice expressing human apoE, the clearance rate of the apoE but not Aβ in mouse brain homogenates was modulated by expression of LDL receptors (LDLR) and ATP-binding cassette A1 (ABCA1)[24]. Mouse brain homogenate Aβ synthesis rate was not modulated by human apoE isoforms[23], consistent with the findings that CSF Aβ kinetics are not modified by apoE isoforms in humans[14].

SOD1 SILK

Heterozygous mutations in the superoxide dismutase 1 (*SOD1*) gene cause about 1-2% of amyotrophic lateral sclerosis (ALS). Methods for simultaneously quantifying mutant and normal SOD1 protein turnover rates in the same participant have been developed[26]. The motivation to develop SOD1 SILK was driven by the recognition that protein turnover rates may be particularly informative in considering novel therapeutic strategies for *SOD1* ALS that focus on lowering levels of mRNA. Understanding SOD1 protein

turnover in human CSF will influence when CSF samples are taken in a clinical trial, the magnitude of SOD1 protein lowering to be expected, and may serve as an early pharmacodynamic marker. SOD1 SILK requires a longer SILK sampling protocol since the half-life of SOD1 in human CNS is approximately 25 days[26]. Studies of SOD1 turnover rates are ongoing in participants with *SOD1* mutations, ALS participants without known genetic cause, and controls. In mouse and rat models, measures of newly generated protein showed earlier pharmacodynamics changes for RNA-lowering therapeutics compared with total protein concentration, suggesting that SILK may be a promising early pharmacodynamics marker for RNA-targeted therapeutics[27] .

Tau SILK

Along with amyloid plaques, tau aggregates in the form of neurofibrillary tangles are a pathological hallmark of AD and other neurodegenerative diseases, collectively termed the tauopathies. Tauopathies are distinguished according to the tau isoforms enriched in aggregates which are differentiated by their number of repeats (R) in the microtubule-binding region: 3R for Pick's disease, 4R for corticobasal degeneration, progressive supranuclear palsy, and a 3R/4R mix for AD tauopathies[28]. In addition to greater deposited aggregated tau, CSF total tau and specific phosphorylated tau (p-tau) species are increased in AD. Therefore, tau SILK was recently developed to test the hypothesis that increased CSF tau/p-tau is due to changes in tau production or clearance rates of tau.

Similar to SOD1[26], longer SILK protocols are required for CNS tau, which has a long half-life of approximately 23 days[29]. To reduce participant's burden, 8, 16, or 24-hr intravenous infusion protocols were developed with CSF sampling being achieved using 4-5 follow up lumbar punctures over several months. Data from 24 participants with and without amyloidosis suggest that tau clearance is not altered by the presence of tau aggregates measured by ^{18}F-flortaucipir PET imaging, but tau production rate is doubled in the presence of amyloid deposition[29].

Tau SILK has also been applied to human induced pluripotent stem cell (iPSC) derived neurons in order to examine *in vitro* tau kinetics. *In vitro* studies offer several advantages including, higher labeling, greater sampling frequency, mechanistic interventions and access to intracellular compartments (Table 1). In cell lysates, more than fifteen tau peptides incorporate $^{13}C_6$-leucine, including 3R/4R specific and phosphorylated tau peptides. Similar tau truncation profiles were found in iPSC-neurons and their media, and brain and CSF, respectively. Despite similar truncation profiles, tau peptides measured in iPSC-derived neurons exhibited half-lives that were shorter than the half-lives of the same peptides measured in human CSF (6 days vs. 23 days). This observation could be explained by the complex interplay of multiple cell types in the CNS (i.e., glia, different neuronal subtypes) and protein transport from brain to CSF on protein kinetics. SILK measurements demonstrated that while full-length release of tau occurs with no time delay, truncated forms of tau are released after 3-days, adding to prior evidence for active truncation and secretion mechanism for tau[30,31]. Those tau proteoforms that are prone to aggregate may be turned over at a different rate: 4R-tau isoforms and some p-tau sites exhibited faster turnover compared to 3R-tau isoforms or unphosphorylated tau. *In vitro* tau SILK has considerable potential to further understanding of tau metabolism in health and disease e.g. using iPSC-derived neurons and other cell types derived from patients with different mutations linked to AD and FTD, and in combination with advanced cell culture systems (i*.e.*, transdifferentiation, organoids, long-term culture, co-culture) and genome editing techniques (*i.e.*, Cas9/CRISPR); as well as providing a means of assess the mechanisms of novel tau targeted therapies[32].

(Table 1 here)

SILK: potential utility

In this section we consider potential practical advantages of SILK over static biomarkers and potential for probing other specific aspects of neurodegeneration.

Comparing SILK to static biomarkers

Static protein biomarkers measure a concentration in a biological fluid, whose equilibrium could be affected by multiple factors. There are several factors that could influence the concentration of any given protein, including increased production and/or secretion, increased release in response to injury/disruption of cellular integrity, or defective clearance from the biological fluid. SILK which provides dynamic measures of production and clearance allows for a more detailed understanding of what a biomarker concentration change actually reflects. CSF tau is a striking example[29]. Previously, increased CSF tau concentration was interpreted as resulting from passive release from dying neurons[33]. Now, results from SILK studies suggest that the bulk of tau in human CSF may be released through an active process that could be stimulated by neuronal exposure to aggregated Aβ[29].

Pre-symptomatic diagnosis

In neurodegeneration characterized by accumulation of misfolded or otherwise defective proteins, altered production or clearance of a protein might be a trait (i.e. lifelong) marker, preceding build-up of the protein in inclusions/aggregates. There may also be informative changes in turnover during progression of disease that could be monitored using SILK; during AD and other neurodegenerative diseases, increased secretion of a protein might be followed by a phase of decreased production due to loss of viable neurons. Similarly, there may be disease stage-dependent changes in clearance of the protein. All could result in altered CSF or plasma concentrations with SILK potentially identifying the cause of the change that could be informative for developing targeted therapies (I,e, targeting production vs clearance), or serving as an earlier biomarker[32] .

Correcting variation

By determining production and clearance rates of multiple proteins, SILK may assist normalization of inter-individual differences in protein concentration. There are a number of additional aspects that should be explored; changes in overall CSF production and clearance rates due to dysfunctions in choroid plexus[34] , blood brain barrier[35], and other causes could affect CSF biomarker concentrations and SILK data for individual proteins. For example, measuring production and clearance rates of choroid plexus-derived molecules could potentially allow for the quantitative measurement of CSF dynamics. Furthermore, paired measurements of turnover rates in CSF and blood may yield information about the relative intrathecal compared to peripheral production of a biomolecule.

Measuring target engagement

SILK may be capable of detecting target engagement by detecting alterations in production rates of a specific protein in CSF or blood in response to a putative disease modifying therapy; such changes in dynamics may occur before a change in the steady state protein concentration is observed[32]. This is particularly important in neurodegenerative diseases where there may be a reservoir of the protein in brain tissue, which might affect the CSF or plasma protein concentration. Refined protein turnover measurements may result in higher statistical power and the need for fewer patients.

Probing other pathways: synaptic degeneration

Synaptic impairment and synapse loss are early features of neurodegenerative diseases including AD, occurring prior to frank neuronal degeneration[36]. This observation has prompted the investigation of synaptic proteins as early-stage biomarkers. Neurogranin (Ng), a dendritic protein enriched in neurons, is the best-established CSF biomarker for synapse loss or dysfunction associated with AD[37-39]. It is presently unclear if changes in the CSF concentrations of synaptic markers reflect synaptic degeneration/injury or

increased secretion because of synaptic dysfunction or altered activity patterns. SILK could help clarify this, at least in part, by determining if the increased CSF Ng concentration in AD is caused by a higher production rate (increased labeling) or release of pre-existing (unlabeled) synaptic proteins from dying synapses.

Markers of axonal degeneration

Neurofilament light chain (NfL), a microfilament component of the neuronal cytoskeleton, is an emerging and promising protein biomarker of axonal damage in a range of diseases. In CSF, studies have consistently shown NfL levels to be elevated across neurodegenerative diseases[40] and in active neuroinflammation[41]. CSF and serum NfL concentrations are significantly higher after traumatic brain injury (TBI), and highest 144 h post-traumatic injury[42]. This delay may be explained in part by the half-life of NfL half-life, but the kinetic prolife of NfL in humans is currently unknown. SILK could be used to quantify CSF and serum NfL kinetics and elucidate the meaning of elevated NfL level, and specifically whether this relates to passive release by damaged axons or upregulated production and secretion to reflect attempted axonal regeneration. Kinetic information is critical to accurately interpret this biomarker and to determine whether an elevated NfL concentration has the same significance across different neurodegenerative and neuroinflammatory diseases.

Markers of neuroinflammation

There is now a substantial body of evidence that neuroinflammation plays an important and early role in neurodegeneration, including the finding that immune receptor genes *TREM2*[43] and *CD33*[44] are associated with AD and other neurodegenerative diseases.

TREM2 is a type-1 protein expressed on the surface of microglia. Its ectodomain is proteolytically cleaved and released into the extracellular space as sTREM2 which can be measured in CSF. sTREM2 is increased

in AD CSF later than alterations in CSF amyloid or tau, but prior to the onset of cognitive symptoms[45]. The function(s) of sTREM2 are unknown, but SILK studies may be able to determine whether alterations in sTREM2 concentration reflects increased proteolytic cleavage of TREM2 (reduced labeling) or increased TREM2 production (increased labeling).

SILK proteomics

Finally, stable isotope labeled proteins/peptides can be detected and quantified using untargeted, shotgun proteomics. This bottom-up approach relies on the proteolytic digestion of all proteins in a sample, the separation of peptides using high performance liquid chromatography and the use of tandem MS for identification. Using optimal preanalytical fractionation of the digested peptides it has been possible to identify up to 2630 different proteins in normal CSF[46]. Preliminary work using LysC/tryptically digested and fractionated SILK labelled CSF samples[47], has used quadrupole time-of flight (Q-TOF) MS to identify a total of 6398 peptides corresponding to 1226 proteins. 4528 of these peptides, corresponding to 1064 proteins, contained leucine and were therefore susceptible to labeling. Approximately 10% of the leucine-containing peptides, corresponding to more than 200 proteins, showed a significant [13C6]leucine incorporation allowing the heavy:light ratio to be quantified. The analysis of kinetic CSF samples from multiple patients requires a sophisticated bioinformatics pipeline with mathematical modeling to extract different turnover patterns. This approach creates an opportunity to discover new dynamic biomarkers that are potentially better than existing static markers. It provides information on relevant metabolic and pathophysiological pathways by analyzing many proteins in parallel. By following multiple peptides belonging to a single protein, it also gives access to the differential kinetic behavior of different protein domains or features (e.g. N- or C-terminus) or different isoforms (e.g ApoE2, 3 or 4). Finally, if applied to both blood and CSF, it can provide relevant information on the transfer of protein between compartments, to explore the blood-brain and blood-CSF barriers function and disorders believe to be important in neurodegenerative diseases.

Limitations and alternatives to SILK

There are a currently a number of limitations to the widespread use of human SILK studies; these include the cost of infusion grade stable isotopes, the availability of suitable MS facilities and the clinical time and effort associated with launching the study, identifying dedicated population of participants, enrolling them as well as participant burden (see Table 1). However, there are important questions that can only be addressed using SILK, and sharing clinical and bench protocols as well as resources (All proteins will be simultaneously labeled) will help in overcoming or mitigating these limitations. There are alternative ways to measure protein turnover, for example radioactive isotopes, such as S_{35}-methionine, and these have been used in *in vitro* systems to determine protein kinetic rates. However, radioactive isotopes have limited use in humans for protein turnover studies, create significant in lab overhead for *in vitro* studies, and do not provide highly specific and precise individual protein estimates compared to SILK. There are also ways of chemically tagging proteins for tracking with a variety of optical and other tags to track turnover and transport[48]. These systems are widely used for *in vitro* techniques: however, have limitations in human or animal model applications and also create the artefact of the label potentially influencing the protein kinetic rates. Subatomic labelling with neutrons in SILK avoids the risks of artefactually altering protein kinetics and is safe and proven in animal and human studies.

Conclusions

SILK is a valuable method for quantifying rates of production and clearance of targeted proteins *in vitro* and *in vivo,* in different compartments including CSF, blood and brain tissue. To date, SILK studies have determined the turnover rates of Aβ, apoE, tau, and SOD1 in the human CNS and described altered patterns of Aβ and tau metabolism in AD. SILK studies have shown that sleep deprivation and age

influence Aβ clearance. This method offers the potential to probe other disease pathways in neurodegeneration and may be useful to demonstrate target engagement by putative disease modifying therapies.

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Contributions

RWP, AG, BL, NB, CS, CL, CH, SL, BWP, TW, KY, TW, NCW, DLE and HZ wrote the first draft of the manuscript. RWP, JDR, JMS, SW, NCF, RJB reviewed and critically edited the manuscript. All authors contributed substantially to discussion of content and review of the manuscript before submission.

Disclosures

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	In vitro SILK (pulsed SILAC)	*In vivo SILK*	
Model	**Cellular**	**Animal**	**Human**
Study Purpose	○ Investigate protein metabolism in cells ○ Evaluate the effect of disease-causing genetic mutations ○ Measure PTM effects on protein kinetics ○ Screen drug effects on protein kinetics	○ Assess drug engagement and effects on protein kinetics (preclinical phase) ○ Evaluate difference of protein kinetics in tissues	○ Investigate protein kinetics in human physiology and pathophysiology ○ Assess target engagement of drug in clinical trials
Labeling	○ Labeling up to 100% TTR allows for better dynamic range of kinetic curves ○ Cost effective (low amounts of tracer required)	*Short half-life proteins:* ○ efficiently labelled at 5-10% TTR via intraperitoneal injection (200mg/kg) *Long half-life protein:* ○ efficiently labelled with up to 20% TTR via labelled chow, water or injection	○ IV: *duration protein dependent* (ex. Tau: 2 to 4mg/kg/hr for 8-24hours) ○ Oral: *duration protein dependent* (ex. Tau: 1g/day for 10 days) *Short half-life proteins:* ○ can label up to 15% TTR *Long half-life proteins:* ○ can label up to 1% TTR
Sampling	*Media:* ○ collected during media exchange (no cell harvest required) allows for secreted proteins to be analysed *Cells:* ○ can be collected as frequently as needed Note: Less abundant proteins may require larger culture (larger wells or organoid cultures)	○ Tissues (eg. brain, spinal cord, liver) can be collected post-labeling ○ Biofluids (CSF, ISF, plasma/serum) can be collected, but will require animal sacrifice for sufficient sample volumes	*CSF::* ○ Short half-life proteins: Catheter preferred to allow hourly sampling (max time: 48hrs) ○ Long half-life proteins: Serial lumbar punctures spanning a few weeks to months *Plasma/Urine/Saliva* ○ Flexible sampling with less regulatory restrictions
Advantages	✓ Human cell lines can be obtained from individuals with disease-relevant genotypes via skin biopsy ✓ Genome=editing with CRISPR/Cas9 allows generation of isogenic lines to remove variability ✓ Storage and expansion of cell lines allow for a virtually limitless supply of material ✓ Differentiation into multiple disease-relevant cell types (neurons, astrocytes, microglia) and co-culture models is possible ✓ Trans-differentiation of fibroblasts directly to neurons maintains molecular ageing signatures of participants ✓ Molecular mechanisms and kinetic responses to genetic and pharmacological manipulations can be easily investigated and in greater detail ✓ Kinetic modelling of tracer/tracee more detailed due to high label incorporation and frequent sampling ✓ Comparison of intracellular vs. extracellular protein kinetics can be investigated	✓ High labeling efficiency ✓ Easy access to tissue for kinetic monitoring ✓ Higher protein content in tissue allows for PTM monitoring ✓ Suitable model to study drug engagement in preclinical phase	✓ Most relevant model to study human biology and abnormal protein kinetics in neurodegenerative diseases ✓ Suitable technique to monitor target engagement in clinical trials ✓ Kinetic data can improve how established static biomarkers are interpreted ✓ SILK could provide greater insight to the role target proteins play in disease etiology and across disease progression and severity
Caveat	× Neuronal cultures lack the different neuronal subtypes, non-neuronal cells and vasculature that contribute to pathology in vivo, thus in vitro systems may only recapitulate partially × iPSC-neurons and glia are more representative of immature cultures than adult cultures × Inter- and intra-patient heterogeneity of iPSC can result in high variability and requires multiple cell lines and/or isogenic lines to compare genotypes	× Limited CSF volume: longitudinal collection from a murine model not possible & potentially problematic for LC-MS analysis × Rat CSF volumes more likely suitable for SILK, however rat disease models are less defined × Disease model protein kinetics in animals may not be fully translatable to humans or human pathologies	× Requires hospital stay for duration of IV labeling study × Although safe, lumbar puncture can cause discomfort for some patients and requires trained practitioner × Infusion grade tracer must meet pharmaceutical criteria × Large doses required for sufficient CNS labeling × Expensive labeling cost limits study design × Limited sampling frequency × Tissue availability depends on donations or surgery × Monitoring low abundant proteins requires high-resolution MS and/or larger sample volumes

Table 1: Pros and cons of different SILK biological models

CSF: cerebrospinal fluid; ISF: interstitial fluid; iPSC: induced pluripotent stem cell; IV: intravenous; LC-MS: liquid chromatography mass spectrometry; PTM: posttranslational modification; SILAC: stable isotope labeling with amino acids in cell culture; SILK: stable isotope labeling kinetics; SIMS: secondary-ion mass spectrometry; TTR: tracer to tracee ratio; 3D: 3 dimensional.

Figure titles and legends:

Fig 1: Overview of stable isotope labeling kinetics (SILK)
In the SILK method, an amino acid labelled with a stable isotope such as $^{13}C_6$-leucine is administered into a biological system. Pre-clinical SILK Models (Left Column): Pre-clinical SILK biological models include rodents (for caveats, see Table 1), cell culture, and induced pluripitent stem cell models (iPSCs). Human SILK (Center Panel): Human participant receiving an infusion of $^{13}C_6$ leucine (chemical structure shown) to measure the turnover rate for the protein of interest. At time intervals determined by the protein turnover rate, blood and cerebrospinal fluid (CSF) samples are collected. $^{13}C_6$ leucine enrichment is measured in blood while CSF samples undergo analysis to determine protein kinetics. SILK Analytic Pipeline (Right Column): To calculate protein kinetics, CSF samples undergo immunoprecipitation and digestion followed by quantification using targeted mass spectrometry (Top Right Panel), calculation of labeled and unlabeled protein concentrations (Center Right Panel), and compartmental modeling to determine protein synthesis and clearance rates (Bottom Right Panel).

Fig. 2: Hypothetical model of leucine enrichment in humans Hypothetical compartmental model tracking leucine enrichment from the site of tracer intravenous infusion (compartment 1) to a final product such as CSF Aβ40 (compartment 3). k_{xy} is the fraction of compartment y converted to compartment x per unit time, where "0" represents loss from the system. The model transforms the shape and magnitude of the plasma leucine enrichment (mol fraction labeled, MFL) into the shape and magnitude of the product MFL. Plasma leucine MFL is defined as a “forcing function” using a linear interpolation between measured time points that defines the availability of tracer for incorporation into product proteins. Compartmental modeling of SILK data consists of constructing a physiologically plausible model structure with optimized rate constants that provide an excellent fit to the Aβ40 SILK data (solid line). In the example shown, gross systematic errors in the fit to Aβ40 SILK result if k_{03} is increased by 20% (dashed line).

Fig. 3: Aβ SILK curves

A and B: Aβ42 turns over faster in amyloid(+) subjects

The enrichment of Aβ peptides (mol fraction labeled, MFL) was normalized to the plasma leucine plateau MFL for each subject, and averaged for 46 amyloid (-) subjects (age 72.8 ± 6.3 StDev; panel A) and 54 age-matched amyloid(+) subjects (age 73.6 ± 6.9 StDev; panel B). Mean ± 95% CI error bars are shown for Aβ40 (green) and Aβ42 (red). Aβ42 shows identical kinetics as Aβ40 in amyloid(-) subjects (panel A), but turns over faster than Aβ40 in amyloid(+) subjects (panel B) as noted by an earlier peak. Solid lines show fits of the data to a comprehensive compartmental model that describes the entire time course. Not shown: Aβ38 curves are superimposable over Aβ40 curves regardless of amyloid status. Data taken from Patterson et al (2015).

C and D: Aβ turnover kinetics slow down with age

Normalized MFLs were averaged for subjects aged < 60 years (blue, n=23), aged 60-70 (red, n=27), and aged >70 (green, n=28) for Aβ40 (C) and Aβ42 (D). Aβ peptides turn over more slowly with increasing age, noted by later, broader, and lower peaks. All subjects are amyloid(-), although the same age-related

trends are found in amyloid(+) subjects. Not shown: Aβ38 shows the same behavior as Aβ40 and Aβ42. Data taken from Patterson et al (2015)[8].

Fig. 4: Imaging *In vivo* incorporation of a stable isotope tracer in brain.

SILK-SIMS allows quantitative imaging of stable isotopes at high spatial resolution[22] (50–100 nm or <1 μm^3 – much smaller than cells), high mass resolution (*e.g.*, ^{13}C, 13.00336 vs. ^{12}CH, 13.00782 amu or 27.00643 13C14N vs. 27.00011 12C15N amu), and high sensitivity (<1% enrichment can be detected)[49,50]. By contrast, PET imaging only obtains average measurements at ~1 centimeter resolution, and confocal microscopy can image at ~0.5–1 micrometer resolution. The result is a nanometer-level histological map of *in situ* incorporation of a stable isotope tracer. **A.** Optical image of neurons stained with Toluidine Blue (T. Blue) 40X. Scale bar, 50 µm. **B**. 12C14N ion map for the same field of view in panel A. Imaging carbon as a polyatomic isotope (*i.e.,* cyanide ion) results in improved image quality and contrast[3] due to the increased count rate per pixel attributable to the high ionization of cyanide ions. Additionally, measuring carbon as a polyatomic cyanide ion produces an enhanced image of biological material due to the abundance of carbon, nitrogen *and* C-N bonds, thus almost entirely removing carbon-rich (and nitrogen poor) signal from the embedding media (*e.g.*, LR White, Epon). **C**. 13C14N/12C14N ion map showing the distribution of 13C in the sample. The natural abundance of 13C is 1.1%. Scale bar for all SILK–SIMS images, 20 µm.

Supplementary Video:

(1) One compartment model of isotope labeling, varying synthesis rates

Each panel in the top row show approximately 10,000 particles in a volume. New particles have certain probability of forming at each time step. Existing particles have a certain probability of being removed at each time step. The greater the probability for formation, the higher the rate of synthesis. The greater the probability of removal, the higher the rate of degradation. Particles in red are unlabeled and particles in blue are labeled. At time = 0, all 10,000 particles are red. Because particle formation or removal are determined using a random number generator, the total number of particles may diverge slightly from 10,000.

The bottom row illustrates fractional labeling or the number of unlabeled and labeled particles. It is assumed that each new particle has a chance of being labeled or unlabeled as dictated by the mole fraction of labeled precursor (green curve). This is justified by the fact that incorporation of labeled or unlabeled amino acids rely on a competition for binding sites on tRNA. Faster synthesis rates result in the production of new particles at a greater rate. However, the overall system is assumed to be at a steady state. If degradation rates are equal, the 'fast synthesis' system simply has more particles than the 'slow synthesis' system. Because the 'slow synthesis' system starts with fewer particles, the slower synthesis of labeled particles is negated when calculating or measuring the mole fraction of labeled particles. Thus,

the kinetic curves for the fast and slow synthesis systems overlap (except for stochastic variations visible due to the relatively small number of particles in the model).

(2) One compartment model of isotope labelling, varying degradation rates

Fewer particles also result from fast degradation (right half) if synthesis rates are the same. Because new particles are synthesized at the same rate, the mole fraction of labeling increases at a greater rate in the case of fast degradation. There are simply fewer unlabeled particles in the fast degrading system. Initially, the number of labeled particles is nearly identical in the fast and slow degradation cases, but the effects of rapid degradation becomes evident at longer times. The net result is that the fast degrading system peaks earlier and higher and falls of faster.

These video illustrate how SILK kinetics directly measures degradation rates, but indirectly measures synthesis rates. Synthesis rates may be determined if the total concentration of 'particles' is known. Quite simply, at steady state, the total concentration of particles is equal to the degradation (turnover) rate constant divided by the synthesis rate constants.

(3) Three compartment model of isotope labelling

In this model, synthesis and degradation rate constants are identical for all three compartments ('APP', 'C99' and 'Aβ2'). New APP particles has a chance of being labeled or unlabeled as dictated by the mole fraction of labeled precursor (blue curve). APP particles are then 'transferred' at a certain rate to the C99 compartment, representing degradation of APP and, at the same time, synthesis of C99. Similarly, C99 is converted to Aβ42, which is degraded at a certain rate, disappearing from the system. The kinetic plot of mole fraction labeling shows that the sequential synthesis results in a delay in the onset of production of labeled C99 and Aβ42. While APP displays a monoexponential rise to plateau, C99 and Abeta42 demonstrate sigmoidal shapes during the labeling phase. After the labeled precursor is removed, APP mole fraction labeling immediately decrease as a monoexponential decay, while C99 and Aβ42 curves fall off as a monoexponential decay with some delay.

This very simple model generally captures the shape of the in vivo SILK Abeta labeling curves, including the sigmoidal rise and monoexponential decay. Most of the SILK Abeta labeling curves are consistent with a single rate limiting compartment connected to about 5 identical compartments in series.