Imaging complex protein metabolism in live organisms by stimulated Raman scattering microscopy with isotope labeling

Lu Wei[1], Yihui Shen[1], Fang Xu[1], Fanghao Hu[1], Jamie K. Harrington[2], Kimara L. Targoff[2], Wei Min[1,3*]

[1]*Department of Chemistry, Columbia University, New York, NY 10027*
[2] *Department of Pediatrics, Columbia University, New York, NY 10032*
[3]*Kavli Institute for Brain Science, Columbia University, New York, NY 10027*

**To whom correspondence should be addressed. Email: wm2256@columbia.edu*

SUPPORTING INFORMATION

SI Methods and Materials

1. Stimulated Raman scattering (SRS) microscopy

An integrated laser (picoEMERALD with custom modification, Applied Physics & Electronics, Inc) was used as the light source for both Pump and Stokes beams. Briefly, picoEMERALD provides an output pulse train at 1064 nm with 6 ps pulse width and 80 MHz repetition rate, which serves as the Stokes beam. The frequency-doubled beam at 532 nm is used to synchronously seed a picosecond optical parametric oscillator (OPO) to produce a mode-locked pulse train (the idler beam of the OPO is blocked with an interferometric filter) with 5~6 ps pulse width. The wavelength of the OPO is tunable from 720 to 990 nm, which serves as the Pump beam. The intensity of the 1064 nm Stokes beam is modulated sinusoidally by a built-in electro-optic modulator (EOM) at 8 MHz with a modulation depth of more than 95%. The Pump beam is spatially overlapped with the Stokes beam with a dichroic mirror inside picoEMERALD. The temporal overlap between Pump and Stokes pulse trains is ensured with a built-in delay stage and optimized by the SRS signal of pure dodecane liquid.

Pump and Stokes beams are coupled into an inverted laser-scanning microscope (FV1200MPE, Olympus) optimized for near IR throughput. A 60X water objective (UPlanAPO/IR, 1.2 N.A., Olympus) with high near IR transmission is used for all cellular level imaging, and a 25X water objective (XLPlan N, 1.05 N.A., MP, Olympus) with both high near IR transmission and large field of view is used for brain tissue and *in vivo* imaging. The Pump/Stokes beam size is matched to fill the back-aperture of the objective. The forward going Pump and Stokes beams after passing through the sample are collected in transmission with a high N.A. condenser lens (oil immersion, 1.4 N.A., Olympus), which is aligned following Köhler illumination. A telescope is then used to image the scanning mirrors onto a large area (10 mm by 10 mm) Si photodiode (FDS1010, Thorlabs) to descan beam motion during laser scanning. The photodiode is reverse-biased by 64 V from a DC power supply to increase both the saturation threshold and response bandwidth. A high O.D. bandpass filter (890/220 CARS, Chroma Technology) is used to block the Stokes beam completely and transmit the Pump beam only. The output current of the photodiode is electronically pre-filtered by an 8-MHz band-pass filter (KR

2724, KR electronics) to suppress both the 80 MHz laser pulsing and the low-frequency contribution due to laser scanning across the scattering sample. It is then fed into a radio frequency lock-in amplifier (HF2LI, Zurich instrument) terminated with 50 Ω to demodulate the stimulated Raman loss signal experienced by the Pump beam. The R-output of the lock-in amplifier is fed back into the analog interface box (FV10-ANALOG) of the microscope.

For HeLa cell imaging and brain tissue imaging, the time constant of the lock-in amplifier is set for 8 μs, and the images are acquired by a 12.5 μs pixel dwell time, corresponding to 3.3 s for a 512-by-512 pixel frame. For neurons and *in vivo* imaging of embryonic zebrafish and mice livers and intestines, the time constant is set to be 20 μs, and the images are acquired by a 40 μs of pixel dwell time, corresponding to 10.5 s for a 512-by-512 pixel frame. Laser powers after 60X IR objective used for cell imaging are: 100 mW for modulated Stokes beam and 112 mW for the Pump beam at 2133 cm^{-1}, 2000 cm^{-1} and 1655 cm^{-1} channels; 50 mW for modulated Stokes beam and 56 mW for Pump beam at 2940 cm^{-1} and 2845 cm^{-1} channels. Laser powers after 25X objective used for tissue and *in vivo* imaging are: 134 mW for modulated Stokes beam; 120 mW for the Pump beam of 2133 cm^{-1}, 2000 cm^{-1} and 1655 cm^{-1} channels; 67 mW for modulated Stokes beam and 60 mW for Pump beam at 2940 cm^{-1} and 2845 cm^{-1} channels.

2. Retrieval of pure CH_3 and CH_2 signals by linear combination between 2940 cm^{-1} and 2845 cm^{-1} channels (for Fig. 3).

The employed equations follow ref. (27, 28). Pure CH_3 signal can be retrieved as $[c]_{protein} \propto$ 5.2*(2940 cm^{-1} signal) – 4.16*(2845 cm^{-1} signal); Pure CH_2 signal can be retrieved as $[c]_{lipid} \propto$ 1.2*(2845 cm^{-1} signal) – 0.3*(2940 cm^{-1} signal). This algorithm was tested with skin tissue samples, yielding similar results as reported in ref. (27, 28).

3. Two-color pulse-chase linear combination algorithm between 2067 cm^{-1} and 2133 cm^{-1} channels (for Fig. 4).

In order to achieve SRS imaging of pure group I D-AA labeled protein distribution and pure group II D-AA labeled protein distribution simultaneously, we construct a robust linear combination algorithm to retrieve the underlying pure concentration information for two-color pulse-chasing imaging similar to the one presented above from ref. (27, 28). Since SRS signals exhibit linear concentration dependence with analyte concentrations, two chemical species with different Raman spectra can be retrieved quantitatively with two-color SRS imaging. Hence, based on the spectra shown in Fig. 4c, we choose to acquire narrow-band SRS images at 2067 cm^{-1} and 2133 cm^{-1} channels, respectively, and perform subsequent linear combination algorithm to remove the spectral cross-talk.

The proper algorithm with the corresponding cross-talk coefficients is constructed with SRS images of standard reference samples, i.e., pure group I D-AA labeled protein and pure group II D-AA labeled protein. To do so, we labeled HeLa cells with only group I D-AA medium and only group II D-AA medium, respectively, and acquired a set of image pairs at 2067 cm^{-1} and 2133 cm^{-1} channels for each cell samples (Supplemental Fig. 1).

For any sample labeled with both groups of D-AAs, the measured SRS signals at 2067 cm^{-1} and 2133 cm^{-1} channels can be written as the following, with linear relationship to group I D-AA and group II D-AA concentrations ($[c]_{group\ I}$ and $[c]_{group\ II}$) :

$$\begin{bmatrix} 2067\,cm^{-1}\ signal \\ 2133\,cm^{-1}\ signal \end{bmatrix} = \begin{bmatrix} i_{group\,I,2067cm^{-1}} & i_{group\,II,2067cm^{-1}} \\ i_{group\,I,2133cm^{-1}} & i_{group\,II,2133cm^{-1}} \end{bmatrix} \begin{bmatrix} [c]_{group\,I} \\ [c]_{group\,II} \end{bmatrix},$$

where $i_{group\,I,2067cm^{-1}}$, $i_{group\,I,2133cm^{-1}}$, $i_{group\,II,2067cm^{-1}}$, $i_{group\,II,2133cm^{-1}}$ are the average pixel intensity recorded inside cells in (a) and (b) of Supplemental Fig. 1.

Thus group I D-AA and group II D-AA concentrations can then be easily solved as:

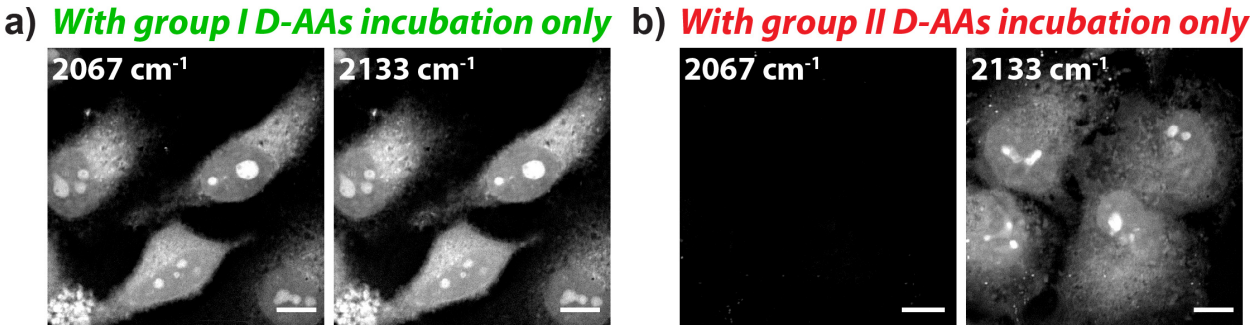
$$[c]_{group\,I} = \frac{i_{group\,II,2133cm^{-1}}(2067\,cm^{-1}\ signal) - i_{group\,II,2067cm^{-1}}(2133\,cm^{-1}\ signal)}{i_{group\,II,2133cm^{-1}} i_{group\,I,2067cm^{-1}} - i_{group\,II,2067cm^{-1}} i_{group\,I,2133cm^{-1}}},$$

$$[c]_{group\,II} = \frac{i_{group\,I,2067cm^{-1}}(2133\,cm^{-1}\ signal) - i_{group\,I,2133cm^{-1}}(2067\,cm^{-1}\ signal)}{i_{group\,II,2133cm^{-1}} i_{group\,I,2067cm^{-1}} - i_{group\,II,2067cm^{-1}} i_{group\,I,2133cm^{-1}}}.$$

Taking the average pixel intensity recording in (a) and (b) from Supplemental Fig. 1 into the above equations, the final linear combination algorithm reads as:

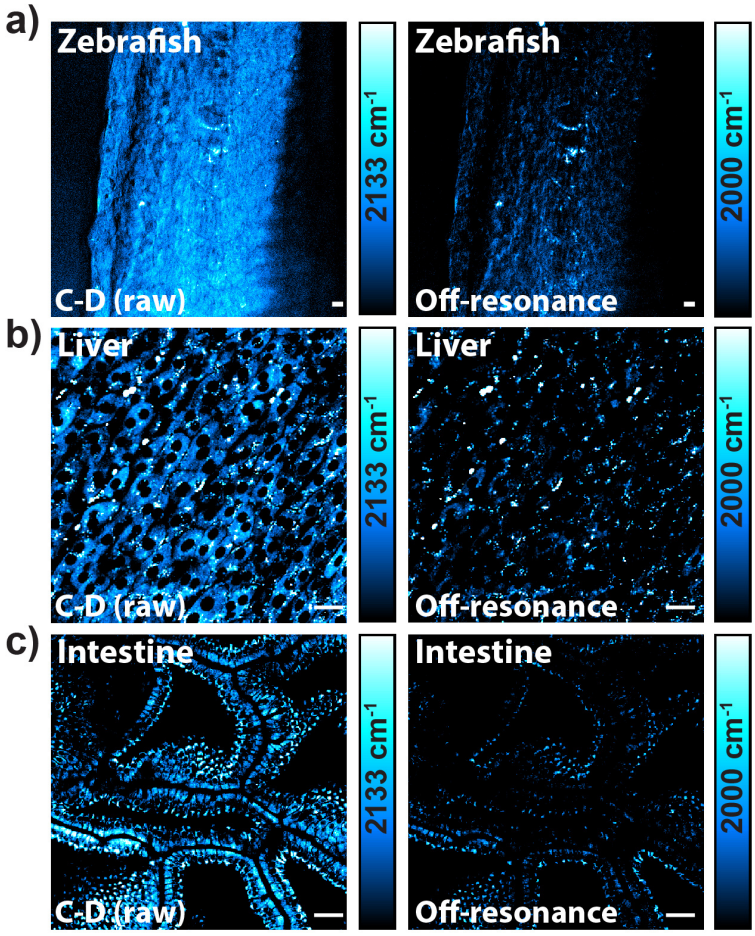
$[c]_{group\ I} \propto 1.06*(2067\ cm^{-1}\ signal) - 0.0047*(2133\ cm^{-1}\ signal)$, (1)

$[c]_{group\ II} \propto (2133\ cm^{-1}\ signal) - 1.15*(2067\ cm^{-1}\ signal)$. (2)

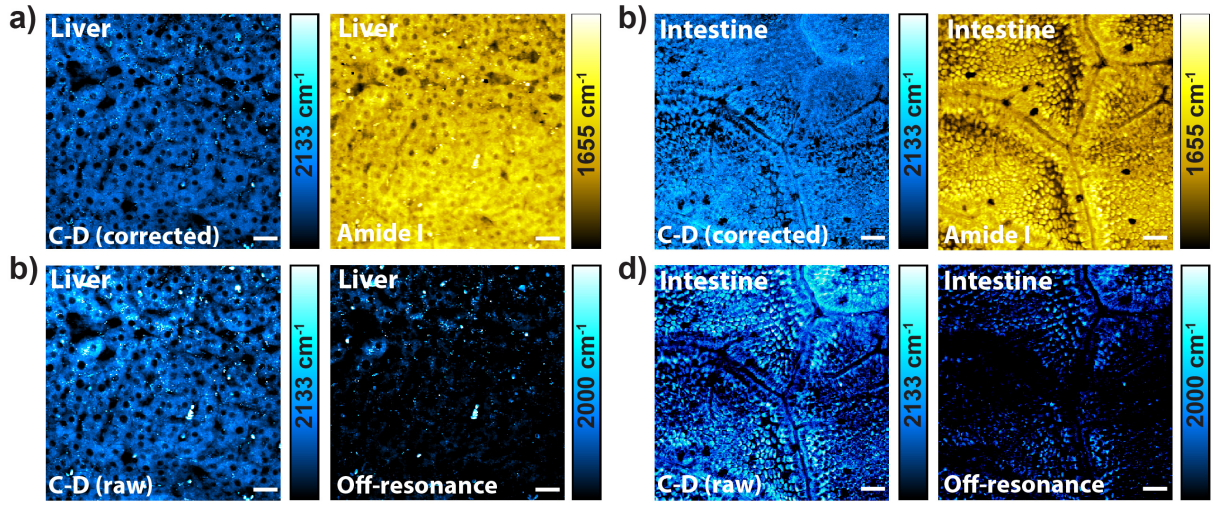


Supplemental Fig. 1. SRS images at 2067 cm^{-1} and 2133 cm^{-1} channels of proteins labeled with group I D-AA only (a) and group II D-AA only (b).

4. In vivo SRS imaging (for Fig. 6).



Supplemental Fig. 2. Raw C-D on-resonance (2133 cm^{-1}) and off-resonance (2000 cm^{-1}) SRS images of newly synthesized proteins *in vivo* in Fig. 6. (a) SRS C-D on-resonance and off-resonance images of a 24 hpf embryonic zebrafish. The difference image between C-D on-resonance and off-resonance (pixel-by-pixel subtraction) shows pure C-D labeled protein distribution in the somites of an embryonic zebrafish tail, as in Fig. 6a. (b-c) SRS C-D on-resonance and off-resonance images of live mouse liver (b) and intestine (c) tissues harvested from the mice after administering with D-AA containing drinking water for 12 days. The difference image between C-D on-resonance and off-resonance (pixel-by-pixel subtraction) shows pure C-D labeled protein distribution in the liver and intestine tissues, shown in Fig. 6b and Fig. 6c, respectively. The residual signals presented in the off-resonance images mainly come from cross-phase modulation induced by highly scattering tissue structures.



Supplemental Fig. 3. SRS imaging for newly synthesized proteins *in vivo* with intraperitoneal injection of mice with D-AA solutions. (a-b) SRS images of live mouse liver (a) and intestine (b) tissues harvested from mice after intraperitoneal injection with D-AA solutions for 36 h. 2133 cm^{-1} channel shows newly synthesized proteins (off-resonance image subtracted) that resemble the distribution of total proteins as shown in the 1655 cm^{-1} image (Amide I). (c-d) Corresponding raw C-D on-resonance (2133 cm^{-1}) and off-resonance (2000 cm^{-1}) images are shown as references for liver (c) and intestine (d) tissues. Scale bar, 10 μm.

5. Materials.

5.1 Regular HeLa cells medium was made of 90% DMEM medium (11965, invitrogen), 10% FBS (10082, invitrogen) and 1X penicillin/streptomycin (15140, invitrogen); regular hippocampal neuron medium was made of Neurobasal A Medium (10888, Invitrogen), 1X B27 serum free supplement (17504, Invitrogen) and 0.5 mM glutamine (25030, Invitrogen).

5.2 Htt-mEos2 plasmid construct and transfection. mHtt94Q-mEos2 plasmid was constructed by replacing CFP gene sequence in pTreTight-Htt94Q-CFP plasmid (Addgene, 23966) with mEos2 gene sequence from pRSETa-mEos2 plasmid (Addgene, 20341). For transfection of mHtt-mEos2 plasmid in HeLa cells, 4μg mHtt94Q-mEos2 plasmid were transfected using Transfection Reagent (FuGene, Promega).

5.3 Recipe for deuterared amino acids (D-AAs) Media

1) D-AA medium (CD-DMEM) for HeLa cells: adapted from regular recipe of DMEM medium (11965, Invitrogen). The D-AA culture medium for HeLa cells was made with 90% CD-DMEM, 10% FBS (10082, invitrogen) and 1X penicillin/streptomycin (15140, invitrogen).

Amino acids components	Concentration (mM)	Product company and catalog number
Glycine-d_5	0.4	DLM-280, Cambridge isotope
L-Arginine·HCl-d_7	0.398	DLM-541, Cambridge isotope
L-Cysteine·2HCl	0.2	C6727, SIGMA (regular)*
L-Glutamine-d_5	4.0	DLM-1826, Cambridge isotope

L-Histidine·HCl·H_2O	0.2	H5659, SIGMA (regular)*
L-Isoleucine-d_{10}	0.802	DLM-141, Cambridge isotope
L-Leucine-d_{10}	0.802	DLM-567, Cambridge isotope
L-Lysine·HCl-d_8	0.798	616214, ALDRICH (Isotech)
L-Methionine-d_3	0.201	DLM-431, Cambridge isotope
L-Phenylalanine-d_8	0.4	DLM-372, Cambridge isotope
L-Serine-d_3	0.4	DLM-582, Cambridge isotope
L-Threonine	0.798	T8441, SIGMA (regular)*
L-Tryptophan	0.078	T8941, SIGMA (regular)*
L-Tyrosine-d_2	0.398	DLM-2317, Cambridge isotope
L-Valine-d_8	0.803	DLM-488, Cambridge isotope
Other components (vitamins, Inorganic Salts and glucose) are exactly the same as in the regular DMEM medium (11965, invitrogen).		

*The reasons these 4 amino acids are remain in their regular forms are because: first, their deuterated forms have limited number of side chain deuterium and are also relatively expensive; second, their occurrence (percentage) in mammalian cell proteins are small (1). Thus the lack of the deuterated version for these 4 amino acids would not influence the general deuterium labeling efficiency for CD-DMEM. Same reason applies to below media.

1. Okayasu T, Ikeda M, Akimoto K, Sorimachi K (1997) The amino acid composition of mammalian and bacterial cells. *Amino Acids* 13:379–391.

2) D-AA medium (CD-Neurobasal A) for hippocampal neuron culture and organotypic brain slices: adapted from regular recipe of Neurobasal A medium (10888, Invitrogen). The D-AAs culture medium for hippocampal neurons was made of CD-Neurobasal A Medium, 1x B27 serum free supplement (17504, Invitrogen) and 0.5 mM glutamine-d_5 (DLM-1826, Cambridge isotope). The CD-Neurobasal A culture medium for organotypic brain slices was made of CD-Neurobasal A Medium, 1X B27 serum free supplement (17504, Invitrogen), 0.5% glucose (15023, invitrogen), 2 mM glutamine-d_5 (DLM-1826, Cambridge isotope) and 1X penicillin/streptomycin (15140, invitrogen).

Amino acids components	Concentration (mM)	Product company and catalog number
Glycine-d_5	0.4	DLM-280, Cambridge isotope
L-Alanine-d_4	0.022	DLM-250, Cambridge isotope
L-Arginine·HCl-d_7	0.398	DLM-541, Cambridge isotope
L-Asparagine-d_8	0.006	672947 ALDRICH (Isotech)
L-Cysteine·2HCl	0.26	C6727, SIGMA (regular)*
L-Histidine·HCl·H_2O	0.2	H5659, SIGMA (regular)*
L-Isoleucine-d_{10}	0.802	DLM-141, Cambridge isotope
L-Leucine-d_{10}	0.802	DLM-567, Cambridge isotope
L-Lysine·HCl-d_8	0.798	616214, ALDRICH (Isotech)
L-Methionine-d_3	0.201	DLM-431, Cambridge isotope
L-Phenylalanine-d_8	0.4	DLM-372, Cambridge isotope
L-Proline-d_7	0.067	DLM-487, Cambridge isotope
L-Serine-d_3	0.4	DLM-582, Cambridge isotope

L-Threonine	0.798	T8441, SIGMA (regular)*
L-Tryptophan	0.078	T8941, SIGMA (regular)*
L-Tyrosine-d_2	0.398	DLM-2317, Cambridge isotope
L-Valine-d_8	0.803	DLM-488, Cambridge isotope
Other components (vitamins, Inorganic Salts and glucose) are exactly the same as in the regular Neurobasal A medium (10888, Invitrogen).		

3) Group I D-AA medium for HeLa cells. The group I D-AA culture medium for HeLa cells was made with 90% group I D-AA medium, 10% FBS (10082, invitrogen) and 1X penicillin/streptomycin (15140, invitrogen).

Amino acids components	Concentration (mM)	Product company and catalog number
Glycine	0.4	50046, SIGMA (regular)
L-Arginine·HCl	0.398	A6969, SIGMA (regular)
L-Cysteine·2HCl	0.2	C6727, SIGMA (regular)
L-Glutamine	4.0	G8540, SIGMA (regular)
L-Histidine·HCl·H_2O	0.2	H5659, SIGMA (regular)
L-Isoleucine-d_{10}	0.802	DLM-141, Cambridge isotope
L-Leucine-d_{10}	0.802	DLM-567, Cambridge isotope
L-Lysine·HCl	0.798	L8662 SIGMA (regular)
L-Methionine	0.201	M5308 SIGMA (regular)
L-Phenylalanine	0.4	P5482 SIGMA (regular)
L-Serine	0.4	S4311 SIGMA (regular)
L-Threonine	0.798	T8441, SIGMA (regular)
L-Tryptophan	0.078	T8941, SIGMA (regular)
L-Tyrosine	0.398	T8566 SIGMA (regular)
L-Valine-d_8	0.803	DLM-488, Cambridge isotope
Other components (vitamins, Inorganic Salts and glucose) are exactly the same as in the regular DMEM medium (11965, invitrogen).		

4) Group II D-AA medium for HeLa cells. The group II D-AA culture medium for HeLa cells was made with 90% group II D-AA medium, 10% FBS (10082, invitrogen) and 1X penicillin/streptomycin (15140, invitrogen).

Amino acids components	Concentration (mM)	Product company and catalog number
Glycine-d_5	0.4	DLM-280, Cambridge isotope
L-Arginine·HCl-d_7	0.398	DLM-541, Cambridge isotope
L-Cysteine·2HCl	0.2	C6727, SIGMA (regular)
L-Glutamine-d_5	4.0	DLM-1826, Cambridge isotope
L-Histidine·HCl·H_2O	0.2	H5659, SIGMA (regular)
L-Isoleucine	0.802	I7403 SIGMA (regular)
L-Leucine	0.802	L8912 SIGMA (regular)
L-Lysine·HCl-d_8	0.798	616214, ALDRICH (Isotech)

L-Methionine-d_3	0.201	DLM-431, Cambridge isotope
L-Phenylalanine-d_8	0.4	DLM-372, Cambridge isotope
L-Serine-d_3	0.4	DLM-582, Cambridge isotope
L-Threonine	0.798	T8441, SIGMA (regular)
L-Tryptophan	0.078	T8941, SIGMA (regular)
L-Tyrosine-d_2	0.398	DLM-2317, Cambridge isotope
L-Valine	0.803	V0513 SIGMA (regular)
Other components (vitamins, Inorganic Salts and glucose) are exactly the same as in the regular DMEM medium (11965, invitrogen).		

5) D-AA medium (CD-MEM) for organotypic brain slice: adapted from regular recipe of MEM medium (11095, Invitrogen). The CD-MEM culture medium for organotypic brain slice was made with 90% CD-MEM, 10% FBS (10082, invitrogen), 0.5% glucose (15023, invitrogen) and 1X penicillin/streptomycin (15140, invitrogen).

Amino acids components	Concentration (mM)	Product company and catalog number
L-Arginine·HCl-d_7	0.597	DLM-541, Cambridge isotope
L-Cysteine·2HCl	0.1	C6727, SIGMA (regular)*
L-Glutamine-d_5	2.0	DLM-1826, Cambridge isotope
L-Histidine·HCl·H_2O	0.2	H5659, SIGMA (regular)*
L-Isoleucine-d_{10}	0.397	DLM-141, Cambridge isotope
L-Leucine-d_{10}	0.397	DLM-567, Cambridge isotope
L-Lysine·HCl-d_8	0.399	616214, ALDRICH (Isotech)
L-Methionine-d_3	0.1	DLM-431, Cambridge isotope
L-Phenylalanine-d_8	0.19	DLM-372, Cambridge isotope
L-Threonine	0.403	T8441, SIGMA (regular)*
L-Tryptophan	0.049	T8941, SIGMA (regular)*
L-Tyrosine-d_2	0.199	DLM-2317, Cambridge isotope
L-Valine-d_8	0.393	DLM-488, Cambridge isotope
Other components (vitamins, **Inorganic Salts and glucose**) are exactly the same as in the regular MEM medium (11095, invitrogen).		

6) For zebrafish: Wild-type zebrafish embryos at the 1-cell stage were injected with 1 nL D-AA solution and allowed to develop normally for another 24 h. The zebrafish embryos at 24 hpf were manually dechorionated before imaging. D-AA solution was made of 150 mg uniformly deuterium-labeled amino acid mix (20 aa) (DLM-6819, Cambridge Isotope) dissolved in 1 mL PBS, with subsequent filtration using Millipore sterile syringe Filters (0.22 μm, SLGV033RS).

7) For mice: 1. Oral administration: 3-week-old mice were fed with D-AA containing drinking water for 12 days before harvesting the liver and intestine tissues. The drinking water was made of 500 mg uniformly deuterium-labeled amino acid mix (20 aa) (DLM-6819, Cambridge Isotope) dissolved in 200 ml PBS, with subsequent filtration using Millipore sterile syringe Filters (0.22 μm, SLGV033RS). 2. Intraperitoneal injection: 3-week-old mice were injected with 500 μl D-AAs solution at the 0^{th} h, 12^{th} h and 24^{th} h. The tissues were then harvested at the 36^{th} h after the

first injection. D-AA solution was made of 500 mg uniformly deuterium-labeled amino acid mix (20 aa) (DLM-6819, Cambridge Isotope) dissolved in 2 ml PBS solutions, with subsequent filtration using Millipore sterile syringe Filters (0.22 μm, SLGV033RS).