

Supplementary Information

Live-cell vibrational imaging of choline metabolites by stimulated Raman scattering coupled with isotope-based metabolic labeling

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Materials. D₉-choline chloride, D₃-methionine and D₁₀-leucine were purchased from Cambridge Isotope Laboratories. DMEM medium (11965), McCoy's 5A medium (16600), fetal bovine serum FBS (10082), penicillin-streptomycin (11540), Neurobasal-A Medium (10888), B27 serum-free supplement (17504) and L-glutamine (25030) were obtained from Invitrogen. Bovine calf serum BCS (Hyclone SH30072), bovine serum albumin BSA (BP9703), CaCl₂, and EDTA were from Fisher Scientific. 2,3-butanedione monoxime, formaldehyde solution, Phospholipase C (Type XIV from *Clostridium perfringens*, P4039), phosphate buffered saline PBS, and tris buffered saline TBS buffer were obtained from Sigma-Aldrich.

Cell culture and sample preparation. Culture medium for HeLa and HEK293T cells was prepared by adding 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin into DMEM medium. NIH3T3 cell culture medium was prepared by adding 10% (v/v) BCS and 1% (v/v) penicillin-streptomycin into DMEM medium. U2OS cell culture medium was made with 90% (v/v) McCoy's 5A medium with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Neuron culture

medium was prepared from Neurobasal-A medium added with 1× B27 serum-free supplement and 0.5 mM L-glutamine.

For mammalian cell lines, cells were seeded on coverslips in petri dishes with 2 mL of culture media and incubated at 37 °C and 5% CO₂ for 20 h. The culture media were then supplemented with 5 or 10 mM D₉-choline (in PBS) for 48 h.

For primary mouse hippocampal neurons, cells were seeded on coverslips in petri dishes with 1 mL of neuron culture medium and incubated at 37 °C and 5% CO₂ for 7-14 days. Then D₉-choline PBS solution was added into the medium with a final concentration of 10 mM for 48 h.

After incubation, the coverslip was taken out, washed gently with 1 mL PBS buffer and assembled into a chamber filled with PBS solution and is ready for spontaneous Raman scattering measurement and SRS imaging.

***C. elegans* microinjection and sample preparation.** Handling of the worms was described by Brenner¹ and the microinjection protocol was described in details by Fire². In brief, live adult worms (3 days from hatching at 20 °C) were immobilized on a dried pad of agarose (Lonza, Rockland, ME) under an oil layer. About 1 ml of 100 mM D₉-choline PBS solution was used to fill the tip (<3 mm in diameter) of the needle and a simple air pressure system is used to facilitate the flow of liquid from the needle to the gonade of the worm. After the injection, worms were saved by dropping M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 ml 1 M MgSO₄, H₂O to 1 liter; sterilize by autoclaving) onto the agarose pad and then transferred to individual Petri plates with nutrient agar and bacteria for food and maintained at 20°C. One day later, L1 larvae of the next generation were collected, immobilized on a pad of 3% agarose with 2,3-butanedione

monoxime and imaged. The microscope used for injection is a Zeiss IM which is equipped for differential interference contrast with a Plan40 lens.

Stimulated Raman scattering microscopy. Both pump and Stokes beams with linear and parallel polarization are produced from an integrated laser system (picoEMERALD, Applied Physics & Electronics, Inc). A 1064 nm fundamental laser (Stokes beam) is generated with 6 ps pulse width and 80 MHz repetition rate, part of which is frequency-doubled to 532 nm and used to seed a picosecond optical parametric oscillator to generate a mode-locked signal (pump beam) with 5-6 ps pulse width (the idler is blocked with an interferometric filter). The wavelength of pump beam can be tuned from 720 to 990 nm. The intensity of the Stokes beam is sinusoidally modulated with a built-in electro-optic-modulator at 8 MHz and the modulation depth is over 95%. Spatial overlap of pump and Stokes beam is adjusted with a dichroic mirror inside the laser system and temporal overlap between two beams is optimized with a built-in delay stage based on the dodecane liquid SRS signal.

Both beams are introduced into an inverted multiphoton laser-scanning microscope (FV1200MPE, Olympus) with maximized near IR output, which are then focused on the cell sample through a 60× water immersion objective (UPlanAPO/IR, 1.2 N.A., Olympus) with high near IR transmission. The beam size of pump and Stokes laser matches with the back-aperture of the objective. After transmission, pump and Stokes beams are collected efficiently with a high N.A. condenser lens (oil immersion, 1.4 N.A., Olympus) in Kohler illumination. The laser-scanning motion is descanned using a telescope onto a large area (1 cm × 1 cm) silicon photodiode (FDS1010, Thorlabs), which is reverse-biased with a 64 DC voltage to enhance the saturation threshold and response bandwidth. To block the Stokes beam and pass only the pump beam, a high O.D. bandpass filter (890/220 CARS, Chroma Technology) is put in front of the

photodiode. The output current of photodiode passes through a 8 MHz electronic bandpass filter (KR 2724, KR electronics) to remove both high frequency component (80 MHz laser pulsing) and low frequency fluctuations due to laser scanning before entering a radio frequency lock-in amplifier (SR844, Stanford Research Systems) terminated with 50 Ω to demodulate the stimulated Raman loss signal from the pump beam. The in-phase signal at the X channel of the lock-in amplifier is inputted to the analog interface box (FV10-ANALOG) of the microscope to generate the SRS image with 512×512 pixels per frame. The imaging dwell time for each pixel is 100 μs and the time constant for demodulation is 10 μs (shortest without additional filtering), achieving an imaging speed of 27 seconds per frame. 130 mW modulated Stokes beam and 120 mW pump beam, measured after the 60 \times water objective, are used to image the sample at all frequencies. All images are acquired using FluoView scanning software and assigned color with ImageJ.

Spontaneous Raman spectroscopy. An upright confocal Raman microscope (Xplora, Horiba Jobin Yvon) with 532 nm diode laser source and 1800 l/mm grating was used to acquire spontaneous Raman scattering spectra at room temperature. The laser is linearly polarized and the excitation power is 12 mW after passing through a 50 \times , air objective (MPlan N, 0.75 N.A., Olympus).

The Raman spectra of D₉-choline, D₃-methionine and D₁₀-leucine (all in PBS solution) were collected with 80 s acquisition time (Figure S1).

The Raman spectra of cell samples were collected with 80 s or 240 s acquisition time.

Enzymatic assay for D₉-choline enrichment in choline phospholipid metabolism. We designed the phospholipase experiment based on previous literature³. HeLa cells incubated with

10 mM D₉-choline for 48 hours were first fixed with 4% PFA and washed with TBS buffer twice. For Figure S3a, cells were incubated with 1 mg/mL BSA in TBS buffer at 37 °C for 1 hour. For Figure S3b, cells were incubated with 1 mg/mL BSA, 0.02 U/mL phospholipase C and 10 mM CaCl₂ in TBS buffer for 1 hour at 37 °C. For Figure S3c, cells were incubated with 1 mg/mL BSA, 0.02 U/mL phospholipase C and 10 mM EDTA in TBS buffer for 1 hour at 37 °C. Then all cells were washed with TBS buffer and ready for SRS imaging.

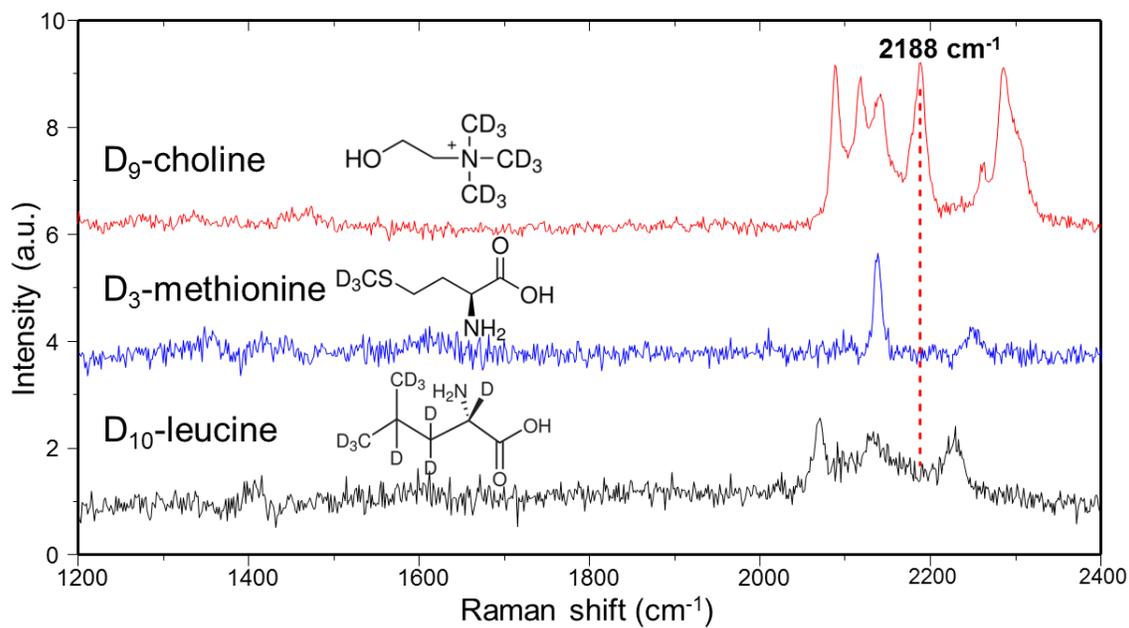


Figure S1. 2188 cm⁻¹ as a characteristic Raman peak for D₉-choline. Methyl C-D vibrational frequency is sensitive to local environment as shown in the comparison between D₉-choline, D₃-methionine and D₁₀-leucine.

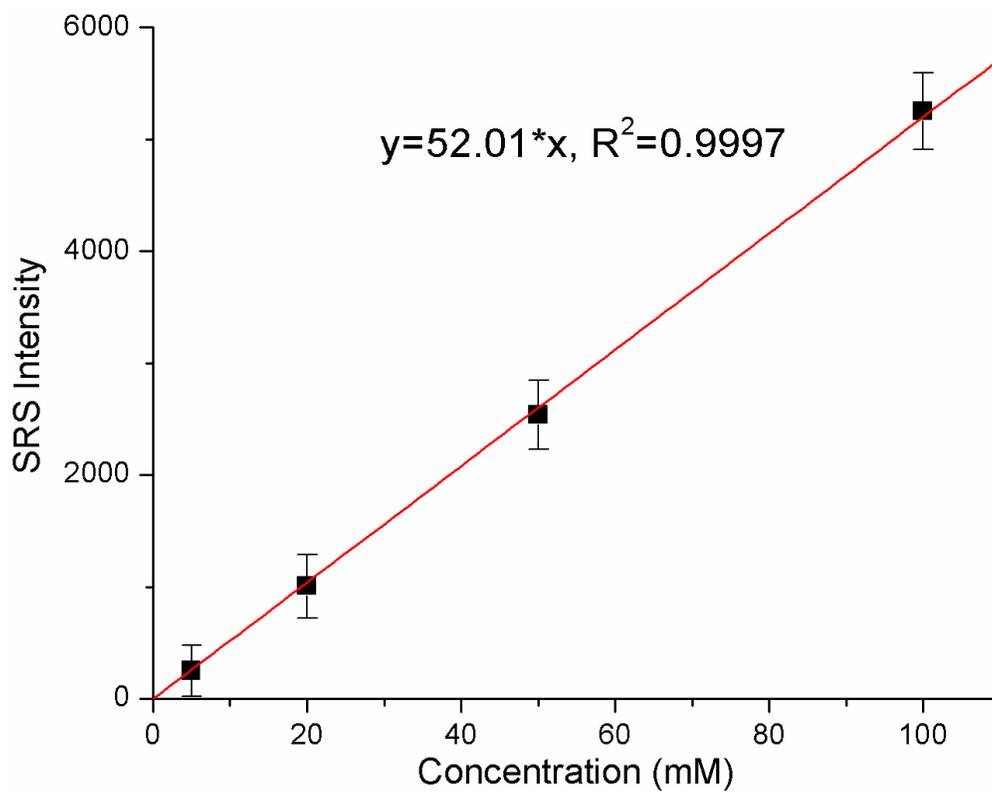


Figure S2. Correlation curve of SRS intensity at 2188 cm^{-1} with D_9 -choline PBS solution at different concentrations.

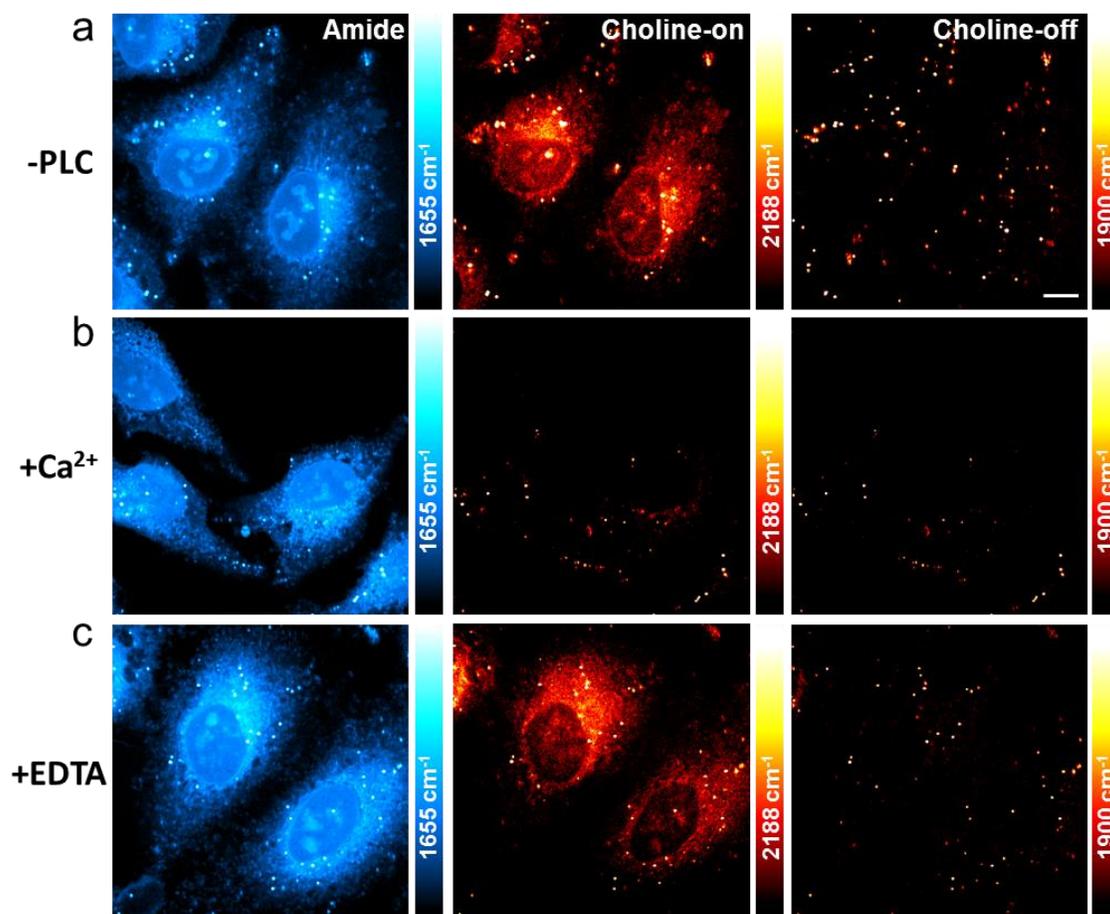


Figure S3. D₉-choline incorporation into phospholipids shown by phospholipase assay. (a) The 2188 cm⁻¹ choline-on image of fixed HeLa cells incubated with 10 mM D₉-choline for 48 hours without the treatment of phospholipase C (PLC). (b) The loss of signal in the 2188 cm⁻¹ choline-on image of fixed HeLa cells incubated with 10 mM D₉-choline for 48 hours which are treated with PLC under Ca²⁺ catalysis. (c) Signal is retained in the 2188 cm⁻¹ choline-on image of fixed HeLa cells incubated with 10 mM D₉-choline for 48 hours which are treated with PLC in the presence of EDTA (inhibiting enzyme activity by chelating Ca²⁺). The 1655 cm⁻¹ amide and the 1900 cm⁻¹ choline-off images display the same area as in the choline-on images. Scale bar: 10 μm.

References

1. Brenner, S. *Genetics* 1974, **77**, 71.
2. Fire, A. *EMBO J.* 1986, **5**, 2673.
3. Jao, C. Y.; Roth, M.; Welti, R.; Salic, A. *Proc. Natl. Acad. Sci. U.S.A.* 2009, **106**, 15332.