Two-color Vibrational Imaging of Glucose Metabolism by Stimulated Raman Scattering

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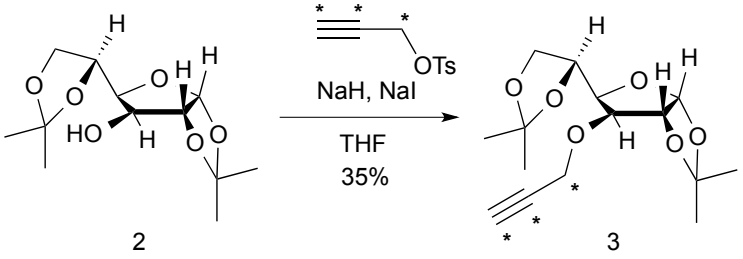
Organic synthesis

General Information. All reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) carried out on glass backed silica gel TLC plates (250 μm) from Silicycle; visualization by UV light, an ethanolic solution of phosphomolybdic acid as staining agent. Reagents and solvents were purchased from Sigma-Aldrich and Cambridge isotopes at the highest commercial quality and used without further purification, unless otherwise stated. Flash chromatography was performed on a Teledyne ISCO CombiFlashRf using RediSepRf silica gel columns. NMR spectra were recorded on either a Brüker Advance 400 (^{1}H: 400 MHz, ^{13}C: 100 MHz). Brüker Advance 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz). High resolution mass spectrometric (HRMS) data were obtained using JMS-HX110A mass spectrometer. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad.

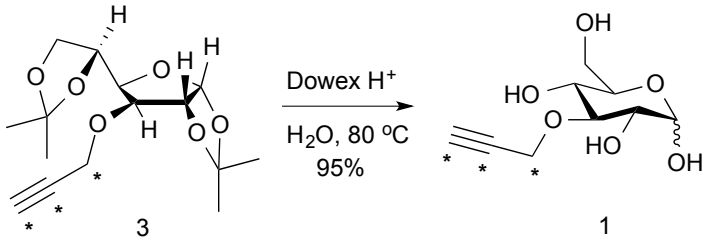
Synthesis of 3-*O*-propargyl-D-glucose-$^{13}C_3$ (3-OPG-$^{13}C_3$, 1)



To a solution of 1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose **2** (260 mg, 1.0 mmol) in 5 mL dry THF was added sodium hydride (60% dispersion in mineral oil, 73 mg, 1.82 mmol) at 0 °C. The solution was stirred at the same temperature for 30 min. To the mixture were added NaI[1] (67 mg, 0.45 mmol) and propargyl tosylate-$^{13}C_3$ (194 mg, 0.91 mmol, prepared by standard tosylation of propargyl alcohol-$^{13}C_3$ with TsCl)[2] at 0 °C, and the mixture was stirred at the room temperature for 13 h. The reaction was quenched by addition of a saturated solution of NH_4Cl (5 mL), and the mixture was extracted with AcOEt (2×10 mL). The combined organic layers were washed with brine (5 mL), and dried with Na_2SO_4. The solvent was removed in vacuum, and the

residue passed through a short pad of silica before further purified with preparative TLC (hexane/ethyl acetate = 5/1) to yield **3** (96 mg, 0.32 mmol, 35%) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.87 (d, J = 3.7 Hz, 1H), 4.62 (d, J = 3.7 Hz, 1H), 4.27 (dm, J_{CH} = 152 Hz, 2 H), 4.31 – 4.21 (m, 1H), 4.13 (dd, J = 7.7, 3.0 Hz, 1H), 4.11 – 4.05 (m, 2H), 3.99 (dd, J = 8.6, 5.4 Hz, 1H), 2.47 (qdd, J = 3.5, 56, 268 Hz, 1 H), 1.49 (s, 3H), 1.42 (s, 3H), 1.34 (s, 3H), 1.31 (s, 3H)
^{13}C NMR ($CDCl_3$, 100 MHz, labeled carbons only) δ ppm: 79.4 (dd, J = 71, 171 Hz), 74.7 (dd, J = 14, 171 Hz), 58.1 (dd, J = 14, 71 Hz)
HRMS (ASAP+) m/z Calcd. for $C_{12}{}^{13}C_3H_{22}O_6$ $[M+H]^+$: 302.1595. Found: 302.1598



To **3** (90 mg, 0.30 mmol) was added water (1.5 mL) and Dowex® 50WX8 hydrogen form (90 mg). The mixture was heated to 80 °C for 20 h before the solvent was removed *in vacuo*. The residue was purified via silica gel flash chromatography (DCM/MeOH = 6/1) to yield **1** (63 mg, 0.285 mmol, 50%) as a white solid. The two diastereomers could not be separated and were characterized as 1.2:1 mixture. (Note: ^{1}H NMR spectra data for 1 not provided here due to isomers and the ^{13}C-H coupling, causing substantial line and peak broadening)

^{13}C NMR ($CDCl_3$, 100 MHz, labeled carbons only) δ ppm: 80.2 (dd, J = 71, 169 Hz), 79.8 (dd, J = 71, 169 Hz), 75.8 (dd, J = 13.7, 169 Hz), 75.6 (dd, J = 12.5, 169 Hz), 60.0 (dd, J = 13.7, 71 Hz), 59.8 (dd, J = 13.7, 71 Hz)
HRMS (APAP+) m/z Calcd. for $C_6{}^{13}C_3H_{14}O_6$ $[M+Na]^+$: 244.0789. Found: 244.0796

Stimulated Raman scattering microscopy

SRS microscopy was performed on an integrated laser system with custom modification (picoEMERALD, Applied Physics & Electronics, Inc.). This system not only produced a Stokes beam (1064 nm, 6 ps), but also generated a tunable Pump beam (720-990 nm, 5-6 ps). Both beams were at 80 MHz repetition rate, also were spatially and temporally overlapped to introduce into an inverted multiphoton laser scanning microscopy (FV1200MPE, Olympus), and then focused onto the sample through a 25X water objective (XLPlan N, 1.05 N.A.,Olympus). The Stokes beam was then blocked with a high O.D. bandpass filter (890/220 CARS, Chroma Technology), and the pump beam was collected onto a large area (10×10 mm) Si photodiode (FDS1010, Thorlabs) reverse-biased at a 64 DC voltage. To detect the stimulated Raman loss with near shot-noise-limited sensitivity, output current was electronically filtered by electronic

bandpass filter (KR 2724, KR electronics), terminated with 50Ω, and demodulated by a radio frequency lock-in amplifier (SR844, Stanford Research Systems). The output of the lock-in amplifier was sent to the microscope through an analogue interface box (FV10-ANALOG, Olympus), and images were reconstructed using Fluoview software (Olympus). The signal from lock-in amplifier was entered into analog interface box (FV10-ANALOG, Olympus) of the microscope, and then the images were generated using Fluoview software (Olympus). Laser powers on sample were measured to be ~150 mW for modulated Stokes beam and ~120 mW for Pump beam. All images were acquired with 30 μs time constant set of lock-in amplifier and 100 μs pixel dwell time for 512×512 pixel field of view.

Spontaneous Raman Spectroscopy

Spontaneous Raman spectra were acquired via a Raman spectrometer (Xplora, Horiba Jobin Yvon) at room temperature. A 532 nm diode laser with the power of 27 mW on samples passing through a 50×, 0.75 N.A. objective (NPLAN EPI; Leica) was applied to excite samples. All the spectra for live cells were captured in 40 s and collected by LabSpec 6 software.

Cell Culture and imaging. All cell lines purchased from ATCC were cultured in 37 °C incubator under the atmosphere with 5% CO_2. Culture media containing 10% (v/v) FBS and 1% (v/v) P&S of EMEM was applied to U87 MG cells. Culture media containing 10% (v/v) FBS and 1% (v/v) P&S of F-12K was applied to PC-3 cell. Culture media containing 10% (v/v) FBS and 1% (v/v) P&S of DMEM was applied to Cos-7 cells. Culture media containing 10ug/ml insulin, 10% (v/v) FBS and 1% (v/v) P&S of EMEM was applied to MCF-7 cell. Culture media containing 0.05 mg/ml BPE, 5 ng/ml EGF and 1% (v/v) P&S of K-SFM was applied to RWPE-1 cell. Primary antibodies mouse anti-E-cadherin and rabit anti-vimentin, and secondary antibodies goat-anti-mouse antibody conjugated with Alexa647 and goat-anti-rabit antibody conjugated with Alexa488 were all purchased from Abcam.

For two-color imaging of cell lines, cells were seeded on coverslips within plates containing 1.0 mL of corresponding complete media, and incubated for 48 hours. The media was firstly replaced with glucose-free DMEM containing 25 mM D7-glucose, supplemented without FBS for 48 hours. Then the media was changed to glucose-free DMEM containing 25 mM 3-OPG-$^{13}C_3$ and further cultured for 48 hours at 37 °C and 5% CO_2 before imaging.

For cross-talk measurement, cells were seeded on coverslips within plates containing 1.0 mL of corresponding complete media, and incubated for 48 hours. The media was firstly replaced with glucose-free DMEM containing 25 mM D7-glucose, supplemented without FBS for 48 hours at 37 °C and 5% CO_2 before imaging.

For co-culture experiment, PC-3 cells and RWPE-1 cell were seeded on the same coverslips within plates containing 1.0 mL media which was consist of 0.5 ml complete media for PC-3 and 0.5 mL complete media for RWPE-1, incubated for 48 hours. The media was firstly replaced with glucose-free DMEM containing 25 mM D7-glucose, supplemented without FBS for 48

hours. Then the media was changed to glucose-free DMEM containing 25 mM 3-OPG-$^{13}C_3$ and further cultured for 48 hours at 37 °C and 5% CO_2 before imaging.

For TVB-3166 treatment experiment, cells were seeded on coverslips within plates containing 1.0 mL of complete media, and incubated for 48 hours. The media was firstly replaced with glucose-free DMEM containing 25 mM D7-glucose and 10 μM TVB-3166, supplemented without FBS for 24 hours. Then the media was changed to glucose-free DMEM containing 25 mM 3-OPG-$^{13}C_3$ and 10 μM TVB-3166, and further cultured for 2 hours at 37 °C and 5% CO_2. The corresponding control cells were studied in parallel for comparison.

For EMT experiment, MCF-7 cells were seeded on coverslips within plates containing 1.0 mL of complete media, and incubated for 48 hours. The media was firstly replaced with inducing medium containing 10ug/ml insulin and 1x StemXVivo EMT inducing media supplement in EMEM, and changed with fresh inducing medium every 3 days. MCF-7 cells turned to mesenchymal after 5-8 days treatment. The inducing media was replaced with glucose-free DMEM containing 25 mM D7-glucose and 10ug/ml insulin for 48 hours. Then the media was changed to glucose-free DMEM containing 25 mM 3-OPG-$^{13}C_3$ and further cultured for 2 hours at 37 °C and 5% CO_2. The control epithelial MCF-7 cell was studied in parallel for comparison.

For immunofluorescence, epithelial and mesenchymal MCF-7 cells were grown on glass coverslip, and stained with primary antibody at 4 °C overnight then stained with secondary antibody for 1 hour at room temperature. Cell nucleus were stained with NucBlue and imaged with 2-photon excitation at 780 nm. The vimentin and E-Cadherin distribution were imaged with fluorescence from excitation at 488 nm and 647 nm, respectively.

After incubation of all cell samples, cells are gently washed with 1 mL PBS buffer and then carried out for SRS imaging and spontaneous Raman scattering measurement.

Tissue Culture and imaging. All mice experiments are performed with approval from Columbia University IACUC. The 300 μm thick brain slices obtained from P2 mice by a Leica vibratome was cultured for 24 hours on Millicell cell culture insert in glucose-free Neurobasal-A medium, supplemented with 2× B27 serum-free supplement, 1 mM L-glutamine, 1 mM sodium pyruvate and 50 mM D7-glucose. Then the media was changed to glucose-free Neurobasal-A medium containing 25 mM 3-OPG-$^{13}C_3$ and further cultured for 2.5 hours at 37 °C and 5% CO_2. Before SRS imaging, the tissue was washed with PBS solution, then transferred to a chamber containing with PBS solution and closed with a coverslip.

Image processing and data analysis. The assigned color was processed though ImagJ. Threshold was applied to the $^{13}C{\equiv}^{13}C$ image for analysis of the C–D/$^{13}C{\equiv}^{13}C$ ratio. The ratio was obtained by dividing C–D images over $^{13}C{\equiv}^{13}C$ images from all cells within the optical field. Statistical analysis was obtained by multiple independent experiments, thus giving the mean and standard deviation for each condition. Data fitting was performed in OriginPro 8.

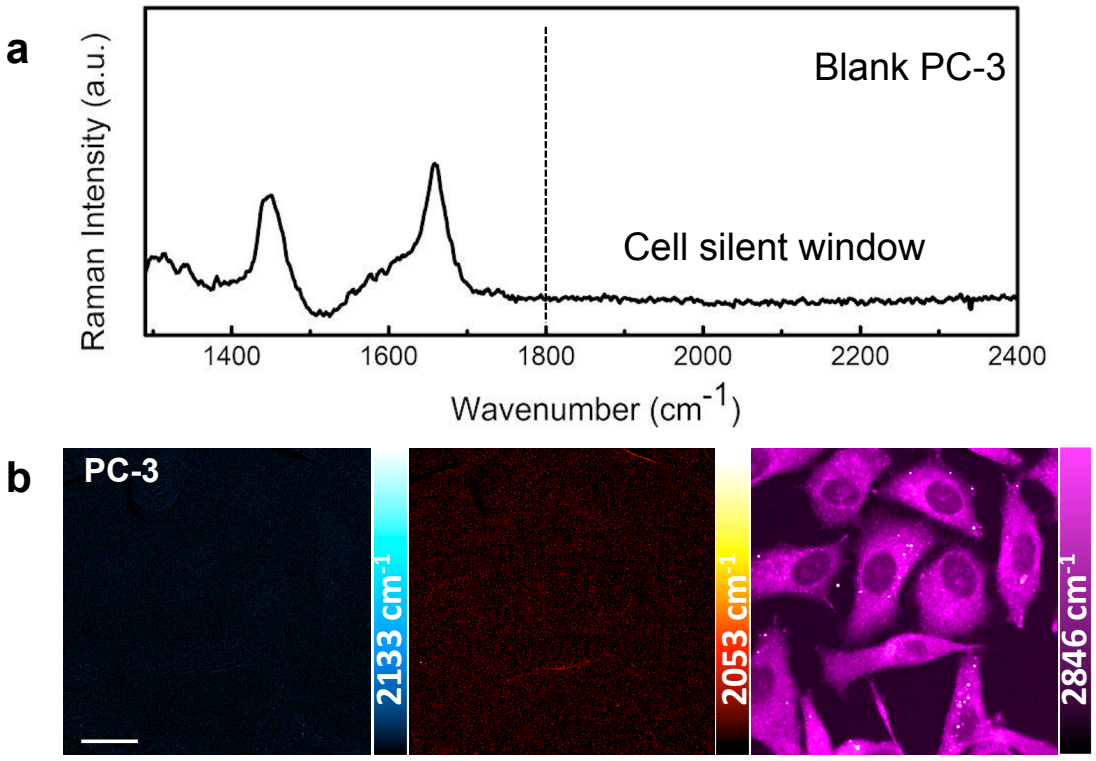


Figure S1. Raman background of live PC-3 cells. (a) Spontaneous Raman spectrum of blank PC-3. (b) SRS images of blank PC-3 confirm the negligible cellular Raman background at 2133 cm^{-1} and 2053 cm^{-1} within the cell silent window. Scale bar: 20 μm.

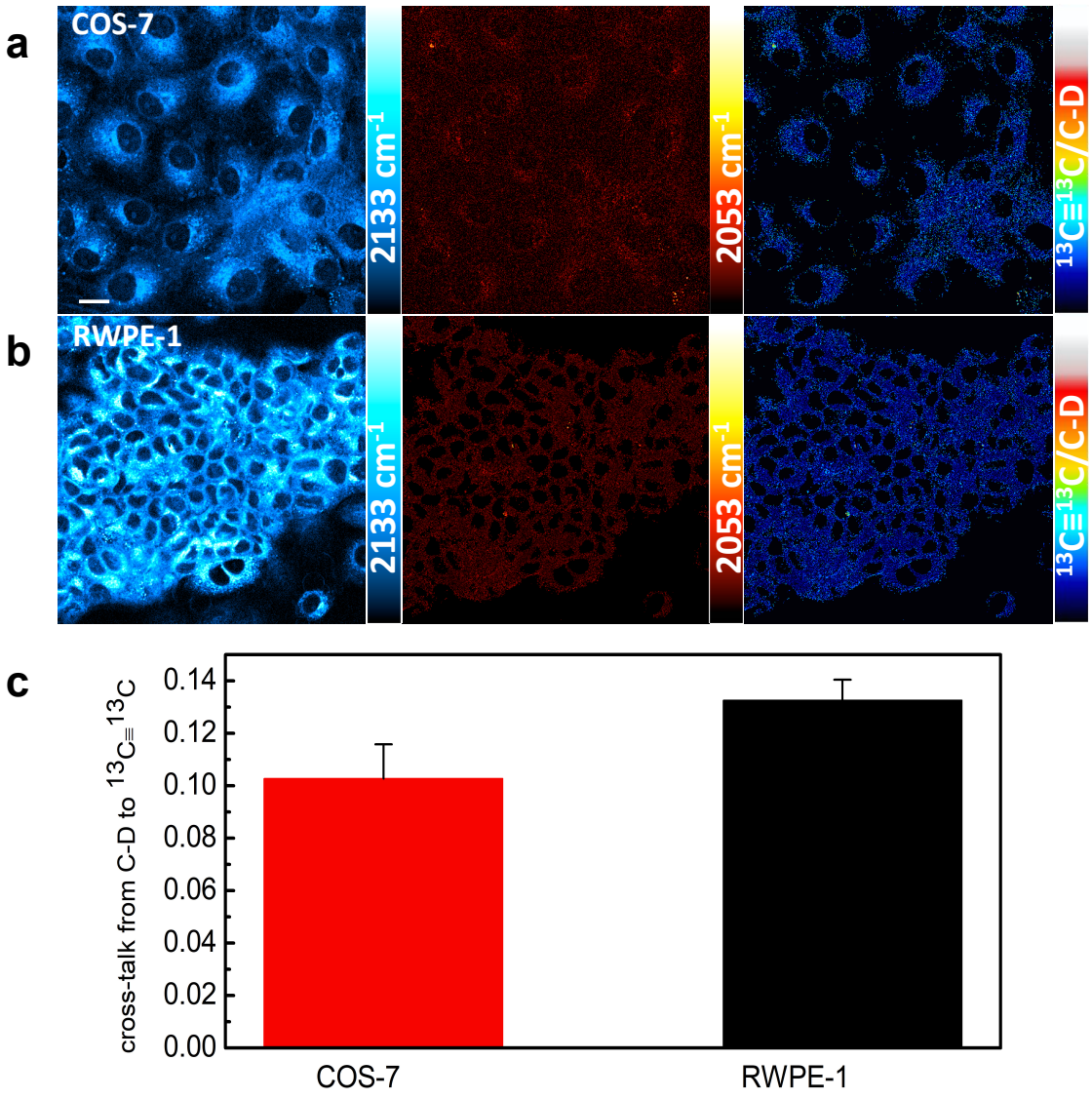


Figure S2. Cross-talk measurement in live mammalian cells. (a) SRS images of COS-7 cells cultured with 25 mM D7-glucose for 48 hours. (b) SRS images of RWPE-1 cells cultured with 25 mM D7-glucose for 48 hours. (c) Quantitative analysis of the ratio of $^{13}C\equiv^{13}C$/C-D. The two peaks are resolved with ~10% cross-talk. Scale bar: 20 μm.

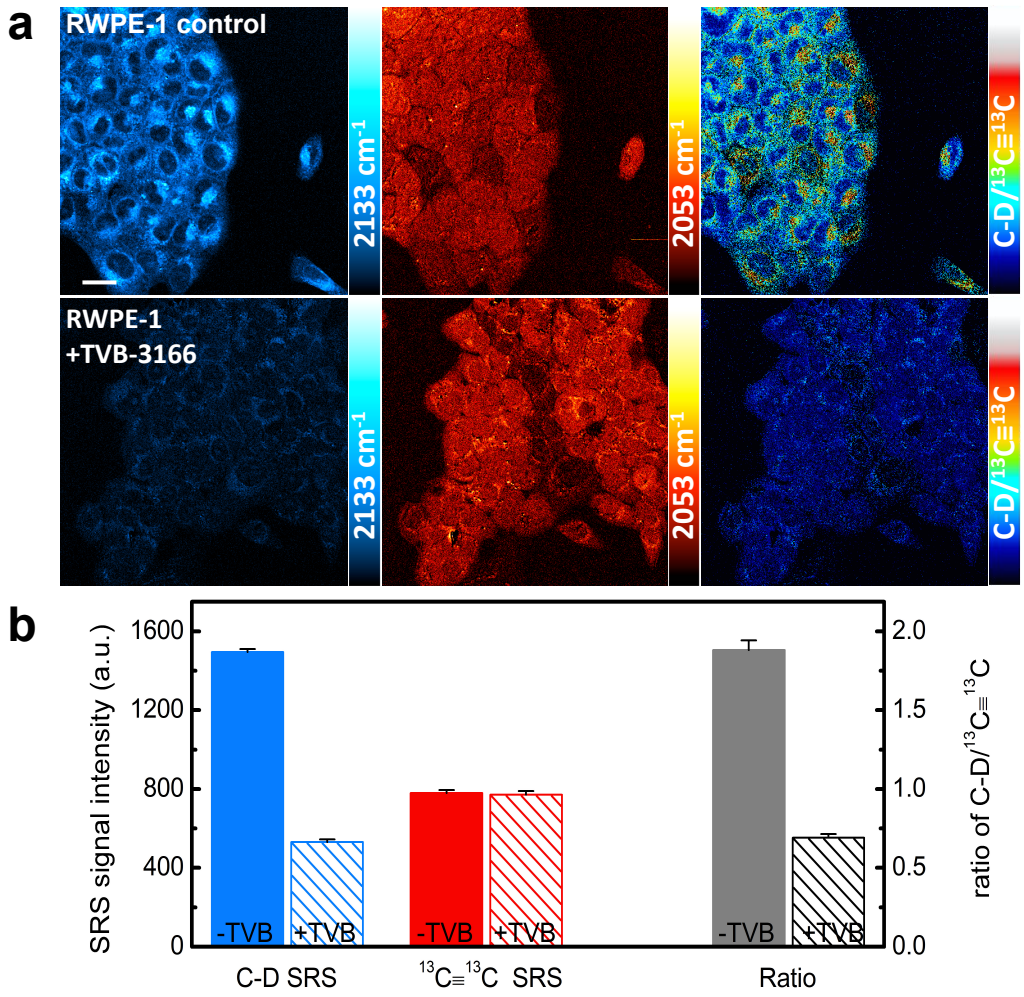


Figure S3. Influence of FASN inhibition. a) Two-color SRS imaging of RWPE-1 cells with or without 10 μM TVB-3166. b) Quantitative analysis of glucose incorporation (C-D), glucose uptake ($^{13}C\equiv^{13}C$) and ratiometric images (C-D/$^{13}C\equiv^{13}C$) with or without TVB-3166 treatment. Data are shown as mean + standard deviation (SD; n ≥ 3 replicates for each group). *p* values of Student's t-test: $p < 0.0001$; $p > 0.6$; $p < 0.0001$ (from left to right). Scale bar: 20 μm.

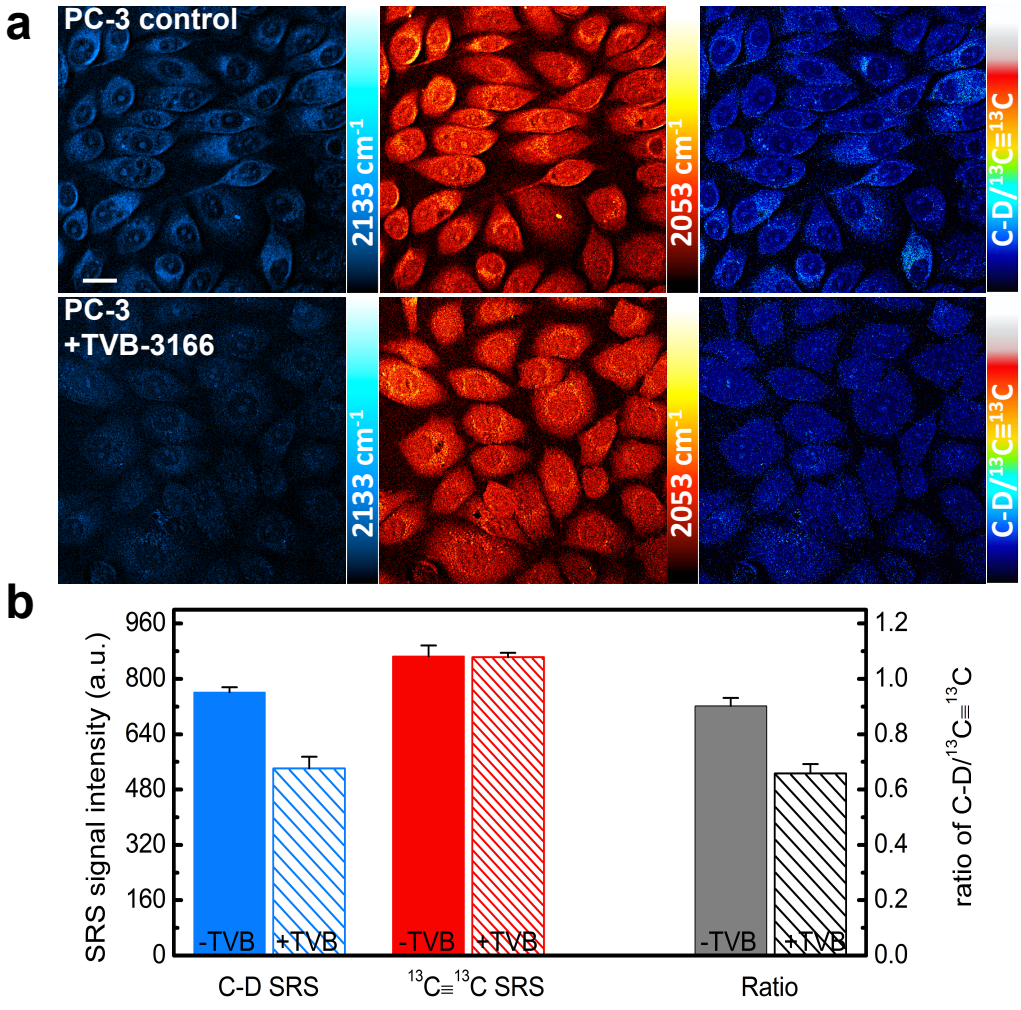


Figure S4. Influence of FASN inhibition. a) Two-color SRS imaging of PC-3 cells with or without 10 μM TVB-3166. b) Quantitative analysis of glucose incorporation (C-D), glucose uptake ($^{13}C\equiv^{13}C$) and ratiometric images (C-D/$^{13}C\equiv^{13}C$) with or without TVB-3166 treatment. Data are shown as mean + standard deviation (SD; n ≥ 3 replicates for each group). *p* values of Student's t-test: $p < 0.0002$; $p > 0.6$; $p < 0.002$ (from left to right). Scale bar: 20 μm.

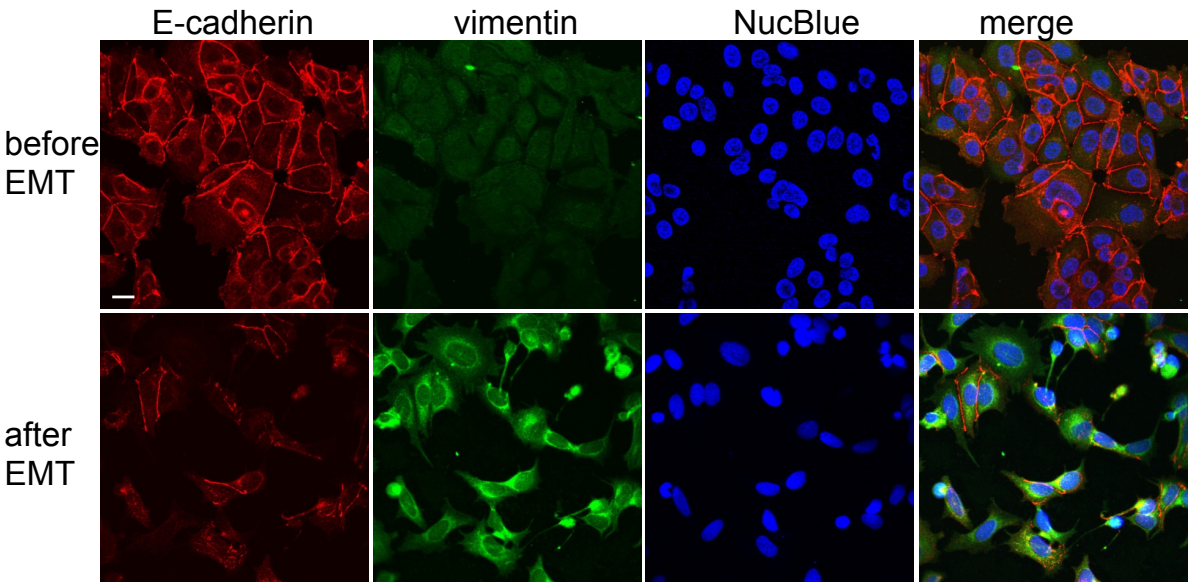


Figure S5. Immunofluorescence of MCF-7 confirms cells undergo EMT. After EMT, cells have decreased E-cadherin (red) expression but increased vimentin (green) expression. Cell nucleus was stained with NucBlue. Scale bars: 20 μm.

Reference

1. Saito, N.; Sugimura, Y.; Sato, Y. *Org Lett* **2010,** *12*, 3494-3497.

2. Neukom, J. D.; Perch, N. S.; Wolfe, J. P. Organometallics **2011**, *30*, 1269-1277.