

Investigating Single-Cell Metabolism with Advanced Stimulated Raman Scattering Microscopy

Thesis by
Adrian Colazo

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The Caltech logo, featuring the word "Caltech" in a bold, orange, sans-serif font.

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Adrian Colazo

ORCID: 0000-0002-0702-0851

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ABSTRACT

Stimulated Raman scattering (SRS) microscopy has emerged as a powerful platform for chemically specific imaging in living systems, enabling minimally perturbative visualization of molecular composition and metabolic dynamics at subcellular resolution. However, its potential for metabolomic studies remains limited by intrinsic challenges in sensitivity, multiplexing applications, and quantitative interpretation of metabolized signals. This thesis addresses these limitations through advances in instrumentation, probe palette design, and computational analysis to expand the capabilities of SRS microscopy for metabolic imaging.

In Chapter 2, visible enhanced stimulated Raman scattering microscopy via frequency-doubling of picosecond pulses, we develop a new form visible-SRS microscopy (VIS-SRS) microscopy through direct frequency-doubling of narrowband picosecond laser sources for a more practical route to multiplexed VIS-SRS imaging. By shifting excitation into the visible regime with picosecond pulses, this approach enhances Raman scattering cross-sections and improves spatial resolution while simplifying instrumental implementation compared to femtosecond-based VIS-SRS modalities. VIS-SRS holistically provides a new route toward increased sensitivity for imaging low-abundance molecular species and opening new opportunities for electronic pre-resonance imaging of new target analytes.

In chapter 3, MetaboRamics: highly multiplexed metabolic imaging using stimulated Raman scattering microscopy for live-cell spatial metabolomics, we introduce a new vibrational palette for live-cell metabolic analysis. By leveraging minimally perturbative,

metabolically active probes in combination with label-free contrast and advanced spectral unmixing, MetaboRamics enables super-multiplexed imaging across multiple metabolic pathways. This platform allows high-dimensional characterization of metabolic states and reveals interconnected metabolic rewiring in response to biological perturbations with single-cell and subcellular resolution.

In chapter 4, Optical Metabolic Imaging of Tricarboxylic Acid Cycle Activity, we introduce MATRIX-SRS, a framework for quantitative imaging of tricarboxylic acid (TCA) cycle metabolism in living systems. By integrating hyperspectral SRS imaging with density functional theory (DFT)-informed spectral modeling, MATRIX-SRS enables new quantitative insights into metabolized probe signals. This approach allows, for the first time, spatially resolved and absolute quantification of metabolically incorporated species using optical imaging.

Together, these advances position SRS microscopy as an increasingly powerful platform for metabolic investigations. By improving sensitivity, enabling super-multiplexed spatial metabolomics, and enhancing chemical insight into metabolic activity, this work opens new opportunities to study cellular heterogeneity, metabolic dynamics, and disease-associated metabolic rewiring.

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Chapter 1

INTRODUCTION

1.1 Raman Scattering and Stimulated Raman Scattering Microscopy

Biological systems are inherently dynamic, with metabolic processes constantly evolving in space and time. As a result, to truly understand how biological systems function, it is essential to study them in their native context with minimal perturbation. Toward this goal, optical approaches based on light-matter interactions provide a powerful foundation for probing biology. Raman scattering, first reported by C. V. Raman in 1928¹, arises from inelastic scattering of light, where photons exchange energy with molecular vibrations and emerge either red-shifted (stokes shifted) or blue-shifted (anti-stokes shifted) relative to the incident frequency. These shifts correspond to vibrational modes of chemical bonds, enabling chemically specific, label-free molecular imaging. However, Raman scattering initially remained limited in its practice due to its low Raman cross-sections and the lack of sufficiently intense laser sources. Although the development of lasers in the 1960s improved their feasibility², spontaneous Raman microscopy remained slow for biological imaging, as the inherently weak signals require long integration times and are often overwhelmed by autofluorescence from endogenous biomolecules.

A major improvement in the applicability of Raman for bio-imaging came with the development of coherent Raman scattering, in which two optical fields amplify the Raman scattering effect when their frequency difference matches a vibrational resonance. This

coherent excitation greatly enhances the Raman transition probability ($\sim 10^5$ – 10^8 -fold) and forms the basis of both coherent anti-Stokes Raman scattering (CARS)³ and stimulated Raman scattering (SRS)⁴. In SRS, energy transfer between synchronized pump and Stokes beams produces either stimulated Raman loss (SRL) in the pump beam or stimulated Raman gain (SRG) in the Stokes beam. While CARS enabled early biomedical imaging⁵⁻⁷, it suffers from non-resonant background and nonlinear signal dependence, complicating quantitative analysis. In contrast, SRS provides a background-free signal with linear dependence on molecular concentration and produces spectra that directly aligns with spontaneous Raman measurements, allowing the SRS field to directly reference previously done spontaneous Raman studies. The modern implementation of SRS microscopy that came in 2008, enabled by high-frequency modulation and lock-in detection, marked a turning point for Raman microscopy by allowing highly sensitive chemical imaging with video-rate acquisition speeds, compatible with live biological systems with minimal autofluorescence background⁸.

Since its modern implementation, SRS microscopy has been widely used to visualize biological systems through both label-free and probe-enabled vibrational contrast. In the label-free regime, SRS has enabled imaging of endogenous biomolecules such as lipids, proteins, and nucleic acids, including live-cell and in vivo DNA imaging based on intrinsic vibrational signatures⁹⁻¹¹. Beyond endogenous contrast, the narrow linewidths of Raman peaks and the availability of the cell-silent spectral window have made SRS especially powerful for multiplexed chemical imaging. Rationally designed vibrational probes, including electronic pre-resonance dyes, have enabled super-multiplexed imaging and barcoding in a single shot at unprecedented numbers^{12,13}. At the same time, minimally

perturbative vibrational tags such as alkynes, nitriles, and deuterium labels have expanded SRS into live-cell metabolic imaging, allowing researchers to track glucose uptake, de novo lipogenesis, protein synthesis, and metabolic remodeling during disease conditions¹⁴⁻¹⁹. SRS has also been extended beyond abundance measurements toward functional environmental readouts, including local environmental sensing through hydrogen-deuterium exchange²⁰ and hyperspectral imaging of protein secondary structure.²¹ Together, these advances demonstrate the promise of SRS microscopy as a platform for label-free imaging, multiplexed probe development, and dynamic metabolic analysis.

1.2 Limitations of the Current SRS Microscopy Landscape

Despite its unique advantages, SRS microscopy still faces several fundamental limitations that constrain its use for comprehensive metabolic imaging in biological systems.

First, sensitivity remains intrinsically limited by the small Raman scattering cross-section. Although SRS provides orders-of-magnitude signal enhancement over spontaneous Raman scattering, the absolute Raman cross-sections of most molecular vibrations are still low compared to absorption cross sections ($\sim 10^{-30}$ cm² vs $\sim 10^{-16}$ cm², respectively), typically restricting detection sensitivity to the millimolar regime under standard conditions. This limitation becomes especially problematic for probing low-abundance metabolites, where native concentrations can often lie in the micromolar regime. While the use of electronic pre-resonance dyes can enhance signal strength, these strategies are not universally applicable and can potentially result in biological perturbations.

Second, while Raman linewidths are inherently narrow and, in principle, well-suited for multiplexed imaging, their application to metabolic super-multiplexing remains underdeveloped. A key challenge arises from the spectral distribution of commonly used bioorthogonal vibrational probes. Although the cell-silent region ($\sim 1800\text{--}2600\text{ cm}^{-1}$) provides a large, uncluttered spectral window, widely used probes such as deuterium (C–D), nitriles (C $\equiv$ N), and alkynes (C $\equiv$ C) tend to cluster within a narrow subregion ($\sim 2100\text{--}2300\text{ cm}^{-1}$). This spectral crowding is particularly severe for highly deuterated probes, which produce broad, overlapping features that rapidly saturate this region, limiting the number of distinguishable channels and complicating multiplexed analysis without robust spectral unmixing.

Finally, these limitations are compounded by challenges in spectral interpretation and quantification in metabolic systems. Upon metabolic incorporation, vibrational probes are often transformed into downstream products with altered chemical environments, leading to peak broadening, spectral shifts, and increased overlap between signals. As a result, the molecular identity of detected signals becomes ambiguous, making it difficult to assign specific biochemical pathways and products or achieve absolute quantification of the measured signal.

Together, these challenges represent key barriers to fully realizing the potential of SRS microscopy for comprehensive metabolic imaging. Addressing these limitations is the central focus of this thesis, with each of the following chapters targeting one of these challenges

through advances in sensitivity, super-multiplexing for spatial metabolomics, and quantitative spectral interpretation on new biosystems.

1.3 Improving Sensitivity Through Instrument Development

To address the intrinsic sensitivity limitations of SRS microscopy, significant efforts have been directed toward increasing Raman signal strength through both sample modification and instrumental advances. One promising strategy is to enhance the Raman scattering cross-section itself by exploiting its positive relationship with shorter wavelengths. This strategy has been successfully implemented in the past through the frequency doubling of femtosecond pulses with high success. However, femtosecond pulses need to be chirped into picosecond pulses for SRS to retain high spectral resolution, introducing instrumental complexity that limits its ability for fast multiplexed imaging across large spectral regions. In this context, we develop visible-enhanced stimulated Raman scattering (VIS-SRS) microscopy through direct frequency-doubling of picosecond laser sources, enabling excitation in the visible regime. This approach enhances non-resonant Raman cross-section, leading to measurable signal gains compared to conventional near-infrared SRS while opening up new probes for electronic pre-resonance SRS imaging. In addition to improved sensitivity, shorter excitation wavelengths also provide increased spatial resolution. By simplifying instrumentation requirements through direct frequency doubling of picosecond pulses, VIS-SRS establishes a practical route for improved SRS sensitivity while retaining high multiplexing capabilities.

1.4 Developing a Live-Cell Compatible Spatial Metabolomics Platform

To further capitalize on the ability of SRS for super-multiplex metabolic imaging, we next develop MetaboRamics, a highly multiplexed vibrational imaging platform for live-cell spatial metabolomics. Cellular metabolism is inherently complex, involving numerous interconnected pathways constantly responding to new stimulus to maintain homeostasis. However, existing approaches to multiplexing focus on engineered Raman probes that require conjugation and cannot be metabolized further to gain further metabolic insight. However, probe metabolism tends to result in clustering of peaks around a narrow portion of the cell silent region ($\sim 2100 - 2300 \text{ cm}^{-1}$), heavily constraining probe selection. MetaboRamics addresses these challenges through careful probe selection, isotope editing, and advanced spectral unmixing, enabling the construction of an expanded metabolic palette spanning multiple pathways, including glucose metabolism, lipid synthesis and uptake, protein synthesis, nucleotide incorporation, and redox state while keeping all metabolic products spectrally separated. By integrating bioorthogonal probes with label-free channels, this platform enables high-dimensional, subcellular metabolic profiling in living systems, providing a framework for super-multiplexed imaging that captures the interconnected nature of metabolic networks.

1.5 Developing Tools for Increased Chemical Insight

Finally, to address challenges in spectral interpretation and quantitative analysis of metabolized probes, we develop MATRIX-SRS, a framework for quantitative imaging of tricarboxylic acid (TCA) cycle metabolism in living systems. The TCA cycle serves as the central hub of cellular metabolism, yet its activity remains difficult to visualize directly due to the low abundance, transient nature, and chemical diversity of its intermediates. While

SRS microscopy has enabled metabolic imaging from TCA substrates such as glucose and glutamine, extending this approach to other TCA substrates has proven quite difficult for the aforementioned reasons. Additionally, once the substrates are incorporated into downstream biomass, their chemical identity becomes ambiguous leading to difficulty in quantifying absolute concentrations. MATRIX-SRS addresses these challenges by combining deuterium-labeled metabolic probes with hyperspectral SRS imaging, complemented by density functional theory (DFT)-informed spectral analysis and isotope tracing validation. This integrated approach enables, for the first time, spatially resolved and quantitatively interpretable imaging of TCA-associated metabolic activity in live cells, bridging the gap between vibrational imaging and quantitative metabolomics.

1.5 Summary and Outlook

SRS microscopy has emerged as a powerful platform over the past two decades for chemically specific imaging in biological systems, enabling minimally perturbative visualization of molecular composition and dynamics in living cells. However, as highlighted in this chapter, key challenges in sensitivity, multiplexing applicability, and quantitative interpretation continue to limit its broader application for comprehensive metabolic imaging.

In this thesis, we address these limitations through three complementary advances: visible-enhanced SRS for improved sensitivity with reduced instrumentation complexity, MetaboRamics for super-multiplexed spatial metabolomics, and MATRIX-SRS for quantitative imaging of TCA cycle activity. Together, these approaches expand the

capabilities of SRS microscopy towards a more comprehensive, quantitative framework for probing metabolism in living systems.

Looking forward, continued integration of advances in instrumentation, probe design, and computational analysis will further extend the reach of SRS microscopy, enabling deeper insights into metabolic regulation, disease progression, and cellular heterogeneity at the single-cell level.

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VISIBLE ENHANCED STIMULATED RAMAN SCATTERING MICROSCOPY VIA FREQUENCY-DOUBLING OF PICOSECOND PULSES

Colazo, A; Miao, K; Lin L.; Chadha, R.; Qian, C.; Bi, X.; Kocheril, P.; Wei, L.¹ Visible enhanced stimulated Raman scattering microscopy via frequency-doubling of picosecond pulses. *Biomed. Opt. Express* **16**, 2430-2439 (2025)

¹ = Division of Chemistry and Chemical Engineering, California Institute of Technology, 6 Pasadena, CA 91125, USA

2.1 Summary

We report visible-enhanced stimulated Raman scattering (VIS-SRS) microscopy by direct frequency-doubling of picosecond pulsed lasers. Compared to previous reports from femtosecond sources, our setup is greatly simplified by removing the need to perform spectral focusing and allows for quick wavelength tuning for sparse multi-channel imaging across a large spectral range. We report a signal enhancement of 3.2-fold for the C–H bond from decane, consistent with theoretical analysis, and up to 483-fold with resonance enhancement. We showcase multi-channel imaging applications in HeLa cells and demonstrate a spatial resolution down to 47 nm from 5xFAD mouse brain tissues coupled with Raman-tailored sample-expansion. Finally, we discuss the future applications and limitations of our picosecond-doubled VIS-SRS.

2.2 Introduction

Raman spectroscopy, probing the vibrational modes of target molecules, allows for investigations of chemical structures in native states and has offered chemical-rich information for materials and biological investigations. However, for biological imaging, spontaneous Raman suffers from extremely slow imaging speed due to the weak signals and high auto-fluorescence background caused by visible excitation. To address this challenge, stimulated Raman scattering (SRS) significantly enhances Raman scattering efficiency by utilizing a secondary laser to drive stimulated emission and has been extensively employed to image live biological samples with high sensitivity, subcellular spatial resolution, and unparalleled chemical specificity¹⁻⁵.

Despite the improvement, SRS still faces a sensitivity bottleneck especially for detecting low concentration ($\sim$ nM - μ M) molecules.³⁻⁶ Consequently, extensive efforts have been directed towards the theory of SRS and methods for improving SRS detection to increase the signal strength. Traditionally, SRS signal is detected by measuring either pump laser intensity loss (stimulated Raman loss, SRL) or Stokes laser intensity gain (stimulated Raman gain, SRG) resulting from stimulated emission, with SRL being preferred due to its higher photosensitivity with available photodiodes in the near-infrared (NIR) region. The SRL process is described as follows¹:

$$SRL \propto -N \cdot \sigma_{\text{Raman}} \cdot \Phi_p \cdot \Phi_S \quad (1)$$

where N denotes the number of bonds in the focal volume, σ_{Raman} is the Raman cross-section, and Φ_p (or Φ_s) are the photon flux of the pump (or Stokes) beams. The Raman cross-section can be further described by equation (2)

$$\sigma_{\text{Raman}} = K \cdot \underbrace{\omega_{\text{pump}} \cdot \omega_{\text{stokes}}^3}_{\text{NR}} \cdot \underbrace{\left(\frac{(\omega_{\text{pump}}^2 + \omega_e^2)}{(\omega_e^2 - \omega_{\text{pump}}^2)} \right)^2}_{\text{Albrecht-A}} \quad (2)$$

Here, K is a sample-dependent constant, the red-boxed terms are the molecule-independent non-resonant (NR) terms, and the blue-boxed term is the molecule-dependent Albrecht-A electronic pre-resonance (EPR) approximation for totally symmetric transitions under a strong dominating electronic transition.⁷ Based on these terms, two strategies harnessing the enhancement in the non-resonance, or the pre-resonance condition can be developed separately or in combination to boost the SRL process.

SRS microscopy is conventionally performed with NIR laser excitation to offer fine biocompatibility with less photodamage in live biological samples.⁸ The pump laser energy is set far from electronic excitation to avoid electronic background (Fig.1 (A), left). One significant strategy to enhance the imaging performance is through pre-resonance SRS imaging.^{9,10} As the pump laser approaches the electronic excitation of the target molecules, the Albrecht-A term in equation (2) predicts a quadratic increase in the bonds' Raman cross-section. Coupling the excitation lasers with NIR absorbing dyes to drive this pre-resonance condition (Fig.1 (A), right), Min et al., pushed the detection limit down to 250 nM.⁹ Another direction for increasing sensitivity is through frequency doubling of the lasers that converts

the NIR laser into the visible laser SRS (VIS-SRS) excitation, which is expected to enhance both the NR terms and the Albrecht-A term for molecules absorbing in the visible or even into the UV regime, such as retinoids, endogenous proteins, and DNA.¹¹⁻¹² Such a strategy also offers much-improved spatial resolution with the shortening of the excitation wavelength. Powered by Fourier reweighting after denoising, resolution down to 86 nm was recently achieved for cellular imaging.¹³

So far, all VIS-SRS instruments are based on the frequency doubling of femtosecond (~ 100 - 200 fs) pulses followed by spectral focusing of temporally-stretched picosecond (ps) laser pulses to retain high spectral resolution, lowered photodamage, and fast spectral tuning within the designated frequency range.¹⁴⁻¹⁵ Spectral-focusing SRS requires highly dispersive media (e.g., rods or gratings) for pulse stretching, and exhibits challenges for multi-frequency imaging across a wide spectral regime due to the wavelength dependence of group delay dispersion, which can cause chirp mismatch and degrade spectral resolution unless compensated appropriately.¹⁵⁻¹⁶ Managing these dispersion-related effects may in practice require adjustments to the optical path and system alignment when tuning across distant spectral regions. In this work, we explore the extension of picosecond-based SRS into the visible spectral regime, given its widespread use in the SRS community for its ease of use, with the goal of establishing a foundation for future VIS-SRS development based on picosecond platforms and providing practical insight into associated challenges.

Here, we report the VIS-SRS modality based on direct frequency-doubling of narrow-band picosecond lasers, bypassing spectral focusing, and greatly accelerating the wavelength

tuning spanning across drastically different bond regions. We implement our system with the high-efficiency picosecond laser compatible conversion strategy and characterize our system by directly comparing the same samples between VIS-SRS and NIR-SRS. Using n-decane solution, we report a 3.2-fold non-resonance signal enhancement. Under our experimental design, we achieved a spatial resolution of 148 nm, and the same spectral resolution offered by NIR-SRS in both the cell-silent and the high-frequency C–H region. We next screened the (pre-) resonant enhancement of commercial dyes, reaching signal enhancements up to 480-fold. Finally, we discuss the nature and severity of photodamage associated with VIS-SRS on biological samples and establish a sample-expansion-based effective solution with VISTA-VIS-SRS.¹⁷ We report an effective 47-nm spatial resolution on expanded 5xFAD mouse brain tissue.

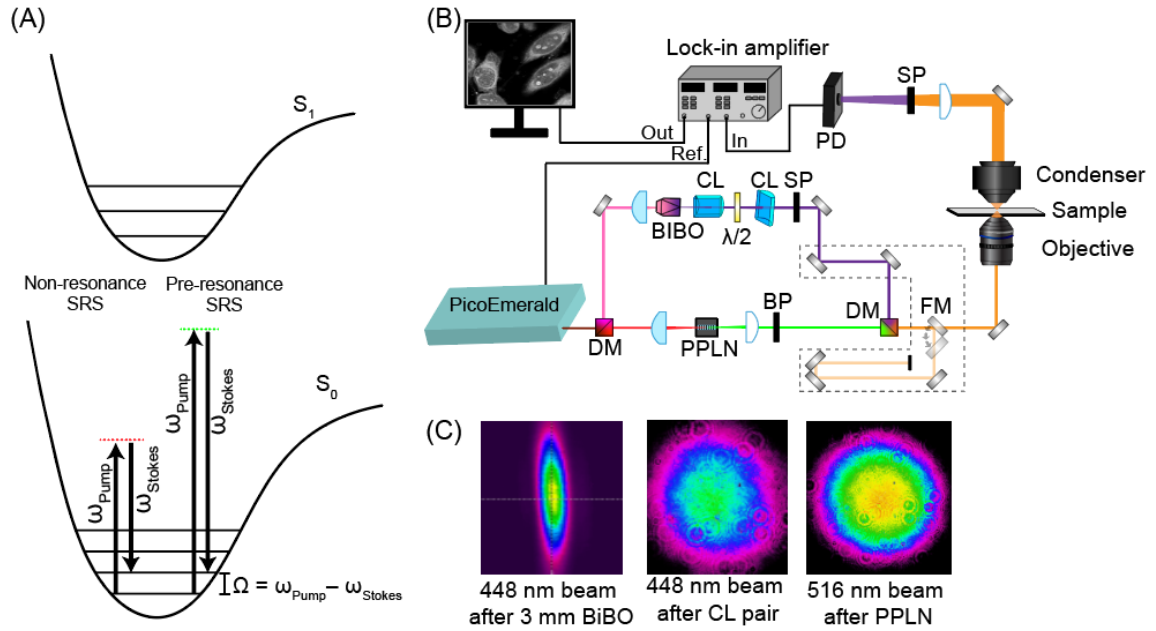


Fig. 1. VIS-SRS microscopy with picosecond pulsed-lasers. (A) Energy diagram for non-resonance and pre-resonance SRS conditions. (B) Schematic of VIS-SRS microscope

through frequency doubling on picosecond lasers. FM: flip mirror. $\lambda/2$: half waveplate.

PD: photodiode. Ref: reference. BIBO: bismuth triborate. PPLN: periodically poled lithium niobate. SP: short-pass filter. BP: bandpass filter. DM: dichroic mirror. CL: cylindrical lens
(C) Beam profile after SHG for pump and Stokes beams. Left: pump beam directly after BIBO, Middle: pump beam after correction with a pair of cylindrical lenses, Right: Stokes beam directly after PPLN.

2.3 Experimental Setup

VIS-SRS microscope with picosecond lasers

A tunable pump (770-990 nm, 2 ps, 80 MHz) and Stokes (1031.2 nm, 2 ps, 80 MHz) beams from a picoEmerald laser system (Applied Physics & Electronics) are split through a long-pass dichroic mirror (Thorlabs, DMLP1000) to perform frequency doubling (Fig. 1 (B)). In spectral-focusing VIS-SRS, thin (<1 mm) Beta-Barium Borate (BBO) and Lithium Borate (LBO) crystals were demonstrated with high conversion efficiency for the short fs pulses. However, these crystals provide very low SHG efficiency for ps pulses. As an alternative, we explored bismuth triborate (Bi_3BO_6 , BIBO, Newlight Photonics, BITC5300-SHG800(I)-AR) for our tunable pump laser.¹⁸ With a 3 mm BIBO crystal, which balances between the conversion efficiency and spatial walk-off effects, we obtained a conversion efficiency of 23% for the pump laser, yielding a total output power of 167 mW (Fig. 1(B)). To correct the astigmatism with a beam divergence ratio of about 1:5 (Fig. 1 (C), left), we employed a pair of cylindrical lenses with focal lengths of 50 mm and 250 mm (Fig. 1 (C), middle). In addition, a half-wave plate is used to correct for the type I SHG polarization change

(Thorlabs, AHP10N-580) (Fig. 1 (B)). For our Stokes beam, a periodically poled lithium niobate (PPLN, Covesion, MSHG1030-0.5-3) crystal was chosen for its high conversion efficiency (50%), ease of phase matching criteria, and desirable type 0 SHG process that directly yields a round beam mode (Fig. 1 (C), right).¹⁹ The remaining fundamental pump & Stokes laser pulses after frequency-doubling are cleaned up with optical filters (FF01-745/SP-25 & FF01-520/15-25, respectively). Due to the slight wavelength dependence of the spatial walk-off effect in the BIBO crystal, a small diagnostic path was added to realign the SHG pump beam and maintain spatial overlap with the Stokes beam. The frequency-doubled beams are then recombined with an additional dichroic mirror (Thorlabs, DMLP490R) before being routed into an Olympus FV3000 microscope (Fig. 1 (B)). Light is focused on the sample with a 60x water immersion objective (UPLSAPO60XW, 1.20 NA, Olympus) and transmitted light is collected with an oil immersion condenser lens (1.40 NA, Olympus). The Stokes beam is filtered out (Semrock, FF01-498/SP-25) and the remaining pump beam is collected with a photodiode (S3590-19, Hamamatsu). The SRL signal from the pump beam is demodulated via lock-in amplification (SR844, Stanford Research Instruments) at the reference modulation frequency (20 MHz). Images were captured in a 16-bit grayscale using Olympus FluoView 3000 software. Images and graphs are visualized using FIJI and GraphPad Prism, respectively. Deconvolution to extract the system PSF was performed in MATLAB by fitting a convolution model to the measured bead profiles using nonlinear least-squares minimization (lsqnonlin, Optimization Toolbox). Imaging parameters for all measurements are tabulated in supplementary tables S2-S4.

2.4 Results

3.1 System characterization

We first characterized the spatial resolution of our system by imaging 100-nm polystyrene (PS) beads (Fig. 2 (A)), targeting the 2904 cm^{-1} band and obtained a full width at half maximum (FWHM) of 162 nm (Fig. 2 (B)). After deconvolving with the size of the beads, we calculate our effective point spread function to be 148 nm, which is in good agreement with the theoretical value of 141 nm, calculated from the Abbe diffraction limit, $D = \frac{0.50\lambda}{\text{N.A.}}$, where λ is the convolved wavelength of the excitation lasers (448.4 nm & 515.6 nm) and numerical aperture (N.A.) is 1.2.

We next calculated our non-resonance signal enhancement using n-decane, a small molecule with minimal electronic absorption far into the UV region,²⁰ by directly comparing signals of the same samples obtained from VIS-SRS and NIR-SRS. Under the same NIR and VIS power conditions, we obtained an optimal 3.2-fold signal enhancement, lower than our theoretically calculated 8.4-fold enhancement (Supplemental Section 1). We reason that the enhancement discrepancy can be potentially attributed to the use of different objectives between measurements. For NIR-SRS, a 25x water immersion objective (XLPLN25XWMP, 1.05 NA, Olympus) is used, which however leads to severe chromatic aberration in the visible region, requiring us to instead use a visible-wavelength optimized objective (UPLSAPO60XW, 1.20 NA, Olympus). In our theoretical calculations, we only accounted for the NA difference, but other practical parameters (e.g. chromatic aberration correction)

may differ. We also note that here we focused on analyzing the pure signal enhancement for quantitative understanding, instead of the signal-to-noise ratio.

With n-decane, we further confirmed that VIS-SRS achieves the same spectral resolution quality in the high-frequency C–H region through wavelength sweeping of the pump laser across the designated region ($\sim 2825\text{--}3000\text{ cm}^{-1}$, Fig. 2 (C)), compared to that obtained by NIR-SRS and spontaneous Raman, indicating the well-maintained bond-selectivity and the pump laser tuning in VIS-SRS. As the cell-silent regime has been proven essential for performing specific dynamic metabolic imaging, we next moved to characterize 5-ethynyl-2'-deoxyuridine (EdU) solution with a sharp alkyne peak centered at 2125 cm^{-1} .²¹ Since EdU has a characteristic absorption peak at 288 nm (Fig. S1), we expect a slight pre-resonance enhancement in VIS-SRS. Indeed, we obtained a 7-fold total signal enhancement between VIS-SRS and NIR-SRS, confirming the presence of weak pre-resonance effects. Further spectral acquisition also confirms that VIS-SRS has comparable spectral resolution in the cell-silent window (Fig. 2 (D), FWHM of 21.4 cm^{-1} (VIS-SRS) vs 20.5 cm^{-1} (NIR-SRS), respectively).

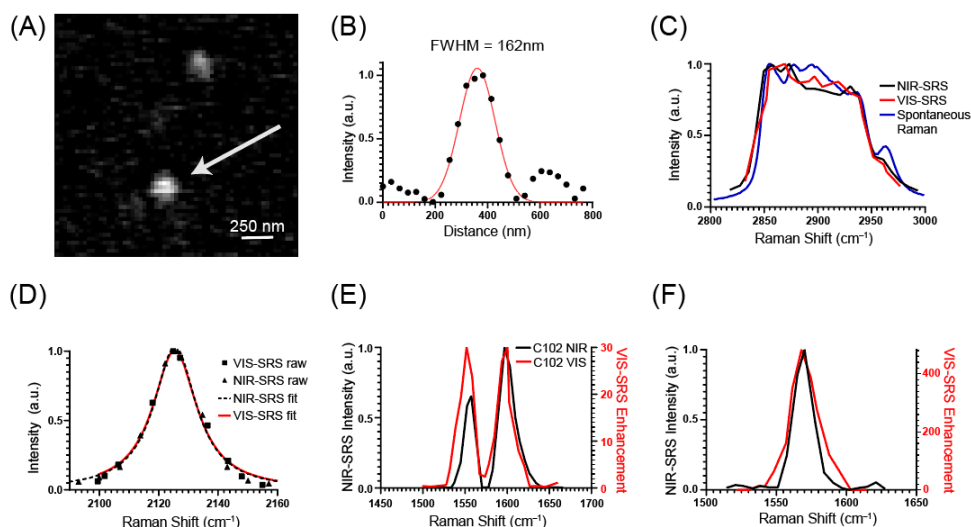


Fig 2. Characterizations of VIS-SRS microscope. (A) A representative VIS-SRS image of 100-nm PS beads. (B) Sectional profile obtained from the bead indicated by the white arrow in (A). A full-width-at-half-maximum (FWHM) of 162 nm is obtained through Gaussian fitting. (C) Raman spectra of the C-H stretching of n-decane measured by spontaneous Raman (blue), VIS-SRS (red), and NIR-SRS (black). (D) SRS spectra of EdU measured using VIS-SRS (squares) and NIR-SRS (triangles). Corresponding Lorentzian fittings are overlaid. (E-F) SRS spectra of Coumarin 102 (E) and 3-Ethynylperylene (F) measured from VIS-SRS (red) and NIR-SRS (black).

Finally, we screened candidates for pre-resonance and resonance imaging experiments by characterizing a series of commercially available dyes with varying absorption maxima. We identified two promising candidates in Coumarin 102 (Fig. S1, $\lambda_e = 396$ nm, $\lambda_{\text{pump}} = 476.3$ nm & 477.4 nm, Fig. 2 (E)), in the pre-resonance region and 3-Ethynylperylene (3-Perylene Fig. S1, $\lambda_e = 459$ nm, $\lambda_{\text{pump}} = 477.1$ nm, Fig. 2 (F)) in the rigorous resonance region. Despite

Coumarin 102 suffering from a large transient absorption (TA) background (Fig. S2), we obtained a 30-fold signal enhancement compared to NIR-SRS when probing the C=C double bond. Notably, the C=C double-bond from 3-Perylene yielded a 483-fold signal enhancement, due to more significant resonance enhancements, with only a minor and flat background (Fig. 2 (F)). However, the alkyne peak of 3-Perylene could not be resolved due to the high electronic background (Fig. S2, $\lambda_{\text{Pump}} = 465.2 \text{ nm}$), likely due to the increased proximity of the pump laser to the absorption maximum. For the importance of alkynes in Raman imaging in the cell silent-region, we further investigated the pre-resonance enhancement for alkynes in VIS-SRS systematically. We synthesized and measured a series of polyynes^{22,23} molecules that absorb in the UV-vis region with varying degrees of resonance enhancement (Table S1). We note that lowered signals for 3- and 4- yne were due to induced higher background.

Bio-imaging using VIS-SRS

We next characterized the biocompatibility of our VIS-SRS system. We first imaged fixed HeLa cells in the high-frequency C–H region targeted at the protein channel (2940 cm^{-1}) (Fig. 3 (A)) and achieved similar performance with enhanced spatial resolution to visualize the cellular network to a previous report.¹¹⁻¹³ Through direct comparison to NIR-SRS, we obtained a 7-fold signal enhancement in the cytoplasm of HeLa cells. The slightly larger enhancement in HeLa cells compared to that of n-decane solution also indicates the presence of pre-resonant effects likely arising from the absorption band around 280 nm from proteins in the cytoplasm.¹¹ We also demonstrated that more clinically relevant samples, such as neuronal cells, benefited from this same resonant enhancement and could be imaged with

strong image fidelity and signal strength (Fig. 3 (B)). We further confirmed that our VIS-SRS system is live-cell compatible by imaging HeLa cells (Fig. 3 (C)) targeted at the CH_3 channel (2940 cm^{-1}) with similar image qualities to NIR-SRS (Fig. 3 (D)). We note, however, live cells showed much-reduced photon tolerance for picosecond visible photons which originates from the higher order absorption induced photo-damage, preventing long-term imaging applications (Table S3).

As rationalized above, we seek to demonstrate the flexibility and wide tunability of our system by performing two-channel imaging of the same cells in the cell-silent and high-frequency C–H regions. We imaged the protein channel (CH_3 , 2940 cm^{-1} , Fig. 3 (E)) mapping the endogenous proteins in HeLa cells and the cell silent region (C–D, 2250 cm^{-1} , Fig. 3 (F)) where incubation with deuterated arachidonic acid ($\text{d}_8\text{-AA}$) results in the accumulation of the polyunsaturated fatty acid (PUFA) within the lipid droplets.²⁴ This set of images, including an off-resonance image at 2700 cm^{-1} for background subtraction, was captured within five minutes, a time frame compatible with many live-cell imaging tasks. We note that while spatial realignment is needed to ensure optimal beam overlap after tuning, our imaging speed is ultimately limited by laser tuning (10-15 seconds for spatial realignment, and 60-90 seconds for laser tuning). Nonetheless, this imaging speed paves the way for metabolic imaging of UV-VIS absorbing target molecules, such as drugs, that are below the detection threshold in NIR-SRS.

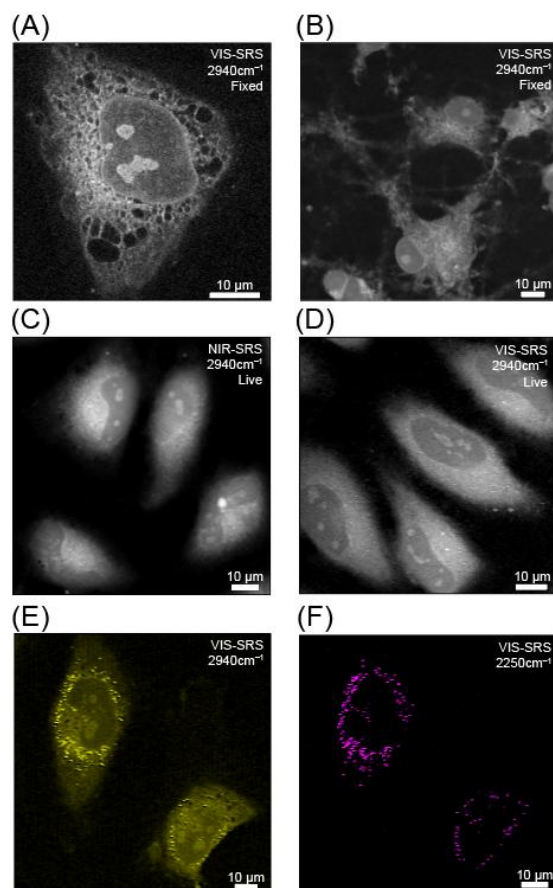


Fig. 3. VIS-SRS imaging of biological samples. (A-B) Protein distribution of a fixed HeLa cell (A) and neurons (B) with fine structural details targeting 2940 cm⁻¹. (C-D) Protein distribution in live HeLa cells by NIR-SRS (C) and VIS-SRS (D). (E-F) Two-channel imaging of HeLa cells under the same field-of-view showing protein distributions (yellow, E, 2940 cm⁻¹) and lipid droplets formed containing d₈-AA in C-D channel (magenta, F, 2250 cm⁻¹).

Mitigating Photodamage, and Tissue Imaging using VIS-SRS

The bluer wavelengths after frequency doubling increase photodamage and must be carefully considered in VIS-SRS for biological samples.¹³ Symptoms of photodamage we observed during cell imaging include nuclei darkening, nucleoli shrinking, and cytoplasm bubbling and burning. One way to mitigate the damage is to use longer pulses. Fujita et al. demonstrated that stretching 2 ps pulses to 7 ps and 14 ps greatly reduced photodamage.²⁵ However, this strategy may suffer from insufficient laser power for high-quality VIS-SRS cell imaging applications. An alternative strategy to circumvent this limitation is to explore sample preparation techniques, such as pre-emptive cooling,²⁶ sample dilution, or homogenization.¹⁷

Therefore, we coupled sample expansion with VIS-SRS as a strategy to mitigate photodamage. We theorized that expanding samples isotropically would decrease sample density and homogenize sample refractive index to minimize local heat generation and facilitate heat dissipation. At the same time, the expanded sample coupled with VIS-SRS should render unprecedented super-resolution imaging capabilities for chemical imaging and allow deep-tissue imaging with optically cleared tissues. Consequently, we employ our previously developed VISTA framework¹⁷ to expand 5xFAD brain tissues. Figure 4 (A) shows a fixed 5xFAD tissue brain image before the expansion by VIS-SRS, with a protein and a lipid rich amyloid-beta aggregate region. After a 3.4-fold expansion, the reduced protein density with the removal of lipids indeed significantly raised the photodamage threshold, allowing us to fully capture an aggregate region (Fig. 4 (B)) in 3D under dramatically higher power conditions compared to cells and unmodified tissues (Table S4). We measured the cross section of a tissue structure (Fig. 4 (C)) and fitted it to the gaussian

profile with a FWHM of 160 nm (Fig. 4 (D)), resulting in the effective spatial resolution of 47 nm after accounting for 3.4-fold sample expansion.

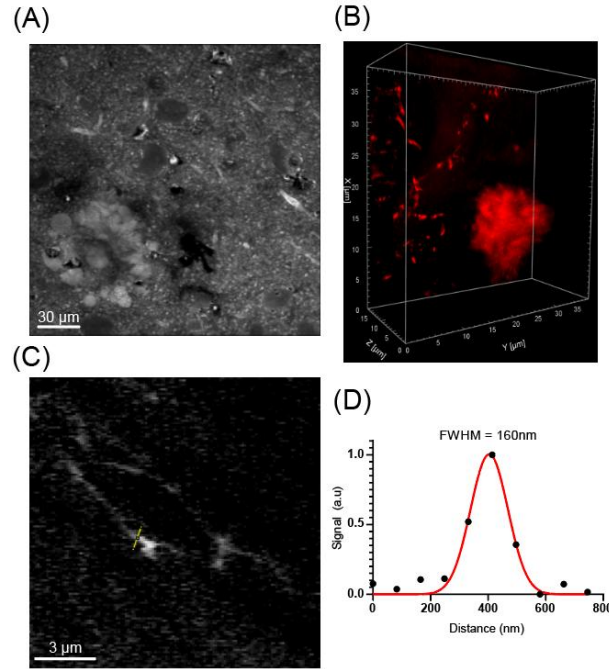


Fig. 4. VIS-SRS imaging on isotropically expanded brain tissues. (A) VIS-SRS image of a 5xFAD mouse brain tissue before sample expansion under 2940 cm^{-1} . (B) 3D VIS-SRS image (2940 cm^{-1}) of 5xFAD mouse brain tissue after 3.4-fold isotropic expansion. (C) A XY-slice of 3.4-fold expanded single frame of a tissue structure in 5xFAD brain tissue. (D) Spatial analysis of the yellow-line highlighted structure from (C).

2.5 Conclusion

In this work, we developed a widely tunable VIS-SRS system based on the frequency doubling directly from a picosecond output laser, which is capable of rapid multi-channel imaging across different bond regions, enabling various biological imaging applications.

Using the picosecond VIS-SRS system, we achieved significant resonance enhancements in Coumarin 102 and 3-Ethynylperylene, highlighting potential targets for pre-resonance enhanced SRS imaging in future applications. We imaged HeLa cells across the high-frequency C–H stretching region and cell-silent region in a timely manner compatible with live-cell investigations. We discussed the destructive nature of photodamage and provided a solution based on the sample expansion, enabling label-free imaging of brain tissues in 3D that achieves an effective spatial resolution of 47 nm.

Compared to spectral-focusing VIS-SRS with fs laser sources, we note that our VIS-SRS has pros and cons, depending on the specific applications. While our VIS-SRS provides excellent multi-channel imaging across a wide range for live-cell metabolic studies, its tuning is slower if the bonds of interest are limited to the bandwidth of fs pulses ($\sim 200 \text{ cm}^{-1}$). Due to the slight wavelength dependence of spatial walk-off in the BIBO crystal, quick optimization of the laser spatial overlap is still necessary after tuning shifts of $7\text{--}10 \text{ cm}^{-1}$. However, with the aid of the diagnostic light path described earlier (Fig. 1 (B), dashed-lined box), realignment can be rapidly performed. Using this approach, two-channel imaging including an off-resonance image were acquired within 5 minutes, with laser tuning as the primary time bottleneck. Implementing a fast feedback loop involving motorized or piezo stage that automatically corrects spatial walk-offs should further speed up the spectral acquisition and make the system more user friendly.

Despite its increased photodamage compared to NIR-SRS, VIS-SRS still offers unique opportunities worth exploring. Most commercial drugs at their physiological concentrations

cannot be detected by NIR-SRS due to the small Raman cross sections without pre-resonance enhancement. However, many drugs absorb in the UV-VIS region and frequently contain biorthogonal alkyne or nitrile moieties, making them promising imaging targets in VIS-SRS. With continuous improvements to this technique and advancements in statistical models predicting resonance dynamics,²⁷ we envision VIS-SRS should find utility in the field of drug development and imaging.

2.6 Supplementary Information

1. Calculating VIS-SRS Enhancement

When performing signal comparisons, the SRL expression is written as:

$$SRL \propto -N \cdot \sigma_{Raman} \cdot \Phi_P \cdot \Phi_S \cdot QE$$

Where σ_{Raman} in the non-resonant case is simplified to:

$$\sigma_{Raman} = K \cdot \frac{1}{\lambda_P} \cdot \frac{1}{\lambda_S^3}$$

N is the number of bonds in the focal volume, σ_{Raman} is the Raman cross-section, K is a sample dependent constant, Φ_P/Φ_S are the pump/stokes photon flux, and QE is the photodiode quantum efficiency.

Our non-resonance term Enhancement Factor (EF) is calculated as:

$$EF = \frac{SRL_{\text{VIS-SRS}}}{SRL_{\text{NIR-SRS}}}$$

Under the same concentration (C), focal volume (V) describes N by the relation $N = C \cdot V$.

$$V_{\text{ellipsoid}} = \frac{4}{3} \pi a b l$$

$$\text{Lateral: } a = b = 0.5 \cdot \left(\frac{\lambda}{2NA} \right) = \frac{\lambda}{4NA}$$

$$\text{Axial: } l = 0.5 \cdot \left(\frac{2\lambda}{NA^2} \right) = \frac{\lambda}{NA^2}$$

$$V_{\text{ellipsoid}} = \frac{4}{3} \pi \cdot \left(\frac{\lambda}{4NA} \right)^2 \cdot \left(\frac{\lambda}{NA^2} \right) = \frac{\pi}{12} \cdot \left(\frac{\lambda^3}{NA^4} \right)$$

For multiphoton processes: $\text{PSF} = \text{PSF}_1 \cdot \text{PSF}_2$

$$\text{FWHM}_{\text{PSF}} = \frac{\lambda_p \lambda_s}{2NA \sqrt{\lambda_p^2 + \lambda_s^2}}$$

$$V_{\text{SRS}} = \frac{\pi}{12} \cdot \frac{\left(\frac{\lambda_p \lambda_s}{\sqrt{\lambda_p^2 + \lambda_s^2}} \right)^3}{NA^4}$$

The flux of our pump & Stokes beams (Φ_p & Φ_s) per pulse can be calculated from:

$$I = \frac{P}{A} = \frac{E_{\text{pulse}} \cdot N}{A} = \left\{ \frac{N}{A} \right\} \cdot \left(\frac{hc}{\lambda} \right) = \{\Phi\} \cdot \left(\frac{hc}{\lambda} \right)$$

$$\Phi = \frac{N}{A} = \frac{\lambda}{A} \cdot \left(\frac{P}{hc} \right)$$

$$A_P = \pi r^2 = \pi \cdot \left(\frac{\lambda_P}{4NA} \right)^2$$

$$\Phi_P = \frac{\lambda_P}{\pi \left(\frac{\lambda_P}{4NA} \right)^2} \cdot \frac{P}{hc} = \frac{NA^2}{\lambda_P} \cdot \left(\frac{16P_P}{\pi hc} \right)$$

$$\text{Similarly: } \Phi_S = \frac{NA^2}{\lambda_S} \cdot \left(\frac{16P_S}{\pi hc} \right)$$

$$SRL \propto V \cdot \sigma_{\text{Raman}} \cdot \Phi_{P,SRS} \cdot \Phi_{S,SRS} \cdot Q$$

$$SRL \propto \left[\frac{\pi}{12} \frac{\left(\frac{\lambda_P \lambda_S}{\sqrt{\lambda_P^2 + \lambda_S^2}} \right)^3}{NA^4} \right] \cdot \left[\frac{1}{\lambda_P \lambda_S^3} \right] \cdot \left[\frac{NA^2}{\lambda_P} \cdot \left(\frac{16\pi P_P}{\pi hc} \right) \right] \cdot \left[\frac{NA^2}{\lambda_S} \cdot \left(\frac{16\pi P_S}{\pi hc} \right) \right] \cdot [QE]$$

$$SRL \propto \frac{64}{3\pi h^2 c^2} \left(\frac{\lambda_P^3 \lambda_S^3}{[\lambda_P^2 + \lambda_S^2]^{3/2}} \cdot \frac{1}{\lambda_P \lambda_S^3} \cdot \frac{P_P}{\lambda_P} \cdot \frac{P_S}{\lambda_S} \right) \cdot QE$$

Finally:

$$SRL \propto \frac{64}{3\pi h^2 c^2} \left(\frac{\lambda_P \cdot P_P \cdot P_S}{\lambda_S \cdot [\lambda_P^2 + \lambda_S^2]^{\frac{3}{2}}} \right) QE$$

SRS enhancement under the same NIR and VIS laser powers is calculated by:

$$\begin{aligned}
 EF = \frac{SRL_{VIS}}{SRL_{NIR}} &= \frac{\frac{64}{3\pi\hbar^2c^2} \left(\frac{\lambda_{P,VIS} \cdot P_{P,VIS} \cdot P_{S,VIS}}{\lambda_{S,VIS} \cdot [\lambda_{P,VIS}^2 + \lambda_{S,VIS}^2]^{\frac{3}{2}}} \right) QE_{VIS}}{\frac{64}{3\pi\hbar^2c^2} \left(\frac{\lambda_{P,NIR} \cdot P_{P,NIR} \cdot P_{S,NIR}}{\lambda_{S,NIR} \cdot [\lambda_{P,NIR}^2 + \lambda_{S,NIR}^2]^{\frac{3}{2}}} \right) QE_{NIR}} \\
 &= \frac{\lambda_{P,VIS} \lambda_{S,NIR} QE_{VIS} [\lambda_{P,NIR}^2 + \lambda_{S,NIR}^2]^{\frac{3}{2}}}{\lambda_{P,NIR} \lambda_{S,VIS} QE_{NIR} [\lambda_{P,VIS}^2 + \lambda_{S,VIS}^2]^{\frac{3}{2}}}
 \end{aligned}$$

When probing the 2940 cm^{-1} stretch, $\lambda_{P,NIR} = 791.3 \text{ nm}$, $\lambda_{S,NIR} = 1031.2 \text{ nm}$, $\lambda_{P,VIS} = 447.7 \text{ nm}$, $\lambda_{S,VIS} = 515.6 \text{ nm}$. From the manufacturer's photosensitivity curves, $QE_{VIS} = 98\%$ (S3590-19, Hamamatsu) and $QE_{NIR} = 91\%$ (S3590-09, Hamamatsu).

Therefore:

$$\text{Signal EF} = 8.40$$

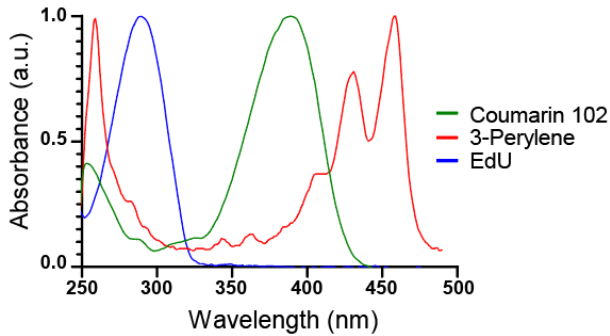


Fig. S1: Normalized absorption spectrum for Coumarin 102, 3-Ethynylperylene (3-Perylene) and 5-ethynyl-2'-deoxyuridine (EdU) measured in DMSO.

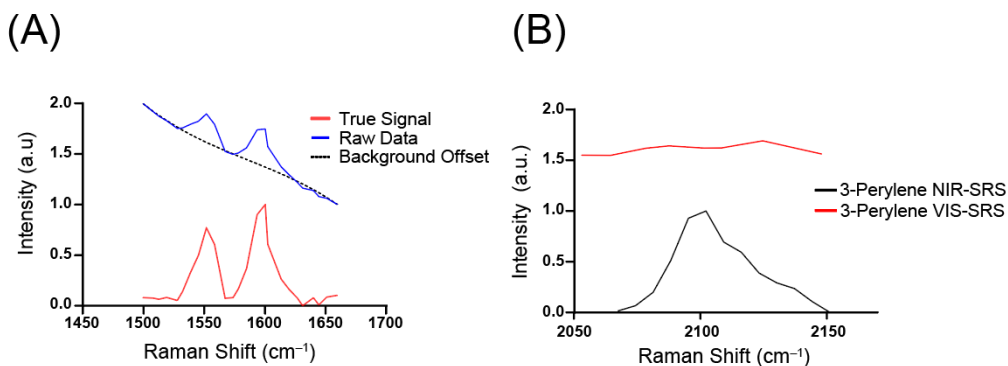


Fig. S2: VIS-SRS spectra of double-bond of Coumarin 102 and triple-bond of 3-Ethynylperylene. (A) Raw Coumarin 102 VIS-SRS spectra (blue) and flat-background subtracted spectra (red) in the fingerprint region. (B) 3-Ethynylperylene alkyne peak investigated by the VIS-SRS (red) compared to NIR-SRS (black) in the cell-silent region.

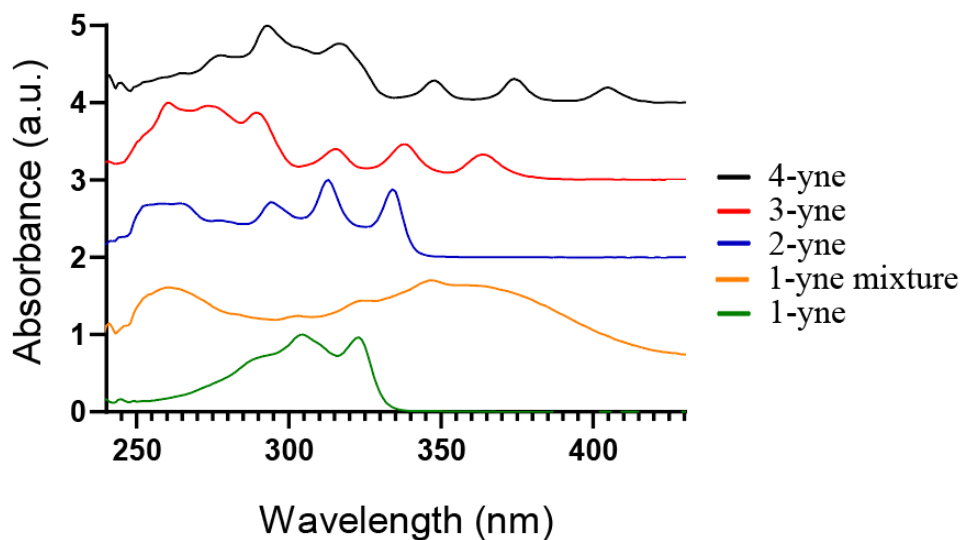


Fig. S3: Absorption spectrum for polyynes measured in DMSO.

Table S1: Characterization of polyene molecules

Material	Structure	Abs. Max	Enhancement
1-yne		325	4.0
1-yne mixture	Major 	345	12.0
	Minor 		
2-yne		335	6.5
3-yne		365	0.3
4-yne		405	5.5

Table S2: SRS Imaging Parameters for all measurements for Fig.2

Imaging Parameters	2.A	2.C	2.D	2.E	2.F
NIR, pump/Stokes (mW)	—	28/23	42/123	42/140	42/140
VIS, pump/Stokes (mW)	3/3	12/16	12/14	8/14	3/4
Pixel number	512/512	256x256	256x256	256x256	256x256
Pixel size (nm/pixel)	31.88	1998 NIR 828 VIS	1998 NIR 828 VIS	1998 NIR 828 VIS	1998 NIR 828 VIS

Pixel dwell time (μ s)	40	2	2	2	2
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Table S3: SRS Imaging Parameters for all measurements for Fig.3

Imaging Parameters	3.A	3.B	3.C	3.D	3.E	3.F
NIR, pump/Stokes (mW)	—	—	42/32	—	—	—
VIS, pump/Stokes (mW)	5/10	9/10	—	6/10	5/11	5/11
Pixel number	512x512	512x512	512x512	512x512	512x512	512x512
Pixel size (nm/pixel)	101	165	397	414	414	414
Pixel dwell time (μ s)	40	40	40	40	40	40

Table S4: SRS Imaging Parameters for all measurements for Fig.4

Imaging Parameters	4.A	4.B	4.C
NIR, pump/Stokes (mW)	—	—	—
VIS, pump/Stokes (mW)	6/13	13/42	12/22
Pixel number	512x512	512x512	512x512
Pixel size (nm/pixel)	165	165	83
Pixel dwell time (μ s)	40	40	40

2.7 References

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METABORAMICS: HIGHLY MULTIPLEXED METABOLIC IMAGING USING STIMULATED RAMAN MICROSCOPY FOR LIVE-CELL SPATIAL METABOLOMICS

3.1 Summary

Cellular metabolism is highly dynamic, intertwined and spatially heterogeneous, yet methods that can simultaneously visualize multiple metabolic pathways in living systems remain limited. Here, we present MetaboRamics— highly multiplexed metabolic imaging using stimulated Raman scattering (SRS) microscopy for live-cell spatial metabolomics. Through rational probe selection and careful optimization, isotope editing, and robust spectral unmixing, we establish a metabolic palette of 16-colors spanning glucose uptake and utilization, lipid uptake and synthesis, choline metabolism, DNA synthesis, and amino acid incorporation in addition to endogenous proteins, lipids and redox signals. Incorporation of organelle-targeted Raman probes further enables spatial interactomics and assessment of organelle activities. Applying MetaboRamics to epithelial-to-mesenchymal transition (EMT), we observe metabolic rewiring in mesenchymal cells, reflected by reduced glucose-derived biomass, lipid turnover, protein synthesis, and altered redox balance. Finally, we perform 16-plex metabolic phenotyping of cellular states under metabolic stress of serum deprivation, nutrient overload (fructose, and saturated fatty acid), inflammation, and pharmacological perturbations to reveal subcellular metabolic changes. This work fully realizes the potential of SRS for super-multiplexed metabolic imaging by establishing, for the first time, a 16-plex platform for live-cell spatial metabolomics.

3.2 Introduction

Metabolism is an inherently dynamic, spatially organized network of chemical reactions that enables essential cellular functions. Changes in metabolic flux often initiate or accompany disease progression, making metabolism a critical lens for investigating disease. Recent advances in high-throughput genomics,^{1,2} transcriptomics^{3,4} including spatial transcriptomics,⁵⁻⁷ and proteomics⁸⁻¹⁰ have transformed our understanding of cellular states by allowing comprehensive, scalable, and increasingly spatially resolved measurements. These advances are largely driven by sequencing-based technologies that leverage molecular amplification and the stability of linear biopolymers such as DNA and RNA. In contrast, metabolites cannot be amplified, are difficult to chemically fix, and exhibit extensive chemical diversity, lability, low abundance, and broad dynamic range.¹¹ Consequently, spatial metabolomics remains a grand challenge, and approaches that can directly visualize multiple metabolic activities in living cells are still highly limited, despite the central role of metabolism in health and disease.

Recently, mass spectrometry imaging (MSI), leveraging matrix-assisted laser desorption/ionization (MALDI),^{12,13} secondary ion mass spectrometry (SIMS),¹⁴ and desorption electrospray ionization (DESI),¹⁵ have significantly expanded the capability for in situ spatial metabolomics. Imaging mass cytometry has also been demonstrated to perform untargeted spatial metabolomics and targeted multiplexed protein imaging using a single workflow.¹⁶ However, despite achieving excellent molecular sensitivity and specificity, these techniques are inherently destructive and thus incompatible with live-cell subcellular

analysis. Moreover, additional sample processing requirements and variable ionization efficiencies pose additional challenges for throughput and quantitative accuracy. Alternatively, fluorescence microscopy enables ultrasensitive live-cell analysis, but relies on bulky fluorophores or genetically encoded reporters that can perturb native biology, are substantially larger than the metabolites of interest, and possess inherently broad spectra, thereby limiting multiplexing for metabolomic applications.¹⁷

To address these limitations, vibrational imaging, particularly Raman microscopy, offers a promising avenue toward live-cell spatial metabolomics. By probing specific vibrational motions of chemical bonds, Raman scattering provides rich chemical information and enables direct fingerprinting of endogenous metabolites. Through molecular fingerprinting and advanced profiling algorithms, label-free strategies have recently enabled multiplexed analysis in live cells and tissues, with demonstrated correlations to functional transcriptomic readouts termed “RamanOmics.”^{18,19} However, label-free approaches alone are largely limited to static profiling, lacking direct access to metabolic dynamics and offering restricted molecular coverage (e.g., proteins, saturated and unsaturated lipids) due to spectral overlap and background interference.

Recent advances have demonstrated that the integration of small biorthogonal tags (e.g., alkynes, deuterium, nitriles that reside in the ideal cell-silent spectral region) with stimulated Raman scattering (SRS) microscopy provides a powerful strategy to overcome these limitations, enabling minimally perturbative quantitative imaging of metabolic dynamics in live cells with target specificity, subcellular spatial resolution, and high temporal fidelity

(Fig. 1a).^{20,21} Indeed, metabolic activity phenotyping using several of these bioorthogonal probes has demonstrated much-improved separation and sensitivity compared to purely label-free imaging.²² Although the intrinsically narrow Raman linewidths (~ 10 cm^{-1}) have enabled super-multiplex Raman imaging (up to ~ 14 channels) through the use of conjugation-based strategies, these probes are typically bulky and are incompatible with labeling small metabolites without perturbing their native functions for imaging and profiling.²³ Therefore, the accessible Raman metabolic palette remains yet to be fully exploited.

Here, we report a robust analytical platform, termed MetaboRamics, for highly multiplexed metabolic imaging using stimulated Raman scattering microscopy for live-cell spatial metabolomics (Fig. 1). We achieve this through rational probe screening and careful selection to ensure high spectral separation, in combination with isotope editing strategies and robust unmixing algorithms. Together, these advances substantially expand the accessible Raman metabolic palette while maintaining minimal perturbation to native cellular processes. We demonstrate a greatly expanded and minimally invasive metabolic palette for 16-plex MetaboRamics in live cells, integrating nine bioorthogonal channels targeting key metabolic pathways- including sugars, choline, DNA, proteins, lipids, and organelle markers- alongside five label-free channels and two autofluorescence channels from two-photon excited fluorescence (TPEF) microscopy within a unified imaging platform (Fig. 1a-b). To fully leverage this multiplexing capability, we further establish a quantitative analysis pipeline with subcellular segmentation, enabling spatially resolved profiling of metabolic states (Fig. 1c).

Following systematic optimization and validation, we apply this framework to probe the metabolic basis of epithelial-mesenchymal transition (EMT), a process that remains incompletely understood. Our multi-dimensional analyses reveal system-wide metabolic remodeling where mesenchymal cells display a globally attenuated metabolic state relative to their epithelial counterparts. We further perform subcellular metabolic profiling under diverse metabolic stressors that mimic pathological conditions, including inflammation, serum deprivation, nutrient overload (e.g., high fructose and saturated fatty acids), and pharmacological treatments to understand their broad effects on metabolic activity. MetaboRamics resolves stressor-specific metabolic rewiring across multiple pathways, revealing both shared and distinct cellular response programs. Collectively, MetaboRamics provides a versatile platform for high-resolution, super-multiplexed imaging of live-cell spatiotemporal metabolomics.

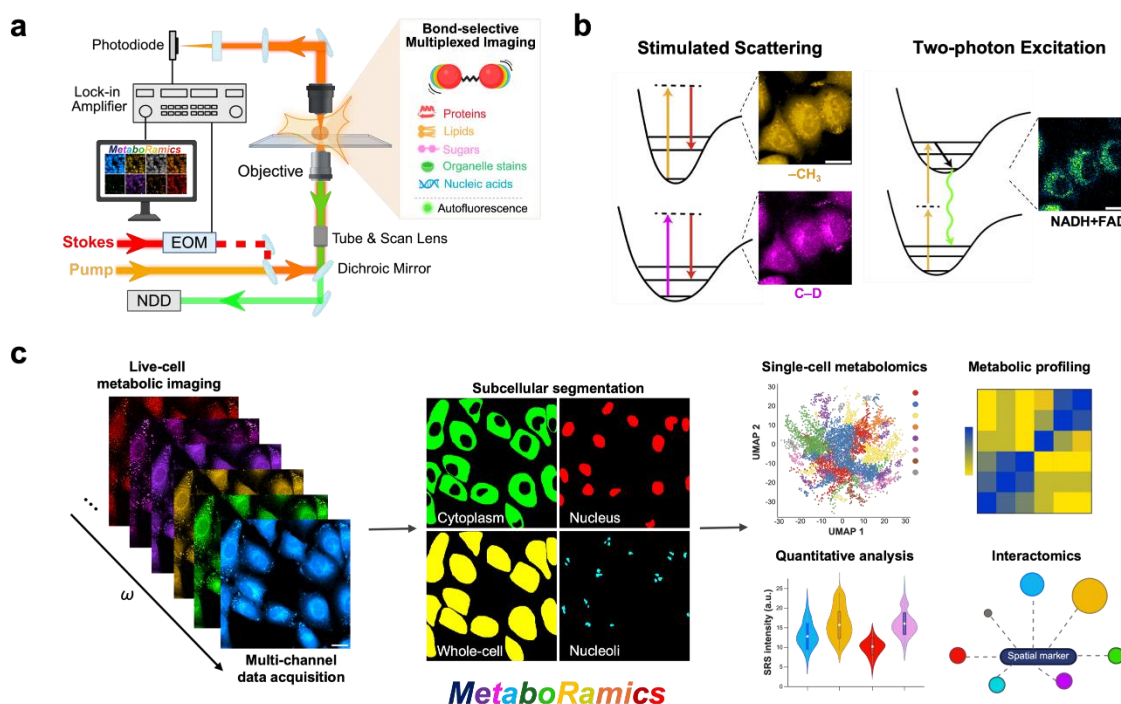


Figure 1. MetaboRamics workflow. (a) SRS microscopy setup for bond-selective multiplexed imaging. EOM = electro-optic modulator, NDD= non-descanned detector; (b) Energy diagram for stimulated Raman scattering (left) and two-photon excitation (right); (c) Workflow for spatial metabolomics using MetaboRamics.

3.3 Results

Design, Rationale, and Implementation of Live-Cell MetaboRamics Platform

An effective targeted metabolic palette should fulfill the following criteria. First, given the complexity and interconnectedness of metabolic pathways, the palette must encompass a broad range of metabolic species while remaining biocompatible, minimally toxic, cost-effective, and robust for spatial metabolomics. Second, these metabolic analogs should have sufficient uptake and/or incorporation into targeted metabolic pathways with high signal-to-

noise ratios (SNR). Third, they should maintain spectral resolvability after incorporation into metabolic pathways. Although the cell-silent region is relatively wide, commonly used vibrational tags (i.e., C–D, alkyne, nitrile) are largely confined to a narrow window ($\sim 2000\text{--}2300\text{ cm}^{-1}$). In particular, highly deuterated probes (e.g., d₇-Glc) exhibit broadened linewidths that fully inundate this region and increase spectral unmixing complexity.

Based on these criteria, we first screened a large set of analogs and isotopologues of glucose, amino acids, lipids and their precursors (Fig. S1), yielding a metabolic palette that spans multiple metabolic pathways (Fig. 2a-b). Our palette exhibited largely resolvable peaks after metabolic incorporation while retaining high SNR, enabling robust linear unmixing used to deconvolve overlapping channels for downstream quantification (Fig. 2b). Specifically, we used 5-ethynyl-2'-deoxyuridine (EdU, 2118 cm^{-1}), a thymidine analogue, to map de novo synthesis of DNA,²⁴ and propargylcholine (PGC, 2142 cm^{-1}) for choline metabolism into cellular phospholipids.²⁵ We further adopted a monitoring strategy for glucose uptake and metabolism, by synthesizing an isotopologue of an alkyne-tagged glucose analogue, ¹³C₃-3-OPG (2054 cm^{-1} , Fig. 2a-b, purple),²⁶ to primarily visualize glucose uptake, while in parallel using d₇-glucose (d₇-Glc) to track its metabolic incorporation into downstream biomass, including lipids (d₇-Glc_L, 2150 cm^{-1}) through de novo lipogenesis (DNL), and proteins (d₇-Glc_P, 2190 cm^{-1}).

Extending into the unoccupied cell-silent spectral region, we incorporated key spatial organelle markers, including Me-Lyso (ML, 2205 cm^{-1}) to label lysosome²⁷ and Mito-BADY (MB, bearing a conjugated diyne, 2220 cm^{-1}) as a mitochondria tracker,²⁸ that enabled us to assess both organelle dynamics and metabolite-organelle interactomics using our platform. To complement the DNL readout from $\text{d}_7\text{-Glc}$, we selected $\text{d}_8\text{-arachidonic acid}$ ($\text{d}_8\text{-AA}$, 2250 cm^{-1}) to assess the uptake of polyunsaturated fatty acid (PUFA). We note that $\text{d}_8\text{-AA}$ was used at low concentrations throughout, as higher concentrations promotes lipid droplet formation, complicating multi-channel analysis due to increased cross-phase modulation (Fig. S2). Lastly, we employed $\text{d}_5\text{-phenylalanine}$ ($\text{d}_5\text{-Phe}$, $\text{C}_{\text{sp}2}\text{-D}$, 2298 cm^{-1} , Fig. 2b, red), an essential amino acid positioned outside congested spectral region, to visualize nascent protein synthesis. To further expand the palette into less crowded spectral windows, a potentially viable design is to exploit deuterated terminal alkynes ($\text{C}\equiv\text{C-D}$), which exhibits a peak at $\sim 1985\text{ cm}^{-1}$.²⁹ However, although we synthesized 17-D-ODYA to probe SFA uptake, we observed rapid hydrogen-to-deuterium exchange (HDX), resulting in conversion back to protonated alkyne ($\text{C}\equiv\text{C-H}$, 2120 cm^{-1}) in cells, rendering it unsuitable for this application (Fig. S3).

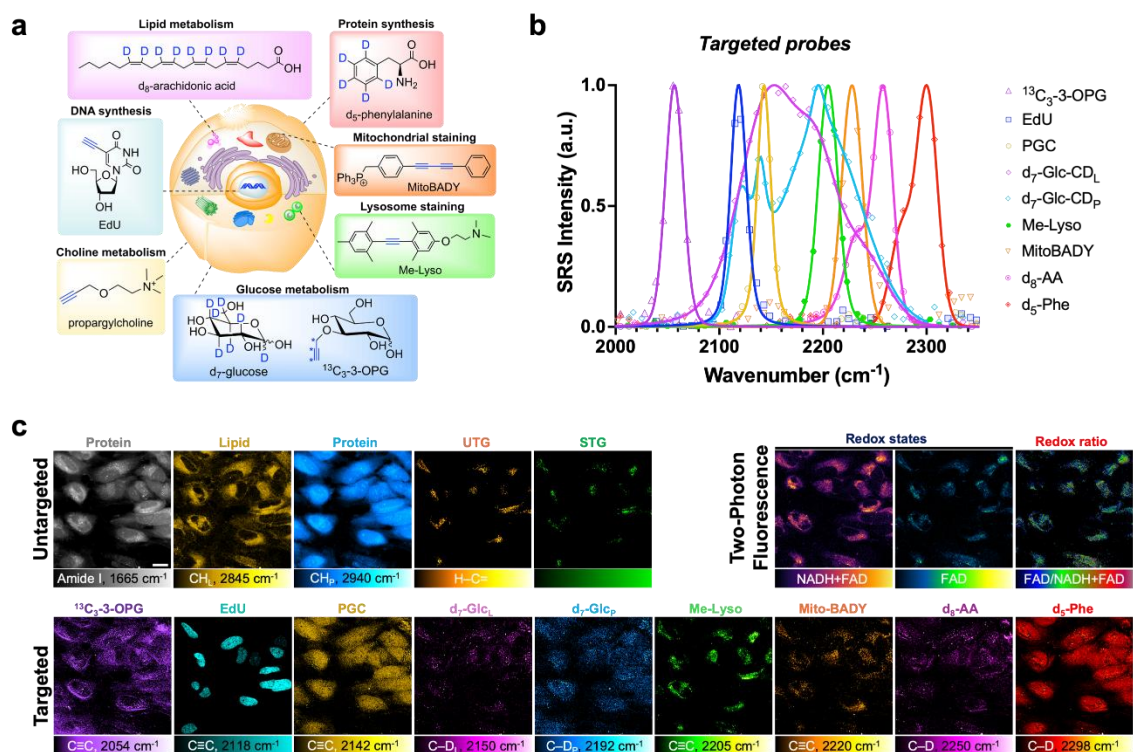


Figure 2. 16-plex metabolic imaging using MetaboRamics in live cells. (a) Metabolic probe palette encompassing a broad range of metabolites for targeted SRS imaging. **(b)** SRS spectra of incorporated Raman probes from live cells; **(c)** Representative 16-plex metabolic images from live HeLa cells using MetaboRamics. Scale bar: 20 μm .

With these nine-color targeted probes enabling the simultaneous interrogation of metabolic dynamics (Fig. 2c, bottom), we next integrated them with established label-free channels, including amide I ($\text{C}=\text{O}$ stretching of peptide backbone, 1665 cm^{-1}), lipids ($-\text{CH}_2$, 2845 cm^{-1}), proteins ($-\text{CH}_3$, 2940 cm^{-1}) and unsaturated lipids ($=\text{C}-\text{H}$, 3022 cm^{-1}). Here, saturated triglycerides (STG) in lipid droplets (LDs) were quantified using a previously established approach, in which unsaturated triglycerides (UTG) are subtracted from the total lipid pool

(CH₂) to yield STGs (Fig. S4), hence providing five label-free channels (Fig. 2c, top left).³⁰ In parallel, label-free TPEF microscopy was employed to optically read out the cellular redox state by capturing endogenous autofluorescence from the redox coenzymes NADH and FAD, using the SRS pump laser for excitation (NADH is excited only at 760 nm; whereas FAD is excited at both 760 nm and 880 nm) and with non-descanned detectors employed for signal collection (Fig. 2c, top right). The resulting NADH (reduced) to FAD (oxidized) ratio serves as a readout of mitochondrial activity, where both markers tend to localize.

With this framework, MetaboRamics enables, for the first time, rapid, high-throughput 16-plex imaging in live HeLa cells (Fig. 2c, Fig. S5). Each high-resolution image set was acquired within ~25 minutes, primarily limited by pump-laser tuning, while inducing negligible photodamage. Control comparison between spectrally unmixed 16-plex images with corresponding single-channel SRS images, in which each probe was administered individually (Fig. S6), confirm faithful preservation of spatial features, demonstrating minimal probe-associated interference and toxicity.

MetaboRamics Reveals Metabolic Reprogramming in EMT

With our ability to perform 16-plex metabolic imaging, we next applied MetaboRamics to investigate the metabolic changes underlying EMT that remain incompletely characterized. EMT is a process by which epithelial (epi) cells become de-differentiated into mesenchymal (mes) cells, losing cell-to-cell adhesion and polarity while becoming more migratory in nature.³¹ Though EMT has been linked with essential processes such as embryonic

development and wound healing, it has also been reported to progress pathologic responses like cancer metastasis.³² However, most studies linking EMT with altered metabolism rely on bulk measurements or invasive methods that do not capture metabolic intracellular heterogeneity in living systems.³³ Recent studies using optical imaging of live cells have provided insights into metabolic changes associated with EMT.^{34,35} However, a more holistic characterization of the metabolic landscape of EMT in situ still remains lacking.

To model EMT, we first induced EMT in A549 epi cells via a commercial cocktail of proteins and antibodies. We successfully performed high-quality, 16-color MetaboRamics imaging on both epithelial and mesenchymal phenotypes (Fig. 3a) and captured clear morphological transition in the label-free protein imaging (CH_2 , 2940 cm^{-1}), where epi cells exhibited a cobble-stone morphology, while mes cells became elongated with increased fenestrations (Fig. 3a). To quantitatively visualize any present metabolic changes, we next constructed a population-averaged heatmap, plotting the log2fold-change ($\log_2\text{FC}$) at the whole cell and subcellular level (segmented by cytoplasm, nuclei, and nucleoli) (Fig. 3b), standardized against A549 epi (control) cells. We found that EMT showed a global reduction by ~30% across all probed metabolic pathways in, consistent with our prior findings on TCA-linked metabolism.³⁵ (Fig. 3b).

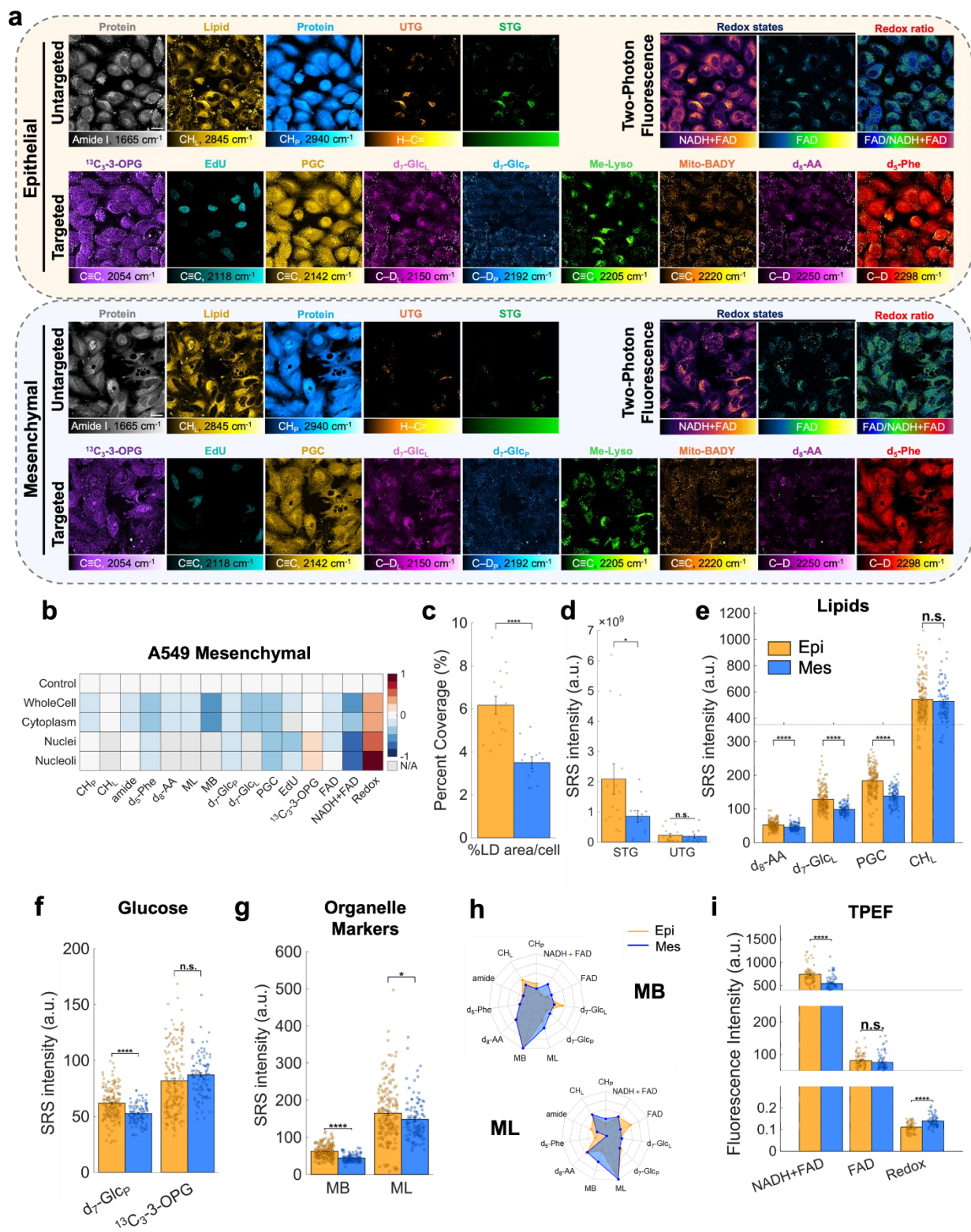


Figure 3. MetaboRamics Reveals Metabolic Reprogramming in EMT. **(a)** Representative 16-plex metabolic images using MetaboRamics in live A549 epithelial (top) and mesenchymal (bottom) cells. Scale bar: 20 μm ; **(b)** Heatmap depicting $\log_2$ fold changes between epithelial and mesenchymal cells profiled by MetaboRamics at the subcellular level (whole-cell, cytoplasm, nuclei and nucleoli). The control comprises of epithelial cells; **(c)** Percent area coverage of LDs per field-of-view (FOV) in EMT; **(d)** Quantification of SRS signal (intensity $\times$ area) per FOV of saturated triglycerides (STG) and unsaturated triglycerides (UTG); **(e)** Quantification of SRS intensity per cell for CH_L (endogenous total lipids), d₈-AA (lipid uptake), d₇-Glc_L (*de novo* lipogenesis) and PGC (choline metabolism) for EMT; **(f)** Subcellular quantification of SRS intensity per cell for d₇-Glc_P (protein synthesis) across the whole-cell, cytoplasm, nuclei and nucleoli; **(g)** Quantification of SRS signal per cell for organelle markers: MB and ML; **(h)** Radial plots for MB (top) and ML (bottom) depicting pairwise interaction networks across channels; **(i)** Quantification of TPEF signal per cell from NADH + FAD, FAD, and redox ratio. Data represented as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was conducted using Welch's two-paired t-test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ and n.s.= no statistical difference.

Surprisingly, label-free lipid channel images (CH_L; Fig. 3a,c and Fig. S7) revealed a marked decrease ($\sim 30\%$) in the number of LDs in the mes phenotype. This trend contrasts with previous observations in MCF7 cells, where mes cells exhibited increased LD abundance relative to their epi counterparts,³⁴ highlighting substantial cell-type-dependent differences

in metabolic reprogramming during EMT. Interestingly, composition analysis showed that STGs in LDs were significantly reduced (~2 fold), whereas UTGs remain relatively constant (Fig. 3d). Our further analysis revealed decreased DNL (i.e. lower d₇-Glc_L signals) and PUFA uptake and metabolism (i.e. lower d₈-AA signal) in mes cells (Fig. 3b,e). In addition, choline metabolism for phospholipid synthesis (i.e. lower PGC signal) was also diminished (Fig. 3e), collectively suggesting a global downregulation of lipid biosynthetic activity. Despite this reduction in DNL and lipid uptake in mes cells, the unchanged intensity from endogenous lipid channel (i.e. CH_L, Fig. 3b,e) indicates that the total lipid biomass is largely maintained. This apparent balance could likely be attributed to our observed enhanced mobilization of lipids from LDs, suggesting that mes cells sustain lipid homeostasis through increased lipid recycling despite a globally attenuated metabolic state.

In addition to lipid remodeling, MetaboRamics also revealed interesting differences in glucose dynamics in EMT. Our data shows that glucose metabolism for both lipid and protein synthesis (d₇-Glc_L and d₇-Glc_P, respectively) is reduced in mes cells (Fig. 3b, e, f), whereas glucose uptake (¹³C₃-3-OPG) remained relatively unchanged (Fig. 3b, f). These results indicate that, despite comparable glucose uptake between epi and mes cells, glucose metabolism via glycolysis in mes cells is less effectively channeled into biosynthetic pathways, consistent with reduced biomass production. As a control, we validated the glucose uptake trend using single-color SRS imaging for EMT with ¹³C₃-3-OPG alone, which yielded consistent results (Fig. S8), confirming minimal cross-channel interference among probes.

Organelle markers further highlighted state-dependent differences during EMT (Fig. 3b,g). We observed a decrease in MB signal in mes cells which may reflect reduced mitochondrial abundance and/or organization (e.g., fragmentation vs. networked structure),³⁶ consistent with our prior observations that EMT attenuates mitochondrial TCA-cycle metabolism.³⁵ Similarly, a modest attenuation in ML signal was detected in mes cells, suggesting potential differences in lysosomal abundance, distribution, and/or size (Fig. 3b,g). Our spatial interactome analysis further uncovered EMT-dependent organelle coupling (Fig. 3h). In epi cells, lysosomes showed stronger association with FAD and protein synthesis markers (d₅-Phe), whereas mitochondria preferentially coupled to lipid-related signals (CH_L and d₇-Glc_L) (Fig. 3h, orange). Consistently, quantification of TPEF signals revealed minimal changes of FAD intensity between epi and mes cells, but a substantial decline in the NADH + FAD mixed channel, indicating reduced NADH levels in mes cells and, consequently a more oxidized mitochondria state³⁷ in EMT (Fig. 3i). Taken together, our multi-channel MetaboRamics analysis reveals that mes cells exhibit reduced glycolytic flux, diminished glucose incorporation into biomass, altered mitochondrial activity and decreased NADH levels, reflecting a global metabolically attenuated state.

High-throughput Metabolic Phenotyping of Stress-induced Cellular Responses

Going beyond EMT, to systematically probe cellular metabolic responses under disease-relevant conditions, we curated a set of nine metabolic stressors spanning nutrient excess, serum deprivation, inflammation, and pharmacological perturbations. To model nutrient overload, we subjected A549 cells to high fructose (FRUC) which contributes to the pathogenesis of cardiometabolic disease such as diabetes, obesity, and metabolic fatty liver

disease.³⁸ Additionally, we chose palmitic acid (PA, as a prototypical SFA) which causes lipotoxicity and endoplasmic reticulum stress.³⁹ Moreover, to mitigate growth-factor-dependent metabolic activity, we used FBS-free media for serum deprivation. Furthermore, cells were subjected to TGF- β and IL1- β to mimic inflammation (INF) and trigger EMT.⁴⁰ Since inflammation often accompanies the ramifications caused by high fructose in other cell types,⁴¹ we also assessed the effect of the combination of both stimuli (FRUC+INF). Lastly, we pharmacologically modulated protein levels by inhibiting synthesis with cycloheximide (CHX),⁴² blocking degradation with MG132⁴³ or bortezomib (BTZ),⁴⁴ and promoting it using CHIR-99021 (CHIR) via GSK-3 inhibition.⁴⁵

As an initial demonstration of our 16-plex metabolic imaging framework, we first implemented a 4-plex imaging strategy (PGC, d₇-Glc, d₈-AA and d₅-Phe) to capture baseline metabolic phenotypes before expanding to higher multiplexing. We constructed a heatmap for all 9 metabolic stressors (Fig. 4a). We found that, from nutrient stress, cells subjected to high fructose (i.e. FRUC) displayed an attenuation in PUFA (i.e. d₈-AA) uptake, likely indicating a lower metabolic demand for fatty acid uptake due to increased DNL from excess fructose. Our data also uncovered that high PA caused a downregulation of lipid-associated metabolism from d₇-Glc and PGC but stimulated an upregulation of PUFA (i.e. d₈-AA) uptake (Fig. 4a). This observation is in agreement with previous reports^{46,47} that unsaturated fatty acids (UFA) may rescue cells from SFA-induced lipotoxicity and ER stress by sequestration of excess SFAs into TGs within LDs. In contrast, serum deprivation (FBS-free) caused a downregulation in protein synthesis (d₅-Phe) and PUFA uptake (d₈-AA) but an increase in biomass synthesis from d₇-Glc and PGC (Fig. 4a-c).

For inflammatory stimulus (INF), we observed that similar to EMT, this condition caused a downregulation in d₇-Glc, PGC and d₅-Phe metabolism but not PUFA (i.e. d₈-AA) uptake (Fig. 4a-c). Interestingly, we found that the combination of FRUC+INF downregulated metabolism of the 4-channels (similar to INF) more profoundly than high fructose (FRUC) alone (Fig. 4a-c), suggesting a compounding effect. Furthermore, we found that the additional label-free CH₃ and CH₂ channels revealed that both INF and FRUC+INF resulted in cellular elongation, resembling that from the EMT condition but also differed from EMT by exhibiting strong lipid accumulation in the cytoplasm, highlighting a potentially different state altogether from the mesenchymal type (Fig. S9).

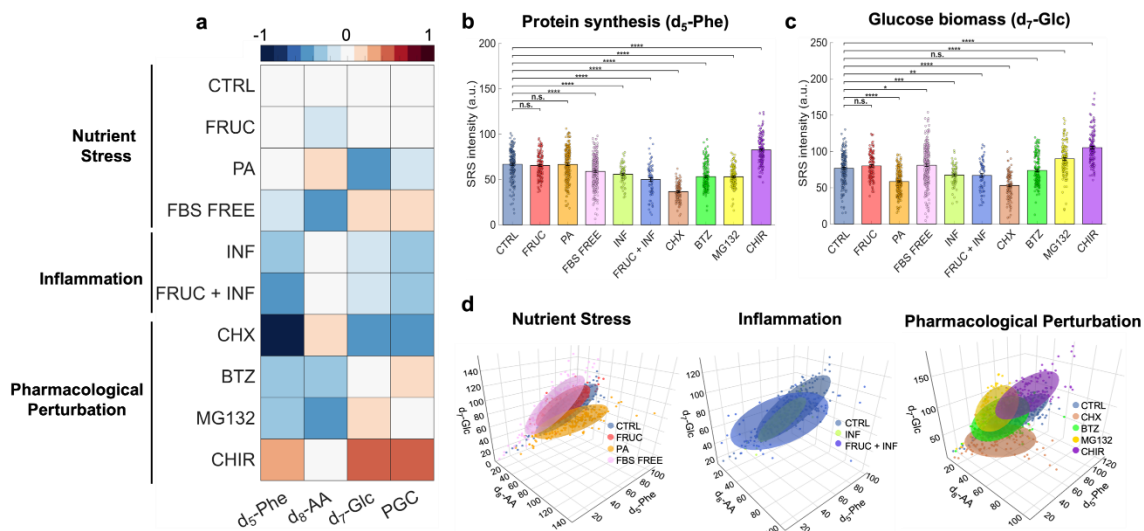


Figure 4. High-content Metabolic Phenotyping of Stress-induced Cellular Responses.

(a) Heatmap showing log₂ fold changes from 4-plex SRS imaging (d₅-Phe, d₈-AA, d₇-Glc, PGC) comparing live A549 control cells with cells exposed to nine metabolic stressors

(FRUC, INF, FRUC+INF, FBS-free, PA, CHX, BTZ, MG132, or CHIR).

Representative SRS images are shown in **Fig. S9**; **(b-c)** Quantification of C–D intensity per cell of **(b)** d₅-Phe incorporation for protein synthesis and **(c)** d₇-Glc for glucose-derived biomass in stressor-treated cells; **(d)** 3D scatter plots representing SRS intensities per cell from d₇-Glc, d₈-AA, and d₅-Phe in control and cells treated with different metabolic stressors. Data represented as mean ± SEM from at least three independent experiments. Statistical analysis was conducted using Welch's two-paired t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and n.s.= no statistical difference.

Pharmacological perturbations led to the most extreme metabolic alterations in A549 cells. We observed significant two-fold decrease in C–D signals from d₅-Phe (2298 cm⁻¹) incorporation upon treatment with protein synthesis inhibitors (CHX, MG132, BTZ) (Fig. 4b, CHX/MG132/BTZ vs CTRL). As a comparison, CHIR treatment displayed broad upregulation, including the largest increase (~25%) in d₅-Phe derived signals across all our series of metabolic stressors (Fig. 4b CHIR vs CTRL). We note that these drugs perturbation indeed have widespread and unexpected metabolic influences. For example, while CHX, MG132, and BTZ are all protein synthesis inhibitors, MG132 upregulates glucose metabolism (Fig. 4c, MG132 vs CTRL), whereas CHX strongly downregulates glucose metabolism (Fig. 4c, CHX vs CTRL) and BTZ had minimal effect on glucose metabolism (Fig. 4c, BTZ vs CTRL), highlighting their fundamentally varied mechanisms of action (MoA) on a larger biological network, which are worth comprehensively investigating when evaluating drug effects, underscoring the value of our platform. Taking advantage of the high dimensionality of our single-cell multiplexed measurements, we then mapped multiple

metabolites into a three-dimensional chemical space (Fig. 4d), visualizing clustering due to the differential responses of d₇-Glc, d₈-AA, and d₅-Phe to each conditions; consistent with previous reports, such clustering again highlight different MoA of the drugs.⁴⁸ Combining the high biocompatibility of small vibrational tags with sensitive SRS microscopy, 4-plex metabolic imaging thus already robustly allows high-throughput screening, enabling systematic characterization of varied drug responses and interactions.

16-plex Mapping of Cellular Stress Responses Using MetaboRamics

Seeking to further disentangle the complex and contrasting metabolic profiles obtained from treatment with CHIR, CHX, and INF, we applied our high-content, 16-plex MetaboRamics platform to thoroughly investigate their metabolic responses in live A549 cells at both single-cell and subcellular levels. We constructed a log₂FC heatmap at both whole-cell and subcellular levels, organized by subcellular compartments (i.e. cytoplasm, nuclei, and nucleoli) and treatment conditions (i.e. CHIR, CHX, and INF) (Fig. 5a). Our whole-cell 16-plex data exhibited excellent agreement with the 4-plex data (Fig. S10), highlighting the robustness and minimal cellular perturbation of our platform. For example, upon treatment with CHIR, we again observe a global upregulation among the metabolic probes reporting de novo synthesis, and uptake (Fig. 5a-c, CHIR, orange). Interestingly, our 16-plex imaging, however, reveals a significant downregulation of both ML and MB (Fig. 5d, left; CHIR, red), suggesting complex perturbation mechanisms to mitochondrial and lysosomal activities. As another example, CHX and INF treatment resulted in similar metabolic profiles across metabolic labels, with general attenuation across the targeted channels, but CHX upregulated

both ML and MB whereas INF did not affect MB signal (Fig. 5d, left). These findings further highlight the need for parallel global organelle-specific analysis during metabolic stress.

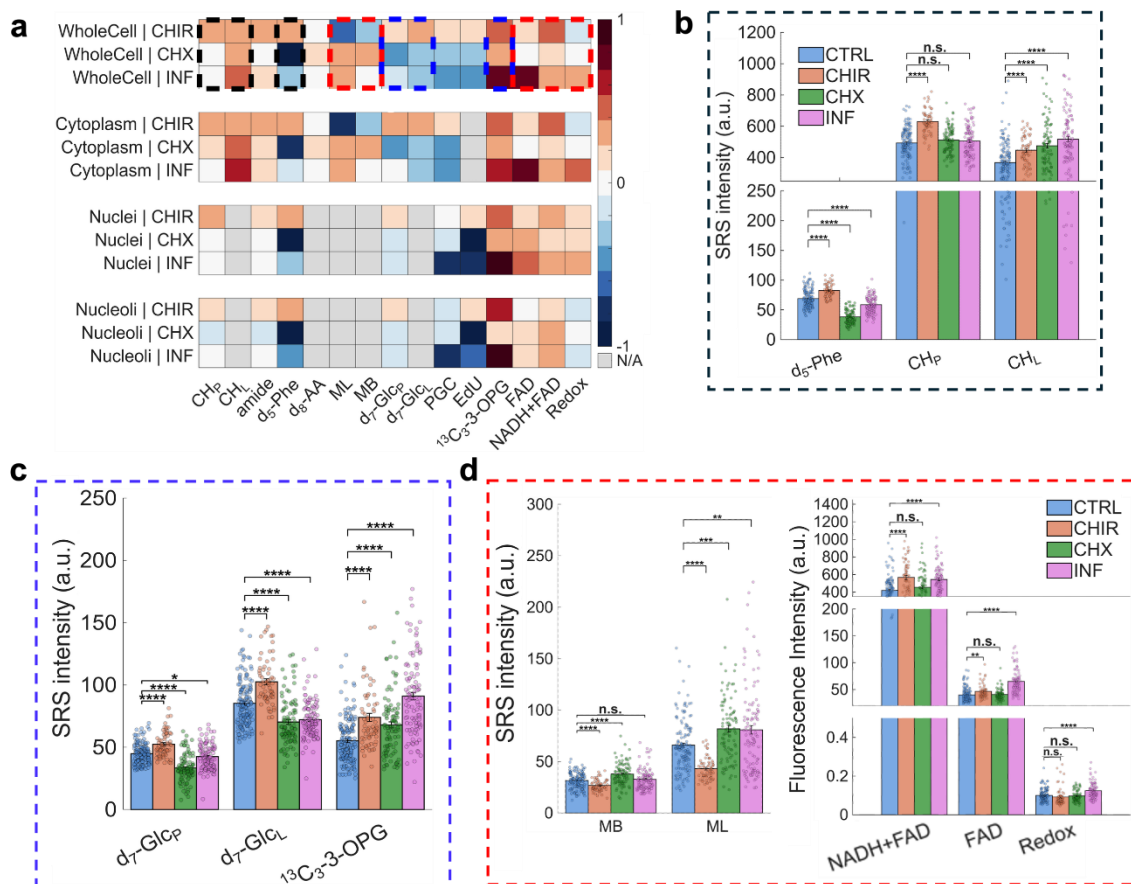


Figure 5. 16-plex Mapping of Cellular Stress Responses Using MetaboRamics. (a)

Heatmap from 16-plex metabolic imaging reflecting log₂ fold changes between the A549 control and cells treated with CHIR, CHX, or INF at the subcellular level (whole-cell, cytoplasm, nuclei and nucleoli); **(b)** Quantification of SRS intensities per cell for (left to right) d₅-Phe, CH_p and CH_l; **(c)** Quantification of SRS intensities per cell for glucose metabolism (d₇-Glc_p and d₇-Glc_l) and glucose uptake (¹³C₃-3-OPG) **(d)** (Left) Quantification

of SRS intensities per cell for organelle markers (MB and ML); (Right) TPEF intensities per cell for NADH+FAD, FAD, and redox ratio. Data represented as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was conducted using Welch's two-paired t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and n.s.= no statistical difference.

Indeed, metabolic labeling enables direct interrogation of dynamic metabolic activity whereas label-free analysis provides complementary insights into steady-state cellular composition. For instance, using d_5 -Phe to probe nascent protein synthesis, we observed a higher inhibitory effect with CHX than with INF. In contrast, the label-free CH_P channel showed no significant change in overall protein content across treatments, suggesting a slower degradation upon the drug treatments (Fig. 5b; CTRL (blue) vs CHX (green) and INF (magenta)). Interestingly, while CHIR upregulated d_5 -Phe metabolic signals, the increase in the label-free CH_P channel was larger (20% vs 27%), suggesting that, in addition to enhanced protein synthesis, protein degradation may also be attenuated (Fig. 5b; CTRL (blue) vs CHIR (orange)). At the subcellular level, we found protein modulation resulted in spatially heterogeneous metabolic changes. For instance, we found CHX uniformly degrades protein synthesis across all cellular compartments (whole-cell, cytoplasm, nuclei and nucleoli) but INF causes pronounced changes in its nucleoli (i.e., higher than the cytoplasm) whereas CHIR had the highest increase in protein upregulation in its nuclear compartments (i.e., both nuclei and nucleoli) (Fig. 5a and S11).

We then examined our full glucose palette (d₇-Glc_P, d₇-Glc_L, ¹³C₃-3-OPG), towards deeper insights into glucose dynamics. Consistent with our 4-plex experiments (Fig. 4a), CHIR upregulates d₇-Glc_P and d₇-Glc_L, while CHX and INF downregulate d₇-Glc_P and d₇-Glc_L (Fig. 5c). Surprisingly, ¹³C₃-3-OPG signal increased for all three metabolic stressors, indicating a relative decoupling of glucose uptake (increased ¹³C₃-3-OPG) and downstream metabolic incorporation (decreased d₇-Glc_P and d₇-Glc_L) for CHX and INF (Fig. 5c). These results further contrast with our EMT measurements (Fig. 3b), where glucose uptake (¹³C₃-3-OPG signal) remained unchanged despite reduced glucose metabolism (decreased d₇-Glc_P and d₇-Glc_L). These observations suggest differences in uptake dynamics and metabolism unique to these perturbations.

Complementary TPEF imaging revealed increases in absolute intensity for both FAD and NADH+FAD in CHIR- and INF-treated cells (Fig. 5 b, d). Analysis of the redox ratios (FAD / (NADH+FAD)) revealed that CHX (green) and CHIR (orange) induced minimal changes, whereas INF (magenta) shifted cells toward a more oxidized state, as reflected by an increased redox ratio (Fig. 5d). This shift is consistent with altered mitochondrial balance, potentially reflecting enhanced oxidative metabolism or changes in electron transport dynamics under INF treatment.

These trends partially align with glucose-derived metabolic readouts (Fig. 5c), where INF treatment is associated with decreased lipid and protein anabolism (d₇-Glc_L and d₇-Glc_P). However, this relationship diverges under CHIR, which exhibits enhanced anabolic activity despite minimal changes in redox ratio, suggesting a partial decoupling between

mitochondrial redox state and biosynthetic output. Together, our results highlight the importance of multiplexed metabolic imaging to capture distinct and partially uncoupled aspects of cellular metabolism.

3.4 Discussion

Metabolism is a highly spatially organized series of chemical reactions, yet most live-cell imaging methods only capture a narrow portion of metabolic activity at a time. To maximize the information rendered from optical imaging methods for spatial metabolomics, it is necessary to simultaneously probe several different metabolic pathways and examine how they interact with each other. Here, we demonstrate MetaboRamics as a super-multiplexed SRS imaging platform that enables simultaneous, spatially resolved mapping of metabolic activity in live cells. In addition to mapping endogenous biomolecules such as proteins and lipids (total lipids, UTG, and STG), our method captures a broad range of metabolic pathways: glucose uptake, glucose metabolism that leads to nascent synthesis of biomass including lipids and proteins, de novo DNA synthesis, choline metabolism, fatty acid uptake, and de novo protein synthesis. With the use of spatial markers for organelles such as lysosomes and mitochondria, our analytical workflow also enables interactomics to understand the interplay between cellular organelles and metabolites. Finally, the highly complementary TPEF provides an optical reporter for the redox states of cells, achieving 16-plex imaging in live HeLa with MetaboRamics.

We next applied our 16-plex platform to investigate the metabolic landscape of EMT in live A549 cells, which remains incompletely characterized. Our multi-dimensional data indicated

a global attenuation of metabolic activity in several metabolic pathways in mes cells compared to their epithelial counterparts. For instance, through a decrease in DNL and PUFA uptake, we found a reduction in lipid turnover in mes cells. Additionally, using two different glucose reporters- we revealed that despite having similar glucose uptake, mes cells have lowered glycolytic flux. By integrating complementary TPEF, we further demonstrated that mes cells accumulate NADH due to reduced NADH oxidation rather than increased glucose flux which is consistent with a change in redox balance, and global metabolic attenuation. Our method highlights the metabolic flexibility displayed by mesenchymal cells to become quiescent which in many cases has been attributed to the drive chemoresistance to therapeutic agents.⁴⁹

We next performed high-content metabolic phenotyping to characterize cellular responses under disease-relevant conditions. MetaboRamics revealed consistent, pathway-specific metabolic adaptations across diverse metabolic stressors. For example, similar to EMT, inflammatory stimuli led to a global downregulation of metabolism in A549 epithelial cells, with select exceptions, including increased ML, glucose uptake and lipid metabolism. Similarly, CHX selectively downregulated de novo protein synthesis in cells but increased PUFA uptake. Therefore, these results demonstrate the ability of our platform to simultaneously capture integrated metabolic responses rather than isolated pathway changes. We also note a few limitations. Although our current probe panel covers a broad range of metabolic pathways, it does not fully encompass the full diversity of cellular metabolism, largely constrained by overlapping Raman spectra and leading to challenging unmixing. Additionally, our imaging speed for 16 channels is limited by laser tuning requirements of

our cavity-based picosecond laser, restricting the total time taken for multiplexing. The use of rapidly tunable lasers could enable faster image and spectral acquisition that would also facilitate the use of more sophisticated unmixing tools such as LASSO.^{50,51} Furthermore, the integration of deep learning algorithms can also assist in faster image acquisition (or image prediction) by drastically increasing the signal-to-noise ratio of SRS images, requiring lower laser power and reducing photodamage as more channels are acquired.⁵² Lastly, despite the use of Raman tags, the sensitivity of SRS is still limited in the low μM to low mM regime.⁵³ This constraint poses challenges for many metabolic species that are present at substantially lower concentrations in cells (in $\sim\text{nM}$ ranges). Future efforts to engineer metabolic probes that leverage epr-SRS⁵⁴ to improve detection limits would greatly expand the ability to visualize sub-micromolar species.

To perform quantitative spatial metabolomics in absolute concentrations, future work should combine MetaboRamics with newly developed frameworks such as MATRIX-SRS35 that can provide absolute concentrations for deuterium-based biomass. Additionally, one can envision a multi-omics platform that leverages the strong complementarity of MetaboRamics with other modalities, such as spatial transcriptomics (e.g., Visium)⁵⁵ or single-cell RNA sequencing,¹⁸ on the same sample to link transcriptional changes with metabolic states and gain deeper systems-level understanding of cellular function. Similarly, coupling MetaboRamics with MSI, such as MALDI,⁵³ could enable deeper metabolome coverage. Lastly, beyond fundamental studies of metabolism, MetaboRamics is also well suited for applications in drug discovery: to probe multiple metabolic outcomes, identify off-target effects, and characterize the MoA of drug candidates.

Overall, our work demonstrates the ability of MetaboRamics to resolve metabolic complexity and spatial heterogeneity in live cells, providing a versatile framework to study metabolic states and rewiring across biological contexts.

3.5 Materials & Methods

Cell Culture and Metabolic Labeling

HeLa cells (ATCC, CCL-2), and A549 cells (gift from the Elowitz lab, Caltech) were each cultured in DMEM (Gibco, Cat. #11965092) supplemented with 1x antibiotic-antimycotic (Gibco, Cat. #15240062) and 10% FBS (Corning, Cat. #35-015-CV) at 37 °C with 5% CO₂. For EMT induction, A549 cells were seeded and cultured in DMEM supplemented with 1x StemXVivo EMT-inducing supplement (R&D Systems, Cat. #CCM017) for 5 days, with media changes every 2-3 days.

Metabolic labels used for MetaboRamics included 1 mM propargylcholine bromide (MedChemExpress, Cat. #HY-129084), 25 mM 1,2,3,4,5,6,6- d₇-D-glucose (Cambridge Isotope Laboratories, Cat. #DLM-2062), 1 mM d₅-L-phenylalanine (Cambridge Isotope Laboratories, Cat. #DLM-1258-5), and 25 mM ¹³C₃-3-OPG (synthesized according to previously reported method²⁶), which were added to cells in custom DMEM with no unlabeled counterparts for 48 hours. Following 24 hours, 200 μM 5-Ethynyl-2'-deoxyuridine (MedChemExpress, Cat. #HY-118411) and 2 μM d₈-arachidonic acid (MedChemExpress, Cat. #HY-109590S) were added for the remaining 24 hours. The Me-Lyso stock solution (in DMSO) was photo-uncaged by irradiation with a 405 nm laser for 10 min prior to incubation

with cells. Finally, 400 nM MitoBADY and 7.5 μ M Me-Lyso (see Supplementary Information for synthetic protocols) were added to the cells for 30 minutes before multi-channel SRS imaging. These concentrations and incubation times were selected following systematic optimization across labels to ensure high imaging signal-to-noise ratios while maintaining high cellular viability.

Metabolic stressors included 25 mM D-fructose (FRUC; Thermo Fisher, Cat. #A17718.0I), 10 ng/mL IL- β 1 (R&D systems, Cat. #201-LB-005) and 10 ng/mL TGF- β (R&D systems, Cat. #7754-BH-005) (INF), 25 mM D-fructose + 10 ng/mL IL- β 1 and 10 ng/mL TGF- β (FRUC + INF), 100 μ M palmitic acid (PA; Sigma-Aldrich, Cat. #506345-25GM), 3.5 μ M CHIR99021 (CHIR; MedChemExpress, Cat. #HY-10182), 5 μ M MG132 (MedChemExpress, Cat. #HY-13259), 1.7 μ M Cyclohexamide (CHX; Sigma Aldrich, Cat. #239763-1GM), 10 nM Bortezomib (BTZ; MedChemExpress, Cat. #HY-10227) and DMEM containing no FBS (FBS-free). For FRUC, INF, and their combinatorial treatment (FRUC + INF), A549 cells were first exposed to these stressors alone for 48 hr before the concomitant addition of metabolic labels for 24 hr. All other stressors were added concomitantly with the metabolic labels and incubated for 24 hr prior to SRS imaging.

SRS Microscopy

A tunable pump beam (700-990 nm, 2 ps, 80 MHz) and Stokes beam (1032.0 nm, 2 ps, 80 MHz, modulated to 20MHz by an Electro-Optic modulator, EOM) from a picoEmerald S laser system (Applied Physics & Electronics) are used as the light source for SRS Microscopy. The spatially and temporally overlapped beams are expanded and introduced

into an inverted multiphoton laser scanning microscope (FV3000, Olympus). Light is focused on the sample with a 25x water immersion objective (XLPLN25XWMP, 1.05 N.A., Olympus) and transmitted light is collected with an oil immersion condenser lens (1.40 N.A., Olympus). The Stokes beam is filtered out (893/209 BrightLine, 25 mm, Semrock) and the remaining pump beam is collected with a large area photodiode (S3590-09, Hamamatsu). A 64 V reverse-bias DC voltage was applied to the photodiode to increase the saturation threshold and reduce the response time. The output current was terminated by a 50 Ω terminator and prefiltered by a 19.2-23.6 MHz band-pass filter (BBP-21.4+, Mini-Circuits) to reduce laser and scanning noise. The SRS signal within the pump beam is demodulated via lock-in amplification (SR844, Stanford Research Instruments) at the reference modulation frequency, and the in-phase X input was fed back to the IO interface box (FV30-ANALOG). Images were captured using Olympus FluoView 3000 software.

Images were acquired with an 80 μ s pixel dwell time to achieve an image acquisition speed of 8.52 s per frame for a 320x320-pixel field of view (FOV) at a 0.497 μ m/pixel resolution. The pump beam wavelength was set to 787.0 nm for the unsaturated triglyceride, 791.8 nm for the protein ($-\text{CH}_3$ channel, 2940 cm^{-1}), 797.8 nm ($-\text{CH}_2$ channel, 2845 cm^{-1}), 834.2 nm (C–D channel for d_5 -phenylalanine, 2298 cm^{-1}), 837.5 nm (C–D channel for d_8 -arachidonic acid, 2250 cm^{-1}), 839.6 nm ($\text{C}\equiv\text{C}$ channel for Mito-BADY, 2220 cm^{-1}), 840.7 nm ($\text{C}\equiv\text{C}$ channel for Me-Lyso, 2205 cm^{-1}), 841.6 nm (C–D protein channel for d_7 -glucose 2192 cm^{-1}), 844.6 nm (C–D lipid channel for d_7 -Glucose, 2150 cm^{-1}), 845.2 nm ($\text{C}\equiv\text{C}$ channel for propargylcholine, 2142 cm^{-1}), 846.6 nm ($\text{C}\equiv\text{C}$ channel for EdU, 2118 cm^{-1}), 851.5 nm ($\text{C}\equiv\text{C}$ channel for $^{13}\text{C}_3$ -OPG, 2054 cm^{-1}), 855.4 nm (off-resonance background image, 2000 cm^{-1}),

880.6 nm (amide I band, 1665 cm^{-1}) All images were processed and color-coded using Fiji (ImageJ 2.16.0; ImageJ 1.54p)⁵⁶ software. For hSRS of solutions, the wavelength of the pump laser was tuned from 834.0 nm to 855.0 nm with a step size of 0.5 nm.

For C–D quantification, on and corresponding off-resonance images were preprocessed with the Fiji ‘nonuniform background removal’ plugin before subtracting them, resulting in the C–D (on-off) images that are used for linear unmixing. For label-free quantification, only the Fiji ‘nonuniform background removal’ plugin was used. For cellular and subcellular quantification, the protein (CH_3) image was used to segment each cell and its compartments utilizing the ‘IdentifyObjectsManually’ module in Cellprofiler 4.2.8, converted to a mask using the ‘ConvertObjectsToImage’ module and saved with the ‘SaveImages’ module. The mask was then fed into MATLAB (R2024b, MathWorks) for identification with the “bwlabel” function and used as a map for quantification for all mask objects. Results are then exported to MS Excel for analysis. Images and graphs are visualized using Fiji, MATLAB, and RStudio.

Linear Combination Algorithm for Spectral Unmixing

While single-frequency SRS imaging maps the distribution of individual Raman peaks with high chemical specificity, many vibrational modes overlap.⁵⁷ To deconvolute discrete molecular signatures from overlapping peaks, we employed a linear combination algorithm. All image sets were unmixed using MATLAB (Mathworks, USA), with image segmentation and processing performed in Fiji (ImageJ 2.16.0; ImageJ 1.54p). hSRS spectra for individual

probes were extracted from hSRS stacks and used to generate the coefficients for the unmixing matrix.

Single-color hSRS stacks for $^{13}\text{C}_3\text{-3-OPG}$ (50mM, 2 day incubation), EdU (200 μM , 1 day), PGC, Me-Lyso (7.5 μM , 30 minutes), MitoBADY (400 nM, 30 minutes), and $\text{d}_5\text{-Phe}$ (400 μM , 12 days) were acquired in live HeLa cells incubated with the respective probes, whereas hSRS stacks for $\text{d}_8\text{-AA}$ (50 μM , 1 day) were acquired in fixed HeLa cells to mitigate LD drift. After processing hyperspectral images (See SRS Microscopy), regions of interest (ROIs) with high SNR were segmented and averaged at each frequency to construct an SRS spectrum for each probe. After treatment with $\text{d}_7\text{-glucose}$ (25mM, 3 days), hSRS images of $\text{d}_7\text{-glucose-derived lipid (CD}_\text{L})$ were acquired in live A549 cells. For $\text{d}_7\text{-glucose-derived protein (CD}_\text{P})$ measurements, fixed HeLa cells were washed with 0.5% Triton X-100 (Sigma, T8787) in phosphate-buffered saline solution for 10 minutes at 4°C . LDs and nucleoli were segmented and averaged at each frequency to construct spectra for CD_L and CD_P , respectively. hSRS spectra were baseline-corrected as needed and fit with Voigt line shapes in Fityk (v1.3.1),⁵⁸ followed by normalization to their maximum intensity. A distinct unmixing matrix was prepared for each image set, as the exact pump wavelength varies during image acquisition. SRS intensities at each Raman shift were extracted from the spectra and used as coefficients for the unmixing matrix. Each image set is then unmixed with a distinct matrix, either 9×9 or 4×4 depending on the sample treatment (Fig. S5).

To ensure the fidelity of unmixed data, any pixels having a negative contribution of $^{13}\text{C}_3\text{-3-OPG}$, PGC, CD_L , $\text{d}_8\text{-AA}$, or $\text{d}_5\text{-Phe}$ were discarded from all channels prior to analysis. The

spatially distinguishable probes—EdU, MB, and ML—were exempted to preserve a data set large enough for analysis, representing a meaningful limitation to the methodology.

For high-frequency C–H stretching region, the lipid channel (CH_L) was generated using a linear combination of the CH_2 and CH_3 images = $5 \cdot [\text{CH}_2] - 0.4 \cdot [\text{CH}_3]$ whereas the protein channel (CH_P) was generated using a linear combination of $[\text{CH}_3] - [\text{CH}_2]$ from the same set of images.⁴⁷

Since the EdU signal is confined to the nucleus, unmixed EdU images were multiplied by nuclear masks to suppress contributions from non-nuclear regions (e.g., cross-phase modulation from LDs) and mitigate background noise.

STG images were calculated according to the workflow described in Fig. S4.

Two-Photon Fluorescence (TPEF) Microscopy

The same picoEmerald used for SRS microscopy was also used to perform TPEF microscopy with the addition of a home-built blackbox. Label-free excitation of FAD and NADH was performed at 760.0 nm, and isolated excitation of FAD was performed at 880.0 nm. The backscattered excitation beam is filtered with a 690.0 dichroic mirror and epi-autofluorescence is collected through a non-descanned detector (NDD). TPEF images were collected at the same spatial dimensions as the SRS images but with a dwell time of 8 μs pixel and averaged 6 times.

Spontaneous Raman Microscopy

Spontaneous Raman spectra of target metabolites were acquired using a Horiba Xplora plus confocal Raman spectrometer equipped with a 532 nm YAG laser (12 mW) and a 100x, 0.9 N.A. objective (MPLAN N, Olympus) with a 500 μm pinhole and a 100 μm slit width. Spectra were collected with a 10 s integration time per frame and averaged over 10 frames using LabSpec6 software. Solvent background was subtracted from the metabolite spectra, followed by baseline correction and normalization.

3.6 Supplementary Information

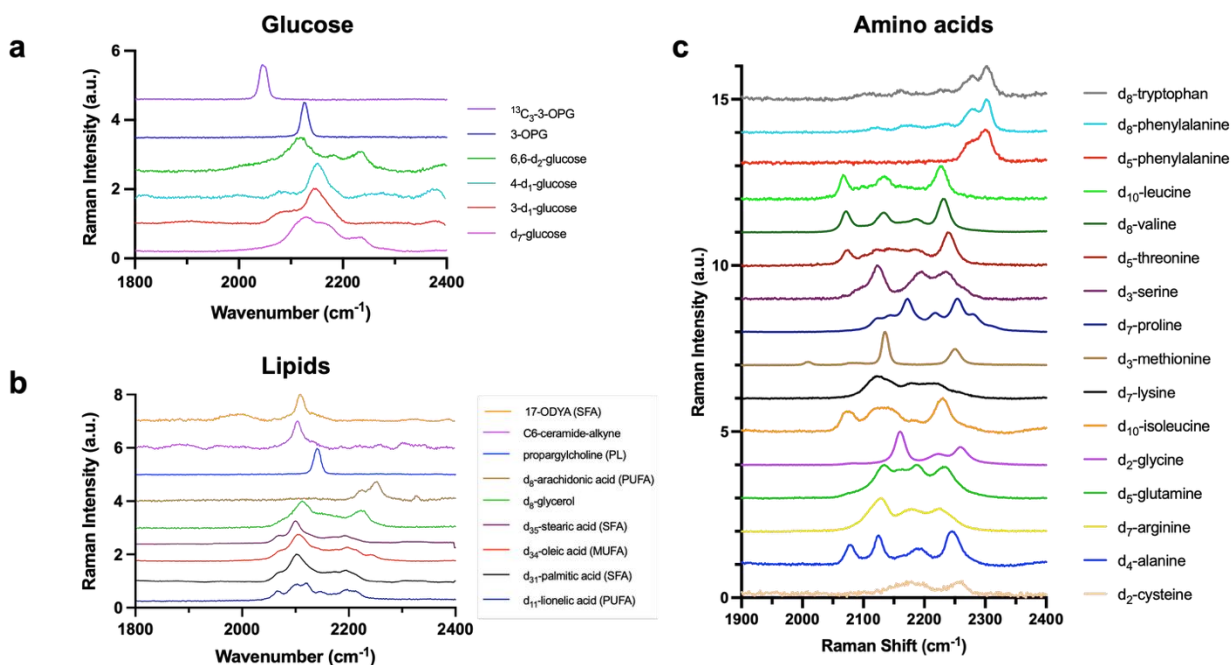


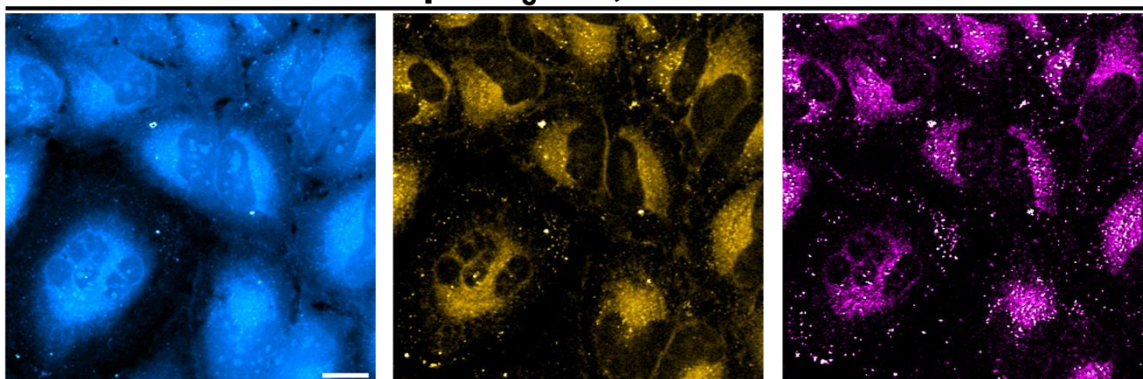
Figure S1. Raman spectra of metabolites.

Spontaneous Raman spectra of different deuterium- or alkyne-based isotopologues or analogues of **a)** glucose; **b)** lipids; and **c)** amino acids; 3-OPG= 3-O-propargyl-d-glucose,

17-ODYA= 17-octadecynoic acid, SFA= saturated fatty acid, PL= phospholipids,

MUFA= monounsaturated fatty acid, PUFA= polyunsaturated fatty acid.

2 μ M d₈-AA, 48 hr



4 μ M d₈-AA, 48 hr

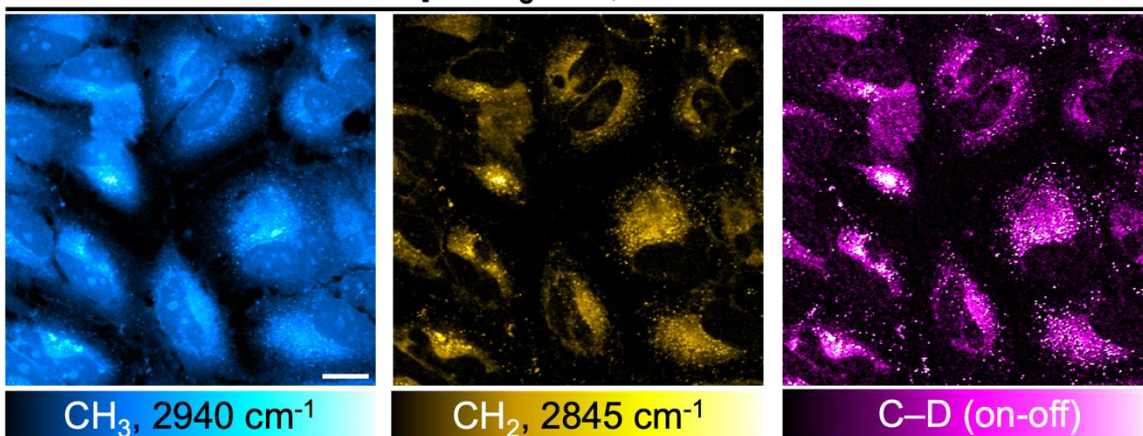


Figure S2. Probe optimization for MetaboRamics.

Representative SRS images in live HeLa cells targeted at the protein (CH₃) and C–D (on-off) channel for cells incubated with only d₈-AA. We notice that an increase in incubation time (from 24 hr (in Fig. S5) to 48 hr, top row) or concentration (from 2 μ M to 4 μ M, bottom row) increases the C–D signal intensity due to increased PUFA incorporation but also leads to formation of more LDs in cells.

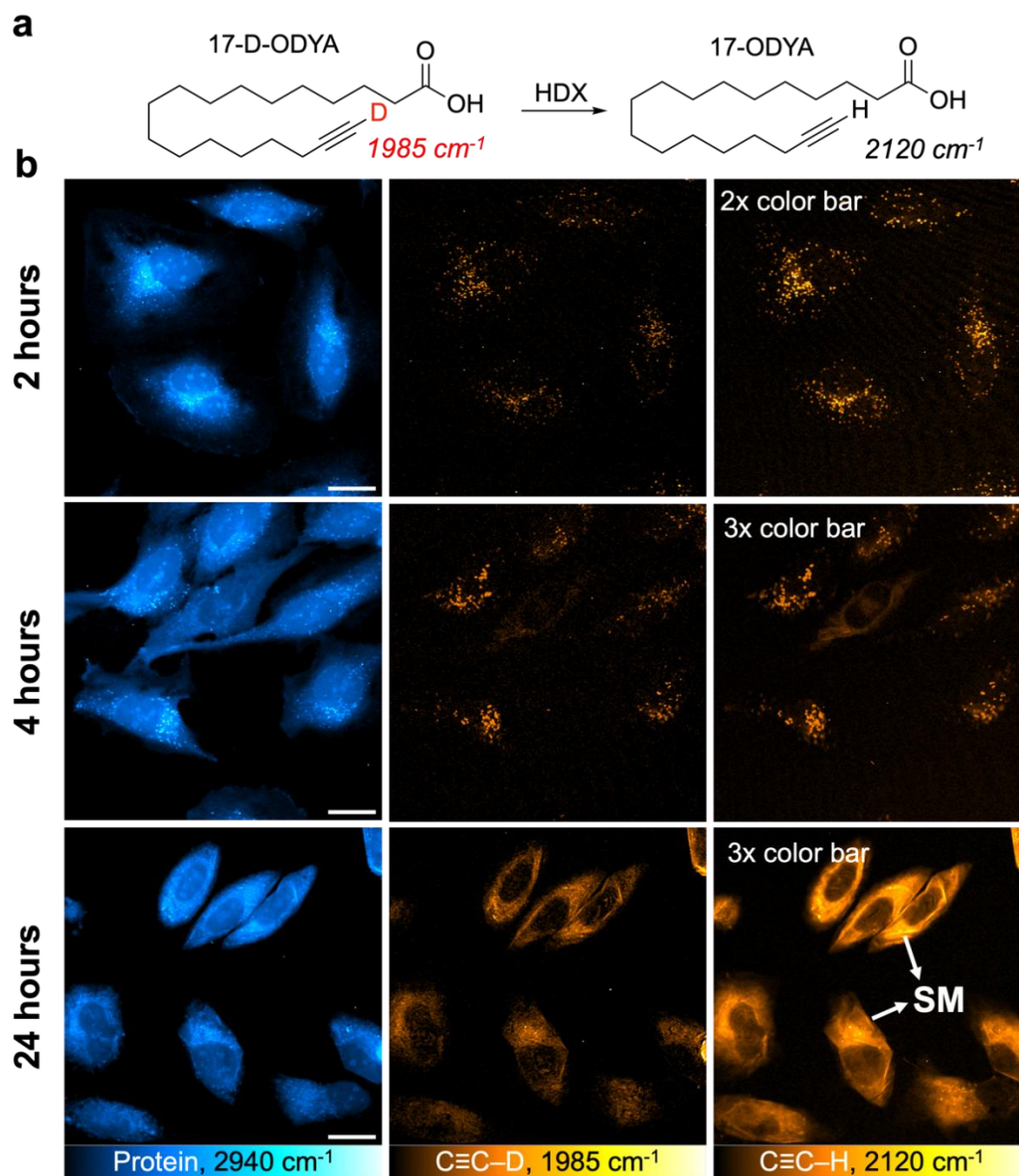


Figure S3. HDX of 17-D-ODYA in live cells.

a) Chemical reaction indicating hydrogen-to-deuterium exchange (HDX) of 17-D-ODYA;

b) Representative SRS images in live HeLa cells targeted at the protein (CH_3), $\text{C}\equiv\text{C}-\text{D}$ (on-off) and $\text{C}\equiv\text{C}-\text{H}$ (on-off) channels. Cells were incubated with $100\ \mu\text{M}$ 17-D-ODYA for 2, 4, or 24 hr before SRS imaging. Significant HDX is already evident by the first imaged time

point (2 hr) under physiological conditions; HDX was observed to begin as early as 30 min regardless of the cell type (data not shown) and increases significantly by 24 hr. Initially enriched in LDs, 17-D-ODYA progressively redistributes to the cytoplasm over time, and ultimately, leads to the formation of rigid solid membrane (SM) structures (white arrows). Scale bar: 20 μm .

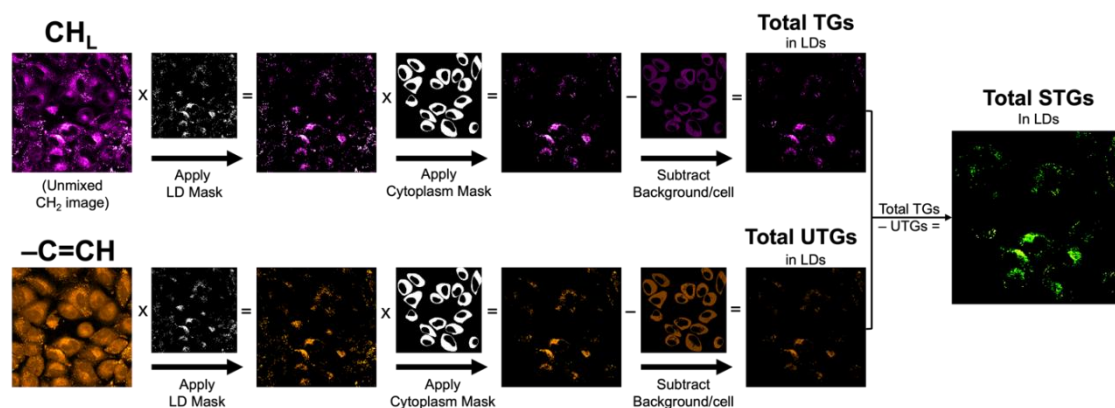


Figure S4. Image processing workflow for calculation of saturated triglycerides (STGs) from SRS images.

STG images were prepared using a modified protocol adapted from a previous report.⁵⁹ Briefly, LDs were segmented to create binary LD masks from the CH_L (unmixed CH_2 image, 2840 cm^{-1}) and U_L ($-\text{C}=\text{C}-\text{H}$, 3022 cm^{-1}) images. These masks were then applied to their corresponding images. The resultant images for both CH_L and U_L were then multiplied with the cytoplasm mask to consider all LDs within the cell. Finally, the masked U_L image was subtracted from the masked CH_L image to yield the STG image (representing STGs obtained by subtracting unsaturated TGs from total TGs) in LDs.

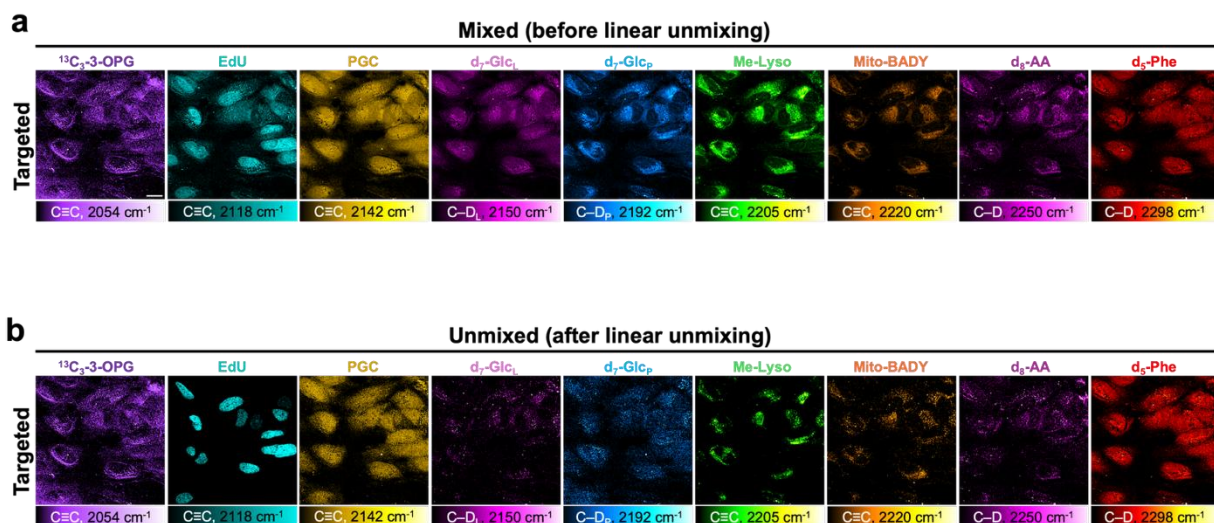


Figure S5. Robust linear unmixing of targeted SRS channels.

Comparison of **a**) mixed (before linear unmixing) and **b**) unmixed (after linear unmixing) SRS channels in live HeLa cells. Scale bar: 20 μm .

Linear unmixing algorithm for 9 targeted SRS channels

Representative linear unmixing of the nine targeted SRS channels above with the exact recorded pump wavelength was performed using the algorithm provided below:

$$\begin{bmatrix} I_{2053} \\ I_{2122} \\ I_{2142} \\ I_{2150} \\ I_{2192} \\ I_{2205} \\ I_{2221} \\ I_{2250} \\ I_{2298} \end{bmatrix} = \begin{bmatrix} 0.69 & 0.00 & 0.00 & 0.02 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 \\ 0.02 & 0.90 & 0.05 & 0.63 & 0.58 & 0.00 & 0.00 & 0.00 & 0.00 \\ 0.01 & 0.15 & 0.97 & 0.94 & 0.68 & 0.01 & 0.00 & 0.01 & 0.00 \\ 0.01 & 0.04 & 0.58 & 1.00 & 0.48 & 0.01 & 0.00 & 0.01 & 0.00 \\ 0.00 & 0.00 & 0.01 & 0.84 & 0.97 & 0.41 & 0.01 & 0.03 & 0.00 \\ 0.00 & 0.00 & 0.01 & 0.69 & 0.88 & 1.00 & 0.07 & 0.06 & 0.00 \\ 0.00 & 0.00 & 0.00 & 0.48 & 0.71 & 0.28 & 0.75 & 0.24 & 0.00 \\ 0.00 & 0.00 & 0.00 & 0.26 & 0.33 & 0.01 & 0.08 & 0.77 & 0.03 \\ 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.98 \end{bmatrix} \times \begin{bmatrix} C \equiv C^{13}_{OPG} \\ C \equiv C_{EdU} \\ C \equiv C_{PGC} \\ CD_{Lipid} \\ CD_{Protein} \\ C \equiv C_{Lyso} \\ C \equiv C_{Mito} \\ CD_{AA} \\ CD_{Phe} \end{bmatrix}$$

inverse

$$\begin{bmatrix} C \equiv C^{13}_{OPG} \\ C \equiv C_{EdU} \\ C \equiv C_{PGC} \\ CD_{Lipid} \\ CD_{Protein} \\ C \equiv C_{Lyso} \\ C \equiv C_{Mito} \\ CD_{AA} \\ CD_{Phe} \end{bmatrix} = \begin{bmatrix} 1.45 & 0.00 & 0.05 & -0.08 & 0.01 & 0.00 & 0.00 & 0.00 & 0.00 \\ -0.03 & 1.11 & 0.10 & -0.26 & -0.95 & 0.40 & -0.03 & 0.02 & 0.00 \\ 0.00 & -0.22 & 1.55 & -0.85 & -0.83 & 0.34 & -0.02 & 0.00 & 0.00 \\ -0.02 & 0.15 & -1.59 & 2.65 & -0.43 & 0.17 & -0.01 & -0.01 & 0.00 \\ 0.02 & -0.13 & 1.43 & -2.41 & 2.02 & -0.83 & 0.05 & -0.02 & 0.00 \\ 0.00 & 0.01 & -0.16 & 0.26 & -1.37 & 1.60 & -0.13 & -0.03 & 0.00 \\ 0.00 & 0.02 & -0.26 & 0.46 & -0.93 & -0.01 & 1.39 & -0.40 & 0.01 \\ 0.00 & 0.00 & -0.05 & 0.08 & -0.61 & 0.28 & -0.16 & 1.35 & -0.04 \\ 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 0.00 & 1.02 \end{bmatrix} \times \begin{bmatrix} I_{2053} \\ I_{2122} \\ I_{2142} \\ I_{2150} \\ I_{2192} \\ I_{2205} \\ I_{2221} \\ I_{2250} \\ I_{2298} \end{bmatrix}$$

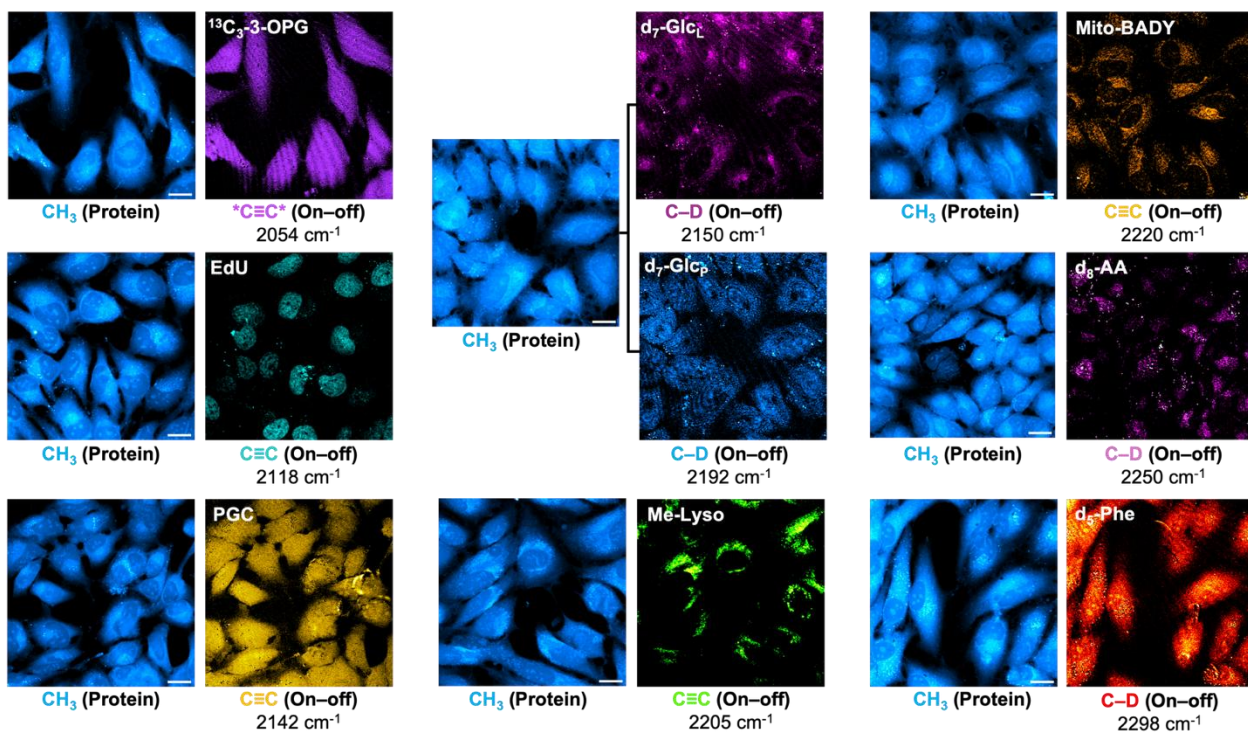


Figure S6. Single-channel targeted SRS imaging.

Representative SRS images of optimized MetaboRamics metabolic probes (administered

individually) alongside their corresponding protein (CH_3) images in live HeLa cells.

As noted in the main text, $\text{d}_7\text{-Glc}$ metabolizes into both lipids ($\text{d}_7\text{-Glc}_\text{L}$) and proteins ($\text{d}_7\text{-Glc}_\text{P}$), resulting in two spectrally distinct channels. Cells were treated using the same probe concentrations and incubation times as in the 16-plex MetaboRamics imaging experiments.

Scale bar: 20 μm .

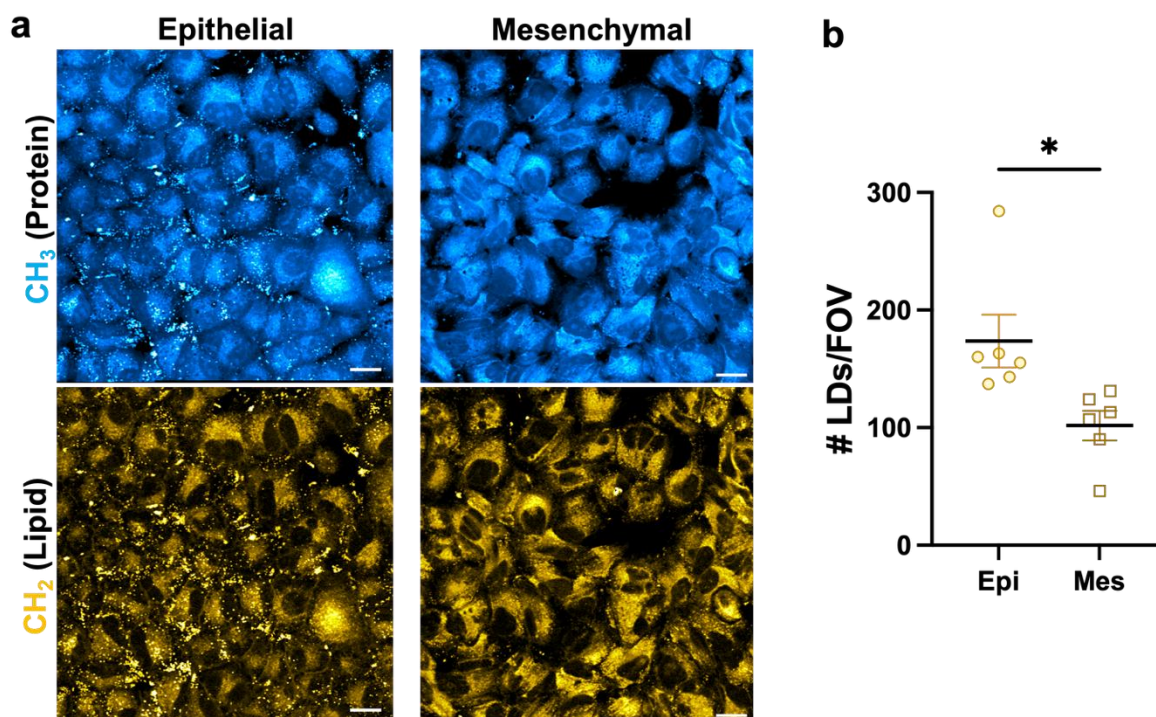


Figure S7. Label-free assessment of lipid droplets (LDs) in EMT.

(a) Representative SRS images targeted at the protein (CH_3 , top row) and lipid (CH_2 , bottom row) channels for live A549 (epithelial and mesenchymal cells). Scale bar: 20 μm ; (b) Quantification of number of LDs per FOV for epithelial and mesenchymal cells. Data represents mean (from different FOVs) $\pm$ SEM. Statistical significance was calculated using two-tailed Student's t-test, * $p < 0.05$.

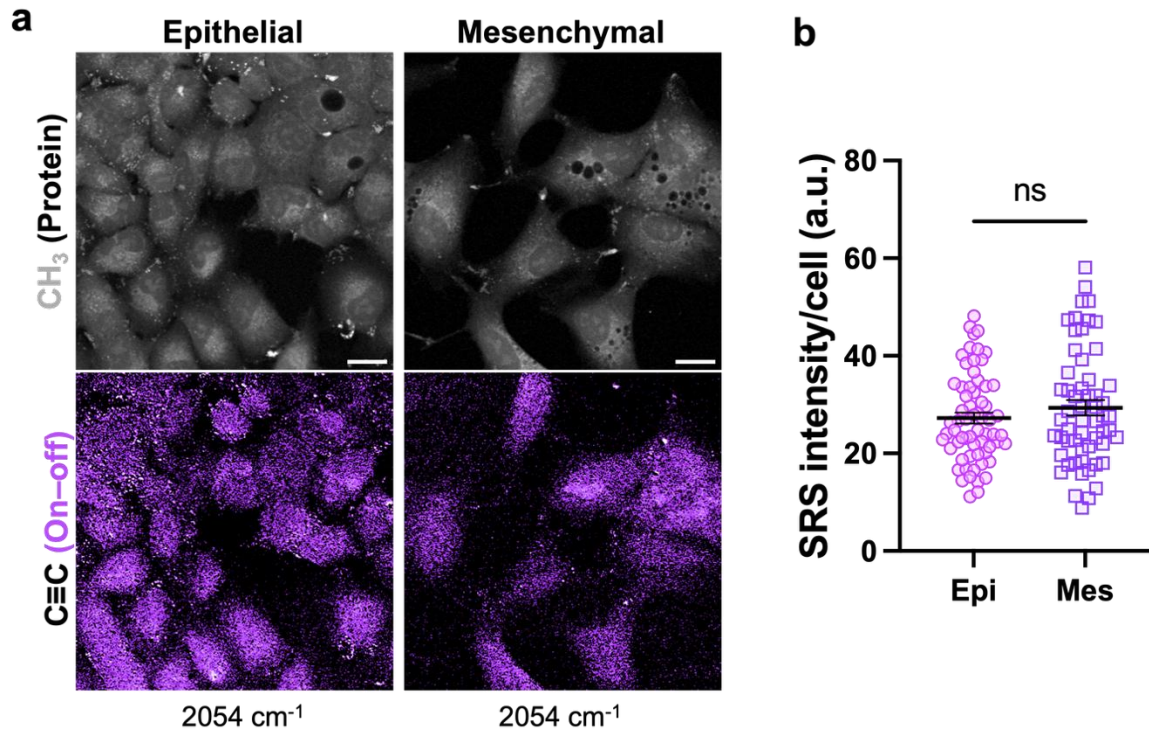


Figure S8. Glucose uptake in EMT.

(a) Representative SRS images targeted at the protein (CH₃, top row) and alkyne (on-off) channels to monitor glucose uptake using 25 mM ¹³C₃-3-OPG in live epithelial and mesenchymal cells for 24 hours. Scale bar: 20 μm; **(b)** Quantification of SRS intensity per cell from alkyne images for epi and mes. Data represents mean ± SEM. Statistical significance was calculated using two-tailed Student's t-test, ns= no statistical difference.

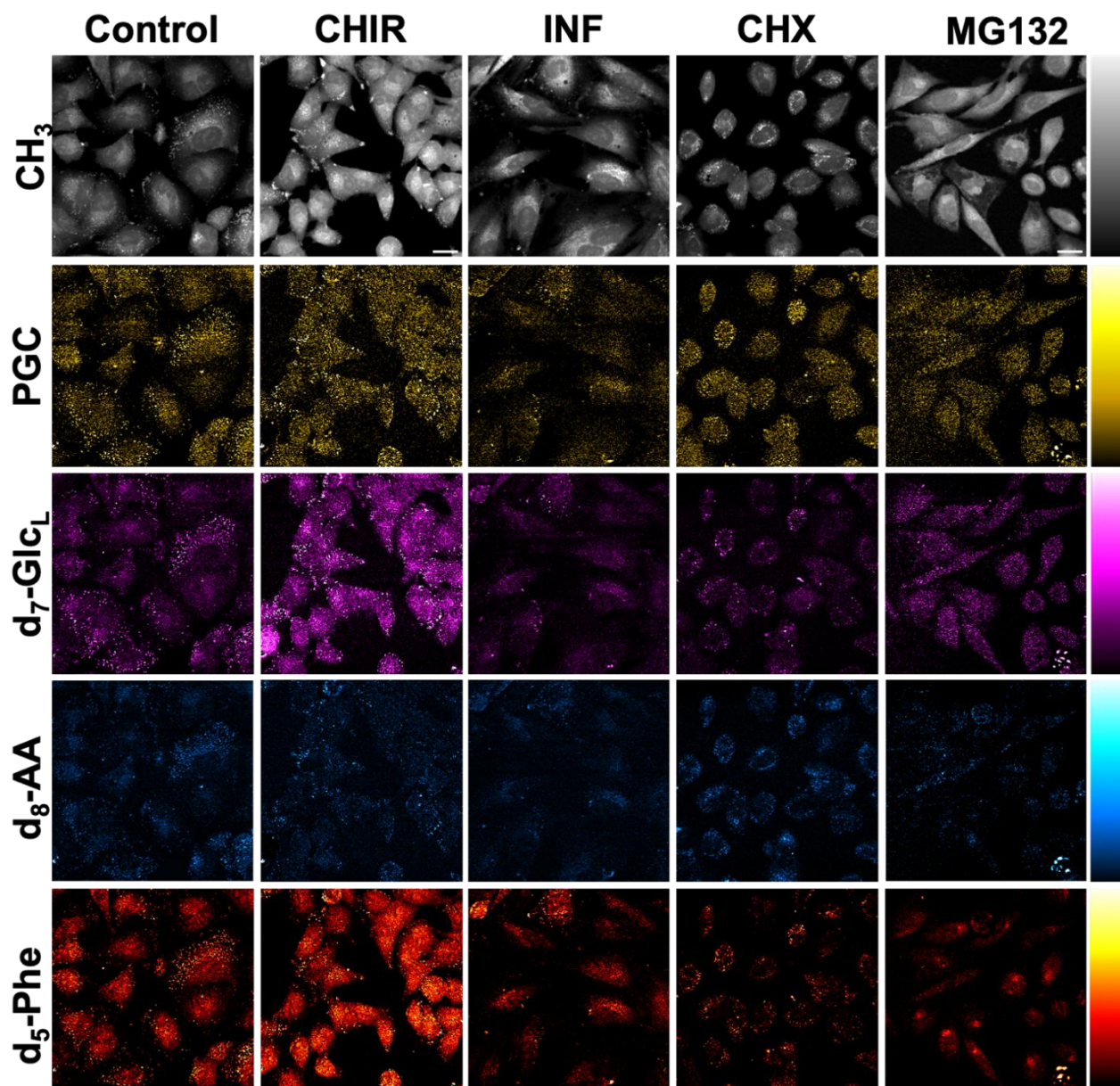


Figure S9. SRS metabolic phenotyping of stress-induced cellular responses.

Select representative SRS images of live A549 cells under control conditions or treated with CHIR, INF, CHX, or MG132 with distinct metabolic signatures across stress states. Scale bar: 20 μm .

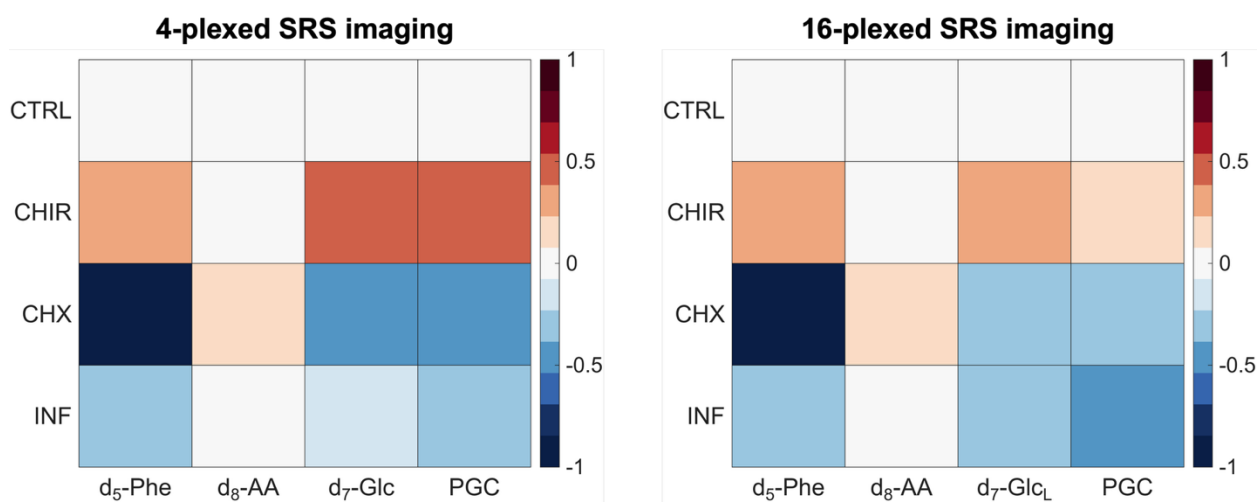


Figure S10. Robustness of MetaboRamics.

Heatmap showing strong agreement between lower-order (4-plexed) and higher-order (16-plexed) imaging datasets, demonstrating the platform's scalability and reproducibility. Data from at least three independent experiments.

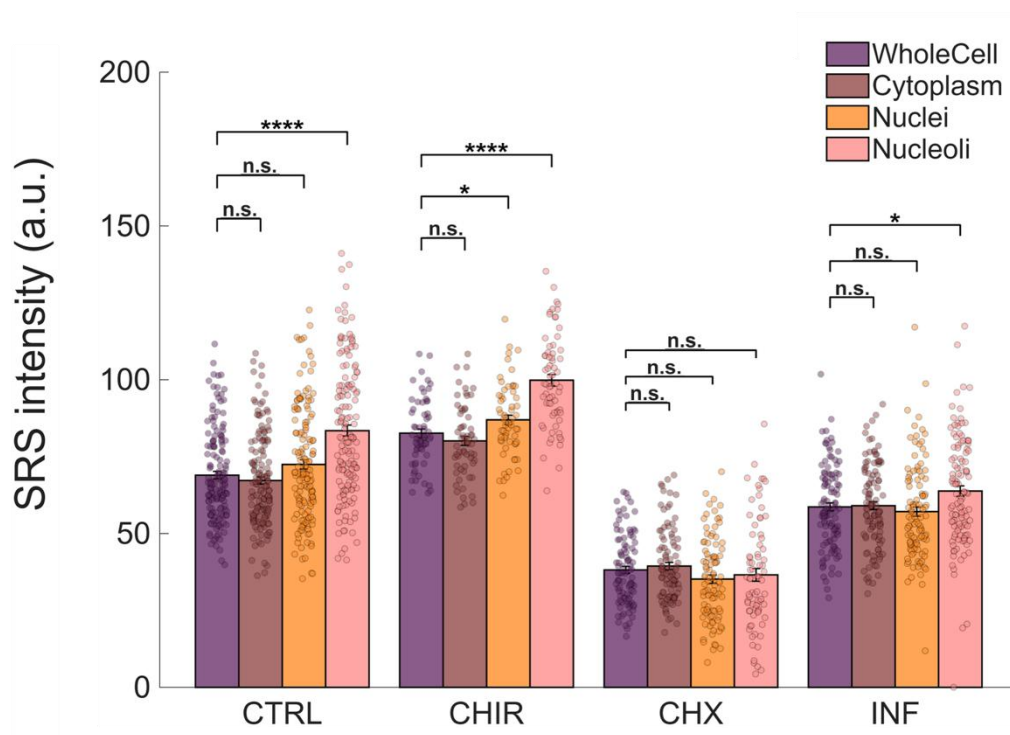


Figure S11. Subcellular analysis of protein synthesis using MetaboRamics.

SRS intensities per cell for d₅-Phe at the subcellular level (whole-cell, cytoplasm, nuclei and nucleoli) for A549 control cells (no drug) or cells treated with INF or pharmacological perturbations: CHIR and CHX. Data represented as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was conducted using Welch's two-paired t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns= no statistical difference.

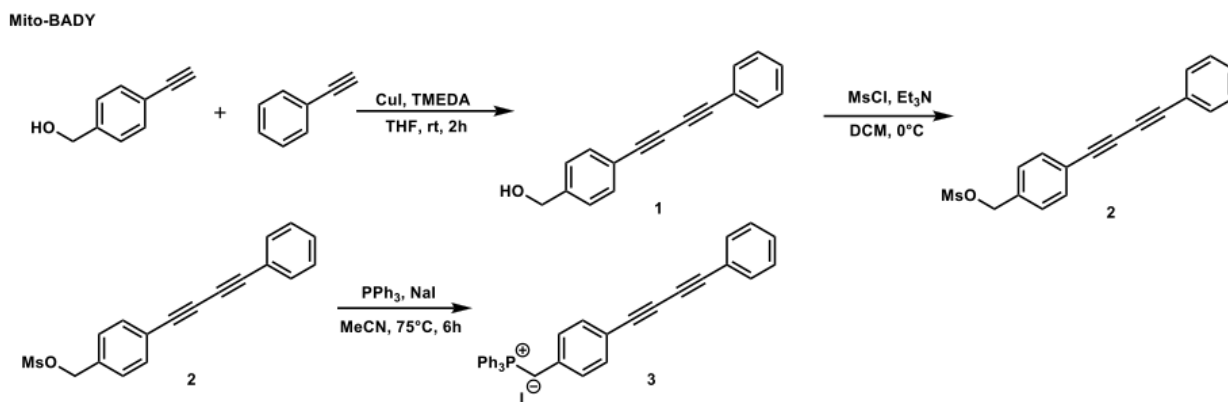
Synthesis of MitoBADY (MB)

MitoBADY was synthesized following a protocol adapted from a previous report.⁶⁰ In a 100 mL flask, CuI (200 mg, 2 mmol) and TMEDA (600 μ L, 4 mmol) were added to 10mL of acetone, and the mixture was bubbled with air for 10 minutes at room temperature. Next, 4-

ethynylbenzyl alcohol (528 mg, 4 mmol, 1.0 eq), and phenylacetylene (1.32 mL, 12 mmol, 1.0 eq) were charged with 50 mL of degassed THF. The reaction was then stirred for 2 hours under air at room temperature. The mixture was concentrated in vacuo and subjected to flash column chromatography with a solvent gradient (0-1% MeOH in DCM), affording compound **1** in 35% yield.

In a 100 mL flame-dried flask, compound **1** is dissolved with 20 mL anhydrous DCM, and subsequently triethylamine (0.58 mL, 4.2 mmol, 1.2 eq) was added. The reaction mixture was then cooled with ice bath, and MsCl (0.17 mL, 2.09 mmol, 1.1 eq) was slowly injected into the reaction mixture, and the reaction was slowly raised to room temperature and allowed to stir at room temperature for 6 hours, and the reaction was monitored with TLC to observe almost full conversion after 6 hours. The reaction was quenched with 20 mL saturated sodium bicarbonate solution, and the reaction mixture was extracted with 50 mL DCM 3 times, and the combined organic phase was dried of Na₂SO₄, and the organic solvent was removed in vacuo, and the crude mixture was purified with flash column chromatography with DCM:MeOH=100:1 to afford the product compound with 42% yield.

In a 100 mL flame-dried flask, compound **2** (660 mg, 1.5 mmol, 1.0 eq) was added with PPh₃ (787 mg, 3.0 mmol, 2.0 eq) NaI (450 mg, 3.0 mmol, 2.0 eq) and dissolved with 25 mL anhydrous MeCN. The reaction was then stirred at 75°C for 6 hours, and the reaction was diluted with 100 mL DCM and filtered with celite, and the organic solvent was removed in vacuo, and the crude mixture was purified with flash column chromatography to afford the final product **3** with 77% yield.



Synthesis of Me-Lyso (ML)

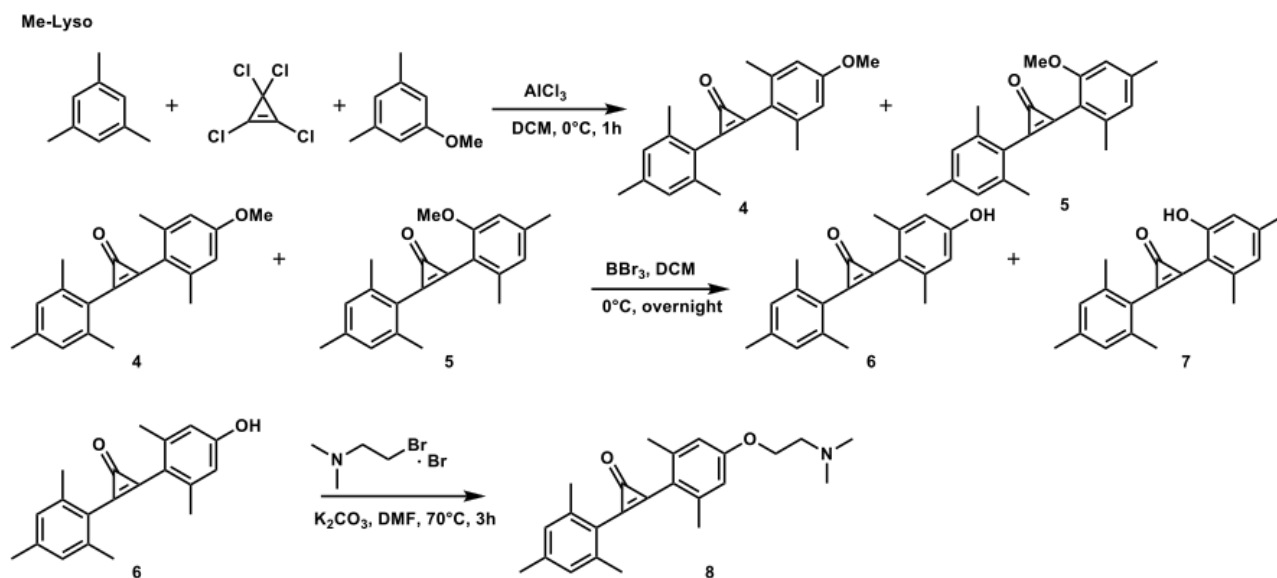
Me-Lyso was prepared using an improved adaptation of our previously reported protocol.⁶¹

In a 250 mL flame-dried flask, 40 mL of anhydrous dichloromethane was charged with AlCl_3 (6.0 g, 44.8 mmol). The mixture was cooled to 0°C with an ice bath, followed by the dropwise addition of tetrachlorocyclopropene (2.0 g, 1.2 mL, 11.2 mmol). After 10 minutes of stirring at 0°C , mesitylene (1.344 g, 11.2 mmol) was added, and the reaction mixture was stirred for 90 minutes at 0°C . Next, 3,5-dimethylanisole (1.524 g, 11.2 mmol) was added, and the mixture was allowed to return to room temperature. After stirring for 1 hour, the mixture was lowered again to 0°C and quenched with NH_4Cl . The organic layer was extracted with dichloromethane, washed with brine, and dried over anhydrous Na_2SO_4 . The crude mixture was purified with flash column chromatography with a solvent gradient (15-45% EA in hexanes), giving a mixture of Compounds **4** and **5** in 52% yield.

A 100 mL flame-dried flask was charged with the mixture of compounds **4** and **5** (1.74 g, 5.8 mmol) and 25 mL of anhydrous DCM. The mixture was lowered to 0°C , and 1M BBr_3

in DCM (15 mL, 15 mmol) was added slowly. The reaction mixture was raised to room temperature and allowed to run overnight. At 0°C, the reaction was slowly quenched with saturated NH₄Cl solution. The organic phase was extracted with DCM, washed with brine, and dried over anhydrous Na₂SO₄. The crude mixture was purified with flash column chromatography with a solvent gradient (5-35% EA in hexanes), giving compounds **6** and **7** (4:1) in 22% yield.

In a 50 mL flame-dried flask was charged compound **6** (100 mg, 0.34 mmol, 1.0 eq) was added with (2-bromoethyl)dimethylamine hydrobromide (159 mg, 0.68 mmol, 2.0 eq) and K₂CO₃ (152 mg, 1.1 mmol, 3.5 eq). The mixture was dissolved with 10 mL dry DMF and the reaction was stirred at 70°C for 3 hours. The reaction was then quenched with 15 mL saturated NH₄Cl aqueous solution and extracted with 30 mL ethyl acetate 3 times, the organic phase was dried over Na₂SO₄ and concentrated in vacuo. The crude mixture was then purified with flash column chromatography with Hex:EA=2:1 to afford the final product **8** as a white solid with 57% yield.



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OPTICAL METABOLIC IMAGING OF TRICARBOXYLIC ACID CYCLE ACTIVITY

Chadha, R.[‡]; Kocheril, P. [‡]; Colazo, A. [‡]; Kapelczak, E.; Suen, B.; Yang, Z.; TeSlaa, T.²; Wei, L.¹ Optical metabolic imaging of the tricarboxylic acid cycle.

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[‡] = Equal Contribution

¹ = Division of Chemistry and Chemical Engineering, California Institute of Technology, 6 Pasadena, CA 91125, USA

² = Department of Molecular and Medical Pharmacology, David Geffen School of Medicine 9 at UCLA, Los Angeles, CA 90095, USA

4.1 Summary

The tricarboxylic acid (TCA) cycle lies at the core of cellular metabolism, integrating energy production, biosynthesis and redox homeostasis, yet direct quantitative imaging of its activity in living systems remains challenging. We introduce MATRIX-SRS (Metabolic Activity TRacing of the trIcarboXylic acid cycle by Stimulated Raman Scattering microscopy), a platform enabling spatially resolved quantification of TCA-linked metabolism in live cells. Using emerging deuterium-labeled probes, MATRIX-SRS visualizes subcellular TCA-linked carbon-deuterium bonds in live cancer cells, and neurons. Complementing isotope-tracing mass spectrometry with SRS, we uncover a global attenuation of TCA activity during epithelial-to-mesenchymal transition, achieving deep molecular insights. We then integrate density functional theory, reaction network mapping, and hyperspectral SRS to construct a robust in situ metabolic quantification pipeline. Applying this framework, we further quantify changes in deuterium-labeled biomass in absolute concentrations, under native and

drug-treated conditions, for the first time. Together, MATRIX-SRS provides a generalizable foundation for live quantitative spatial metabolomics.

4.2 Introduction

The TCA cycle, also known as the Krebs cycle or the citric acid cycle, is regarded as the central metabolic engine of cells.¹ Beyond their role in energy production, TCA intermediates function as precursors for biomacromolecule synthesis and are replenished through anaplerotic reactions to sustain the metabolic balance in cells.² However, dysregulation of TCA cycle intermediates, often resulting from mutations in their regulatory enzymes, has been implicated in neurodegenerative diseases such as Alzheimer's disease^{3,4} and Parkinson's disease,⁵ metabolic disorders such as metabolic-associated fatty liver disease,⁶ and various cancers.^{7,8}

Significant advancements have been made toward visualizing metabolites in biological systems, but the enabling methods each have intrinsic limitations. Fluorescence microscopy allows ultrasensitive dynamic visualization of metabolites at the subcellular level but may perturb their native behavior, since it usually requires labeling with bulky fluorophores that are much larger than the metabolites themselves. Genetically encodable biosensors are highly compatible with live cells, but they require specific expression that may perturb native cellular states, can exhibit bias from other environmental factors (such as pH, temperature, and viscosity),⁹ and are limited to only a handful of targets.¹⁰ Mass spectrometry imaging allows spatial mapping with high chemical specificity but remains destructive and hence incompatible for live-cell analysis.¹¹ Positron emission tomography and magnetic resonance

imaging (MRI) are highly biocompatible and non-invasive but have not yet reached subcellular resolution and still suffer from poor sensitivity and metabolic resolvability.^{12,13} Therefore, there is a critical need for tools that would enable quantitative subcellular mapping of TCA-linked metabolic activity.

Stimulated Raman scattering (SRS) microscopy has emerged as a powerful live-cell metabolic imaging platform over the past decade. Leveraging the $\sim 10^8$ -fold stimulated emission signal amplification compared to spontaneous Raman scattering, SRS has achieved imaging sensitivity of small metabolites down to the μM level, with a spatial resolution of ~ 400 nm and imaging speeds up to video rate. SRS has allowed label-free imaging by targeting the unique vibrations of chemical bonds from endogenous metabolites.^{14–18} With small vibrationally labeled (e.g., deuterium-labeled) metabolites, metabolic dynamics can be further probed by hyperspectral SRS (hSRS) while maintaining high biomolecular fidelity, owing to the minimal change of physico-chemical properties of the molecules from small vibrational tags. Notably, by coupling SRS with deuterated glucose (i.e., d_7 -glucose) labeling, glucose metabolism has been investigated for assessing newly synthesized biomass, including lipids, proteins, nucleic acids (DNA and RNA), and glycogen (each with unique Raman spectra), from live cells to animals.^{19–22} While SRS studies with glucose have revealed new insights into cellular metabolism, studying other key metabolites involved in the TCA cycle with SRS remains challenging, mostly due to their low intracellular abundance, transient nature, and chemical heterogeneity.²³ Another limitation of current spectral interpretations of metabolized biomolecular species is their largely empirical nature and limited resolvability. A physically grounded Raman spectral analysis would therefore

substantially improve both precision and quantitative accuracy over the prior state-of-the-art.^{19–22}

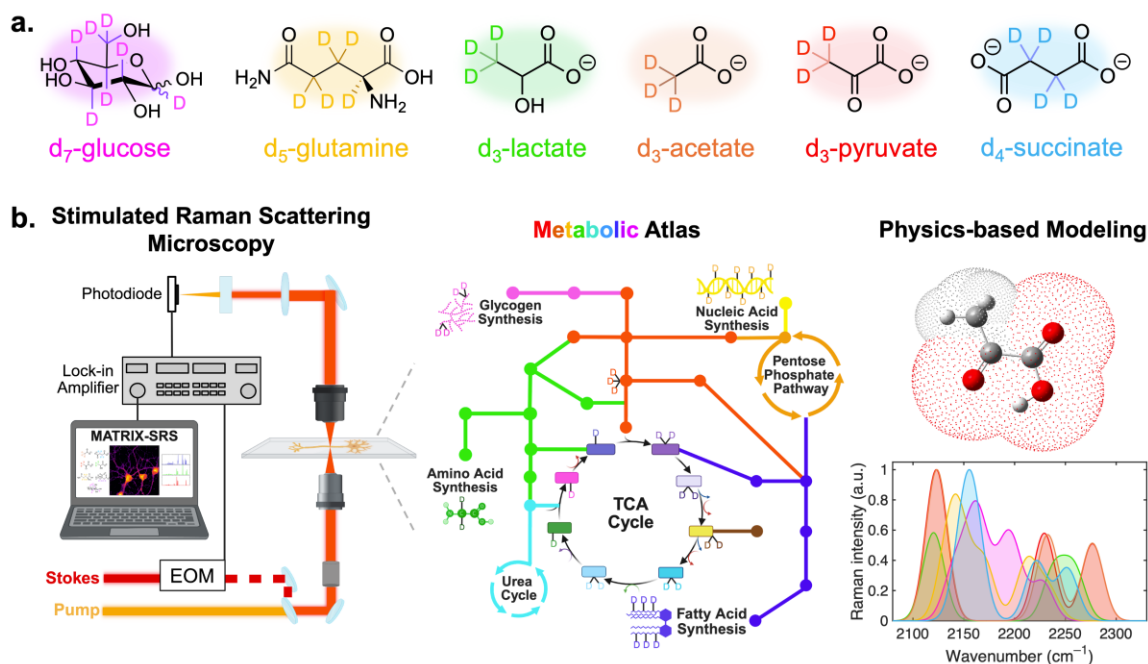


Figure 1. MATRIX-SRS workflow. (a) Chemical structures of TCA-linked Raman probes; (b) Left to right: schematic of the stimulated Raman scattering (SRS) microscopy setup; metabolic atlas tracing using deuterium-labeled probes; and physics-based modeling with density functional theory (DFT) for Raman peak assignments and quantification.

In this work, we describe MATRIX-SRS, Metabolic Activity TRacing of the trIcarboXylic acid cycle via SRS microscopy (**Fig. 1**). Through careful optimization, we first expand the palette of TCA probes accessible to metabolic SRS imaging with deuterium labeling, visualizing TCA-linked metabolic activity from glucose to glutamine, lactate, acetate, pyruvate, and succinate in live neurons and cancer cells (**Fig. 1a**). With detailed

spectroscopy, we demonstrate that our TCA probes are generally compatible in multiplex imaging applications. We next demonstrate that these probes reveal globally altered metabolism underlying epithelial-to-mesenchymal transition (EMT), a process whose metabolic basis remains highly elusive. We further dissect carbon flow into the TCA cycle by focusing on pyruvate, which is uniquely positioned at a branching point between the TCA cycle, glycolysis/gluconeogenesis, amino acid synthesis, and *de novo* lipogenesis (DNL). To gain deeper molecular-level insights, we complement our SRS imaging with stable isotope-labeled (SIL) tracing using liquid chromatography-mass spectrometry (LC-MS), observing excellent quantitative agreement in metabolic trends in EMT. Based on these results, we develop a theoretical foundation for quantitative interpretation of biomass contributions arising from deuterated metabolites, using physics-based modeling built on density functional theory (DFT) (**Fig. 1b**). This model provides a general and accurate framework for *in situ* compositional profiling, transcending the conventional empirical assignments from SRS alone. Finally, through pharmacological perturbation, we assess the lipid and protein biomass derived from d₃-pyruvate incorporation, linking live-cell SRS imaging with biochemical flux measurements. Integrating experiments, theory, and modeling, MATRIX-SRS provides a unique live-cell optical strategy toward intracellular quantitative spatial metabolomics.

4.3 Results

Visualization of TCA cycle metabolism in live cells using MATRIX-SRS

We first screened stable and non-toxic TCA-related deuterium probes compatible with cell culture, from established d₇-glucose (d₇-glc)^{19–22} to emerging TCA probes including d₅-

glutamine (d_5 -gln),^{22,24} d_3 -lactate,²² d_3 -acetate, d_3 -pyruvate (d_3 -pyr), and d_4 -succinate (**Fig. 1a** and **Fig. S1**). Recently, SRS imaging of lactate and glutamine was demonstrated in gluconeogenic conditions,²² but our current work demonstrates quantitative imaging of these probes under native conditions, fueling new insights. After incubation for 72 hours, we directly visualized the biomass produced from each metabolite at the subcellular level with SRS in live HeLa cells (**Fig. 2a**) and live primary neuronal co-cultures (**Fig. 2b**), demonstrating the broad applicability of MATRIX-SRS imaging. We also successfully imaged d_4 -citrate in live HeLa cells (**Fig. S2**) but did not achieve similar image quality in neurons, highlighting the need for careful probe selection and optimization to obtain robust imaging results (**Fig. 2a-b**).

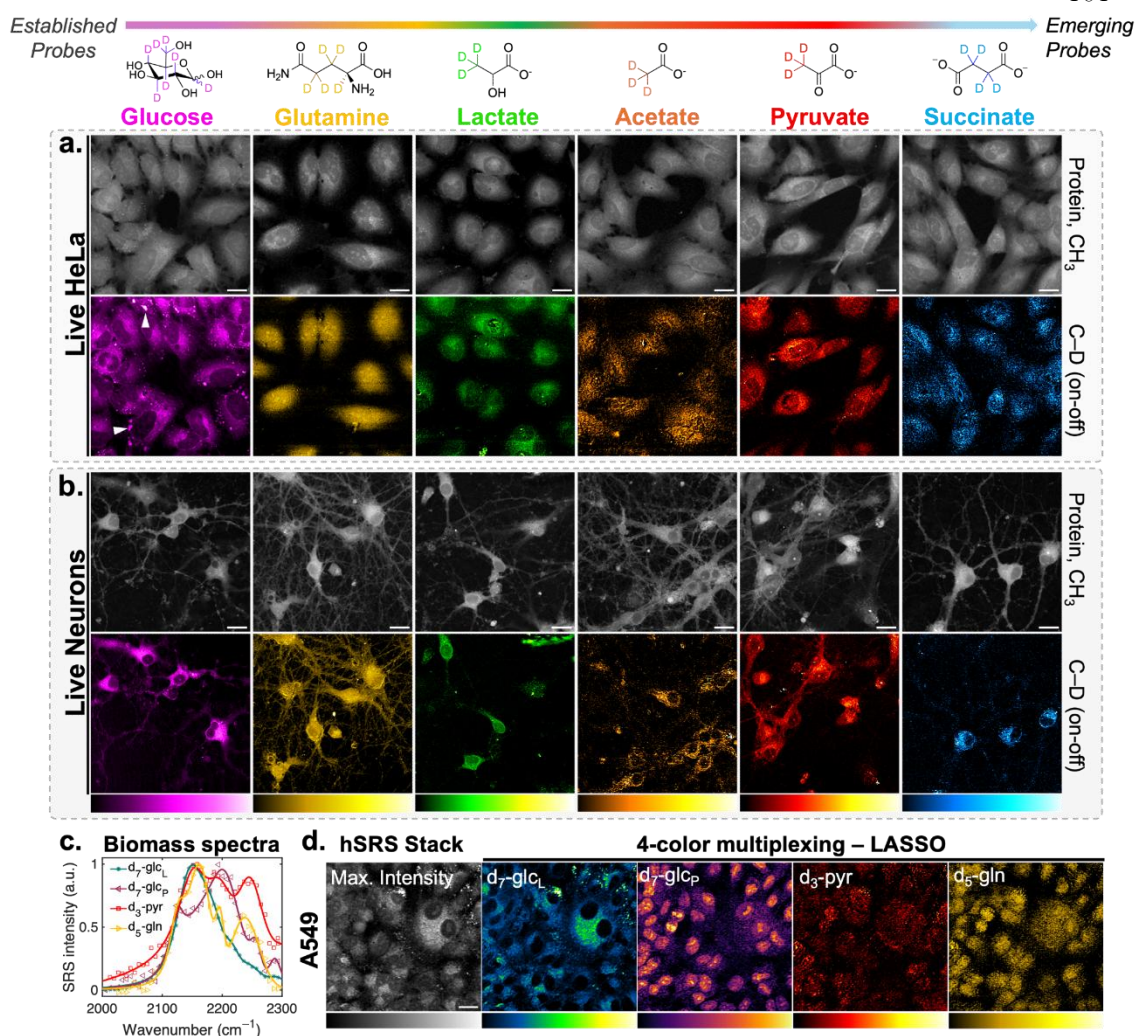


Figure 2. Live-cell subcellular metabolic imaging of TCA-linked activity using MATRIX-SRS. SRS images targeted at the $-\text{CH}_3$ channel (protein, 2940 cm^{-1}) and C–D (on-off) for six metabolic probes: d_7 -glucose, d_5 -glutamine, d_3 -lactate, d_3 -acetate, d_3 -pyruvate and d_4 -succinate in live **(a)** HeLa and **(b)** neuronal co-cultures. White arrowheads indicate subcellular glycogen pools. **(c)** Normalized hSRS spectra of the biomass from i) d_7 -glucose-derived lipids ($\text{d}_7\text{-glc}_L$; blue), ii) d_7 -glucose-derived proteins ($\text{d}_7\text{-glc}_P$; purple), iii) d_3 -pyruvate ($\text{d}_3\text{-pyr}$; red), and iv) d_5 -glutamine ($\text{d}_5\text{-gln}$; yellow); **(d)** Four-color multiplex

imaging of glucose, glutamine and pyruvate metabolism in A549 cells using MATRIX-SRS and LASSO unmixing; Scale bar: 20 μm .

Interestingly, SRS images also revealed distinct spatial patterns between these cell types. For instance, consistent with prior reports,²¹ we found subcellular glycogen reservoirs in live HeLa cells, but not in neurons. Additionally, in HeLa, d_3 -acetate and d_4 -succinate imaging showed a largely homogenous distribution across the entire cell, whereas in the neuronal co-culture, C–D signal from these probes was predominantly cytoplasmic. In contrast, d_3 -lactate imaging showed appreciable biomass in neuronal nuclei, demonstrating that, despite being closely chemically related, each probe can report unique subcellular metabolic information. Since different probes can report on different metabolic processes and dynamics, complex metabolic interactions are best visualized by multiplex imaging of multiple species. It is well established that molecular vibrations act as unique ‘fingerprints,’ and using the frequency-dependence of SRS signal (i.e., hSRS) allows for direct, *in situ* profiling of these spectral fingerprints. After measuring hSRS spectra of A549 cells treated individually with each of our TCA probes, we indeed observe unique spectral fingerprints in the biomass derived from each probe (**Fig. 2c**). These biomass spectra are also substantially different from the Raman spectra of the probes in solution (**Fig. S1**), as evidence that the probes have been significantly metabolized and converted into downstream biomolecular species.

Although the biomass spectra are significantly overlapped (**Fig. 2c**), we hypothesized that the slight differences between the spectra originating from these TCA probes could still enable multiplexing. To test this hypothesis, we treated A549 lung carcinoma epithelial cells

with a mixture of d₇-glc, d₃-pyr, and d₅-gln. We then acquired an hSRS image stack, tuning through several vibrational frequencies, and demonstrated robust unmixing performance with the least absolute shrinkage and selection operator²⁵ (LASSO; **Fig. S3**), differentiating between the biomass originating from d₃-pyr and d₅-gln, and lipids and proteins derived from d₇-glc (d₇-glc_L and d₇-glc_P, respectively; **Fig. 2d**). The observed spatial distributions of each unmixed species correlate well with our single-probe SRS images (**Fig. 2a-b**), and with previous work on d₇-glc and d₅-gln, further validating these results.^{20,21} We achieved similarly robust unmixing in a mixture of d₇-glc, d₃-pyr, and d₃-lactate (**Fig. S4**), demonstrating that these probes are broadly compatible in complex mixtures. MATRIX-SRS therefore allows direct comparison of TCA-derived biomass generation and spatial patterns between cell types for probing subcellular metabolic distribution.

MATRIX-SRS reveals metabolic differences in epithelial-to-mesenchymal transition

We next applied MATRIX-SRS to investigate the TCA-related metabolic changes underlying EMT, which remains elusive at the metabolic level.²⁶ During the EMT process, epithelial cells lose cell-cell adhesion and polarity while acquiring migratory and invasive mesenchymal properties,^{27,28} bearing deep relevance in understanding cancer progression and therapeutic resistance (**Fig. 3a**). Although recent studies have identified select metabolic alterations during EMT,²⁹ most approaches rely on bulk measurements,³⁰ transcriptional readouts,³¹ or other invasive methods³² that do not capture the spatiotemporal metabolic heterogeneity or rewiring at the subcellular level in living systems.

To model EMT, we first induced A549 epithelial cells (epi) to adopt a mesenchymal (mes) phenotype using a commercial cocktail of proteins and antibodies, as confirmed by characteristic morphological changes such as cellular elongation (protein channel, **Fig. 3b**) and immunostaining (**Fig. S5**). Consistent with prior literature on MCF7 cells,²⁹ metabolic imaging of d₇-glc in live A549 cells showed reduced glucose metabolism in mesenchymal cells compared to epithelial cells (~36%, **Fig. 3b-c**). Furthermore, MATRIX-SRS imaging revealed a global reduction in TCA-associated metabolic activity into the newly synthesized biomass during EMT, as indicated by the attenuated C–D signal for d₅-gln (~51%), d₃-lactate (~42%), d₃-acetate (~46%), d₃-pyr (~29%) and d₄-succinate (~36%) (**Fig. 3b-c**). These results highlight the potential of MATRIX-SRS in quantitatively investigating subcellular TCA-linked metabolism. In general, we observed a ~30-40% reduction in total C–D signal in the biomass corresponding to the metabolic probes in EMT. Since many metabolic changes underlying EMT depend on the model system and remain controversial,^{26,33} we highlight the potential of MATRIX-SRS to better understand these longitudinal changes at the subcellular level in different systems.

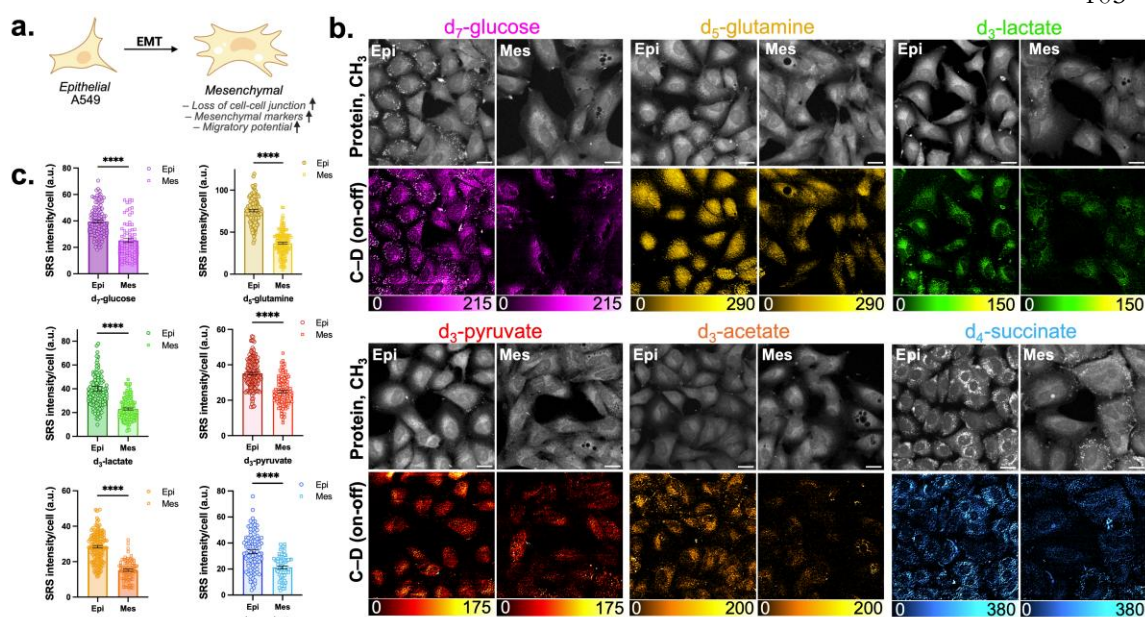


Figure 3. MATRIX-SRS for visualization of metabolic changes underlying EMT. (a) illustration showing epithelial-to-mesenchymal transition of A549 cells that is indicated by profound morphological changes, an increase in mesenchymal markers and migratory potential; (b) Live-cell SRS images targeted at the $-CH_3$ channel (proteins, 2940 cm^{-1}) and C-D (on-off) for epithelial (epi) and mesenchymal (mes) cells for the six metabolic probes: d_7 -glucose, d_5 -glutamine, d_3 -lactate, d_3 -acetate, d_3 -pyruvate, and d_4 -succinate. Scale bar: $20\text{ }\mu\text{m}$; (c) Quantitative analysis of SRS signal of C-D bonds per cell. Data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-tests. **** $p < 0.0001$.

Complementary LC-MS SIL tracing provides molecular insight into reduced pyruvate utilization during EMT

To more accurately characterize the metabolic fate of the deuterium labeled probes and provide complementary confirmation of our SRS imaging results, we leveraged the high

synergy between SRS microscopy and mass spectrometry.²² We chose d₃-pyr, as a central intermediate in cellular metabolism uniquely positioned between the TCA cycle, glycolysis, protein synthesis, and DNL, for complementary *in vitro* tracing via LC-MS for EMT. We incubated the two cell types (epi and mes) using d₃-pyr for 24 hours (**Fig. 4a**). As a metabolically active intermediate, pyruvate is known to anaplerotically incorporate into other small molecules through the TCA cycle. Given the enzymatic nature of these reactions, starting with d₃-pyr should lead to the production of TCA intermediates (**Fig. 4b**) and many other biological molecules (**Fig. S6**) with highly specific deuterated motifs.

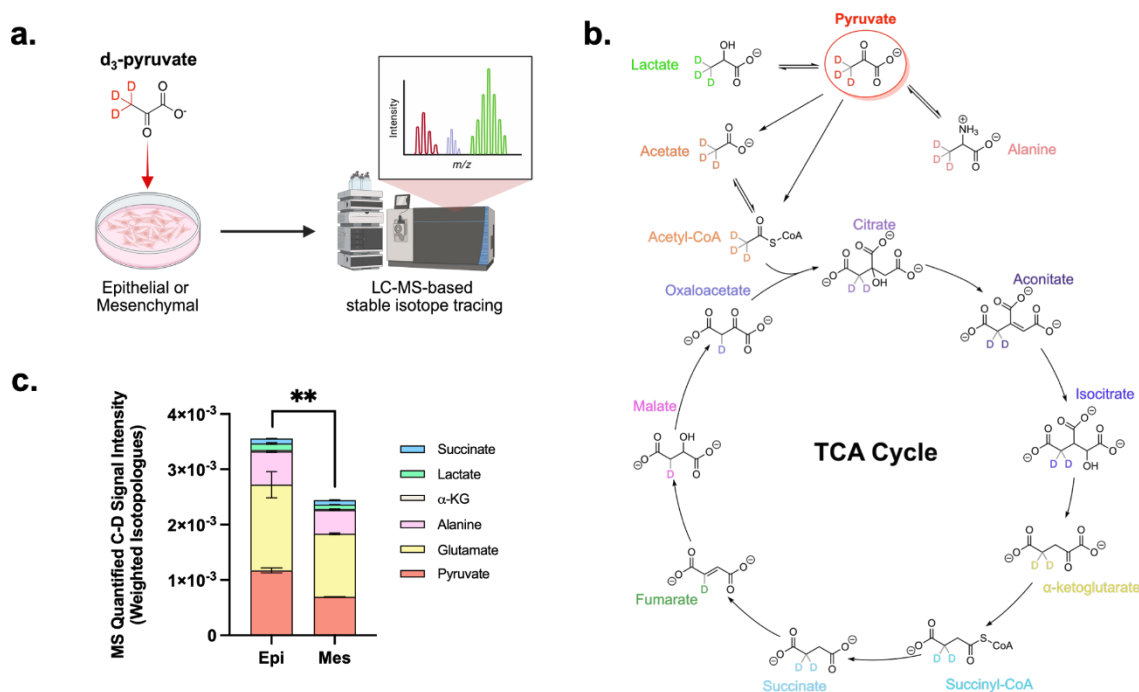


Figure 4. [3,3',3''-²H₃]-pyruvate *in vitro* tracing using LC-MS. **(a)** Illustration showing the *in vitro* tracing workflow using d₃-pyruvate in A549 (epithelial or mesenchymal) cells; **(b)** Schematic of the flow of d₃-pyruvate into the TCA cycle. **(c)** MS-quantified C-D incorporation (weighted isotopologues) of d₃-pyr in TCA cycle intermediates. Data are

presented as mean $\pm$ SEM from three independent experiments. Statistical significance was analyzed using two-tailed unpaired Welch's t-test. $**p < 0.01$. Fractional isotopologue distributions are shown in **Fig. S7**.

Our *in vitro* LC-MS tracing data revealed that mesenchymal cells exhibit ~30% lowered intracellular d_3 -pyr when compared to epithelial cells (**Fig. 4c**), showing excellent consistency with our SRS findings. More specifically, SIL tracing further indicated extensive conversion of d_3 -pyr into downstream metabolic species such as lactate, alanine, and citrate (**Fig. S7**), with the largest differences between epithelial and mesenchymal cells stemming from total glutamate and total pyruvate (**Fig. 4c**). Furthermore, examining the isotopologue distributions of individual metabolites, mesenchymal cells showed a significant attenuation of the M+3 and M+2 labeled fractions for acetylcarnitine (proxy for acetyl-CoA³³) as well as citrate (M+1 and M+2 labeled fractions) which feeds into DNL,³⁴ compared with their epithelial counterparts. Furthermore, pyruvate, which can be transaminated to alanine for protein synthesis,³⁵ showed a reduced contribution to all alanine isotopologues in mesenchymal cells, indicating attenuated protein synthesis derived from d_3 -pyr. Taken together, these LC-MS tracing results complement our findings from MATRIX-SRS and provide deeper molecular specificity in understanding the metabolic fate of d_3 -pyr.

Hyperspectral MATRIX-SRS and DFT-derived spectral profiling

Inspired by the molecular specificity gained by LC-MS, we sought to develop a quantitative framework to determine concentration maps of metabolized C–D probes with hSRS. Upon close examination, we noted that the biomass spectra exhibited peaks at similar positions,

differing primarily in their relative amplitudes (**Fig. 2c**). Such observations have been made previously with hSRS but remain poorly understood, with spectral assignments being largely empirical.²⁰ Quantum mechanical calculations, such as DFT, have significant potential to aid in understanding experimental spectra, but these tools have not yet been applied to interpreting biomass spectra for two main reasons. First, DFT is scarcely used in SRS, due to the difficulty in determining an appropriate computational method and level of theory, the complexity of the target species (e.g., molecules with multiple possible protonation states) and a general perception that DFT is not quantitatively accurate for Raman spectroscopy.³⁴ Second, to interpret *in situ* biomass spectra, computations must be performed at large scales, with the ability to calculate accurate spectra for dozens (if not hundreds) of diverse intermediate molecules, necessitating significant work in the implementation of DFT for metabolic SRS.

To address these challenges, we developed AutoDFT 2.0: a fully automated computational pipeline for calculating Raman spectra of selectively deuterated molecules requiring only ChemDraw structures as inputs (**Fig. S8**). AutoDFT 2.0 builds upon our previous work with automating DFT for calculations of quantitatively accurate vibrational spectra. In our previous work computing vibronic spectra of near-infrared organic dyes,³⁵ we observed a significant improvement in computed spectral accuracy by using the solvation model with density (SMD) as an implicit solvent model.³⁶ Compared with standard solvent continuum models, SMD is unique because it includes additional terms for short-range intermolecular interactions, such as acidity/basicity and surface tension, in addition to the dielectric description of most standard solvent models. Applying these same optimized methods, we

calculated a theoretical Raman scattering spectrum for d₃-pyr, obtaining excellent agreement with our experimental results (**Fig. 5a**). This high accuracy also generalizes to other molecules; we observed strong agreement across our TCA probes in frequency ($R^2 = 0.94$; **Fig. 5b**) and similarly strong agreement in intensity ($R^2 = 0.93$; **Fig. S9**). After performing detailed metabolic deuterium mapping (**Fig. S6**), we applied AutoDFT 2.0 to our entire library of mapped metabolites, totaling 69 unique structures (**Fig. S10**), generating comprehensive, physically informed knowledge about the vibrational features of myriad deuterium-labeled chemical structures.

Upon analyzing our DFT-computed spectra, we observed a striking clustering of spectral fingerprints based on the chemical structures of the deuterated molecules (**Fig. 5c**). Singly deuterated intermediates (d₁; **Fig. 5c**, purple) exhibit a local C–D stretching mode at $\sim 2200\text{ cm}^{-1}$. For molecules with deuterated methylenes (d₂; **Fig. 5c**, pink), the C–D stretching band is split into a redshifted, symmetric stretching band ($\sim 2160\text{ cm}^{-1}$) and a blueshifted, asymmetric stretching band ($\sim 2240\text{ cm}^{-1}$). Fully deuterated methyl groups (d₃; **Fig. 5c**, orange) exhibit a further redshifted symmetric stretching band ($\sim 2120\text{ cm}^{-1}$) and two tightly clustered asymmetric stretching bands (both $\sim 2240\text{ cm}^{-1}$). Computed spectra for deuterated fatty acids (of the form $-(\text{CDHCH}_2)_n-$ from our reaction mapping; **Fig. S6**) exhibit a single strong band at $\sim 2160\text{ cm}^{-1}$ (**Fig. 5c**, teal). Furthermore, the fatty acid scattering intensity is highly correlated with the number of deuteriums in the chain ($R^2 = 0.93$; **Fig. S11**), allowing us to readily quantify lipid signals on a per-deuterium basis (CDHCH₂).

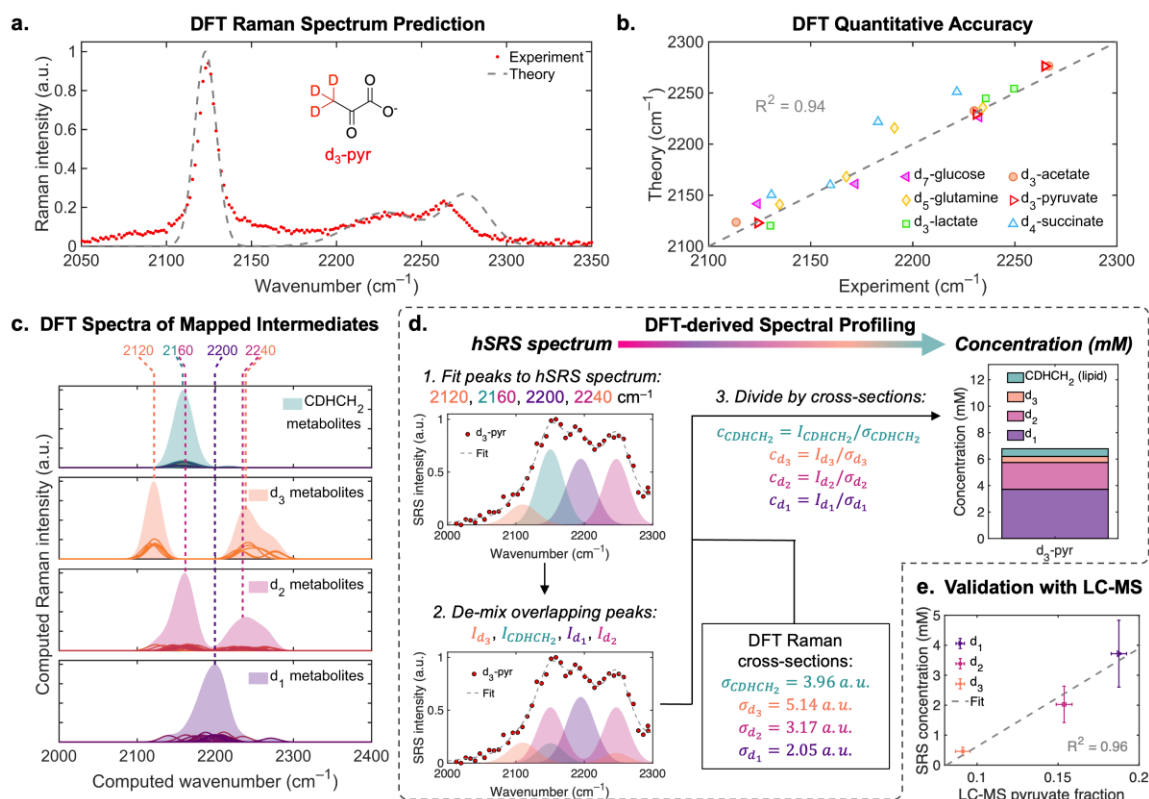


Figure 5. DFT spectral validation and compositional profiling.

(a) Comparison of DFT-computed Raman spectrum for d_3 -pyr and an experimental Raman spectrum of d_3 -pyr in solution. **(b)** Comparison of calculated and experimental vibrational frequencies across all TCA probes in solution. **(c)** Aggregated DFT spectra of mapped deuterated TCA intermediates. Four distinct bands at 2120, 2160, 2200, and 2240 cm^{-1} are observed, characteristic of different labeled species. **(d)** DFT-derived spectral profiling. Fitting hSRS spectra at 2120, 2160, 2200, and 2240 cm^{-1} and dividing by DFT-derived cross-sections (**Fig. S12**) allows for *in situ* compositional profiling and absolute concentration determination for TCA metabolites. **(e)** Correlation between DFT-derived SRS concentrations and LC-MS-detected pyruvate fractions. Error bars are $\pm$ one standard deviation for LC-MS and $\pm 30\%$ (estimated DFT error; see **Fig. S12**) for SRS.

To convert from hSRS spectra to concentration, we leveraged that SRS signal is proportional to the product of the concentration of an analyte (within the focal volume) and the scattering cross-section of the analyte. With the consistency achieved from DFT computed intensities with experimental data, we further derived average scattering cross-sections for each of the four deuterated motifs (d_1 , d_2 , d_3 , and $CDHCH_2$), which we divide by our experimentally measured intensities to determine concentrations (**Fig. 5d**; see also **Fig. S12**). Applying this framework for d_3 -pyr, we obtained a total deuterated biomass concentration of 6.8 ± 2.0 mM. On a per-species basis, we find that most of the deuterium biomass is found in d_1 species (~ 4 mM) with progressively less in d_2 and d_3 species (**Fig. 5d**; see also **Table S1**). Relatively little deuterium biomass (<1 mM) is found in the internal positions in aliphatic lipid tails ($CDHCH_2$), but an appreciable amount of the measured d_1 , d_2 , and d_3 biomass may still originate from lipids due to head group functionalization and the terminal methyl groups of fatty acids, with the remainder likely originating from proteins (**Fig. S6**). To our knowledge, this is the first time that absolute concentrations of metabolized deuterium probes have been determined *in situ* by SRS microscopy.

In principle, our SRS quantification results should be comparable with our LC-MS-quantified isotopologue distributions, where the found $M+1$ peak corresponds to d_1 species, $M+2$ corresponds to d_2 , and $M+3$ corresponds to d_3 . Upon attempting this comparison, we found surprisingly excellent agreement ($R^2 = 0.96$; **Fig. 5e**), with LC-MS similarly indicating globally lower abundances for more highly deuterated species (**Fig. S7**). These results validate our DFT-derived hSRS spectral profiling as a means of determining absolute *in situ*

concentrations of metabolized C–D probes with quantitative accuracy. We also applied this quantitative pipeline to d₇-glc, a much more well-studied Raman metabolic probe, allowing for direct quantification of the compositions of d₇-glc_L and d₇-glc_P (**Fig. S13**).

Biochemically speaking, the conversion of our starting material, d₃-pyr, into d₂ and d₁ species indicates the active metabolism of d₃-pyr (where unreacted d₃-pyr would exhibit purely d₃ biomass). For example, the conversion of d₃-pyr into d₂-citrate (through acetyl-CoA; **Fig. 4b**) necessitates a deuterium abstraction, leading to the loss of d₃ biomass and gain of d₂ biomass. Similarly, the dehydrogenation of d₂-succinate to produce d₁-fumarate converts d₂ biomass into d₁ biomass. These TCA intermediates can then be subsequently incorporated into stable downstream proteins or lipids, such as the feeding of citrate into DNL or conversion into amino acids, leading to build-up of deuterated biomass from d₃-pyr turnover. For the first time, MATRIX-SRS allows for live-cell-compatible quantification of deuterium biomass. In tandem with LC-MS to obtain metabolite-specific isotopologue distributions, our integrative approach provides a uniquely detailed perspective of global cellular metabolism. Moreover, the enrichment of different C–D species could imply the preference of one metabolic pathway over another for a given metabolic precursor. For example, observing appreciable d₃ biomass in newly synthesized proteins could indicate rapid conversion of d₃-pyr into d₃-alanine via transamination (**Fig. 4c**), while conversion into other amino acids generally entails deuterium abstraction (**Fig. S6**), meaning d₁ and d₂ species are expected to be the dominant sources of total protein biomass (as with d₇-glc_P; **Fig. S13**). Moreover, the conversion of d₃-pyr into d₃-lactate (mediated by NADH) can report the redox

state of the cell. Our approach could hence facilitate quantitative comparisons between different treatment conditions to investigate the intricate metabolic network.

Pharmacological perturbation of d₃-pyruvate metabolism

To quantitatively investigate metabolic perturbation, we next pharmacologically treated these metabolic pathways to visualize their outcomes using SRS imaging in live A549 cells incubated with d₃-pyr. We first inhibited ATP-citrate lyase (ACLY), the enzyme that converts cytosolic citrate into acetyl-CoA (and subsequently fatty acids) following a series of biochemical reactions in DNL³⁷ (**Fig. 6a**). Surprisingly, pharmacological perturbation of ACLY using three distinct small molecule inhibitors (SMIs) – bempedoic acid (BA, or ETC-1002)³⁸, SB-204490³⁹, and BMS-303141⁴⁰ – did not significantly attenuate the C–D signal from d₃-pyr in live A549 cells when compared to a control group of untreated cells (**Fig. S14**). These results are consistent with previous findings suggesting that pyruvate may be converted into acetate and then into acetyl-CoA through ACLY-independent pathways.^{41,42} We next perturbed acetyl-CoA carboxylase (ACC), the enzyme that mediates fatty acid synthesis by converting acetyl-CoA into malonyl-CoA, using two specific SMIs (PF05175157⁴³ and ND-646⁴⁴). SRS imaging showed a dramatic decrease in d₃-pyr signal in A549 cells treated with these inhibitors compared to the control (**Fig. 6b-c**). Furthermore, inhibition of fatty acid synthase (FASN), which catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA,³⁷ using denifanstat⁴⁵ and C75⁴⁶ also resulted in decreased d₃-pyr signal. These results indeed suggest that pyruvate metabolizes through ACC and FASN enzyme activity. Lastly, inhibition of stearoyl-CoA desaturase 1 (SCD1), which converts saturated fatty acids (SFAs) to monounsaturated fatty acids (MUFAs), using CAY10566,

elicited an altered response; we found that the C–D intensity in these cells remained comparable to control cells (**Fig. 6b–c**). Interestingly, these cells exhibited enriched C–D signal in cytoplasmic pools, which we classify as solid membrane (SM) domains, as discovered previously in mesenchymal melanoma cancer cells.⁴⁷ The SM formation results from SCD1-induced imbalance between saturated and unsaturated fatty acids (due to increased SFAs) and is characterized by its distinct Raman spectrum (**Fig. S15a**). Taken together, our findings corroborate that a significant portion of d₃-pyr gets incorporated into downstream lipids via DNL.

Since d₃-pyr is also expected to contribute to newly synthesized proteins, we next modulated protein synthesis by either inhibiting it using cycloheximide (CHX)⁴⁸ or promoting it using CHIR-99021 (CHIR) through inhibition of glycogen synthase kinase 3 (GSK-3).⁴⁹ As expected, inhibition of protein synthesis with CHX significantly decreased the C–D signal in cells, whereas upregulation of protein synthesis with CHIR increased the overall C–D intensity (**Fig. 6d–e**). Additionally, hSRS imaging revealed an increase in the ~2200 cm⁻¹ and ~2240 cm⁻¹ bands in cells treated with CHIR (**Fig. S15b**), which are attributed to d₁- and (primarily) d₂-labeled metabolites including proteins, in contrast to cells that primarily exhibited lipid-rich subcellular SM pools (SCD1 inhibition in **Fig. 6b**, **Fig. S15a**) attributed to lipid (CDHCH₂) metabolites.

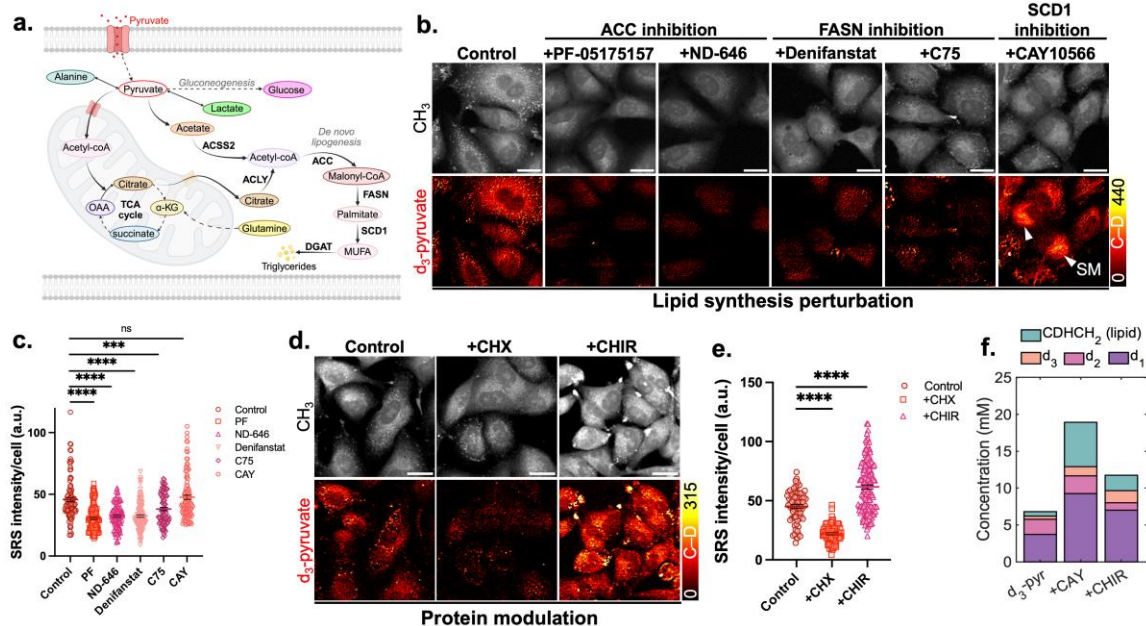


Figure 6. Pharmacological intervention of pyruvate metabolism. (a) Schematic of pyruvate metabolism in cells leading to *de novo* lipogenesis (DNL). ACC = acetyl-CoA carboxylase, ACLY = ATP-citrate lyase, ACSS2 = acyl-CoA synthetase short-chain family member 2, FASN = fatty acid synthase, SCD1 = stearoyl-CoA desaturase 1, DGAT = diacylglycerol acyltransferase, MUFA = monounsaturated fatty acid, SFA = saturated fatty acid; (b) Live-cell SRS images of A549 cells incubated with 30 mM d₃-pyruvate for 48 hours targeted at the protein channel (–CH₃, 2940 cm^{–1}) and C–D (on-off) for (i) control (no drugs), (ii) ACC inhibition (using 1 μM PF-05175157, or 1 μM ND-646), (iii) FASN inhibition (using 10 μM denifanstat, or 75 μM C75), or (iv) SCD1 inhibition (using 1 μM CAY10566) that resulted in SFA-induced solid membrane (SM); (c) Quantification of C–D intensity per cell upon lipid metabolism perturbation using SMIs (from b); (d) Live-cell SRS images of A549 cells incubated with 30 mM d₃-pyruvate for 48 hours targeted at the protein channel (–CH₃, 2940 cm^{–1}) and C–D (on-off) for (i) control (no drugs), (ii) 1 μM cycloheximide, or

(iii) 3.5 μ M CHIR-99021 inhibition; (e) Quantification of C–D intensity per cell upon protein metabolism perturbation using SMIs (from d). Data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-tests. ns = no statistical difference, *** p <0.001, **** p <0.0001; (f) DFT-derived quantification of d_3 -pyr-derived biomass (untreated), SFA-enriched SM domains (+CAY), and cytoplasmic d_3 -pyr-derived biomass following CHIR treatment. All scale bars: 20 μ m.

We further quantified the deuterium distribution in these SFA-rich SM domains (**Fig. S15a**) as well as in the cytoplasm (**Fig. S15b**) of CHIR-treated A549 cells using our DFT-derived MATRIX-SRS workflow. In SM domains (**Fig. 6f**, +CAY), we observed a tenfold increase in deuterium biomass in lipids (6.0 ± 1.8 mM, CDHCH₂, teal) and appreciable increases in d_1 (purple) and d_3 (orange) metabolite concentrations compared with the d_3 -pyr control (**Fig. 6f**, d_3 -pyr). The increase in d_3 (orange) concentration is especially interesting and may indicate faster conversion of d_3 -pyr into d_3 -acetyl-CoA, and subsequent incorporation into the terminal methyl groups in fatty acids (**Fig. S6**). On the other hand, in cells treated with CHIR (**Fig. 6f**, +CHIR), we found increased concentrations of d_1 (purple), d_3 (orange, most likely from the conversion of d_3 -pyr to d_3 -alanine; **Fig. 4b**), and lipid (CDHCH₂, teal) deuterium, but a surprising reduction in d_2 biomass (pink) compared with the d_3 -pyr control (**Fig. 6f**, d_3 -pyr). We reason that the reduction in the d_2/d_1 ratio may be an indicator of overall higher TCA cycle activity, where the progression of the cycle naturally leads to deuterium abstraction when proceeding through fumarate (**Fig. 4b**). Therefore, the decreased relative d_2 abundance observed upon treatment with CHIR is consistent with broadly upregulated

metabolic activity. In this manner, MATRIX-SRS holds significant potential as a live-cell-compatible method to investigate the impacts of drugs on metabolism by directly visualizing and quantifying metabolome-wide changes in absolute concentrations.

4.4 Discussion

Targeted SRS microscopy using small vibrational tags enables visualization of dynamic metabolic turnover and the incorporation of labeled substrates into distinct cellular biomacromolecules.^{11,16} However, the inherent complexity, rapid dynamics and low abundance of intermediate metabolites connecting the initial probe to the final biomass limit our ability to fully map the metabolic atlas. This highlights the need for expanding the repertoire of probes capable of visualizing these transient metabolic species, and a robust and quantitative analysis platform with generally comparable units (e.g., absolute concentration) across multiple experiments and model systems.

We demonstrate the broad applicability of MATRIX-SRS in visualizing both TCA-related anabolism and catabolism in live HeLa cells and neuronal co-cultures. We then applied this methodology to investigate the metabolic reprogramming underlying EMT in live A549 adenocarcinoma cells. MATRIX-SRS revealed a ~30-40% global attenuation of TCA-associated metabolic incorporation of d₇-glucose, d₅-glutamine, d₃-lactate, d₃-acetate, d₃-pyruvate and d₄-succinate in mesenchymal cells compared to their epithelial counterparts. Importantly, we established a physics-informed MATRIX-SRS quantification, employing high-throughput density functional theory calculations to obtain absolute concentrations of metabolized deuterium biomass based on hSRS spectra.

We focused on pyruvate, which occupies a central position between sugar processing, the TCA cycle, amino acid synthesis, and lipid synthesis (**Fig. S6**), to further dissect its metabolic role. We first complemented our SRS imaging results with SIL tracing via LC-MS, which confirmed a ~30-40% downregulation of pyruvate utilization on a per-metabolite basis in mesenchymal cells and demonstrating the high synergy between LC-MS and SRS. We further applied quantitative MATRIX-SRS analysis to d₃-pyr, achieving excellent consistency with SIL tracing, granting previously inaccessible pathway-dependent insights derived from precise biomass quantification.

SRS intensity depends on both the abundance and the scattering cross-section of the analyte molecule. For metabolically stable probes (e.g., alkyne-tagged molecules like 5-ethynyl-2'-deoxyuridine, EdU), absolute concentrations can be determined *in situ* by generating a calibration curve in solution. However, for metabolically active probes that undergo metabolic turnover (e.g., deuterium-labeled metabolites, as with our TCA probes), the observed biomass comprises metabolites of both unknown concentrations and unknown cross-sections, resulting in an underdetermined system. These downstream products are highly heterogeneous, with hundreds of possible structures of varying levels of deuteration (**Fig. S10e**). Moreover, solution reference spectra are not readily acquired for these species, which are transient, low-abundance, and immensely challenging to selectively isolate or prepare synthetically on large scales. As such, SRS on metabolically active probes has been

largely qualitative in intensity, instead focusing on spatial heterogeneity or unmixing from empirically derived spectral differences.²⁰

In principle, computational methods have long been capable of complementing experimental data to provide cross-section estimates for transient species. Compared with empirical modeling, physics-based calculations are derived from an underlying, internally consistent set of fundamental principles, facilitating much deeper insights and broader generalization and prediction. However, the application of DFT calculations for Raman spectra of small molecule metabolites faces several barriers, including method selection, validation, and application to dozens of diverse metabolites (effectively necessitating automation), leading to largely empirical spectral assignments and limited interpretability.³⁴

Our work shows that careful optimization of the computational methods (including the choice of an accurate but cost-effective solvent model³⁶) can allow for quantitatively accurate computations of both Raman scattering frequencies and intensities (**Fig. 5b** and **Fig. S9**). Based on these findings, we applied DFT calculations at large scale to nearly 70 TCA-linked metabolites (**Fig. S10**) and derived cross-section estimates for four spectrally distinct metabolite classes (d_1 , d_2 , d_3 , and $CDHCH_2$), allowing for *in situ* quantification of metabolically active deuterium probes in absolute concentrations with SRS for the first time. Moreover, we expect these quantification methods and metabolite classes to generalize broadly to other metabolically active deuterium probes, since these motifs are

highly conserved through the metabolic reaction network surrounding the TCA cycle

(**Fig. S6**).

Encouragingly, we observe remarkably good agreement between the LC-MS M+1/M+2/M+3 pyruvate fractions and our SRS-derived $d_1/d_2/d_3$ concentrations (**Fig. 5e**), laying a foundation for future *in situ* metabolomic quantification with MATRIX-SRS. A unique strength of SRS-based spatial metabolomics is the potential for dynamic live-cell imaging, where subcellular metabolism could be observed directly by watching the evolution of the $d_1/d_2/d_3/CDHCH_2$ distribution over time.

We should also note a few key limitations. In our current work, we employ slightly elevated concentrations of our TCA probes to achieve robust C–D signals with high signal-to-noise ratios, although the concentrations used (e.g., 10–30 mM for pyruvate) are still lower than those typically required for other techniques, such as MRI (~69 mM).⁵⁰ We carefully characterized cell states following probe incubation and observed no obvious toxicity effects. Importantly, our methods (tandem SRS and LC-MS, informed by physics-based modeling) are broadly generalizable to Raman metabolic imaging, and continued technical developments⁵¹ may grant much improved sensitivity to allow investigations at more physiologically relevant concentrations (such as ~5.8 mM for pyruvate²³). Additionally, our current study focuses on late-time endpoints to study bulk metabolism (and allow sufficient time to overcome deuterium kinetic isotope effects), while the dynamics of the TCA cycle remain relatively underexplored in metabolic imaging. Lastly, our DFT-derived quantification of deuterated biomass currently only allows for bulk

classification into four classes of metabolites (d_1 , d_2 , d_3 , and $CDHCH_2$). Although we complement our SRS imaging with LC-MS SIL tracing to gain deeper molecular specificity, an ideal live-cell spatial metabolomics approach would grant both subcellular localization and per-molecule identification from imaging alone. Multimodal imaging modalities,⁵² imaging techniques based on higher-dimensional spectroscopies,⁵³ and new approaches integrating SRS and machine learning have significant potential to achieve greater molecular-level detail towards this ideal.

In the future, we also envision expanding MATRIX-SRS to other metabolic probes, such as fumarate, malate, and oxaloacetate, in living systems. Additionally, we expect that deeper functional investigations of our emerging TCA probes, as we have done here with d_3 -pyr, will also prove highly informative; for example, detailed study of d_3 -lactate could yield fascinating insights into the lactate shuttle hypothesis. By harnessing characteristic Raman spectral features and incorporating novel metabolic probes, we envision quantitative spatial metabolomics for longitudinal analyses. MATRIX-SRS, incorporating both high-resolution spectro-microscopy and DFT calculations, provides a uniquely quantitative basis for far-field imaging of metabolism with minimally perturbative probes, complementing existing strategies for metabolic profiling of living systems.

4.5 Materials & Methods

Cell Culture and Metabolic Labeling

HeLa cells (ATCC, CCL-2), and A549 cells (gift from the Elowitz lab, Caltech) were each cultured in DMEM (Gibco, Cat. #11965092) supplemented with 10% FBS (Corning, Cat. #35-015-CV) and 1x antibiotic-antimycotic (Gibco, Cat. #15240062) at 37 °C with 5% CO₂. To induce EMT, A549 cells were seeded and cultured in DMEM containing 1x StemXVivo EMT-inducing supplement (R&D systems, Cat. #CCM017) for 5 days, with media refreshed every 2-3 days.

For neuronal co-culture, primary hippocampal neurons were prepared from neonatal (P0-P1) Sprague-Dawley rat pups (CD (Sprague-Dawley), IGS rat, Charles River) in accordance with an IACUC-approved protocol (IA22-1835). Following dissection, mice brains were transferred into ice-cold Hank's balanced salt solution (Gibco, Cat. #14-025-092). Hippocampi were isolated, cut into ~0.5 mm fragments, and digested with 5 mL of 0.25% Trypsin-EDTA (Gibco, Cat. #25200056) at 37 °C and 5% CO₂ for 20 min. Digestion was quenched with 5 mL of DMEM containing 10% FBS. The tissue was transferred into 2 mL of neuronal culture medium (Neurobasal A supplemented with B-27, 2 mM GlutaMAX, and 1x penicillin-streptomycin; Thermo Fisher), then pelleted by brief centrifugation and resuspended in neuronal culture medium. Cells were dissociated by gentle trituration and diluted to a final density of 1×10^5 cells/mL. For plating, 0.7 mL of the cell suspension was added per well of a 24-well plate containing 12 mm circular coverslips pre-coated with poly-D-lysine and laminin. Coated coverslips were prepared in advance by incubating sterile coverslips with 100 $\mu\text{g mL}^{-1}$ poly-D-lysine (Sigma) at 37 °C for 24 h, rinsing twice with ddH₂O, and subsequently coating with 10 $\mu\text{g/mL}$ laminin (Gibco) overnight under the same conditions. Cell cultures were maintained at 37 °C and 5% CO₂, and half of the medium was

replaced every five days with a pre-warmed neuronal medium. The metabolic probes were added on ~DIV11 at their respective concentrations and for the specified duration of time.

The metabolic probes used for SRS imaging were 5 mM 2,3,3,4,4-d₅-L-glutamine (Cambridge Isotope Laboratories, Cat. #DLM-1826), 30 mM 3,3,3-d₃-sodium L-lactate (Cambridge Isotope Laboratories, Cat. #DLM-9071), 10-30 mM d₃-sodium pyruvate (Cambridge Isotope Laboratories, Cat. #DLM-6068), 30 mM d₃-sodium acetate (Cambridge Isotope Laboratories, Cat. #DLM-3126) and 30-50 mM d₄-succinic acid, disodium salt (Cambridge Isotope Laboratories, Cat. #DLM-2307) and 25 mM 1,2,3,4,5,6,6-d₇-D-glucose (Cambridge Isotope Laboratories, Cat. #DLM-2062) in cell media containing no unlabeled counterparts of the respective metabolic probes for the designated time points.

For metabolic imaging of EMT, deuterium-labeled probes were added 3-4 days after cell seeding (for either epithelial or mesenchymal cells) on cover slips, at approximately ~50-60% cell confluency. The concentrations and incubation time points of the metabolic probes were adjusted to 25 mM d₇-glucose for 24 hours, 2 mM for d₅-glutamine for 24 hours, 20 mM for d₃-pyruvate for 24 hours, 30 mM d₃-acetate for 48 hours, 30 mM d₃-lactate for 48 hours, and 30 mM d₄-succinate for 5 days in cell media containing no unlabeled counterparts of the corresponding probes.

Drug Perturbation of Lipid and Protein Metabolism

For pharmacological perturbation of lipid metabolism, A549 cells were treated with 10 μM ETC-1002 (MedChemExpress, Cat. #HY-12357), 20 μM SB-204490 (MedChemExpress, Cat. #HY-16450), 2 μM BMS-303141 (MedChemExpress, Cat. #16107), 75 μM C75 (MedChemExpress, Cat. #HY-12364), 10 μM Denifanstat (MedChemExpress, Cat. #HY-112829), 1 μM PF-05175157 (MedChemExpress, Cat. #HY-12942), 1 μM ND-646 (MedChemExpress, Cat. #HY-101842), or 1 μM CAY10566 (MedChemExpress, Cat. #HY-15823) for 48 hr concurrently with 30 mM d_3 -pyruvate labeling.

For protein modulation experiments, A549 cells were treated with 1 μM cycloheximide (CHX, Sigma-Aldrich, Cat. #C7698-1G) or 3.5 μM CHIR99021 (MedChemExpress, Cat. #HY-10182) for 48 hr with concurrent d_3 -pyruvate labeling.

SRS Microscopy

The SRS microscopy system was configured as described previously.⁵⁴ Briefly, images were acquired with an 80 μs pixel dwell time to achieve an image acquisition speed of 8.52 s per frame for a 320x320-pixel field of view (FOV) at a 0.497 $\mu\text{m}/\text{pixel}$ resolution. The pump beam wavelength was set to 791.3 nm for the protein ($-\text{CH}_3$ channel, 2940 cm^{-1}), 797.3 nm ($-\text{CH}_2$ channel, 2845 cm^{-1}), 842.9 nm (C–D channel for d_5 -glutamine, 2167 cm^{-1}), 844.0 nm (C–D channel for d_7 -glucose/ d_3 -pyruvate/ d_3 -acetate/ d_4 -succinate/ d_4 -citrate, 2150 cm^{-1}), 845.6 nm (C–D channel for d_3 -lactate, 2129 cm^{-1}) and 855.0 nm (off-resonance, 2000 cm^{-1}).

For hSRS, the wavelength of the pump laser was tuned from 834.0 nm to 855.0 nm with a step size of 0.5 nm. The hSRS spectrum for d₅-gln was reproduced from a previous report.²² All images were processed and color-coded using ImageJ software.

For C–D quantification, on-resonance images were processed by subtracting the corresponding off-resonance images to result in the C–D (on-off) images. C–D (on-off) images were further processed with the Fiji ‘nonuniform background removal’ plugin before performing a uniform background subtraction on each image. The protein image was used to generate a mask for each cell utilizing the ‘IdentifyPrimaryObjects’ module in CellProfiler 4.2.8⁵⁵ with the ‘Minimum Cross-Entropy’ thresholding method. The mask was then used as a reference to quantify the final processed C–D image utilizing the ‘MeasureObjectIntensity’ module and exported to Excel using the ‘ExportToSpreadsheet’ module. MS Excel, GraphPad Prism, and MATLAB (R2024b, MathWorks) were used for data processing and plotting.

Spontaneous Raman Microscopy

Spontaneous Raman spectra of analytes were acquired using a Horiba Xplora plus confocal Raman spectrometer equipped with a 532 nm YAG laser (12 mW) and a 100x, 0.9 N.A. objective (MPLAN N, Olympus) with a 500 µm hole and a 100 µm slit. Data were collected with a 10 s integration time and averaged over 10 accumulations using LabSpec6 software. Background signals from solvents were subtracted from the analyte spectra, followed by baseline correction and normalization.

Immunofluorescence

Immunostaining of vimentin in epithelial and mesenchymal cells was performed as described previously.²⁹ Cell nuclei were labeled with DAPI (Thermo Fisher, Cat #. D1306) and imaged using an excitation of 358 nm and an emission peak around 461 nm. Correlative SRS images were acquired at the protein channel (2940 cm^{-1}) as described above using the same microscope.

Multiplex Image Unmixing

Multiplex images were unmixed using the least absolute shrinkage and selection operator (LASSO) as previously described (**Supplementary Figure 6**). hSRS stacks were pre-processed by non-local means denoising⁵⁶ and non-uniform background removal.⁵⁷ After pre-processing, hSRS stacks were power-corrected and background-corrected, then unmixed via LASSO ($\lambda = 0.3$) on a per-pixel basis.

DFT Calculations

Density functional theory (DFT) calculations were carried out using our AutoDFT computational pipeline (<https://github.com/pkocheril/AutoDFT>). ChemDraw (v23.1.2) was used to prepare structure files for each metabolite of interest. Through AutoDFT 2.0, we generated 3D geometries with selective deuteration and performed a classical energy minimization with OpenBabel.⁵⁸ We then automatically generated and submitted job files for Gaussian 16 (rev. B.01, Gaussian Inc.)⁵⁹, performing a coarse geometry optimization (HF/STO-3G), followed by fine optimization and harmonic frequency calculations (B3LYP/6-31G(d,p)) with implicit solvation in dimethyl sulfoxide using the solvation model

with density (SMD).³⁶ Natural bond orbital analysis was employed and symmetry was disabled. Computed normal mode frequencies were scaled by 0.9658. Raman spectra were calculated by applying Gaussian broadening (25 cm⁻¹ full-width at half-maximum; comparable to biomass spectral linewidths) to each of the calculated peaks. GaussView (v6.1.1) was used for additional visualization.

DFT-derived SRS Quantification

SRS intensity can be modeled as $SRS \propto c \cdot \sigma \cdot I_{\text{pump}} \cdot I_{\text{Stokes}}$, where c is the concentration of scattering molecules within the focal volume, σ is the scattering cross-section, and I_{pump} and I_{Stokes} , are the powers of the pump and Stokes beams, respectively. Thus, we can relate concentration to SRS intensity as $c = \frac{SRS}{k \cdot \sigma \cdot I_{\text{pump}} \cdot I_{\text{Stokes}}}$, where k is a constant of proportionality encompassing various experimental factors such as scattering collection efficiency and photodiode sensitivity. Here, we use DFT to calculate the mean scattering cross-sections across our four classes of deuterated metabolites (d_1 , d_2 , d_3 , and lipids (CDHCH₂)), calculated by summing the total computed intensity and dividing by the number of metabolites in the class (in a.u.). To obtain k , we measured an SRS spectrum of a 1 M aqueous solution d_3 -pyr (for our system, $k = 0.12 \text{ mW}^{-2} \text{ M}^{-1} (\text{a.u.})^{-1}$), thus allowing us to convert between SRS intensity and molar concentration (**Fig. S12**).

[3,3',3''-²H₃]-pyruvate SIL Tracing Using LC-MS

A549 cells were cultured in DMEM containing 10% dialyzed FBS (Gibco, Cat.# A3382001) on 6-cm cell culture dishes. After EMT induction, epi and mes cells were treated with 10

mM d₃-pyr. Following 24 hours, cell metabolism was quenched by rapidly aspirating the medium, rinsing with 150 mM NH₄OAc (pH adjusted to 7.3, Fisher Scientific, Cat. #ICN19384880) and adding 80% ice-cold methanol containing 1 µM norvaline (Millipore Sigma, Cat. #53721-100MG) as the internal standard. Samples were incubated at -80 °C for 30 min, scraped into low-bind tubes and centrifuged at maximum speed for 30 min. Supernatants were stored at -80 °C until LC-MS analysis. Prior to injection, extracts were thawed on ice, centrifuged for 10 min, and clarified for LC-MS.

Metabolites were separated using a Waters BEH z-HILIC column (150 x 2.1 mm, 2.7 µm) connected to a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer with a heated electrospray ionization (H-ESI) source. The column temperature was maintained at 25° C, and the autosampler at 4° C. The mobile phase consisted of Solvent A: 10 mM ammonium bicarbonate in water (pH ~9.1) and Solvent B: 95% acetonitrile/5% water. Chromatographic separation was conducted at 0.18 mL/min with the following gradient: 0-1 min, 95% B; 1-15 min, linear decrease to 45% B; 15-19 min, 45% B; 19-22 min, ramp back to 95% B; 22-34.5 min, 95% B for re-equilibration.

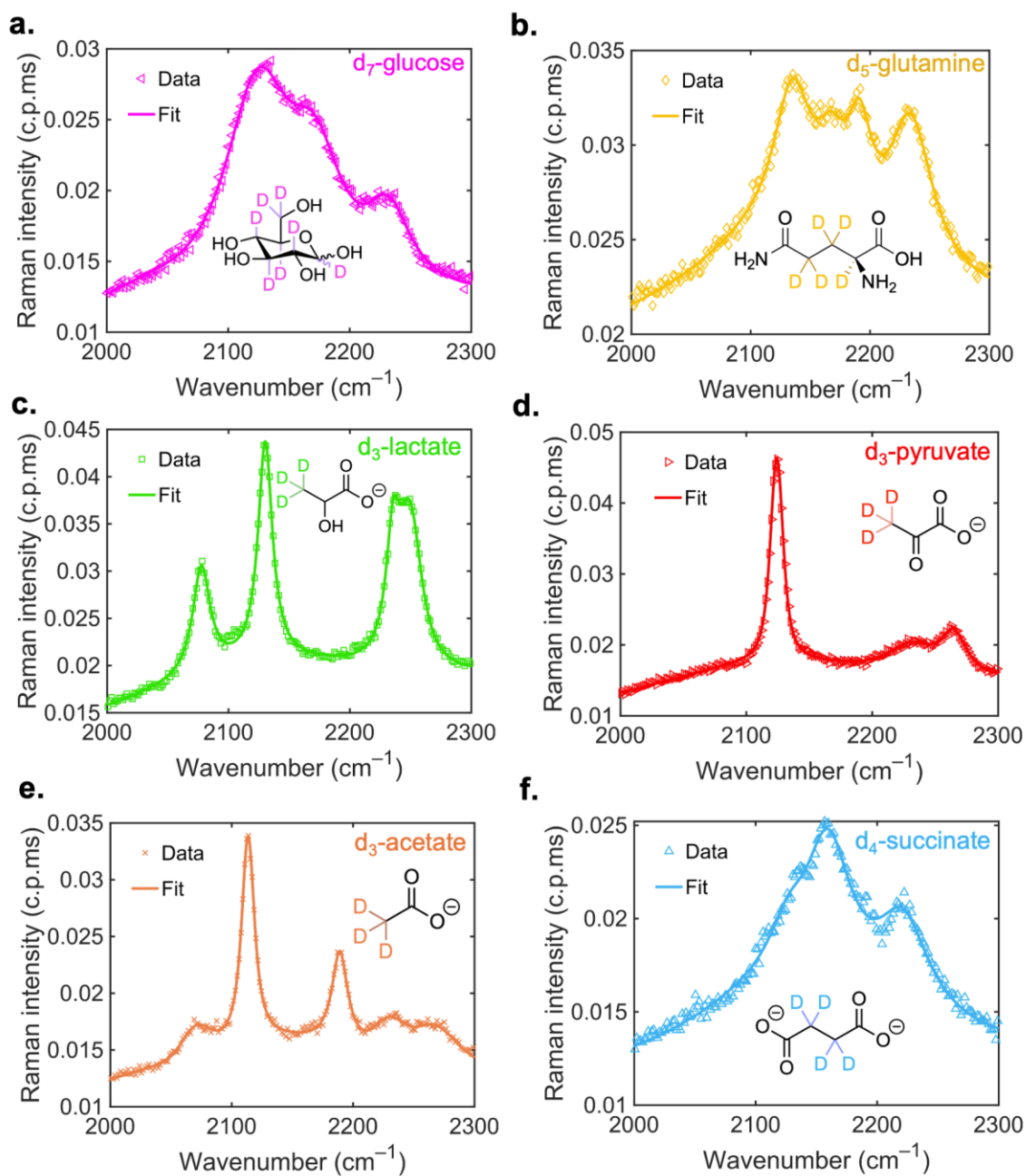
MS data were acquired in data-dependent acquisition (DDA) mode with fast polarity switching. Full MS¹ scans were collected at 120,000 resolution (m/z 70-1000) with an AGC target of 1 x 10⁶. MS/MS scans were acquired using a TOP2 ddMS² method with higher-energy collisional dissociation (HCD) at stepped collision energies of 10% and 40%, resolution 30,000, and isolation window 0.7 m/z. Dynamic exclusion, isotope exclusion,

monoisotopic precursor selection, and mild ion trapping were enabled to enhance spectral quality.

Raw LC-MS data were converted to mzXML format using MSConvert and processed in EI MAVEN (v0.12.0). Peak detection, chromatogram alignment, and automated isotope annotation were performed with default settings. Retention times were normalized using external standards, ion intensities corrected for matrix effects, and peaks manually curated as needed. Processed peak tables were exported in CSV format for analysis of isotopologue distributions.

To account for the contribution of each labeling state, isotopologues were weighted by the number of incorporated labeled atoms (e.g., M+1 by 1, M+2 by 2, M+3 by 3, etc.). This weighting demonstrates the relative contribution of each isotopologue to the overall C–D signal. These weighted values were then multiplied by previously published metabolite concentrations²³ and summed to determine the total metabolite C–D burden. This approach ensures that mass spectrometric quantification accurately reflects the extent of deuterium incorporation, mirroring the Raman-derived signal. Final data were expressed either as the fraction of the total signal from labeled species or as relative isotopologue abundances, depending on the downstream analysis.

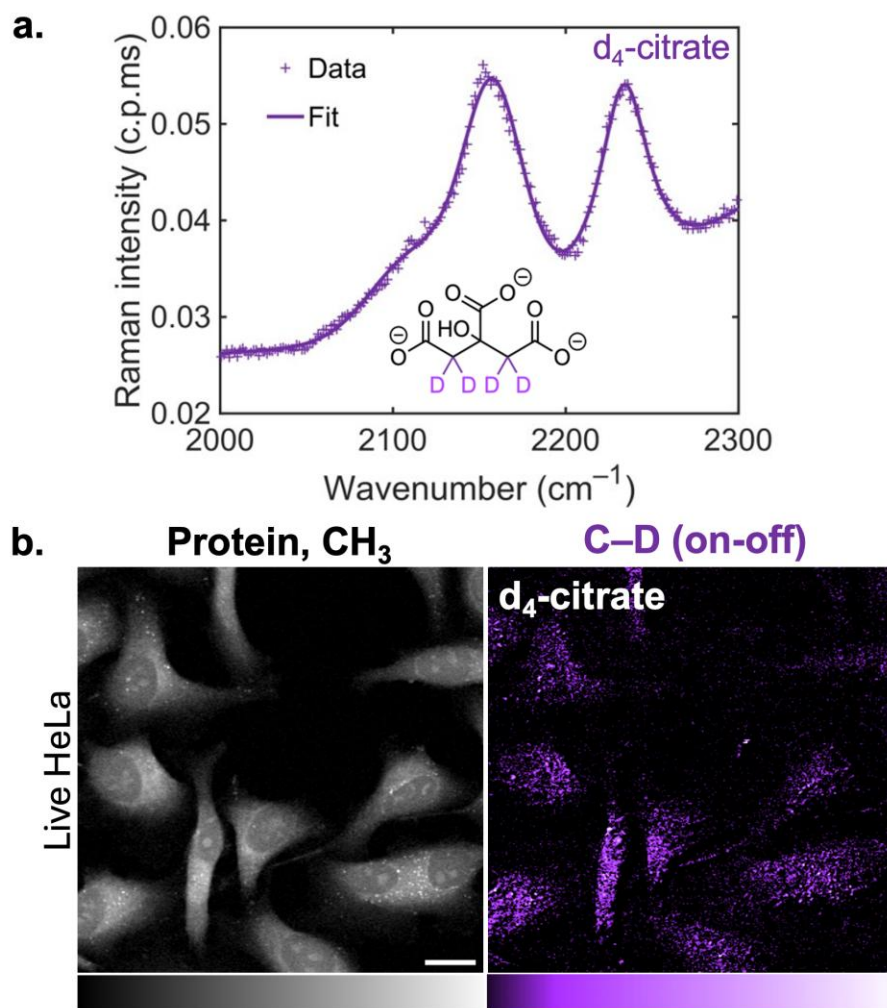
4.6 Supplemental Information



Supplementary Figure 1. Raman spectra of TCA probes.

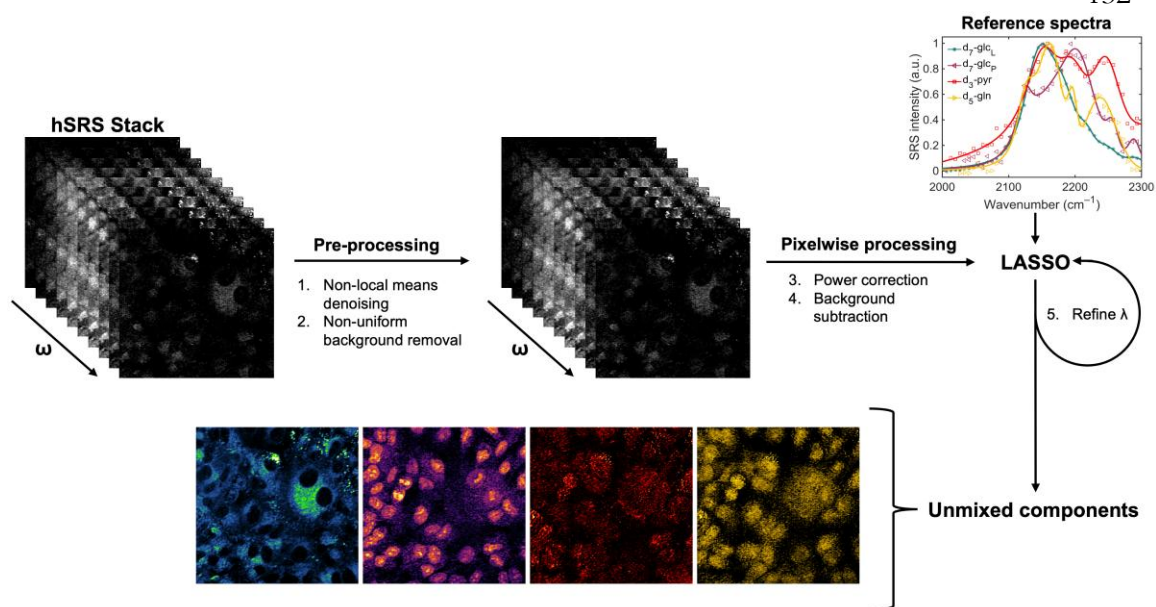
Spontaneous Raman spectra of 200 mM aqueous solutions of **(a)** d₇-glucose, **(b)** d₅-glutamine, **(c)** d₃-lactate, **(d)** d₃-pyruvate, **(e)** d₃-acetate, and **(f)** d₄-succinate (structures inset; c.p.ms, counts per millisecond). A handful of peaks are observed that are not predicted by

harmonic DFT calculations (2076 cm^{-1} in d_3 -lactate; 2070 cm^{-1} and 2190 cm^{-1} in d_3 -acetate). We attribute these peaks to CD-bending combination bands, similar to previous assignments of Fermi resonances for other deuterated probes.⁶⁰



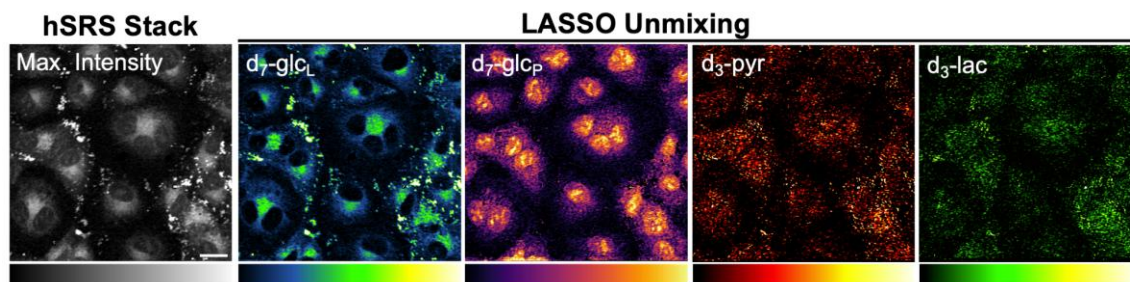
Supplementary Figure 2. Raman spectra and imaging of d_4 -citrate.

(a) Spontaneous Raman spectrum of d_4 -citrate at 1 M in aqueous solution; **(b)** Live-cell SRS imaging of d_4 -citrate-treated HeLa cells targeted at the protein (CH_3 , 2940 cm^{-1}) and C–D (on-off) channels. Scale bar: $20\text{ }\mu\text{m}$.



Supplementary Figure 3. LASSO unmixing workflow.

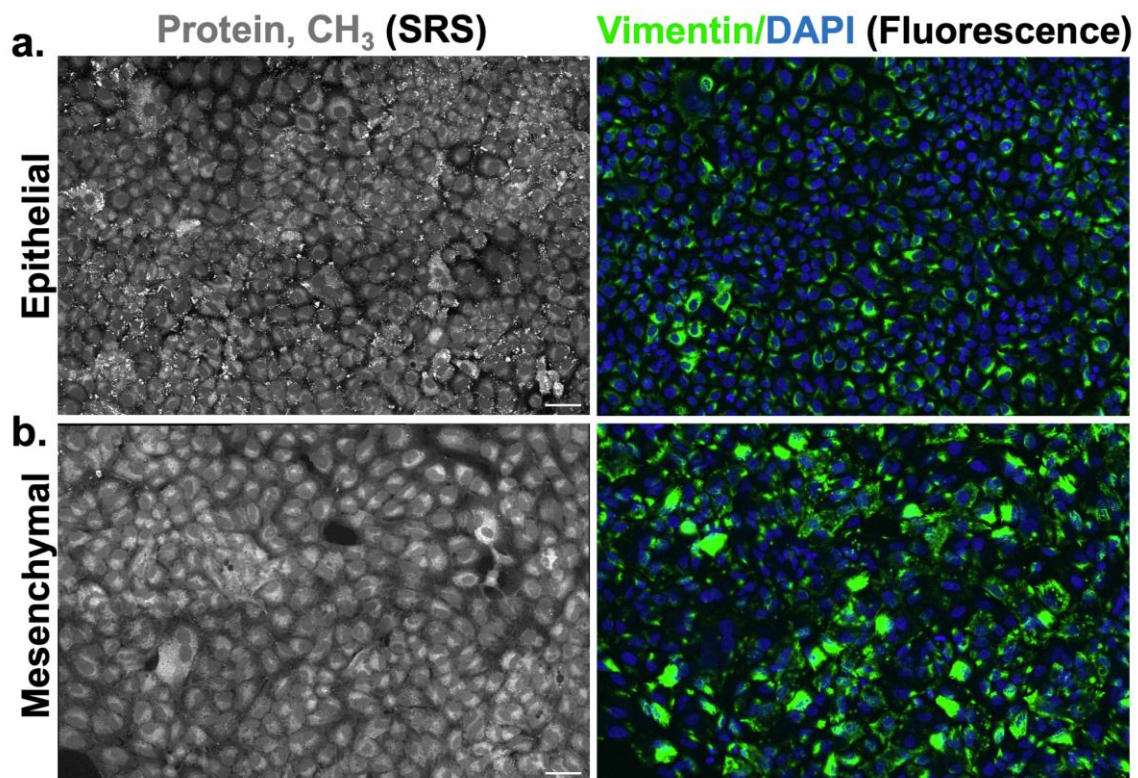
Raw hSRS stacks were pre-processed prior to pixelwise power correction, background subtraction, and unmixing via LASSO.



Supplementary Figure 4. Four-color multiplex imaging with d_7 -glucose, d_3 -pyruvate, and d_3 -lactate.

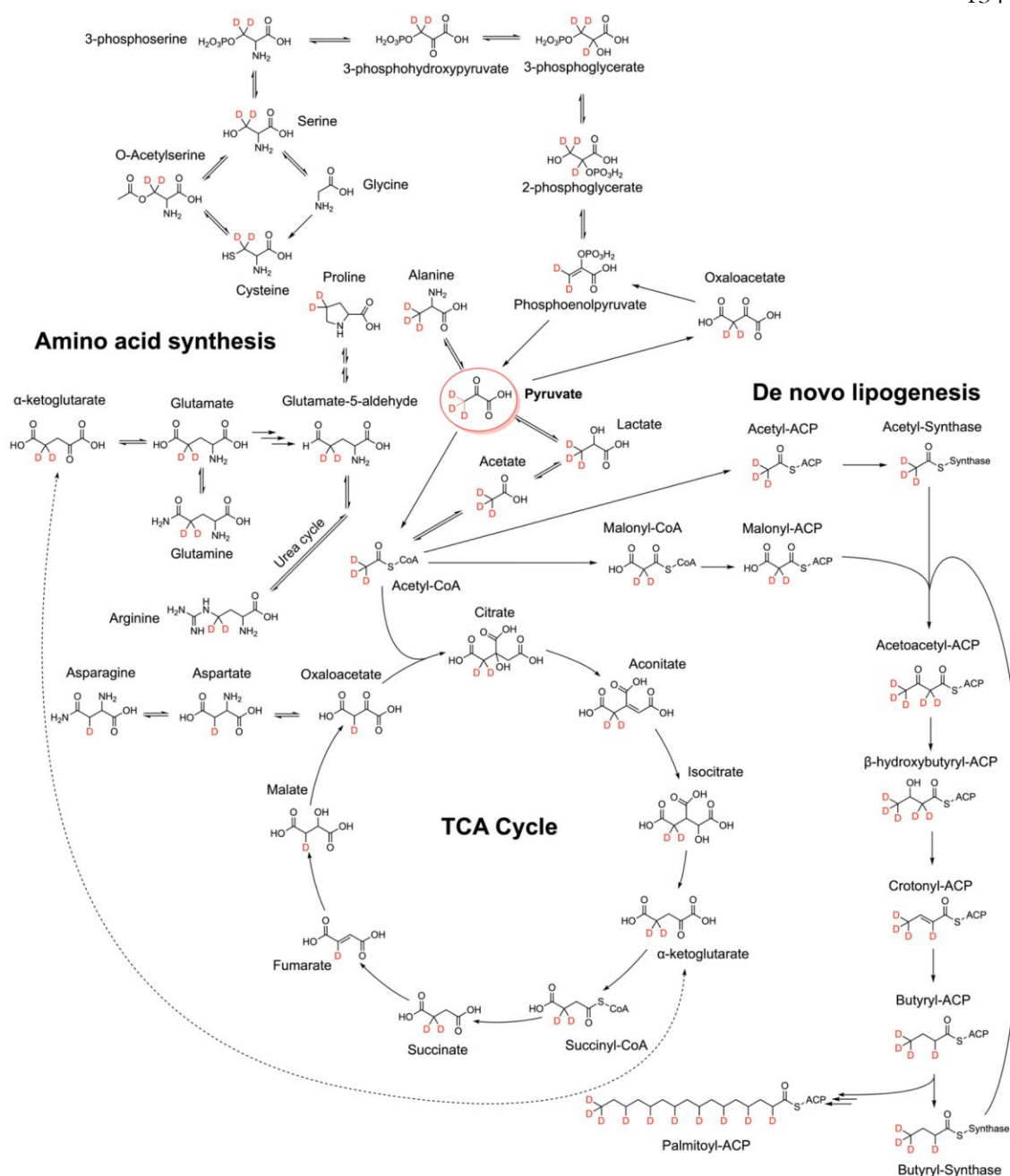
Using the same LASSO unmixing pipeline (Supplementary Figure 3), we again observe robust unmixing of $\text{d}_7\text{-glc}_L$, $\text{d}_7\text{-glc}_P$, $\text{d}_3\text{-pyr}$ biomass, and $\text{d}_3\text{-lactate}$ biomass. These results

serve to demonstrate the broad compatibility of our TCA probes in multiplex imaging with validated chemical unmixing strategies. Scale bar: 20 μm .



Supplementary Figure 5. Validation of EMT by immunostaining.

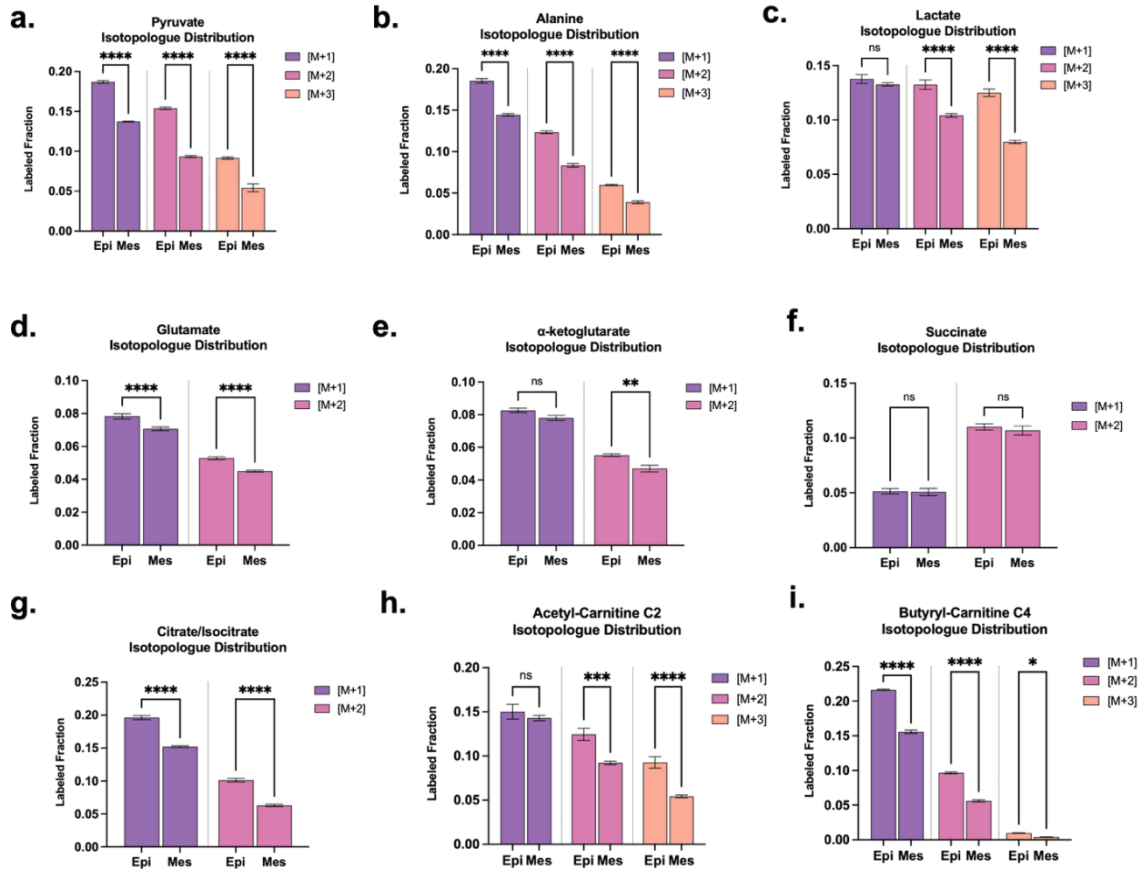
Correlative SRS (protein channel, 2940 cm^{-1}) and Vimentin/DAPI (merged) immunostaining images for **(a)** epithelial and **(b)** mesenchymal cells. Mesenchymal cells exhibit increased vimentin expression. Scale bar: 20 μm .



Supplementary Figure 6. Detailed metabolic reaction mapping.

d_3 -pyruvate (red circle) is metabolically incorporated into both amino acids and lipids, with specific structural motifs obtained from the mechanisms of the associated reactions. Here, we show maximal deuteration, and we assume that no deuterium is incorporated from the

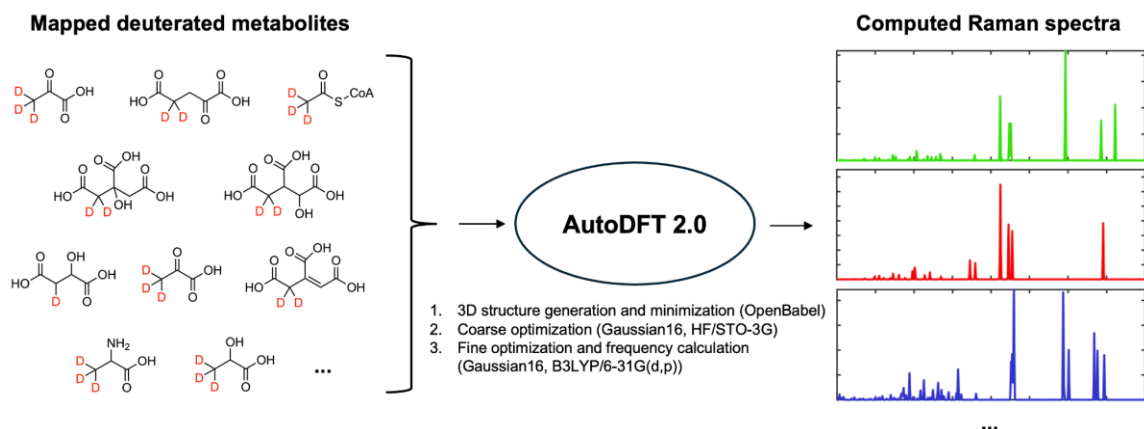
water in the surrounding media or reducing cofactors, since these would require a massive amount of deuterium in the bulk system to out-compete hydrogen sources.



Supplementary Figure 7. LC-MS quantification of individual metabolites from d₃-pyruvate labeling.

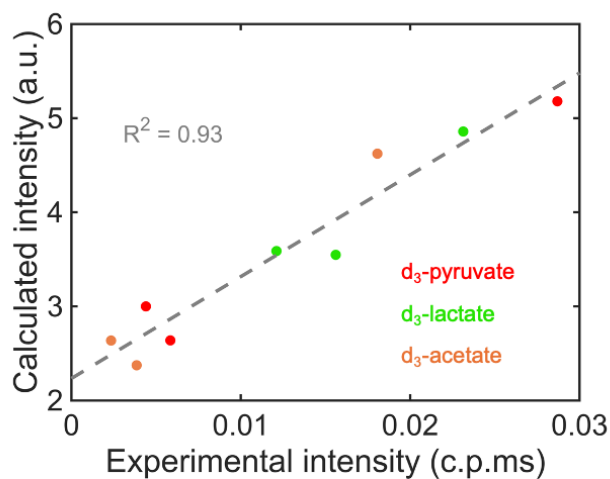
Fractional M+1, M+2, and M+3 isotopologue distributions for (a) pyruvate, (b) alanine, (c) lactate, (d) glutamate, (e) α-ketoglutarate, (f) succinate, (g) citrate/isocitrate, (h) acetyl-carnitine C2, and (i) butyryl-carnitine C4 in epithelial (epi) and mesenchymal (mes) A549 cells. Data from three independent experiments. Data are presented as mean ± SEM from at least three independent experiments. Statistical significance was analyzed using two-way

ANOVA test. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = no statistical difference.



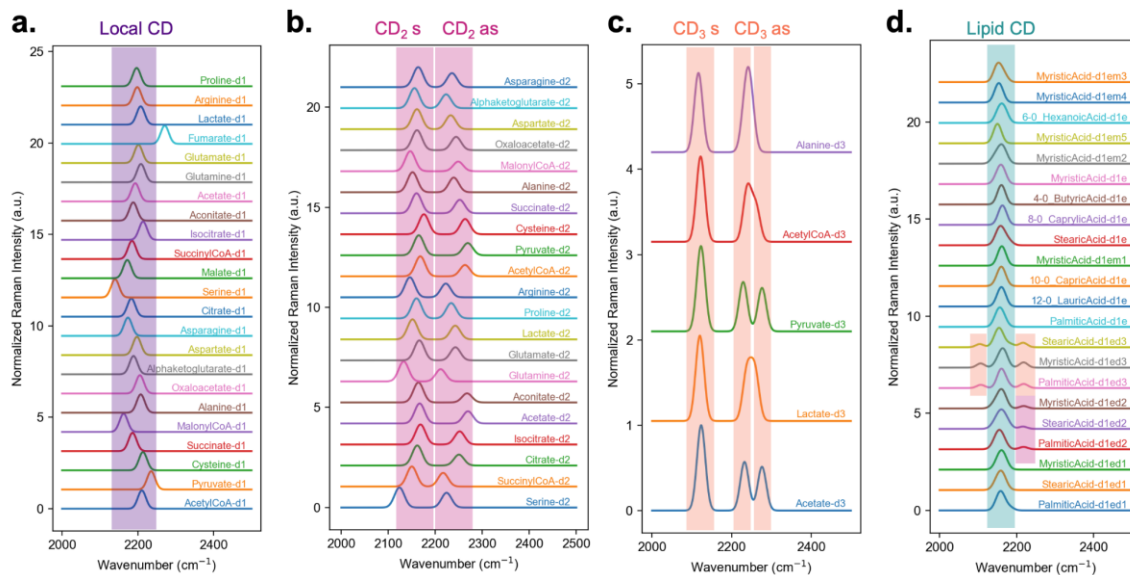
Supplementary Figure 8. AutoDFT 2.0 workflow.

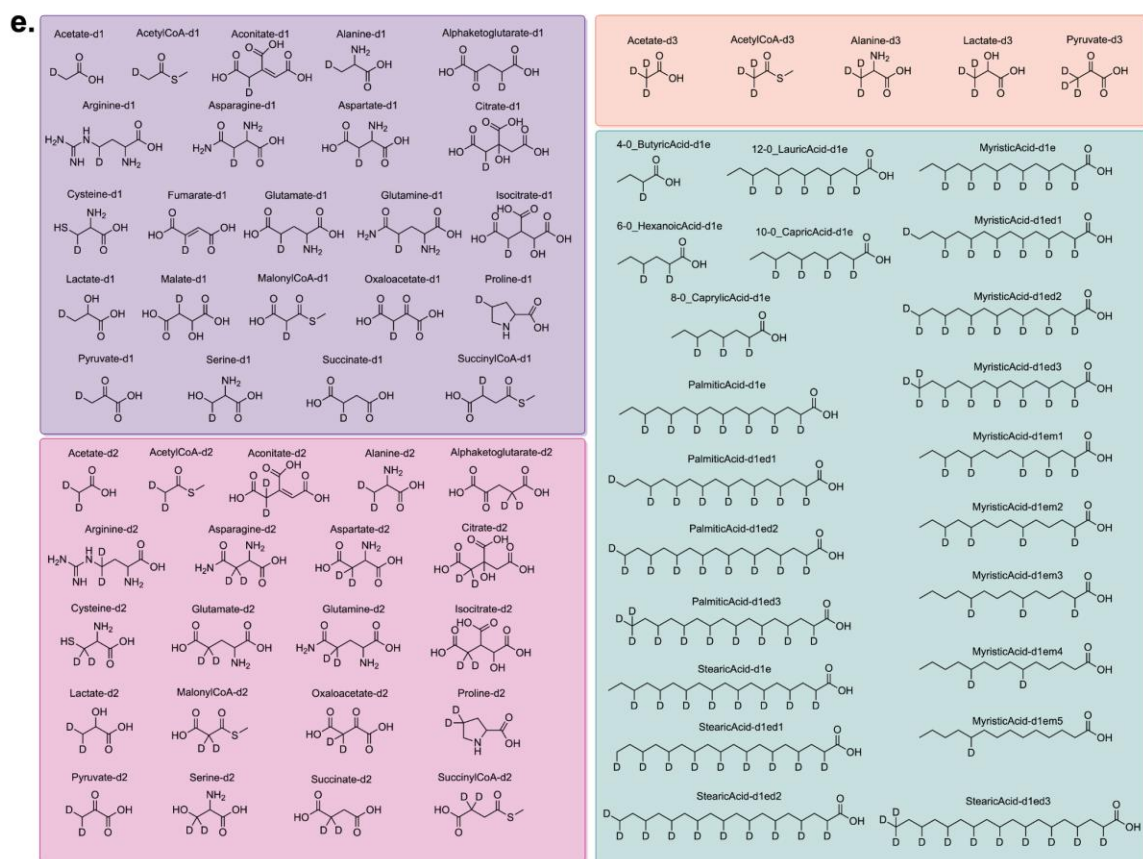
Automated density functional theory calculations were carried out with our AutoDFT computational pipeline, with AutoDFT 2.0 now introducing support for selective deuteration and computing Raman scattering activities.



Supplementary Figure 9. Validation of DFT intensities.

DFT-calculated Raman spectra were validated by comparison to experimental intensities, obtained by analyzing the spectra shown in **Supplementary Figure 1**. Only the d_3 species were used because they exhibited distinct, well-separated bands that allowed confident quantitation. For the more highly deuterated probes, highly overlapping and broad peaks prohibited confident experimental peak quantification (fits were sensitive to initial conditions and yielded inconsistent intensities, while the frequencies were more robust).



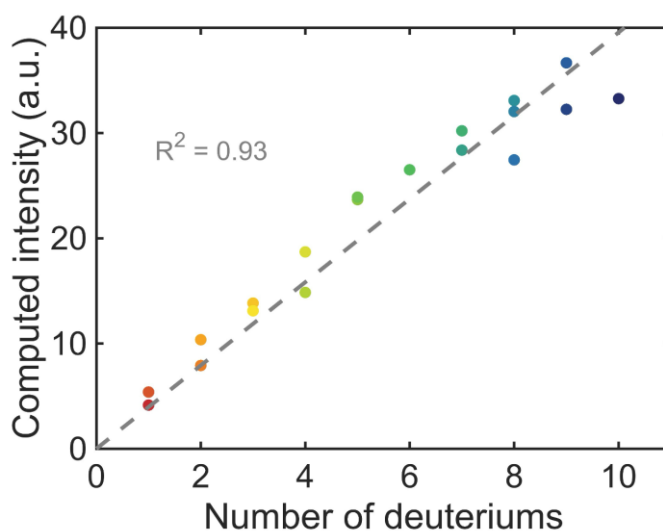


Supplementary Figure 10. DFT-computed spectra of mapped metabolites from d₃-pyr.

(a) Computed spectra of d₁ metabolites, showing a local C–D mode at ~2200 cm⁻¹ (purple shaded area). The main exception is d₁-fumarate, which contains a C_{sp2}–D bond and is blueshifted relative to the C_{sp3}–D local stretching band at 2200 cm⁻¹; **(b)** Computed spectra of d₂ metabolites, showing symmetric (CD₂ s) and asymmetric (CD₂ as) stretching bands at ~2160 and ~2240 cm⁻¹, respectively (pink shaded areas); **(c)** Computed spectra of d₃ metabolites, showing a symmetric stretching band (CD₃ s) at ~2120 cm⁻¹ and two asymmetric stretching bands (CD₃ as) at ~2230 and ~2250 cm⁻¹ (orange shaded areas), often clustering into a single band at ~2240 cm⁻¹; **(d)** Computed spectra of aliphatic chains (CDHCH₂), displaying a coupled CD band at ~2160 cm⁻¹ (teal shaded area). For lipids with

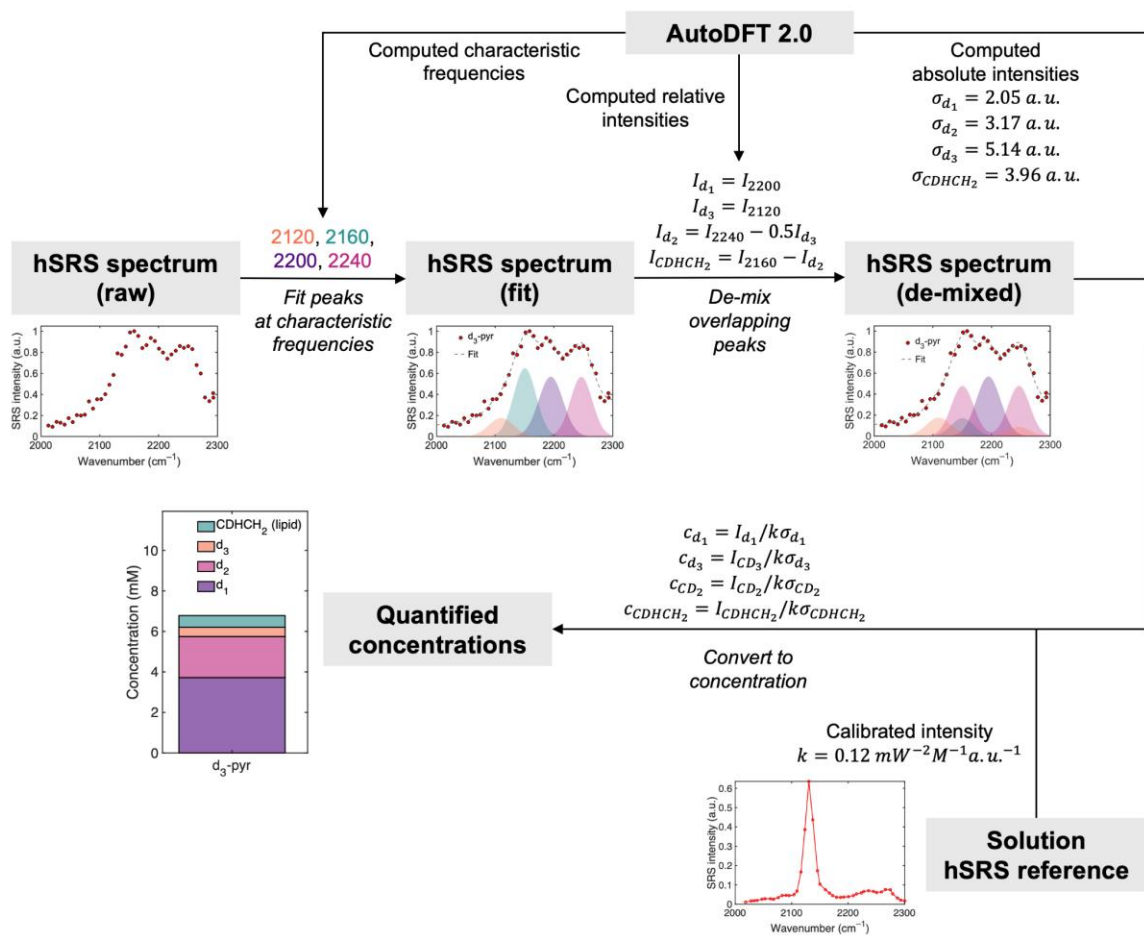
d_2 and d_3 methyl groups, the symmetric and asymmetric bands of the methyl groups are observed at the same characteristic frequencies as the d_2 and d_3 metabolites (highlighted regions in orange and pink); **(e)** Chemical structures of all calculated metabolites, grouped by deuteration (d_1 , purple; d_2 , pink; d_3 , orange; d_4 , teal). CoA units were truncated and replaced by methyl substituents.

Mapped metabolites are named by their deuteration status, broadly grouped as d_1 , d_2 , d_3 , and aliphatic chains. The aliphatic chains all exhibit the $(CDHCH_2)_n$ deuteration motif, which we derived from our reaction network mapping (**Supplementary Figure 6**) and label here as “ $d1e$ ” in shorthand, indicating a single deuterium on every other carbon in the chain. To account for the large potential heterogeneity of lipid biomass, we varied the chain length (ranging from 4 carbons to 18 in increments of 2) and the deuteration of the terminal methyl group (labeled “ $d1ed1$,” “ $d1ed2$,” or “ $d1ed3$ ”). We also explored the effects of missing deuteriums across a set of myristic acid structures (labeled “ $d1em\#$,” where $\#$ is the number of deuteriums missing, relative to the “ $d1e$ ” structure).



Supplementary Figure 11. Lipid computed cross-sections as a function of deuterium content.

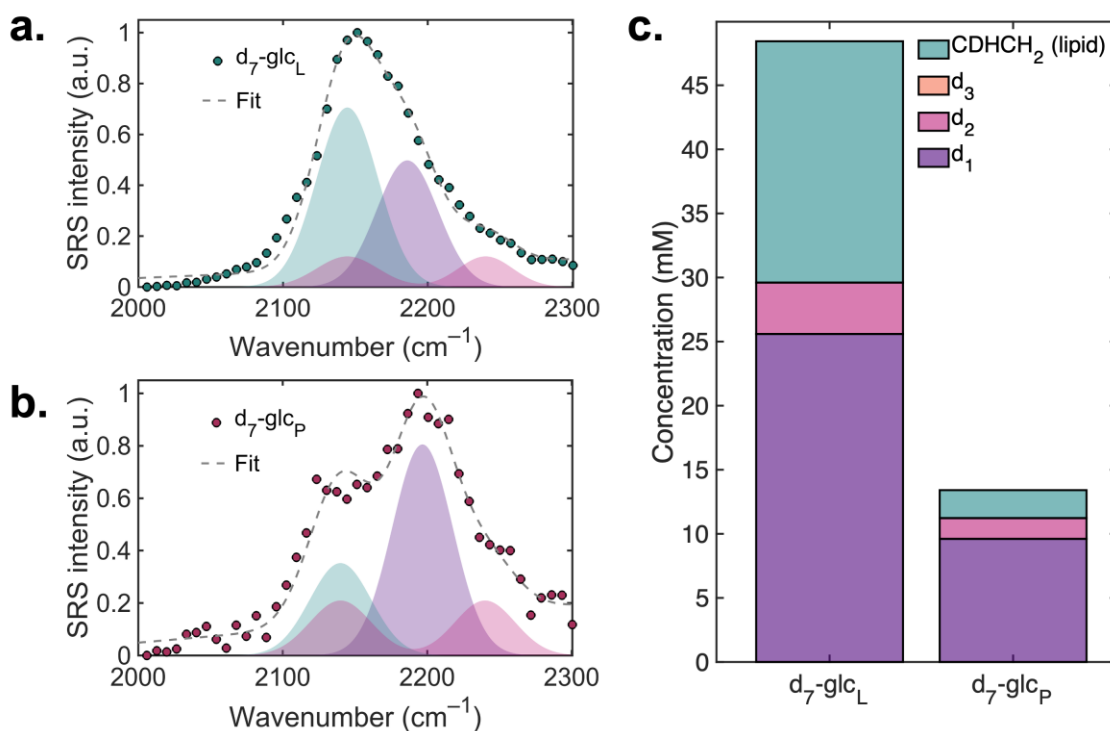
The calculated Raman scattering cross-sections for mapped lipids (CDHCH_2) are strongly linearly correlated with the number of deuteriums in the lipid, allowing us to quantify total deuterium in aliphatic chains (at 2160 cm^{-1}) on a per-deuterium basis.



Supplementary Figure 12. DFT-derived spectral analysis workflow.

The obtained hSRS spectrum from a given sample is fit to four Gaussian peaks at the characteristic frequencies obtained by DFT (allowed to float within 10-15 cm^{-1}). The fit amplitudes (I_{2200} , etc.) at each frequency are de-mixed into amplitudes for each metabolite class (I_{d_1} , etc.) by accounting for spectral mixing (assuming a 1:2 ratio for d_3 species at 2120 and 2240 cm^{-1} and a 1:1 ratio for d_2 species at 2160 and 2240 cm^{-1}). De-mixed amplitudes are then converted to abundances by dividing by the mean cross-sections of each metabolite class (σ) and converted to absolute concentrations from a solution reference measurement of d_3 -pyruvate in water (k). To estimate error, we calculated the root-mean-square error in

intensity in our DFT validation (**Supplementary Figure 9**), which is 0.29 a.u. (corresponding to 6-15% error). Taking this error as σ and propagating through the conversion to concentration yields an estimated 30% (2σ) error in SRS-derived quantification, which we use for all derived concentrations.

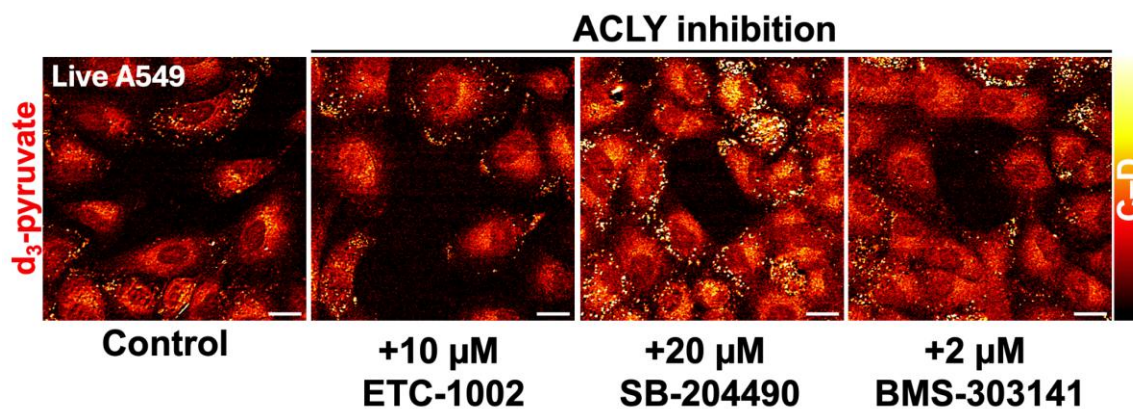


Supplementary Figure 13. SRS quantification of d₇-glucose-derived biomass.

Fitting of hSRS **(a)** d₇-glc_L and **(b)** d₇-glc_P spectra. **(c)** Quantification of d₁, d₂, and CDHCH₂ biomass. Glucose-derived biomass exhibits impressively high total concentrations of 48 ± 15 mM in d₇-glc_L and 13 ± 4 mM in d₇-glc_P (spectra obtained from lipid droplets and nucleoli, respectively; concentrations tabulated in **Supplementary Table 1**), despite the cell media containing only 25 mM d₇-glc. However, considering that d₇-glc contains multiple deuterium equivalents per molecule (i.e., through glycolysis, two molecules of d₂-pyruvate are

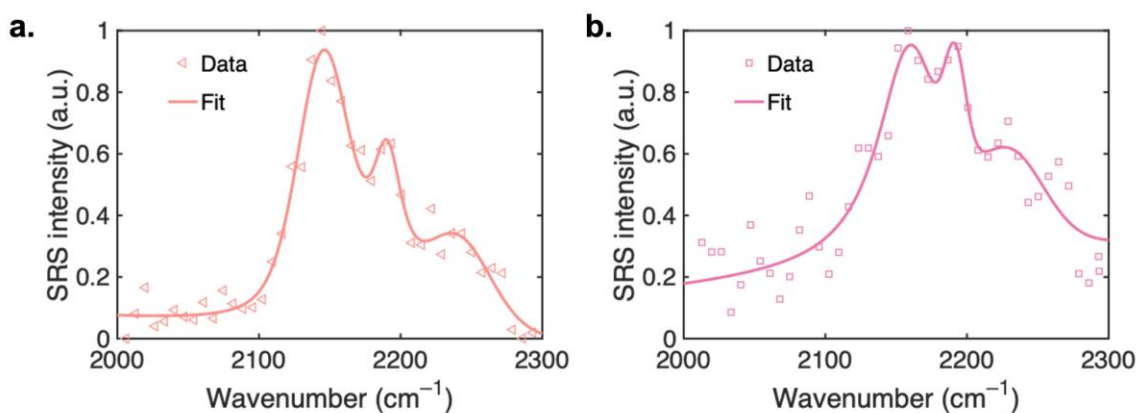
generated per molecule of d₇-glc, setting an estimated upper-bound at 50 mM), these results appear quite reasonable, with near-maximal incorporation efficiency for d₇-glc in lipid droplets.

With our DFT-derived spectral profiling, we can additionally examine the distributions of d₁, d₂, d₃ and lipid (CDHCH₂) deuterium in each of the spectra. Both d₇-glc_L and d₇-glc_P exhibit no d₃ concentration, since d₇-glc contains no deuterated methyl groups. The d₇-glc_P spectrum is dominated by d₁ species (10±3 mM), indicating that nucleolar proteins are synthesized relatively slowly compared with deuterium abstraction (i.e., d₃ → d₂ → d₁), as by the TCA cycle and other related reactions (**Supplementary Figure 6**). More interestingly, our lipid droplet-derived biomass (d₇-glc_L) exhibits a d₁ concentration of 26±8 mM (which is spectrally distinct from lipid-associated deuterium, even at the singly deuterated level; **Supplementary Figure 10**). Although some of this signal results from single deuteration of the terminal methyl group, the d₁ concentration exceeds that of aliphatic deuterium in lipid droplets (19±6 mM). These results suggest that a significant amount of deuterium biomass in lipid droplets may in fact originate from phospholipid head groups (e.g., serine or choline) or lipid droplet-associated proteins, such as the perilipin family, rather than comprising purely fatty acid-associated deuterium. Further work is needed to fully understand these results.



Supplementary Figure 14. ACLY inhibition of d₃-pyruvate metabolism in live A549 cells.

Inhibition of ACLY via three separate drugs (ETC-1002, SB-204490, and BMS-303141) yielded no obvious attenuation of d₃-pyruvate biomass in A549 cells, which is consistent with d₃-pyruvate shuttling into lipids via ACSS2 (bypassing the inhibited pathway). Scale bar: 20 μ m.



Supplementary Figure 15. Pyruvate biomass spectra under pharmacological perturbation.

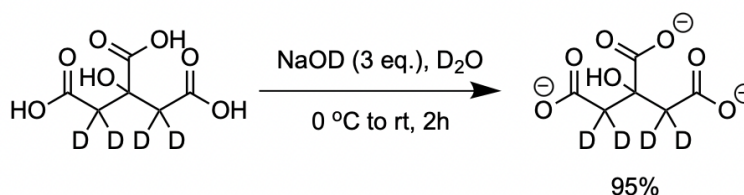
(a) hSRS spectrum of the rigid solid membrane (SM) obtained with inhibition of SCD1 with CAY10566; (b) hSRS spectrum of total cytoplasmic biomass obtained with upregulation of protein by CHIR99021.

Supplementary Tables

Supplementary Table 1. Quantified concentrations from biomass spectra.

Conc. (mM)	d ₁	d ₂	d ₃	CDHCH ₂	Total
d7-glcl	25.6 ± 7.7	4.0 ± 1.2	0	18.8 ± 5.6	48.4 ± 14.5
d7-glcp	9.6 ± 2.9	1.6 ± 0.5	0	2.2 ± 0.7	13.4 ± 4.0
d3-pyr	3.7 ± 1.1	2.0 ± 0.7	0.5 ± 0.1	0.6 ± 0.2	6.8 ± 2.0
d3-pyr SM	9.2 ± 2.8	2.4 ± 0.7	1.2 ± 0.4	6.0 ± 1.8	18.9 ± 5.7
d3-pyr+CHIR	7.0 ± 2.1	1.0 ± 0.3	1.6 ± 0.5	2.1 ± 0.6	11.7 ± 3.5

Preparation and imaging of d₄-citrate.



For metabolic imaging of d₄-citrate, we first formed a trisodium salt using commercially available 2,2,4,4-d₄-citric acid (1.92 g, 10.0 mmol; Cambridge Isotope, Cat. #DLM-3487-0.5) that was added to a flame-dried flask in 10 mL deuterium oxide as the solvent. Following this, NaOD 50% mass/D₂O solution (2.4 g, 30.0 mmol) was slowly added to the reaction mixture on an ice bath. The reaction was then allowed to react at room

temperature for 2h and its completion was monitored using H-NMR. Upon full conversion, the solvent was evaporated with a rotatory evaporator to obtain the final product d₄-citrate-trisodium salt, as a white solid with 95% yield.

For metabolic labeling, HeLa cells were incubated with 30 mM d₄-citrate for 48 hours before SRS imaging.

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