

Stimulated Raman Imaging for Spatial Metabolomics: From Metabolite Mapping to Cellular Function

Thesis by
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My Ph.D. Advisor and Committee

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*Dedicated to
my family and friends*

ABSTRACT

Metabolism encompasses the full network of chemical reactions sustaining life- the creation, utilization, and storage of biologically active small molecules known as metabolites. Understanding how these molecules are organized in space and real time, at the subcellular level, has long been a fundamental bottleneck in advancing our knowledge of cellular metabolism. In recent years, stimulated Raman scattering (SRS) microscopy has emerged as a powerful approach to visualize metabolism directly in living systems, offering high sensitivity, chemical selectivity, and a non-destructive imaging modality uniquely suited to longitudinal studies. Despite these attributes, metabolic imaging with SRS remains in its early stages relative to the well-established fluorescence-based and mass spectrometry imaging methods that dominate the field.

Therefore, this thesis aims to push the boundaries of SRS microscopy toward quantitative, spatially resolved metabolomics to enable direct visualization of metabolic activity in living systems. By applying emerging Raman probes across diverse biological systems, this work reveals metabolic vulnerabilities that deepen insight into disease mechanisms and can inform therapeutic development.

In Chapter 1, we introduce metabolic imaging using SRS microscopy and underscore its unique advantages for longitudinal imaging in living biosystems. We cover both label-free (untargeted) and labeled (targeted) approaches using SRS to probe metabolism in situ. We discuss both the advantages and current limitations of SRS microscopy and propose strategies for integrating it with complementary functional “-omics” techniques to gain deeper insight into cellular metabolism.

In Chapter 2, we explore the use of untargeted SRS microscopy to non-invasively characterize the chemical landscape of engineered living materials (ELMs) in real-time. By correlating spectral data with the mechanical properties of genetically modified biofilms, we find multiscale metabolic heterogeneity within these systems. This approach enables quantitative, real-time monitoring of ELMs that would enable improved design in applications spanning biomedicine, sustainability, and responsive materials.

In Chapter 3, we utilize targeted SRS microscopy to tackle critical questions in cardiovascular health with a focus on understanding the metabolic basis of diabetes. In collaboration with the Chen Lab at City of Hope Medical Center and the TeSlaa lab at UCLA, we observe glycogen accumulation in live endothelial cells under diabetic stress. By imaging glutamine and lactate metabolism using Raman probes for the first time in this system, we reveal how intracellular glycogen pools shape early metabolic adaptations in the endothelium during glucose deprivation- providing new insight into the vascular ramifications of hyperglycemia.

In Chapter 4, we introduce MATRIX-SRS (**M**etabolic **A**ctivity **T**Racing of the **t**ricarbo**X**ylic acid cycle by **S**timulated **R**aman **S**cattering microscopy), a platform for spatially resolved, quantitative imaging of TCA cycle activity in live cells. By combining deuterium-labeled metabolic probes with hyperspectral stimulated Raman scattering microscopy, we directly visualize and map TCA-associated metabolism at subcellular resolution. We next integrate density functional theory (DFT) with reaction network modeling to develop a robust in situ quantification pipeline in live cells. Using this approach, we identify a global attenuation of TCA activity during epithelial-to-mesenchymal transition (EMT) and, for the first time, achieve absolute quantification of deuterium-labeled biomass in live cells under native and drug-treated conditions, establishing a general framework for live-cell spatial metabolomics.

In Chapter 5, we present *MetaboRamics*, highly multiplexed **metabolic** imaging using stimulated **Raman** for spatial metabolomics in live cells. Through rational probe design, isotope editing, and robust spectral unmixing, we establish a 16-color metabolic palette spanning key pathways alongside endogenous protein, lipid, and redox signals, with organelle-targeted probes enabling spatial interactomics. We apply this high-content platform to EMT to observe a global metabolic rewiring in mesenchymal cells. We further resolve subcellular adaptations under diverse metabolic stress conditions in live epithelial cells. *MetaboRamics* enables 16-plex, subcellular metabolomics in living systems and opens new avenues for applications in drug discovery and clinical diagnostics.

Through these studies, I demonstrate the power of stimulated Raman imaging for subcellular, spatiotemporal metabolomics to visualize metabolism in living systems and uncover its roles in both health and disease.

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Chapter 1

INTRODUCTION

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1.1. Chemical Imaging of Biological Heterogeneity

Chemical imaging techniques have emerged as powerful tools for studying the heterogeneity observed in biological systems across different scales. Heterogeneity in chemical distribution throughout a biological system shapes development, function, physiology, and pathological responses.^{1,2} Among imaging methods, fluorescence microscopy is one of the most widely used tools owing to its high sensitivity, resolution, and selectivity.³ However, despite recent advancements in fluorescence imaging, this modality poses some key challenges for studying small biomolecules, e.g., metabolites, in living systems, especially systems that are not yet genetically tractable or lack a whole genome sequence for developing a reporter system.⁴ In the absence of reporter systems, challenges include the utilization and delivery of bulky fluorophores that can perturb the biological system, photobleaching, toxicity, and poor multiplexing due to broad fluorescent spectra causing spectral bleed-through.⁵ On the other hand, mass spectrometry imaging

(MSI) offers excellent sensitivity and specificity but is inherently destructive and requires extensive sample preparation steps.⁶

1.2. Raman Microscopy as an Alternative to Conventional Imaging Modalities

In contrast, Raman spectro-microscopy non-destructively probes the unique contrast from molecular vibrations and offers rich chemical information to gain insights into the composition and spatiotemporal distributions of biomolecules within heterogeneous biosystems.⁷ However, the conventional “spontaneous” Raman process has an inherently small transition cross-section. Hence, the microscopy suffers from low detection sensitivity with rather limited suitability for probing live cell dynamics. Additionally, interference from auto-fluorescence in biological samples also affects the Raman signal, which further hinders the analysis.

Recent efforts in the field of coherent Raman scattering (CRS) microscopy, including techniques such as coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), have successfully addressed many of these challenges. Specifically, in SRS microscopy, two spatially and temporally overlapping laser beams (pump and Stokes) excite the sample coincidentally. When the difference between the laser frequencies ($\Delta\omega = \omega_p - \omega_s$) matches the vibrational frequency Ω of a specific chemical bond, a phenomenon called stimulated Raman excitation occurs. The signal is subsequently detected as intensity gain (Stokes) or intensity loss (pump). Conversely, when the frequency difference does not correspond to the targeted vibrational frequency, the SRS process does not take place. This

results in a high degree of molecular specificity and selectivity with minimal non-resonance background. The utilization of near-infrared lasers makes SRS compatible with live cells with low photodamage, offers high spatial resolution in a diffraction-limited manner (~ 400 nm in lateral; ~ 1 - 2 μ m in axial), and provides 3-D sectioning capability for tissue imaging.⁸ In less scattering tissues, volumetric imaging achieves a depth of 300-500 μ m whereas in highly scattering tissues, such as brain tissue, the imaging depth is about 100 μ m.⁷ However, recent SRS-tailored tissue clearing or expansion strategies have enabled imaging depth up to ~ 1.1 mm and pushed the resolution down to sub-50 nm for either brain volumetric imaging or super-resolution investigations.⁹⁻¹¹

1.3. Advantages and Versatility of SRS for Live-Cell Metabolic Imaging

In comparison to spontaneous Raman scattering, SRS provides an amplification of Raman scattering up to 10^6 - 10^8 times and allows for imaging at speeds more than 1000 times faster.¹² SRS microscopy hence enables rapid molecular imaging at a video rate, making it well-suited for the study of dynamic cellular processes in live cells.^{13,14} For example, high-throughput SRS imaging of metabolites has been demonstrated in highly motile *E. gracilis* cells.¹³ Unlike CARS, SRS offers background-free chemical contrast, linear dependence on the analyte concentration, and higher detection sensitivity. As a result of these technical advancements, SRS microscopy has gained widespread recognition as one of the most powerful vibrational imaging modalities for quantitatively investigating metabolic processes.

By utilizing endogenous vibrational signatures or coupling with minimally perturbative and bio-orthogonal vibrational probes including alkyne and isotopes, SRS can function as both an untargeted (unlabeled) and a targeted (labeled) approach. The labeled approach yields SRS a higher sensitivity, better molecule specificity, and expanded molecular resolving power. The inclusion of additional molecular species and probe classes will further expand the scope of SRS-based single-cell metabolomics. Nonetheless, this versatile technique has already found abundant new opportunities for analyzing a wide range of metabolites (e.g. amino acids, fatty acids, nucleic acids, glucose, cholesterol, choline, glycogen, etc.) and uncovered many previously unknown regulatory and disease-related roles spanning from cell biology,^{15,16} and cancer biology¹⁷⁻¹⁹ to neuroscience,²⁰⁻²⁴ microbiology²⁵⁻²⁸ and even plant biology.²⁹⁻³¹

Another advantage of SRS microscopy is the minimal sample preparation requirements. This property enables the analysis of live or fixed samples without the need for any specific pretreatments. The high specificity of the SRS signal makes it compatible with a wide range of biological samples including standard tissue preparation methods such as fresh frozen tissues, paraformaldehyde-fixed tissues, and formalin-fixed paraffin-embedded (FFPE) tissues. In the case of paraffin which causes an increase in background signals in the fingerprint region, samples can first be deparaffinized before imaging.³² Alternatively, targeted SRS imaging can be performed to eliminate the need for imaging the fingerprint region. Due to the non-invasive nature of SRS imaging, many live samples including cells and organisms (such as *C. elegans*), can be retrieved after imaging which

allows performing longitudinal studies on the same sample. Additionally, most samples labeled with fluorophores can also be imaged in tandem with SRS microscopy with negligible crosstalk between the SRS signal and fluorescence. These properties underscore the high biocompatibility of SRS microscopy with different sample types.

For transmission-detection SRS imaging with optimal signals, samples are usually mounted between thin glass coverslips and glass slides using a spacer and imaging media (e.g., phosphate buffered saline, PBS) while avoiding air bubbles. Epi-detection SRS was also developed previously for non-transparent tissue imaging (e.g. live mouse brain), but at the sacrifice of signal levels. Proper sample hydration with the imaging media ensures heat dissipation and maintenance of optimum refractive indices during image acquisition.³³

1.4. SRS untargeted metabolic imaging

Originally developed as a label-free modality, SRS microscopy facilitates highly specific, non-invasive imaging of endogenous molecules, encompassing various chemical bonds such as C–H, C=O, S=O, O–P–O, O–H, and amide which appear in the fingerprint region ($600\text{--}1800\text{ cm}^{-1}$) and high-frequency C–H stretching region ($2800\text{--}3100\text{ cm}^{-1}$). Untargeted metabolite SRS imaging has been applied to visualize various biomolecules such as proteins,¹⁷ lipids,^{34–37} nucleic acids,^{38,16} retinoids,³⁹ cholesterol,⁴⁰ cell walls (xylan),⁴¹ neurotransmitters,²¹ pharmaceutical compounds (PCs),⁴² and biofuels^{43–45} such as limonene and pinene in a variety of biospecimens (**Table S1**). Label-free SRS imaging

has also been used for histopathology in the clinical setting to capture diagnostic hallmarks for diseases such as cancer.

Despite the advantages associated with label-free imaging, SRS imaging of small molecules remains challenging owing to the presence of interference from other cellular components with overlapping Raman peaks in the crowded fingerprint region that limits its specificity. The use of chemometrics allows spectral deconvolution but adds significant complexity to data processing. Additionally, a label-free approach poses challenges for the visualization of dynamic metabolic processes such as the uptake, localization, and turnover of small biomolecules. Therefore, to improve chemical specificity, enhance detection contrast, and fully probe cellular dynamics, bioorthogonal vibrational tags have been introduced, extending detection beyond the realm of label-free SRS microscopy.

1.5. SRS targeted metabolic imaging

Devoid of the endogenous peaks from biological samples, the cell-silent region ($\sim 1800\text{--}2800\text{ cm}^{-1}$) offers a strategic opportunity to use vibrational tags (targeted SRS imaging) based on chemical bonds with vibrational frequencies that fall within this range. Using bioorthogonal Raman tags containing moieties such as alkynes ($\text{C}\equiv\text{C}$), nitriles ($\text{C}\equiv\text{N}$), and stable isotopes (such as deuterium and C^{13}) has not only enhanced sensitivity and specificity but has also enabled visualization of spatiotemporal dynamics of small biomolecules, a capability not achieved through label-free SRS imaging. These metabolites include (but are not limited to) amino acids,⁴⁶ fatty acids,^{47,19} sterols,⁴⁸ glucose,^{49–52}

glycan,⁵³ choline,⁵⁴ neurotransmitters,⁵⁵ and pharmaceutical compounds such as ferrostatin⁵⁶ and anisomycin⁵⁷ (**Figure 1**).

In addition to tracking the uptake of stable isotope labeled (SIL) metabolites, SRS imaging has been instrumental in observing their synthesis and turnover. For example, deuterated glucose has been coupled with SRS microscopy to visualize its full metabolism—from its catabolism, and anabolic utilization to macromolecular synthesis *in situ*. This probe gets metabolically incorporated into different glucose-related biosynthesis pathways, resulting in distinct C–D Raman peaks that are influenced by the local chemical environment of the biomacromolecule. Through spectral unmixing, the different C–D spectra can be resolved into chemical maps of their corresponding biomacromolecules, enabling visualization of glucose metabolism.⁵⁰ Furthermore, this strategy has enabled the visualization of the subcellular enrichment of glycogen, shedding light on the metabolic reprogramming of glycogen in cancer,⁵¹ as well as hyperglycemic endothelial cells.⁵² In tandem with alkyne–glucose analog (3-OPG) for assaying glucose uptake, two-color glucose imaging for simultaneous investigation of glucose uptake and metabolism, central to metabolic pathways, in live cells has been made possible.⁵⁸

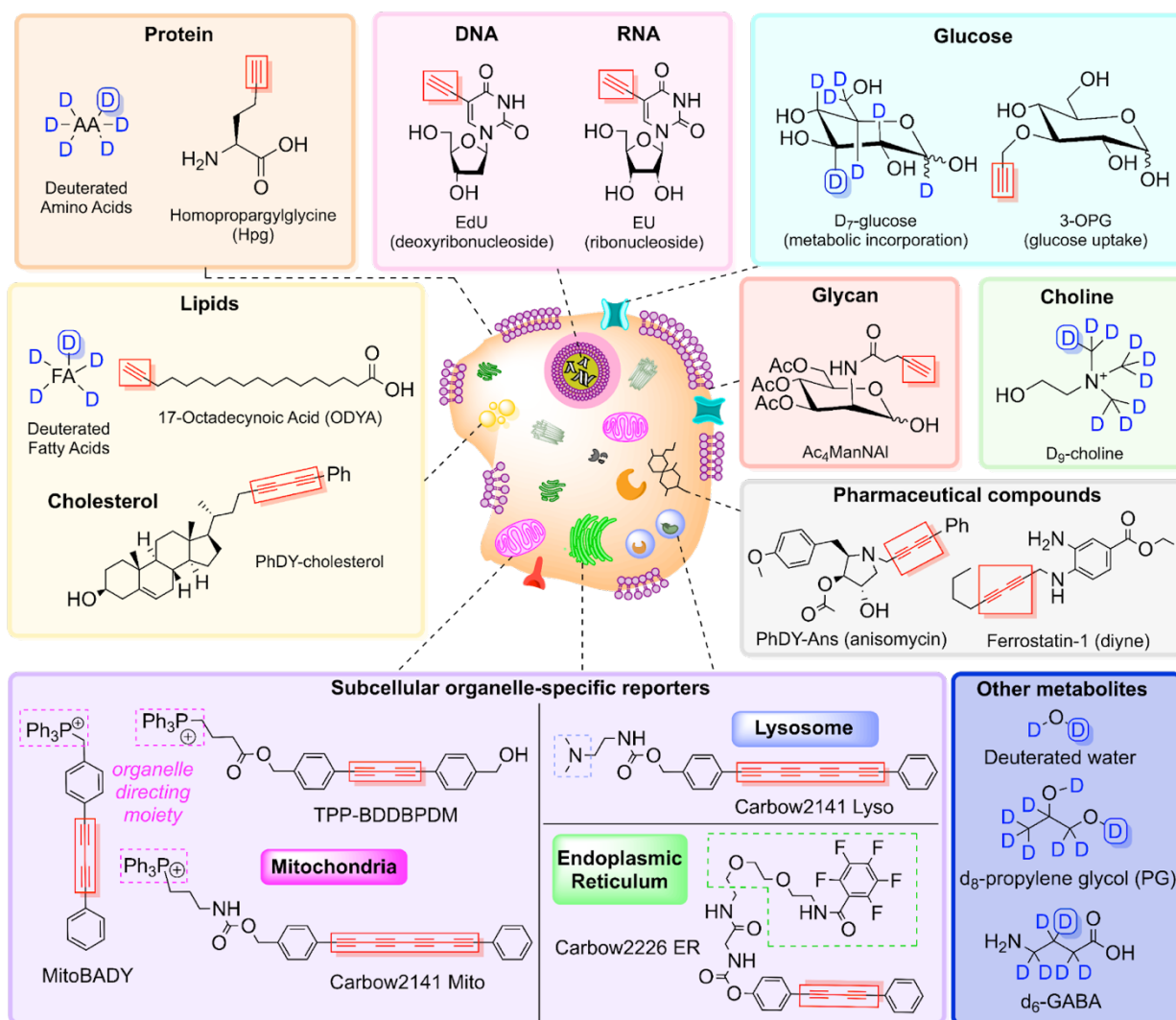


Figure 1. Stimulated Raman imaging of metabolites. Representative vibrational tags for targeted SRS imaging of metabolites such as lipids, glucose, choline, glycan, and more.

Apart from parallel phenotyping of multiple metabolic species, SRS imaging enables multiplexing of the same metabolic species through selective labeling of metabolic probes based on their structural and spectroscopic differences. For instance, Wei et al. achieved real-time visualization of the total complex proteome metabolism by employing a two-color pulse-chase imaging of proteins.⁵⁹ Using two structurally different subsets of

deuterated amino acids, proteins were labeled at different times resulting in distinct Raman signals for multiplexed protein imaging. This approach could be extended to metabolites in dynamic processes by labeling different functional groups with stable isotopes. Similarly, isotopic labeling on alkynes and diynes has enabled the labeling of different metabolites, increasing the possibilities for multicolor SRS imaging.⁶⁰ It is worth noting that any label other than a stable isotope may alter the biological function of a metabolite so it would be important to validate the altered metabolite prior to use in an imaging experiment. For instance, 3-OPG which cannot enter the glycolysis pathway, serves as an effective probe to assay short-term glucose uptake. In contrast, deuterated glucose which can be metabolically incorporated in cells, is a better probe for monitoring long-term glucose metabolism.

Organelle-targeted SRS reporters for subcellular imaging of cellular structures such as mitochondria,^{61,62} lysosomes,^{63,64} and the endoplasmic reticulum⁶⁴ have also been developed. For instance, Bae et al. previously utilized an aryl-diyne-based Raman tag called TPP-BDDBPDM to develop a quantitative model to link mitochondrial membrane potential, a key indicator for mitochondria-mediated metabolic activities, with key pharmacokinetic properties (such as uptake rate and intracellular concentrations of TPP) in live cells.⁶⁵ Taking advantage of the narrow line width of the Raman bands ($<10\text{-}20\text{ cm}^{-1}$), Raman sensors for the detection of intracellular environments such as pH,⁶⁶ metal ions,⁶⁷ small signaling molecules such as hydrogen sulfide,⁶⁸ as well as enzymatic activity have also been developed.⁶⁹

1.6. Advances in Multiplexing and Multi-Omics Integration: Toward Systems-Level Imaging

Enhancing SRS imaging sensitivity would allow more low-abundant metabolites to be detected. The use of these Raman tags has facilitated μM levels of sensitivity of metabolites. For example, the analytical sensitivity of the alkyne-containing thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU) is $200\ \mu\text{M}$ in live cells.¹⁵ Current efforts in the development of highly selective, sensitive, and multiplexed Raman palettes have further increased the detection sensitivity to the high nM range. These include xanthene-based, electronic pre-resonance-enhanced Manhattan Raman Scattering (MARS) dyes and polyynes-based 'Carbow' dyes.⁷⁰ To expand the range of the current palette of Raman tags, novel metallacarborane probes that appear in the 'unchartered territory' of the cell-silent window ($2300\text{-}2800\ \text{cm}^{-1}$) have also been recently reported.⁷¹

Due to its strong complementarity with other modalities, integration of SRS with fluorescence has enabled faster imaging and expanded multiplexing, achieving up to 8-plex imaging in live cells. Additional strategies, including polyyne-based probes, electronic preresonance-SRS (epr-SRS), and sample tailoring approaches such as tissue clearing, have further extended SRS imaging to 10 colors in live cells⁷² and 11 colors in tissues.⁷³ However, despite these advances, the accessible metabolic palette remains constrained, particularly by spectral overlap among deuterium-based species, which limits chemical specificity and poses challenges for super-multiplexed metabolic imaging.

Therefore, strategies that combine rational probe design with high molecular resolvability are critical to fully realize the potential of SRS.

Importantly, when integrated with complementary -omics approaches, including transcriptomics (e.g., bulk and single-cell RNA sequencing), LC-MS-based metabolomics and lipidomics, and stable isotope label-resolved fluxomics, SRS-based metabolic imaging offers a powerful framework to link spatially resolved metabolic activity with systems-level molecular information. Moreover, emerging efforts in spontaneous Raman spectroscopy, such as Raman2RNA⁷⁴ and RamanOmics,⁷⁵ highlight the potential to infer gene expression profiles directly from vibrational signatures. While these frameworks currently employ spontaneous Raman rather than SRS, they point toward an exciting convergence where SRS-based spatial metabolomics could be integrated with transcriptomic inference frameworks to bridge chemical imaging and molecular profiling. Collectively, such multimodal integration of SRS holds significant promise for uncovering disease mechanisms and identifying novel therapeutic targets.

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Chapter 2

QUANTITATIVE REAL-TIME ANALYSIS OF LIVING MATERIALS BY STIMULATED RAMAN SCATTERING MICROSCOPY

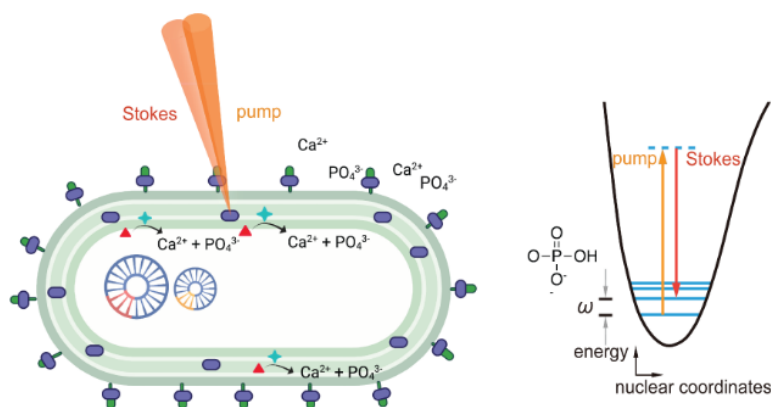
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2.1. Summary

Composite materials built in part from living organisms have the potential to exhibit useful autonomous, adaptive, and self-healing behavior. The physicochemical, biological, and mechanical properties of such materials can be engineered through genetic manipulation of their living components. Successful development of living materials will require not only new methods for design and preparation, but also new analytical tools that are capable of real-time noninvasive mapping of chemical compositions. Here we establish a strategy based on stimulated Raman scattering microscopy to monitor phosphatase-catalyzed mineralization of engineered bacterial films in situ. Real-time label-free imaging elucidates the mineralization process, quantifies both the organic and inorganic components of the material as functions of time, and reveals spatial heterogeneity at multiple scales. In addition, we correlate the mechanical performance of films with the

extent of mineralization. This work introduces a promising strategy for quantitatively analyzing living materials, which should contribute to accelerated development of such materials in the future.



2.2. Introduction

In nature, bacterial cells are often found in complex biofilms, where they interact through cell adhesion, matrix secretion and quorum sensing. Biofilms provide excellent examples of “living materials” in which bacterial survival is enhanced under conditions of environmental stress. Inspired by natural biofilms, several laboratories have reported the development of engineered living materials (ELMs) that contain live cells and polymeric matrices.¹ Genetic manipulation of the constituent cells in ELMs allows control of composition, structure, growth dynamics, and cellular interactions, and provides a powerful tool for design of materials with potential applications in robotics, tissue engineering, drug delivery, etc.²

To date, our understanding of ELMs has been limited by a dearth of non-destructive analytical methods that can be applied *in situ*.³ Traditional methods such as

thermogravimetric analysis (TGA)⁴ lack spatial and temporal resolution. Imaging based on Fourier-transform infrared spectroscopy (FTIR)⁵ has low resolution and sensitivity, and high water background. Secondary ion mass spectrometry (SIMS)^{6,7} and electron microscopy (TEM and SEM)⁸ are intrusive, cannot be applied *in situ*, and have low throughput. Fluorescence microscopy offers substantial advantages, but requires labels or stains that can alter sample behavior.^{9,10}

To address these challenges, Raman spectro-microscopy has emerged to target specific vibrational modes and visualize the distribution of selected molecules and structures within living cells and macromolecular scaffolds.¹¹ In particular, nonlinear stimulated Raman scattering (SRS) microscopy (Figure 1a) retains the advantages of conventional spontaneous Raman imaging while achieving improved spatial and temporal resolution, significantly higher sensitivity (below millimolar), faster imaging throughput (up to video rate), and deeper sample penetration (up to $\sim 10^2$ μm).¹²⁻¹⁴ The spectral resolvability of SRS microscopy allows fine structural analysis of target materials, and can reveal changing compositions, bonding conditions, conformations, and details of the extracellular microenvironment.¹⁵ The linear dependence of SRS intensity on target concentration enables highly desirable quantitative analysis.¹⁶ SRS imaging has been shown to be applicable to the study of biofilms^{17,18} and calcification processes¹⁹. Therefore, SRS imaging presents an ideal combination of features for non-intrusively and quantitatively analyzing the components of ELMs, allowing real-time observation of ELM growth and the investigation of functional connections between the properties of materials and their

chemical, structural and morphological characteristics, paving the way for high-throughput materials screening. The fast feedback provided by SRS microscopy also helps form and test hypotheses that can inspire and accelerate imaging-guided design of new ELMs.

Here, we report the use of SRS microscopy to track and quantify phosphatase-catalyzed mineralization of engineered bacterial systems *in situ*. We first established SRS spectral-imaging as an effective tool to benchmark bacterial mineralization levels in both single cells and bacterial films. Applying this tool to ELM design, we tested the hypothesis that the mechanical performance of mineralized biofilms is determined by two factors: (1) the concentration of the inorganic component; and (2) the spatial distribution of the organic (living cells) and inorganic (scaffolds or matrices) components. By combining SRS imaging-analysis and hydraulic bulge tests, we successfully correlated the mechanical performance of the mineralized biofilms with their inorganic-to-organic ratios. We observed that the biofilms exhibit Young's moduli of tens of kPa and can endure relatively large strains (≥ 0.1) at early mineralization stages, and that moduli and elongation behavior can be tuned through control of the inorganic-to-organic ratio. All these changes were observed within minutes of initiating mineralization. This study illustrates the value of *in situ* SRS imaging in accelerating ELM analysis, design and development.

2.3. Results and Discussion

Analysis of Alkaline Phosphatase (PhoA)-catalyzed Calcium Phosphate Formation by *in situ* SRS Imaging.

Scaffolds based on bioceramics such as calcium phosphates are preferred candidates for medical implants.²⁰ To explore the integration of bioceramics into ELMs, we employed a bacterial mineralization system based on a modified version of the well-known phosphatase PhoA.²¹ Three engineered variants of *Escherichia coli* strain DH10B were examined: PhoA, E6/PhoA and Triblock/PhoA. All of them contain a plasmid that carries a gene encoding PhoA under control of an arabinose-inducible promoter, with an N-terminal leader peptide that guides the enzyme to the periplasm (**Figure 1a**, sequence design in **Figure S1, Table S1**). Two of the variants have an additional plasmid that encodes constitutive expression of a surface display protein (**Figure 1a**, sequence design in **Figure S1**). E6/PhoA's surface protein has six repeats of an elastin-like peptide (E, 25-aa). Triblock/PhoA's surface protein has three E3 domains spaced by two copies of a 16-aa peptide sequence known to mediate calcium phosphate mineralization.²² Upon induction, all three variants gain periplasmic phosphatase activity, which converts organic phosphates into inorganic phosphate (**Figure 1b**). When the cells are cultured in buffer supplemented with calcium glycerophosphate (CGP), PhoA converts glycerophosphate to phosphate anion and thereby initiates formation of calcium phosphate minerals (which we designate bio-calcium phosphate, BCP) (**Figure 1b**). Because PhoA resides in the

periplasm, we expect mineralization to be confined primarily to the periplasm and cell surface.

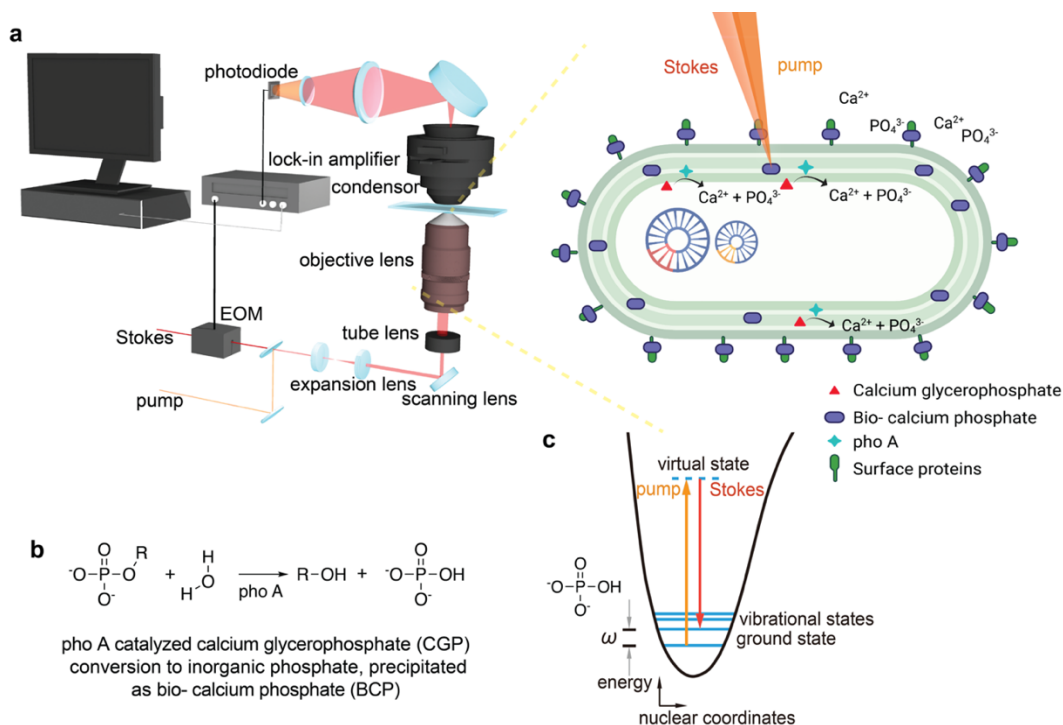


Figure 1. Label-free tracking of the engineered *E. coli* bacterial mineralization. (a) Schematics of the stimulated Raman scattering (SRS) imaging setup and an engineered *E. coli* cell for biomineralization. **(b)** PhoA-catalyzed conversion of calcium glycerophosphate (CGP) into bio-calcium phosphate (BCP). **(c)** Energy scheme of the phosphate vibrational modes probed by SRS microscopy. The SRS signal is detected when the energy difference between the pump photons and the Stokes photons matches the frequency of the vibrational mode of phosphate (ω).

In a typical *in situ* SRS imaging setup for probing PhoA-catalyzed calcium phosphate formation, two spatially and temporally overlapped pulsed laser beams, pump and Stokes, are sent into a confocal-type microscope and focused on the sample (Figure 1a, left). When the energy difference between the pump and Stokes laser photons matches the vibrational frequency of target chemical bonds (e.g. the phosphate bonds from BCP, Figure 1c), those

bonds are efficiently driven from the vibrational ground state to the corresponding vibrational excited state, creating stimulated Raman loss in the pump beam, which is subsequently detected by a photodiode (**Figure 1a, 1c**). Series of chemical maps can then be efficiently generated by raster-scanning the laser focus throughout the samples in 3D.

Raman Characterization and SRS Imaging of *E. coli* Mineralization.

Because the phosphate vibrational frequency varies among common forms of calcium phosphate, we first used spontaneous Raman spectroscopy to identify the Raman signals characteristic of BCP (**Figure 2a**, blue): the most distinctive band is at 961 cm^{-1} , which we assign to the ν_1 symmetric stretching mode of PO_4^{3-} (BCP- ν_1 - PO_4^{3-}).^{23,24} This band is also observed for hydroxyapatite (HA, **Figure 2a**, green), suggesting structural similarity between BCP and HA. In contrast, β -tricalcium phosphate (β -TCP, **Figure 2a**, magenta), another form of calcium phosphate widely used in bioceramic implants, presents its own unique spectrum with clear splitting in the frequency region of the PO_4^{3-} symmetric stretching. The peaks at 949 cm^{-1} and 970 cm^{-1} reflect significant differences in the intratetrahedral P-O bond lengths for different nonequivalent PO_4^{3-} ions of the β -TCP structure.²⁴ These results indicate that the mineral precipitated through the action of PhoA is a non-stoichiometric HA rather than β -TCP, consistent with previous reports.²¹ The fact that the ν_1 symmetric stretching mode of PO_4^{3-} is absent from the spectrum of the substrate

CGP (CGP, **Figure 2a**, red) enables clear distinction between substrate (CGP) and product (BCP) in the course of SRS imaging and tracking of the mineralization process.

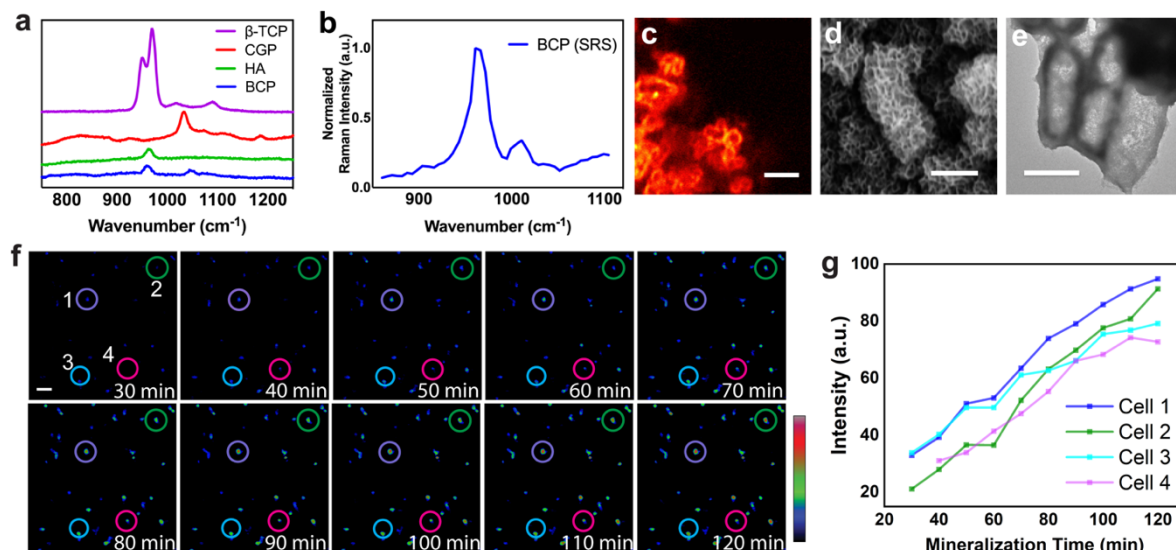


Figure 2. Raman spectral characterization, SRS imaging and real-time tracking of PhoA-catalyzed mineralization of *E. coli* cells. (a) Spontaneous Raman spectra of the various forms of phosphates: the organic precursor calcium glycerophosphate (CGP, red), β -tricalcium phosphate (β -TCP, purple), hydroxyapatite (HA, green), and bio-calcium phosphate formed by bacterial mineralization (BCP, blue). (The spectra suggest that the mineralization product BCP most resembles HA among all common forms of inorganic phosphate. (b) The SRS spectrum of BCP showing the characteristic phosphate peak at 961 cm⁻¹. (c) An SRS image showing the phosphate formed on the surface of mineralized bacterial cells, exhibiting hollow structures similar to those in the TEM image. Scale bar, 5 μ m. d-e, SEM (d) and TEM (e) images showing mineralized bacterial cells expressing PhoA in the periplasm are wrapped in a mesh of calcium phosphate. Scale bars, 2 μ m (d) and 1 μ m (e). (f) In-situ phosphate-channel SRS images of a liquid culture of *E. coli* cells during mineralization over a period of 2 h, showing the real-time accumulation of inorganic phosphate at the single-cell level. Scale bar: 5 μ m. (g) Phosphate-channel SRS intensity of four arbitrarily selected *E. coli* cells (circled in f) showing increasing phosphate deposition with cell-to-cell heterogeneity.

We next obtained the SRS hyper-spectrum from a BCP sample (**Figure 2b**) and verified that the characteristic SRS band at 961 cm^{-1} could be detected. We then employed SRS microscopy to capture the spatial distribution of BCP- ν_1 - PO_4^{3-} (denoted as phosphate, 961 cm^{-1}) in live *E. coli* cells subjected to mineralization conditions (**Figure 2c**). Consistent with our expectation that mineralization should occur near the periplasm and cell surface, SRS images of mineralized bacteria revealed clear core-shell structures associated with individual cells, indicating that each cell is wrapped in a mesh of calcium phosphate (**Figure 2c, Figure S2**). These results are consistent with scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of the corresponding samples (**Figure 2d-e**). Notably fluorescence microscopy images (**Figure S3**) of calcein-stained samples suffer from high background and non-specific staining, which precluded quantitative analysis (Experimental details in **Supplementary Note 6**).

Once a standard workflow for SRS imaging of mineralized bacterial films was established, we undertook *in situ* time-lapse tracking of the *E. coli* mineralization process. We first demonstrated tracking at the single cell level by analysis of *E. coli* liquid cultures. Cells were suspended in a nutrient-free HEPES buffer (pH=7.4) containing CGP as substrate and diluted to an appropriate optical density (details in Materials and Methods). Time-lapse images of single cells over the course of 2 h of mineralization showed an increase in the phosphate-channel SRS intensity (**Figure 2f**) with cell-to-cell heterogeneity in the extent of mineralization (**Figure 2g**), showing a difference up to 50% at early stages. By taking advantage of the proportionality between the SRS signal intensity and the concentration of BCP, we determined that the amount of phosphate deposited could triple

or quadruple between 30 and 120 min of mineralization (**Figure 2g**). After 2 h, the mineralization process slows, indicating that it is close to completion. To our knowledge, this is the first report of visualization of such rapid mineralization in a label-free and non-destructive way in a bacterial system.

Real-time SRS Imaging of Mineralization in Bacterial Films. To prepare bacterial films for mineralization, overnight *E. coli* cultures in LB media were dropped on polycarbonate membrane filters with 0.2 μm diameter pores and vacuum filtered. Filters were transferred to fresh LB agar plates containing suitable antibiotics daily for six days to allow proliferation and constitutive expression of surface-displayed proteins and then moved to LB agar plates with suitable antibiotics and 0.1% L-arabinose for one day to induce expression of PhoA. The bacterial films and supporting filters were then immersed in mineralization buffer containing CGP for periods of a few hours to 24 h. Detailed procedures used for bacterial film growth and mineralization can be found in Materials and Methods. After 24 h, mineralized films were stiff and tough enough to be peeled off their filter supports for imaging and analysis (**Figure 3a**, **Figure S4**).

SRS images of mineralized bacterial films recorded at 961 cm^{-1} (phosphate, **Figure 3c**) and at 2940 cm^{-1} (CH_3 , **Figure 3d**), and fluorescence images of calcein-stained films (**Figure 3b**) showed co-localization of phosphate ions, *E. coli* cells and calcium ions, respectively, confirming the utility of SRS imaging of mineralized samples. It is worth noting that 961 cm^{-1} signals in SRS images of calcein-stained samples were much weaker

than those derived from unstained samples (**Figure 3c** vs **Figure 3e** at 105 min). This difference was due to a competing reaction during fluorescence staining: dissolution of inorganic phosphate occurs as the calcein-AM dye chelates calcium cations and replaces the phosphate anions. This effect was also evident in images of isolated cells (Figure S5), illustrating the value of imaging methods that do not rely on stains.

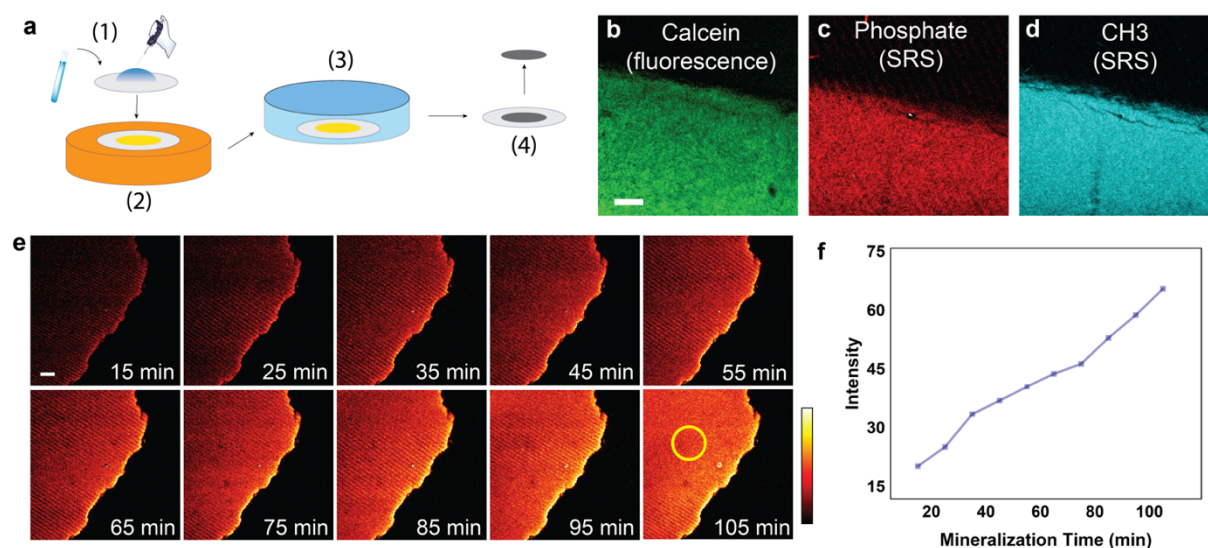


Figure 3. SRS imaging and real-time tracking of *E. coli* bacterial film mineralization.

(a) Preparation of a typical bacterial film (growth and mineralization). (1) Overnight *E. coli* culture inoculated in LB media was dropped on polycarbonate membrane filter with 0.2 μm diameter pores and vacuum filtered. (2) The membrane filter carrying bacteria was transferred to an LB agar plate containing suitable antibiotics and inducer and grown for several days to allow the surface-displayed protein and PhoA to express. (3) The mature film on polycarbonate filter was immersed in mineralization buffer containing calcium glycerophosphate for intervals ranging from a few hours to one day. (4) The mineralized film, hard and self-standing, can be peeled from the polycarbonate filter for imaging and analysis. **(b)-(d)** Microscopy images of a typical mineralized *E. coli* biofilm: fluorescence image, calcein stained **(b)**, SRS image, phosphate channel **(c)**, and SRS image, CH₃ channel **(d)**. Scale bar, 25 μm . **(e)** *In-situ* phosphate-channel SRS images of a typical *E.*

coli biofilm during mineralization over a period of 105 min, showing the real-time accumulation of inorganic phosphate in films. Scale bar, 20 μm . f, Phosphate-channel SRS intensity of an arbitrarily selected region during mineralization in the *E. coli* biofilm.

To visualize the dynamics of mineralization in bacterial films by SRS, *E. coli* films were grown and induced as above, then transferred to a sealed imaging chamber and immersed in mineralization buffer (**Figure 3a(3)**). Time-lapse SRS images were recorded every 10 min with an acquisition time of 8.6 s/frame in the phosphate channel (961 cm^{-1}) (**Figure 3e**). A clear increase in the phosphate intensity indicated a rapid rise in the mineralization level over the course of 105 min (**Figure 3f**, intensities recorded from the areas circled in yellow in Figure 3e to avoid intensity heterogeneity from the edge to the center of the film). The rate of mineralization is remarkably high in comparison to previous reports in which mineral deposition occurred over periods ranging from days to weeks.^{10,21,22} With the quantitative nature of SRS imaging, we also observed an “edge effect”^{25,26} characteristic of many reaction systems in heterogeneous environments: contact between cells and substrate is enhanced at the edge of the biofilm, leading to faster mineralization at the edge. As shown in Figure S6, SRS signal intensity in the phosphate channel (961 cm^{-1}) at the edge is much higher compared to that in the interior region of a bacterial film after 120 min of mineralization, while the background signals showed negligible difference. This result confirms that mineralization was faster at the edge during mineralization.

Morphology of Mineralized Biofilms

Owing to its high sensitivity and good temporal resolution, SRS microscopy allows the recording of time-lapse images of phosphate deposition even in the early stages of bacterial mineralization. In addition, the fine spatial resolution of SRS microscopy provides insight into the morphological characteristics of mineralized films and the relations between structure and properties. With that objective in mind, we first benchmarked the morphology of unmineralized films by label-free imaging in the organic channel (Figure 4a-b, CH₃, different cross-section views). We then prepared mineralized films based on *E. coli* strains that displayed different cell-surface proteins that we anticipated would modulate mineral deposition and mechanical properties.

Inspired by previous work of Prieto *et al*²², we examined the role of cell-surface polypeptides in controlling the structures and properties of mineralized bacterial films. We prepared three types of *E. coli* cells (shown schematically in **Figure 4c**): (i) one free of surface-displayed polypeptides (PhoA, described above), (ii) a second that displays E6 peptides (E6/PhoA), and (iii) a third with surface expressed triblock peptides (triblock/PhoA). The E6 peptide contains six 25-residue elastin-like repeats (**Table S2**) and facilitates intercellular adhesion. When bacterial cells display E6 at the surface, they aggregate, forming clusters and precipitating out of the culture medium. They also form cohesive soft films due to intercellular adhesion. The E6 peptides contain glutamic acid residues that can bind to Ca²⁺ and serve as potential nucleation sites for calcium phosphate. The triblock peptide also contains nucleation sequences known to facilitate calcium phosphate mineralization.²² Compared with E6, the triblock peptide appears to cause weaker intercellular adhesion. Films grown from bacterial cells that displayed the triblock

peptide exhibited reduced mechanical strength and were less cohesive than E6 films, consistent with our observation that the triblock/PhoA strain doesn't generate aggregates as strongly as E6 in planktonic culture (**Figure S7**). Parallel 3D volume SRS images of both the organic component (cells, CH₃ channel, **Figure 4d/g/j**) and the inorganic component (calcium phosphate, phosphate channel, **Figure 4e/h/k**) were acquired for bacterial film samples from all three constructs. Excellent spatial co-localization was seen for cells and deposited phosphate materials. Cross-sectional views are presented in **Figure 4f/i/l**.

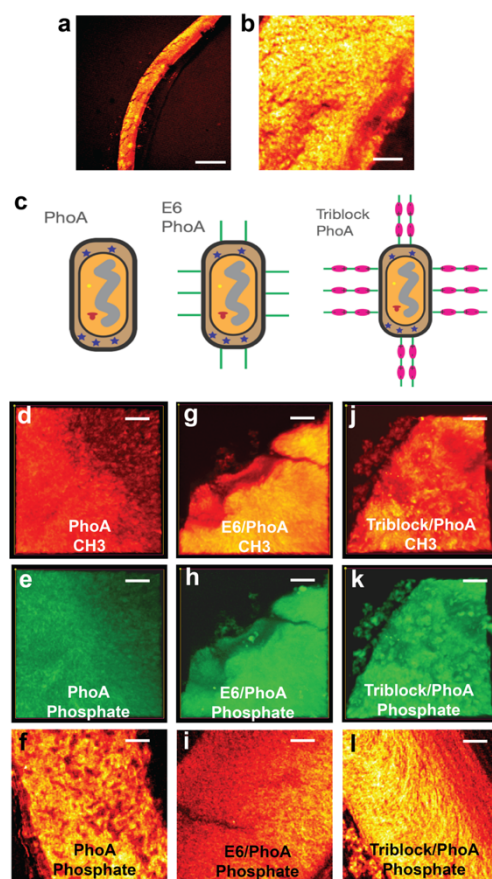


Figure 4. SRS images of *E. coli* biofilms with different surface protein constructs. (a)-(b) Cross-sectional views of a typical unmineralized *E. coli* biofilm at different magnifications. **(c)** Three types of engineered *E. coli* cells with different surface constructs: bare surface type (PhoA), with surface expressed E6 peptides (E6/PhoA) and with surface

expressed Triblock peptides (Triblock/PhoA). Detailed sequence information about E6 and Triblock peptides is included in supplementary data. d-f, SRS images of a PhoA-type *E. coli* biofilm: CH₃ channel, axial-view of 3D volume image (**d**), phosphate channel, axial-view of 3D volume image (**e**) and phosphate channel, cross-sectional view (**f**). (The PhoA-type *E. coli* biofilms exhibit heterogeneous spatial distribution of organic and inorganic materials, showing individual cells clearly. **(g)-(i)**, SRS images of a E6/PhoA-type *E. coli* biofilm: CH₃ channel (**g**), phosphate channel (**h**) and phosphate channel, cross-sectional view (**i**). The E6/PhoA-type *E. coli* biofilms exhibit more homogeneous spatial distribution of phosphate. **j-l**, SRS images of a Triblock/PhoA-type *E. coli* biofilm: CH₃ channel (**j**), phosphate channel (**k**) and phosphate channel, cross-sectional view (**l**). The Triblock/PhoA-type *E. coli* biofilms exhibit heterogeneous spatial distribution of organic and inorganic components with clear surface roughness. Scale bars: a, 100 μm ; b, d-l, 10 μm .

We found clear differences in film morphology among the three engineered strains. The PhoA strain (**Figure 4d-f**) yielded sparse distributions of both cells and phosphate materials, with spatially detached cells arrayed across the film. The triblock/PhoA construct (**Figure 4j-l**) exhibited a different type of heterogeneity. Larger blocks of materials, apparently formed by small aggregates of cell/phosphate hybrid structures, made the film heterogeneous and coarse in texture, especially near the surface, where roughness was observed ubiquitously. In contrast, in the E6/PhoA (**Figure 4g-i**) construct, cells and phosphate were distributed more homogeneously. We believe that in this case E6 proteins displayed at the cell surface act as intercellular adhesives that bind the cells, forming a compact and dense structure, which was maintained during the mineralization process. SEM images (**Figure S8**) and calcium-dependent fluorescence images (**Figure S9**) yielded similar results, confirming the morphological differences among the three types of films.

We also found that the surface protein influenced cell viability within the film during late-stage mineralization (Figure S10, characterized as colony forming units (CFUs) per unit area). Unmineralized films of PhoA, E6/PhoA and triblock/PhoA started at 10^6 CFU/mm²; this value remained nearly unchanged during the first hour of mineralization. After 1 h, however, the CFU per unit area started to decline slowly for all three types of films. After 2 h, E6/PhoA samples contained roughly 10^5 CFU/mm² with a minor decrease of cell viability, while triblock/PhoA samples dropped to 10^4 CFU/mm², signaling a more substantial loss of viability.

Correlating Biofilm Mechanical Properties with Phosphate Levels Quantified by SRS.

A central objective of this work was to explore the role of mineralization in increasing the stiffness of engineered bacterial films. The morphological differences observed by SRS microscopy led us to anticipate a range of mechanical properties in the three bacterial strains described in the preceding section. We noticed immediately, when handling biofilms at different stages of mineralization, that there were readily discernible differences in mechanical properties among the strains. We then undertook a systematic study of mechanical behavior as a function of the extent of mineralization, in which we performed parallel SRS imaging and hydraulic bulge testing experiments on film samples exposed to mineralization conditions for periods of time ranging from 20 to 120 min.

We used the molar phosphate/CH ratio in each sample determined by SRS imaging to characterize the extent of mineralization. These molar ratios could be achieved by

calculations based on the SRS intensities in the phosphate and CH channels, and the known Raman cross sections of phosphate P-O bond and C-H bond vibrations (details in **Supplementary Note 7** and **Table S3**).

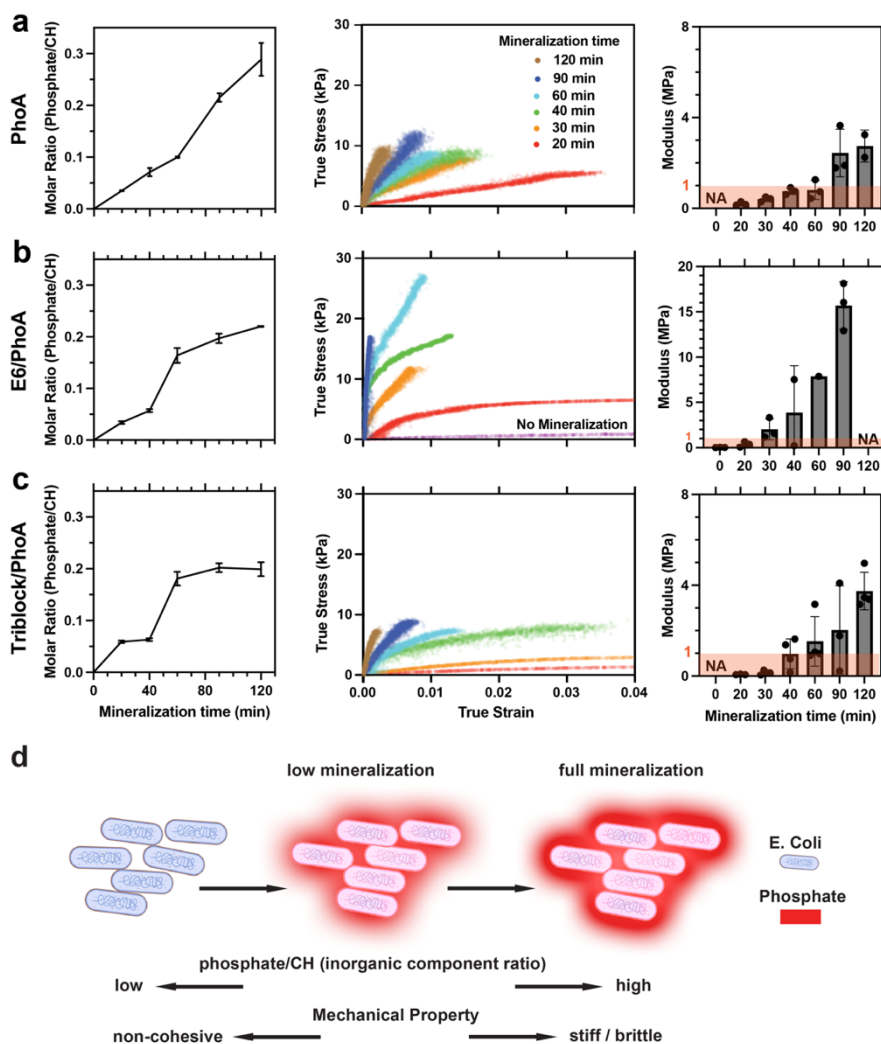


Figure 5. Inorganic phosphate levels and mechanical properties of *E. coli* biofilms during mineralization. (a)-(c) Parallel studies on three types of biofilms, based on PhoA (a), E6/PhoA (b), and the Triblock/PhoA (c) constructs, during 2-hour mineralization, showing increasing molar ratios of phosphate/CH (left column), clear transitions in stress-strain relations (center column), and moduli calculated from the stress-strain data (right column). (PhoA and Triblock/PhoA films transitioned from non-cohesive at 0 min to cohesive during the mineralization process, while E6/PhoA films transitioned from

cohesive soft yielding films into stiff non-yielding films in the end. After 2 h of mineralization E6/PhoA films became stiff and brittle, and cannot be picked up without fracture. Therefore, the modulus could not be determined at 120 min. Moduli of the biofilms increased during mineralization. **(d)** Schematics showing the relations between phosphate level and mechanical properties of the biofilms according to the observations from handling the films.

Films were mounted in a fluidic device and subjected to a bulge/inflation test; deformation was recorded by optical coherence tomography (OCT) imaging (**Figure S11**, details in **Supplementary Note 8**). True stress- true strain curves were constructed for each film assuming membrane deformation. Young's modulus was calculated using the initial linear portion of each curve (**Figure 5a-c**, middle column, and **Figure S12**). Notably, the E6/PhoA construct (**Figure 5b**) exhibited marked increases in stiffness as mineralization proceeded; unmineralized E6/PhoA films were soft, exhibiting Young's moduli of tens of kilopascals (kPa). Upon initial mineralization (20-30 min) the Young's modulus increased by as much as to two orders of magnitude. Films exposed to mineralization conditions for less than 40 min exhibited yielding behavior and eventual film failure, behavior that was absent at longer mineralization times within the limits of stress imposed by our tests. Compared to the free PhoA (**Figure 5a**) and the triblock/PhoA constructs (**Figure 5c**), E6/PhoA biofilms (**Figure 5b**) exhibited a 4- to 5-fold enhancement in Young's modulus (**Figure 5a-c**, right column). Notably, SRS quantification showed that marked changes in the mechanical behavior of E6/PhoA films required only modest levels of mineralization. For example, after only 20 min of mineralization, the molar ratio of phosphate/CH was less than 10% for biofilms with the PhoA and E6/PhoA constructs; nevertheless, the Young's

moduli of the films increased from the kPa level to the MPa level, indicating at least a 100-fold increase in stiffness. The result suggests that even a relatively small change in the inorganic matrix content can change the mechanical properties of biofilms dramatically (**Figure 5d**). We note that the degree of mechanical property enhancement varies among different bacterial constructs (PhoA, E6/PhoA, triblock/PhoA) with similar degree of mineralization. Such variations among different constructs could be attributed to the structures and morphologies of the minerals formed in different constructs (**Figure 4d-l**, **Figures S8-S9**). One distinctive feature of E6/PhoA films is their relative homogeneity in mineral distribution. In this case, cells in the E6/PhoA films appear to be wrapped in a relatively homogeneously distributed extracellular mineral matrix. However, PhoA and triblock/PhoA films are more heterogeneous, exhibiting discrete individual cells not tightly connected to one another by the deposited mineral. Therefore, the mechanical strengths of PhoA and triblock/PhoA films were less enhanced by the mineralization network.

2.4. Conclusion

In this work, we demonstrate the utility of SRS microscopy for quantitative investigation of engineered living materials, specifically for *in situ* tracking of rapid mineralization of bacterial systems. By correlating quantitative SRS imaging and mechanical testing data, we have gained new insight into the structural origins of the mechanical behavior of mineralized bacterial films, exhibiting a imaging-guided design strategy of engineered living materials. In searching for an optimally performing *E. coli* - calcium phosphate hybrid film, we found especially striking increases in the moduli of

films bearing the E6 surface protein even at modest levels of mineralization (less than 10% in terms of the phosphate-to-CH molar ratio). This molar ratio of around 10% represents a sweet spot of mineralization level that led to a mechanically superior hybrid biomaterial. We also discovered that different surface proteins yielded different mineralization morphologies, which exhibited different mechanical behaviors at comparable mineralization levels. More generally, we illustrate the value of combining microscale morphological information, quantitative chemical imaging, and mechanical testing in the analysis and design of engineered living materials.

2.5. Materials and Methods

Preparation of Bacterial Films

Individual colonies harvested from LB plates were grown overnight to stationary phase in liquid LB medium supplemented with 100 mg/L ampicillin and 25 mg/L chloramphenicol to maintain plasmid stability. The resulting cultures were then diluted to an optical density at 600 nm (OD_{600}) of approximately 0.8. Diluted cultures were loaded on UV-sterilized track-etched polycarbonate filters (Nuclepore Whatman, 0.2 μ m pore size) mounted in vacuum filter units (Nalgene Rapid-flow Thermo-Fisher). For 2.5 cm diameter polycarbonate filters, 200 μ L culture volumes were loaded; 2 mL volumes were loaded on 4.7 cm diameter filters. After filtration was complete, filters were transferred to fresh LB plates. Bacterial films were grown in a 37 °C incubator. Filters were moved to fresh LB agar plates containing suitable antibiotics every day for 6 days. On the seventh day the

filters (and supported biofilms) were moved to a special LB plate containing 100 mg/L L-arabinose (Sigma-Aldrich) for 1 day to induce expression of PhoA. (More details in Supplementary Notes 1-3)

***In situ* Imaging of Mineralization of Planktonic Bacterial Cultures**

Overnight cultures of *E. coli* were diluted 100-fold and grown to OD₆₀₀ of 0.4–0.6 prior to induction with 0.1% L-arabinose. Expression was allowed to proceed for 90 min, after which the culture was centrifuged and the cells resuspended in 20 mM HEPES buffer (pH 7.4) containing 40 mM calcium glycerophosphate. A 3 µL volume of resuspended cells was immediately pipetted into a 120 µm thick plastic chamber (Thermo-Fisher) sandwiched between a glass slide and a cover slip coated with poly-L-lysine (Neuvitro Corporation) for Raman microscopy. No shaking was involved in this mineralization experiment. The experiments were performed under static conditions. (More details in Supplementary Note 4)

Time-Dependent Mineralization of Bacterial Films

Bacterial films were prepared as described in the above sections. Films prepared on 4.7 cm diameter polycarbonate filters were cut with a razor blade into seven pieces of nearly identical size. One piece was used as an unmineralized control sample. Other pieces were transferred to 6-well plates (Falcon) with each well filled with 5 mL of 20 mM HEPES buffer (pH 7.4) containing 30 mM calcium glycerophosphate. At time points of 20, 30, 40, 60, 90 and 120 mins, pieces of film were removed from mineralization buffer and placed

in another 6-well plate with each well containing 5 mL 20 mM HEPES buffer to stop mineralization. These samples were used to determine mechanical properties and extent of mineralization by bulge testing and quantitative Raman microscopy, respectively. (More details in Supplementary Note 4)

Spontaneous Raman Spectroscopy

Spontaneous Raman spectra were acquired using an upright confocal Raman spectrometer (Horiba Raman microscope; Xplora plus). A 532 nm YAG laser was used to illuminate the sample with a power of 12 mW on sample through a 100 $\times$, N.A. 0.9 objective (MPLAN N; Olympus). Raman shift ranges from 690 to 1300 cm^{-1} were acquired to cover the Raman peaks of interest (characteristic of phosphate species). Data acquisition was performed with 10 s integration by the LabSpec6 software. Spontaneous Raman spectra were organized and presented by Excel and GraphPad, respectively.

Stimulated Raman Scattering (SRS) Spectroscopy and Microscopy

A picoEmerald laser system (Applied Physics & Electronics) was used as the light source for SRS microscopy. This system produces 1.8 ps pump (tunable from 770 nm – 990 nm, bandwidth 0.5 nm, spectral bandwidth $\sim 7 \text{ cm}^{-1}$) and Stokes (1031.2 nm, spectral bandwidth 10 cm^{-1}) beams with 80 MHz repetition rate. The Stokes beam was modulated at 20 MHz by an internal electro-optic modulator. The spatially and temporally overlapped pump and Stokes beams were introduced into an inverted multiphoton laser scanning microscope (FV3000, Olympus), and then focused onto the sample by a 25X water

objective (XLPLN25XWMP, 1.05 N.A., Olympus) for imaging. Transmitted pump and Stokes beams were collected by a high N.A. condenser lens (oil immersion, 1.4 N.A., Olympus) and passed through a bandpass filter (893/209 BrightLine, 25 mm, Semrock) to filter out the Stokes beam. A large area (10×10 mm) Si photodiode (S3590-09, Hamamatsu) was used to measure the pump beam intensity. A 64 V reverse-bias DC voltage was applied on the photodiode to increase saturation threshold and reduce response time. The output current was terminated by a 50-Ω terminator and pre-filtered by a 19.2-23.6-MHz bandpass filter (BBP-21.4+, Mini-Circuits) to reduce laser and scanning noise. The signal was then demodulated by a lock-in amplifier (SR844, Stanford Research Systems) at the modulation frequency. The in-phase X output was fed back to the Olympus IO interface box (FV30-ANALOG) of the microscope. Image acquisition speed was limited by the 30 μs time constant set for the lock-in amplifier. Correspondingly, we used an 80 μs pixel dwell time, which gives a speed of 21s per frame for a 512-by-512-pixel field of view. The pump laser was tuned to 938.3 nm for imaging the phosphate vibrational mode at 961 cm⁻¹. Laser power on sample was measured to be 30 mW for the pump beam and 200 mW for the modulated Stokes beam. 16-bit grey scale images were acquired by Olympus Fluoview 3000 software. Volumetric images were acquired by collecting a z-stack with a step size of 1 micron in the z-direction. SRS spectra were acquired by fixing the Stokes beam at 1031.2 nm and scanning the pump beam through the designated wavelength range point by point. SRS spectra were processed and presented by Excel and GraphPad. (More details in Supplementary Note 5)

Image Processing and Data Analysis

Image color-coding, intensity profiling and intensity normalizations of the z-stacks were done by ImageJ. 3D rendering of z-stacks was done in either ImageJ or Olympus cellSens. Data plotting and analysis, including spectral plots and Gaussian fitting were performed in OriginLab and Prism (GraphPad).

2.6. References

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Supplementary Information

QUANTITATIVE REAL-TIME ANALYSIS OF LIVING
MATERIALS BY STIMULATED RAMAN SCATTERING
MICROSCOPY

Supplementary Notes 1-3: Cell Strains, Reagents and Materials

Supplementary Note 1: Cell Strains, Reagents and suppliers

All mineralization experiments were conducted in *E. coli* strain DH10B (Invitrogen, Carlsbad, CA). Restriction enzymes, ligase, and Q5 DNA polymerase were purchased from New England Biolabs (Beverly, NJ). DNA oligos and G-blocks were purchased from Integrated DNA Technologies (Coralville, IA).

Supplementary Note 2: Plasmid subcloning

Recombinant fusion proteins were produced by standard recombinant DNA technology.

E. coli strain DH10B was used for all cloning steps and material preparation.

Genes encoding the autotransporter protein along with elastin solubility/stability tags have been previously cloned by our group into modified pQE-80L plasmids. (*1*)

G-blocks encoding PhoA without its natural leader peptide were ordered with 5' BamHI and 3' HindIII sites. G-blocks were digested with BamHI and HindIII and inserted into a similarly digested pQE-Empty plasmid containing pelB leader peptide described in previous work (*1*) to make a pelB-PhoA construct that directs PhoA to the periplasm. Using Gibson assembly, the pelB-PhoA construct was placed under control of the araBAD promoter in pBAD33.

pX-E6 containing six 25-residue elastin-like-peptide repeats reported in previous work (1) was digested with BamHI and XhoI and inserted into a similarly digested pAT-Empty vector to make pAT-E6. The T5 promoter of the plasmid drives constitutive expression of protein E6-AT.

pX-ST encoding the SpyTag peptide was mutated into pX-N encoding nucleation peptide SN15 (N) (2) by PCR. Recursive ligation (3) was used to assemble pX-triblock peptide from pX-N and pX-E3. pX-triblock was digested with BamHI and XhoI and inserted into a similarly digested pAT-Empty vector to make pAT-triblock. The T5 promoter of the plasmid drives constitutive expression of protein triblock-AT.

Supplementary Note 3: Buffer recipe

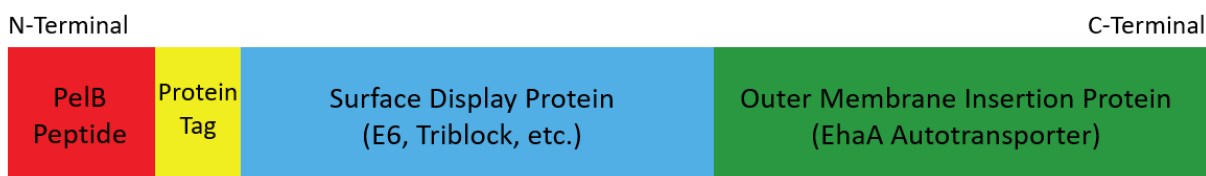
The 20 mM HEPES buffer used in this work doesn't contain any PO_4^{3-} . The buffer contains 20 mM HEPES, 115 mM NaCl, and 1.2 mM MgCl_2 buffered at pH 7.4. The mineralization buffer contains calcium glycerophosphate at concentrations ranging from 10 mM to 60 mM.

Table S1: Plasmids used in this study

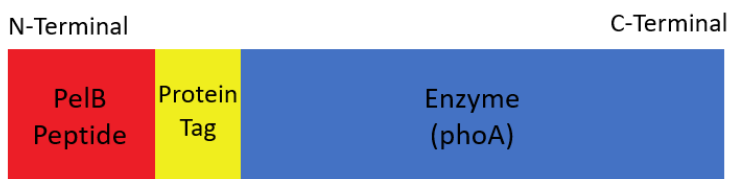
Name	Backbone/origin/promoter	Purpose
pQE-Empty	pQE80l/colE1/T5	Empty plasmid for cloning and ampicillin resistance
pelB-PhoA	pQE80l/colE1/T5	Constitutive expression of PhoA in periplasm
pBAD-PhoA	pBAD33/p15a/araBAD	Arabinose-inducible expression of PhoA in periplasm
pAT-E6	pQE80l/colE1/T5	Constitutive expression of E6-AT protein on cell surface
pAT-Triblock	pQE80l/colE1/T5	Constitutive expression of triblock-AT surface displayed nucleation peptide
pAT-Empty	pQE80l/colE1/T5	Cloning of autotransporter fusion proteins
pX-E6	pQE80l/colE1/T5	Cloning of pAT-E6
pX-E3	pQE80l/colE1/T5	Cloning of pAT-triblock
pX-ST	pQE80l/colE1/T5	Cloning of pAT-triblock
pX-N	pQE80l/colE1/T5	Cloning of pAT-triblock

Table S2: Protein sequences

Protein:	Sequence
pelB-PhoA	MKYLLPTAAAGLLLLAAQPA MAMRGS HHHHHH GSVDRTPEMPVLENRAA QGDITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNY AEGAGGFFKGIDALPLTGQYTHYALNKKTGKPDYVTDASAATAWSTGVK TYNGALGVDIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTS RKCYGPSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFAETAT AGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNMP VRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMMDKAIELLSKNEKG FFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVT ADHAHASQIVAPDTKAPGLTQALNTKDGA VMVMSYGNSEEDSQEHTGSQ RIAAYGPHAANVVGLTDQTDLFYTMKAALGLK
E6-AT	MKYLLPTAAAGLLLLAAQPA MAMRGS HHHHHH GSVD VPGAGVPGAGVPG EGVPGAGVPGAGVPGAGVPGAGVPGEGVPGAGVPGAGVPGAGVPG GEGVPGAGVPGAGLDVPGAGVPGAGVPGEGVPGAGVPGAGVPGAGVPG GVPGEGVPGAGVPGAGVPGAGVPGAGVPGAGVPGAGVPGAGLE TPTPGPD LNVDNDLRPEAGSYIANLAAANTMFTTRLHERLGNTYYTDMVTGEQKQTT MWMRHEGGHNKWRDGSQGLKTQSNRYVLQLGGDVAQWSQNGSDRW HV GVMAGYGNSDSKTISSRTGYRAKASVNGYSTGLYATWYADDES RNGAYLD SWAQYSWFDNTVKGDDLQSESYKSKGFTASLEAGYKHKLAEFNGSQGTRN EWYVQPPAQVTWMGVKADKHRESNGTLVHSNGDGNVQTRLGVKTWLKS HHKMDDGKSREFQPFVEVNWLHNSKDFSTSM DGVSVTQDGARNIAEIKTG VEGQLNANLNVWGNVGVQVADRGYNDTSAMVGIKWQF
Triblock-AT	MKYLLPTAAAGLLLLAAQPA MAMRGS HHHHHH GSVD VPGAGVPGAGVPG EGVPGAGVPGAGVPGAGVPGAGVPGEGVPGAGVPGAGVPGAGVPG GEGVPGAGVPGAGLD DDDEEKFLRRIGRFG LDVPGAGVPGAGVPGEGVPG AGVPGAGVPGAGVPGAGVPGEGVPGAGVPGAGVPGAGVPGAGVPG GAGVPGAGLD DDDEEKFLRRIGRFG LDVPGAGVPGAGVPGEGVPGAGVPG AGVPGAGVPGAGVPGEGVPGAGVPGAGVPGAGVPGAGVPGAGVPG GAGLE TPTPGPDLNVDNDLRPEAGSYIANLAAANTMFTTRLHERLGNTYYT DMVTGEQKQTTMWMRHEGGHNKWRDGSQGLKTQSNRYVLQLGGDVAQ WSQNGSDRW HVGVMAGYGNSDSKTISSRTGYRAKASVNGYSTGLYATWY ADDES RNGAYLDSWAQYSWFDNTVKGDDLQSESYKSKGFTASLEAGYKH KLAEFNGSQGTRNEWYVQPPAQVTWMGVKADKHRESNGTLVHSNGDGN VQTRLGVKTWLKSHHKMDDGKSREFQPFVEVNWLHNSKDFSTSM DGVSV TQDGARNIAEIKTGVEGQLNANLNVWGNVGVQVADRGYNDTSAMVGIKW QF



Autotransporter Fusion protein



Periplasm enzyme

Fig. S1. Protein sequence design of autotransporter fusion protein and periplasmic enzyme.

The N-terminal pelB leader peptide directs the translated protein into the periplasm of *E. coli* and is then cleaved from the protein. The C-terminal autotransporter sequence is recognized and inserted into the outer membrane of *E. coli* while the N-terminal part is displayed at the cell surface. PhoA is transported to the periplasm. (Corresponding color code was applied to **Table S2**.)

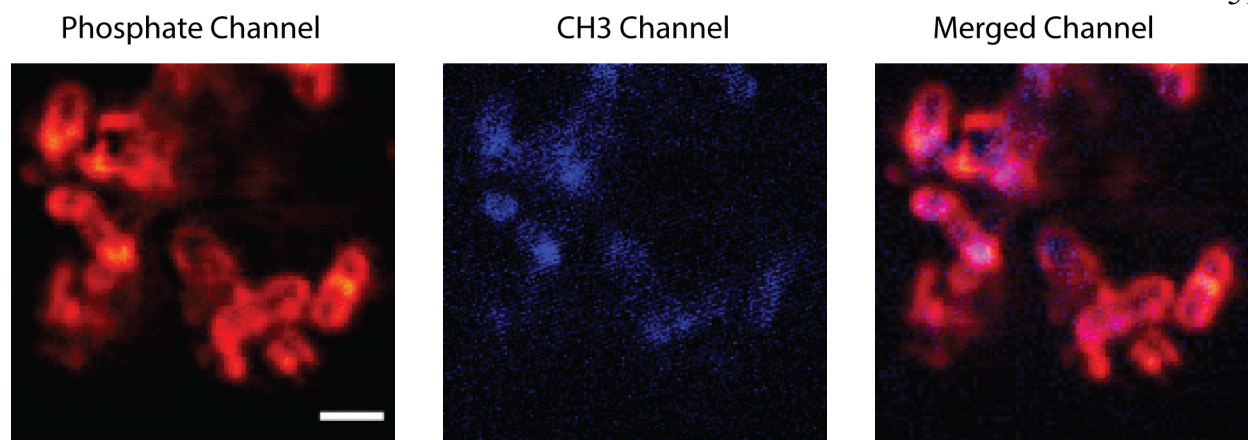


Fig. S2. SRS images of mineralized bacteria in liquid culture in Phosphate, CH₃ channel.

The merged image showed core-shell structure in which CH₃-containing organics are distributed inside the cell body while phosphate are forming a shell at the cell periphery. Scale bar: 2 μm .

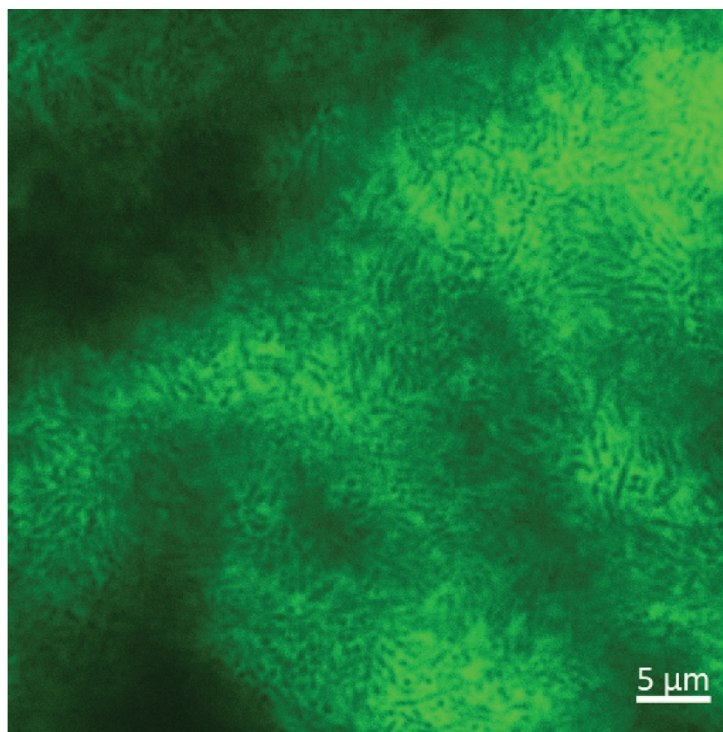


Fig. S3. Fluorescence microscopy image of 7 days old (1 day induction with 0.1% arabinose for PhoA expression) PhoA bacterial film expressing periplasmic PhoA after 24 hrs of mineralization in 10 mM CGP, stained by the calcium binding dye calcein. The cell interior is not stained by calcein, consistent with mineralization outside the inner membrane of bacterium. Calcein binds to calcium cation and becomes fluorescent once binds to calcium, meaning that it binds non-specifically and cannot distinguish between BCP and other molecules that contains calcium. Scale bar, 5 μm .



Fig. S4. A Triblock/PhoA bacterial film after 24 hrs of mineralization in 10 mM CGP, stiff and self-standing in a 20 mL scintillation vial, already removed from its polycarbonate filter for imaging and analysis. Diameter of the film is around 2 cm.

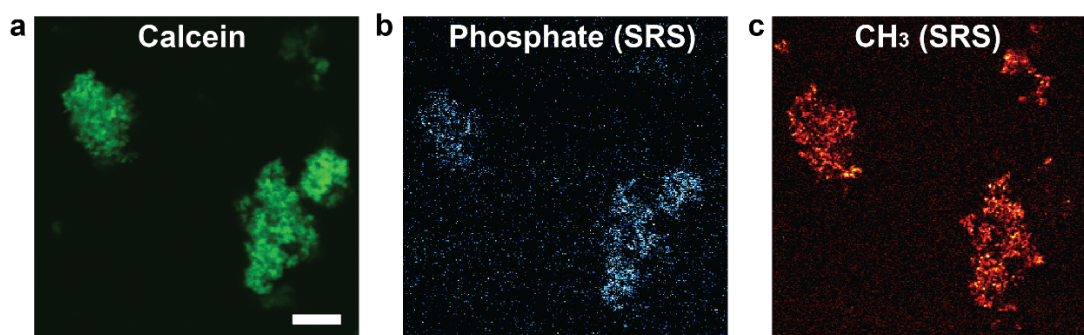


Fig. S5. *E. coli* cell liquid culture fully mineralized and stained by calcein (calcium tracker) and imaged by fluorescence (**a**, calcein) and SRS microscopy (**b**, phosphate channel and **c**, CH₃ channel). The phosphate channel showed weak SRS signal compared to unstained samples because phosphate anions were displaced by the calcein-AM dye which chelates calcium ions. Scale bars, 25 μm .

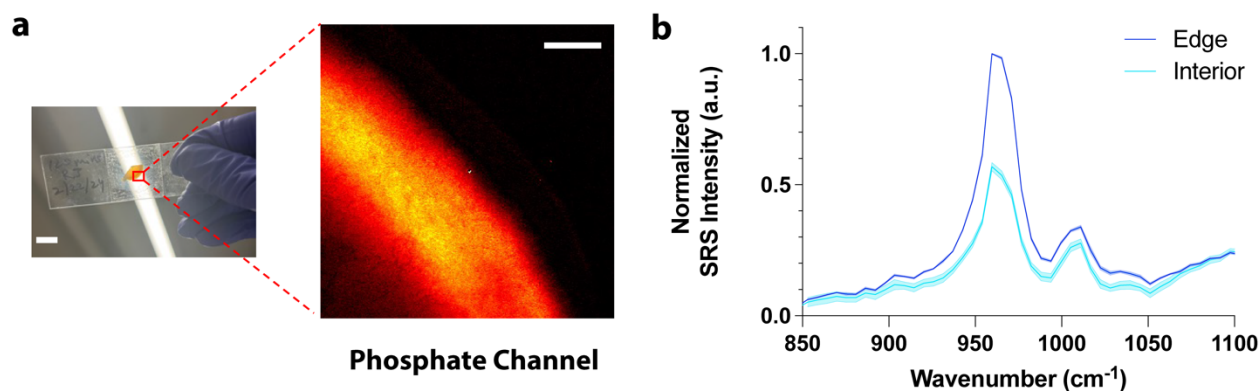


Fig. S6. a, A photo and an SRS image of a E6/PhoA film after 120 min mineralization.

The edge was imaged under an SRS microscope. The SRS image was taken in the phosphate channel (ON – OFF). It is clear that the edge of the film has high intensity of phosphate signal compared to the interior regions. Scale bars: 1 cm (left, photo), 100 μ m (right, SRS image). **b**, SRS spectra of an E6/PhoA film (after 120-min mineralization) at the edge (blue) and the interior (cyan) regions, normalized to the highest point in of the spectrum at the edge. The phosphate peak at 961 cm⁻¹ is significantly higher at the edge, compared to that from the interior, while the background levels and shapes remain identical. Number of replicates for each sample: 5. Standard error of mean is represented by the shaded error bands.

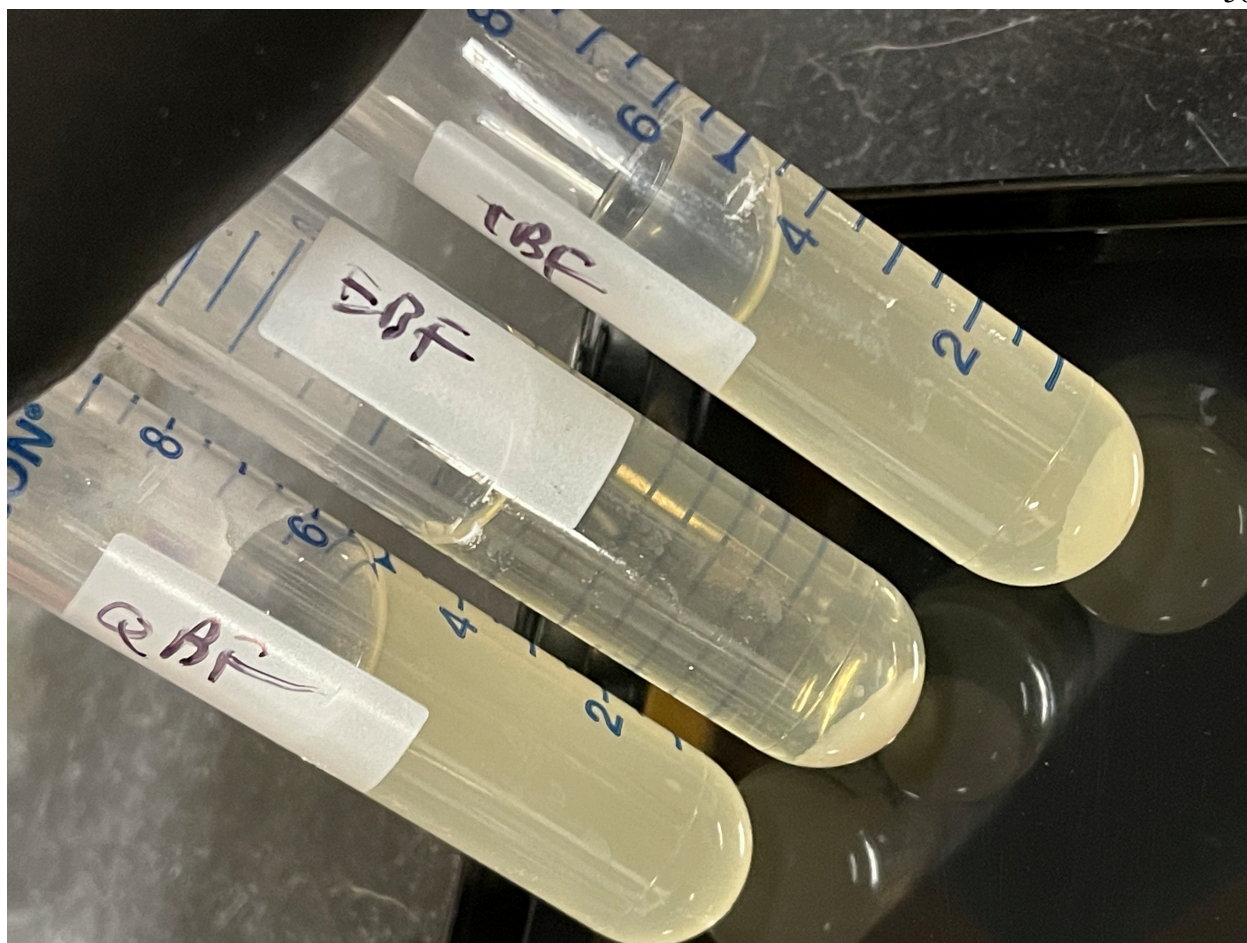


Fig. S7. Images of PhoA, E6/PhoA and triblock/PhoA cultures. Left bottom tube contains a PhoA culture. Without surface protein expression, the PhoA culture stays turbid and cells are freely floating. The middle tube is an E6/PhoA culture. E6 increases intercellular adhesion, causing cells to aggregate and precipitate, settling to the bottom of the tube. Top right tube is triblock/PhoA. Many cells are still suspended in the culture, but some precipitate and settle to the tube bottom.

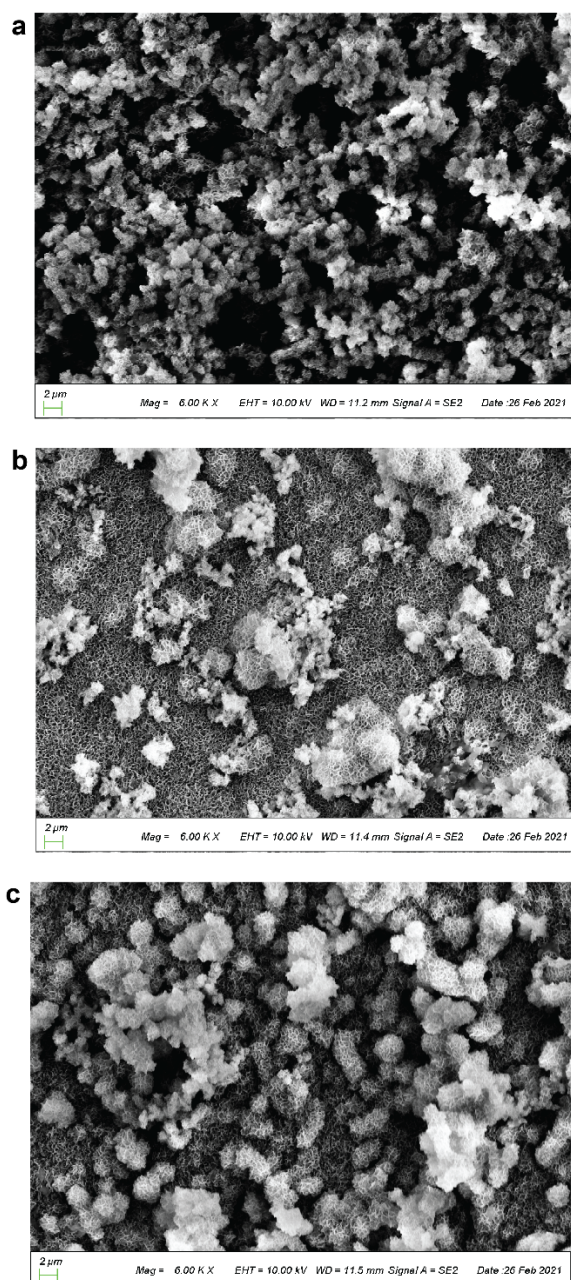


Fig. S8. SEM images of three types of 7 days old (1 day induction with 0.1% arabinose for PhoA expression) engineered *E. coli* films with different surface protein constructs after 24 hrs of mineralization in 10 mM CGP: **a**, no surface-displayed peptide (PhoA); **b**, with surface-displayed E6 peptides (E6/PhoA); **c**, with surface-displayed triblock peptides (Triblock/PhoA). Scale bars, 2 μm.

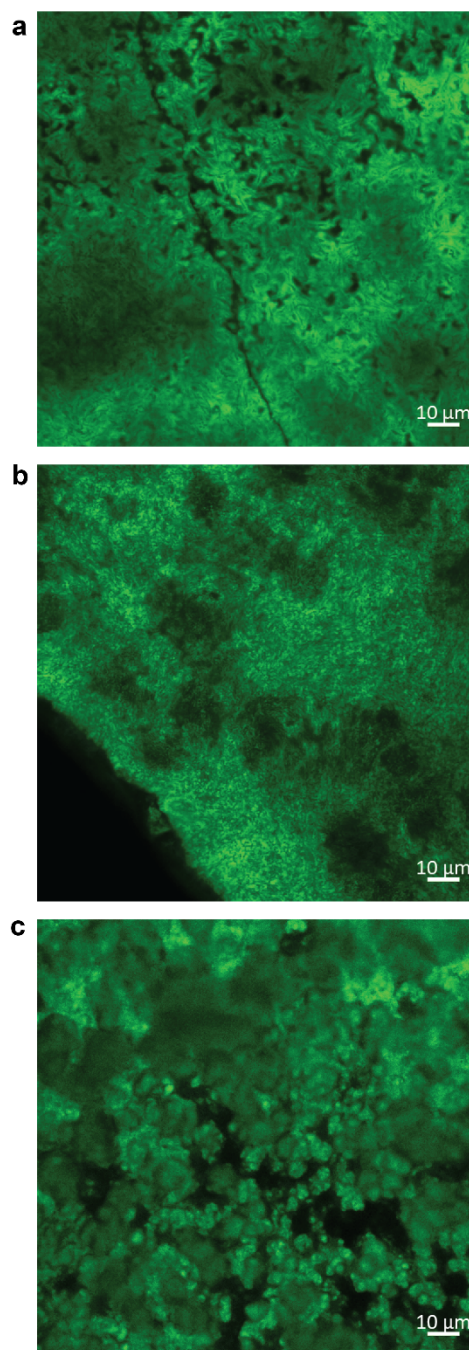


Fig. S9. Calcium-tracking fluorescence images of three types of 7 days old (1 day induction with 0.1% arabinose for PhoA expression) engineered *E. coli* films with different surface protein constructs after 24 hrs of mineralization in 10 mM CGP: **a**, no surface-displayed peptide (PhoA); **b**, with surface-displayed E6 peptides (E6/PhoA); **c**, with surface-displayed triblock peptides (Triblock/PhoA). Scale bars, 10 μ m.

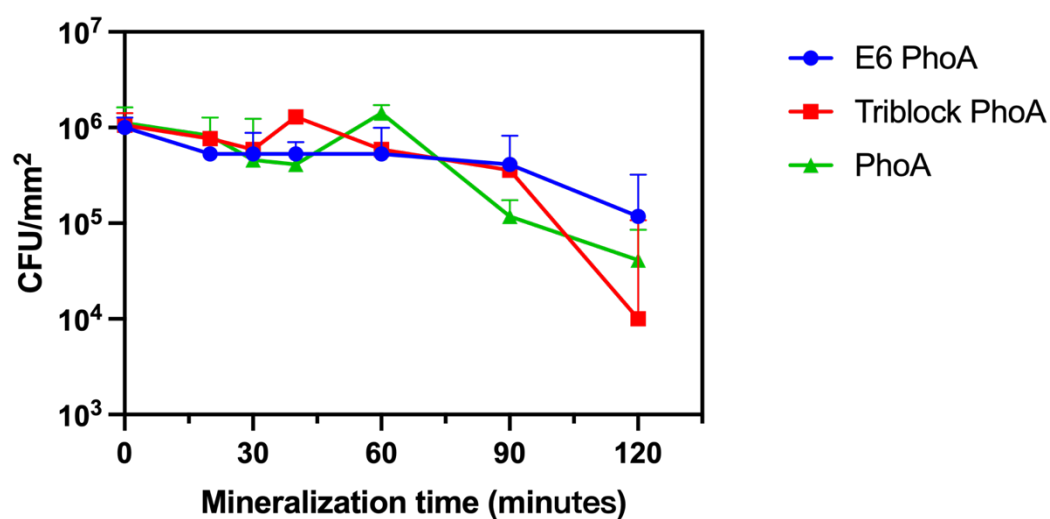


Fig. S10. Colony-forming units (CFU) per unit area of the three types of films examined in this work. Little change in viability was observed during the first hour of mineralization. Relative large decrease in viability was observed after 90 min of mineralization. Triblock/PhoA has least viability across all three constructs after 2 h of mineralization.

Supplementary Notes 4: Raman cross section data for quantification of phosphate/CH (molar ratio)

To get the phosphate/CH ratio in each sample from SRS imaging data, we used the SRS intensity of both the phosphate and CH channels, as well as the known Raman cross sections of phosphate P-O bond and C-H bond vibrations (**Table S3**).

$$\frac{I_{\text{Phosphate}}}{\sigma_{\text{Phosphate}}} \text{ molar ratio} = \frac{I_{\text{CH}}}{\sigma_{\text{CH}}}$$

I: SRS intensity; *σ*: Raman cross section (**Table S3**)

Table S3: Reported Raman cross sections of phosphate and C-H bond vibrations (4, 5)

Target	Raman Cross Section (cm ⁻²)
Phosphate	8.10E-30
CH	1.00E-29

Supplementary Notes 5: Fluorescence Imaging

The fluorescence images of processed samples with fluorescent labels were obtained with a 25X, 1.05 N.A. water-immersion objective with the Olympus Fluoview system. Single-photon confocal laser scanning imaging was performed with a 488-nm laser (Coherent OBIS), and emission in the Calcein-AM channel ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 495/515 nm). The images were visualized and analyzed with Fiji or Imaris Viewer.

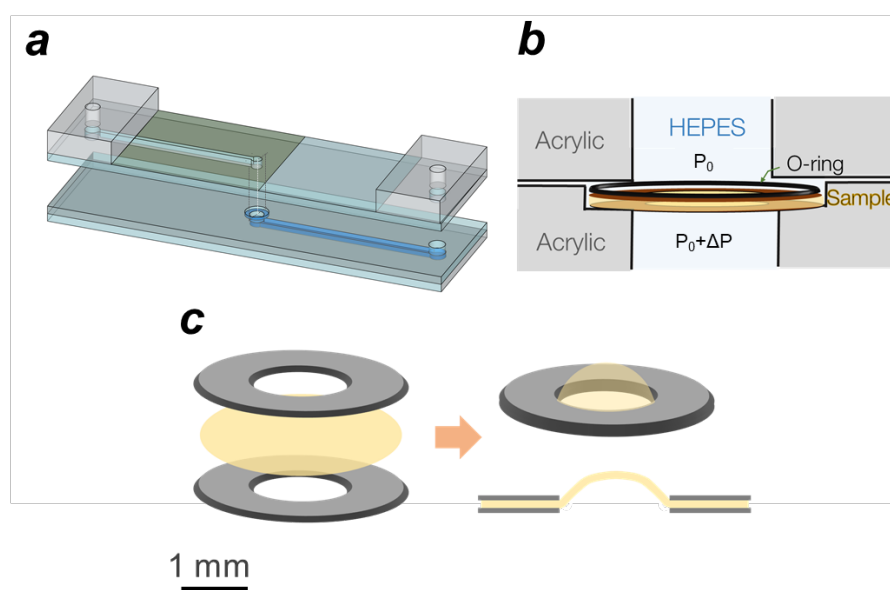


Fig. S11. **a**, Schematic of bulge test device, fabricated as two parts that are separated to load a sample in the central chamber at the center of the device. When sealed, each external port connects, via a channel, to a reservoir of fluid that is used to control pressure on the top or bottom face of the sample respectively (reservoirs not shown). Gray layers are acrylic; green thin layer is cover slip glass; layers are bonded using epoxy. Horizontal channels are longer than shown. **b**, Schematic diagram of central chamber. Bacterial film sample is supported by two washer-shaped disks. A thin O-ring seals this “sandwich” to the top half of the device. **c**, Schematic showing a disk of bacterial film sample clamped

between two washer-shaped supports; when the supports and specimen are loaded in the central chamber and $p_{\text{bottom face}} > p_{\text{top face}}$, the bacterial film bulges upward (right).

Supplementary Notes 6: Mechanical property obtained by bulge/inflation tests

Mechanical properties of the biofilms were estimated using a bulge/inflation test, a method that has been used extensively in the characterization of sheet metals, polymers, and some biological tissues (6-17). A millifluidic device was constructed using laser cut acrylic sheets to adapt the bulge test to the Pa-kPa pressures needed to deform the biofilms. The final device construct consisted of a sealed central chamber housing the biofilm sample as a 3 mm diameter disk, sandwiched between two washer-shaped supports (**Fig. S9a, b**). Each face of the sample was exposed to a chamber filled with HEPES buffer whose hydrostatic pressure could be independently controlled. Thus, by applying different hydrostatic pressures to the top and bottom faces of the biofilm, the film could be “inflated” through the washer aperture into a dome-like shape (**Fig. S9c**). Hydrostatic pressures were applied across the film by varying the height of HEPES in two separate reservoirs connected to external ports in the device. The reservoirs themselves were 60 ml syringes of known internal diameter (BD Scientific, ID = 26.72 mm, cross sectional area 5.61 cm²). Thus, the addition of 858 μ l of HEPES buffer increased the reservoir level by 1.53 mm, which increased the hydrostatic pressure on the corresponding face of the sample by 15.0 Pa (density assumed constant at 1 g/ml).

Sample loading

Prior to mechanical testing, a bacterial film with its polycarbonate support was submerged in HEPES buffer and a 3 mm dia. biopsy punch (Integra Biosciences) was used to gently make a circular cut through the bacterial film but not the polycarbonate. The sample support disk (copper TEM disk, 1.5 mm diameter circular aperture, 3.05 mm outer diameter, thickness 25 μ m, Ted Pella) was then slid in between the bacterial film and polycarbonate and used to gently separate the film from the polycarbonate. Once the edge

of the film was freed, the film freely floated away from the polycarbonate and could be loaded into the device (**Fig. S9b**). The device channels were then filled with HEPES and the syringe reservoirs attached via luer slip connectors threaded into the device ports.

Bulge Test and Mechanical Characterization

A millifluidic device was constructed using laser cut acrylic sheets to adapt the bulge test to the Pa-kPa pressures needed to deform the biofilms. The device consisted of a sealed central chamber housing the biofilm sample as a 3 mm diameter disk, sandwiched between two washer-shaped supports. Each face of the sample was exposed to a chamber filled with HEPES buffer whose hydrostatic pressure could be independently controlled. Thus, by applying different hydrostatic pressures to the top and bottom faces of the biofilm, the film could be “inflated” through the washer aperture into a dome-like shape. Hydrostatic pressures were applied across the film by varying the height of HEPES in two separate reservoirs connected to external ports in the device. The reservoirs were 60 ml syringes of known internal diameter (BD Scientific, ID = 26.72 mm, cross sectional area 5.61 cm²). Thus, the addition of 858 μ l of HEPES buffer increased the reservoir level by 1.53 mm, which increased the hydrostatic pressure on the corresponding face of the sample by 15.0 Pa (density assumed constant at 1 g/ml). Prior to mechanical testing, a bacterial film with its polycarbonate support was submerged in HEPES buffer and a 3 mm diameter biopsy punch (Integra Biosciences) was used to make a circular cut through the bacterial film but not the polycarbonate. The sample support disk (copper TEM disk, 1.5 mm diameter circular aperture, 3.05 mm outer diameter, thickness 25 μ m, Ted Pella) was then inserted between the bacterial film and polycarbonate and used to gently separate the film from the polycarbonate. Once the edge of the film was freed, the film freely floated away from the polycarbonate and could be loaded into the device. The device channels were then filled with HEPES and the syringe reservoirs attached via luer slip connectors threaded into the device ports. A syringe pump (KD Scientific) was used to add HEPES buffer to the reservoir connected to the bottom face of the biofilm at a flow rate of 70 ml/min with a final volume of 55 ml. Biofilm deformation was imaged using optical coherence

tomography (OCT, Thorlabs, GAN210 base unit: 930 nm central wavelength, 6 μm axial resolution. OCTP-900 scan head, OCT-LK3-BB scan lens: 36 mm FL, 8 μm lateral resolution). A-Scan/Line Rate was 36 kHz (acquisition time = 19 ms).

Operation and analysis

A syringe pump (KD Scientific) was used to add HEPES buffer to the reservoir connected to the bottom face of the biofilm at a flow rate of 70 ml/min with a final volume of 55 ml. The biofilm deformation was imaged using optical coherence tomography (OCT, Thorlabs, GAN210 base unit: 930 nm central wavelength, 6 μm axial resolution. OCTP-900 scan head, OCT-LK3-BB scan lens: 36 mm FL, 8 μm lateral resolution). A-Scan/Line Rate was 36 kHz (acquisition time = 19 ms).

Analysis protocols: Biofilm thickness ranged from 55-120 μm with a majority of films under 80 μm in thickness. Thus, the ratio of aperture diameter to film thickness ranged from 12.5 to 27.2. Flexural stiffness was neglected and only membrane stresses and strains were considered. Analysis of 2D images further required the spherical cap assumption: that there is equibiaxial stress and strain everywhere in the film and that the bulge radius of curvature is the same in both radial and circumferential directions – these assumptions are generally only satisfied at the center of the film and fail near the clamped edge where the circumferential strain vanishes.

Analysis of 2D images: 2D OCT datasets were exported as tiff files and cropped using ImageJ. The resulting images were processed with in-house MATLAB scripts: the images were binarized; the top and bottom surfaces of the film were automatically detected and fit to fourth degree polynomials which were then used to estimate arc lengths of the top and bottom surfaces of the film. The arc lengths were used to calculate true strain ϵ averaged over the top and bottom surfaces of the biofilm.

True stress was calculated with the equation for stress in a thin-walled spherical pressure vessel:

$$\sigma = \frac{PR}{2t}$$

Where σ is film stress, P is the applied pressure, R is bulge radius of curvature, and t is instantaneous film thickness. Further, $R = \sqrt{a^2 + h^2}$ where a is radius of aperture of the support disk and h is deflected height of the center of the film, directly measurable by OCT. Young's modulus was estimated using the initial linear regime of each stress-strain trace.

Supplementary Notes 7: Mineralization Protocols

Mineralization of Planktonic Bacterial Cultures

Overnight *E. coli* cultures were diluted 100-fold and grown to OD₆₀₀ of 0.4–0.6 prior to induction with 0.1% L-arabinose. Expression was allowed to proceed for 90 min, after which the culture was centrifuged and resuspended in 20 mM HEPES buffer (pH 7.4) containing 10 mM calcium glycerophosphate. The detailed buffer recipe is documented in Supplementary Note 3. Mineralization was allowed to proceed for at least 1 h under shaking at 250 rpm until cells precipitated as white clusters. Clusters were resuspended and a 3 μ L aliquot of resuspended mineralized cells was transferred to a plastic chamber (120 μ m depth, Thermo-Fisher) sandwiched between a glass slide and a cover slip for Raman microscopy.

Full Mineralization of Bacterial Films

Bacterial films were prepared as described in the above sections and transferred on their polycarbonate filters to a 6-well plate (Falcon) with each well filled with 5 mL of 20 mM HEPES buffer (pH 7.4) containing 10 mM calcium glycerophosphate. Four 3 mm diameter glass beads were placed at the edge of each polycarbonate filter to keep the films flat during the mineralization process. To achieve full mineralization, films remained in mineralization buffer for 24 h.

Microtomy of Fully-Mineralized Bacterial Films

Fully-mineralized films were removed from the mineralization buffer, washed with 5 mL of 20 mM HEPES buffer for 5 min and then cut with a 6 mm diameter biopsy punch (Miltex). The circular section of film was embedded in Tissue-Tek® resin (Sakura) and frozen at - 20 °C overnight. The frozen piece was microtomed at - 20 °C with each section of 50 µm thickness. Microtomed sections were placed either on glass slides (for Raman microscopy) or on Al stubs (for SEM imaging).

Supplementary Notes 8: Sample Mounting and Imaging for SRS

Cell or biofilm samples were kept in HEPES buffer for imaging. Grace Bio-Labs SecureSeal™ spacers with appropriate opening sizes were used as spacers between microscope slides (1 mm, VWR) and coverslips (12 mm, #1.5, Fisher). Confocal images were obtained by the Olympus FluoView™ FV3000 confocal microscope with the SRS setup described above.

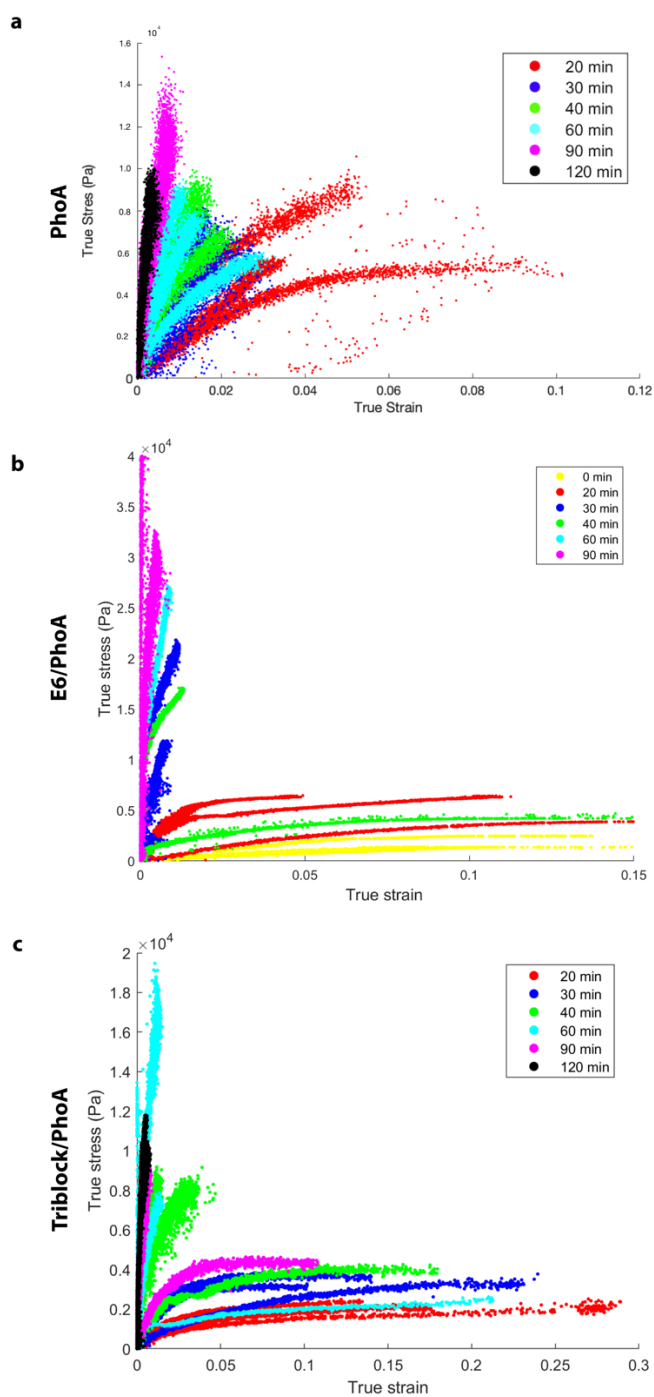


Fig. S12. **a**, True stress vs. true strain curves of all 7 days old PhoA films mineralized at different time points. Number of replicates for each time point: 20 min, $n = 3$; 30 min, $n = 3$; 40 min, $n = 3$; 60 min, $n = 3$; 90 min, $n = 3$; 120 min, $n = 2$. **b**, True stress vs. true strain

curves of all 7 days old E6/PhoA films mineralized at different time points. Number of replicates for each time point: 0 min, n = 3; 20 min, n = 3; 30 min, n = 3; 40 min n = 2; 60 min, n = 1; 90 min, n = 3. **c.** True stress vs. true strain curves of all 7 days old Triblock/PhoA films mineralized at different time points. Number of replicates for each time point: 20 min, n = 3; 30 min, n = 3; 40 min, n = 4; 60 min n = 4; 90 min, n = 3; 120 min, n = 4.

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SINGLE-CELL METABOLIC IMAGING REVEALS GLYCOGEN-DRIVEN ADAPTATIONS IN ENDOTHELIAL CELLS

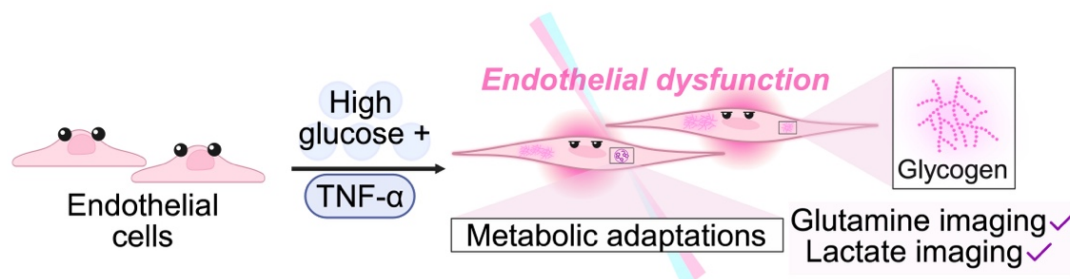
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<https://doi.org/10.1002/advs.202508517>.

3.1. Summary

Endothelial dysfunction (ED) is a defining feature of diabetes mellitus (DM) and a key contributor to many metabolic and cardiovascular diseases. Endothelial cells (ECs) are known to be highly glycolytic and primarily rely on glucose to meet their energy demands. However, the role of glycogen metabolism in ECs remains poorly characterized due to a lack of suitable tools. Here, we utilize stimulated Raman scattering (SRS) microscopy to investigate glycogen metabolism in live ECs exposed to a diabetes-mimicking milieu—high glucose and tumor necrosis factor (TNF- α). We show that ECs divert excess glucose toward subcellular glycogen storage, and that this storage capacity is significantly enhanced by the inhibition of glycogen synthase kinase 3 (GSK3). Pulse-chase experiments uncover glycogen dynamics and reveal rapid mobilization under glucose starvation, highlighting its role as an immediate energy reserve. We further extend the capabilities of SRS imaging to visualize glutamine and lactate metabolism for

the first time, directly showcasing EC reliance on alternative substrates during glucose deprivation. ECs containing glycogen exhibit reduced immediate metabolic demand for these gluconeogenic substrates. These findings suggest that glycogen may play a regulatory role in modulating stress-responsive metabolic adaptations and may offer therapeutic opportunities to address diabetes-induced ED and related cardiometabolic diseases.



3.2. Introduction

Diabetes mellitus (DM) affects over 500 million people globally, with cardiovascular complications being a leading cause of disability and morbidity.¹ A central driver of these complexities is endotheliopathy, or the dysfunction of endothelial cells (ECs), signified by a pro-inflammatory activation and a decreased nitric oxide production.² ECs are strategically located, lining up the interior walls of all vascular networks. This positioning not only allows them to serve as a physical barrier between blood and tissues, but also as regulators of essential processes such as vascular homeostasis, transport of macromolecules, and recruitment of leukocytes.^{3,4} When exposed to pathophysiological stimuli such as pro-inflammatory cytokines, hyperglycemia, and oxidative stress, ECs can become dysfunctional and contribute to a myriad of cardiometabolic diseases, including but not limited to atherosclerosis, pulmonary arterial hypertension (PAH), and diabetes.^{5–8} So far, the dysregulation of metabolites that drive ED progression remains largely unexplored at the subcellular level.^{9,10} This knowledge gap highlights the need to investigate the metabolic basis of ED under pathophysiological conditions such as diabetes, which can identify potential therapeutic strategies for ameliorating ED and related metabolic and cardiovascular diseases (CVDs).

The highly glycolytic nature of ECs makes them reliant primarily on glucose metabolism to meet their biosynthetic needs and energy demands.¹¹ However, excess glucose and elevated inflammation, as in the case of diabetes, can induce endotheliopathy and exacerbate metabolic dysfunction.¹² One potential metabolic outcome of excessive glucose is the formation of glycogen, a hyperbranched polymer of glucose.¹³ In cancer cells, high glucose has been observed to promote subcellular accumulation of glycogen which in turn accelerates liver tumor initiation.^{14,15} However, in ECs, the role of glycogen remains poorly understood.¹⁶ Existing techniques face significant limitations for specific subcellular detection of glycogen: magnetic resonance imaging (MRI) with ¹³C-glucose lacks subcellular resolution¹⁷; positron emission tomography (PET) with [¹⁸F]-2-fluoro-2-deoxy-D-glucose (FDG) and fluorescence microscopy using 2-NBDG employ glucose tracers that are neither specific for glycogen¹³ nor capable of incorporation to glycolysis or glycogenesis pathways^{18,19}; mass-spectrometry-based imaging methods are inherently destructive and incompatible with live-cell studies or longitudinal analyses.²⁰

To address these challenges, stimulated Raman scattering (SRS) microscopy combined with deuterated metabolites offers a powerful, non-invasive method for specifically probing cellular metabolism in living systems by targeting the C–D vibration peaks corresponding to the metabolized biomass structures.^{21–24} In contrast to spontaneous Raman microscopy, SRS offers significantly enhanced sensitivity and rapid acquisition times that allow efficient mapping of these dynamic metabolites in living systems.^{25,26} Unlike conventional fluorescence microscopy, SRS imaging circumvents the need for bulky fluorophores or genetically encoded reporters, which can perturb biological systems and are prone to photobleaching.²⁷ Compared to a purely label-free approach, the use of vibrational tags provides dynamic information on the incorporation, growth, and turnover of metabolites.²⁰ Additionally, the compatibility of SRS with tissue imaging and linear concentration dependence for quantitative analysis render it a uniquely powerful tool for visualizing and studying subcellular metabolism with high spatial resolution in live cells, both in health and disease.

In this work, we leveraged SRS microscopy with deuterated glucose tracing to visualize glycogen metabolism in ECs. In a model of diabetic milieu (high glucose and TNF- α ; HT),²⁸ we captured the subcellular formation of glycogen pools in live ECs. HT induced significant glycogen accumulation, which was further amplified by inhibiting glycogen synthase kinase 3 (GSK3)– resulting in glycogen covering up to more than 50% of the cell area and highlighting the unexpectedly high glycogenic capacity of ECs under metabolic stress. Pulse-chase experiments showed dynamic glycogen turnover and spatial growth, supporting its role as a back-up energy reserve. To evaluate the functional significance of glycogen stores, glucose-starvation assays directly visualized the metabolic adaptation of ECs to lactate (Lac) and glutamine (Gln)– two alternative carbon substrates that fuel EC metabolism via truncated gluconeogenesis (GNG; reverse glycolysis) in the absence of extracellular glucose.²⁹ This is also the first time that SRS metabolic imaging of lactate and glutamine was achieved. Notably, ECs with elevated glycogen levels showed reduced reliance on Lac and Gln metabolism, suggesting a role for glycogen in buffering the immediate metabolic demand in ECs during glucose-deprived conditions.

3.3. Results

Visualization of glycogen in live endothelial cells

We first established an *in vitro* model of EC dysfunction by exposing human umbilical vein endothelial cells (HUVECs) to high glucose and TNF- α (25 mM D-glucose and 5ng/mL TNF- α ; termed “HT”) for three days to mimic hyperglycemia and chronic inflammation. As a normoglycemic and osmolarity control, cells were maintained in 25 mM D-mannitol (osmotic control; termed “NM”). As demonstrated previously,^{12,28} this combined HT treatment not only causes a strong induction of pro-inflammatory response but also an endothelial-mesenchymal transition phenotype, evidenced by the marker gene expression and cell morphology (**Figure 1a**). Concomitant with the induction of pro-inflammatory genes *ICAM1* and *VCAM1*, quantitative PCR (qPCR) revealed an increased expression of *GYS1*, an isoform of glycogen synthase (GS)³⁰ and

the key rate-limiting enzyme of glycogen synthesis,³¹ in HT-exposed ECs (**Figure 1b**). This led us to examine glycogenesis in dysfunctional ECs using live-cell SRS imaging. Specifically, we utilized a glucose isotopologue (d₇-glucose) as a functional substitute for glucose, where all carbon atoms are labeled by deuterium. Unlike FDG and 2-NBDG, d₇-glucose is fully metabolized through native glucose metabolism pathways, allowing it to label the major biomolecules such as lipids, proteins, nucleic acids, and glycogen for chemical mapping (**Figure 1c**). Each of these biomacromolecules is characterized by a distinct C–D Raman spectral signature arising from their local chemical environments.^{13,32}

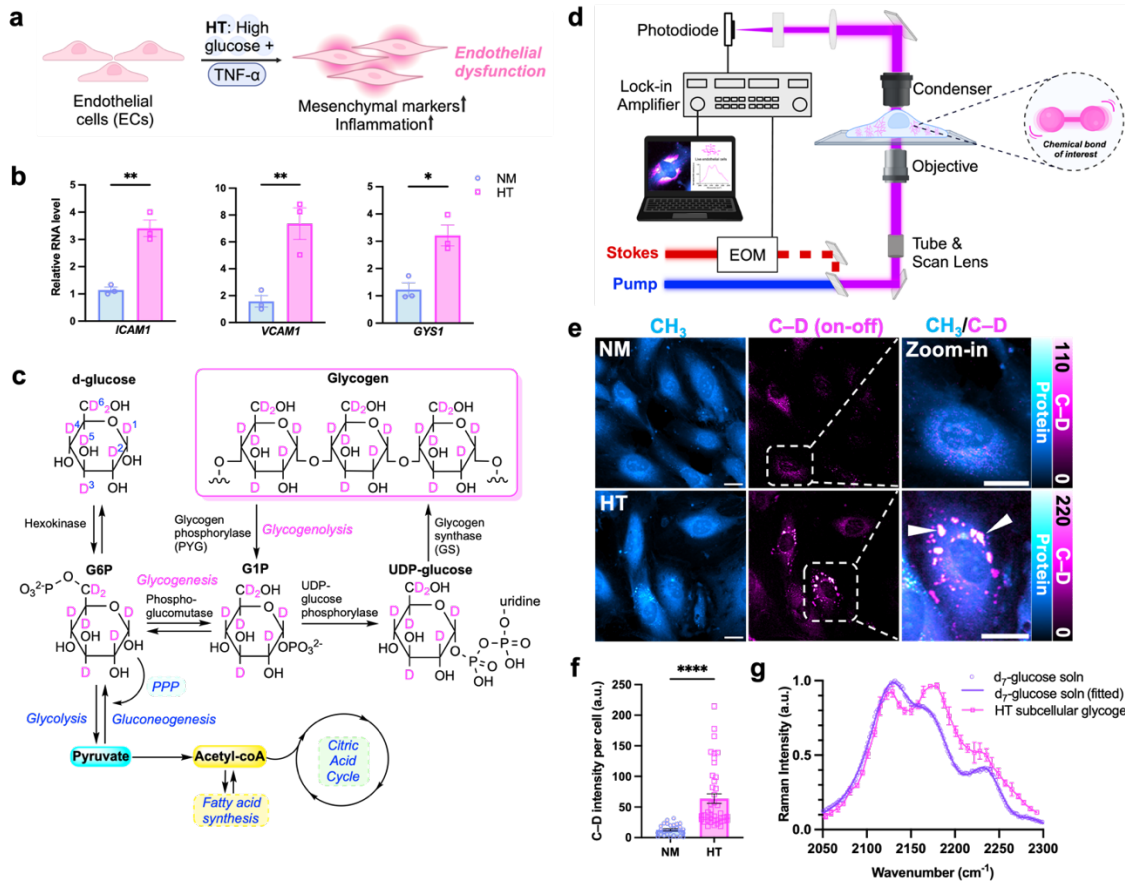


Figure 1. Visualization of subcellular glycogen in endothelial cells under HT. (a) Illustration of ECs exposed to high glucose and TNF- α (HT) and undergoing dysfunctional changes, characterized by an increase in inflammation and mesenchymal markers; (b) mRNA levels of *ICAM1*, *VCAM1* and *GYS1* in (osmotic control, NM) vs HT-treated HUVECs for 3 days; (c) Scheme of metabolic incorporation pathways of d₇-glucose, including the glycogen synthesis (glycogenesis) pathway. PPP= Pentose Phosphate Pathway; (d) Schematic of the SRS microscopy configuration. EOM: Electro-optic modulator; (e) Representative SRS images of live HUVECs treated with NM or HT containing d₇-glucose for 3 days, targeted at the -CH₃ (protein) and C-D (on-off resonance) channel. The third column represents merged (CH₃/C-D) channels for the zoomed-in view of ECs (boxed) in the corresponding C-D images. White arrowheads represent subcellular glycogen pools. Scale bar: 20 μ m. (f) Quantitative analysis of SRS C-D signal per cell for NM and HT-treated cells (n=29 cells for NM and 42 cells for HT). (g) Normalized spontaneous Raman spectrum of d₇-glucose solution (soln) (purple circles, 300 mM; purple solid line, fitted data). Normalized SRS spectrum of subcellular glycogen from HT-treated ECs (magenta squares with solid line). Data in all bar plots (b, f) is presented as mean $\pm$ SEM from at least three independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-tests. *p<0.05, **p<0.01, ****p<0.0001.

SRS imaging (**Figure 1d**) clearly revealed the appearance of subcellular glycogen pools labeled with d₇-glucose as localized puncta (**Figure 1e**, white arrowheads) in HT-exposed ECs, indicated by a pronounced increase in the overall C–D signal per cell (**Figure 1f**). Because glycogen is composed of polymerized glucose units, its identity can be directly confirmed in live cells using hyperspectral SRS (hSRS). The spectral features of the observed subcellular puncta match those of d₇-glucose with all major peaks (e.g. 2120 and 2172 cm⁻¹) consistent with known spectra of glucose solution (**Figure 1g**).^{13,32}

Since downstream glucose-derived biomolecules like lipids and proteins produce overlapping but distinct C–D peaks, we applied two complementary chemometric methods— spectral phasor³³ and least absolute shrinkage and selection operator (LASSO)³⁴— to specifically resolve and unmix C–D signal arising from glycogen in dysfunctional ECs (**Figure S1**). These methods confirmed that the enriched spots in the C–D channel indeed correspond to glycogen. Moreover, these two methods provide a robust approach for glycogen phenotyping in biosystems.

To further validate these findings, we performed correlative SRS imaging with an established glycogen histochemical staining method, Periodic Acid-Schiff (PAS) staining using bright-field microscopy. Our results demonstrated a high correlation of identified glycogen deposits between SRS imaging and PAS staining (**Figure S2**). Additionally, we verified that the observed glycogen reservoirs did not arise from the aggregation of d₇-glucose by detecting glycogen in ECs exposed to HT containing unlabeled D-glucose using PAS staining (**Figure S3**). However, we caution that fixation, as required in PAS detection, induced extracellular vesicles (fi-EVs) enriched with glycogen (**Figure S3–S4**), representing a fixation artifact consistent with our previous observations in cancer cells.¹³ Such vesicles are absent in all live-cell measurements, underscoring the rigor and advantage of performing unbiased live-cell investigations. Collectively, we successfully visualized and characterized C–D imaging of glycogen in ECs. To the best of our knowledge, this is the first report of subcellular glycogen visualization in live ECs.

Pharmacological modulation of GSK3 reveals a high capacity for glycogen storage in ECs

We next sought to modulate glycogen accumulation by targeting glycogen synthase kinase 3 (GSK3), a serine/threonine kinase that phosphorylates GS to inhibit glycogen synthesis³⁵ (**Figure 2a**). In its active form, GSK3 phosphorylates glycogen synthase (GS) to make it less active for glycogen synthesis. Inhibition of GSK3 causes dephosphorylation of GS, thereby making it more active for glycogen synthesis. We treated HT-exposed ECs with two small-molecule inhibitors of GSK3, CHIR-99021³⁶ or SB-216763³⁷ (hereafter CHIR and SB, respectively). Compared to ECs exposed to HT alone, HT+CHIR resulted in a striking increase in d₇-glucose-tracked glycogen accumulation, with glycogen pools covering over 50% of the cellular area (**Figure 2b-c**). Similar results were confirmed by using 6,6-d₂-glucose (hereafter d₂-glucose), another glucose isotopologue, bearing fewer deuterium labels (**Figure 2d-e**) and a different Raman spectral signature (**Figure S5**). Such drastic increase in glycogen storage was consistent with the effect of SB, the other GSK3 inhibitor (**Figure S6**).

To test whether this glycogenic response is conserved across other endothelial subtypes, we next examined human aortic endothelial cells (HAECs) under the same conditions. Similar to HUVECs, we observed subcellular glycogen pools in HAECs under HT and HT+CHIR, but not NM (**Figure S7**). To complement our single-cell SRS observations, we performed a bulk glycogen assay using enzyme-coupled luminescence reactions, which confirmed a ~2.3-fold total increase in glycogen concentration in ECs treated with HT+CHIR compared to HT alone (**Figure S8, Table S1**). Our results imply a surprisingly high storage capability for ECs under GSK3 inhibition. By leveraging GSK3 inhibition to enhance glycogen accumulation, this model also provides a robust platform to investigate the spatial growth and subcellular distribution of glycogen clusters in greater detail.

Next, to examine the functional consequences of glycogen accumulation using orthogonal assays, we conducted Seahorse profiling of HUVECs under NM, HT, and HT+CHIR conditions.

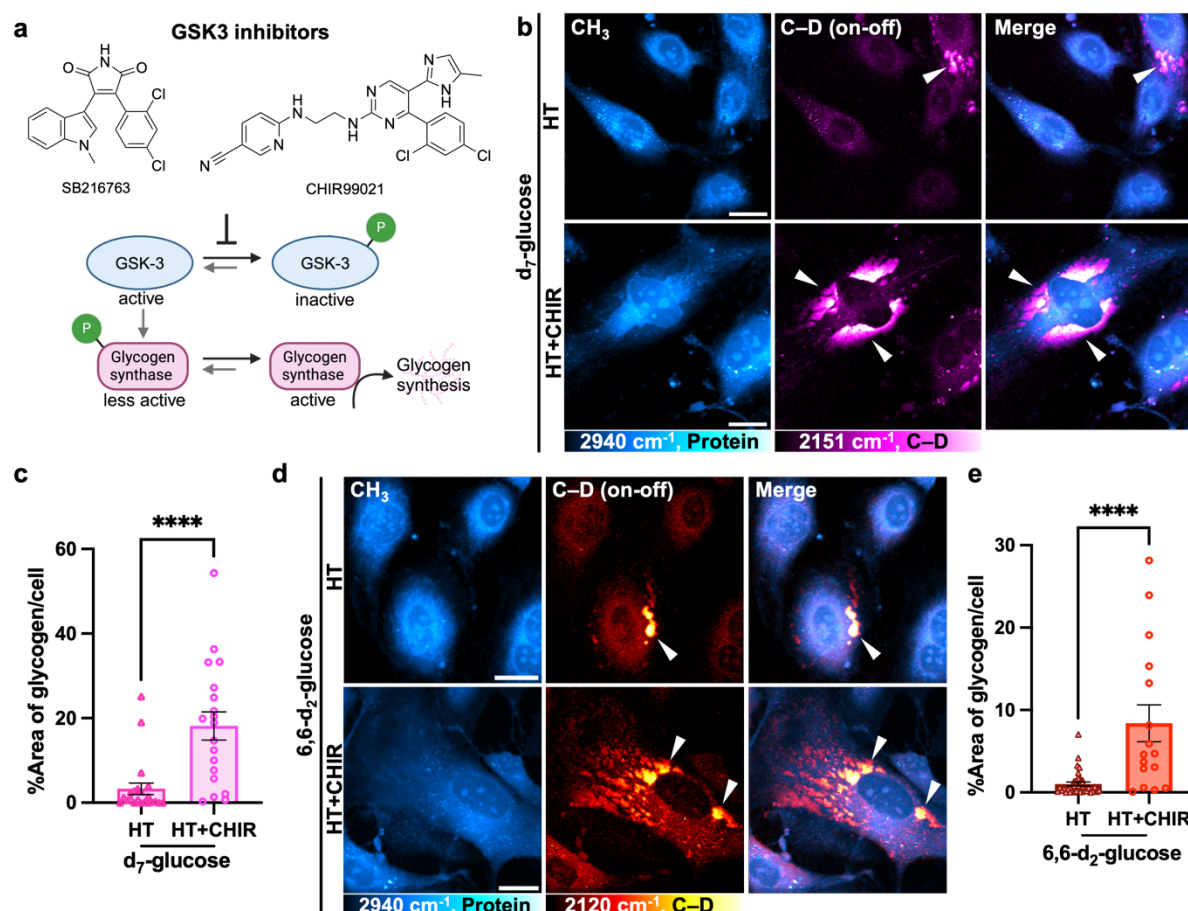


Figure 2. Pharmacological inhibition of GSK3 reveals a high capacity for glycogen storage in ECs. (a) Chemical structures of small molecule inhibitors of GSK3, SB216763 and CHIR99021; and the mechanistic scheme for inhibition of GSK-3 to activate glycogen synthesis. (b) Left to right: representative live-cell SRS images at the $-CH_3$ (protein), C-D (on-off), merged (CH_3 /C-D) channels for HUVECs treated with HT, or HT+CHIR containing d_7 -glucose for three days; (c) Quantification of the percentage of glycogen area per cell from HT and HT+CHIR-treated ECs ($n=22$ and 19 cells, respectively.); (d) Left to right: representative live-cell SRS images at the $-CH_3$ (protein), C-D (on-off), merged (CH_3 /C-D) channels for HUVECs treated with HT, or HT+CHIR containing 6,6- d_2 -glucose for three days. White arrowheads represent subcellular glycogen pools; (e) Quantification of the percentage of glycogen area per cell from HT and HT+CHIR-treated ECs ($n=36$ and 16 cells, respectively). Data (c, e) presented as mean $\pm$ SEM from at least three independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-tests. **** $p < 0.0001$. Scale bar: $20 \mu m$.

Consistent with our SRS data, diabetes-exposed ECs (HT) displayed elevated glycolysis as reflected by higher extracellular acidification rates (ECAR) without key changes in mitochondrial respiration (OCR) (NM vs. HT, **Figure S9**), indicating a Warburg-like metabolic shift. In contrast, HT+CHIR cells showed reductions in both ECAR and OCR, suggesting glucose carbons were diverted into glycogen rather than fueling bioenergetics. This redistribution was also captured by our SRS data (**Figure 2 and 3a**). Such global suppression of glycolytic and mitochondrial activity is consistent with reports that GSK3 inhibition enhances glycogen synthesis by redirecting glucose-6-phosphate away from glycolysis^{38,39} and attenuates mitochondrial respiration.⁴⁰ However, future studies are warranted to dissect the mechanisms underlying glycolytic attenuation in this condition.

To determine whether these metabolic changes correlate with endothelial function, we performed qPCR of representative markers of vascular homeostasis (*eNOS*), adhesion (*VE-cadherin*) and inflammation (*ICAM1*) to link metabolic changes with endothelial function (**Figure S10**). Both HT and HT+CHIR showed attenuated vascular homeostasis as indicated by the downregulation of eNOS as well as reduced adhesion marker expression (VE-cadherin) compared with NM. In contrast, both HT and HT+CHIR had elevated levels of ICAM1 as compared to NM, indicating heightened inflammation.

Finally, to spatially map mitochondria relative to glycogen, we performed correlative mitochondrial fluorescence staining with SRS imaging of d₇-glucose (**Figure S11**). Our data revealed spatial heterogeneity between NM, HT, and HT+CHIR. Specifically, in HT and HT+CHIR conditions, cellular regions enriched in glycogen (identified using the C–D channel) showed limited colocalization with mitochondrial signals that highlight a heterogeneous subcellular distribution of mitochondria relative to glycogen accumulation.

Dynamics of glycogen formation, spatial organization, and degradation in endothelial cells

To advance the understanding of glycogen synthesis (glycogenesis) and degradation (glycogenolysis) in ECs, we performed longitudinal SRS imaging in live ECs pulsed with HT and d₇-glucose. We observed increased glucose incorporation into biomass in ECs over time, including progressive accumulation of subcellular glycogen reservoirs (**HT; Figure 3a, S12**). While a previous study based on stable isotope labeling and mass spectrometry reported mobilization of glycogen reserves in bulk and lysed ECs under hypoglycemia,⁴¹ our imaging approach directly visualized glycogen breakdown at the subcellular level in live ECs: our chase experiment, following a glucose starvation chase, observed rapid glycogenolysis, with most of the subcellular pools depleted by 48 hours in ECs pre-exposed to HT (**HT; Figure 3b, S12**).

We further investigated glycogen turnover under HT+CHIR conditions, which markedly increased glycogen storage capacity (**HT+CHIR; Figure 3a, S12**). Similar to HT, glycogen pools in HT+CHIR also underwent rapid glycogenolysis under glucose deprivation with both conditions depleting their glycogen pool by 48 hours (**HT+CHIR; Figure 3b, S12**). Taken together, these results indicate the highly dynamic nature of these glycogen reservoirs and support their role as immediate energy reservoirs in ECs under glucose-limited conditions.

Building on these findings, we next investigated glycogen initiation and localization by performing multi-channel SRS imaging in HUVECs treated with HT+CHIR for 8 hours. Interestingly, we observed glycogen cluster initiation at multiple sites in close proximity to lipid droplets (LDs) (**Figure 3b**). In parallel experiments, we noted a striking increase in the LD abundance in ECs treated with HT compared to NM, consistent with other models of EC dysfunction.^{42–44} In other closely related cell types, such as brown hepatocytes, glycogen dynamics have been linked to driving lipid droplet (LD) biogenesis during cellular differentiation.⁴⁵ While further investigation is needed, these observations raise the intriguing possibility of potential metabolic crosstalk between LDs and glycogen dynamics in dysfunctional ECs.

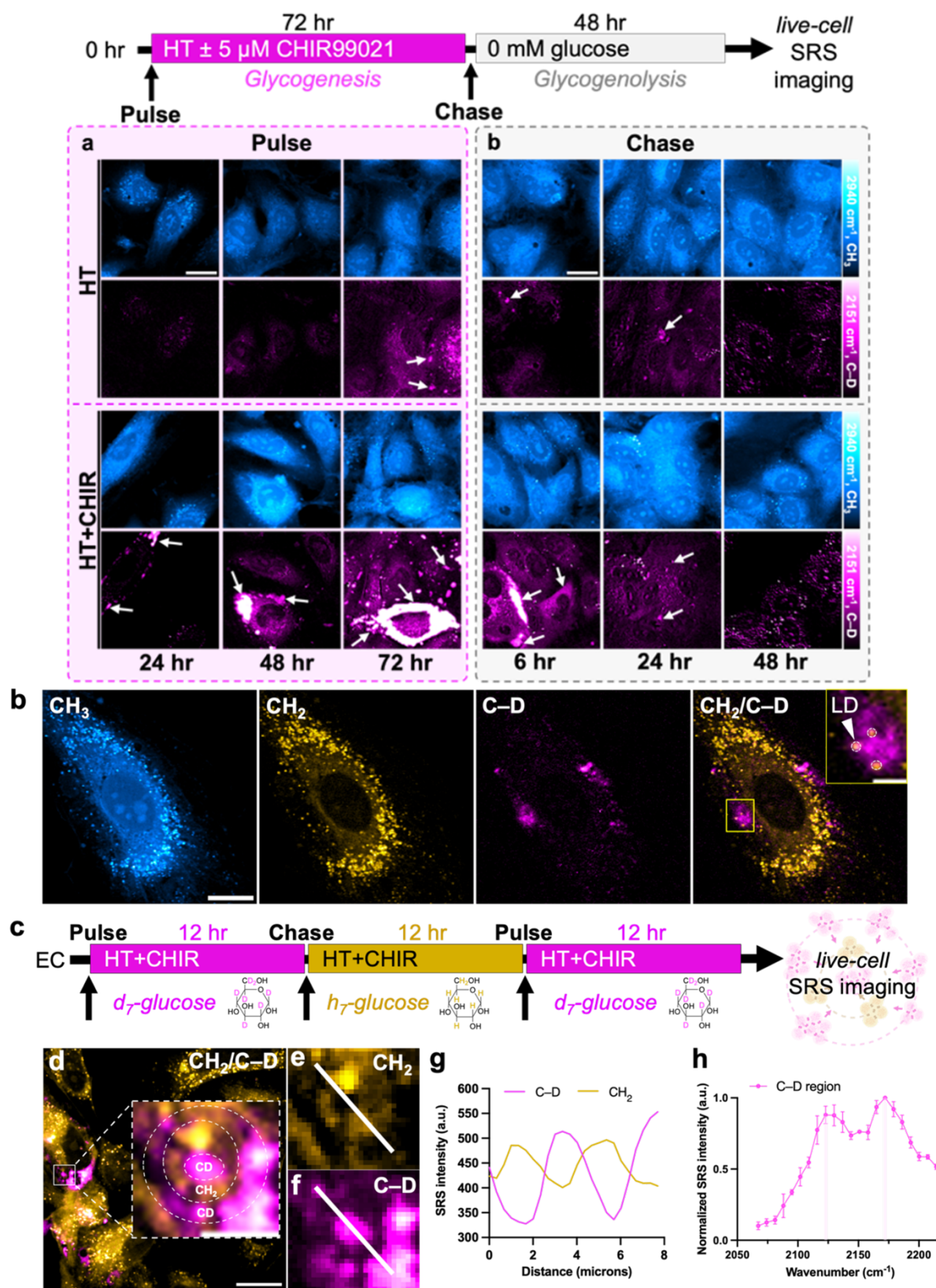


Figure 3. Glycogen dynamics, spatial organization, and degradation in endothelial cells. **(a)** Experimental design for the pulse-chase experiment to probe glycogenesis and glycogenolysis. Representative SRS images targeting the $-\text{CH}_3$ (protein) and C–D (on-off) channel after 24-, 48- and 72-hr of pulse with HT or HT+CHIR-treated HUVECs (top) and after 6-, 24- and 48-hr of chase in glucose-free media (bottom). White arrows indicate glycogen pools; **(b)** Multi-channel SRS images at the $-\text{CH}_3$ (protein), $-\text{CH}_2$ (lipid) and C–D (on-off) channels for HUVECs treated with HT+CHIR for 8-hr. Scale bar: 20 μm . Overlay images of CH_2 and C–D with an inset indicate lipid droplets (LDs; yellow circles) colocalized with glycogen clusters. Inset scale bar: 1 μm ; **(c)** Design of two-color glycogen experiment; **(d)** Overlay of CH_2 and C–D channel. Scale bar: 20 μm . Inset: a zoomed-in region of a bullseye pattern of glycogen formed from alternating concentric rings (dotted white circles) of glucose isotopologues. Inset scale bar: 5 μm ; **(e-f)** CH_2 (e) and C–D (f) image of the inset in (d), indicating a glycogen ring formed from unlabeled H-glucose (e) and the two concentric rings of glycogen formed from d_7 -glucose (f); **(g)** Intensity profile across the white solid line in (e) and (f); **(h)** Normalized SRS spectrum across the white-line in (f) confirming glycogen composition. Data are plotted as mean $\pm$ SEM.

To visualize glycogen cluster assembly and expansion in real space and time, we performed a two-color pulse-chase experiment using sequential labeling of d_7 -glucose and normal glucose media. HUVECs were first pulsed with d_7 -glucose for 12 hours, chased with unlabeled D-glucose for another 12 hours, and then pulsed again with d_7 -glucose for 12 hours during CHIR treatment (**Figure 3c**). SRS microscopy revealed a bullseye pattern of concentric rings, reflecting alternating incorporation of labeled and unlabeled glucose within glycogen clusters. This pattern suggests that glycogen protrusions expand radially from a central origin and can span over several microns under hyperglycemic stress (**Figure 3d-h**). While diffraction-limited SRS microscopy (~ 400 nm spatial resolution)²⁰ does not resolve ultrastructural features such as β - or α -particles seen by transmission electron microscopy (TEM),⁴⁶ it uniquely captures the spatial dynamics of glycogen synthesis and organization in live ECs.

SRS metabolic imaging of glutamine and lactate reveals metabolic shifts in ECs containing glycogen

The glycogen accumulation in ECs under HT conditions led us to hypothesize that this adaptation may confer a survival advantage during glucose deprivation. Surprisingly, HUVEC viability remained largely unaffected even in glycogen-deficient HUVECs (NM) after 72 hours of glucose starvation (**Figure S13**). In contrast, cervical cancer-derived HeLa cells which are also highly glycolytic,⁴⁷ showed significantly decreased viability under the same conditions (**Figure S13**). Building on our observation that glycogen serves as an energy reserve in ECs and exhibits rapid turnover (*vide supra*), we hypothesized the elevated presence of glycogen may initially affect the metabolic demand for alternative carbon sources when extracellular glucose is scarce. To investigate the metabolic adaptations that enable ECs to sustain their energy requirements in glucose-deprived environments, we focused on glutamine and lactate metabolism, two key metabolites known to undergo truncated GNG in ECs to generate lower glycolytic intermediates that support serine and glycerophospholipid biosynthesis pathways.²⁹

Glutamine, the most abundant freely circulating amino acid in the body, can fuel metabolism in various cell types including cancer cells under glucose stress.⁴⁸ It contributes to the tricarboxylic (TCA) cycle, nucleotide and nonessential amino acid (NEAA) synthesis,⁴⁹ and can fuel GNG by converting to glutamate and subsequently α -ketoglutarate (**Figure 4a**).⁵⁰ In ECs, glutamine is also essential for vascular expansion and proliferation.^{51,52} To visualize glutamine metabolism, we coupled SRS microscopy with d₅-glutamine, which exhibits a distinct C–D Raman peak at 2165 cm⁻¹ (**Figure 4b**, **Figure S14a**). HUVECs were pulsed for three days with NM (negligible glycogen), HT (moderate glycogen), or HT+CHIR (high glycogen), followed by a 24-hour chase with 5 mM d₅-glutamine in glucose-free medium.

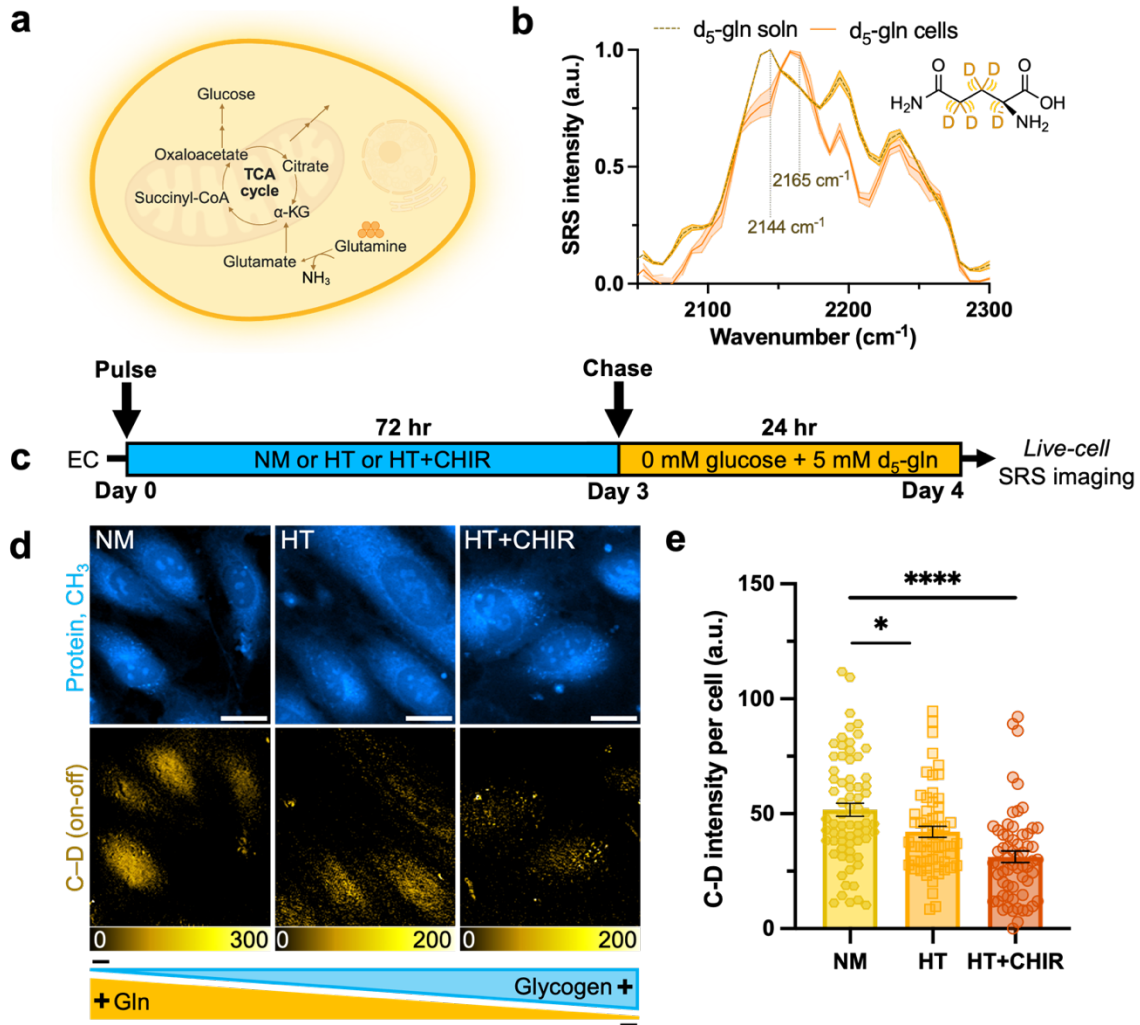


Figure 4. SRS microscopy reveals a decreased metabolic reliance on glutamine in ECs containing glycogen. (a) Schematic illustration of the gluconeogenic pathway of glutamine; (b) Normalized SRS spectra of 100 mM d_5 -gln solution, and of HUVECs incubated with 30 mM d_5 -gln for three days; (c) Schematic of pulse-chase experiment to probe glutamine metabolism; (d) Representative SRS images at the $-\text{CH}_3$ and C-D (on-off) for NM, HT and HT+CHIR. Scale bar: 20 μm ; (e) Quantitative analysis of SRS signal of C-D bonds per cell ($n=69, 59, 62$ cells for NM, HT, and HT+CHIR, respectively). 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.

**** $p<0.0001$, * $p<0.05$.

Strikingly, in the absence of extracellular glucose, ECs containing moderate to high contents of glycogen (both HT and HT+CHIR) showed reduced glutamine incorporation, compared to NM-treated cells, indicating a lower metabolic demand for glutamine in the presence of glycogen (**Figure 4c-d**). As a control, we tested glutamine metabolism in all three conditions after glycogen stores were mostly exhausted by performing a longer chase in glucose-free medium and found that this difference diminished over time (**Figure S11**). This result aligns with the metabolic shift seen in the liver where GNG increases to maintain euglycemia (normal glucose levels) as glycogen is exhausted.^{53,54}

To determine whether this decrease in glutamine signal was due to altered protein synthesis, we traced d₅-phenylalanine metabolism, an essential amino acid, as a proxy. SRS imaging targeted at the C–D channel at 2298 cm⁻¹ (**Figure S15a**) showed no significant differences in phenylalanine-derived protein synthesis among NM, HT, and HT+CHIR (**Figure S15b-c**). These results support the previous hypothesis that the reduction in glutamine signal in glycogen-rich conditions should be mostly contributed from glutamine metabolism itself, and not due to a reduced demand for protein synthesis.

We next moved to examine lactate metabolism— another key alternative carbon source.⁵⁵ Once considered a metabolic waste product in mammals, lactate is now recognized as a major circulating metabolic fuel, especially during glucose deprivation.⁵⁶ As a critical GNG precursor via pyruvate (**Figure 5a**),^{57,58} lactate is traditionally studied using genetically encoded fluorescent sensors^{59,60} which face limitations in their dynamic range, genetic accessibility, and applicability in primary cells like ECs. To address these challenges, we employed SRS microscopy coupled with d₃-lactate, targeting the C–D channel at 2129 cm⁻¹ (**Figure 5b, Figure S14b**). HUVECs pretreated with NM, HT, or HT+CHIR for 3 days were chased in 30 mM d₃-lactate for 24 hours in glucose-free media. Similar to glutamine, lactate incorporation was reduced in glycogen-rich ECs (HT and HT+CHIR) compared to NM (**Figure 5c-d**). This difference was also mitigated over time as glycogen was consumed (**Figure S17**), further reinforcing the idea that glycogen

modulates the immediate metabolic adaptations of ECs to alternative fuels during glucose starvation. Notably, this also marks the first time that glutamine and lactate metabolism have been visualized in live cells using SRS imaging.

To further validate our SRS metabolic imaging results, we performed complementary [3,3,3- $^2\text{H}_3$] lactate tracing combined with LC-MS-based targeted metabolomics in HUVECs using the same workflow. Stable isotope tracing enables quantification of isotope incorporation into downstream metabolic pathways.⁶¹ Consistent with SRS imaging, lactate showed higher labeling in NM compared to HT and HT+CHIR (**Figure 5f**). However, in the TCA metabolites, HT decreased labeling from lactate as compared to NM, but HT-CHIR increased it (**Figure 5g**). Therefore, to further evaluate the sources of our Raman signal, we estimated contributions of individual metabolites based on reported concentrations and the measured fraction labeled of each metabolite weighted by number of C–D bonds detected.^{62,63} While some small-molecule metabolites such as TCA-derived metabolites did not fully recapitulate the Raman trends, several downstream products did such as acetyl-carnitine, palmitoleic acid and oleic acid (**Figure S18**). These compounds share acetyl groups derived from acetyl-CoA which can feed into *de novo* lipogenesis (DNL) to make fatty acid chains or can be used for protein acetylation. We therefore hypothesize that the lactate Raman signal reflects not only labeling in intermediary metabolites but also incorporation of DNL derived fatty acids into acetylated lipids and other downstream products. Collectively, our Raman data

represent the total cellular lactate flux as it captures both direct metabolite labeling and incorporation into downstream products.

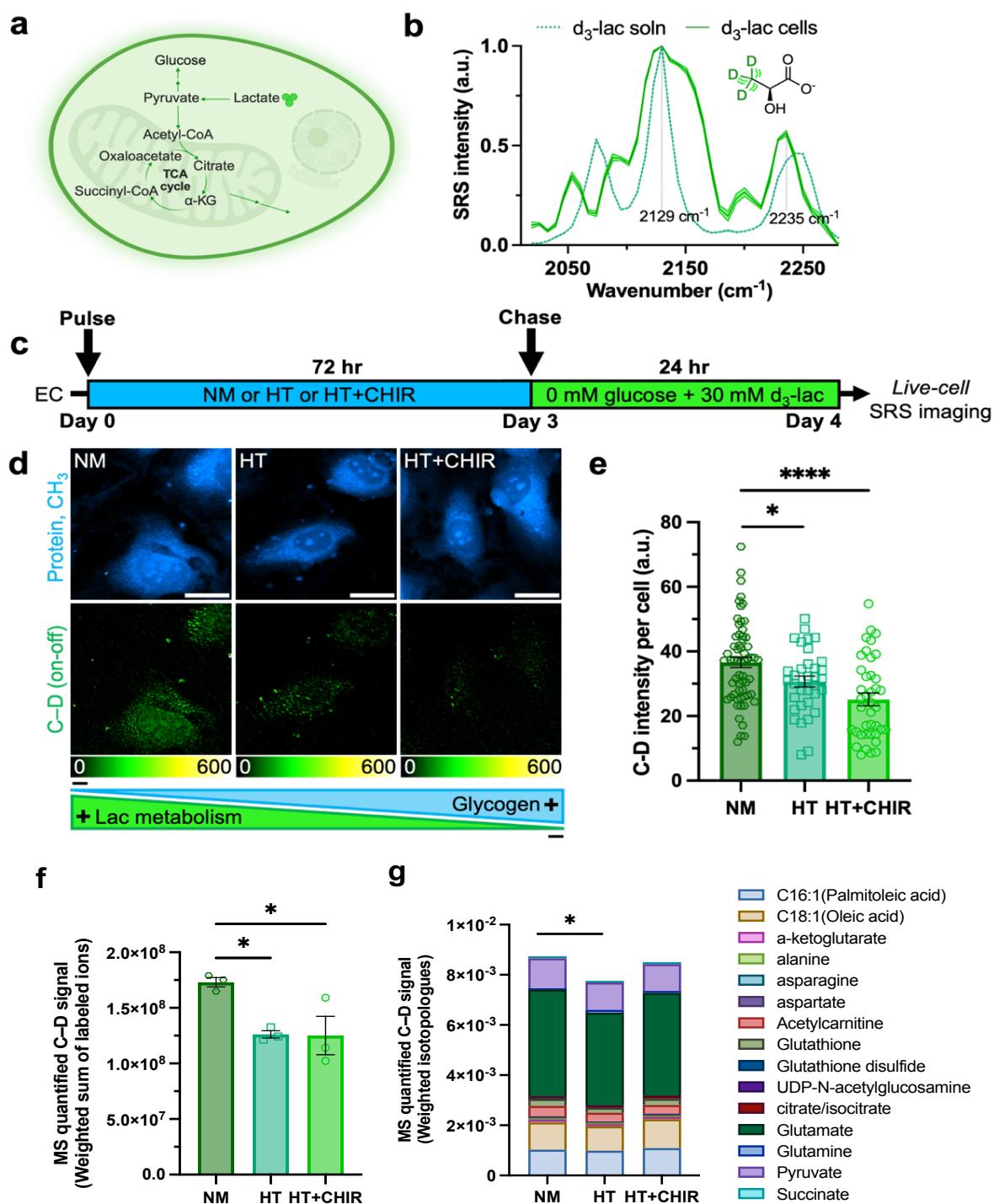


Figure 5. SRS and mass spectrometry-based stable isotope tracing reveal reduced lactate utilization in glycogen-rich ECs. (a) Schematic illustration of the gluconeogenic pathway of lactate in cells; (b) Normalized SRS spectra of 30 mM d₃-lac solution, and of HUVECs incubated with 30 mM d₃-lac for three days; (c) Schematic of pulse-chase experiment to probe lactate metabolism; (d) Representative SRS images at the –CH₃ and C–D (on-off) for NM, HT and HT+CHIR. Scale bar: 20 μm; (e) Quantitative analysis of SRS signal of C–D bonds per cell (n=62, 34, 40 cells for NM, HT and HT+CHIR, respectively); (f) MS-based quantification of d₃-lactate labeling (weighted sum of labeled ions) in HUVECs; (g) MS-based quantification of C–D incorporation in TCA cycle intermediates and downstream products including lipids (weighted isotopologues). Data are presented as mean ± SEM from at least three independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-tests (e) or one-way ANOVA (f), or two-way ANOVA tests (g). ****p<0.0001, *p<0.05.

3.4. Discussion

Diabetes-induced vascular dysfunction is driven by metabolic stressors such as hyperglycemia and hypoxia, which disrupt regulation of metabolites such as glucose across various cell types.^{64,65} However, the role of glycogen metabolism in endothelial dysfunction remains poorly understood. Our study addresses this gap by revealing the dynamic nature of glycogen metabolism in ECs under diabetes-related metabolic stress. By leveraging longitudinal SRS microscopy with non-perturbative deuterium labeling, we provide direct visualization of subcellular glycogen pools in live ECs and track their rapid turnover in response to hyperglycemia and glucose deprivation. Furthermore, SRS imaging of glutamine and lactate demonstrates that glycogen reserves initially modulate ECs' reliance on these alternative carbon sources under glucose starvation. These findings offer new insights into the metabolic adaptations that accompany diabetes-associated endothelial dysfunction.

Building on these findings, given the ability of ECs to crosstalk with other cell types including immune cells,⁶⁶ perivascular cells,⁶⁷ and adipocytes,⁶⁸ it would be compelling to explore whether EC-derived glycogen can be transferred to other cells to better understand metabolic crosstalk. Correlative studies combining SRS imaging with spatial transcriptomics could further elucidate whether glycogen-rich regions reflect distinct EC subpopulations or local microenvironmental influences. Finally, beyond glutamine and lactate, future studies should also explore the role of other key GNG substrates such as pyruvate, glycerol, alanine, etc.⁶⁹ in driving metabolic adaptations in ECs.

While our study provides new insights, certain limitations should be addressed in future investigations. For instance, although lactate supplementation was necessary to probe its metabolism, supraphysiological concentrations could also influence endothelial cell biology, potentially triggering endothelial-to-mesenchymal transition (EndoMT) under metabolic stress.⁷⁰ However, toxicity at these concentrations is not a concern for short-term imaging as trypan blue viability assays confirmed no significant changes in cell survival (**Figure S19**). Furthermore, vascular ECs are continuously exposed to biomechanical forces that influence their function and metabolism. For instance, physiological laminar or pulsatile flow has been shown to suppress whereas pathophysiological disturbed flow increases glycolysis and inflammatory response.^{71–74} Therefore, future studies should consider how these biomechanical cues may modulate glycogen dynamics under diabetes.

Overall, our work shows that dysfunctional ECs respond to hyperglycemic conditions by forming glycogen pools that serve as short-term energy backup during metabolic stress. Beyond this conventional role, these dynamic stores also initially influence the metabolic reliance on alternate carbon sources during the absence of extracellular glucose. Moreover, our demonstration of SRS metabolic imaging of lactate and glutamine in living cells unlocks new possibilities for visualizing the underlying

metabolic changes of diabetes-induced endothelial dysfunction and related pathologies.

3.5. Materials and Methods

Cell Culture, Metabolic Labeling and Imaging

HUVECs (passages 5-8; Cell Applications, Cat. #200-05n) were tested negative for mycoplasma contamination and cultured at 37°C with 5% CO₂ in commercial endothelial cell growth media (ECGM; Cell Applications, Cat. #211-500), supplemented with 10% FBS (Corning, Cat. #35-015-CV) and 100x antibiotic-antimycotic (Gibco, Cat. #15240062). HeLa cells (ATCC, CCL-2) were cultured in DMEM (Corning, Cat. #10-017-CV) with the same supplementation as above. For SRS imaging experiments, HUVECs were seeded on glass coverslips pre-coated with attachment factor solution (Cell Applications, Cat. #123-500) for 2 hours. The HT condition was induced by adjusting the final concentration to 25 mM D-glucose (Thermo Fisher, Cat. #A16828-36), d₇-glucose (Cambridge Isotope Laboratories, Cat. #DLM-2062), or 6,6-d₂-glucose (Cambridge Isotope Laboratories, Cat. #DLM-349), along with 5 ng/mL TNF- α (Gibco, Cat. #PHC3015). The NM condition included addition of 25 mM mannitol (Thermo Fisher, Cat. #125340025) as an osmolarity control in ECGM containing 5 mM baseline D-glucose. For GSK3 inhibition, 5 μ M CHIR-99021 (MedChemExpress, Cat. #HY-10182), or 5 μ M SB-216763 (MedChemExpress, Cat. #HY-12012) was added concomitantly with the HT condition containing labeled or unlabeled glucose for the specified duration.

For the glycogen dynamics experiments, HUVECs were treated with HT (using DMSO as a vehicle) or HT+CHIR in medium containing labeled d₇-glucose for the specified duration. To probe glycogenolysis, cells were subsequently chased in custom glucose-free ECGM (Cell Applications, Cat. #211AG-500) for the respective time points before live-cell SRS imaging.

For the two-color glycogen experiment, HUVECs were first pulsed with HT (containing labeled d₇-glucose)+CHIR for 12 hours, then chased with HT (containing unlabeled

glucose)+CHIR for 12 hours and finally pulsed again with HT (containing labeled d₇-glucose)+CHIR for another 12 hours before SRS imaging.

For the glutamine and lactate chase experiments, HUVECs were treated with NM, HT, or HT+CHIR containing unlabeled glucose for three days, followed by a chase with the labeled probes added at the desired concentration for 24 hours in custom media containing 0 mM glucose, 0 mM unlabeled L-glutamine and 0 mM unlabeled lactate (Cell Applications, Cat. #211AG-500). The labeled probes used for metabolic imaging were 5 mM 2,3,3,4,4-d₅-L-glutamine (Cambridge Isotope Laboratories, Cat. #DLM-1826) and 30 mM 3,3,3-d₃ sodium L-lactate (Cambridge Isotope Laboratories, Cat. #DLM-9071) for glutamine and lactate, respectively. The metabolic label used for phenylalanine imaging was d₅-L-phenylalanine (Cambridge Isotope Laboratories, Cat. #DLM-1258).

SRS Microscopy

The SRS microscopy system was configured as described previously.⁷⁵ **Figure 1d** depicts the corresponding microscope configuration. Briefly, images were acquired with an 80 μ s pixel dwell time, achieving an image acquisition speed of 8.52 s per frame for a 320x320-pixel field of view at a 0.497 μ m/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}), 844.0 nm (C–D channel for d₇-glucose, 2150 cm^{-1}), 846.2 nm (C–D channel for 6,6-d₂-glucose, 2120 cm^{-1}), 842.9 nm (C–D channel for d₅-glutamine, 2167 cm^{-1}), 845.6 nm (C–D channel for d₃-lactate, 2129 cm^{-1}), 833.7 nm (C–D channel for d₅-phenylalanine, 2297 cm^{-1}) and 867.0 nm (off-resonance, 1837 cm^{-1}). For hSRS, the wavelength of the pump laser was tuned from 833.5 nm to 853.5 nm with a step size of 0.5 nm unless indicated otherwise. All images were analyzed 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. Individual cells were then manually segmented, and the SRS intensity per cell was quantified following background subtraction. MS Excel, GraphPad Prism, and MATLAB (R2024b, MathWorks) were used for data processing and plotting.

Spectral Phasor Analysis

The hSRS sweeps across the range 2067–2235 cm^{-1} (25 images with a step size of $\sim 7 \text{ cm}^{-1}$) were imported into ImageJ as a stack. A maximum intensity projection was created and spectral phasor analysis was performed using a spectral phasor plugin (<http://www.spechron.com/Spectral%20Phasor-Download.aspx>) as described previously.³³ Segmentation of the phasor plot was performed manually to discretely map glycogen.

Least Absolute Shrinkage and Selection Operator (LASSO)

The same set of aforementioned hSRS images were also unmixed using least absolute shrinkage and selection operator (LASSO)^{34,76} in MATLAB (R2024b, MathWorks) on a per-pixel basis following nonuniform background removal and non-local means denoising in ImageJ.^{77,78}

Spontaneous Raman Microscopy

Spontaneous Raman spectra were acquired using a Horiba Xplora plus upright confocal Raman spectrometer with a 532 nm YAG laser at 12 mW through a 100x, 0.9 N.A. objective (MPLAN N, Olympus) with a 500 μm hole, and a 100 μm slit. Data were collected with an integration time of 10 s and averaged 10 times using LabSpec6 software. The background signal from the solvents was subtracted from the spectra of the target analytes followed by baseline correction, and normalization.

Periodic Acid-Schiff (PAS) Staining

HUVECs were incubated under HT treatment (containing labeled or unlabeled glucose) for three days, fixed with 4% paraformaldehyde for 15 minutes, and washed three times using Dulbecco's phosphate-buffered saline (DPBS, Gibco, Cat. #14190144). Cells were then treated with 1% periodic acid (Sigma Aldrich, Cat. #3951) for 30 mins at room temperature and washed five times using deionized water. After incubation with Schiff's reagent (Sigma Aldrich, Cat. #3952016) for 30 mins and additional washes with

deionized water, PAS-stained cells were imaged on an Olympus FV3000 microscope using a color CMOS camera (Thorlabs, CS165CU) in tandem with SRS microscopy.

Mitochondria Imaging using Fluorescence Microscopy

HUVECs cultured under NM, HT, or HT+CHIR for 72 hours using d₇-glucose were treated with 80 nM MitoTracker™ Deep Red FM (Invitrogen, Cat. #M22426) for 30 minutes, followed by three washes with DPBS. Fluorescence images were acquired in tandem with SRS microscopy using the same Olympus FV3000 confocal microscope using excitation at 640 nm and emission collected at 665 nm for the mitochondria dye.

Cell Viability Assay

For the viability assay under glucose starvation, cells cultured on 24-well plates were switched to glucose-deficient media and chased for 1-3 days. Relative cell viability was calculated using trypan blue staining and normalized to the control that consisted of cells grown in the corresponding baseline glucose-containing media. To assess toxicity from metabolic labeling, HUVECs were treated with the respective probes for 24-72 hr and relative viability was determined in the same manner, with normalization to HUVECs maintained in baseline ECGM as the control.

RNA Extraction and Quantitative PCR

RNA extraction was performed using Trizol reagent (Ambion, Cat. #15596018). RNA concentration was measured using Nanodrop spectrophotometer (Thermo Fisher). PrimeScript™ RT Master Mix contained both Oligo-dT and random hexamer primers. Quantitative PCR was performed using SYBR Green Master Mix (Bio-Rad) following the manufacturer's suggested protocol, using the Bio-Rad CFX Optus PCR System (Bio-Rad). The primer sequences used are listed in **Table 1**. Relative expression levels were determined using the comparative CT method to normalize target genes to *18S* internal controls.

Table 1. List of primer sequences used for qPCR.

qPCR primers	Forward	Reverse
ICAM1	GTGTCCTGTATGGCCCCCGACT	ACCTTGCGGGTGACCTCCCC
VCAM1	GTCAATGTTGCCCCCAGAGA	TTTTCGGAGCAGGAAAGCCC
GYS1	CCGCTATGAGTTCTCCAACAAGG	AGAAGGCAACCACTGTCTGCTC
eNOS	TGATGGCGAAGCGAGTGAAG	ACTCATCCATACACAGGACCC
VE-cadherin	GTTCACCTTCTGCGAGGATATG	GATGGTGAGGATGCAGAGTAAG
18-s	TGTGCCGCTAGAGGTGAAATT	TGGCAAATGCTTTCGCTTT

Glycogen Luminescence Assay

In preparation for glycogen detection, HUVECs cultured on 6-cm dishes were treated with HT, or HT+CHIR for three days. Glycogen detection was performed using the Glycogen-GloTM Assay kit (Promega, Cat. #J5051) following manufacturer's protocol. Luminescence was measured on a SpectraMax M3 multi-mode microplate reader (Molecular Devices) in relative light units (RLU). The sample buffer with no cells was used as a negative control. A control with no glucoamylase was also used to detect residual glucose in the samples. All samples were run as both technical and biological triplicates. Glycogen and glucose calibration curves were plotted in the concentration ranges of 0 to 20 $\mu\text{g/mL}$ and 0 to 2.5 $\mu\text{g/mL}$, respectively. Glycogen and glucose concentrations in samples were calculated using the corresponding calibration plots in MS Excel and plotted in Prism GraphPad.

Seahorse Assay

HUVECs were assayed using a Seahorse XFe24 Analyzer (Agilent Technologies) according to the manufacturer's instructions. Briefly, cells were seeded at 60,000 cells/well in XFe24 cell culture plates and allowed to adhere overnight. Cells were then treated for 3 days with one of the following conditions: 25 mM mannitol (normal-osmolarity control), 25 mM D-glucose + 5 ng/mL TNF- α , or 25 mM D-glucose + 5

ng/mL TNF- α + 5 μ M CHIR-99021, as described above. On the day of the assay, culture medium was replaced with Seahorse XF DMEM base medium (Agilent, Cat. #103334-100) without phenol red, supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM sodium pyruvate, adjusted to pH 7.4. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF Cell Mito Stress Test Kit (Agilent, #103015-100) following sequential injections of 1.5 μ M oligomycin, 1 μ M FCCP, and 0.5 μ M rotenone/antimycin A.

3,3,3-²H₃-Lactate Tracing using Targeted Metabolomics

HUVECs were cultured under NM, HT, or HT+CHIR for three days in ECGM containing 10% dialyzed FBS (Gibco, Cat.# A3382001), followed by a 24-hour chase with 2 mM d₃-lactate in glucose-free ECGM. Cell metabolism was quenched by rapidly aspirating the medium, rinsing with 150 mM NH₄OAc (adjusted to pH 7.3, Fisher Scientific, Cat. #ICN19384880) and adding 80% ice-cold methanol containing 1 μ M norvaline (Millipore Sigma, Cat. #53721-100MG) as an internal standard. Samples were incubated at -80 °C for 30 minutes, scraped into low-bind tubes and centrifuged at maximum speed for 30 minutes. Supernatants were stored at -80 °C until LC-MS analysis. Prior to injection, extracts were thawed on ice, centrifuged for 10 minutes, and clarified for LC-MS.

Metabolites were separated on a Waters BEH z-HILIC column (150 $\times$ 2.1 mm, 2.7 μ m) coupled 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 performed at 0.18 mL/min using 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 180,000 resolution (m/z 70-1000) with an

AGC target of 1×10^6 . MS/MS scans were acquired using a TOP3 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 using a window of 2 ppm.

To quantify the C–D signal with our mass spectrometry data, isotopologues were weighted by the number of C–D bonds detected (e.g., M+1 by 1, M+2 by 2, M+3 by 3, etc.). This weighting shows the relative contribution of each isotopologue to the overall C–D signal. To account for the total C–D signal detected in the metabolite pool, these weighted values were then multiplied by previously published intracellular metabolite concentrations^{62,63} and summed. Final data were expressed either as the fraction of total signal from labeled species or as relative isotopologue abundances, depending on the downstream analysis.

Statistical Analysis

All data are represented as mean $\pm$ SEM unless otherwise noted. Statistical significance was analyzed using two-tailed unpaired Student's t-tests, or one-way/two-way ANOVA (for LC-MS measurements), using GraphPad Prism 10. As indicated above, for statistically significant differences, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, whereas ns (no statistical difference) indicates $p > 0.05$.

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Supplementary Information

SINGLE-CELL METABOLIC IMAGING REVEALS
GLYCOGEN-DRIVEN ADAPTATIONS IN ENDOTHELIAL
CELLS

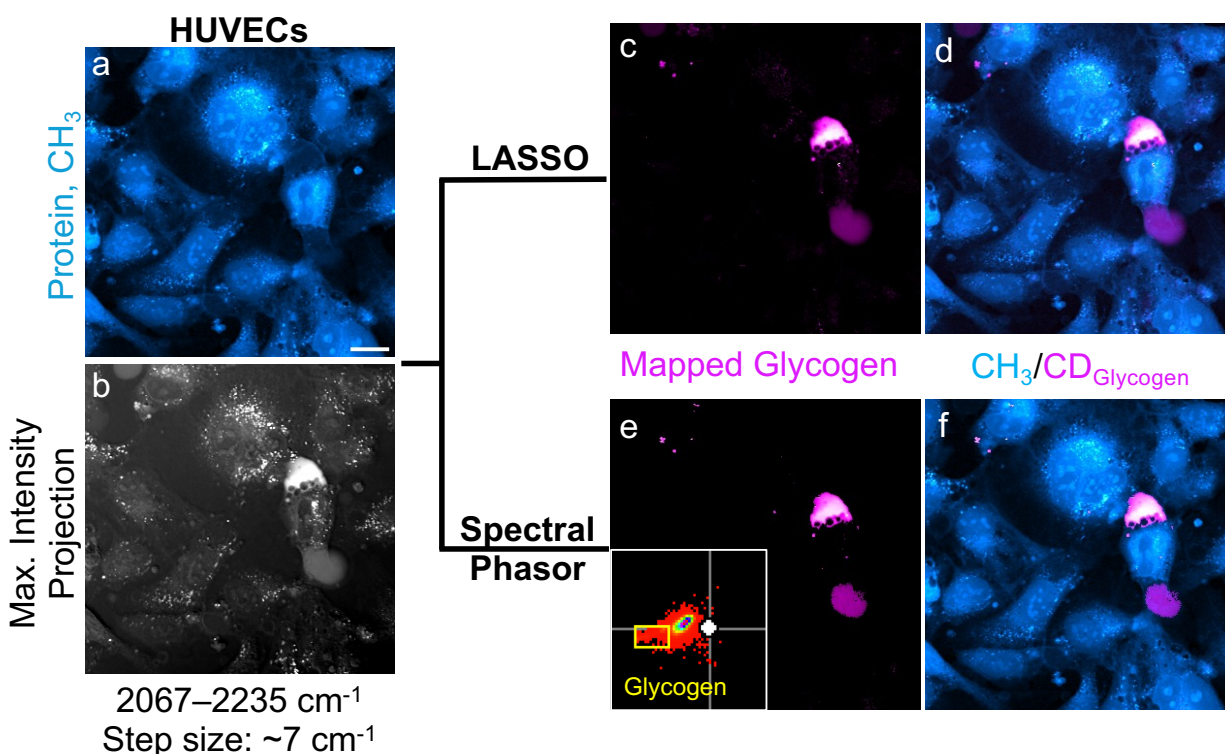


Figure S1. Chemometric approaches for glycogen phenotyping in ECs. (a) SRS image targeting the $-\text{CH}_3$ channel (protein) in ECs under hyperglycemic conditions; (b) Maximum intensity projection of a hSRS stack comprising 25 images acquired across $2067\text{--}2235\text{ cm}^{-1}$ with a step size of 7 cm^{-1} used for chemometric analysis; (c) Mapped glycogen image generated using LASSO ($\text{CD}_{\text{Glycogen}}$); (d) Overlay of CH_3 , and $\text{CD}_{\text{Glycogen}}$ image derived from LASSO analysis; (e) Mapped glycogen image generated using spectral phasor analysis ($\text{CD}_{\text{Glycogen}}$). The inset shows the phasor plot where the boxed area (yellow) represents pixels attributed to glycogen; (f) Overlay of CH_3 , and $\text{CD}_{\text{Glycogen}}$ image generated using spectral phasor analysis. Scale bar: $20\text{ }\mu\text{m}$.

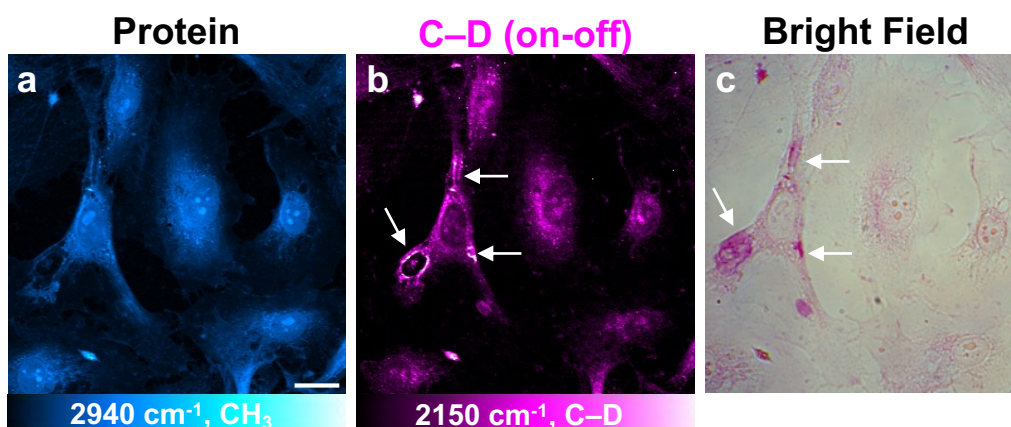


Figure S2. Correlative PAS staining of glycogen pools in ECs. (a, b) Representative SRS images targeted at the -CH_3 (protein) and C-D (on-off) channels, respectively, for HT-treated HUVECs; (c) Bright field image of the same set of cells after PAS staining. White arrows represent glycogen pools. Scale bar: 20 μm .

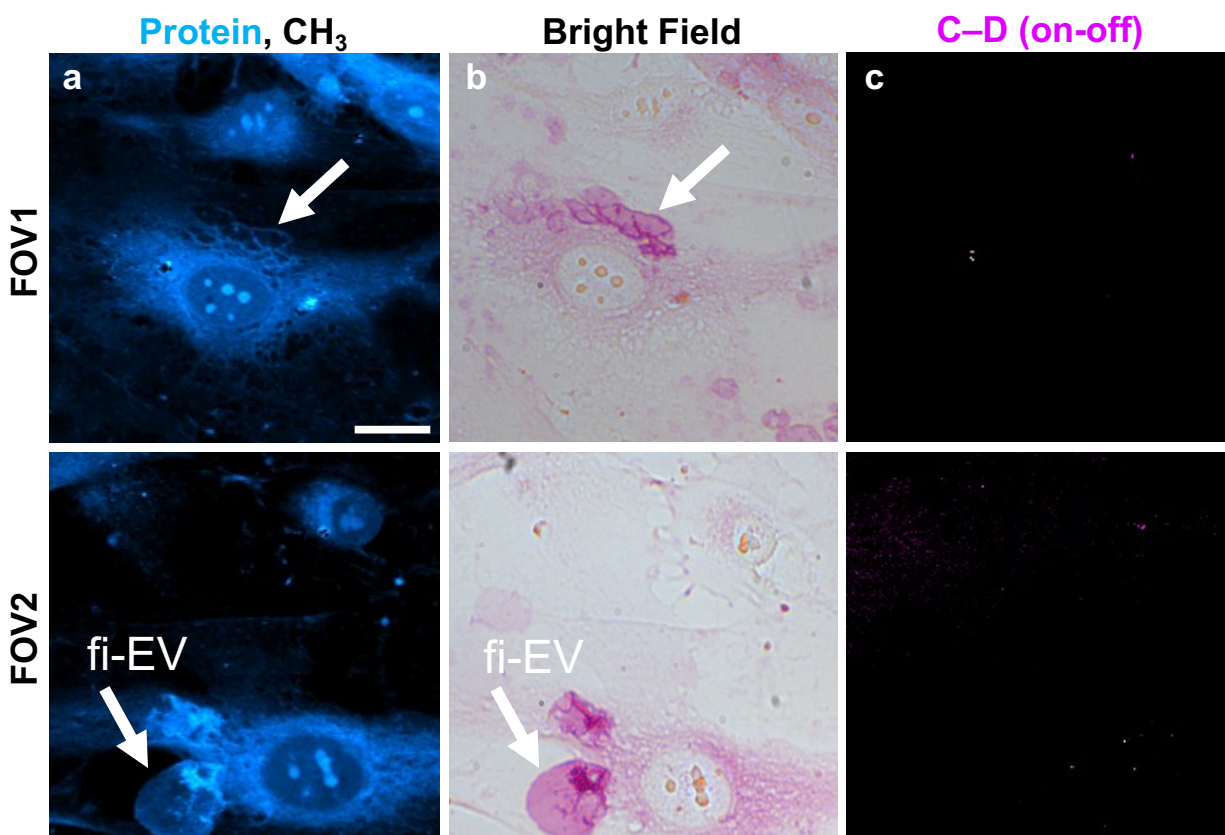


Figure S3. Glycogen pools in ECs are not an artifact of glucose isotopologues. (a) Representative SRS image targeted at the $-\text{CH}_3$ (protein) for HT-treated HUVECs containing unlabeled glucose (h_7 -glucose); (b) Bright field image of the same set of cells after PAS staining; (c) C–D images (on–off) of the same corresponding field of views (FOVs). White arrows represent glycogen pools; fi-EV = fixation-induced extracellular vesicle. Scale bar: 20 μm .

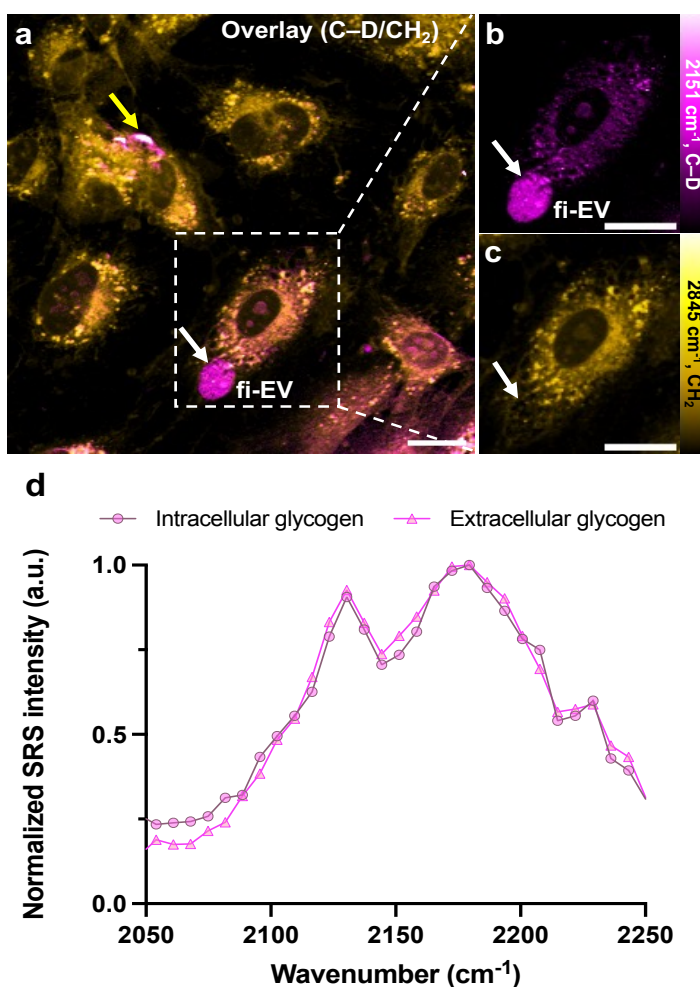


Figure S4. PFA fixation induces glycogen-enriched extracellular vesicles. (a) Representative overlay image (C–D/CH₂ channels) of fixed HUVECs treated with HT using d₇-glucose for three days. Yellow arrow represents intracellular glycogen reserves, while the white arrow indicates the extracellular glycogen in fixation-induced EVs (fi-EVs) after PFA fixation; (b) Zoomed-in image of the boxed region in (a), targeted at the C–D channel (on–off) indicating glycogen-enriched fi-EV (white arrow); (c) Corresponding zoomed-in CH₂ (lipid) image of the boxed region in (a); (d) Normalized SRS spectrum of intracellular and extracellular glycogen regions from (a). Scale bar: 20 μ m.

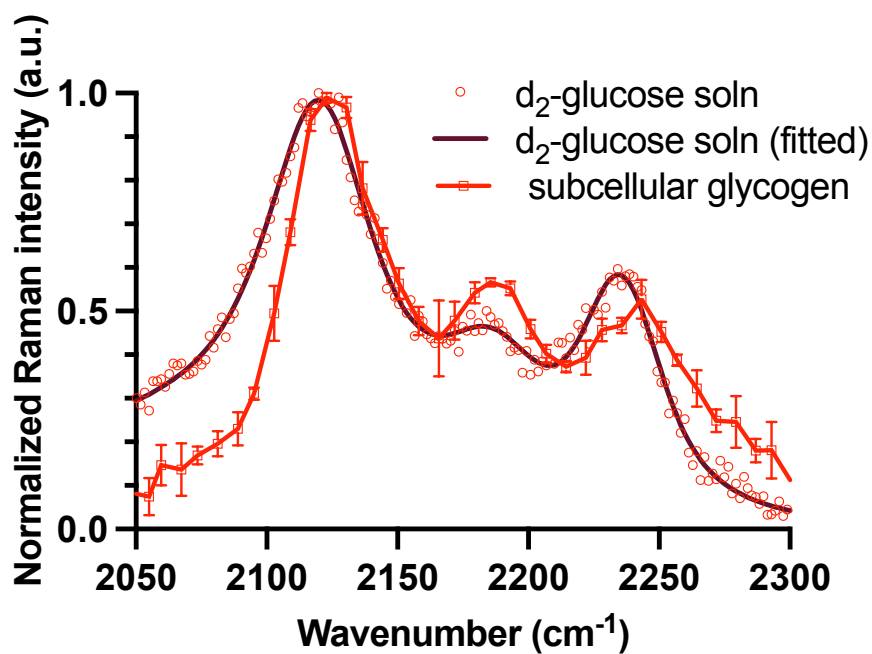


Figure S5. Raman spectra of glucose isotopologue, 6,6-d₂-glucose. Normalized spontaneous Raman spectrum of 100 mM solution (soln) of 6,6-d₂-glucose in water (red circles) with corresponding fitted data (maroon solid line). Normalized SRS spectrum of subcellular glycogen from HT-treated ECs incubated with d₂-glucose (red squares with solid line). Data are presented as mean $\pm$ SEM.

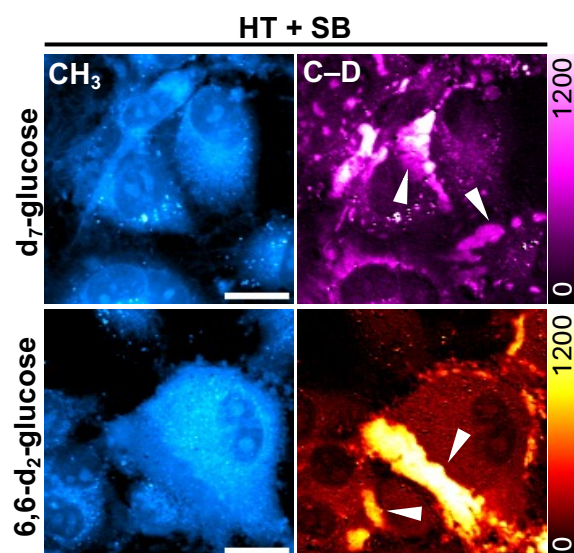


Figure S6. GSK3 inhibition using an alternative small molecule inhibitor, SB-216763. Representative SRS images targeting the $-\text{CH}_3$ (protein) and C–D (on-off) channel for live HUVECs treated with HT+SB incubated with either d_7 -glucose, or d_2 -glucose. White arrowheads represent subcellular glycogen pools. Scale bar: 20 μm .

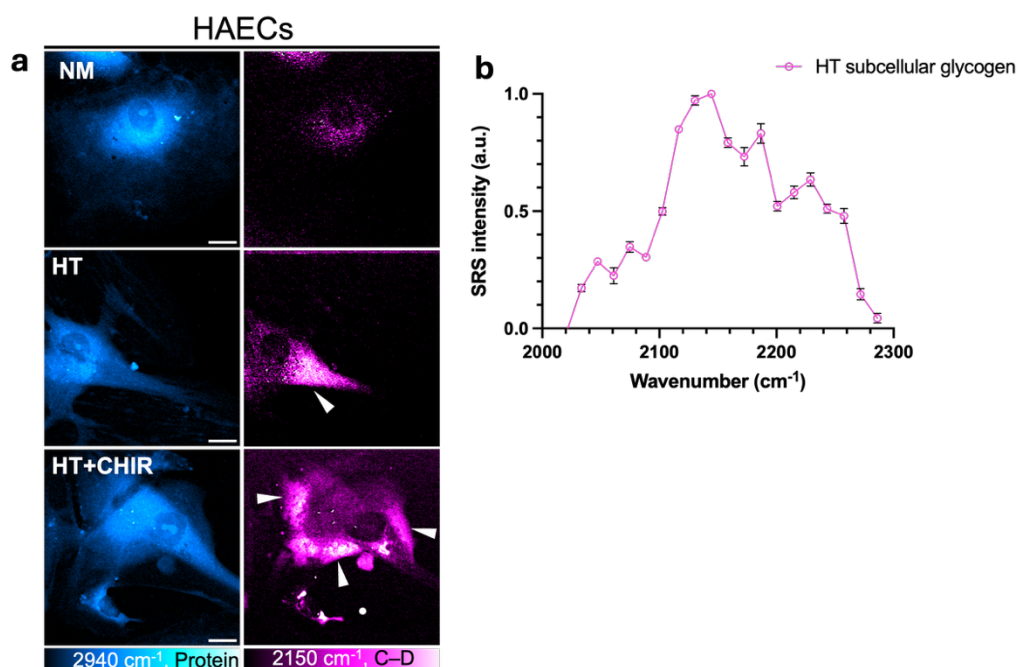


Figure S7. Glycogen phenotyping in live human aortic endothelial cells (HAECs). (a) SRS images targeted at the protein channel ($-\text{CH}_3$, left) and C–D (on–off, right) channels for HAECs exposed to NM, HT, or HT+CHIR for 72 hr. White arrowheads indicate subcellular glycogen pools. Scale bar: 20 μm ; (b) Hyperspectral SRS of the subcellular pools in HT-treated cells (panel a) confirms the chemical signature of glycogen. Data represented as mean $\pm$ SEM.

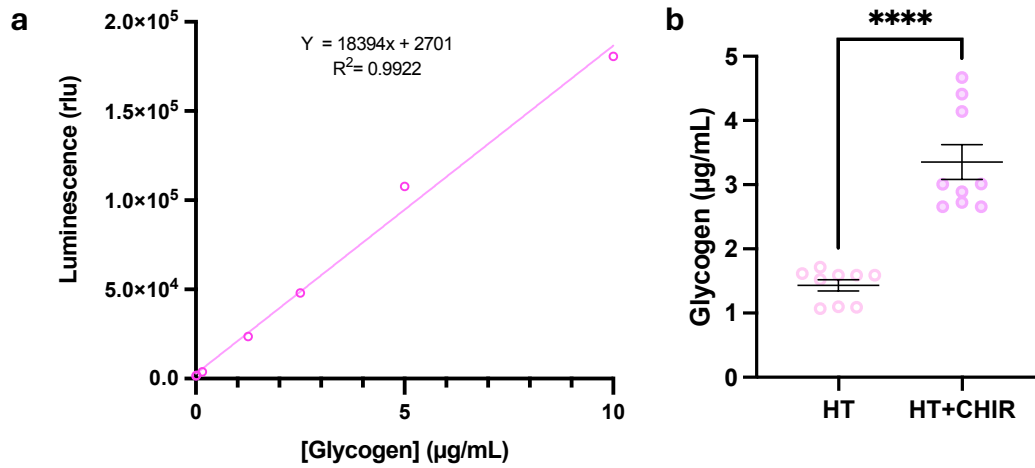


Figure S8. Glycogen detection in bulk ECs using luminescence. (a) Calibration curve for glycogen with different concentrations ($\mu\text{g/mL}$) measured by luminescence (rlu); (b) Glycogen concentration ($\mu\text{g/mL}$) in ECs treated with HT, or HT+CHIR for three days, quantified using the calibration curve in (a) (n=9 technical replicates from three independent experiments). Statistical significance was analyzed using two-tailed unpaired Student's t-test, ****p<0.0001. Data are presented as mean $\pm$ SEM.

Table S1. Glycogen concentration detected in bulk HUVECs using luminescence.

Mean concentration of glycogen ($\mu\text{g/mL}$) for the corresponding treatments detected using the luminescence assay (in Figure S7) and their standard error of mean (SEM). The analytical sensitivity (γ) of the assay is calculated to be 18,394 rlu/ $(\mu\text{g/mL})$.

Condition	Glycogen conc. ($\mu\text{g/mL}$)	SEM ($\mu\text{g/mL}$)
HT	1.43	0.08
HT+CHIR	3.35	0.27

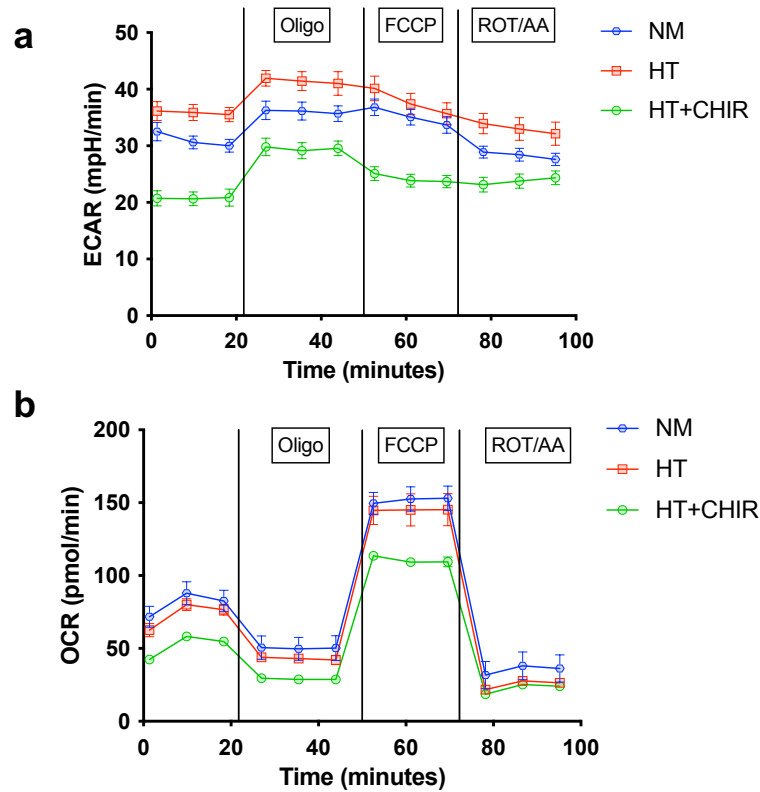


Figure S9. Bioenergetics profiling of HUVECs. (a) Extracellular acidification rate (ECAR) profile of ECs under NM, HT, or HT+CHIR conditions measured with the Seahorse assay; (b) Oxygen consumption rate (OCR) profile of endothelial cells (ECs) under the same conditions. Mitochondrial respiration modulators were sequentially injected (oligomycin, FCCP, rotenone, and antimycin A). Data represented as mean $\pm$ SEM.

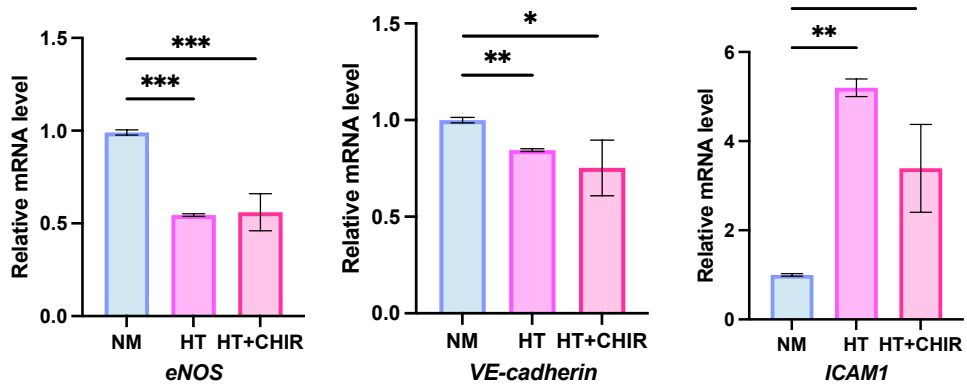


Figure S10. mRNA levels of eNOS, VE-cadherin and ICAM1 in NM-, HT-, or HT+CHIR-treated HUVECs for 3 days. Data represented as mean $\pm$ SD. Statistical significance was analyzed using two-tailed unpaired Student's t-tests. ***p<0.001, **p<0.01, *p<0.05.

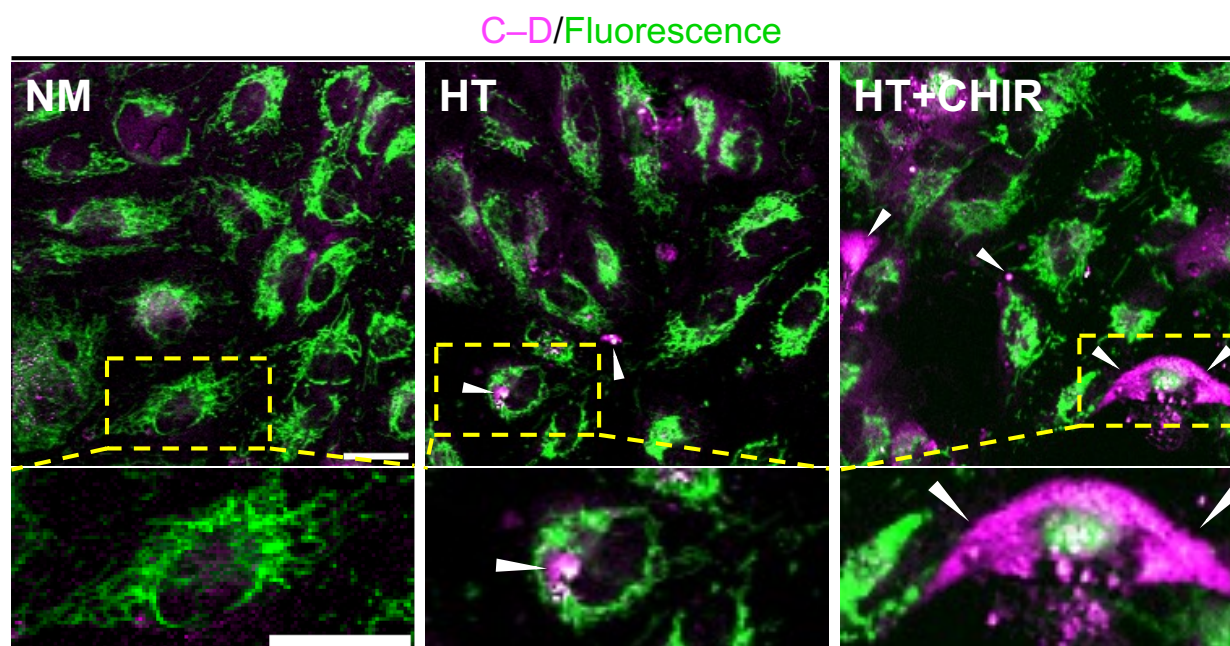


Figure S11. Correlative Mitochondrial staining (Fluorescence) and d₇-glucose imaging (SRS) of NM-, HT-, and HT+CHIR-exposed live ECs. Representative overlay images of fluorescence (MitoDeepRed) and SRS (C–D) of live HUVECs are shown. The bottom panel represents selected zoomed-in regions from the top panel (yellow box). White arrowheads indicate subcellular glycogen pools. Scale bar: 20 μ m.

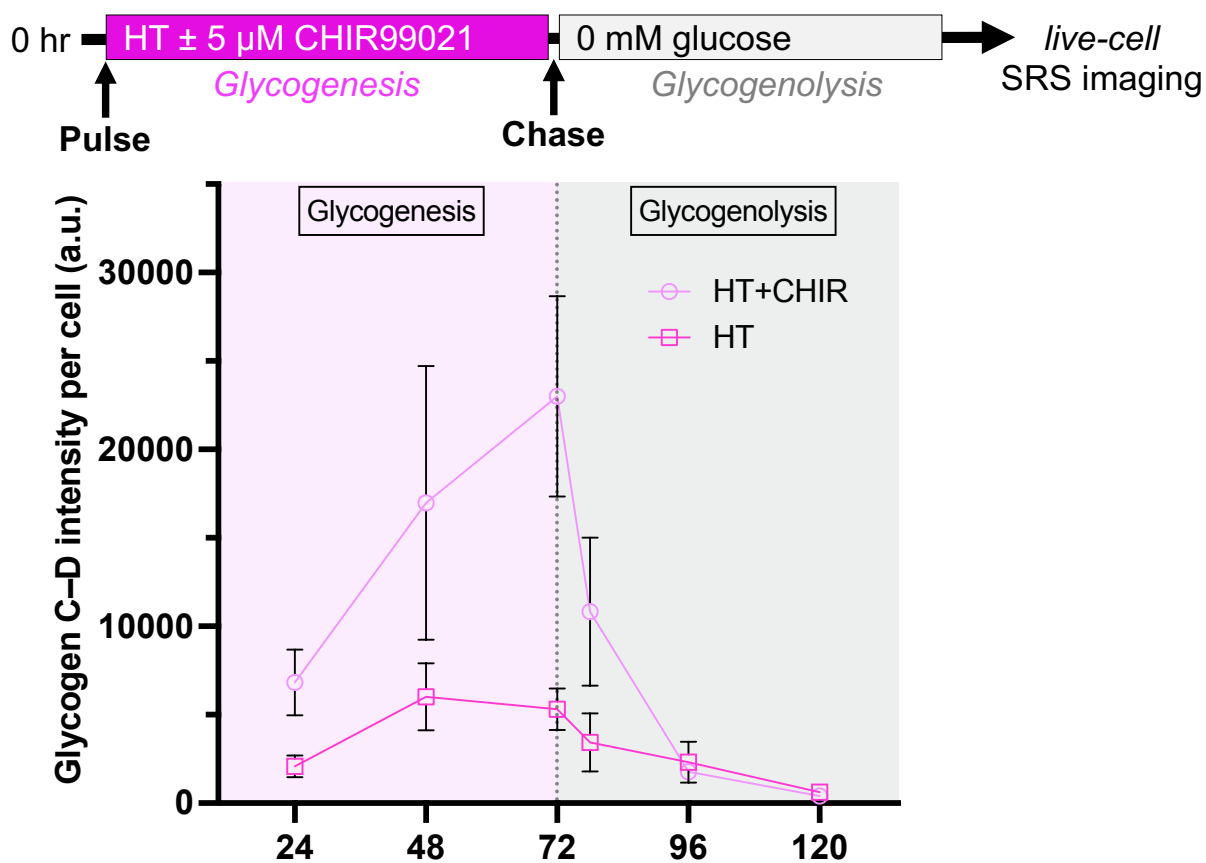


Figure S12. Longitudinal imaging of glycogen metabolism in HUVECs. SRS intensities of segmented glycogen C–D (on-off) per cell pulsed with HT, or HT+CHIR for 72 hr, and chased in glucose-free media for 48 hr. Data are presented as mean $\pm$ SEM from at least three independent experiments.

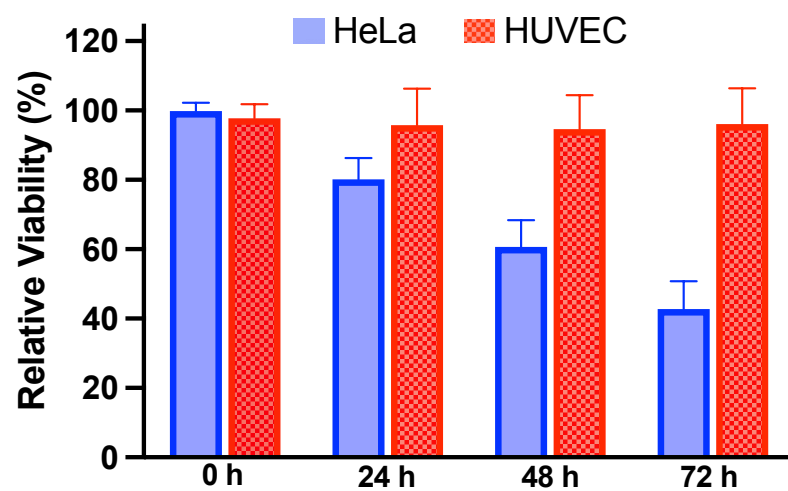


Figure S13. Viability assay for HeLa and HUVEC under glucose starvation. Relative viability of HeLa and HUVECs cultured in glucose-deficient media for 72 hours. Data are presented as mean $\pm$ SEM from three biological replicates.

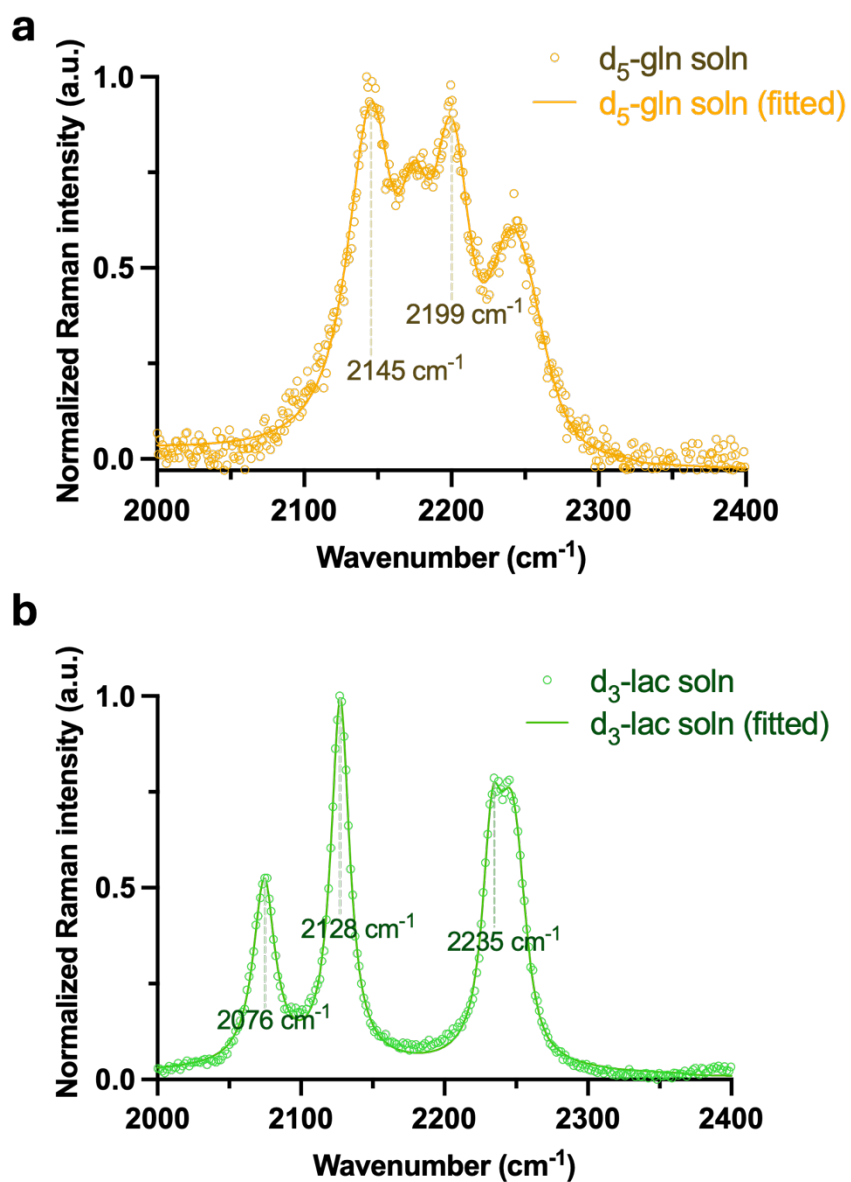


Figure S14. Spontaneous Raman spectra of **(a)** 200 mM of d_5 -glutamine solution (soln) in water (experimental, yellow circles; fitted, yellow solid line) and **(b)** 25 mM d_3 -lactate solution (soln) in water (experimental, green circles; fitted, green solid line).

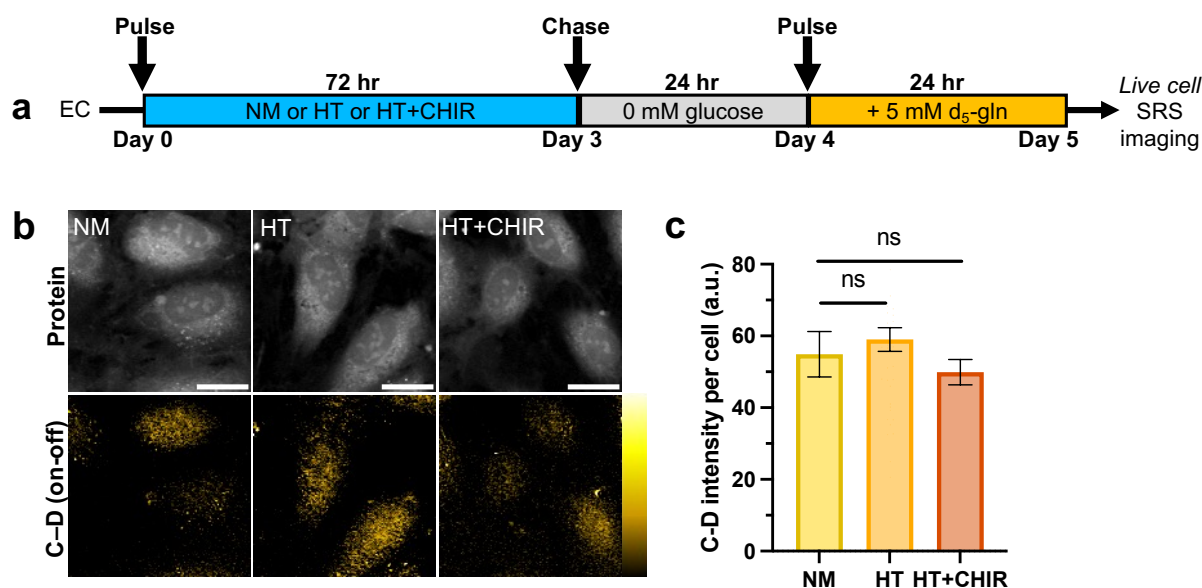


Figure S15. No differences in glutamine reliance upon glycogen depletion in ECs. (a) Illustration of pulse-chase experiment. After 72 hr of corresponding treatments, HUVECs were starved for 24 hr in glucose-free media followed by a chase with 5 mM d₅-gln in glucose-free media for 24 hr; **(b)** Representative SRS images at the Protein (–CH₃) and C–D (on-off) channels. Scale bar: 20 μm; **(c)** Quantitative analysis of SRS signal of C–D bonds per cell (n=38, 42, 39 cells for NM, HT, and HT+CHIR, respectively). Data are presented as mean ± SEM. Statistical significance was analyzed using two-tailed unpaired Student's t-tests, ns: no statistical difference.

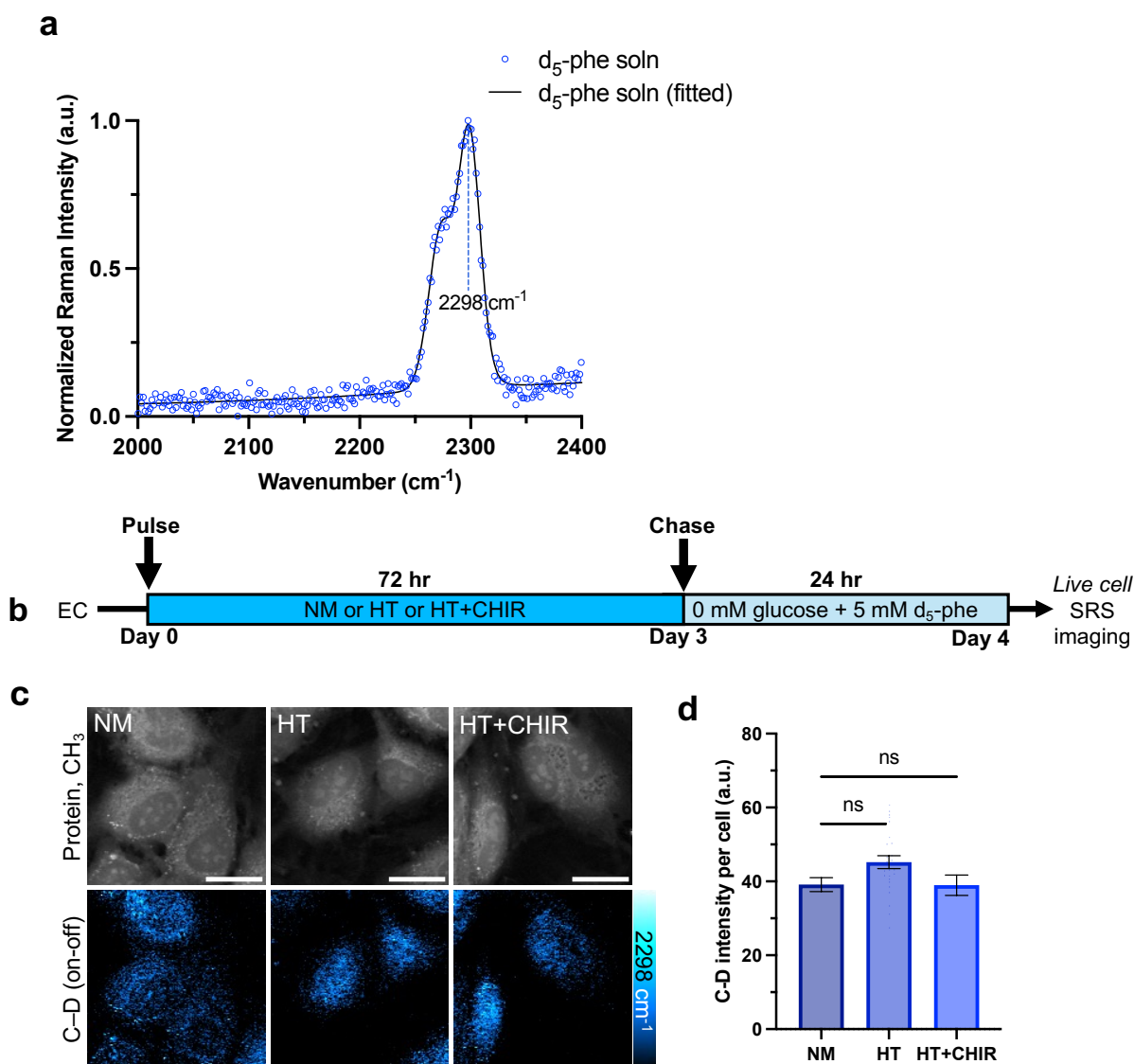


Figure S16. Presence of glycogen influences specific metabolic demands in HUVECs.

(a) Normalized spontaneous Raman spectrum of 50 mM d_5 -phenylalanine solution (soln) in water (experimental, blue circles; fitted, blue solid line); **(b)** Schematic of pulse-chase experiment with 5 mM d_5 -phenylalanine in glucose-free ECGM. **(c)** Representative SRS images at the Protein ($-\text{CH}_3$) and C-D (on-off) channels. Scale bar: 20 μm ; **(d)** Quantitative analysis of SRS signal of C-D bonds per cell ($n=33$, 25, 28 cells for NM, HT, and HT+CHIR, respectively). Data are presented as mean $\pm$ SEM. Statistical significance was analyzed using two-tailed unpaired Student's *t*-tests, ns: no statistical difference.

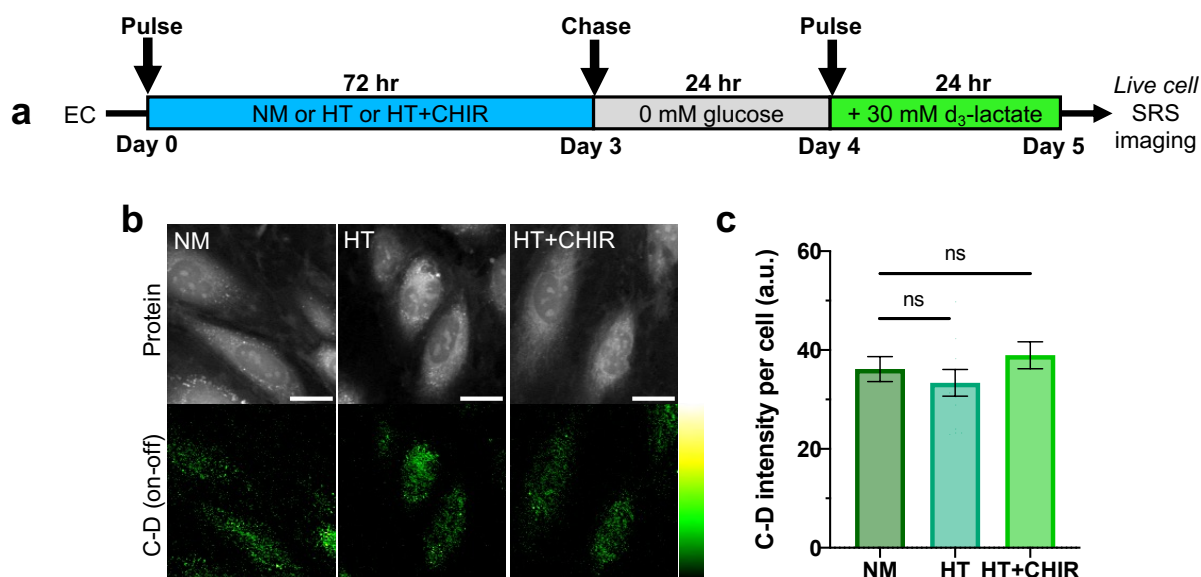


Figure S17. No differences in lactate reliance upon glycogen depletion. (a) Schematic of pulse-chase experiment. After 72 hr of corresponding treatments, HUVECs were starved for 24 hr in glucose-free media followed by a chase with 30 mM d₃-lac in glucose-free media for 24 hr; (b) Representative SRS images at the Protein (–CH₃) and C–D (on-off) channels. Scale bar: 20 μm; (c) Quantitative analysis of SRS signal of C–D bonds per cell (n=16, 14, 11 cells for NM, HT, and HT+CHIR, respectively). Data are presented as mean ± SEM. Statistical significance was analyzed using two-tailed unpaired Student's t-test, ns: no statistical difference.

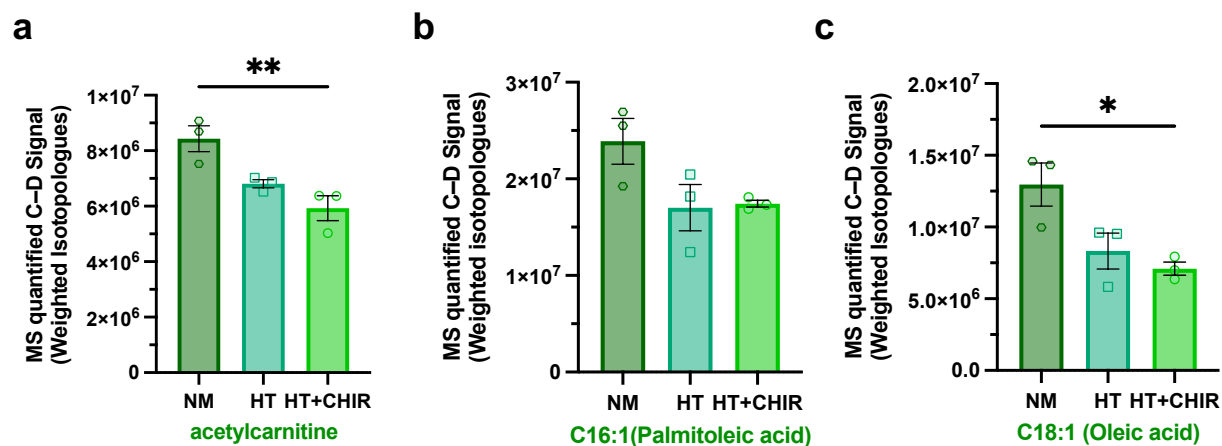


Figure S18. D₃-lactate tracing in downstream metabolites of HUVECs. MS-based quantification of C–D incorporation in **(a)** acetyl carnitine; **(b)** palmitoleic acid (C16:1); and **(c)** oleic acid (C18:1). Data are presented as mean (weighted isotopologues) ± SEM from at least three independent experiments. Statistical significance was analyzed using one-way ANOVA tests. **p<0.01, *p<0.05. n = 3 biological replicates.

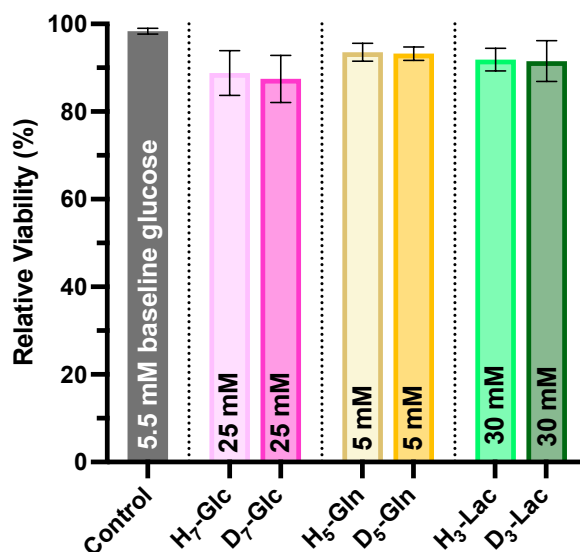


Figure S19. Cellular viability of HUVECs treated with Raman probes. Relative viability (%) of HUVECs incubated with deuterium-labeled Raman probes compared to cells with no probes: glucose (d₇- vs h₇-) for 72 hr, glutamine (d₅- vs h₅-) for 24 hr, or lactate (d₃- vs. h₃-) for 24 hr, normalized to the baseline regular glucose incubation for 24 hr (control). Data are presented as mean $\pm$ SEM. n = 3 biological replicates.

OPTICAL METABOLIC IMAGING OF THE TRICARBOXYLIC ACID CYCLE

The content of this chapter has been published in:

- (1) Chadha, R. S.[‡]; Kocheril, P. A.[‡]; Colazo, A.[‡]; Kapelczak, E. D.; Suen, B.; Yang, Z.; TeSlaa, T. A.; Wei, L. Optical Metabolic Imaging of the Tricarboxylic Acid Cycle. *bioRxiv*. **2026**, p 2026.01.28.702395. <https://doi.org/10.64898/2026.01.28.702395>.

[‡]denotes equal contribution.

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}

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

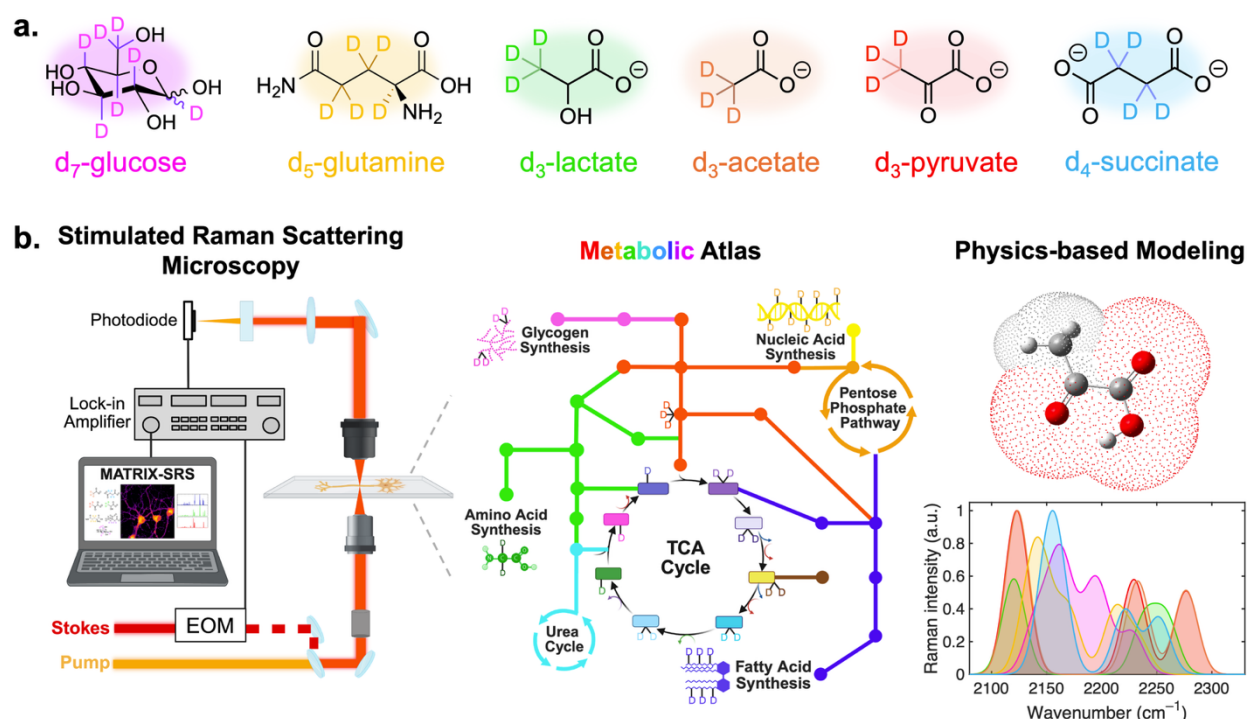


Figure 1. MATRIX-SRS workflow. (a) Chemical structures of TCA-linked Raman probes; (b) Left to right: schematic of the stimulated Raman scattering (SRS) microscopy setup; metabolic atlas tracing using deuterium-labeled probes; and physics-based modeling with density functional theory (DFT) for Raman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

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 d_3 -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

I. 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 (d₅-gln),^{22,24} d₃-lactate,²² d₃-acetate, d₃-pyruvate (d₃-pyr), and d₄-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 d₄-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**). 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, d₃-acetate and d₄-succinate imaging showed a largely homogenous distribution

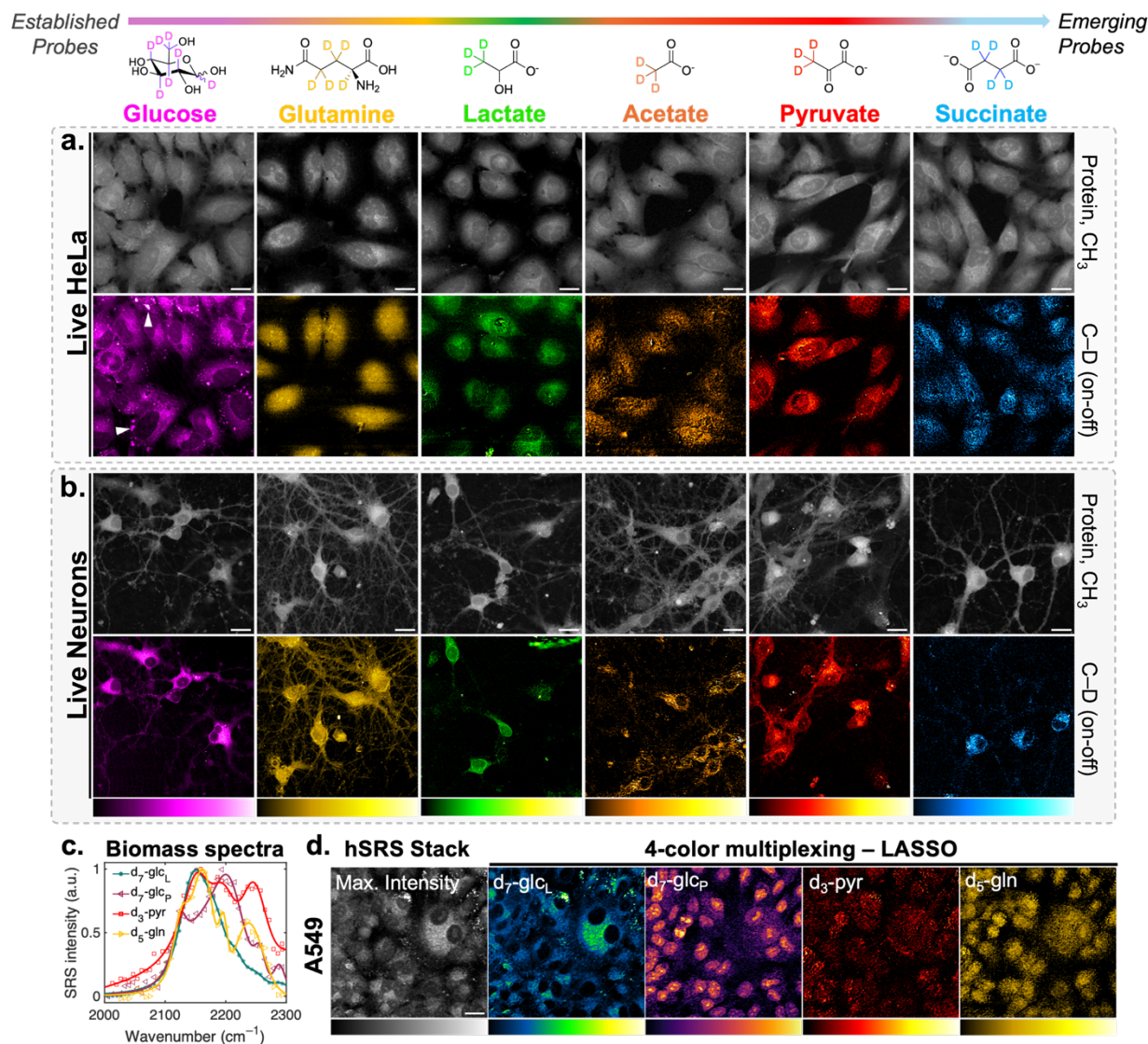


Figure 2. Live-cell subcellular metabolic imaging of TCA-linked activity using MATRIX-SRS. SRS images targeted at the -CH₃ channel (protein, 2940 cm⁻¹) and C-D (on-off) for six metabolic probes: d₇-glucose, d₅-glutamine, d₃-lactate, d₃-acetate, d₃-pyruvate and d₄-succinate in live (a) HeLa and (b) neuronal co-cultures. White arrowheads indicate subcellular glycogen pools. (c) Normalized hSRS spectra of the biomass from i) d₇-glucose-derived lipids (d₇-glc_L; blue), ii) d₇-glucose-derived proteins (d₇-glc_P; purple), iii) d₃-pyruvate (d₃-pyr; red), and iv) d₅-glutamine (d₅-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.

across the entire cell, whereas in the neuronal co-culture, C–D signal from these probes was predominantly cytoplasmic. In contrast, d₃-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.

II. 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

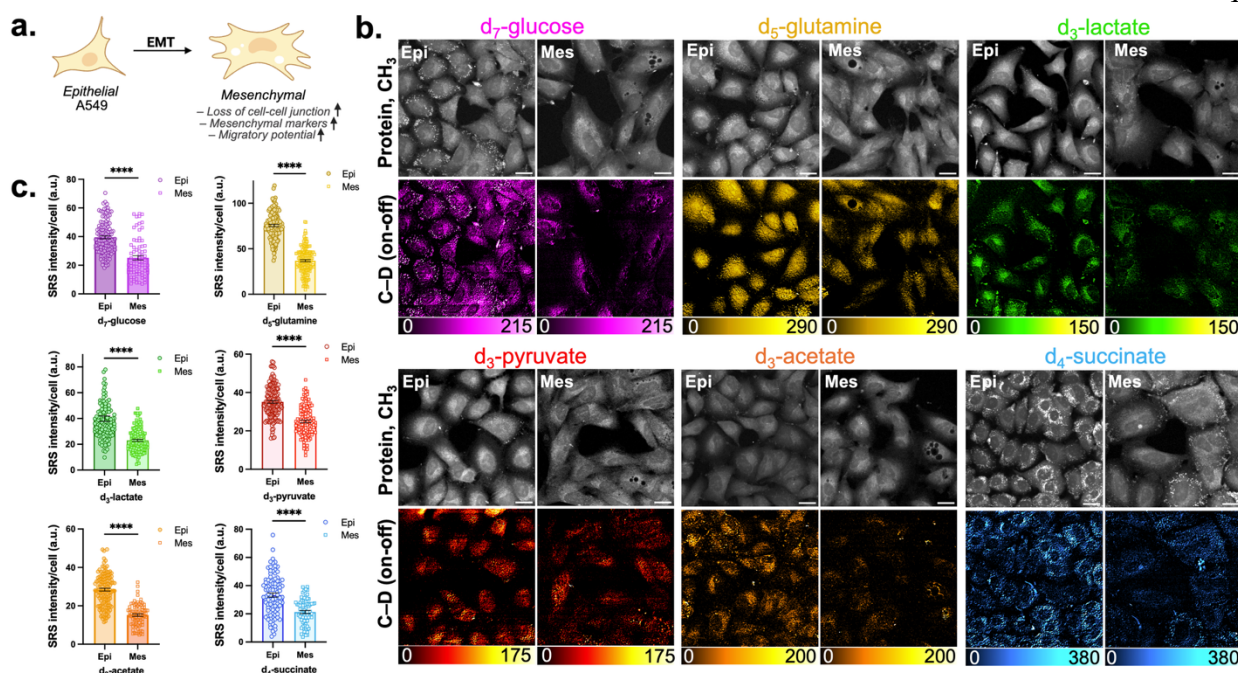


Figure 3. MATRIX-SRS for visualization of metabolic changes underlying EMT. (a)

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 $-\text{CH}_3$ 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. **** $p < 0.0001$.

MCF7 cells,²⁹ metabolic imaging of d_7 -glc in live A549 cells showed reduced glucose metabolism in mesenchymal cells compared to epithelial cells ($\sim 36\%$, **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 d_5 -gln ($\sim 51\%$), d_3 -lactate ($\sim 42\%$), d_3 -acetate ($\sim 46\%$), d_3 -pyr ($\sim 29\%$) and d_4 -succinate ($\sim 36\%$) (**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.

III. 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. Our *in vitro* LC-MS tracing data revealed that mesenchymal cells exhibit ~30% lowered intracellular d₃-pyr when compared to epithelial cells (**Fig. 4c**), showing excellent consistency with our SRS findings. More specifically, SIL tracing further indicated extensive conversion of d₃-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**).

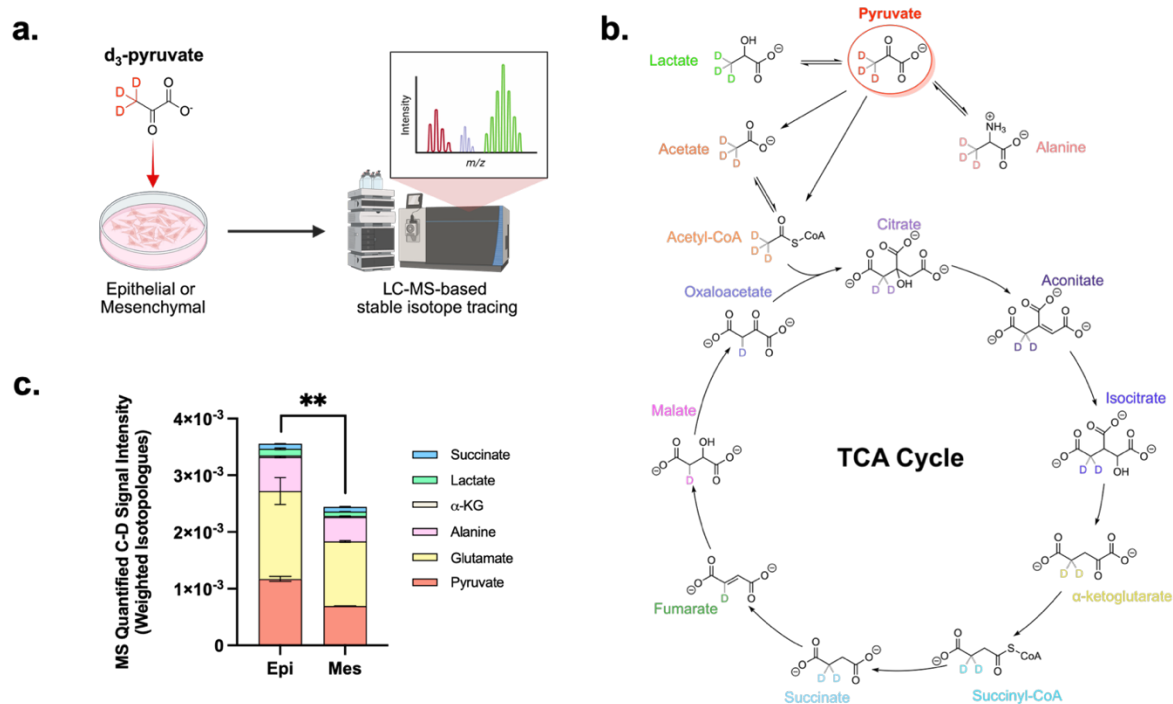


Figure 4. $[3,3',3''\text{-}^2\text{H}_3]$ -pyruvate *in vitro* tracing using LC-MS. **(a)** Illustration showing the *in vitro* tracing workflow using d_3 -pyruvate in A549 (epithelial or mesenchymal) cells; **(b)** Schematic of the flow of d_3 -pyruvate into the TCA cycle. **(c)** MS-quantified C–D incorporation (weighted isotopologues) of d_3 -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**.

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.

IV. 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.

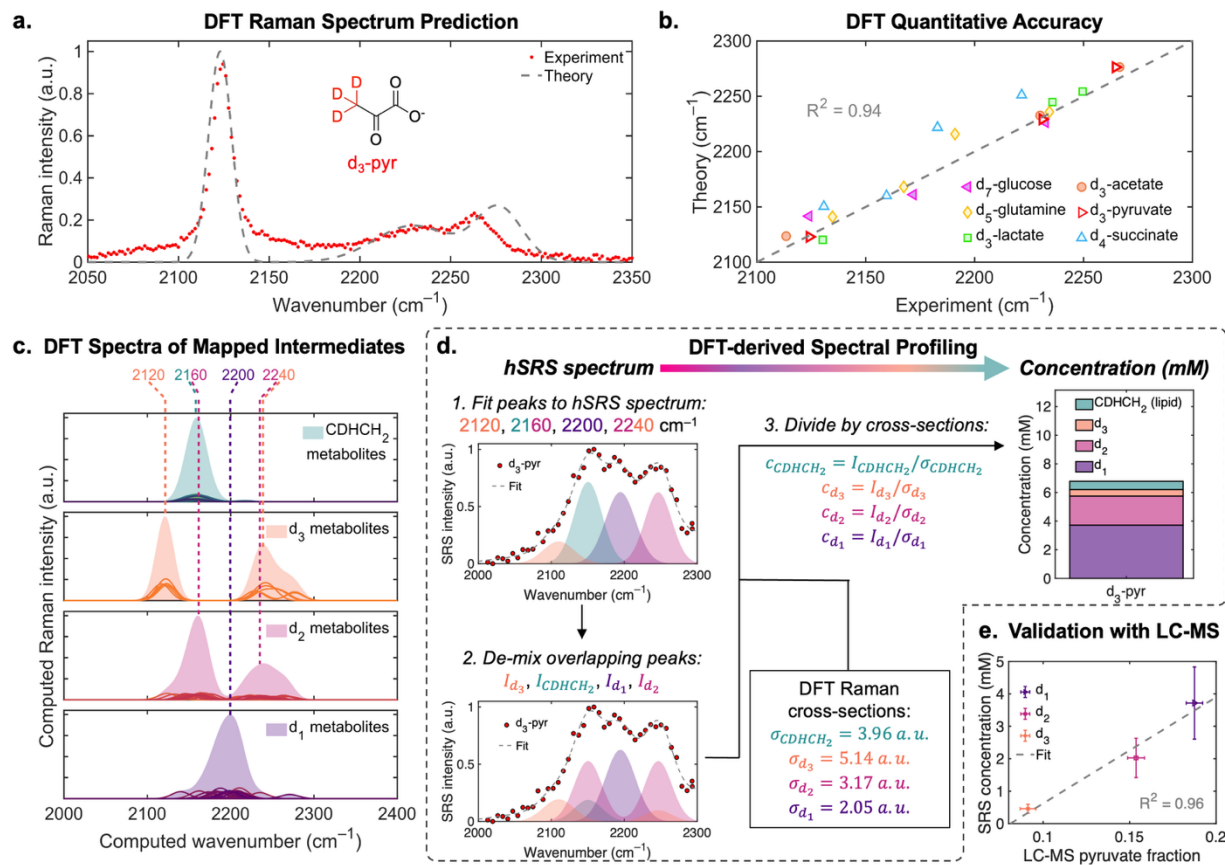


Figure 5. DFT spectral validation and compositional profiling. (a) Comparison of DFT-computed Raman spectrum for d₃-pyr and an experimental Raman spectrum of d₃-pyr in solution. (b) Comparison of calculated and experimental vibrational frequencies across all TCA probes in solution. (c) Aggregated DFT 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 ± one standard deviation for LC-MS and ± 30% (estimated DFT error; see Fig. S12) for SRS.

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 (d_1 ; **Fig. 5c**, purple) exhibit a local C–D stretching mode at $\sim 2200\text{ cm}^{-1}$. For molecules with deuterated methylenes (d_2 ; **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 (d_3 ; **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 (CDHCH_2).

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 $6.8 \pm 2.0\text{ mM}$. On a per-species basis, we find that most of the deuterium biomass is found in d_1 species ($\sim 4\text{ mM}$) with progressively less in d_2 and d_3 species (**Fig. 5d**; see also **Table S1**). Relatively little deuterium biomass ($<1\text{ mM}$) 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 d₁ species, M+2 corresponds to d₂, and M+3 corresponds to d₃. 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

d_2 species are expected to be the dominant sources of total protein biomass (as with d_7 -glcP; **Fig. S13**). Moreover, the conversion of d_3 -pyr into d_3 -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.

V. Pharmacological perturbation of d_3 -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 d_3 -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 d_3 -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 d_3 -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 d_3 -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

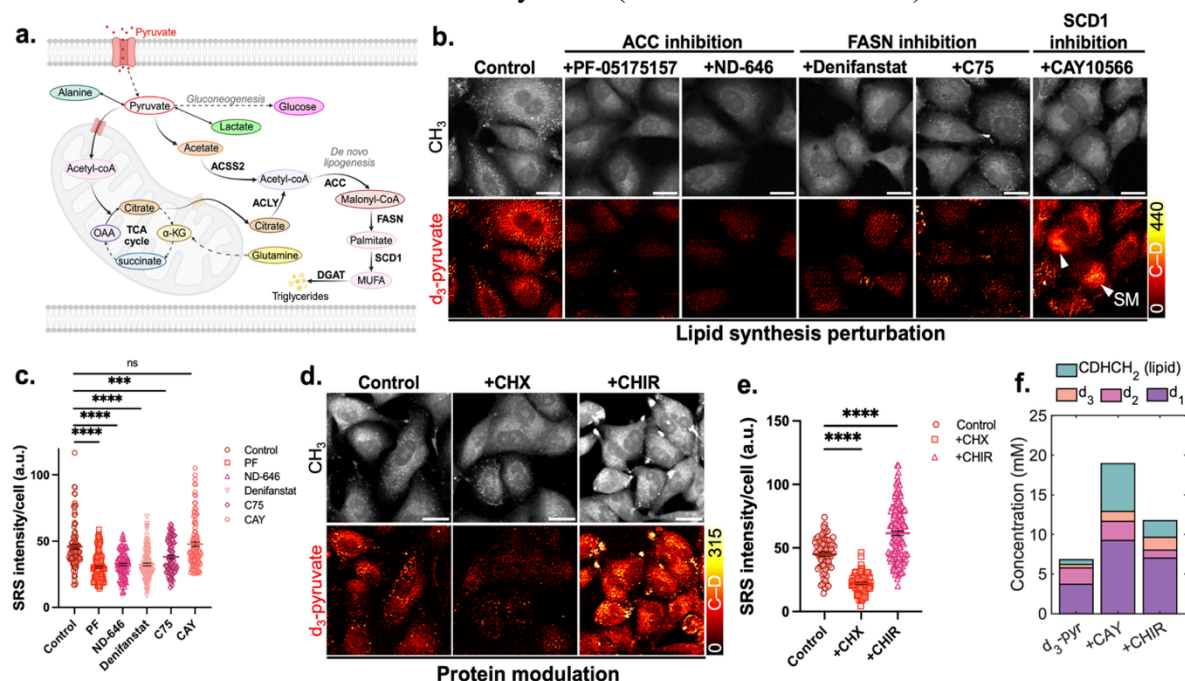


Figure 6. Pharmacological intervention of pyruvate metabolism. (a) Schematic of pyruvate metabolism in cells leading to *de novo* lipogenesis (DNL). ACC = acetyl-CoA carboxylase, ACLY = ATP-citrate lyase, ACSS2 = acyl-CoA synthetase short-chain family member 2, FASN = fatty acid synthase, SCD1 = stearoyl-CoA 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 μM CHIR-99021 inhibition; (e) Quantification of C–D intensity per cell upon protein metabolism perturbation using SMIs (from d). Data are presented as mean ± 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 d₃-pyr-derived biomass (untreated), SFA-enriched SM domains (+CAY), and cytoplasmic d₃-pyr-derived biomass following CHIR treatment. All scale bars: 20 μm.

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.

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 d₁ (purple) and d₃ (orange) metabolite concentrations compared with the d₃-pyr control (**Fig. 6f**, d₃-pyr). The increase in d₃ (orange) concentration is especially interesting and may indicate faster conversion of d₃-pyr into d₃-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 d₁ (purple), d₃ (orange, most likely from the conversion of d₃-pyr to d₃-alanine; **Fig. 4b**), and lipid (CDHCH₂, teal) deuterium, but a surprising reduction in d₂ biomass (pink) compared with the d₃-pyr control (**Fig. 6f**, d₃-pyr). We reason that the reduction in the d₂/d₁ 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 d₂ 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 and 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 1x10⁵ 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 µg mL⁻¹ poly-D-lysine (Sigma) at 37 °C for 24 h, rinsing twice with ddH₂O, and subsequently coating with 10 µg/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 μ M ETC-1002 (MedChemExpress, Cat. #HY-12357), 20 μ M SB-204490 (MedChemExpress, Cat. #HY-16450), 2 μ M BMS-303141 (MedChemExpress, Cat. #16107), 75 μ M C75 (MedChemExpress, Cat. #HY-12364), 10 μ M Denifanstat (MedChemExpress, Cat. #HY-112829), 1 μ M PF-05175157 (MedChemExpress, Cat. #HY-12942), 1 μ M ND-646 (MedChemExpress, Cat. #HY-101842), or 1 μ M CAY10566 (MedChemExpress, Cat. #HY-15823) for 48 hr concurrently with 30 mM d₃-pyruvate labeling.

For protein modulation experiments, A549 cells were treated with 1 μ M cycloheximide (CHX, Sigma-Aldrich, Cat. #C7698-1G) or 3.5 μ M CHIR99021 (MedChemExpress, Cat. #HY-10182) for 48 hr with concurrent d₃-pyruvate labeling.

SRS Microscopy

The SRS microscopy system was configured as described previously.⁵⁴ Briefly, 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 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 d₅-glutamine, 2167 cm^{-1}), 844.0 nm (C–D channel for d₇-glucose/d₃-pyruvate/d₃-

acetate/d₄-succinate/d₄-citrate, 2150 cm⁻¹), 845.6 nm (C–D channel for d₃-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 $c = \frac{SRS}{k \cdot \sigma \cdot I_{Pump} \cdot I_{Stokes}}$,

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 ($CDHCH_2$)), 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''- $^2\text{H}_3$]-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 d_3 -pyr. Following 24 hours, cell metabolism was quenched by rapidly aspirating the medium, rinsing with 150 mM NH_4OAc (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 1×10^6 . 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. References

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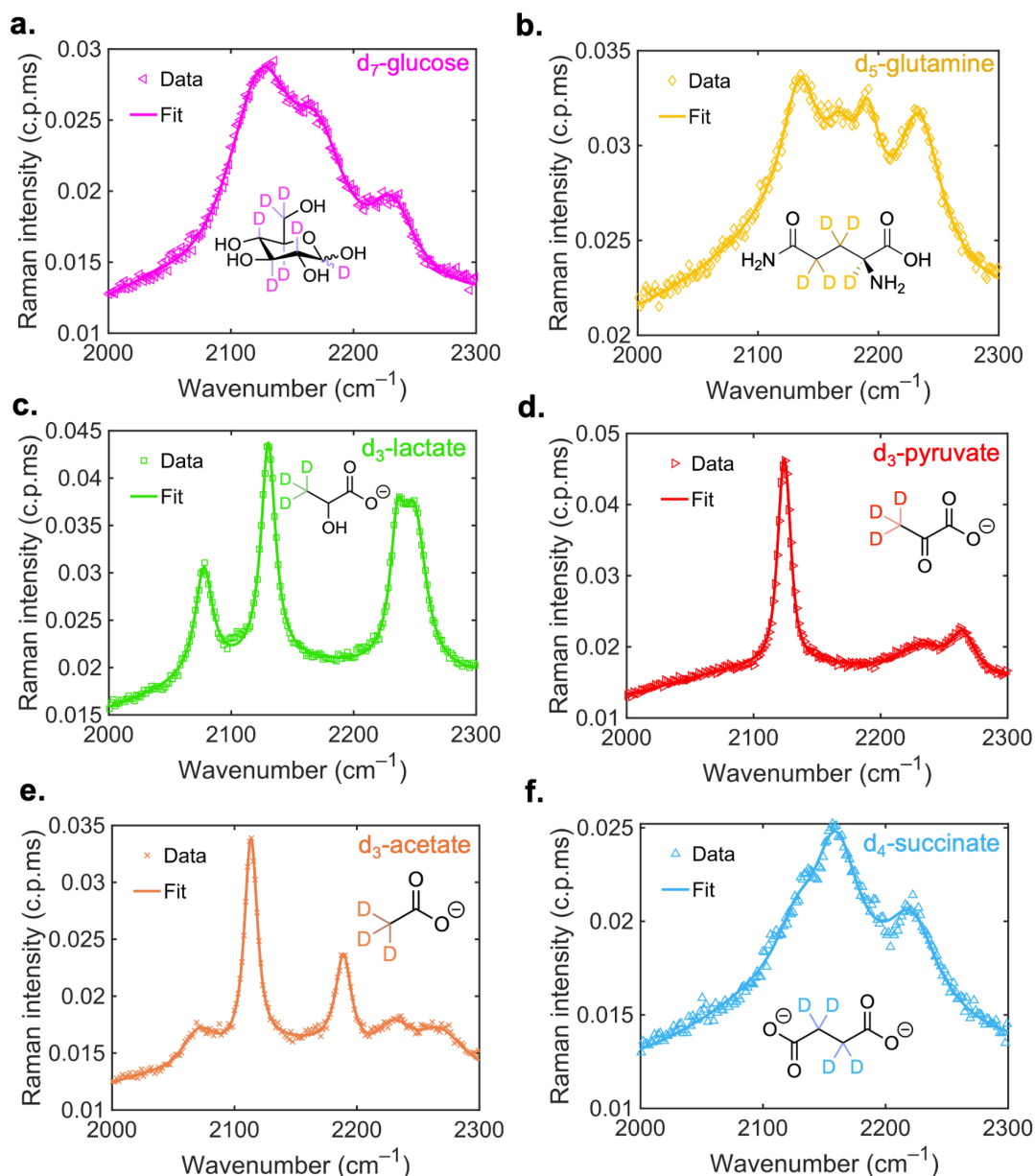
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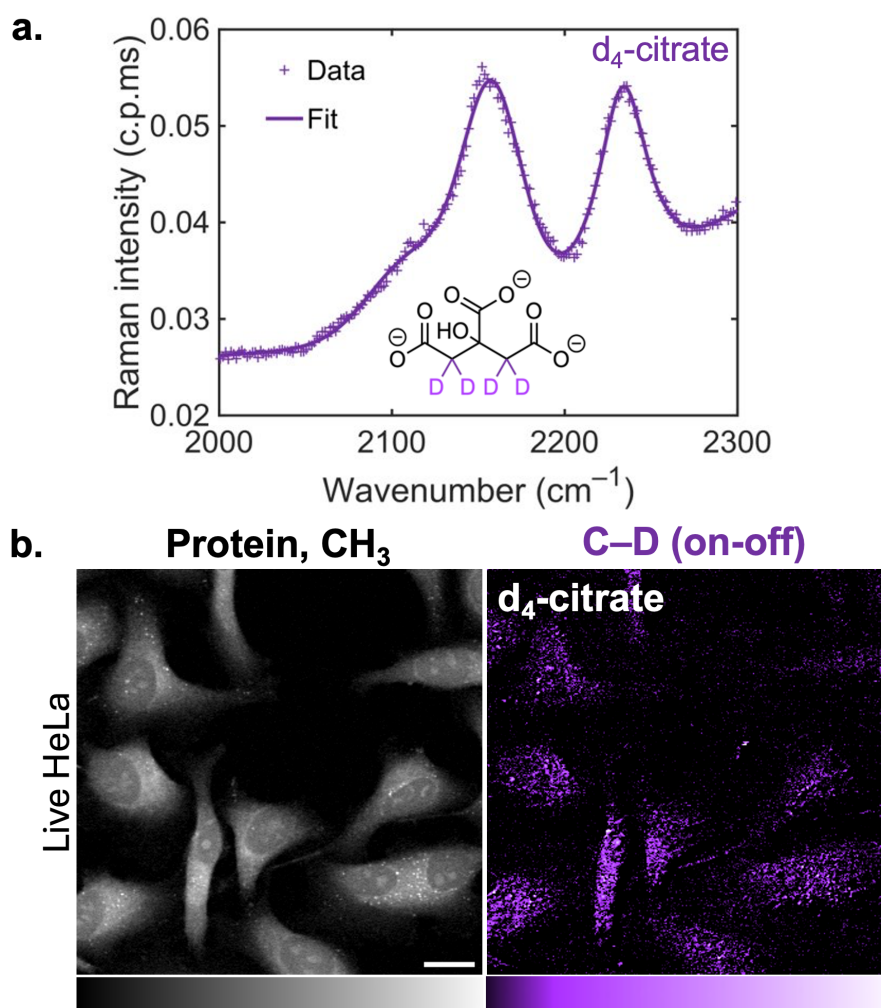
Supplementary Information

OPTICAL METABOLIC IMAGING OF THE TRICARBOXYLIC ACID CYCLE



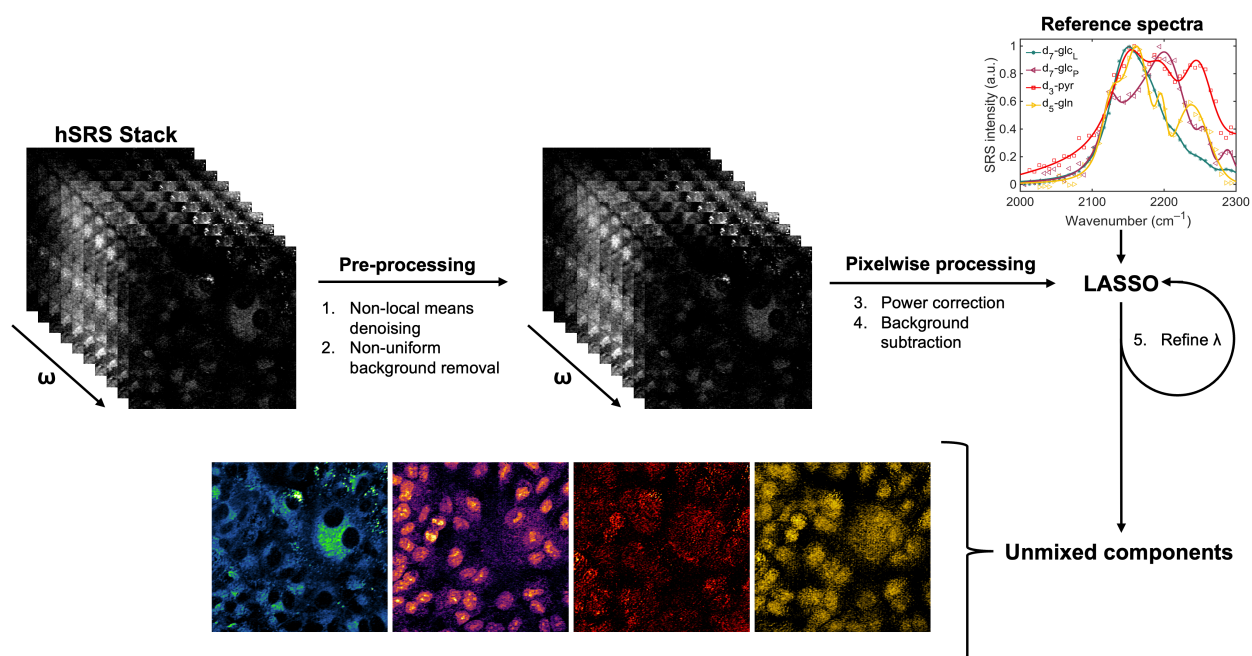
Supplementary Figure 1. Raman spectra of TCA probes.

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 d₃-lactate; 2070 cm⁻¹ and 2190 cm⁻¹ in d₃-acetate). We attribute these peaks to CD-bending combination bands, similar to previous assignments of Fermi resonances for other deuterated probes.



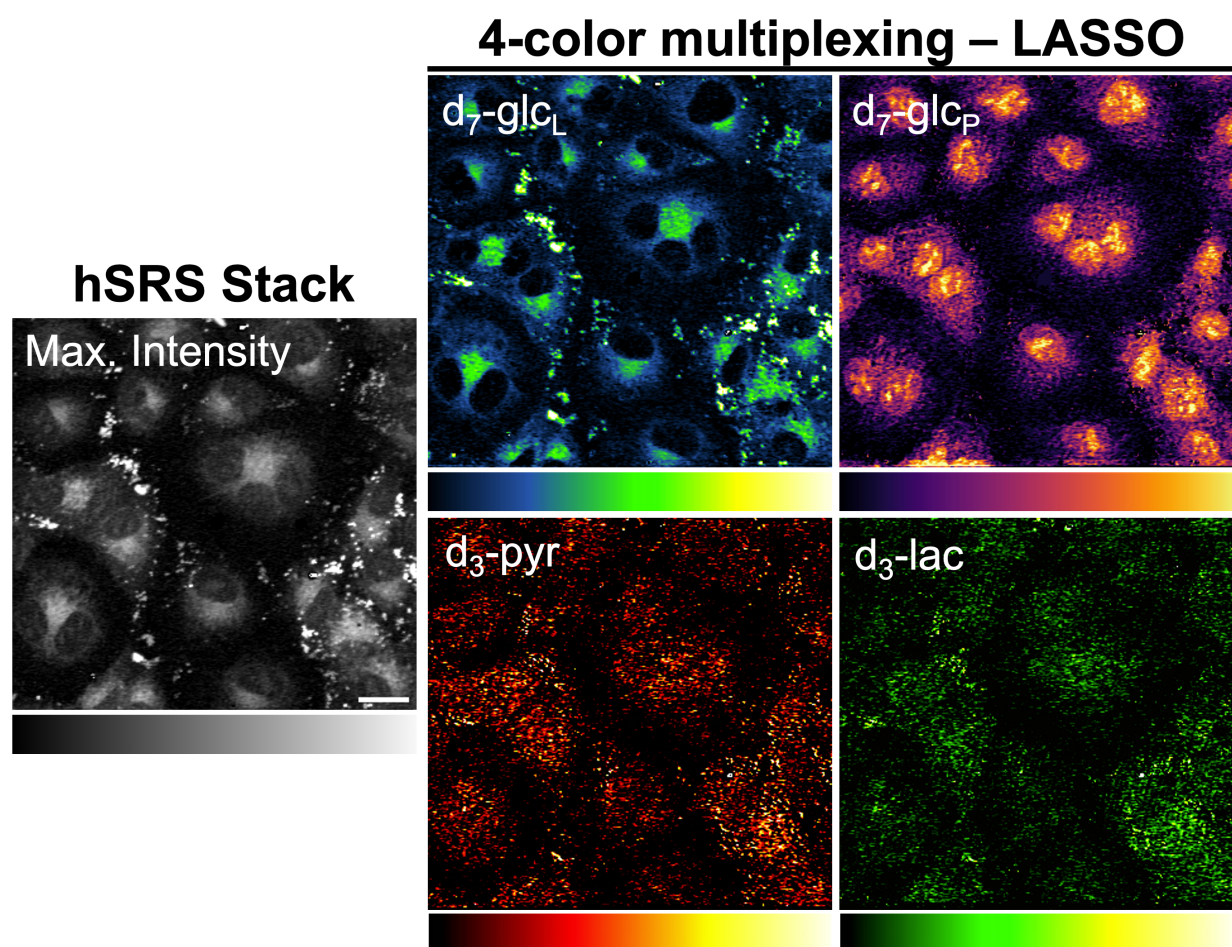
Supplementary Figure 2. Raman spectra and imaging of $\text{d}_4\text{-citrate}$.

(a) Spontaneous Raman spectrum of $\text{d}_4\text{-citrate}$ at 1 M in aqueous solution; **(b)** Live-cell SRS imaging of $\text{d}_4\text{-citrate}$ -treated HeLa cells targeted at the protein (CH_3 , 2940 cm^{-1}) and C–D (on-off) channels. Scale bar: $20\text{ }\mu\text{m}$.



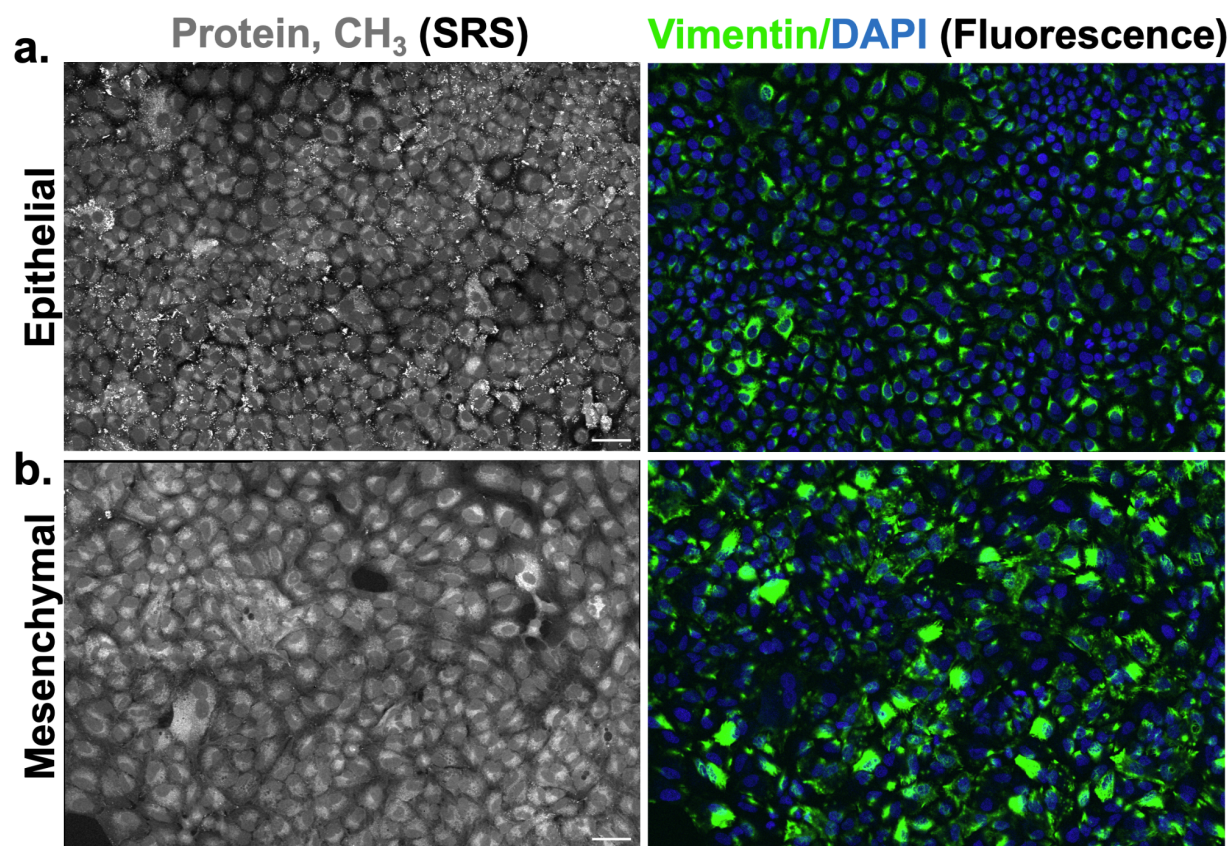
Supplementary Figure 3. LASSO unmixing workflow.

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 d₇-glucose, d₃-pyruvate, and d₃-lactate.

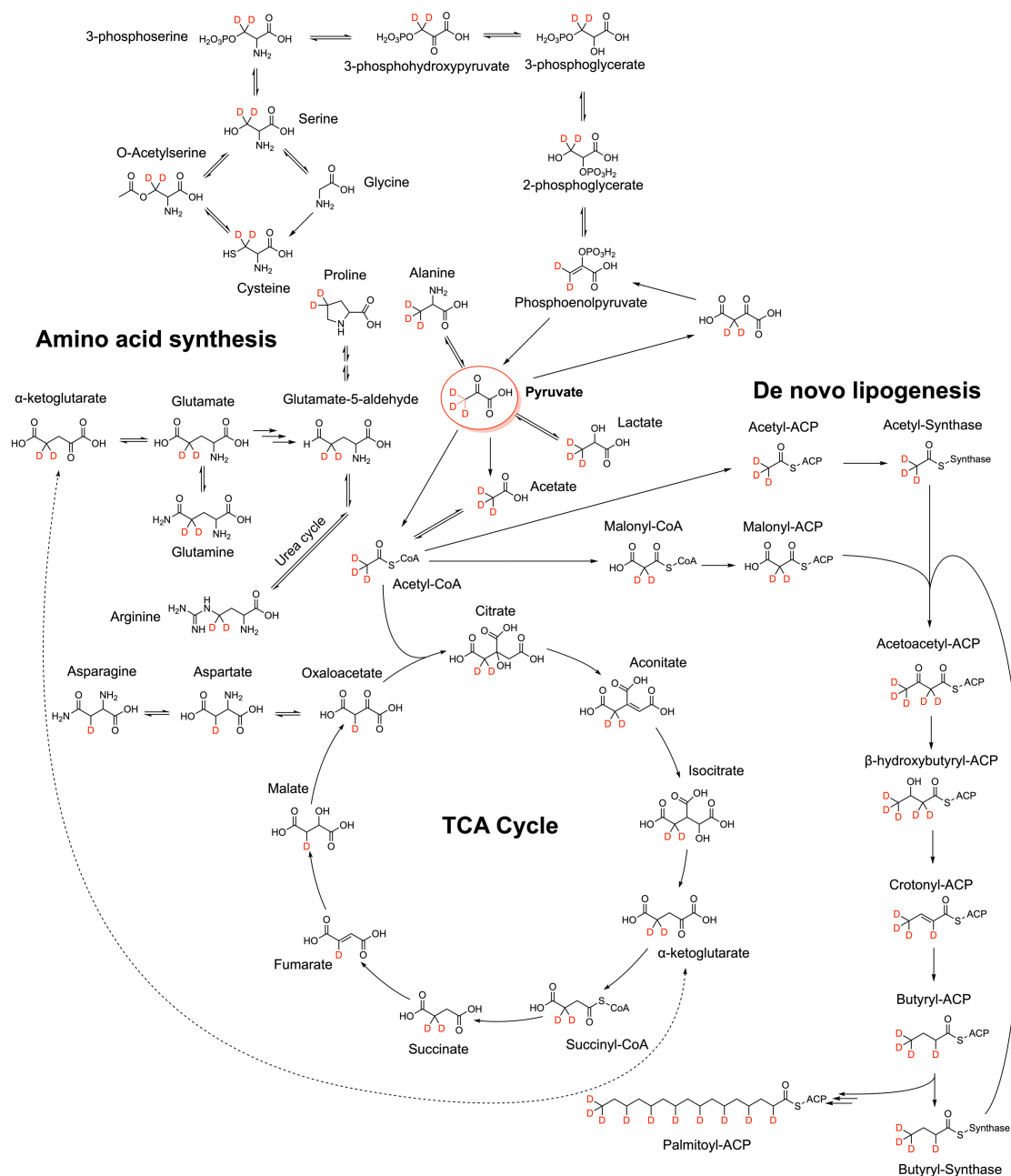
Using the same LASSO unmixing pipeline (**Supplementary Figure 3**), we again observe robust unmixing of d₇-glc_L, d₇-glc_P, d₃-pyr biomass, and d₃-lactate biomass. These results serve to demonstrate the broad compatibility of our TCA probes in multiplex imaging with validated chemical unmixing strategies. Scale bar: 20 μ m.



Supplementary Figure 5. Validation of EMT by immunostaining.

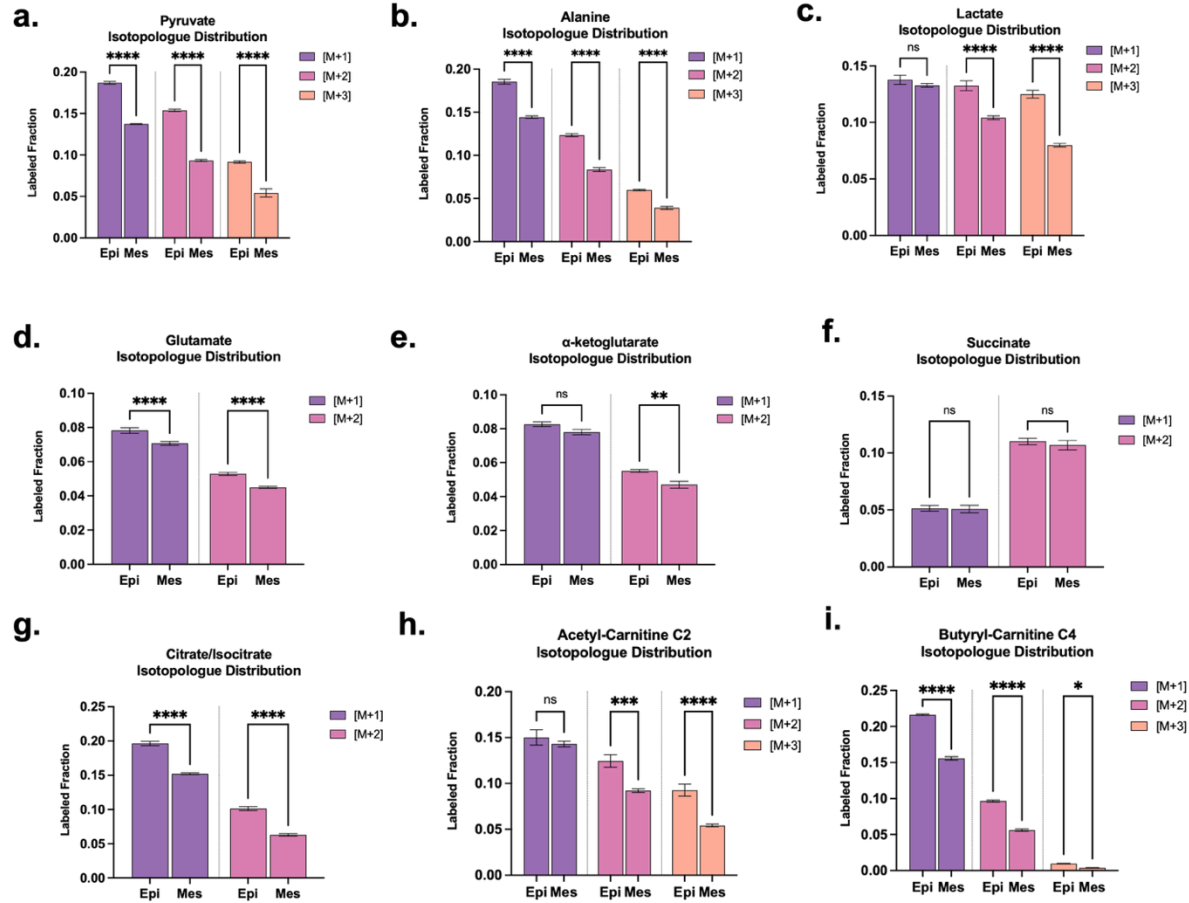
Correlative SRS (protein channel, 2940 cm^{-1}) and Vimentin/DAPI (merged)

immunostaining images for **(a)** epithelial and **(b)** mesenchymal cells. Mesenchymal cells exhibit increased vimentin expression. Scale bar: $20\text{ }\mu\text{m}$.



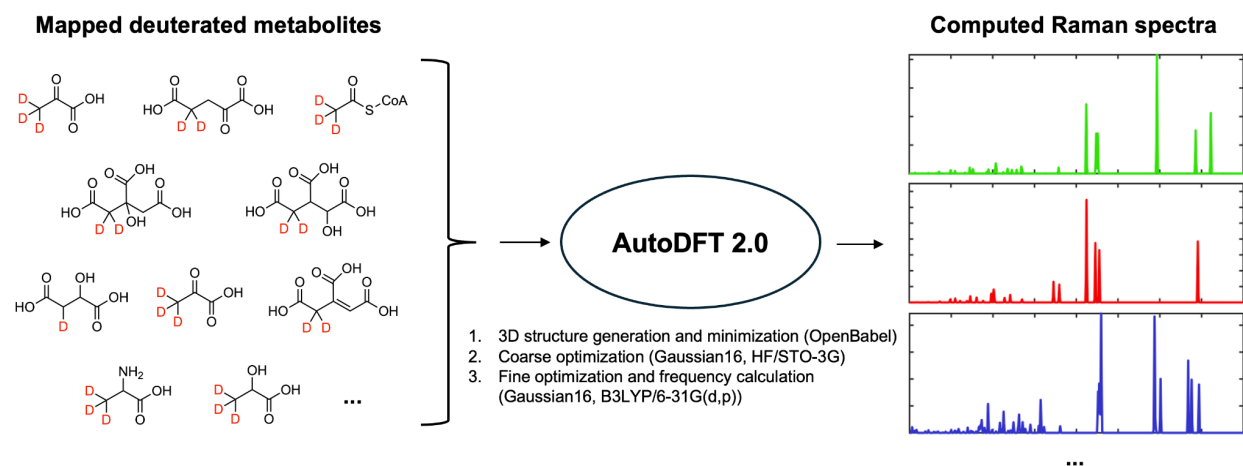
Supplementary Figure 6. Detailed metabolic reaction mapping.

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.



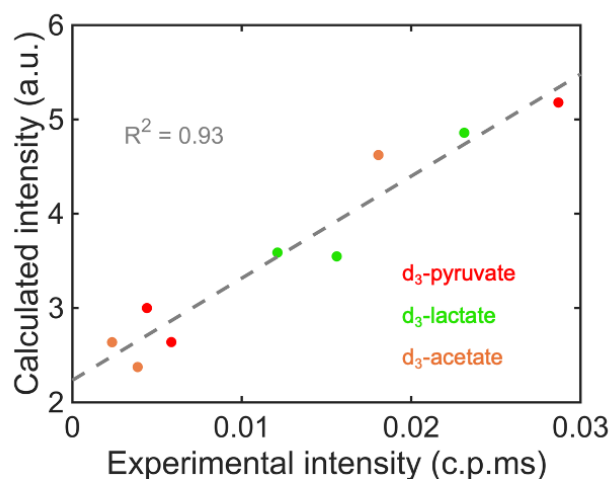
Supplementary Figure 7. LC-MS quantification of individual metabolites from d₃-pyruvate labeling.

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 $\pm$ 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.

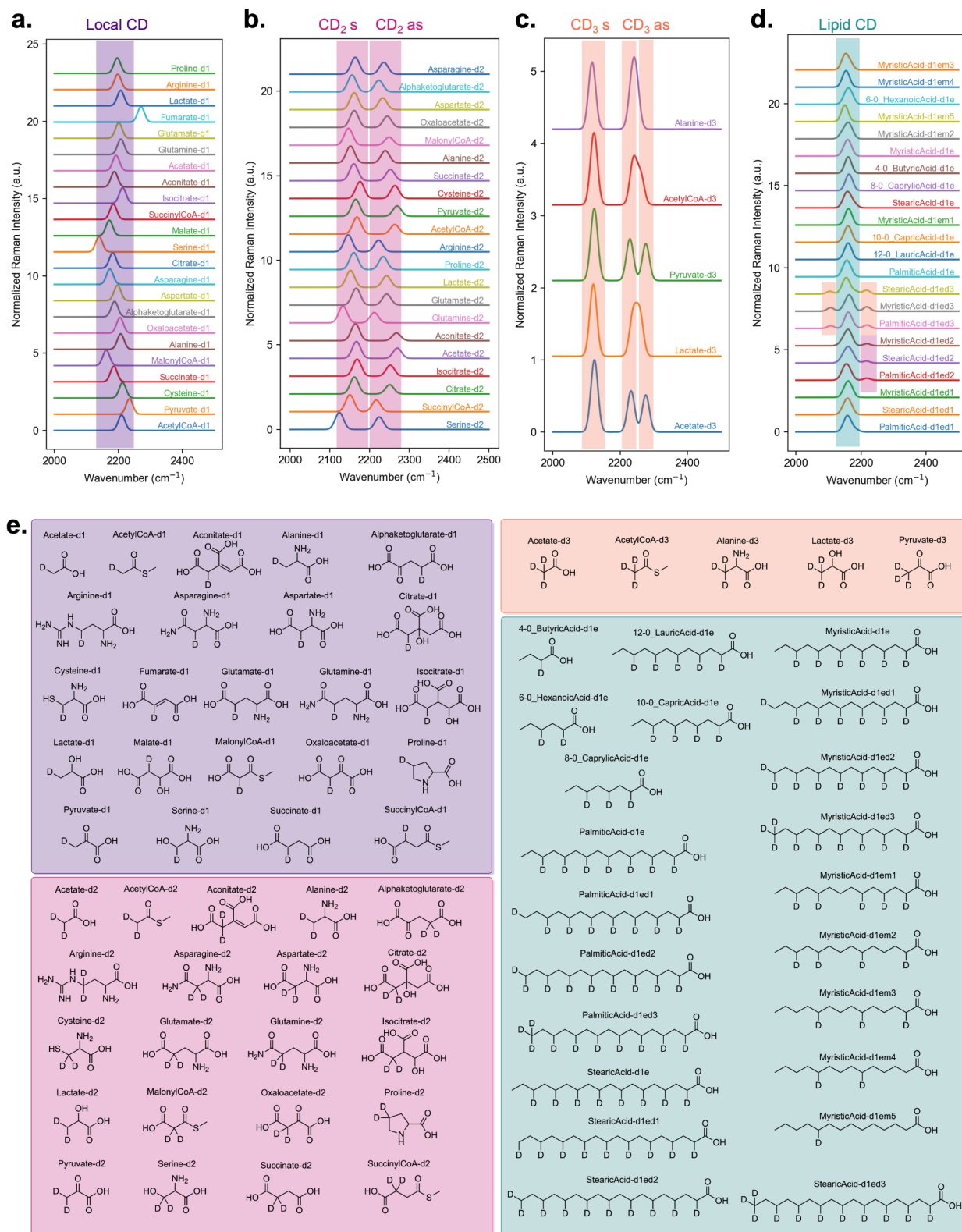
Automated density functional theory calculations were carried out with our AutoDFT computational pipeline, with AutoDFT 2.0 now introducing support for selective deuteration and computing Raman scattering activities.



Supplementary Figure 9. Validation of DFT intensities.

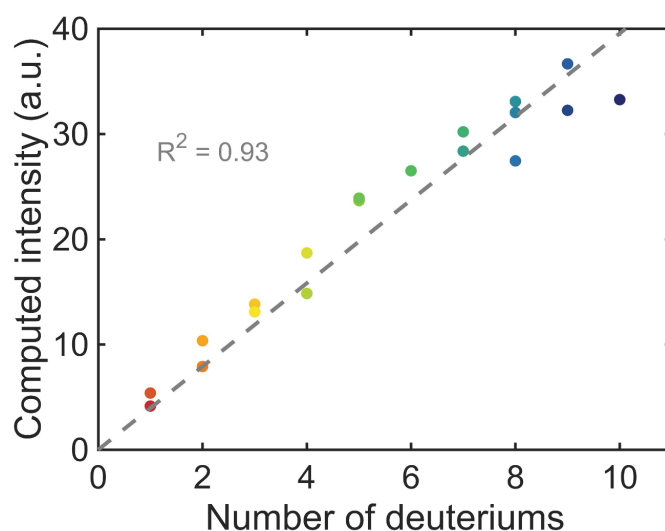
DFT-calculated Raman spectra were validated by comparison to experimental intensities, obtained by analyzing the spectra shown in **Figure S1**. Only the d₃ species were used for intensity validation because they exhibited distinct, well-separated bands that allowed confident quantitation. For the more 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).

We expect this to have a minimal impact on the quantitative analysis presented in the main text (e.g., **Fig. 5d**), since the internal consistency of DFT means that this accuracy should generalize to more than simple d₃ species. Additionally, since we apply our spectral profiling only to four well-resolved spectral bands (2120, 2160, 2200, and 2240 cm⁻¹), our quantitation of deuterated biomass is expected to be more robust.



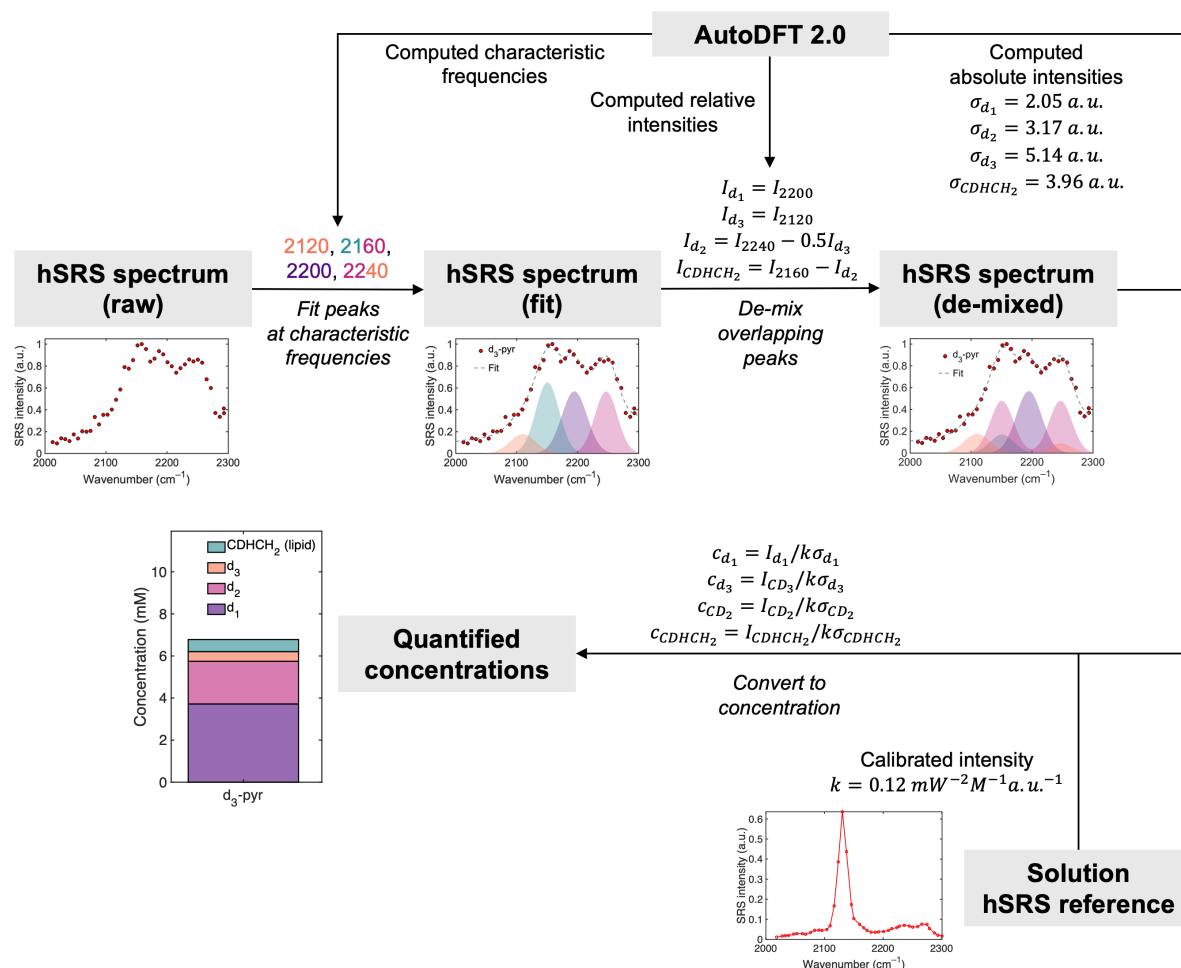
Supplementary Figure 10. DFT-computed spectra of mapped metabolites from d₃-pyr.

(a) Computed spectra of d₁ metabolites, showing a local C–D mode at $\sim 2200\text{ cm}^{-1}$ (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 $\sim 2240\text{ cm}^{-1}$, respectively (pink shaded areas); **(c)** Computed spectra of d₃ metabolites, showing a symmetric stretching band (CD₃ s) at $\sim 2120\text{ cm}^{-1}$ and two asymmetric stretching bands (CD₃ as) at ~ 2230 and $\sim 2250\text{ cm}^{-1}$ (orange shaded areas), often clustering into a single band at $\sim 2240\text{ cm}^{-1}$; **(d)** Computed spectra of aliphatic chains (CDHCH₂), displaying a coupled CD band at $\sim 2160\text{ cm}^{-1}$ (teal shaded area). For lipids with d₂ and d₃ methyl groups, the symmetric and asymmetric bands of the methyl groups are observed at the same characteristic frequencies as the d₂ and d₃ metabolites (highlighted regions in orange and pink); **(e)** Chemical structures of all calculated metabolites, grouped by deuteration (d₁, purple; d₂, pink; d₃, orange; fatty acids, teal). CoA units were truncated and replaced by methyl substituents. All DFT calculations were performed on neutral molecules (as drawn). Mapped metabolites are named by their deuteration status, broadly grouped as d₁, d₂, d₃, and aliphatic chains. The aliphatic chains all exhibit the (CDHCH₂)_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.

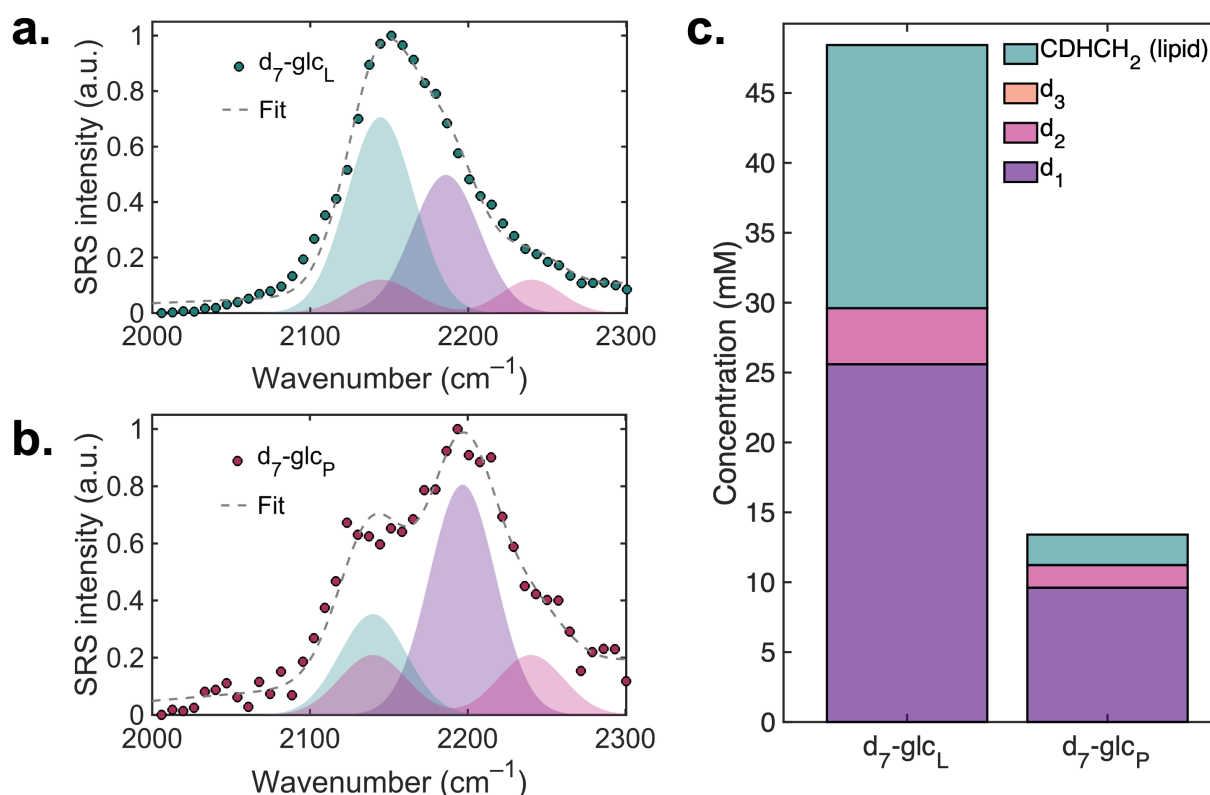
The calculated Raman scattering cross-sections for mapped lipids (CDHCH_2) are strongly linearly correlated with the number of deuteriums in the lipid, allowing us to quantify total deuterium in aliphatic chains (at 2160 cm^{-1}) on a per-deuterium basis.



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 d₃ species at 2120 and 2240 cm^{-1} and a 1:1 ratio for d₂ 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 d₃-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.

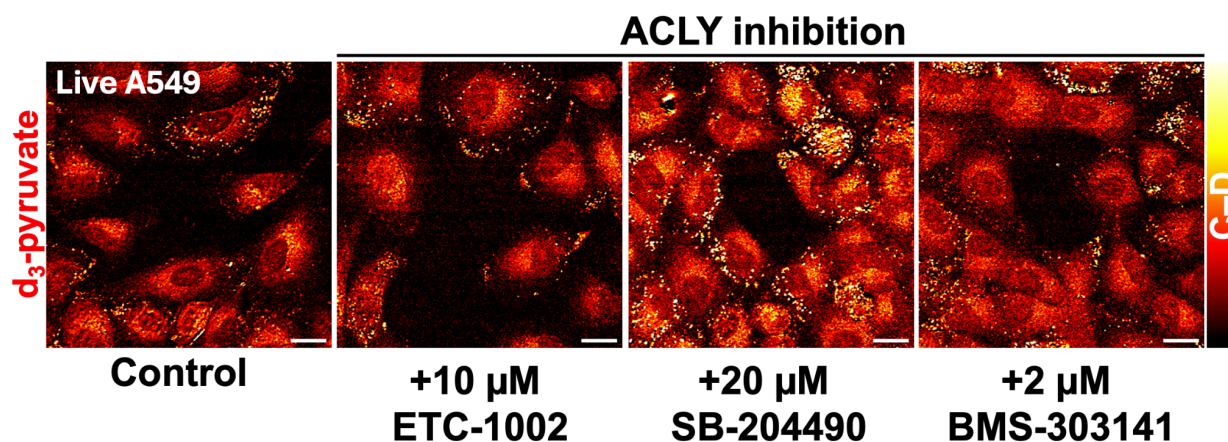


Supplementary Figure 13. SRS quantification of d₇-glucose-derived biomass.

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.

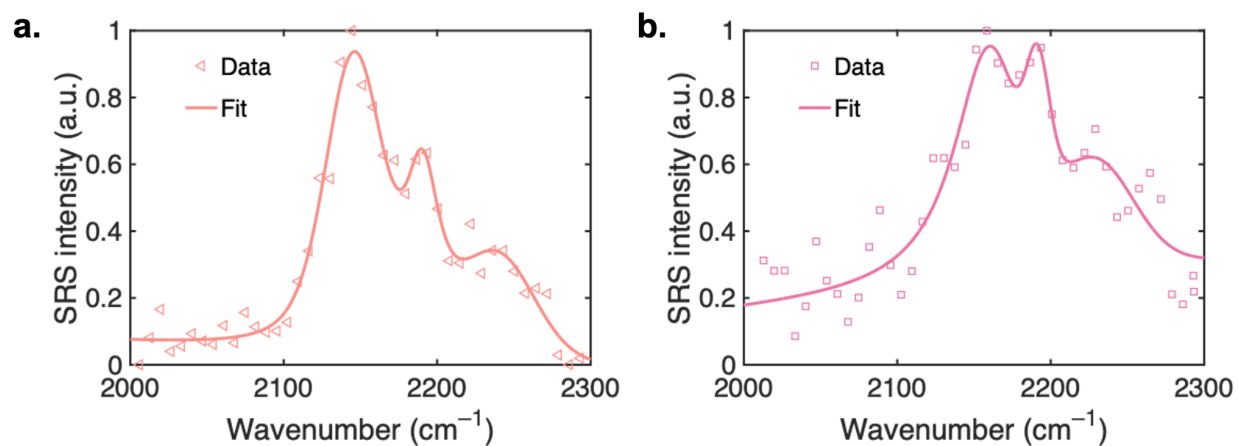
With our DFT-derived spectral profiling, we can additionally examine the distributions of d₁, d₂, d₃ and lipid (CDHCH₂) deuterium in each of the spectra. Both d₇-

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.

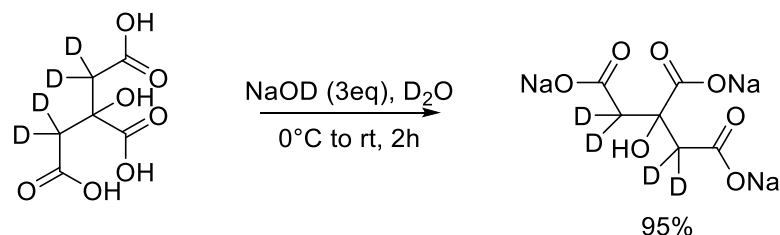
Supplementary Tables

Supplementary Table 1. Quantified concentrations from biomass spectra.

Conc. (mM)	d ₁	d ₂	d ₃	CDHCH ₂	Total
d₇-glc_L	25.6 ± 7.7	4.0 ± 1.2	0	18.8 ± 5.6	48.4 ± 14.5
d₇-glc_P	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

Supplementary Materials & Methods

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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METABORAMICS: HIGHLY MULTIPLEXED METABOLIC IMAGING BY STIMULATED RAMAN FOR SPATIAL METABOLOMICS IN LIVE CELLS

The content of this chapter is currently under preparation for submission:

Rahuljeet S. Chadha[‡], Adrian Colazo[‡], Joseph A. Ambarian, Ziguang Yang, Philip A. Kocheril, Benjamin Yang, and Lu Wei*. MetaboRamics: Highly Multiplexed Metabolic Imaging by Stimulated Raman for Spatial Metabolomics in Live Cells. *bioRxiv*. May 26, 2026, p 2026.05.21.727012. <https://doi.org/10.64898/2026.05.21.727012>.

[‡]denotes equal contribution.

5.1. Summary

Cellular metabolism is highly dynamic, intertwined and spatially heterogenous, 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 MetaboRomics 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.

5.2. 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 bioorthogonal 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 ($\sim 10\text{ cm}^{-1}$) 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**).

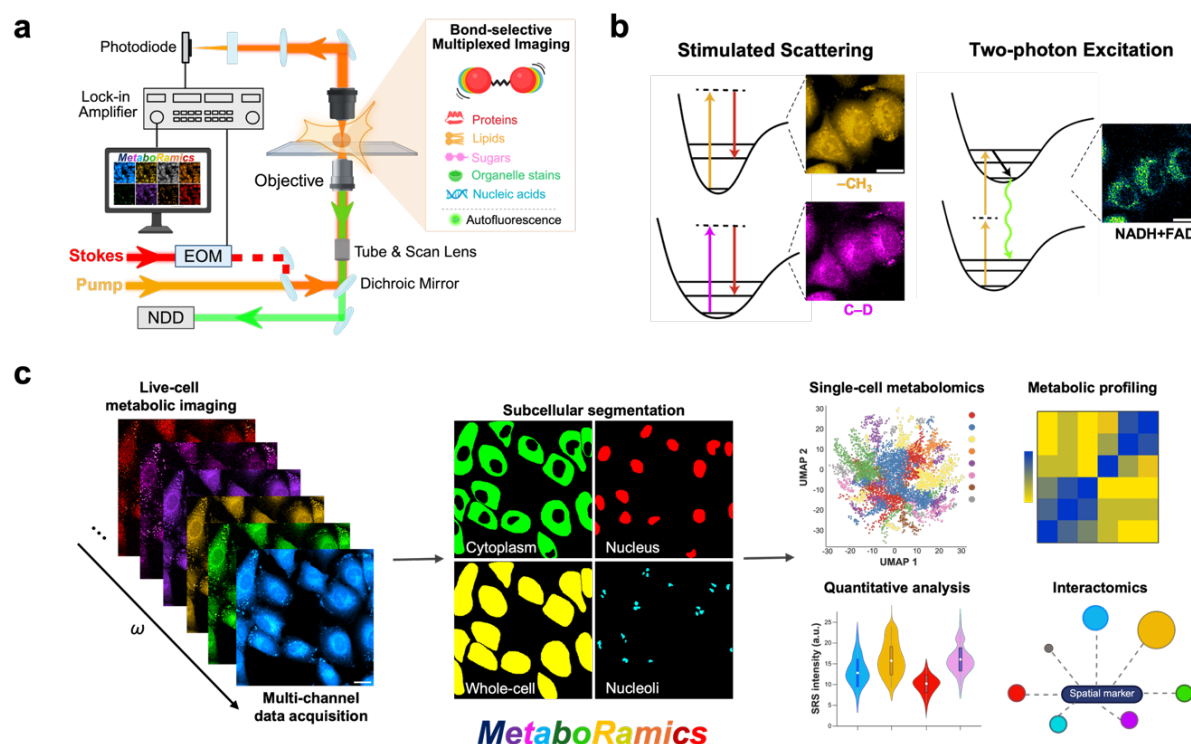


Figure 1. MetaboRamics workflow. (a) SRS microscopy setup for bond-selective multiplexed imaging. EOM = electro-optic modulator, NDD= non-descanned detector; (b) Energy diagram for stimulated Raman scattering (left) and two-photon excitation (right); (c) Workflow for spatial metabolomics using MetaboRamics.

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.

5.3. Results

I. 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., $d_7\text{-Glc}$) 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

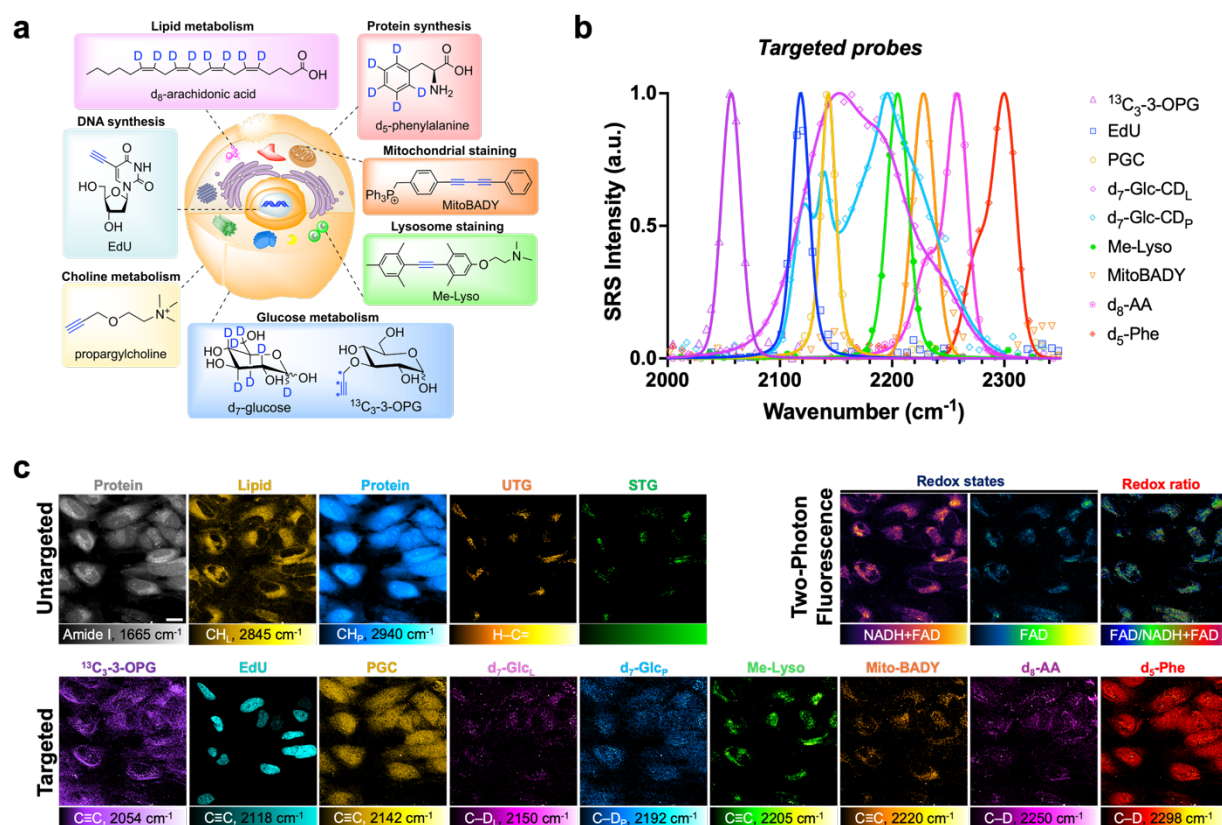


Figure 2. 16-plex metabolic imaging using MetaboRamics in live cells. (a) Metabolic probe palette encompassing a broad range of metabolites for targeted SRS imaging. **(b)** SRS spectra of incorporated Raman probes from live cells; **(c)** Representative 16-plex metabolic images from live HeLa cells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

We further adopted a monitoring strategy for glucose uptake and metabolism, by synthesizing an isotopologue of an alkyne-tagged glucose analogue, $^{13}\text{C}_3\text{-3-OPG}$ (2054 cm^{-1} , **Fig. 2a-b**, purple),²⁶ to primarily visualize glucose uptake, while in parallel using $\text{d}_7\text{-glucose}$ ($\text{d}_7\text{-Glc}$) to track its metabolic incorporation into downstream biomass, including lipids ($\text{d}_7\text{-Glc}_L$, 2150 cm^{-1}) through *de novo* lipogenesis (DNL), and proteins ($\text{d}_7\text{-Glc}_P$, 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**).

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 (C=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 ($=\text{C-H}$, 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 (CH_2) 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.

II. 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 (CHP, 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 (log₂FC) 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**).

Surprisingly, label-free lipid channel images (CH_L; **Fig. 3a,c** and **Fig. S7**) revealed a marked decrease (~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**).

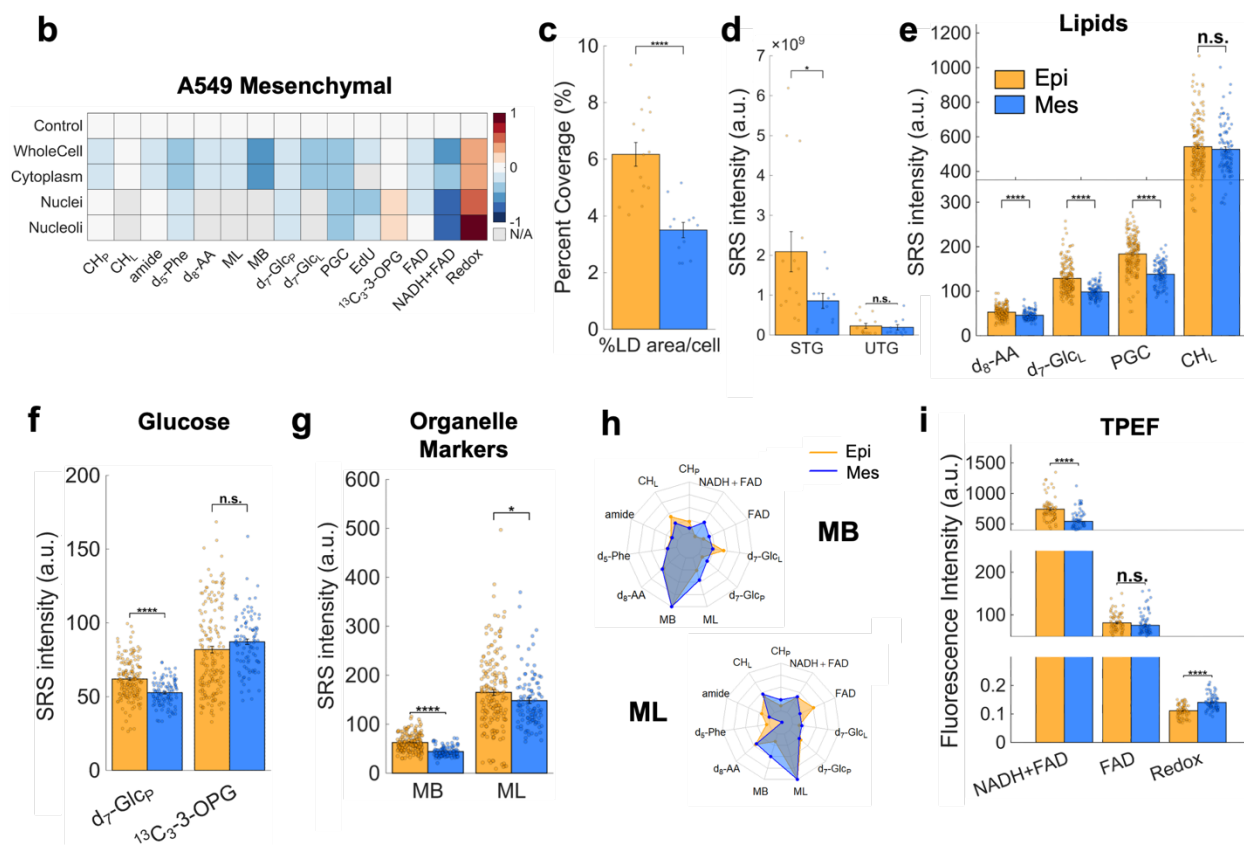
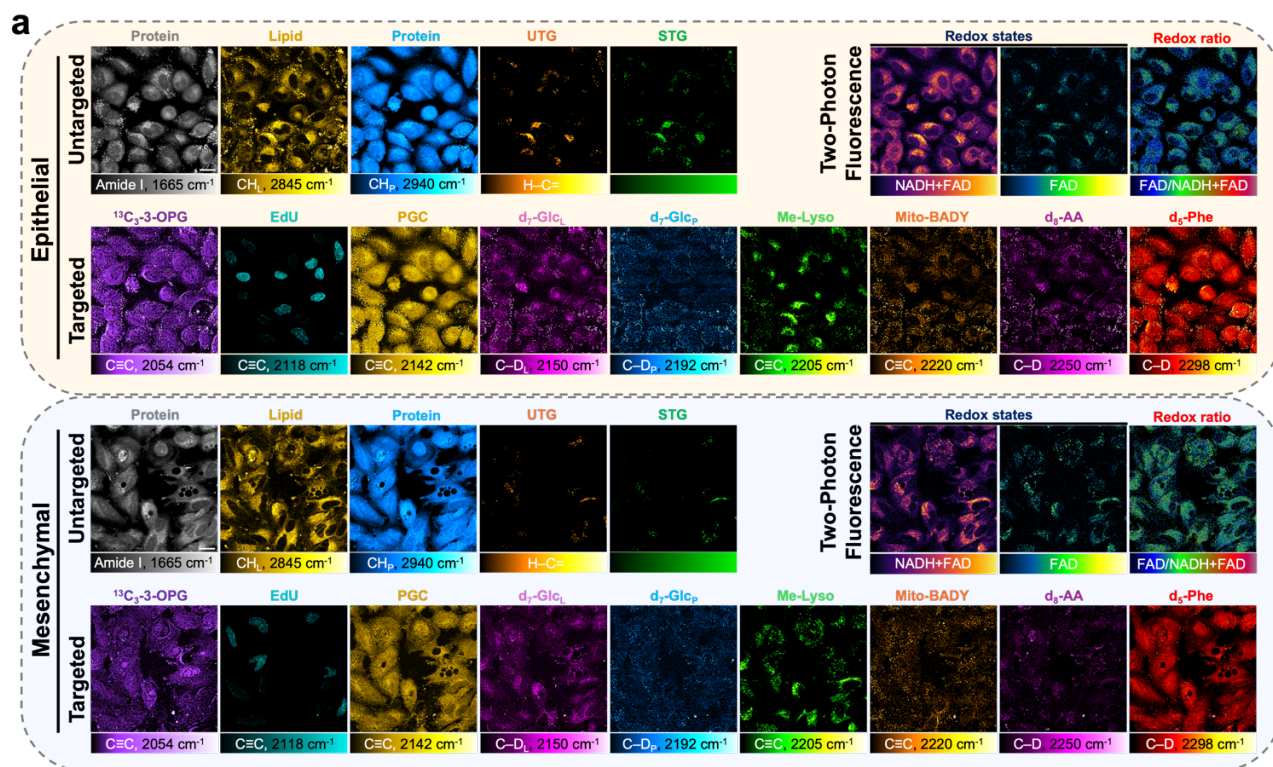


Figure 3. MetaboRamics Reveals Metabolic Reprogramming in EMT. (a) Representative 16-plex metabolic images using MetaboRamics in live A549 epithelial (top) and mesenchymal (bottom) cells. Scale bar: 20 μm ; (b) Heatmap depicting $\log_2$ fold changes between epithelial and mesenchymal cells profiled 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 x area) per FOV of saturated triglycerides (STG) and unsaturated triglycerides (UTG); (e) Quantification of SRS intensity per cell for CH_L (endogenous total lipids), $\text{d}_8\text{-AA}$ (lipid uptake), $\text{d}_7\text{-Glc}_\text{L}$ (*de novo* lipogenesis) and PGC (choline metabolism) for EMT; (f) Subcellular quantification of SRS intensity per cell for $\text{d}_7\text{-Glc}_\text{P}$ (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.

Our further analysis revealed decreased DNL (i.e. lower $\text{d}_7\text{-Glc}_\text{L}$ signals) and PUFA uptake and metabolism (i.e. lower $\text{d}_8\text{-AA}$ 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 ($\text{d}_7\text{-Glc}_\text{L}$ and $\text{d}_7\text{-Glc}_\text{P}$, respectively) is reduced in mes cells (**Fig. 3b, e, f**),

whereas glucose uptake ($^{13}\text{C}_3\text{-3-OPG}$) 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 $^{13}\text{C}_3\text{-3-OPG}$ 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 ($\text{d}_5\text{-Phe}$), whereas mitochondria preferentially coupled to lipid-related signals (CH_L and $\text{d}_7\text{-Glc}_\text{L}$) (**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.

III. 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

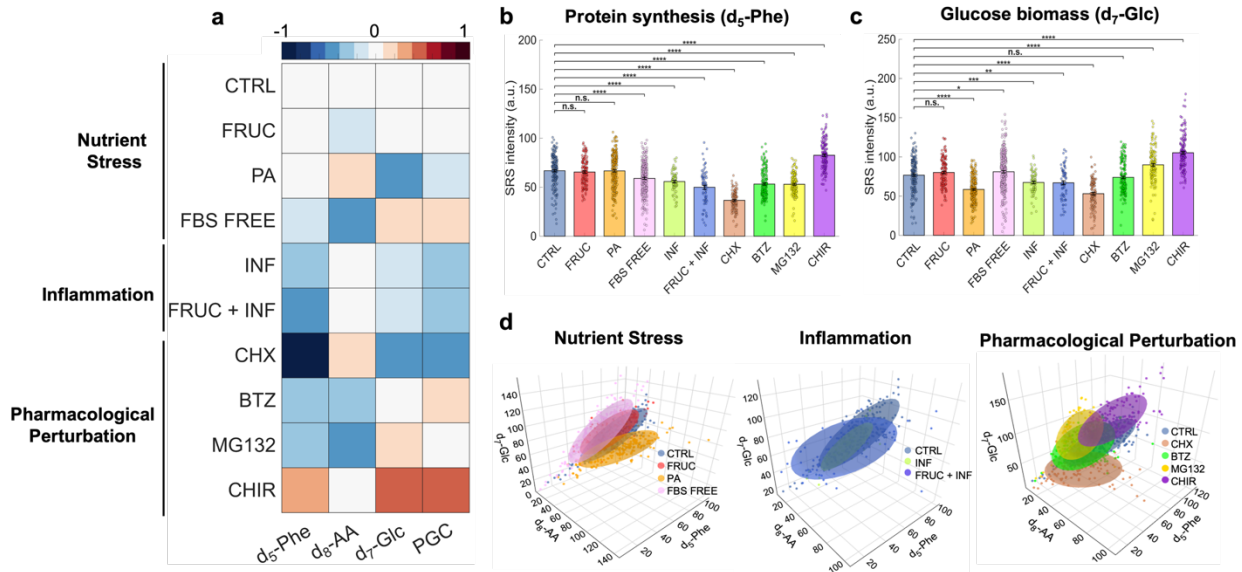


Figure 4. High-content Metabolic Phenotyping of Stress-induced Cellular Responses.

(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.

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**).

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.

IV. 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).

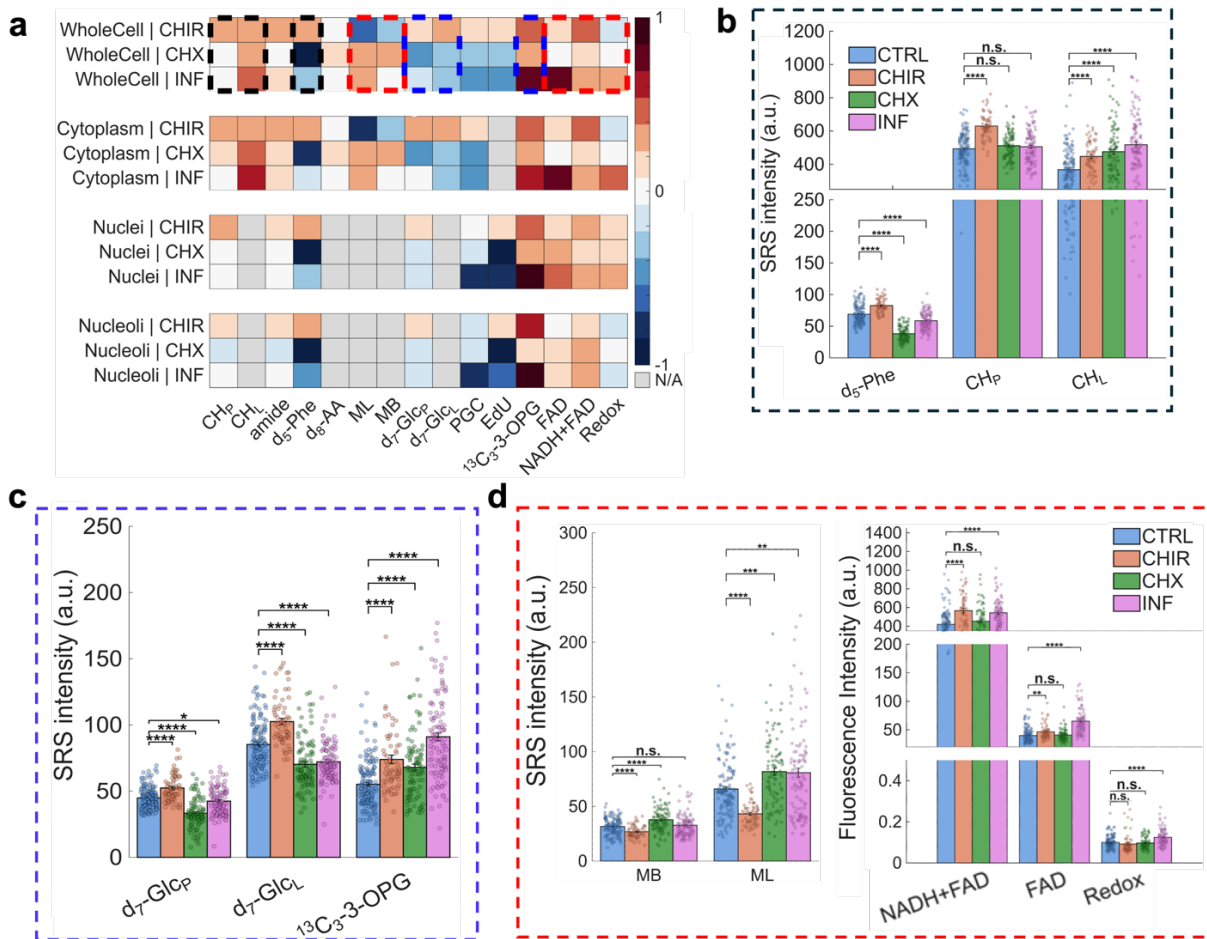


Figure 5. 16-plex Mapping of Cellular Stress Responses Using MetaboRamics. (a) Heatmap from 16-plex metabolic imaging reflecting log₂ fold changes between the A549 control and cells treated with CHIR, CHX, or INF at the subcellular level (whole-cell, cytoplasm, nuclei and nucleoli); (b) Quantification 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 ± 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.

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.

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 d₅-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 d₅-Phe metabolic signals, the increase in the label-free CHP 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 ($^{13}\text{C}_3$ -3-OPG signal) remained unchanged despite reduced glucose metabolism (decreased $\text{d}_7\text{-Glc}_\text{P}$ and $\text{d}_7\text{-Glc}_\text{L}$). 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 ($\text{FAD} / (\text{NADH} + \text{FAD})$) 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 ($\text{d}_7\text{-Glc}_\text{L}$ and $\text{d}_7\text{-Glc}_\text{P}$). 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.

5.4. 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 sequencing¹⁸, 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.

5.5. Methods & Materials

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-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 μM 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 $^{13}\text{C}_3$ -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 d_5 -Phe (400 μM , 12 days) were acquired in live HeLa cells incubated with the respective probes, whereas hSRS stacks for d_8 -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 d_7 -glucose (25mM, 3 days), hSRS images of d_7 -glucose-derived lipid (CD_L) were acquired in live A549 cells. For d_7 -glucose-derived protein (CD_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 x 9 or 4 x 4 depending on the sample treatment (**Fig. S5**).

To ensure the fidelity of unmixed data, any pixels having a negative contribution of $^{13}\text{C}_3$ -3-OPG, PGC, CD_L , d_8 -AA, or d_5 -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 = $5 \cdot [CH_2] - 0.4 \cdot [CH_3]$ whereas the protein channel (CH_P) was generated using a linear combination of $[CH_3] - [CH_2]$ 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 μ s 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 μ m pinhole and a 100 μ m 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.

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Supplementary Information

MetaboRamics: Highly multiplexed metabolic imaging
using stimulated Raman for spatial metabolomics in live
cells

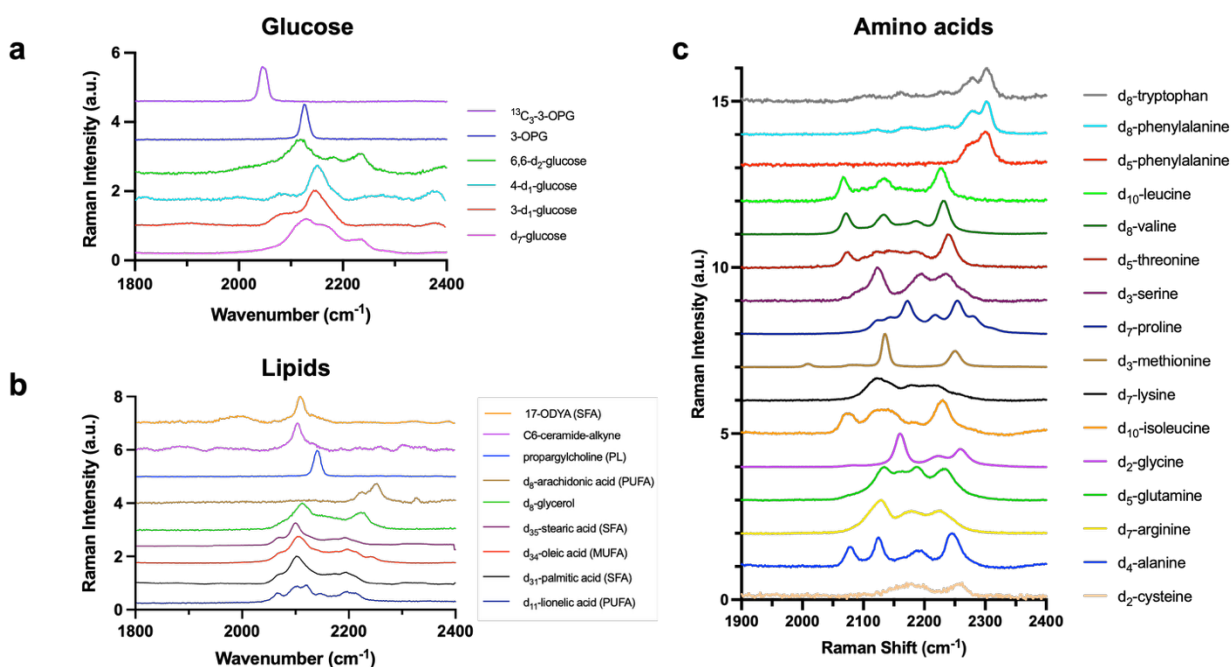


Figure S1. Raman spectra of metabolites.

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.

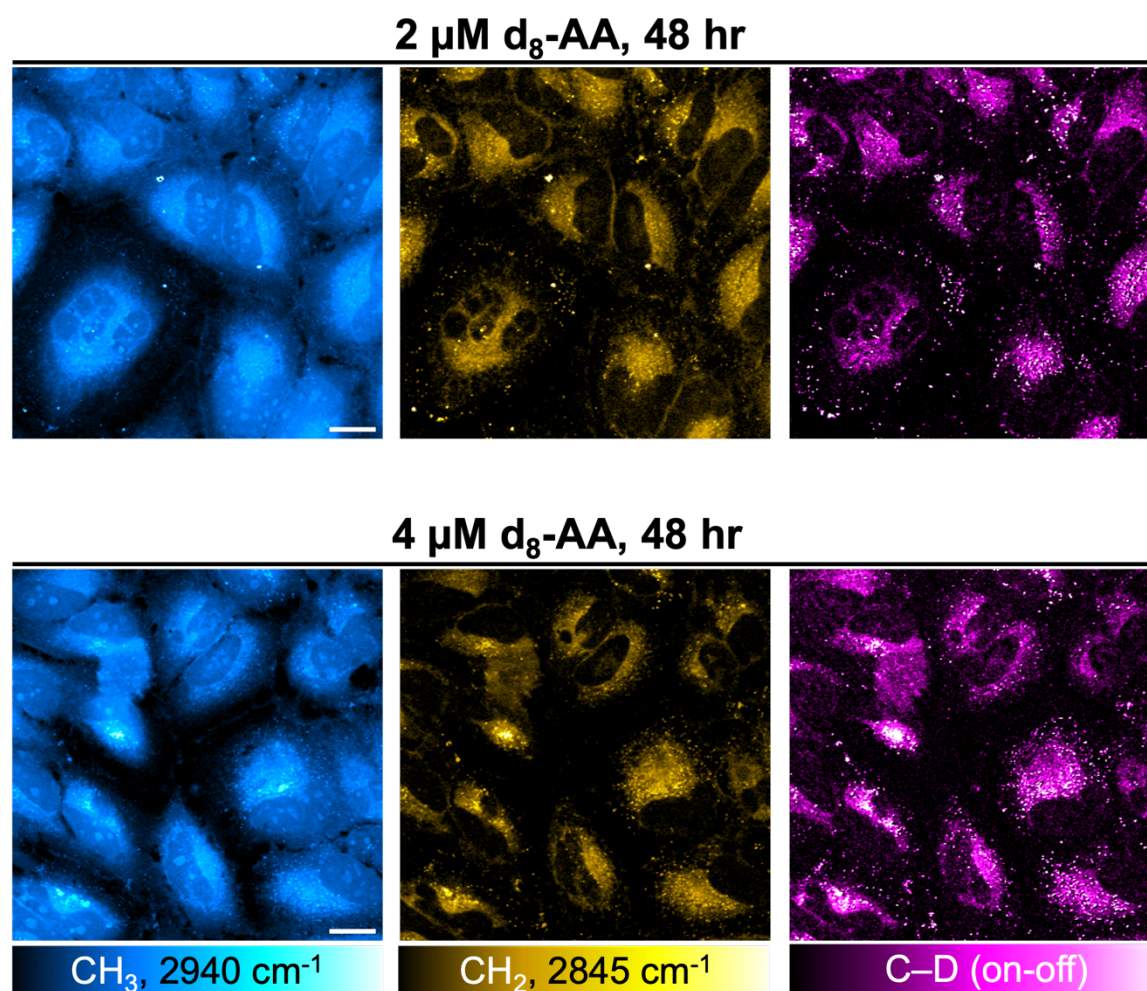


Figure S2. Probe optimization for MetaboRamics.

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.

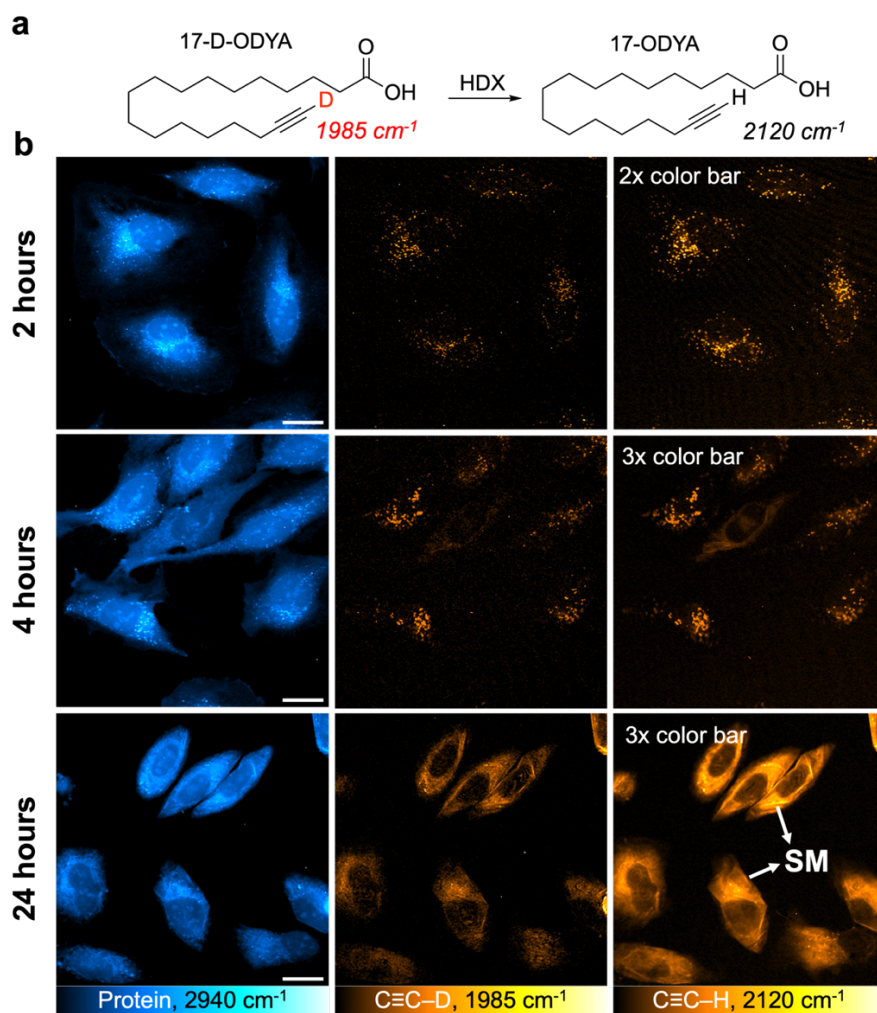


Figure S3. HDX of 17-D-ODYA in live cells.

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: $20\ \mu\text{m}$.

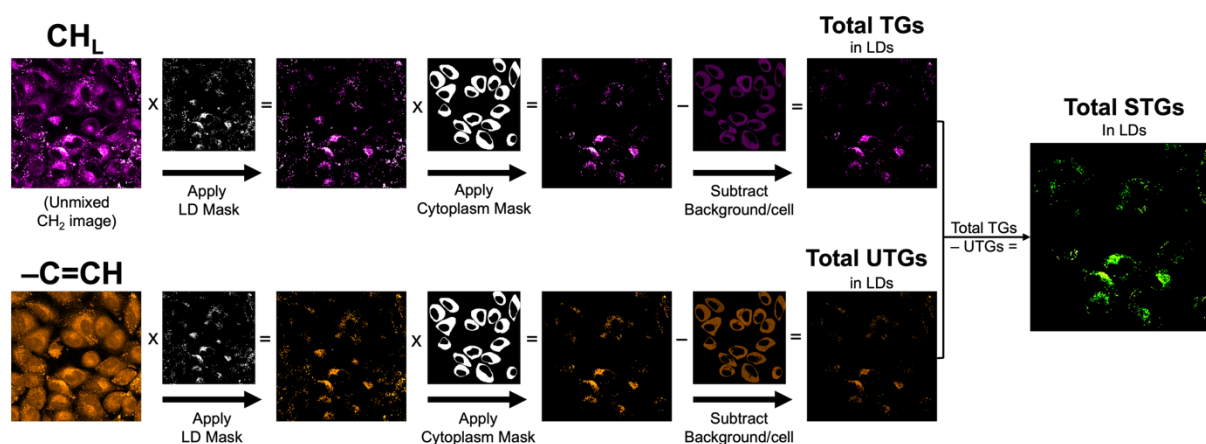


Figure S4. Image processing workflow for calculation of saturated triglycerides (STGs) from SRS images.

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

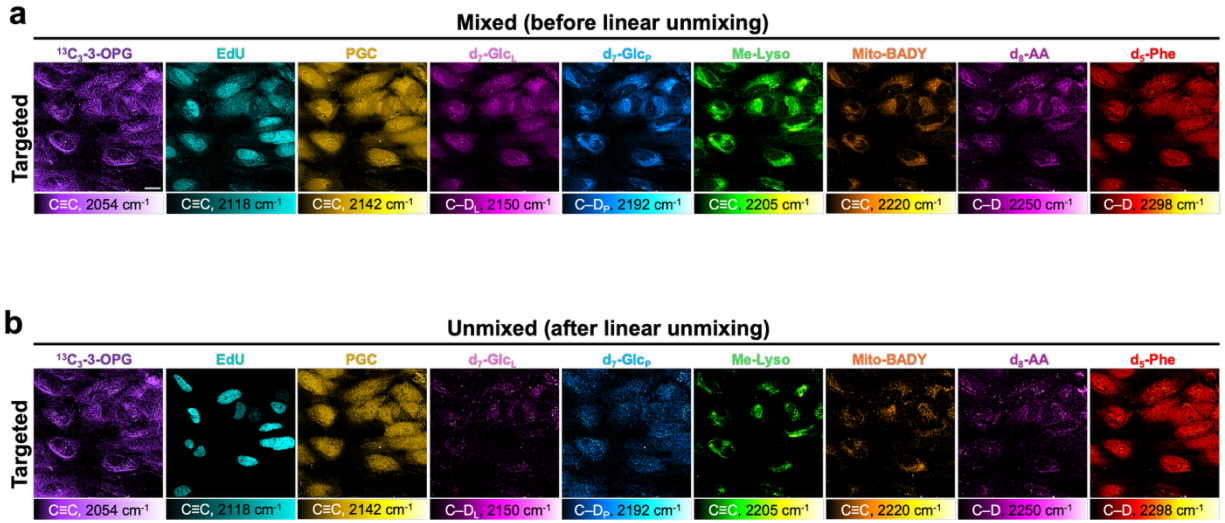


Figure S5. Robust linear unmixing of targeted SRS channels.

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:

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

inverse

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

Representative SRS images of optimized MetaboRamics metabolic probes (administered individually) alongside their corresponding protein (CH₃) images in live HeLa cells. As noted in the main text, d₇-Glc metabolizes into both lipids (d₇-Glc_L) and proteins (d₇-Glc_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.

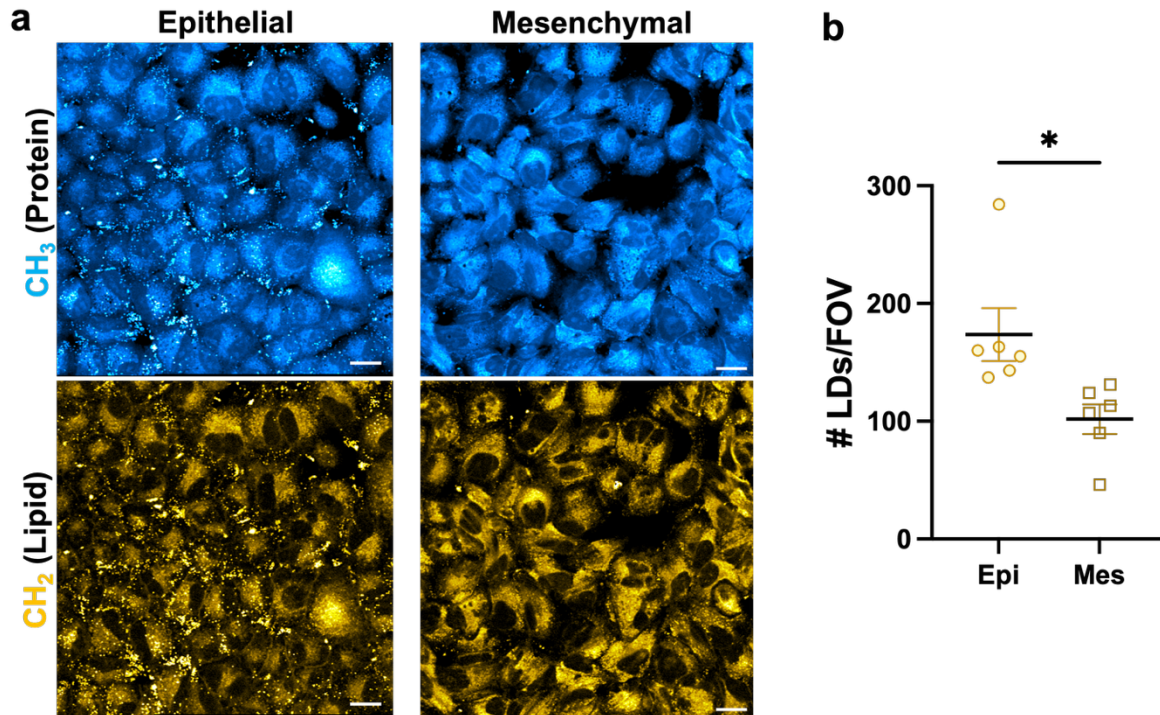


Figure S7. Label-free assessment of lipid droplets (LDs) in EMT.

(a) Representative SRS images targeted at the protein (CH₃, top row) and lipid (CH₂, bottom row) channels for live A549 (epithelial and mesenchymal cells). Scale bar: 20 μ m;

(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, * $p < 0.05$.

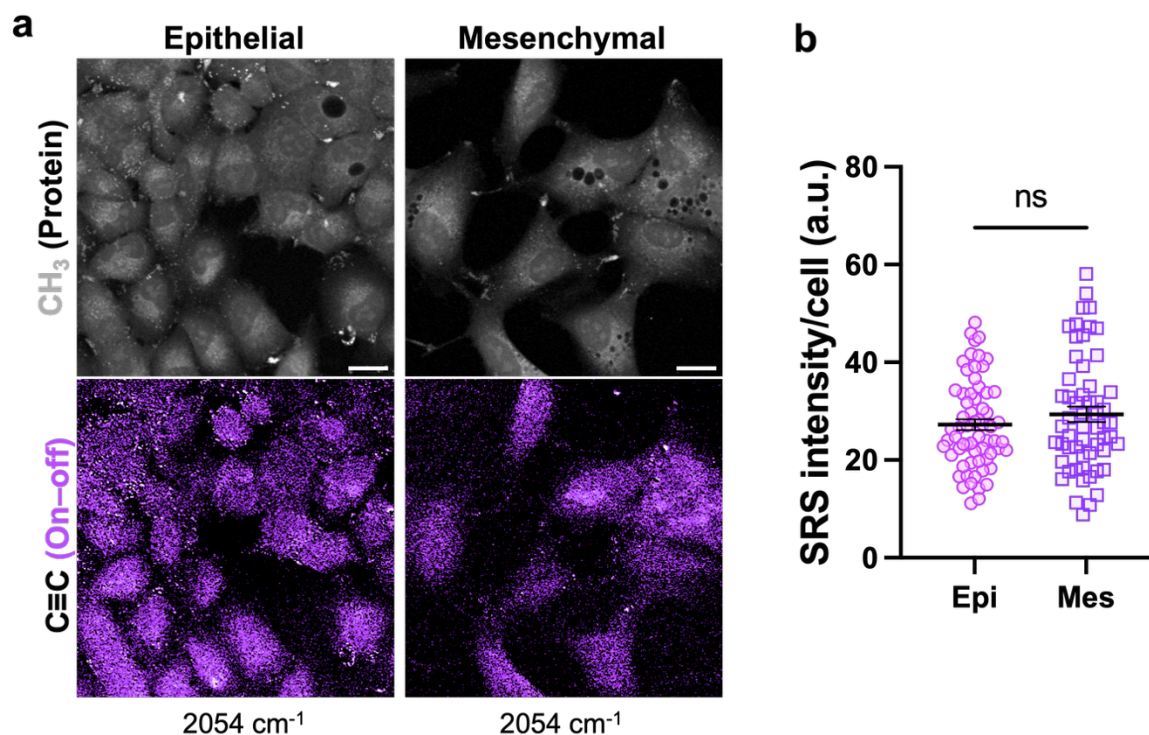


Figure S8. Glucose uptake in EMT.

(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.

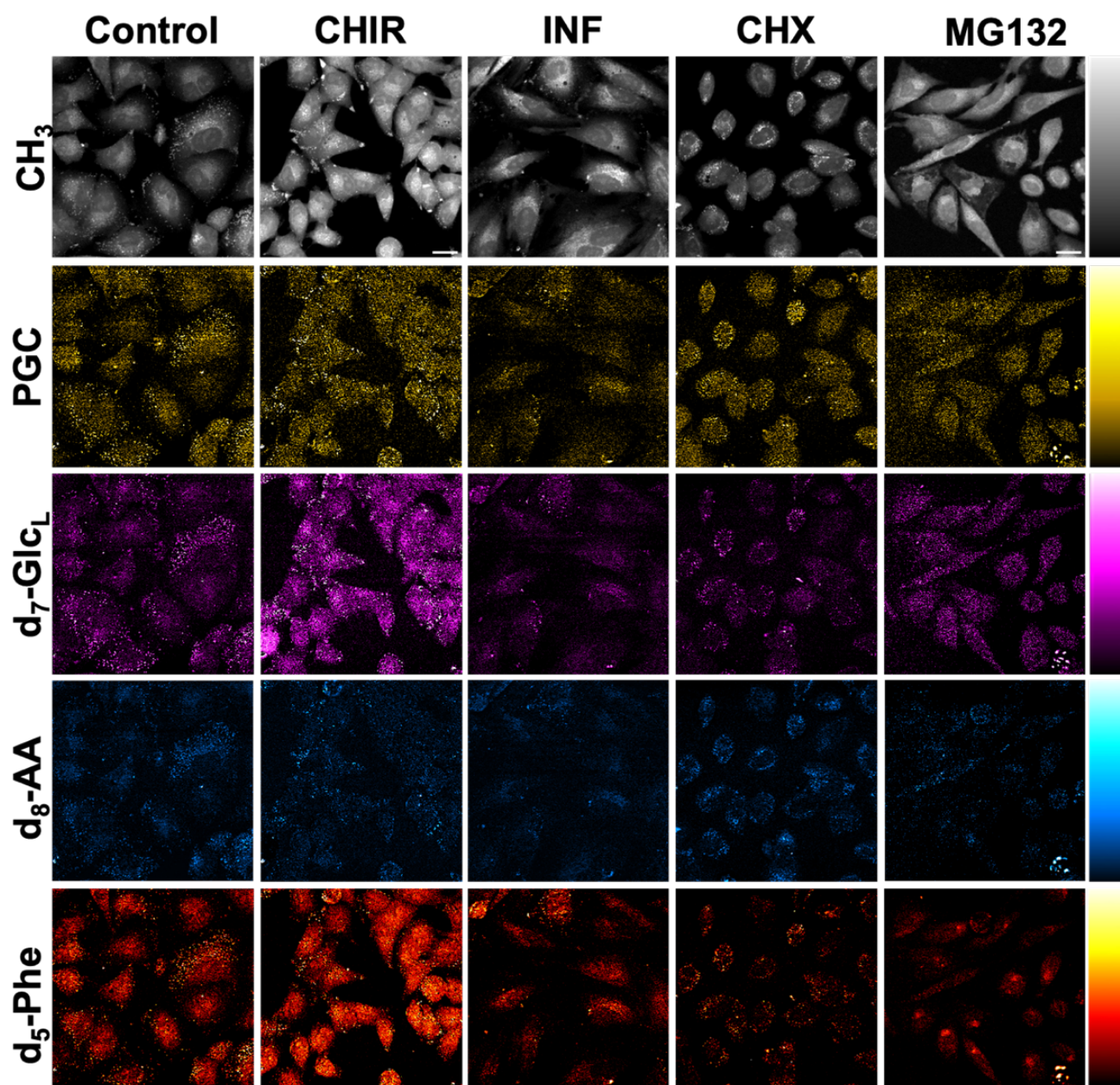


Figure S9. SRS metabolic phenotyping of stress-induced cellular responses.

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.

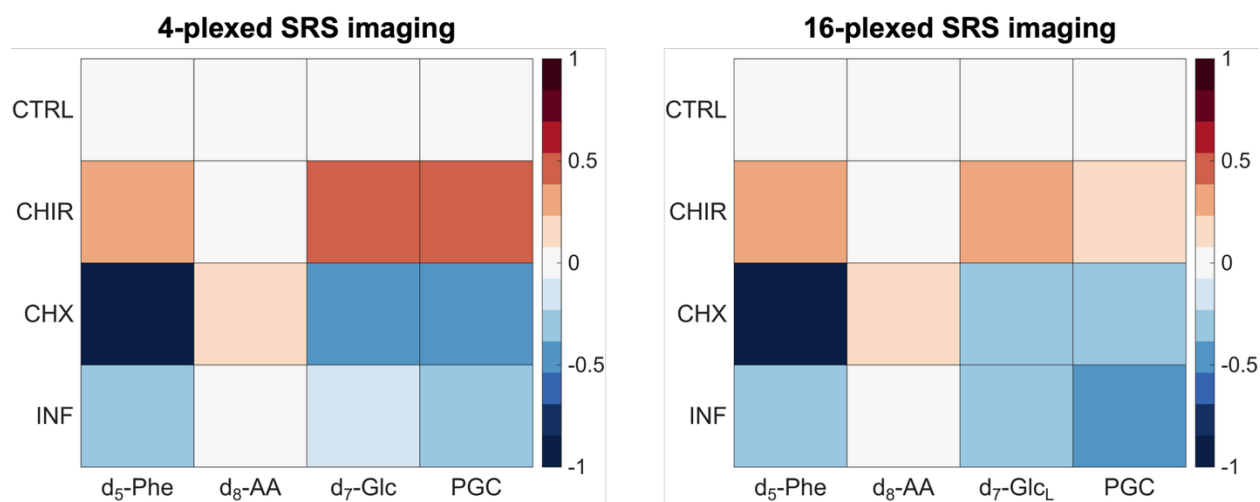


Figure S10. Robustness of MetaboRamics.

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.

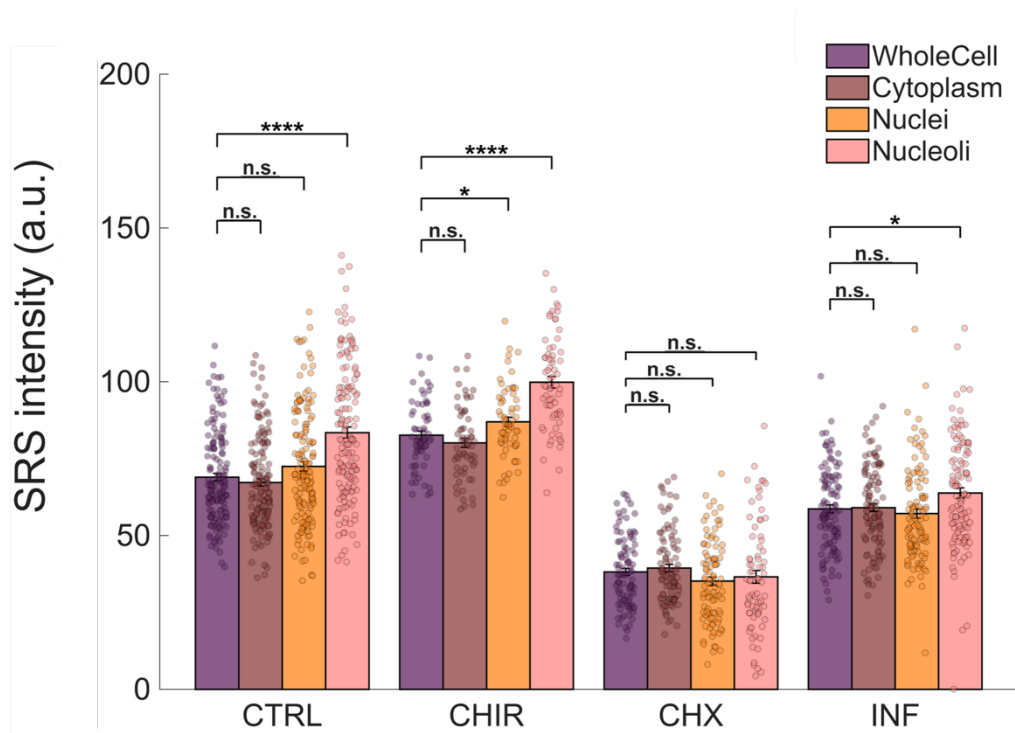


Figure S11. Subcellular analysis of protein synthesis using MetaboRamics.

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.

Supplementary Materials and Methods

1. Synthesis of MitoBADY (MB)

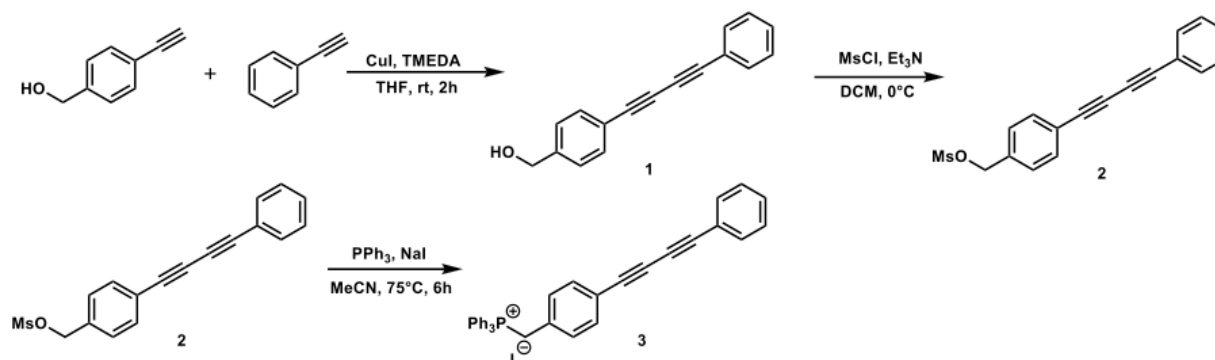
MitoBADY was synthesized following a protocol adapted from a previous report.² In a 100 mL flask, CuI (200 mg, 2 mmol) and TMEDA (600 μ L, 4 mmol) were added to 10 mL of acetone, and the mixture was bubbled with air for 10 minutes at room temperature. Next, 4-ethynylbenzyl alcohol (528 mg, 4 mmol, 1.0 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.

Mito-BADY



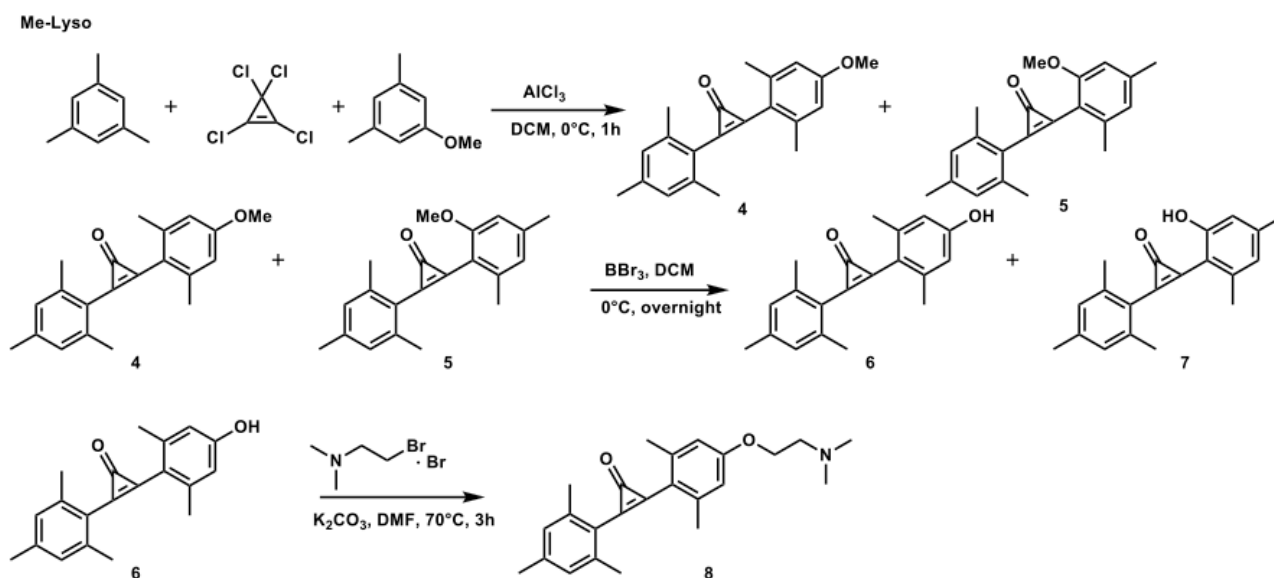
2. Synthesis of Me-Lyso (ML)

Me-Lyso was prepared using an improved adaptation of our previously reported protocol.³ 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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