nature portfolio

Peer Review File

Simultaneous Dual-Raman-Shift Scanning-Free Stimulated Raman Scattering Microscopy for Label-Free Biomolecular Imaging

Corresponding Author: Professor Liwei Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript presents a dual Raman shift, scanning free SRS (SDRSRS) approach that uses birefringent crystals and orthogonal polarizations to acquire two vibrational channels simultaneously. The authors claim that this configuration provides fast imaging, high chemical specificity, and high quality dual channel information without spectral scanning. They apply the system to living cells, mouse liver cancer models, and human colorectal cancer samples.

The optical strategy is interesting, but several claims in the manuscript are not fully supported when compared with the current state of SRS technology. Modern SRS systems already achieve one shot CH window coverage, high speed dual color imaging, and rapid wavelength tuning in commercially available configurations. These technologies are not discussed or compared, which affects the accuracy of the framing of the field.

The biological demonstrations primarily rely on the CH2 lipid channel. Similar applications have been shown extensively using standard dual color SRS, broadband SRS, or CARS. No biological mechanism, temporal process, or diagnostic interpretation is demonstrated that specifically requires SDRSRS acquisition. As a result, the biological relevance and added value of SDRSRS remain unclear.

Based on these points, I believe the manuscript does not yet demonstrate the level of technical or biological advancement needed for publication in its current form. It would benefit from clearer benchmarking, more rigorous comparison with existing technologies, and biological studies that specifically leverage the SDRSRS technology.

I have read the manuscript carefully and provide line indexed examples that illustrate the issues:

Overstatements: 1) Lines 16-18, lines 67-71 claim unprecedented performance despite the existence of single shot CH window and dual color platforms, also commercially available. 2) Current state of the art and commercially available SRS technologies are not acknowledged. 3) No quantitative metrics are provided that demonstrate improvement over standard spectral focusing in terms of imaging speed or temporal resolution. 4) Speed and temporal claims are not benchmarked against existing fast SRS platforms, and it is not shown that the observed biological dynamics require this specific speed.

Incremental technical advance: 1) Lines 106 to 116 present theoretical benefits (idle light use, energy efficiency) without quantitative evidence or experimental evidence. 2) The optical design is a variation on known dual color or a limited version of broadband configurations rather than a substantial technical innovation.

Biological insight is not advanced by SDRSRS acquisition: 1) Lines 150 to 203: lipid droplet dynamics rely only on CH2. 2) Lines 205 to 224: lipid rich liver tissue is a routine SRS proof-of-concept sample with extended literature available. 3) Lines 229 to 231 and 273 to 281: interpretations are speculative and lack proper statistical support. 4) The imaging data are largely presented as single representative examples, and the structure of biological and technical replicates is not clearly described. It is difficult to assess how many cells, sections, animals, or patients underlie each conclusion, or whether the statistical

analyses adequately capture biological variability.

Reviewer #2

(Remarks to the Author)
This is a nice demonstration of dual color SRS for histology applications. As dual color SRS has shown great potential for histology, this work presents a new step towards clinical translation of SRS. The authors demonstrated intra-operative data in their final figure! with this said, the following should be discussed in depth.

(1) Recent work by Ji Group showed that single color is enough to generate H&E image via machine learning. Other groups have used phase image to recover H&E without any chemical information. Their results diminish the value of dual color SRS. I would request that the authors discuss the value of two-color SRS. Is it really necessary for clinical applications?

(2)This paper used a femtosecond laser, like insight laser or similar. Fast tuning picosecond fiber laser has been widely used for SRS with balanced detection. The authors should discuss the value of their elegant yet complicated method versus fiber laser SRS.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
In the manuscript "Simultaneous Dual-Raman-Shift Scanning-Free Stimulated Raman Scattering Microscopy for Label-Free Biomolecular Imaging" the authors present a simultaneous dual-Raman-shift scanning-free SRS (SDRSRS) microscopy, which captures different distinct molecular vibrational signatures simultaneously without spectral scanning. The authors achieve this through a combination of birefringent-crystal–induced optical path differences, polarization control, and dual-phase electrical demodulation, enabling simultaneous two-color SRS imaging with more than a tenfold increase in acquisition speed while preserving chemical specificity.
The approach is interesting, particularly because of its use of “idle” polarization states and a custom-built dual-channel phase-shift detector. The manuscript is well written and provides a comprehensive validation across a wide range of samples, including living cells, murine liver cancer models, and human colorectal cancer tissues. These experiments convincingly demonstrate the method’s potential for real-time metabolic imaging, dynamic lipid tracking, and label-free histopathological assessment.
Nevertheless, there are several issues, which must be addressed to improve the manuscript. First, although the authors cite earlier dual-phase and multicolor SRS approaches, the manuscript does not clearly articulate how SDRSRS differs conceptually or technically from prior work, particularly He et al. (Optica 2017), nor does it adequately specify what limitations of these methods it overcomes. Additionally, a closely related approach was recently published by Xin & Huang (2025) (Simultaneous Dual-Color Stimulated Raman Scattering Biomolecular Imaging with Tailored Supercontinuum Generationand Phase-Sensitive Detection), which also demonstrates simultaneous dual-color SRS via phase-sensitive detection, though using PCF-based supercontinuum generation targeting the C–H and cell-silent windows. It would benefit the authors to position their work more clearly relative to this, highlighting the advantages of the birefringent-crystal–based OPD strategy in terms of stability, simplicity, and specificity.
In addition, the fixed Raman-shift separation determined by the birefringent crystal length is a significant constraint that restricts flexibility and limits broader applicability; this limitation is not sufficiently acknowledged, and potential mitigation strategies are not discussed. The biological interpretation of CH_2/CH_3 contrast in cancer tissues is occasionally overstated, as these ratios are influenced by multiple confounding factors such as stromal composition, extracellular matrix content, fibrosis, necrosis, and sample-preparation variability, and therefore should be described more cautiously. In most Raman-publication this ratio is references. One wonders about the true specificity.
The statistical analysis in the mouse immunotherapy study raises concerns because the reported sample size largely reflects pixel-level data rather than biological replicates, which inflates the apparent separation in PCA, UMAP, and ROC analyses; per-animal statistical treatment is required to substantiate claims about treatment effects. Furthermore, the system’s long-term stability, i.e. phase stability, drift under experimental conditions, and susceptibility to depolarization in thick or scattering tissues is not quantified, though this stability is essential. Finally, some figure sections are dense and could be clarified with simplified schematics or improved structuring, and the terminology regarding “healthy” versus “unhealthy” cells or lipid-driven cellular deterioration requires clearer definition and more conservative phrasing.
In summary, the work represents a promising and innovative contribution to high-speed vibrational imaging, and the overall methodology is strong, but some revisions are necessary to more clearly position the technical novelty, appropriately biological conclusions, strengthen statistical rigor, and document stability and performance limitations.

Minor issue:
• Please correct grammar throughout the manuscript
• Second paragraph introduction -> usinga ->using
• Introduction -> we exploits-> we exploit

• Results -> biomoleculer -> biomolecular
• Abbreviations should be introduced when used first: HWP and QWP
• The authors clame that the improvement is 10-fold, but I could not identify any location how this was particularily assessed.
•

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The manuscript has been sufficiently revised. I have no further questions.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I have carefully reviewed the revised manuscript and the authors' point-by-point responses. The authors have adequately addressed all of my previous concerns, including clarification of the methodological novelty, discussion of limitations, and refinement of the biological interpretation and statistical analysis. The revisions have improved the clarity, rigor, and positioning of the work within the context of existing dual-color SRS approaches. In its current form, the manuscript is scientifically sound and clearly presented, and I believe it is suitable for publication as is.

(Remarks on code availability)

Dear reviewers,

Thank you very much for your careful review of our manuscript and additional feedback. The comments are insightful and highly helpful for revising our manuscript and our next work. We have studied the comments and revised our manuscript carefully. Our reply to the reviewers' comments is shown in blue. Our revised text is shown in red here and highlighted in the manuscript. Please find below our point-by-point responses to the comments.

Response 1 (Reviewer #1)

This manuscript presents a dual Raman shift, scanning free SRS (SDRSRS) approach that uses birefringent crystals and orthogonal polarizations to acquire two vibrational channels simultaneously. The authors claim that this configuration provides fast imaging, high chemical specificity, and high quality dual channel information without spectral scanning. They apply the system to living cells, mouse liver cancer models, and human colorectal cancer samples.

[Reply] We appreciate the reviewer for the comments on our work and have made substantial revisions to address the reviewer's concern:

1. *The optical strategy is interesting, but several claims in the manuscript are not fully supported when compared with the current state of SRS technology. Modern SRS systems already achieve one shot CH window coverage, high speed dual color imaging, and rapid wavelength tuning in commercially available configurations. These technologies are not discussed or compared, which affects the accuracy of the framing of the field.*

[Reply] We thank the reviewer for this constructive comment. We agree that modern SRS platforms that already provide one-shot CH-window coverage, high-speed dual-color imaging, and rapid wavelength tuning. Using the references already cited in our manuscript and Supplementary Information, we have now added explicit comparison and clarified how SDRSRS is positioned relative to these technologies.

Introduction:

"… Modern SRS platforms already provide several of the capabilities that motivate our work. Super-multiplex vibrational imaging has been demonstrated with single-shot coverage of tens of Raman bands across the CH stretching window using tailored excitation and frequency-domain detection, enabling rapid acquisition of rich spectral information[1]. Hyperspectral and fingerprint-region SRS based on ultrafast tuning and spatial-spectral learning achieves microsecond-scale spectral scanning and rapid wavelength hopping across broad windows[2, 3]. Multiplex and broadband SRS implementations that use parallel detection or single-pixel reconstruction can

capture the CH window in a single shot[4-7], but require spectrometers, multi-channel detection, specialized lasers, or computational reconstruction. Although CARS offers fast vibrational contrast, its strong non-resonant background, nonlinear signal dependence, dispersive line shapes, and sensitivity to sample heterogeneity hinder accurate quantification of CH_3/CH_2 ratios. Multiband CARS imaging also requires separate anti-Stokes detection and complex phase retrieval, making synchronous two-wavenumber acquisition difficult to achieve on femtosecond platforms. In parallel, multicolor and dual-color SRS schemes using synchronized picosecond sources or rapidly tunable optical parametric oscillators provide fast two-wavenumber imaging[8-10]. Dual-phase SRS leveraging polarization and phase-sensitive detection has further shown real-time two-color contrast in spectral-focusing configurations[11]. However, these techniques based on optical phase delay require substantial additional optical path deployment (approximately four meters) and precise time delay matching for the vibration energy of two target molecules. … Building on these advances, fibre-laser-based SRS architectures[12-16] have been translated into clinically compatible systems for intraoperative and histological applications.

Against this backdrop, we provide a complementary, low-complexity approach: robust and strictly simultaneous two-channel contrast on a standard femtosecond spectral-focusing microscope. We exploit different polarization states of light, refractive index differences of varying wavelengths in media, and electrical phase delays to achieve simultaneous imaging of major biomolecules within the high wave-number C-H window. Our technology has two modes: rapid dual-color imaging and balanced detection on a spectral focusing-based SRS imaging system, with applications in real-time observation of living cells and clinicopathological tissues. The system achieves dual-phase modulation excitation and orthogonal signal demodulation through carefully designed dual-channel phase-shift photodetectors, along with optimized optical path design and circuit control. By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. …”

2. *The biological demonstrations primarily rely on the CH2 lipid channel. Similar applications have been shown extensively using standard dual color SRS, broadband SRS, or CARS. No biological mechanism, temporal process, or diagnostic interpretation is demonstrated that specifically requires SDRSRS acquisition. As a result, the biological relevance and added value of SDRSRS remain unclear.*

Based on these points, I believe the manuscript does not yet demonstrate the level of technical or biological advancement needed for publication in its current form. It would benefit from clearer benchmarking, more rigorous comparison with existing technologies, and biological studies that specifically leverage the SDRSRS technology.

[Reply] We thank the reviewer for this important comment. We agree that CH_2 contrast is widely used in vibrational imaging, and that its biological relevance must be demonstrated beyond standard two-color SRS. We have revised the manuscript to sufficiently articulate *why* simultaneous dual-Raman-shift acquisition is essential for several biological processes and diagnostic analyses and to provide direct benchmarking.

1) Biological analyses in this study rely on *simultaneous* CH_3-CH_2 measurement rather than CH_2 alone

Although CH_2 contrast highlights lipid-rich structures, our biological conclusions, including lipid-protein coupling dynamics, lipid-wave propagation, therapy-induced biochemical normalization, and CRC classification, depend on ratiometric and temporal information between CH_3 and CH_2 channels, not CH_2 alone.

Sequential acquisition with spectral scanning produces inter-frame temporal offsets of 1–3 seconds (depending on FOV and dwell time). For dynamic processes such as: lipid droplet growth and fusion, micron-scale lipid-wave propagation, rapid cell swelling or division, and time-resolved treatment-induced biochemical changes, this sequential offset introduces errors in CH_3/CH_2 ratios, particularly for living cells with nonstationary morphology. SDRSRS eliminates this error by providing **strictly synchronous dual-channel acquisition**, enabling reliable biochemical ratios at each pixel and each time point.

We now emphasize this requirement in the manuscript and explicitly contrast SDRSRS with sequential two-color acquisition.

Results: Label-free cell chemical mapping and dynamics:

"Many cellular processes probed in this study, e.g. lipid droplet growth, fusion, micron-scale lipid-wave propagation, and cell swelling or division, evolve on sub-second to second timescales. To obtain quantitative insight into lipid-protein remodeling, strict synchrony between the CH_3 and CH_2 channels is required, because sequentially acquired Raman bands introduce 1–3 s inter-frame mismatch under typical scanning parameters. Such offsets distort pixel-wise CH_3/CH_2 ratios in living cells undergoing morphological changes. SDRSRS acquires CH_3 and CH_2 signals within a single exposure, enabling accurate temporal and spatial pairing of both signals, thereby preserving

biochemical ratios at every pixel and every time point. This simultaneity enables reliable quantification of lipid-protein coupling dynamics and lipid-wave transport, enabling non-invasive, near real-time monitoring of intracellular chemical component changes.
”

Methods: Statistics and reproducibility

“All diagnostic analyses in this work, including CH_3/CH_2 ratiometry, PCA/UMAP clustering, and ROC discrimination, rely on accurate pairing of the two Raman channels. When two wavenumbers are acquired sequentially, tissue drift, hydration changes, and subtle deformations during raster scanning can artificially alter CH_3/CH_2 ratios and bias statistical classification. By ensuring strict simultaneity of the two channels, SDRSRS avoids these artifacts and provides temporally and spatially registered biochemical measurements that are beneficial for robust therapy-response quantification (Fig. 4) and CRC tissue stratification (Fig. 5). ”

Discussion:

“Many biologically relevant contrasts arise not from individual Raman bands but from their ratios or co-evolution over time. With identical imaging parameters (512 × 512 pixels, 1 µs dwell), standard spectral-focusing SRS requires two sequential scans to collect two Raman bands, resulting in a per-dual-color acquisition time of ~1 s. SDRSRS acquires both bands strictly simultaneously within a single raster, reducing dual-channel acquisition time to 0.26 s. Compared to full hyperspectral CH-window scanning typically requiring ~18–28 s per stack, SDRSRS provides more than 10-fold acceleration. Sequential two-color SRS suffers from inter-frame delays, which degrade the fidelity of CH_3/CH_2 ratios when samples undergo metabolic motion or morphological fluctuations. This problem is particularly acute in living cells, where lipid droplets move, fuse, and redistribute on similar timescales. By acquiring the two Raman shifts strictly simultaneously, SDRSRS preserves pixel-level biochemical correspondence and enables quantitative tracking of lipid-protein coupling, lipid-wave propagation, and treatment-induced biochemical normalization. … These capabilities are distinct from conventional CH_2-only lipid imaging and cannot be replicated by sequential dual-color SRS or CARS approaches. “

2) In the Introduction, we added text acknowledging: that dual color SRS, broadband SRS, or CARS can capture the CH window in one shot, but require spectrometers, multi-channel detection, specialized lasers, strong non-resonant background, nonlinear signal dependence, or computational reconstruction.

“… Modern SRS platforms already provide several of the capabilities that motivate our work. Super-multiplex vibrational imaging has been demonstrated with single-shot coverage of tens of Raman bands across the CH stretching window using tailored excitation and frequency-domain

detection, enabling rapid acquisition of rich spectral information[1]. Hyperspectral and fingerprint-region SRS based on ultrafast tuning and spatial-spectral learning achieves microsecond-scale spectral scanning and rapid wavelength hopping across broad windows[2, 3]. Multiplex and broadband SRS implementations that use parallel detection or single-pixel reconstruction can capture the CH window in a single shot[4-7], but require spectrometers, multi-channel detection, specialized lasers, or computational reconstruction. Although CARS offers fast vibrational contrast, its strong non-resonant background, nonlinear signal dependence, dispersive line shapes, and sensitivity to sample heterogeneity hinder accurate quantification of CH_3/CH_2 ratios. Multiband CARS imaging also requires separate anti-Stokes detection and complex phase retrieval, making synchronous two-wavenumber acquisition difficult to achieve on femtosecond platforms. In parallel, multicolor and dual-color SRS schemes using synchronized picosecond sources or rapidly tunable optical parametric oscillators provide fast two-wavenumber imaging[8-10]. Dual-phase SRS leveraging polarization and phase-sensitive detection has further shown real-time two-color contrast in spectral-focusing configurations[11]. However, these techniques based on optical phase delay require substantial additional optical path deployment (approximately four meters) and precise time delay matching for the vibration energy of two target molecules. … Building on these advances, fibre-laser-based SRS architectures[12-16] have been translated into clinically compatible systems for intraoperative and histological applications.

3. *I have read the manuscript carefully and provide line indexed examples that illustrate the issues:*

   Overstatements: 1) Lines 16-18, lines 67-71 claim unprecedented performance despite the existence of single shot CH window and dual color platforms, also commercially available. 2) Current state of the art and commercially available SRS technologies are not acknowledged.

[Reply] We thank the reviewer for pointing this out. We have revised the manuscript to avoid overstated claims and to accurately position SDRSRS within the broader landscape of modern SRS technologies. In the Introduction, we now explicitly acknowledge that single-shot CH-window coverage, fast dual-color imaging, and rapidly tunable SRS platforms already exist, including commercially available systems, and we clarify that our method is not intended to replace these approaches. Instead, SDRSRS provides a complementary, low-complexity solution that enables strictly simultaneous two-wavenumber acquisition on standard femtosecond spectral-focusing microscopes without requiring specialized picosecond dual-color lasers. We have removed “unprecedented” language and revised these sections to accurately reflect the scope and contribution of our work.

Revised Abstract (Lines 16-18)

Original:

"This technology advances SRS from a slow spectroscopic tool to a high-speed, high-specificity molecular imaging platform suitable for live metabolic monitoring, clinical pathology, and drug discovery applications."

Revised:

"This technique enables rapid and simultaneous dual vibrational contrast, providing chemically specific imaging for live metabolic monitoring, clinical pathology, and drug-response studies."

Revised Introduction (Lines 67–71)

Original:

"… By leveraging the optical path difference (OPD) of different wavelengths in the medium, our technique enables simultaneous collection of bimolecular vibration information. This technology promises to advance label-free optical imaging with unprecedented capabilities: high speed (eliminating the need for spectral scanning), high specificity (targeting specific biomolecules), and high quality (switchable collinear balanced detection). Our approach represents a step forward in high-speed, label-free molecular imaging for biomedical applications and clinical diagnostics."

Revised:

"… By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. This approach offers a complementary solution that provides fast two-channel molecular contrast without spectral scanning and integrates a switchable collinear balanced-detection mode. This design facilitates high-speed, label-free molecular imaging for biomedical and clinical applications while maintaining compatibility with widely used two-photon-based SRS microscopes."

3) No quantitative metrics are provided that demonstrate improvement over standard spectral focusing in terms of imaging speed or temporal resolution. 4) Speed and temporal claims are not benchmarked against existing fast SRS platforms, and it is not shown that the observed biological dynamics require this specific speed.

[Reply] We thank the reviewer for raising this critical point. We agree that quantitative benchmarking is essential to justify both the claimed speed improvement and the biological need for strictly simultaneous dual-color acquisition and have addressed these concerns.

Under our standard spectral focusing configuration (single Raman shift), acquiring two wavenumbers requires two sequential scans:
Standard SRS (single shift): 512 × 512 pixels, 1 µs dwell → ~0.26 s per frame (which was previously given in the Supplementary Note 8). With SDRSRS, both channels are acquired *simultaneously*, so: SDRSRS: 0.26 s per dual-channel frame. Sequential dual-color SRS: two frames acquired consecutively → ~1 s (including controling the time delay linear stage by the sofeware and XY scanner returning to the starting point). Thus, under identical imaging parameters. Measured improvement: near 4× reduction in per-frame acquisition time.

But importantly, full spectral scanning (typical for CH-window SRS, 2740–3080 cm^{-1}) requires: Step size ~10–15 cm^{-1} → 23–35 spectral steps→ ~18–28 seconds per hyperspectral stack. Therefore: measured improvement over spectral scanning: ~70–100× faster.

We have now added these quantitative comparisons directly to the Discussion sections.

Discussion
"…With identical imaging parameters (512 × 512 pixels, 1 µs dwell), standard spectral-focusing SRS requires two sequential scans to collect two Raman bands, resulting in a per-dual-color acquisition time of ~1 s. SDRSRS acquires both bands strictly simultaneously within a single raster, reducing dual-channel acquisition time to 0.26 s. Compared to full hyperspectral CH-window scanning typically requiring ~18–28 s per stack, SDRSRS provides more than 10-fold acceleration. …"

We now explicitly compare SDRSRS to representative state-of-the-art systems:
Supplementary Table 1 Comparison of representative fast SRS systems.

Platform	Temporal Resolution	Limitations Compared to SDRSRS
Microsecond spectral-tuning polygon systems[2]	~2–5 µs per step, but full CH-window still requires multi-step tuning	Not simultaneous; requires 20–30 steps for spectral scanning and deep learning
Fast-tuning picosecond fiber-laser SRS[12, 15]	Tens of microseconds to sub-millisecond tuning; dual-color acquisition achieved by rapid wavelength hopping or interleaved pulses	Non-simultaneous acquisition of multiple Raman bands; requires dual-wavelength synchronization hardware; potential temporal

		mismatch between channels for dynamic samples
Single-shot broadband multiplex SRS[4-7]	Single burst (μs)	Requires multi-channel photodiodes / spectrometer; not easily retrofitted to standard 2P microscopes
Commercial dual color SRS (DC-SRS), e.g., deltaEmerald, APE Inc.	N.A.	Only fixed 85 cm^{-1} energy difference set to address CH_2 & CH_3 at their peak position.
Dual-phase SRS[11]	Simultaneous 2-color	Requires ~4 m of free-space delay lines, sensitive alignment

In contrast, SDRSRS offers: 1) **Strict simultaneity** with no frame-to-frame delay; 2) **No spectral tuning**; 3) **No spectrometer or multi-pixel detector**; 4) **No long optical delay lines**; 5) **Compatibility with standard femtosecond spectral focusing microscopes.**

Discussion:

" Supplementary Table 1 summarizes representative fast SRS platforms, including microsecond spectral-tuning systems, multiplex broadband detection, commercial dual-color SRS, dual-phase SRS, and fast-tuning picosecond fiber-laser architectures. Although each of these approaches has enabled significant progress, they rely on either multi-step spectral tuning, spectrometer-based detection, fixed Raman-shift separations, rapid wavelength hopping with interleaved pulses, or long free-space delay paths. These constraints lead to non-simultaneous acquisition of Raman bands or increased optical and electronic complexity, which can introduce temporal mismatch and reduce quantitative robustness in dynamic or intra-operative settings. In contrast, SDRSRS provides strictly simultaneous dual-Raman-shift acquisition without spectral tuning, spectrometers, synchronized dual-wavelength sources, or extended delay lines, and can be retrofitted onto standard femtosecond spectral-focusing microscopes. This positions SDRSRS as a complementary operating point optimized for simplicity, robustness, and reliable biochemical quantification in real-time clinical environments."

We also added the need that biological dynamics require simultaneous imaging as explained above. Although CH_2 contrast highlights lipid-rich structures, our biological conclusions, including lipid-protein coupling dynamics, lipid-wave propagation, therapy-induced biochemical normalization, and CRC classification, depend on ratiometric and temporal information between CH_3 and CH_2 channels, not CH_2 alone.

Sequential acquisition with spectral scanning produces inter-frame temporal offsets of 1–3 seconds (depending on FOV and dwell time). For dynamic processes such as: lipid droplet growth and fusion, micron-scale lipid-wave propagation, rapid cell swelling or division, and time-resolved treatment-induced biochemical changes, this sequential offset introduces errors in CH_3/CH_2 ratios, particularly for living cells with nonstationary morphology. SDRSRS eliminates this error by providing **strictly synchronous dual-channel acquisition**, enabling reliable biochemical ratios at each pixel and each time point.

We now emphasize this requirement in the manuscript and explicitly contrast SDRSRS with sequential two-color acquisition.

Results: Label-free cell chemical mapping and dynamics:

"Many cellular processes probed in this study, e.g. lipid droplet growth, fusion, micron-scale lipid-wave propagation, and cell swelling or division, evolve on sub-second to second timescales. To obtain quantitative insight into lipid-protein remodeling, strict synchrony between the CH_3 and CH_2 channels is required, because sequentially acquired Raman bands introduce 1–3 s inter-frame mismatch under typical scanning parameters. Such offsets distort pixel-wise CH_3/CH_2 ratios in living cells undergoing morphological changes. SDRSRS acquires CH_3 and CH_2 signals within a single exposure, enabling accurate temporal and spatial pairing of both signals, thereby preserving biochemical ratios at every pixel and every time point. This simultaneity enables reliable quantification of lipid-protein coupling dynamics and lipid-wave transport, enabling non-invasive, near real-time monitoring of intracellular chemical component changes."

Methods: Statistics and reproducibility

"All diagnostic analyses in this work, including CH_3/CH_2 ratiometry, PCA/UMAP clustering, and ROC discrimination, rely on accurate pairing of the two Raman channels. When two wavenumbers are acquired sequentially, tissue drift, hydration changes, and subtle deformations during raster scanning can artificially alter CH_3/CH_2 ratios and bias statistical classification. By ensuring strict simultaneity of the two channels, SDRSRS avoids these artifacts and provides temporally and spatially registered biochemical measurements that are beneficial for robust therapy-response quantification (Fig. 4) and CRC tissue stratification (Fig. 5). "

1. *Incremental technical advance: 1) Lines 106 to 116 present theoretical benefits (idle light use, energy efficiency) without quantitative evidence or experimental evidence. 2) The optical design is a variation on known dual color or a limited version of broadband configurations rather than a substantial technical innovation.*

[Reply] We thank the reviewer for raising this important point. We now have provided quantitative evidence to support the stated advantages, clearly differentiate SDRSRS from existing dual-color and broadband SRS designs.

Our method used ~**92%** of Stokes power across the two polarization channels (power of Stoke beam before BFCs: 100 mW, after BFC: 92 mW; power of pump beam before BFCs: 100 mW, after BFC: 97 mW), **compared to <50%** effective use in the widely used standard single-polarization spectral-focusing configuration because it needs intensity modulation for signal demodulation.

The non-co-path dual-Raman-shift scheme (Supplementary Note 1 and Fig. S1) is also our optional design.

As explained above, we compared dual-phase SRS, broadband multiplex SRS, and commercial dual-color SRS, clarifying that the key innovation is not the concept of two-color SRS, but the integration of birefringent-crystal-induced OPD with same-frequency hetero-phase modulation and dual-channel electrical demodulation to create a compact, alignment-insensitive, spectrometer-free module compatible with standard femtosecond spectral-focusing SRS systems.

These additions clarify that SDRSRS is not intended to replace broadband architectures but rather offers a minimal-complexity, plug-and-play solution for strict simultaneity of dual Raman shifts on widely used two-photon-based SRS microscopes.

Revised text (for Lines 106–116)

"As a contrast, our optional non-co-path dual-Raman-shift scheme (Supplementary Note 1 and Fig. S1), …

… Measurement of Stokes power before and after BFCs showed that ~92% of the total modulated Stokes energy is delivered through BFC, recovering the "idle" polarization that is unused in standard single-polarization spectral-focusing SRS, which typically uses only <50% of the available power. Unlike conventional spectral focusing systems, our approach does not waste either polarization mode."

2. *Biological insight is not advanced by SDRSRS acquisition: 1) Lines 150 to 203: lipid droplet dynamics rely only on CH2. 2) Lines 205 to 224: lipid rich liver tissue is a routine SRS proof-of-concept sample with extended literature available. 3) Lines 229 to 231 and 273 to 281: interpretations are speculative and lack proper statistical support. 4) The imaging data are*

largely presented as single representative examples, and the structure of biological and technical replicates is not clearly described. It is difficult to assess how many cells, sections, animals, or patients underlie each conclusion, or whether the statistical analyses adequately capture biological variability.

[Reply] We thank the reviewer for highlighting the need to more clearly articulate the biological insight enabled by SDRSRS and to ensure that our interpretations are statistically supported. We have substantially revised the relevant sections to clarify 1) why CH_3-CH_2 simultaneity, rather than CH_2 alone, underpins all biological conclusions; 2) why liver tissue and immunotherapy analyses in our work extend beyond routine CH_2 lipid imaging; and 3) how statistical rigor has been strengthened to avoid speculative interpretation.

1) Although lipid-rich structures can be visualized with CH_2 contrast alone, the biological analyses presented in our study deliberately rely on paired CH_3-CH_2 information. Cellular dynamics such as lipid droplet growth, fusion, intracellular redistribution, swelling, division, and lipid-wave propagation involve coupled lipid-protein remodeling. These processes cannot be quantified from a single band because CH_2 intensity varies with droplet packing and lipid composition, whereas CH_3 provides complementary information on protein-rich structures, cytoskeletal deformation, and lipid-protein coupling. Meaningful quantities such as the CH_3/CH_2 ratio, its temporal evolution, and pixel-level coupling patterns require strictly simultaneous dual-Raman-shift acquisition to avoid inter-frame mismatch. Sequential SRS, even with modest time offsets, introduces inconsistencies when imaging non-stationary living cells; therefore, SDRSRS enables biochemical insights that cannot be achieved with CH_2-only measurements or sequential two-color SRS. We now explicitly emphasize in the revised text that all dynamic conclusions—lipid-wave speed estimation, droplet maturation, and protein-lipid coupling during mitosis—are derived from synchronized CH_3-CH_2 readouts rather than CH_2 alone.

2) We agree that liver tissue is often used as a proof-of-concept model in SRS imaging. In our revised manuscript, we clarify that our goal is not to re-establish known lipid-rich anatomy but to demonstrate the diagnostic value of simultaneous dual-band biochemical information for differentiating therapeutic states. Standard CH_2 imaging alone indeed highlights lipid-rich hepatocytes, but it fails to distinguish cancer, CCR8-treated, and PD-1-treated tissues. By quantifying paired CH_3 and CH_2 intensities and their ratios, we show that cancer reduces both lipid and protein abundance, while therapeutic interventions partially restore these biochemical components in distinct patterns. These treatment-induced biochemical signatures were detectable only when both Raman bands were measured synchronously; a single Raman shift (e.g., CH_2) could not resolve treatment responders from non-responders. We have revised the presentation to focus on this treatment-specific biochemical separation and removed any language that could imply novelty in using liver lipids per sec.

3) Regarding correlation analyses, we acknowledge the reviewer's concern about potential overinterpretation. We have strengthened the statistical framework by adding explicit sample sizes and confdence intervals. These additions ensure that statements regarding biochemical divergence between cancer and normal tissues are supported by quantitative evidence rather than qualitative trends. We have also revised the textual interpretation to avoid implying causal or mechanistic relationships; the revised text states the statistical findings clearly, without conjecture about biological pathways or immune mechanisms.
These revisions clarify that the biological insights in our study arise from synchronous two-band biochemical quantification rather than CH_2 imaging alone, that liver immunotherapy analysis leverages paired Raman information to differentiate therapeutic states, and that all statistical interpretations now rest on rigorous quantitative evaluation rather than speculation.

1. Revision to Lines 150–203 (Label-free cell chemical mapping and dynamics):
"Many cellular processes probed in this study, e.g. lipid droplet growth, fusion, micron-scale lipid-wave propagation, and cell swelling or division, evolve on sub-second to second timescales. To obtain quantitative insight into lipid-protein remodeling, strict synchrony between the CH_3 and CH_2 channels is required, because sequentially acquired Raman bands introduce 1–3 s inter-frame mismatch under typical scanning parameters. Such offsets distort pixel-wise CH_3/CH_2 ratios in living cells undergoing morphological changes. SDRSRS acquires CH_3 and CH_2 signals within a single exposure, enabling accurate temporal and spatial pairing of both signals, thereby preserving biochemical ratios at every pixel and every time point. This simultaneity enables reliable quantification of lipid-protein coupling dynamics and lipid-wave transport, enabling non-invasive, near real-time monitoring of intracellular chemical component changes."
"

After original text describing lipid droplet dynamics, we inserted clarification sentence:
"… Although the CH_2 band highlights lipid-rich structures, the interpretation of lipid droplet dynamics presented here is based on the co-evolution of CH_3 and CH_2 signals, which provides the biochemical basis for quantifying lipid-protein coupling during droplet growth and redistribution."

2. Revision to Lines 205–224 (Liver cancer and immunotherapy imaging):
"By simultaneously quantifying total intensities at 2930 cm^{-1} and 2843 cm^{-1} in normal liver, liver cancer, CRT, and PDT tissues (Fig. 4c), SDRSRS captures treatment-induced biochemical shifts that are not visible from a single Raman band. We therefore analyzed paired CH_3-CH_2 intensities across normal liver, liver cancer, CRT, and PDT tissues. Liver cancer tissues showed significant reductions in both CH_3 and CH_2 signals, with CH_2 experiencing a more pronounced decrease, consistent with lipid degradation and metabolic disruption. CRT and PDT treatments produced distinct recovery patterns in the CH_3-CH_2 space, enabling biochemical discrimination of therapeutic states. These differences could be resolved when both Raman shifts were measured

synchronously, whereas single-band CH_2 imaging may lead to diagnostic bias due to insufficient information."

3. Revision to Lines 229–231 (Correlation interpretation)

"We quantified biochemical similarity among the four tissue groups by computing Pearson correlation coefficients (95% confidence interval) using paired CH_3 (2930 cm^{-1}) and CH_2 (2843 cm^{-1}) intensities across all samples. The resulting matrix (Fig. 4d) showed low correlations between normal and cancer tissues reflecting substantial biochemical deviation in the cancer state. Correlations between cancer and CRT tissues and between cancer and PDT tissues were moderate but remained significantly lower than within-group correlations. CRT and PDT exhibited partial similarity to each other but did not overlap fully, indicating that the two therapeutic interventions produce distinguishable biochemical signatures. These statistical summaries describe dataset similarity and do not imply mechanistic relationships."

4. Revision to Lines 273–281 (Cancer vs. paracancerous PCA and clustering)

"We evaluated biochemical differences between cancer and paracancerous tissues by analyzing paired CH_3-CH_2 intensities from SDRSRS imaging. PCA revealed statistically significant separation between the two groups (variance explained by the first two principal components: 0.97), with cancer and paracancerous data points occupying distinct regions of the biochemical space (Fig. 5c). K-means clustering yielded consistent group assignments (Supplementary Fig. 7b,c). The correlation matrix (Fig. 5d) showed a Pearson coefficient of 0.28 with 95% confidence interval of 0.07 to 0.46 between cancer and paracancerous tissues, indicating limited biochemical similarity. These quantitative metrics describe the statistical separability of the two tissue types without implying causal or mechanistic interpretation."

> *4) The imaging data are largely presented as single representative examples, and the structure of biological and technical replicates is not clearly described. It is difficult to assess how many cells, sections, animals, or patients underlie each conclusion, or whether the statistical analyses adequately capture biological variability.*

[Reply] We thank the reviewer for this important comment. We have revised the manuscript to clearly state the number of biological and technical replicates associated with each experiment, including the number of cells, tissue sections, animals, and patient samples. Statistical analyses have been updated to reflect these replicate structures and to ensure that variability across biological replicates, rather than single fields of view, supports all quantitative conclusions. We have updated figure captions to explicitly list sample sizes. These revisions ensure that all analyses are transparent, statistically valid, and supported by sufficient biological replicates.

"Statistics and reproducibility"

"All biological and technical replicates used in this study are reported in the figure captions. For cultured-cell imaging, quantitative analyses were performed across n = 16 cells acquired from three independent cultures. For mouse liver cancer and immunotherapy experiments, measurements were obtained from n = 12 mice in total (normal: 3; cancer: 3; CRT: 3; PDT: 3), with 4–6 imaging regions per animal and three tissue sections per condition. For human CRC studies, SDRSRS imaging was performed on n = 21 patients, each contributing paired cancer and paracancerous tissues. PCA, k-means clustering, correlation matrices, and ROC analyses were conducted on 84 tissue regions sampled across these patients. Each imaging dataset included technical replicates obtained from repeated acquisitions of the same field to verify instrument stability. All statistical tests were performed on these biological replicates rather than single images. Measures of central tendency and confidence intervals are reported in the main text and figures."

Add to Fig. 3 caption:
(e) Comparison of component intensity and component ratio at 2930 cm^{-1} and 2843 cm^{-1} between cells with low lipid content and lipid-rich cells (n = 16).

Figure 4 and 5 already have sample sizes.

Overall, we have substantially revised both the text and data presentation.
First, we now explicitly situate SDRSRS within the landscape of modern SRS technologies, including single-shot CH-window, broadband, dual-color, dual-phase, and commercial systems, and clearly position our method as a complementary, low-complexity solution that enables strictly simultaneous two-wavenumber acquisition on standard femtosecond spectral-focusing microscopes. Overstated claims have been removed, the Abstract and Introduction have been toned down, and quantitative benchmarks are now provided. We gave a new comparison table summarizing differences from representative fast SRS platforms.
Second, we clarify that all biological conclusions rely on synchronous CH_3-CH_2 measurements rather than CH_2 alone, and we explicitly explain why strict simultaneity is necessary for quantifying lipid-protein coupling, lipid-wave propagation, therapy-induced biochemical normalization, and CRC tissue stratification. For the liver cancer immunotherapy experiments, we emphasize that the novelty lies in dual-band, ratiometric discrimination of therapeutic states rather than in imaging lipid-rich liver tissue per se.
Third, we provide quantitative evidence for the technical advantages of the optical design, including ~92% utilization of modulated Stokes power and the integration of birefringent-crystal-induced OPD with same-frequency hetero-phase modulation and dual-channel electrical demodulation in a compact, spectrometer-free, retrofit module.

Finally, we strengthen statistical rigor by explicitly reporting biological and technical replicate numbers, adding confidence intervals and clarified correlation/PCA interpretations, and updating the Statistics and reproducibility section and figure captions so that all conclusions are transparently supported by appropriate sample sizes and analyses rather than by single representative images.

Response 2 (Reviewer #2)

This is a nice demonstration of dual color SRS for histology applications. As dual color SRS has shown great potential for histology, this work presents a new step towards clinical translation of SRS. The authors demonstrated intra-operative data in their final figure! with this said, the following should be discussed in depth..

[Reply] We thank the reviewer for the positive assessment of our dual-color SRS demonstration and for recognizing the clinical potential of our SDRSRS platform, particularly in the context of histology and intra-operative imaging. We have made revision to address the reviewer's concern.

1. *Recent work by Ji Group showed that single color is enough to generate H&E image via machine learning. Other groups have used phase image to recover H&E without any chemical information. Their results diminish the value of dual color SRS. I would request that the authors discuss the value of two-color SRS. Is it really necessary for clinical applications?*

[Reply] We thank the reviewer for raising this important point regarding whether two-color SRS remains necessary for clinical translation in light of recent virtual histology studies. We have carefully read the cited works from the Ji group before, and note that although their methods are described as single-shot, the underlying SRS signals still contain both lipid- and protein-derived vibrational information. In their Science Advances study (Sci. Adv.10,eadn3426, 2024), the excitation scheme embeds dual biochemical contrast into the acquired image, and the deep-learning model reconstructs histological features by leveraging this mixed protein-lipid information rather than relying on a single chemical channel. Likewise, in their Nature Communications report (Nat Commun 13, 4050, 2022), the femtosecond excitation produces broadband vibrational responses that include contributions from both CH_3 and CH_2 bands, which are then separated computationally. In both cases, the success of virtual H&E generation relies fundamentally on access to protein and lipid contrast, whether these components are acquired explicitly as two channels or implicitly as mixed broadband signals that must later be unmixed. These studies therefore do not diminish the value of dual-color SRS; rather, they reinforce the conclusion that accurate histological transformation and diagnostic interpretation depend on the joint biochemical content encoded by the CH_3 and CH_2 vibrations.

We also acknowledge that purely structural approaches, such as phase-based virtual H&E, can recover broad tissue morphology but inherently lack biochemical specificity. Clinical scenarios that require quantifying metabolic remodeling, distinguishing viable tumor from necrotic or fibrotic regions, assessing treatment response, or evaluating subtle stromal alterations depend on biochemical contrast that cannot be inferred from morphology alone. For these reasons, explicit and synchronous access to CH_3 and CH_2 information remains important for robust, interpretable,

and generalizable clinical decision-making. Our SDRSRS approach addresses this need by providing strictly simultaneous measurement of these two essential biochemical signals on a compact, clinically compatible femtosecond platform, reducing motion-induced artifacts and improving quantitative reliability in intra-operative environments. These considerations have been incorporated into the revised Discussion to clarify that SDRSRS does not compete with structural-only methods but instead provides the biochemical foundation required for clinically meaningful SRS histopathology.

Introduction:

"Recent advances in data-driven virtual histology[17-19] have demonstrated that H&E-like contrast can be predicted from two-color SRS acquisitions. For example, the recent virtual histology through femtosecond-excited SRS images[17] in which lipid- and protein-derived vibrational contributions are jointly embedded and subsequently separated by deep learning. Although acquired in a single frame, the physical signal contains both CH_3 and CH_2 contrast, and the computational reconstruction relies on this combined biochemical information rather than on a single chemical component[18]. In contrast, structural methods, such as phase-based virtual H&E, provide architectural features but lack the biochemical specificity required for assessing metabolic alterations, necrosis versus viability, stromal remodeling, or subtle treatment-induced biochemical normalization. For clinical translation, especially in intra-operative environments, synchronous access to CH_3 and CH_2 signals provides robust and interpretable biochemical readouts. "

Although CH_2 contrast highlights lipid-rich structures, our biological conclusions, including lipid-protein coupling dynamics, lipid-wave propagation, therapy-induced biochemical normalization, and CRC classification, depend on ratiometric and temporal information between CH_3 and CH_2 channels, not CH_2 alone.

Results: Label-free cell chemical mapping and dynamics:

"Many cellular processes probed in this study, e.g. lipid droplet growth, fusion, micron-scale lipid-wave propagation, and cell swelling or division, evolve on sub-second to second timescales. To obtain quantitative insight into lipid-protein remodeling, strict synchrony between the CH_3 and CH_2 channels is required, because sequentially acquired Raman bands introduce 1–3 s inter-frame mismatch under typical scanning parameters. Such offsets distort pixel-wise CH_3/CH_2 ratios in living cells undergoing morphological changes. SDRSRS acquires CH_3 and CH_2 signals within a single exposure, enabling accurate temporal and spatial pairing of both signals, thereby preserving biochemical ratios at every pixel and every time point. This simultaneity enables reliable quantification of lipid-protein coupling dynamics and lipid-wave

transport, enabling non-invasive, near real-time monitoring of intracellular chemical component changes. ”

Methods: Statistics and reproducibility

“All diagnostic analyses in this work, including CH_3/CH_2 ratiometry, PCA/UMAP clustering, and ROC discrimination, rely on accurate pairing of the two Raman channels. When two wavenumbers are acquired sequentially, tissue drift, hydration changes, and subtle deformations during raster scanning can artificially alter CH_3/CH_2 ratios and bias statistical classification. By ensuring strict simultaneity of the two channels, SDRSRS avoids these artifacts and provides temporally and spatially registered biochemical measurements that are beneficial for robust therapy-response quantification (Fig. 4) and CRC tissue stratification (Fig. 5). ”

2. *This paper used a femtosecond laser, like insight laser or similar. Fast tuning picosecond fiber laser has been widely used for SRS with balanced detection. The authors should discuss the value of their elegant yet complicated method versus fiber laser SRS.*

[Reply] We thank the reviewer for raising this important question regarding the relationship between our femtosecond-based SDRSRS approach and fast-tuning picosecond fiber-laser SRS systems. In the revised manuscript, we now clarify the complementary advantages and intended operating regime of SDRSRS. While tunable picosecond fiber lasers have indeed become a powerful platform for balanced-detection SRS and have shown strong applicability in clinical and translational imaging, these systems rely on wavelength tuning or sequential dual-wavelength excitation to access multiple Raman shifts. Even with recent advances in rapid-tuning architectures, two-color acquisition typically involves either fast hopping or interleaved scans, which introduce temporal offsets between spectral channels. For applications focused primarily on static tissues, such offsets may be acceptable; however, for biochemical quantification in dynamic or intra-operative environments, where tissue motion, hydration changes, and morphological fluctuations occur on sub-second to second timescales, strict simultaneity is essential to preserve accurate CH_3-CH_2 pairing and prevent distortion in protein-lipid ratios. Our method addresses this by enabling two Raman shifts to be captured within the same femtosecond excitation and the same pixel dwell, without wavelength tuning, phase-delay lines, or spectrometer-based detection.

Moreover, picosecond fiber lasers, though robust and increasingly accessible, have fixed pulse durations and require complex synchronization hardware when dual-color excitation is needed. By contrast, SDRSRS builds on the femtosecond spectral-focusing framework that is already widely available on commercial two-photon microscopes and in many clinical research centers. Our birefringent-crystal-induced OPD and dual-phase modulation/demodulation module is compact, passive, and easily retrofittable, providing tunable Raman-shift separation simply by exchanging

crystal lengths. This modularity avoids the need for specialty dual-wavelength fiber sources, tunable oscillators, or long optical delay paths, and allows researchers to integrate dual-color SRS capability into existing femtosecond-based imaging systems without reconfiguring their laser architecture.

We have added a paragraph in the Discussion clarifying that SDRSRS is not intended to replace picosecond fiber-laser SRS but to provide a complementary solution optimized for scenarios where strict channel simultaneity, plug-and-play integration with existing femtosecond microscopes, and avoidance of complex tunable-laser infrastructures are advantageous. This distinction reinforces the translational relevance of SDRSRS while acknowledging the strengths of fiber-laser-based SRS systems.

Discussion:
" Supplementary Table 1 summarizes representative fast SRS platforms, including microsecond spectral-tuning systems, multiplex broadband detection, commercial dual-color SRS, dual-phase SRS, and fast-tuning picosecond fiber-laser architectures. Although each of these approaches has enabled significant progress, they rely on either multi-step spectral tuning, spectrometer-based detection, fixed Raman-shift separations, rapid wavelength hopping with interleaved pulses, or long free-space delay paths. These constraints lead to non-simultaneous acquisition of Raman bands or increased optical and electronic complexity, which can introduce temporal mismatch and reduce quantitative robustness in dynamic or intra-operative settings. In contrast, SDRSRS provides strictly simultaneous dual-Raman-shift acquisition without spectral tuning, spectrometers, synchronized dual-wavelength sources, or extended delay lines, and can be retrofitted onto standard femtosecond spectral-focusing microscopes. This positions SDRSRS as a complementary operating point optimized for simplicity, robustness, and reliable biochemical quantification in real-time clinical environments."

Supplementary Table 2 Comparison of representative fast SRS systems.

Platform	Temporal Resolution	Limitations Compared to SDRSRS
Microsecond spectral-tuning polygon systems[2]	~2–5 μs per step, but full CH-window still requires multi-step tuning	Not simultaneous; requires 20–30 steps for spectral scanning and deep learning
Fast-tuning picosecond fiber-laser SRS[12, 15]	Tens of microseconds to sub-millisecond tuning; dual-color acquisition achieved by rapid wavelength hopping or interleaved pulses	Non-simultaneous acquisition of multiple Raman bands; requires dual-wavelength synchronization hardware; potential temporal mismatch between channels for dynamic samples

Single-shot broadband multiplex SRS[4-7]	Single burst (μs)	Requires multi-channel photodiodes / spectrometer; not easily retrofitted to standard 2P microscopes
Commercial dual color SRS (DC-SRS), e.g., deltaEmerald, APE Inc.	N.A.	Only fixed 85 cm^{-1} energy difference set to address CH_2 & CH_3 at their peak position.
Dual-phase SRS[11]	Simultaneous 2-color	Requires ~4 m of free-space delay lines, sensitive alignment

Response 3 (Reviewer #3)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

This MS reports a dual-color SRS imaging platform aiming to achieve simultaneous SRS imaging at two wavenumbers without spectral scanning. The major principle is to use polarization and birefringent crystal to generate a pair of pulses at different time delays. In addition, the authors introduced in line balanced detection to improve the signal to noise ratio. While the goal of improving imaging speed and simplifying SRS operation is great, I am afraid that the methodology does not show clear novelty or advantage over the existing methods, nor does it demonstrate compelling biomedical applications to meet the publication standard of Nature Communications. I have some specific concerns as follows:

[Reply] We thank the reviewer for the careful evaluation of our manuscript and for summarizing the core components of our dual-color SRS approach. We appreciate the concern regarding methodological novelty and the strength of biomedical application, and we have substantially revised the manuscript to clarify both aspects. While our work draws inspiration from earlier polarization- and phase-based schemes, the key contribution of SDRSRS lies in integrating birefringent-crystal-induced optical-path separation with same-frequency hetero-phase modulation and dual-channel electrical demodulation in a compact, alignment-insensitive module that operates on femtosecond spectral-focusing microscopes. This design eliminates the need for tunable picosecond sources, long free-space delay lines, or spectrometer-based detection, and enables strictly simultaneous acquisition of two Raman shifts within a single raster scan. We have also added quantitative benchmarking against state-of-the-art fast SRS platforms to demonstrate measurement fidelity, speed improvement, and SNR characteristics under identical imaging conditions.

On the biological side, we have strengthened the presentation to emphasize analyses that explicitly require synchronous dual-band acquisition, including lipid-protein coupling dynamics in live cells, biochemical normalization associated with immunotherapy response in mouse liver tumors, and CH_3-CH_2-based stratification of human colorectal cancer tissues. These processes depend on accurate pixel-level pairing of CH_3 and CH_2 signals, and cannot be reliably assessed using CH_2-only imaging or sequential two-color acquisition. To ensure robustness, we now provide explicit sample sizes, replicate structures, confidence intervals, and statistical validation across all datasets. Together, these revisions clarify both the technical distinctness and the biomedical relevance of SDRSRS within the context of clinically oriented vibrational imaging.

1. *The use of birefringent crystals for path-length differentiation and orthogonal polarization modulation builds upon established dual-phase SRS methods (e.g., He et al., Optica 2017, ref 33 in the MS). Also, the wavenumber difference between the two channels is fixed, further limiting the versatility of the method.*

[Reply] We thank the reviewer for this comment and agree that birefringent crystals and polarization-based schemes have been explored previously in dual-phase SRS. We have revised the manuscript to clarify that the contribution of our work is not the conceptual use of birefringence itself but the development of an integrated optical-electronic architecture that enables strictly simultaneous dual-Raman-shift acquisition on a femtosecond spectral-focusing microscope. Unlike earlier dual-phase approaches such as He et al. (Optica 2017), which require meter-scale free-space delay lines, picosecond synchronization, and alignment-sensitive optical paths, our method combines birefringent-crystal-induced optical path separation with same-frequency hetero-phase modulation and dual-channel electrical demodulation in a compact, passive, and alignment-insensitive module that directly retrofits into widely used femtosecond two-photon/SRS systems. This integration eliminates the need for external delay stages, dual-color picosecond laser platforms, or spectrometer-based multiplex detection, thereby reducing system complexity while enabling real-time two-band acquisition on hardware already common in biomedical laboratories. Moreover, our system inherently possesses a collinear balanced mode.

We also note that the reviewer's impression that the wavenumber separation is fixed does not reflect the operational design of SDRSRS. In the revised manuscript we describe the practical strategies we have implemented to address it. Although the Raman-shift difference in SDRSRS is set by the optical-path delay of the birefringent crystal, this separation is not fixed by the system design. In practice, we fabricated a set of interchangeable crystals with lengths of 1 cm and 2 cm, which can be used individually or cascaded to provide effective optical-path differences equivalent to 1–4 cm. This enables users to select the desired Raman-shift separation in a plug-and-play manner without modifying the laser source, beam geometry, or detection module. For applications requiring different spectral separations, such as shifts between CH_3-CH_2 or selected bands, crystals of alternative thicknesses can be manufactured with high reproducibility and swapped within seconds. The impedance of fine-grained tuning is therefore practical rather than fundamental, and the passive nature of the birefringent module preserves long-term alignment stability, which we found advantageous for biological and intra-operative imaging. We have added text in the Introduction to explicitly clarify this tunability, stating that our approach provides adjustable separations through interchangeable crystal lengths, unlike fixed-wavelength dual-color schemes. This revision makes clear that SDRSRS is not limited to a single Raman-shift separation and that its flexibility can be extended according to application requirements.

These revisions highlight that SDRSRS is not a straightforward extension of dual-phase SRS but rather a new operational solution that brings adjustable, strictly simultaneous dual-wavelength SRS into a compact and clinically compatible femtosecond platform. This represents a distinct technical regime compared with prior birefringence-based implementations and provides practical advantages for both biological imaging and translational deployment.

Introduction:

"… Dual-phase SRS leveraging polarization and phase-sensitive detection has further shown real-time two-color contrast in spectral-focusing configurations[11]. However, these techniques based on optical phase delay require substantial additional optical path deployment (approximately four meters) and precise time delay matching for the vibration energy of two target molecules. …

Against this backdrop, we provide a complementary, low-complexity approach: robust and strictly simultaneous two-channel contrast on a standard femtosecond spectral-focusing microscope. We exploit different polarization states of light, refractive index differences of varying wavelengths in media, and electrical phase delays to achieve simultaneous imaging of major biomolecules within the high wave-number C-H window. Our technology has two modes: rapid dual-color imaging and balanced detection on a spectral focusing-based SRS imaging system, with applications in real-time observation of living cells and clinicopathological tissues. The system achieves dual-phase modulation excitation and orthogonal signal demodulation through carefully designed dual-channel phase-shift photodetectors, along with optimized optical path design and circuit control. By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. …"

Discussion:

"… The Raman-shift separation ($\Delta\Omega$) is defined by the BFC length and is therefore discretely configurable rather than continuously tunable. However, this separation can be readily adapted in a plug-and-play manner by interchanging or cascading crystals of different lengths, providing practical flexibility while preserving optical stability and simplicity. …"

2. *The reported "11 dB SNR enhancement and 10-fold speed improvement" are not directly supported by numerical comparisons. Without side-by-side comparison against state-ofthe-art spectral-focusing or multiplex SRS, the performance claims remain questionable.*

[Reply] We thank the reviewer for raising this important point, and we have revised the Supplementary Note 7 to provide explicit numerical evidence supporting the reported SNR enhancement. Specifically, we conducted a controlled quantitative analysis by recording ten consecutive SRS spectra at demodulation times of 4 μs and 1 μs. For each measurement, a 10-ms integrated spectrum was used as a high-SNR approximation to the noise-free Raman response, allowing us to calculate the noise level of each single-shot spectrum before and after applying our collinear balanced-detection procedure. Because the two orthogonally polarized components propagate through the same optical and electronic pathways, their differential demodulation naturally suppresses correlated electronic noise and laser RIN without requiring an external reference arm or precise phase-locking. Across all independent spectra, this intrinsic balanced-detection configuration yielded a mean SNR improvement of 11 dB. These results are shown in Supplementary Fig. 8 and detailed in Supplementary Note 7. This quantitative benchmarking directly supports our SNR claim and establishes that the improvement arises from higher effective Stokes energy utilization together with robust suppression of common-mode fluctuations. The revised Supplementary Note now provides a clear methodological pathway and the numerical basis for this enhancement.

Supplementary Note 1 Collinear balanced detection

Without adjusting phase shifts between the two heterodyne demodulation channels (π/2), we naturally achieved collinear balanced detection with a phase difference of π, thereby enabling subtraction of common-mode noise in the electronic domain (Supplementary Fig. 8a). This configuration exploits the fact that both orthogonally polarized pump components share the same optical path and experience identical laser-intensity fluctuations, allowing their differential detection to suppress RIN without requiring dual-beam alignment or matched reference arms.

To quantitatively assess noise suppression, we recorded ten consecutive SRS spectra at demodulation times of 4 μs (Supplementary Fig. 8b) and 1 μs (Supplementary Fig. 8c). Each spectrum acquired with 10 ms total integration time was treated as a high-SNR approximation to the noise-free Raman response and used as the reference for estimating per-spectrum noise levels. For each of the ten spectra, the SNR was computed before and after balanced-detection processing. The balanced-detection configuration yielded a mean SNR improvement of 11 dB, arising from both higher effective energy utilization and robust rejection of correlated electronic and RIN fluctuations.

In contrast to traditional optical balanced-detection schemes that require precise phase matching between a sample arm and an external reference arm, which often necessitate active stabilization or additional optical hardware. Our approach achieves phase matching intrinsically because the two polarization channels originate from the same modulated Stokes beam and propagate through the exact same optical and electronic pathways. This collinearity eliminates drift-prone differential phase errors and substantially improves experimental robustness, especially during long acquisitions or when imaging turbid biological tissues.

Importantly, the collinear balanced-detection module also preserves the ability to demodulate two Raman frequency shifts (Ω_1 and Ω_2) simultaneously, although it does not separate them chemically at the detection stage. Instead, Ω_1 and Ω_2 separation occurs upstream via the birefringent-crystal-induced OPD. The resulting dual-channel demodulated signals thus reflect two vibrational resonances obtained under identical excitation and noise environments with improved SNR.

For imaging speed, under our standard spectral focusing configuration (single Raman shift), acquiring two wavenumbers requires two sequential scans:
Standard SRS (single shift): 512 × 512 pixels, 1 μs dwell → ~0.26 s per frame (which was previously given in the Supplementary Note 8). With SDRSRS, both channels are acquired *simultaneously*, so: SDRSRS: 0.26 s per dual-channel frame. Sequential dual-color SRS: two frames acquired consecutively → ~1 s (including controling the time delay linear stage by the sofeware and XY scanner returning to the starting point). Thus, under identical imaging parameters. Measured improvement: near 4× reduction in per-frame acquisition time.

But importantly, full spectral scanning (typical for CH-window SRS, 2740–3080 cm^{-1}) requires: Step size ~10–15 cm^{-1} → 23–35 spectral steps→ ~18–28 seconds per hyperspectral stack. Therefore: measured improvement over spectral scanning: ~70–100× faster. We have now added these quantitative comparisons directly to the manuscript.

Discussion
"…With identical imaging parameters (512 × 512 pixels, 1 μs dwell), standard spectral-focusing SRS requires two sequential scans to collect two Raman bands, resulting in a per-dual-color acquisition time of ~1s. SDRSRS acquires both bands strictly simultaneously within a single raster, reducing dual-channel acquisition time to 0.26 s. Compared to full hyperspectral CH-window scanning typically requiring ~18–28 s per stack, SDRSRS provides more than 10-fold faster acceleration. …"

We agree that modern SRS platforms that already provide one-shot CH-window coverage, high-speed dual-color imaging, and multiplex SRS. Using the references already cited in our manuscript and Supplementary Information, we have now added explicit comparison and clarified how SDRSRS is positioned relative to these technologies.

Introduction:

"… Modern SRS platforms already provide several of the capabilities that motivate our work. Super-multiplex vibrational imaging has been demonstrated with single-shot coverage of tens of Raman bands across the CH stretching window using tailored excitation and frequency-domain detection, enabling rapid acquisition of rich spectral information[1]. Hyperspectral and fingerprint-region SRS based on ultrafast tuning and spatial-spectral learning achieves microsecond-scale spectral scanning and rapid wavelength hopping across broad windows[2, 3]. Multiplex and broadband SRS implementations that use parallel detection or single-pixel reconstruction can capture the CH window in a single shot[4-7], but require spectrometers, multi-channel detection, specialized lasers, or computational reconstruction. Although CARS offers fast vibrational contrast, its strong non-resonant background, nonlinear signal dependence, dispersive line shapes, and sensitivity to sample heterogeneity hinder accurate quantification of CH_3/CH_2 ratios. Multiband CARS imaging also requires separate anti-Stokes detection and complex phase retrieval, making synchronous two-wavenumber acquisition difficult to achieve on femtosecond platforms. In parallel, multicolor and dual-color SRS schemes using synchronized picosecond sources or rapidly tunable optical parametric oscillators provide fast two-wavenumber imaging[8-10]. Dual-phase SRS leveraging polarization and phase-sensitive detection has further shown real-time two-color contrast in spectral-focusing configurations[11]. However, these techniques based on optical phase delay require substantial additional optical path deployment (approximately four meters) and precise time delay matching for the vibration energy of two target molecules. … Building on these advances, fibre-laser-based SRS architectures[12-16] have been translated into clinically compatible systems for intraoperative and histological applications.

Against this backdrop, we provide a complementary, low-complexity approach: robust and strictly simultaneous two-channel contrast on a standard femtosecond spectral-focusing microscope. We exploit different polarization states of light, refractive index differences of varying wavelengths in media, and electrical phase delays to achieve simultaneous imaging of major biomolecules within the high wave-number C-H window. Our technology has two modes: rapid dual-color imaging and balanced detection on a spectral focusing-based SRS imaging system, with applications in real-time observation of living cells and clinicopathological tissues. The system achieves dual-phase modulation excitation and orthogonal signal demodulation through carefully designed dual-channel phase-shift photodetectors, along with optimized optical path design and circuit control. By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables

simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. …"

Discussion

"…With identical imaging parameters (512 × 512 pixels, 1 μs dwell), standard spectral-focusing SRS requires two sequential scans to collect two Raman bands, resulting in a per-dual-color acquisition time of ~1s. SDRSRS acquires both bands strictly simultaneously within a single raster, reducing dual-channel acquisition time to 0.26 s. Compared to full hyperspectral CH-window scanning typically requiring ~18–28 s per stack, SDRSRS provides more than 10-fold faster acceleration. …"

" Supplementary Table 1 summarizes representative fast SRS platforms, including microsecond spectral-tuning systems, multiplex broadband detection, commercial dual-color SRS, dual-phase SRS, and fast-tuning picosecond fiber-laser architectures. Although each of these approaches has enabled significant progress, they rely on either multi-step spectral tuning, spectrometer-based detection, fixed Raman-shift separations, rapid wavelength hopping with interleaved pulses, or long free-space delay paths. These constraints lead to non-simultaneous acquisition of Raman bands or increased optical and electronic complexity, which can introduce temporal mismatch and reduce quantitative robustness in dynamic or intra-operative settings. In contrast, SDRSRS provides strictly simultaneous dual-Raman-shift acquisition without spectral tuning, spectrometers, synchronized dual-wavelength sources, or extended delay lines, and can be retrofitted onto standard femtosecond spectral-focusing microscopes. This positions SDRSRS as a complementary operating point optimized for simplicity, robustness, and reliable biochemical quantification in real-time clinical environments."

Supplementary Table 3 Comparison of representative fast SRS systems.

Platform	Temporal Resolution	Limitations Compared to SDRSRS
Microsecond spectral-tuning polygon systems[2]	~2–5 μs per step, but full CH-window still requires multi-step tuning	Not simultaneous; requires 20–30 steps for spectral scanning and deep learning
Fast-tuning picosecond fiber-laser SRS[12, 15]	Tens of microseconds to sub-millisecond tuning; dual-color acquisition achieved by rapid wavelength hopping or interleaved pulses	Non-simultaneous acquisition of multiple Raman bands; requires dual-wavelength synchronization hardware; potential temporal

		mismatch between channels for dynamic samples
Single-shot broadband multiplex SRS[4-7]	Single burst (μs)	Requires multi-channel photodiodes / spectrometer; not easily retrofitted to standard 2P microscopes
Commercial dual color SRS (DC-SRS), e.g., deltaEmerald, APE Inc.	N.A.	Only fixed 85 cm^{-1} energy difference set to address CH_2 & CH_3 at their peak position.
Dual-phase SRS[11]	Simultaneous 2-color	Requires ~4 m of free-space delay lines, sensitive alignment

3. *The cell and tissue imaging results are limited to the well-established lipid-protein contrast in the CH region. No new biological mechanism, diagnostic threshold, or biomarker discovery is reported in the MS..*

[Reply] We appreciate the reviewer's concern regarding the biological scope of the manuscript. Our intention in this work is not to propose new molecular biomarkers beyond the well-established CH_3-CH_2 contrast, but to demonstrate that several biologically and clinically relevant analyses require strictly simultaneous acquisition of these two vibrational channels—an operating regime that is not easily achieved with conventional spectral-scanning, sequential dual-color, or CARS-based approaches. While the lipid-protein contrast in the CH region is indeed well characterized, many of the biological conclusions presented here, including lipid-protein coupling dynamics in live cells, treatment-induced biochemical normalization in liver cancer immunotherapy, and stratification of human colorectal cancer tissues, depend on pixel-level ratiometry and temporal correspondence between CH_3 and CH_2 signals. These analyses cannot be reliably performed when the two channels are acquired seconds apart, as is the case with sequential or tunable systems, because cell morphology, hydration state, and tissue microstructure change on comparable timescales.

By enabling strictly synchronous acquisition within a single exposure, SDRSRS provides the biochemical stability necessary to quantify dynamic intracellular processes and to obtain diagnostically meaningful CH_3-CH_2 metrics in heterogeneous clinical tissues. Rather than aiming to discover a new mechanism or threshold, our work clarifies the methodological requirement for accurate use of existing biochemical markers and demonstrates how simultaneous dual-band SRS opens previously inaccessible applications in biological dynamics and intra-operative pathology.

We have revised the manuscript to make this purpose explicit and to highlight the translational significance of robust, simultaneous CH_3-CH_2 quantification.

We now emphasize this requirement in the manuscript and explicitly contrast SDRSRS with sequential two-color acquisition.

Results: Label-free cell chemical mapping and dynamics:

"Many cellular processes probed in this study, e.g. lipid droplet growth, fusion, micron-scale lipid-wave propagation, and cell swelling or division, evolve on sub-second to second timescales. To obtain quantitative insight into lipid-protein remodeling, strict synchrony between the CH_3 and CH_2 channels is required, because sequentially acquired Raman bands introduce 1–3 s inter-frame mismatch under typical scanning parameters. Such offsets distort pixel-wise CH_3/CH_2 ratios in living cells undergoing morphological changes. SDRSRS acquires CH_3 and CH_2 signals within a single exposure, enabling accurate temporal and spatial pairing of both signals, thereby preserving biochemical ratios at every pixel and every time point. This simultaneity enables reliable quantification of lipid-protein coupling dynamics and lipid-wave transport, enabling non-invasive, near real-time monitoring of intracellular chemical component changes."

Methods: Statistics and reproducibility

"All diagnostic analyses in this work, including CH_3/CH_2 ratiometry, PCA/UMAP clustering, and ROC discrimination, rely on accurate pairing of the two Raman channels. When two wavenumbers are acquired sequentially, tissue drift, hydration changes, and subtle deformations during raster scanning can artificially alter CH_3/CH_2 ratios and bias statistical classification. By ensuring strict simultaneity of the two channels, SDRSRS avoids these artifacts and provides temporally and spatially registered biochemical measurements that are beneficial for robust therapy-response quantification (Fig. 4) and CRC tissue stratification (Fig. 5). "

Response 4 (Reviewer #4)

In the manuscript "Simultaneous Dual-Raman-Shift Scanning-Free Stimulated Raman Scattering Microscopy for Label-Free Biomolecular Imaging" the authors present a simultaneous dual-Raman-shift scanning-free SRS (SDRSRS) microscopy, which captures different distinct molecular vibrational signatures simultaneously without spectral scanning. The authors achieve this through a combination of birefringent-crystal-induced optical path differences, polarization control, and dual-phase electrical demodulation, enabling simultaneous two-color SRS imaging with more than a tenfold increase in acquisition speed while preserving chemical specificity.

The approach is interesting, particularly because of its use of "idle" polarization states and a custom-built dual-channel phase-shift detector. The manuscript is well written and provides a comprehensive validation across a wide range of samples, including living cells, murine liver cancer models, and human colorectal cancer tissues. These experiments convincingly demonstrate the method's potential for real-time metabolic imaging, dynamic lipid tracking, and label-free histopathological assessment.

[Reply] We thank the reviewer for the thoughtful and encouraging evaluation of our work. We appreciate the recognition of the methodological contributions—including the use of birefringent-crystal-induced optical-path separation, exploitation of previously unused polarization states, and the design of the dual-channel phase-shift detector—as well as the reviewer's positive assessment of the biological and clinical demonstrations. We are pleased that the reviewer found the manuscript well written and the validation across living cells, murine liver cancer models, and human colorectal cancer tissues convincing. We have carefully considered the reviewer's subsequent comments and have revised the manuscript accordingly to further clarify the novelty, technical rigor, and translational relevance of SDRSRS.

1. *First, although the authors cite earlier dual-phase and multicolor SRS approaches, the manuscript does not clearly articulate how SDRSRS differs conceptually or technically from prior work, particularly He et al. (Optica 2017), nor does it adequately specify what limitations of these methods it overcomes. Additionally, a closely related approach was recently published by Xin & Huang (2025) (Simultaneous Dual-Color Stimulated Raman Scattering Biomolecular Imaging with Tailored Supercontinuum Generationand Phase-Sensitive Detection), which also demonstrates simultaneous dual-color SRS via phase-sensitive detection, though using PCF-based supercontinuum generation targeting the C-H and cell-silent windows. It would benefit the authors to position their work more clearly relative to this, highlighting the advantages of the birefringent-crystal-based OPD strategy in terms of stability, simplicity, and specificity.*

[Reply] We thank the reviewer for highlighting the need to more explicitly distinguish SDRSRS from earlier dual-phase and multicolor SRS implementations. We have now added context to

clarify the conceptual and technical distinctions. Although dual-phase SRS, such as the method reported by He et al. (Optica 2017), demonstrated real-time two-color imaging using polarization multiplexing and phase-sensitive detection, the underlying mechanism relies on meter-scale free-space delay lines to generate picosecond-level temporal offsets. This architecture requires careful phase matching, long-path stability, and precise environmental control. Even small drifts in temperature or beam pointing introduce timing fluctuations that degrade signal fidelity. In contrast, SDRSRS generates optical-path differences (OPDs) through passive birefringent-crystal dispersion, which produces femtosecond-level delays that are intrinsically stable and do not require mechanical delay scanning or external synchronization. Because the two polarization channels share the same beam path, SDRSRS does not need alignment sensitivity and phase drift—limitations that are intrinsic to conventional dual-phase designs.

Furthermore, SDRSRS integrates birefringent OPD generation with same-frequency hetero-phase modulation and dual-channel electrical demodulation, enabling simultaneous retrieval of two Raman shifts without wavelength tuning, synchronized picosecond sources, or spectrometer-based detection. This is a different operational regime from prior dual-color SRS methods, which typically rely on two synchronized lasers, tunable OPOs, or sequential wavelength switching. The plug-and-play nature of the birefringent-crystal module also provides adjustable Raman-shift separations simply by exchanging crystal lengths, whereas earlier dual-phase schemes offer fixed energy differences provided by commercial laser such as deltaEmerald, APE Inc.

We also appreciate the reviewer's reference to the recent work by Xin & Huang (2025), which demonstrates simultaneous dual-color SRS using tailored supercontinuum generation (SCG) and phase-sensitive detection. Their approach is technically elegant and broadens the spectral reach of simultaneous SRS into both CH and cell-silent regions. However, SCG-based systems depend on nonlinear broadening in photonic crystal fibers, high peak-power excitation, spectral filtering to isolate usable components, and careful management of spectral coherence. These requirements increase system complexity and impose sensitivity to laser fluctuations and fiber coupling conditions. In contrast, SDRSRS is explicitly designed for mechanical robustness, spectral purity, and seamless integration with standard femtosecond spectral-focusing microscopes already used in biology and clinical research. By avoiding SCG altogether, SDRSRS maintains fixed excitation spectra, minimizes wavelength crosstalk, and ensures that both Raman shifts are generated under identical excitation conditions—an advantage for quantitative ratiometric analyses, metabolic dynamics, and intra-operative tissue evaluation. We have revised Introduction to present these distinctions clearly and to position SDRSRS as a complementary alternative that prioritizes stability, simplicity, and compatibility with widely available imaging platforms.

Introduction:

"... In parallel, multicolor and dual-color SRS schemes using synchronized picosecond sources or rapidly tunable optical parametric oscillators provide fast two-wavenumber imaging[8-10]. Dual-phase SRS leveraging polarization and phase-sensitive detection has further shown real-time two-color contrast in spectral-focusing configurations[11]. However, these techniques based on optical phase delay require substantial additional optical path deployment (approximately four meters) and precise time delay matching for the vibration energy of two target molecules. A recently reported simultaneous dual-color SRS imaging[20] synthesizes two vibrational excitation bands from a photonic-crystal-fiber-based supercontinuum and achieves simultaneous detection through spectrally filtered demodulation. This requires high peak-power excitation, precise control of nonlinear broadening, spectral filtering to stabilize output coherence, and careful management of fiber coupling conditions, increasing system complexity and sensitivity to environmental perturbations. …"

Against this backdrop, we provide a complementary, low-complexity approach: robust and strictly simultaneous two-channel contrast on a standard femtosecond spectral-focusing microscope. We exploit different polarization states of light, refractive index differences of varying wavelengths in media, and electrical phase delays to achieve simultaneous imaging of major biomolecules within the high wave-number C-H window. Our technology has two modes: rapid dual-color imaging and balanced detection on a spectral focusing-based SRS imaging system, with applications in real-time observation of living cells and clinicopathological tissues. The system achieves dual-phase modulation excitation and orthogonal signal demodulation through carefully designed dual-channel phase-shift photodetectors, along with optimized optical path design and circuit control. By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. This approach offers a complementary solution that provides fast two-channel molecular contrast without spectral scanning and integrates a switchable collinear balanced-detection mode. This design facilitates high-speed, label-free molecular imaging for biomedical and clinical applications while maintaining compatibility with widely used two-photon-based SRS microscopes. …"

2. *In addition, the fixed Raman-shift separation determined by the birefringent crystal length is a significant constraint that restricts flexibility and limits broader applicability; this limitation is not sufficiently acknowledged, and potential mitigation strategies are not discussed.*

[Reply] We thank the reviewer for raising this important point regarding the flexibility of Raman-shift separation. In the revised manuscript we describe the practical strategies we have implemented to address it. Although the Raman-shift difference in SDRSRS is set by the optical-path delay of the birefringent crystal, this separation is not fixed by the system design. In practice, we fabricated a set of interchangeable crystals with lengths of 1 cm and 2 cm, which can be used individually or cascaded to provide effective optical-path differences equivalent to 1–4 cm. This enables users to select the desired Raman-shift separation in a plug-and-play manner without modifying the laser source, beam geometry, or detection module. For applications requiring different spectral separations, such as shifts between CH_3-CH_2 or selected bands, crystals of alternative thicknesses can be manufactured with high reproducibility and swapped within seconds. The impedance of fine-grained tuning is therefore practical rather than fundamental, and the passive nature of the birefringent module preserves long-term alignment stability, which we found advantageous for biological and intra-operative imaging. We have added text in the Introduction to explicitly clarify this tunability, stating that our approach provides adjustable separations through interchangeable crystal lengths, unlike fixed-wavelength dual-color schemes. This revision makes clear that SDRSRS is not limited to a single Raman-shift separation and that its flexibility can be extended according to application requirements.

Introduction:

"... By leveraging birefringent-crystal-induced optical path difference (OPD) , our technique enables simultaneous collection of bimolecular vibration information. Unlike fixed-wavelength dual-color schemes, the Raman-shift separation in our system is readily adjustable by interchanging or cascading birefringent crystals of different lengths (e.g., 1–4 cm), providing a plug-and-play means to select distinct vibrational pairs without updating the laser source. This passive OPD module, together with a compact dual-channel phase-sensitive detector, can be retrofitted into most SRS systems that share components with two-photon microscopes, avoiding the need for complex system reconfiguration. …"

Discussion:

"… The Raman-shift separation ($\Delta\Omega$) is defined by the BFC length and is therefore discretely configurable rather than continuously tunable. However, this separation can be readily adapted in a plug-and-play manner by interchanging or cascading crystals of different lengths, providing practical flexibility while preserving optical stability and simplicity. …"

3. *The biological interpretation of CH_2/CH_3 contrast in cancer tissues is occasionally overstated, as these ratios are influenced by multiple confounding factors such as stromal composition, extracellular matrix content, fibrosis, necrosis, and sample-preparation variability, and*

therefore should be described more cautiously. In most Raman-publication this ratio is references. One wonders about the true specificity.

[Reply] We thank the reviewer for this important comment. We fully agree that CH_2/CH_3 contrast and their ratios should not be interpreted as uniquely specific biomarkers, because they are influenced by multiple confounding factors, including stromal and immune-cell composition, extracellular matrix and collagen content, fibrosis, edema, necrosis, tissue architecture, and sample-preparation variability. Our intention is not to claim that the CH_2/CH_3 ratio represents a single, mechanistically specific marker of cancer, but rather that it provides a composite biochemical contrast that, when acquired strictly simultaneously and analyzed statistically, helps to differentiate tissue states within a well-defined experimental context. In response to the reviewer's concern, we have carefully revised the text in the liver cancer and colorectal cancer sections as well as in the Discussion to remove any language that might be read as overclaiming biological specificity. We now explicitly describe CH_2/CH_3 contrast as a relative, context-dependent biochemical index that reflects the combined contributions of cellular and stromal compartments and clearly state that it must be interpreted together with histopathology and clinical information.

Concretely, in the main text we have removed phrases that could imply that CH_2/CH_3 ratios alone define diagnostic thresholds or single-pathway mechanisms. We instead emphasize statistical separability and relative biochemical trends, and we now acknowledge that changes in these ratios may arise from variations in tumor cell density, stromal infiltration, fibrosis, necrotic regions, and section preparation, in addition to intrinsic metabolic reprogramming. In the revised Discussion we further clarify that the present work does not propose CH_2/CH_3 as a standalone biomarker, but rather demonstrates that synchronous dual-band acquisition is necessary to use this widely referenced ratio in a quantitatively reliable way. We also highlight that future studies combining SDRSRS with more detailed histological annotation and molecular profiling are required to disentangle the specific contributions of individual microenvironmental components. These revisions ensure that our interpretation of CH_2/CH_3 contrast is cautious, transparent, and aligned with the broader Raman literature.

Section: Label-free chemical distinction of liver cancer immunotherapy
Original:
"These data provide important references for understanding sample changes under different treatment conditions."
Revised:
"These data provide a relative biochemical contrast between groups under different treatment conditions. We note that CH_3/CH_2 ratios represent a composite signal arising from both tumor cells

and surrounding stroma, and may be influenced by extracellular matrix content, fibrosis, necrosis, and section preparation. Accordingly, these ratios should be interpreted as statistical descriptors of group-level biochemical differences rather than as specific molecular biomarkers."

Section: Label-free chemical diagnosis of human colorectal cancer (CRC)
Original:
"This difference likely reflects distinct metabolic and biosynthetic pathways between cancerous and non-cancer tissues."
Revised:
"This difference is consistent with altered metabolic and biosynthetic activity between cancerous and non-cancer tissues, but the CH_3/CH_2 ratio is not uniquely specific to any single pathway. It reflects the combined contributions of tumor cells, stromal and immune components, extracellular matrix, and tissue processing, and therefore should be viewed as a composite biochemical index rather than a standalone molecular marker."

Original:
"The observed differences indicate that cancer tissues undergo significant alterations in relative protein and lipid content. Cancer tissues demonstrate either relatively higher lipid content or inhibited protein expression, which likely correlates with metabolic abnormalities and alterations in biosynthetic pathways characteristic of cancerous states."
Revised:
"The observed differences indicate that cancer tissues, together with their associated stroma, exhibit altered relative protein-lipid contrast compared to paracancerous regions. While such shifts are compatible with known metabolic abnormalities in cancer, the CH_3/CH_2 contrast measured here integrates multiple microenvironmental factors, including tumor cell density, stromal remodeling, fibrosis, and local necrosis. As such, it should be interpreted cautiously and in conjunction with histopathological assessment rather than as a single, highly specific biochemical signature."

Discussion:
Added text:
"An additional and important consideration concerns the biological interpretation of CH_3/CH_2 contrast in heterogeneous tissues. Although this ratio is widely used in Raman imaging as a proxy for relative protein and lipid content, it is inherently composite rather than molecularly specific. In complex tissues, CH_3/CH_2 values reflect the combined contributions of tumor cells, stromal and immune components, extracellular matrix and collagen, fibrotic remodeling, necrotic regions, and potential variability introduced during tissue processing and sectioning. Accordingly, in this study we use CH_3/CH_2 primarily as a quantitative, group-level biochemical contrast to differentiate conditions, such as cancer versus paracancerous tissue or pre- versus post-treatment states.

Differences are interpreted in terms of statistical separability and reproducibility, rather than ascribing a unique mechanistic meaning to any absolute ratio value. Within this framework, the primary contribution of SDRSRS is to provide strictly simultaneous and spatially registered CH_3 and CH_2 measurements, which are essential for reliable use of such composite biochemical indices. Definitive biological specificity will ultimately require integration with histopathology, immunohistochemistry, and complementary molecular assays."

4. *The statistical analysis in the mouse immunotherapy study raises concerns because the reported sample size largely reflects pixel-level data rather than biological replicates, which inflates the apparent separation in PCA, UMAP, and ROC analyses; per-animal statistical treatment is required to substantiate claims about treatment effects.*

[Reply] We thank the reviewer for raising this critical point regarding statistical rigor. We agree that treating pixel-level measurements as independent samples can artificially inflate apparent separation in multivariate analyses. In fact, we performed biological replication at the animal level to substantiate conclusions about treatment effects. We now have substantially revised the manuscript to clarify the replicate structure and to ensure that all statistical analyses are grounded in biological, not pixel-level, replication.

Specifically, in the revised manuscript we now explicitly distinguish biological replicates (animals, tissue sections) from technical replicates (fields of view and pixels). For the mouse liver cancer and immunotherapy experiments, all quantitative conclusions are based on n = 12 mice in total (normal: 3; cancer: 3; CRT: 3; PDT: 3). For each animal, 4–6 imaging regions were acquired from three independent tissue sections, and pixel-level values were first aggregated within each region and then averaged per animal before downstream statistical analysis. Thus, the effective unit of replication for PCA, UMAP, correlation analysis, and ROC evaluation is the animal images, not individual pixels.

To make this explicit and transparent, we have revised the Statistics and reproducibility section to state clearly that "all statistical tests were performed on biological replicates rather than single images or pixels," and we now report sample sizes consistently in the figure captions. The PCA, UMAP and ROC plots shown in Fig. 4g,h reflect animal-level averaged features derived from paired CH_3-CH_2 measurements, ensuring that the observed group separation reflects inter-animal biochemical variability rather than within-image redundancy.

"Statistics and reproducibility"

"All biological and technical replicates used in this study are reported in the figure captions. For cultured-cell imaging, quantitative analyses were performed across n = 16 cells acquired from

three independent cultures. For mouse liver cancer and immunotherapy experiments, measurements were obtained from n = 12 mice in total (normal: 3; cancer: 3; CRT: 3; PDT: 3), with 4–6 imaging regions per animal and three tissue sections per condition. For human CRC studies, SDRSRS imaging was performed on n = 21 patients, each contributing paired cancer and paracancerous tissues. PCA, k-means clustering, correlation matrices, and ROC analyses were conducted on 84 tissue regions sampled across these patients. Each imaging dataset included technical replicates obtained from repeated acquisitions of the same field to verify instrument stability. All statistical tests were performed on these biological replicates rather than single images. Measures of central tendency and confidence intervals are reported in the main text and figures."

Add to Fig. 3 caption:
(e) Comparison of component intensity and component ratio at 2930 cm^{-1} and 2843 cm^{-1} between cells with low lipid content and lipid-rich cells (n = 16).

Figure 4 and 5 already have sample sizes.

5. *Furthermore, the system's long-term stability, i.e. phase stability, drift under experimental conditions, and susceptibility to depolarization in thick or scattering tissues is not quantified, though this stability is essential.*

[Reply] We thank the reviewer for highlighting the importance of long-term system stability, including phase drift and susceptibility to depolarization, which are important for quantitative SRS imaging and clinical translation. We have revised the text to clarify both the intrinsic stability of the SDRSRS architecture and its current limitations.

In SDRSRS, the two Raman channels originate from the same pump-Stokes pulse pair and propagate collinearly through identical optical elements before detection. The birefringent-crystal-induced OPD is entirely passive and does not rely on mechanically scanned delay lines or long free-space interferometric arms, which are known sources of phase drift in dual-phase SRS systems. As a result, the relative phase between the two channels is intrinsically stable against thermal fluctuations, vibration, and air turbulence. This stability is further reinforced by orthogonal electrical demodulation and, when enabled, collinear balanced detection, which suppresses common-mode intensity and phase noise.

In the revised manuscript, we now provide explicit quantification of system stability over extended imaging sessions. During continuous operation over 2 hours, we observed <2% intensity drift in both demodulated channels, <0.5° deviation in relative phase, and no measurable drift in the Raman-shift separation $\Delta\Omega$ under standard laboratory conditions. These measurements confirm

that SDRSRS is intrinsically phase-locked and stable over biologically relevant timescales. Consistent with this, long-term time-lapse imaging of living cells showed no detectable drift in CH_3/CH_2 ratios or degradation of channel separation.

We also acknowledge that polarization-dependent approaches, including SDRSRS, may be affected by depolarization in thick or highly scattering tissues. In the present study, all tissue experiments were performed on thin sections, where polarization scrambling is minimal and stable dual-channel separation is preserved. We now explicitly state this limitation in the Discussion and clarify that extending SDRSRS to thick or *in vivo* tissues will require additional strategies, such as polarization-diversity detection or epi-SRS geometries, which are beyond the scope of the present work. These revisions provide a more balanced and transparent description of system stability, robustness, and applicability.

Section: Label-free cell chemical mapping and dynamics
Original:
"After extended observation, no weakening of the SRS signal was detected, and component ratios remained unchanged (Supplementary Fig. 5)."
Revised:
"After extended observation, no weakening of the SRS signal was detected, and CH_3/CH_2 component ratios remained unchanged (Supplementary Fig. 5), indicating stable relative phase and preserved channel separation during long-term time-lapse imaging under typical experimental conditions."

Section: Discussion
Original:
"In highly scattering or thick tissues, sample-induced depolarization may reduce signal contrast, and fully in vivo SRS imaging remains technically challenging, as is the case for most transmission-mode SRS implementations."
Revised:
"In highly scattering or thick tissues, sample-induced depolarization may reduce signal contrast, and fully *in vivo* SRS imaging remains technically challenging, as is the case for most transmission-mode SRS implementations. All tissue experiments in this study were performed on thin sections, where polarization scrambling is minimal and stable dual-channel separation is preserved. The BFC-induced OPD is entirely passive and provides intrinsically stable phase relations without mechanically scanned delay lines, mitigating long-term drift under typical experimental conditions (Supplementary Note 9). "

Methods:

"Surgically resected tissue samples were rapidly frozen in liquid nitrogen and stored at -80°C until sectioning into 25 μm slices using a cryogenic sectioning machine …"

Supplementary Note 9 Long-term stability characterization

To quantitatively evaluate long-term system stability, we monitored the relative phase, Raman-shift separation ΔΩ, and signal intensity of both demodulated channels during continuous operation over a 2-hour period. Measurements were performed under standard laboratory conditions without active environmental stabilization. The relative phase deviation between the two channels remained below 0.5°, while the normalized signal intensity exhibited <2% fluctuation over the entire duration. No measurable drift in ΔΩ was observed, confirming the stability of the birefringent-crystal–induced OPD. These results demonstrate that SDRSRS is intrinsically phase-locked and robust against long-term drift.

6. *Finally, some figure sections are dense and could be clarified with simplified schematics or improved structuring, and the terminology regarding "healthy" versus "unhealthy" cells or lipid-driven cellular deterioration requires clearer definition and more conservative phrasing.*

[Reply] We appreciate the reviewer's constructive suggestions regarding figure clarity and terminology. We agree that several multipanel figures contain dense information, and we have revised their layout to improve readability.

Regarding the terminology used to describe cellular states, we agree that the phrases "healthy" and "unhealthy" were overly informal and could imply biological conclusions not directly supported by our measurements. In the revised manuscript, we have replaced these terms with more precise descriptors such as "cells exhibiting low Raman-lipid burden" and "cells exhibiting elevated lipid accumulation," and we now describe biochemical changes explicitly rather than inferring cellular deterioration. We also emphasize that SDRSRS quantifies lipid–protein ratios as biochemical signatures rather than diagnostic endpoints, and we present changes in CH_2/CH_3 contrast as correlates of cellular phenotype rather than categorical indicators of health status. This more conservative phrasing reflects the inherent multifactorial nature of Raman contrasts, which can be shaped by organelle composition, metabolic activity, and environmental conditions.

Section: Label-free cell chemical mapping and dynamics
We Replace: "healthy cells" / "unhealthy cells" with: "cells with low lipid content" / "lipid-rich cells" in the main text, Figure 3e and figure caption.

Original:
"We compared the component normalized intensities and component ratios at 2930 cm^{-1} and 2843 cm^{-1} between healthy cells (Supplementary Fig. 2a) and unhealthy cells (Supplementary Fig. 2b), as shown in Fig. 3e. This revealed differences in SRS signal between healthy and unhealthy cells. Changes in the component ratio may indicate important shifts in cellular health status. This suggests that excessive lipid accumulation may lead to cellular deterioration, or alternatively, declining cell condition may increase lipid accumulation[21]."
Revised:
"We compared the normalized component intensities and CH_3/CH_2 ratios at 2930 cm^{-1} and 2843 cm^{-1} between cells with low lipid content (Supplementary Fig. 2a) and lipid-rich cells (Supplementary Fig. 2b), as summarized in Fig. 3e. Lipid-rich cells exhibited systematically altered CH_3/CH_2 ratios relative to lipid-poor cells, reflecting coordinated changes in protein- and lipid-associated vibrational signals. Although these ratios are not specific to a single biochemical pathway, such variations are consistent with differences in cellular metabolic state and structural composition reported in prior studies[21]. This observation may indicate a correlation between cellular condition and relative lipid abundance, highlighting the utility of simultaneous dual-band SRS measurements for capturing phenotypic biochemical heterogeneity in living cells. "

Minor issue:

Please correct grammar throughout the manuscript

1. *Second paragraph introduction -> usinga ->using*
2. *Introduction -> we exploits-> we exploit*
3. *Results -> biomoleculer -> biomolecular*
4. *Abbreviations should be introduced when used first: HWP and QWP*
5. *The authors clame that the improvement is 10-fold, but I could not identify any location how this was particularily assessed.*

[Reply] We thank the reviewer for pointing out these grammatical and formatting issues. We have carefully proofread the entire manuscript and corrected all identified errors including typographical mistakes.

For imaging speed, under our standard spectral focusing configuration (single Raman shift), acquiring two wavenumbers requires two sequential scans:
Standard SRS (single shift): 512 × 512 pixels, 1 µs dwell → ~0.26 s per frame (which was previously given in the Supplementary Note 8). With SDRSRS, both channels are acquired *simultaneously*, so: SDRSRS: 0.26 s per dual-channel frame. Sequential dual-color SRS: two frames acquired consecutively → ~1 s (including controling the time delay linear stage by the

sofeware and XY scanner returning to the starting point). Thus, under identical imaging parameters. Measured improvement: near 4× reduction in per-frame acquisition time.

But importantly, full spectral scanning (typical for CH-window SRS, 2740–3080 cm^{-1}) requires: Step size ~10–15 cm^{-1} → 23–35 spectral steps→ ~18–28 seconds per hyperspectral stack. Therefore: measured improvement over spectral scanning: ~70–100× faster. We have now added these quantitative comparisons directly to the manuscript.

Discussion

"…With identical imaging parameters (512 × 512 pixels, 1 μs dwell), standard spectral-focusing SRS requires two sequential scans to collect two Raman bands, resulting in a per-dual-color acquisition time of ~1s. SDRSRS acquires both bands strictly simultaneously within a single raster, reducing dual-channel acquisition time to 0.26 s. Compared to full hyperspectral CH-window scanning typically requiring ~18–28 s per stack, SDRSRS provides more than 10-fold faster acceleration. …"

> *In summary, the work represents a promising and innovative contribution to high-speed vibrational imaging, and the overall methodology is strong, but some revisions are necessary to more clearly position the technical novelty, appropriately biological conclusions, strengthen statistical rigor, and document stability and performance limitations.*

[Reply] We thank the reviewers for the thoughtful and constructive summary, and we agree that clearer positioning of technical novelty, cautious biological interpretation, rigorous statistical treatment, and explicit discussion of system stability are essential for strengthening the manuscript. In response, we have substantially revised the text to address each of these points.

First, we have clarified the conceptual and technical novelty of SDRSRS relative to prior dual-phase and dual-color SRS approaches by explicitly emphasizing its strictly simultaneous dual-Raman-shift acquisition on a standard femtosecond spectral-focusing platform, without spectral scanning, long free-space delay lines, synchronized dual-wavelength sources, or spectrometer-based detection. We have strengthened the Introduction and Discussion to clearly position SDRSRS as a complementary operating point optimized for robustness, simplicity, and quantitative fidelity in dynamic and clinical settings, and explicitly discussed recent related work to delineate differences in system complexity, stability, and operating principles.

Second, we have revised the biological interpretation throughout the manuscript to adopt a more cautious and statistically grounded tone. In particular, CH_3/CH_2 contrast is now consistently

described as a composite biochemical readout influenced by multiple factors rather than a specific biomarker. All interpretations have been reframed in terms of correlation, statistical separability, and consistency with prior literature, without implying unique mechanisms or diagnostic thresholds. This applies to both the cell studies and the cancer tissue analyses.

Third, we have strengthened statistical rigor by clearly distinguishing biological replicates from pixel-level data. The "Statistics and reproducibility" section has been expanded to explicitly report the number of independent cells, animals, tissue sections, and patients, and all PCA, UMAP, correlation, and ROC analyses are now stated to be performed on biological replicates rather than single images. Figure captions have been updated accordingly to ensure transparency and avoid inflation of statistical significance.

Finally, we have explicitly addressed system stability and limitations. The revised Discussion now clarifies the intrinsic phase stability of SDRSRS arising from its collinear, passive birefringent-crystal–based OPD design, while also acknowledging limitations related to depolarization in highly scattering or thick tissues and the current focus on the C–H stretching region. These considerations are now clearly stated to define the present scope and future directions without overstating applicability.

We believe these revisions more clearly articulate the technical contribution of SDRSRS, appropriately constrain biological conclusions, and transparently document system limitations. We hope that the revised manuscript now more accurately reflects the strengths and scope of the work and meets the standards expected for publication in Nature Communications.

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Dear reviewers,

We sincerely thank all reviewers for their careful evaluation of our manuscript and for the constructive feedback provided throughout the review process.

We are pleased that the reviewers find that all concerns have been adequately addressed and that the revised manuscript is now suitable for publication. As no further questions or revisions have been raised, we have no additional changes to report.

We appreciate the reviewers' positive assessment of the methodological clarification, improved analysis, and overall presentation of the work.

This MS reports a dual-color SRS imaging platform aiming to achieve simultaneous SRS imaging at two wavenumbers without spectral scanning. The major principle is to use polarization and birefringent crystal to generate a pair of pulses at different time delays. In addition, the authors introduced in line balanced detection to improve the signal to noise ratio. While the goal of improving imaging speed and simplifying SRS operation is great, I am afraid that the methodology does not show clear novelty or advantage over the existing methods, nor does it demonstrate compelling biomedical applications to meet the publication standard of Nature Communications. I have some specific concerns as follows:

1. The use of birefringent crystals for path-length differentiation and orthogonal polarization modulation builds upon established dual-phase SRS methods (e.g., He et al., Optica 2017, ref 33 in the MS). Also, the wavenumber difference between the two channels is fixed, further limiting the versatility of the method.

2. The reported “11 dB SNR enhancement and 10-fold speed improvement” are not directly supported by numerical comparisons. Without side-by-side comparison against state-of-the-art spectral-focusing or multiplex SRS, the performance claims remain questionable.

3. The cell and tissue imaging results are limited to the well-established lipid-protein contrast in the CH region. No new biological mechanism, diagnostic threshold, or biomarker discovery is reported in the MS.