Multi-window sparse spectral sampling stimulated Raman scattering microscopy

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Abstract: Stimulated Raman scattering (SRS) is a nondestructive and rapid technique for imaging of biological and clinical specimens with label-free chemical specificity. SRS spectral imaging is typically carried out either via broadband methods, or by tuning narrowband ultrafast light sources over narrow spectral ranges thus specifically targeting vibrational frequencies. We demonstrate a multi-window sparse spectral sampling SRS (S₄RS) approach where a rapidly-tunable dual-output all-fiber optical parametric oscillator is tuned into specific vibrational modes across more than 1400 cm⁻¹ during imaging. This approach is capable of collecting SRS hyperspectral images either by scanning a full spectrum or by rapidly tuning into select target frequencies, hands-free and automatically, across the fingerprint, silent, and high wavenumber windows of the Raman spectrum. We further apply computational techniques for spectral decomposition and feature selection to identify a sparse subset of Raman frequencies capable of sample discrimination. Here we have applied this novel method to monitor spatiotemporal dynamic changes of active pharmaceutical ingredients in skin, which has particular relevance to topical drug product delivery.

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1. Introduction

Optical microscopy is an invaluable tool to visualize and quantify responses to environmental perturbations with subcellular resolution. Conventional microscopic methods are well suited to characterize changes in morphology and structure, but only provide limited contrast regarding chemical composition, primarily manifesting as changes in scattering and absorption. The development of microscopy methods that are inherently sensitive to chemical composition to generate spectral contrast in images has dramatically increased the information that can be extracted from biological and clinical specimens at high spatiotemporal resolution. In particular, coherent Raman imaging methods have become widely applied as non-destructive methods to investigate biological systems [1,2]. Among these methods, stimulated Raman scattering (SRS) microscopy is particularly appealing as a tool that can be implemented at video-rate with minimal background and a signal intensity that is linear with molecular concentration [3,4]. Furthermore, as a multiphoton microscopy method, SRS is inherently depth sensitive, which provides the opportunity for the acquisition of volumetric imaging data over time with specific molecular contrast. Compared to coherent anti-Stokes Raman scattering (CARS) approaches, SRS is free of nonresonant background generation that degrades image contrast (Fig. 1). Additionally, the SRS spectrum is similar to that of spontaneous Raman, as the SRS process is parametric and only generates signal at real vibrational resonances (Fig. 1(a)). Initially demonstrated by Freudiger et al., SRS can be implemented with fiber-based light sources to enhance the potential for translation into biological, medical, and pharmaceutical applications [5]; the ongoing development of these instruments is an active area of research [6–8].

Label-free microscopy methods with high spatiotemporal resolution that are sensitive to compositional differences, such as SRS, may provide a valuable means to visualize and quantify
the dynamic interactions of drug formulations in local tissue environments. The estimation of the active pharmaceutical ingredient (API) dose that penetrates the stratum corneum and permeates into deeper skin stratifications has been investigated through a multitude of methodologies ranging from non-invasive to semi-invasive [9–14]. Each of these methodologies provides macroscale, or bulk, cutaneous bioavailability, and potentially bioequivalence - estimates that are helpful in the comparison of two topically applied drug products. On the other hand, in preclinical drug development it is advantageous to understand the localization and exposure of the API at the intended local site(s) of action, and thus the microscale bioavailability (i.e., local bioavailability within structures, such as corneocytes or potentially sebaceous glands). SRS imaging affords the opportunity to provide insight on the API's route of percutaneous permeation [10], yet, to date, has primarily targeted vibrational frequencies in the silent region of the Raman spectrum to avoid confounding tissue signals [15]. Along with the APIs, these drug products also contain inactive ingredients (i.e., excipients) that can improve the delivery of the API to the desired location and as such it is important to quantify the inactive ingredients’ and APIs’ concentration-time profiles. Measuring an API and its formulation’s inactive ingredients with respect to tissue signatures requires multi-window spectral information that essentially spans the entire Raman vibrational spectrum from the fingerprint to the high wavenumber windows, but collecting this data rapidly for real-time concentration quantification is not feasible with current tools; these dynamic changes occur on the order of minutes where the time required for a full spectral sweep would miss vital information. The development of an automated system to rapidly tune between specific Raman frequencies spanning the entire spectrum would allow targeted, dynamic studies to improve our understanding of how various inactive ingredients ultimately influence the topical bioavailability/bioequivalence of the API under investigation.

SRS is inherently a multi-frequency process. Most simply, two fields of different frequencies that are copropagated and are in resonance with vibrational frequencies in a sample generate signal (Fig. 1(b,c)). SRS imaging is commonly implemented for a single frequency target by manually tuning the temporal delay between these fields to obtain selective contrast. However, by varying the frequency difference between these fields, distinct vibrations can be excited and visualized within a sample, which is known as spectral or hyperspectral SRS. As a multi-frequency process, there are a number of methods that have been used to achieve spectral excitation.

The simplest implementation of spectral SRS is achieved by frequency tuning [3,5,15]. Here, a series of SRS images is acquired by changing the laser frequency (Δω) of one or more fields. Given that the time required to tune laser frequencies to a vibration of interest directly limits the speed of imaging, the use of a high-speed frequency-tunable laser is paramount. Ideally for rapid tuning, the time requirement for frequency tuning would be significantly less than the image acquisition time. Frequency tuning provides flexibility for spectral SRS data acquisition,
enabling collection of continuous spectra or discrete sampling of target vibrations with *a priori* knowledge.

Another widely adopted method for spectral SRS imaging is based on spectral focusing [16–20], in which chirped femtosecond pulses are used. As shown in Fig. 1(d), the optical frequency difference between pump and Stokes pulses can be maintained for several picoseconds, conferring high spectral resolution comparable to the case of picosecond excitation. An important advantage of spectral focusing is that tuning the frequency difference is achieved by changing the optical delay ($\Delta \tau$), which is traditionally less challenging from a technical perspective than frequency tuning for most coherent Raman imaging systems.

A further method for hyperspectral SRS is based on multiplexing wavelengths. This is most commonly achieved through the use of a picosecond Stokes pulse and broadband pump pulses that have a much shorter pulse duration. This approach excites multiple SRS wavelengths simultaneously and uses a spectrometer equipped with photodiode array and lock-in amplification for concurrent detection of multiple SRS signals [21]. Alternatively, spectrometer-free approaches have also been reported that trade detection system complexity for challenges associated with photodetector saturation [22] or through the use of modulation-frequency-division that modulates individual spectral components at unique frequencies [23]. These methods, along with alternative approaches that utilize more than two colors (either with time-gating while frequency tuning, additional single color fields, or with multicolor broadband sources) have been investigated to increase the speed of spectral imaging (in some cases 30 frames/second or higher), increase the spectral resolution of the acquired spectral data, or to expand and control the probed spectral window [24–30]. Despite the improvements in imaging speed and spectral resolution, systems have a finite spectral tuning range without manual reconfiguration; this limits the range of spectral acquisition ($\sim 300 \text{ cm}^{-1}$) based upon the excitation laser bandwidth. Among these methods, Figueroa et al. demonstrated the broadest spectral tuning range ($\sim 680 \text{ cm}^{-1}$) to date, without the requirement of manually changing frequencies, based on the incorporation of a parabolic fiber amplifier [28]. Incorporating this technology with spectral focusing offers expanded spectral availability, yet still prohibits spectral tuning between the feature-dense fingerprint window and the intense, high wavenumber window of the Raman spectrum without user intervention.

The advent of spectral SRS imaging has also necessitated the development of computational methods for extracting spectral features from the data; specific spectral features provide the ability to map concentrations and localize structures based on distinct vibrational signatures. Numerous multivariate approaches for spectral decomposition and mapping have been applied, often based on modified multivariate tools investigated with spontaneous Raman scattering or hyperspectral imaging methods [20,24,26,27,31]. Several approaches including principal component analysis (PCA), independent component analysis (ICA), multivariate curve resolution (MCR), and vertex component analysis (VCA) have been implemented and directly compared with varying levels of success [32]. These methods consistently enable sample separation; however, some methods such as MCR [20] and modified ICA [26] extract spectral profiles that more accurately recapitulate the Raman spectra of pure components based on the user-defined number of sample components. For many biological and clinical samples, acquiring full spectral data may not be necessary for characterization. Some Raman spectral features exhibit multicollinearity while others do not provide data that can differentiate signals of interest. By implementing efficient SRS spectral sampling via a sparse subset of features, the relevant changes in samples can be imaged with a small number of frequencies while providing the same information as full-spectrum datasets. Furthermore, by using feature selection to minimize the number of frequencies imaged and rapidly tuning between these sparse spectral features, multicolor live SRS imaging may be achieved, which improves the temporal resolution to measure rapid, dynamic changes. Of the many reported computational approaches for spectral SRS, the "factorization into susceptibilities and concentrations of the chemical components" (FSC$^3$) [31] and sparse partial least squares
discriminant analysis (sPLSDA) [33–35] algorithms are among the few that specifically focus on sparse feature selection from spectral data and implement multistep approaches for decomposition and feature extraction [31]. Such approaches may have limited performance in complex biological or clinical samples: in sPLSDA, users must predefine the number of features to extract and the performance of the FSC\(^3\) algorithm may underperform for diffuse, low concentration signals based on error minimization methods; however, continued investigation and benchmarking is needed. Here, we have employed an alternative method developed for multivariate analysis and commonly applied to compressive sensing that utilizes spectral signatures for feature selection. Least Absolute Shrinkage and Selection Operator (LASSO) regression is an approach that generates sparse regression models based on a regularization penalty that favors solutions with the fewest non-zero coefficients [36]. LASSO regression has been widely applied for statistical data mining and compressive sensing; extensive descriptions on parameter tuning and optimization for various models are available for greater detail [37]. LASSO and more complicated Elastic Net variants are, in certain instances, capable of producing an exact minimal subset of sparse features that are necessary and sufficient to describe a system within a linear regression. Furthermore, LASSO regression for feature extraction can be combined with a broad range of techniques for spectral decomposition as well as sample classification and prediction, and represents a new approach for spectral data analysis. This was recently demonstrated by Lin et al. who used pixel-based LASSO calculation to decompose SRS spectral images into concentration maps for a predefined number of components [38]. The complexity of biological systems coupled with the ongoing development of spectral SRS instrumentation will likely require continued advancements in algorithmic development for multivariate data analysis of large spectral imaging datasets.

In this manuscript, we introduce a new multi-window sparse spectral SRS system based on a turn-key fiber laser system, recently reported for CARS microscopy [6], capable of all electronic tuning, covering a range of 700-3200 cm\(^{-1}\), in under 5ms regardless of tuning jump. Compared to previous efforts to broadly tune across the fingerprint (700-1800 cm\(^{-1}\)), silent (2000-2400 cm\(^{-1}\)), and high wavenumber (2700-3300 cm\(^{-1}\)) spectral windows, the spectral resolutions and accessible ranges that could be probed have been historically limited by available laser technology or system complexity. Using this new fiber optical parametric oscillator (FOPO) based approach, spectra can be acquired in automated XYZT\(\lambda\) data sets as a defined sweep or selectively, at predefined frequencies of interest. Unlike the previous demonstration that implemented only CARS microscopy, here stimulated Raman Gain imaging is achieved by the use of a single filter for optical detection, removing the need for multiplex detection or user interaction. A program was developed to control both the laser-scanning microscope and optical parametric oscillator wavelength tuning to enable full-speed, multi-area timelapse, completely automated sparse spectral sampling SRS hyperspectral imaging across multiple regions of interest in tissues.

Furthermore, as this system enables specific tuning regardless of frequency change, sparse spectral sampling SRS (S\(^4\)RS) is demonstrated to enable the imaging and analysis of mixtures ranging from simple static polymer mixtures to the complex dynamics of topically applied APIs in mouse skin. Many biomedical systems are inherently complex from a Raman spectral standpoint and thus require computational feature selection to identify the optimal number of unique Raman bands to adequately track dynamic changes over time. Here we further demonstrate the use of several multivariate spectral decomposition algorithms and the LASSO approach to propose a potential workflow (Fig. 2) as an unbiased means to select the optimal number of unique wavenumbers for sparse spectral sampling SRS imaging, which can further be used to monitor dynamic changes such as cutaneous pharmacokinetics.

The rapid and selective tuning demonstrated across multiple model samples in addition to the computational approaches to select an optimal number of Raman bands to characterize a complex system now paves the way for future investigations: specifically those complex dynamic systems that cannot be readily probed through conventional hyperspectral SRS systems capable
Fig. 2. Proposed workflow to obtain a sparse feature set from \textit{a priori} knowledge or acquired multi-window spectral sweeps for new compounds. FP: Fingerprint region (700-1800 cm$^{-1}$), SR: silent region (2000-2400 cm$^{-1}$), HWN: High wavenumber (2700-3300 cm$^{-1}$).

of measuring 300 or 600 cm$^{-1}$ ranges without adjustment. To the best of our knowledge, this is the first report of SRS microscopy that can achieve tuning from fingerprint to high wavenumber Raman spectral regions between continuous imaging frames.

2. Methods

2.1. S$^4$RS microscope

A dual output rapidly tunable all-fiber OPO light source (Picus Duo, Refined Laser Systems, Germany) was used for imaging similar to our previous work, which only demonstrated the applicability of this laser to CARS imaging [6]. Briefly, the amplified output pulses of the FOPO (tunable from 780 to 980 nm) were used as pump pulses and the amplified pulses from the pump laser (tunable between 1020 and 1060 nm) were used as Stokes pulses for simultaneous CARS and SRS microscopy. A resonant amplitude modulated electro-optic modulator (EOM) was placed inline with the amplified output of the FOPO pump beam. A function generator (DS345 Stanford Research Instruments, USA) supplying a 20MHz 1Vpp sinusoidal signal provides input to a gain block (ZHL-32A+, Mini-Circuits, USA) that was used to drive the EOM for optimized amplitude modulation depth as determined when the system was built; these parameters were held constant during imaging. Pump and Stokes pulses were overlapped in space and in time (Di02-R980-25x36 980 nm dichroic beam splitter, Semrock, USA) and both beams were independently conditioned for divergence with telescopes before coupling into a commercial inverted microscope (IX-83, Olympus, Japan), which was equipped with a confocal scanner (FV3000, Olympus, Japan). Due to the small temporal difference between the synchronized pulses, the physical beam path was specifically designed to be compact and was assembled using mounted 1 inch diameter optical posts to provide extra stability. When possible, mirrors
were glued to their mounts using optical-grade epoxy, further hardening the beampath and removing the need for daily adjustment and alignment of the system. The excellent beam pointing stability of the fiber laser, which does not change with wavelength tuning, and this compact, hardened beampath resulted in a setup that was used for weeks without any need for adjustment or realignment. Coherent Raman imaging was performed by relaying the overlapped pump and Stokes pulses to the back focal plane of a 20×0.8 NA microscope objective (UPLXAP020X, Olympus, Japan). The anti-Stokes signal was collected in the epi-direction, optically filtered with a shortpass dichroic filter (ZT775sp-2p-UF1, Chroma, USA) and a 45 nm bandpass filter centered at 650 nm (ET650/45x, Chroma, USA), and focused onto a photomultiplier tube (H7422PA-50, Hamamatsu Photonics, Japan) that was amplified with an RF amplifier (TIA60, Thor Labs, USA) for analog detection. Stimulated Raman gain (SRG) images were acquired in the forward direction, such that light was coupled through the condenser lens, through a 980 nm longpass filter (LP02-980RU-25, Semrock, USA) and onto a NIR optimized photodiode (SRS detector set, 1064nm optimized, APE GmbH, Germany). Beam scanning and image acquisition were realized through commercial Olympus Fluoview (FV10-ASW) software. Images of 1024×1024 pixels (or 512×512 pixels in the case of Visualization 1 & Visualization 2 files) were acquired with 2 µs pixel dwell time and 3 Kalman averages per line. Three-dimensional (3D) images were acquired by moving the objective lens in the z-direction with uniform step sizes between consecutive frames. Tissue imaging was performed using a stage-top incubator (Tokai Hit, Shizuoka-ken, Japan) to maintain sample temperature (32 °C) and humidity during time-course studies. The entire system, including the laser and automated microscope, was controlled via a custom Python interface using the Olympus remote development kit (FV_RDK, Olympus, Japan). This Python interface was developed as a central control program for the microscope, and is capable of orchestrating laser startup and tuning, microscope scanning parameters, and microscope image data acquisition. The control sequences for the FOPO and Olympus RDK are publicly available and were incorporated here into a program specifically for hands-off operation. During system setup for an imaging experiment, the Python control program is used to initialize communication with the FOPO via serial commands while the Olympus RDK is controlled in the same Python program using XLMRPC via a networked IP address. Both microscope and laser source are robust and completely turn-key, requiring no specific adjustments on a daily basis for multi-window spectral SRS imaging. By integrating control for both the light source and the microscope into a single program, synchronized automated multi-window tuning across the Raman spectrum is achieved between consecutive frames. Specifically, after setup for desired imaging parameters within the Fluoview software and defining the program variables for the Raman frequencies of interest (either as a range for spectral mapping or as a list of discrete features for S4RS) XYZT,λ data was acquired such that the frequency was tuned after each XY or XYZ series was completed, depending upon the desired imaging protocol. The number of frequencies and the tuning step size between frequencies was defined before imaging and dictated the number of λ slices in the S4RS dataset. The acquired images were analyzed using JupyterLab via the LOCI bioformats library [39] or with the open-source Fiji package [40]. Multispectral images were false-colored and the "Merge Channels" tool in Fiji was used to display different spectral components in a composite image using the despeckle filter. Volumes were projected into 2D images by color-coding of the consecutive frames with an "ice" lookup table using the "Temporal Color-code" function.

### 2.2. Sample preparation

Acetaminophen (Acet - ≥99.0%), dichloromethane (DCM - ≥99.5%), isotretinoin (Iso - ≥98%), retinoic acid (RA - ≥98%), tazarotene (Tiz - ≥98%), ibuprofen (Ibu - 99.6%), polyethylene glycol (PEG200 - ≥99%), poly(ethyl methacrylate) (PEMA), and poly(vinyl alcohol) (PVA) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Poly(methyl methacrylate) (PMMA)
and polystyrene (PS) microspheres were purchased from Polysciences Inc (Warrington, PA, USA). Poly(methyl methacrylate) powder was purchased from Scientific Polymer Products Inc (Ontario, NY, USA). Acet and Ibu were imaged in powder form. The other APIs (Iso, RA, and Taz) were individually dissolved in pure DCM at 1 mg/mL solution. Two μL of the DCM/API solution was placed on a microscope slide (FisherBrand Hampton, NH, USA - 25 x 75 x 1.0 mm) and allowed to dry at room temperature. The physical mixtures of PMMA/PEME/PVA and PMMA/PS microspheres were prepared and imaged.

Mouse skin was collected following euthanasia under an institutionally approved tissue collection protocol. The ears from nude nu/nu albino mice were harvested and washed in sterile PBS buffer, and then stored at −20 °C until use. Mouse ear skin was then thawed at 32 °C for 10 minutes in an incubation chamber (Tokai Hit, Shizuoka-ken, Japan) prior to use. Taz was formulated in 50 mM solution in 100% PEG200 by 10 minutes of sonication at 30 °C. 10 μL of the formulation was then uniformly pipetted on the nude mouse ear skin and imaged. The intensity vs. time (i.e., flux profiles) were extracted from the individual images for two regions of interest (ROI) by the use of a U-NET as previously described [15].

2.3. Spectral decomposition and feature selection analyses

All of the analyses presented here were implemented in Python 3.7 [41–45] and are directly applicable to other spectral SRS imaging methodologies. Spectral decomposition via PCA, ICA, VCA, and MCR were each implemented while spectral classification and prediction were performed with ordinary least squares (OLS) regression, non-negative least squares (NNLS) regression, LASSO regression, and Elastic Net regression. Sparse feature selection was implemented via LASSO regression. The model was initially trained through cross validation (LassoCV and LassoLarsCV) to yield conventional penalized-likelihood criteria for feature inclusion (Akaike and Bayesian information criteria, AIC and BIC, respectively), as well as with a less restrictive shrinkage parameter to promote sparsity while still including a small number of less relevant terms. Using the selected spectral features based on LASSO regression, individual spectral SRS λ frames were extracted and predicted using a standard OLS regression for comparison.

3. Results and discussion

3.1. Spectral SRS

To demonstrate the advantage of this S⁴RS system, spectra from various retinoid compounds were acquired via both spectral scanning acquisition and sparse selection modes. Targeting the fingerprint spectral window near 1590 cm⁻¹ to capture the variation in C=C stretching vibrations of isomers of retinoic acid [46], Fig. 3 depicts three retinoid compounds used in topical applications. The spectra presented are directly extracted from the spectral SRS XYλ data from a single field of view and the image was false-colored using the SRS intensity image from an individual frame per compound (Iso: 1568 cm⁻¹, RA: 1582 cm⁻¹, Taz: 1594 cm⁻¹). The tunable, controlled spectral sampling of individual features is recorded in Visualization 1, where eight discrete features (nIso=3, nRA=3, and nTaz=2) were selectively probed. The closely spaced, sharp, distinct features of the retinoic acid isomers in this region of the spectrum are ideal targets for the demonstration of the system’s spectral resolution and ability to directly image closely related chemical structures with rapid electronic frequency tuning. No adjustments to the system were made for acquisition of either the spectral data or the S⁴RS data; the user must define desired frequencies in the control interface as a continuous range or a discrete list, respectively. Also, these spectra closely match those obtained by spontaneous Raman spectroscopy further confirming the suitability of this approach [46].
To further validate the tuning capabilities of the system and demonstrate the potential to extract consistent spectral profiles from image structures, samples comprising physical mixtures of pharmaceuticals or polymer microspheres were investigated using spectral tuning SRS. Figure 4 demonstrates $XY\lambda$ datasets acquired from both physical mixtures with the extracted spectra from individual components. Data acquisition across both fingerprint and high wavenumber regions was achieved, confirming the expected multi-window frequency tuning by the FOPO without the need for physical adjustment or user interaction. As a widely used spontaneous Raman spectroscopy calibration standard, acetaminophen validates tuning performance with distinct spectral features from ibuprofen. Direct calculation of the signal-to-noise ratio (SNR, defined as $\mu_{signal}/\sigma_{noise}$) for the spectral peaks of acetaminophen spanning both fingerprint and high wavenumber spectral windows (depicted in Fig. 4(b)) achieved values of 3.4-4.9 for SRG imaging using a pixel dwell time of 2 $\mu$s. Both of these APIs, along with other systemically delivered non-steroidal anti-inflammatory drugs, have the potential to be utilized in topical formulations; further exploration may suggest topical formulations could be an alternative to systemic non-steroidal anti-inflammatory drugs for localized pain management to provide high target-site concentrations and reduce unwanted systemic side effects compared with systemic delivery [47]. The multicolor images presented are merged from sum intensity projections of spectral vibration tuned images acquired with 2 $\mu$s per pixel and the spectra are extracted from individual features, demonstrating consistent and accurate recapitulation of the expected lineshapes for bulk pharmaceuticals and polymers and, consequently, confirming the performance of this system. The lineshapes of recorded spectra presented here may be impacted by power fluctuations when tuning between frequencies, but power compensation during measurement acquisition may further improve this performance. Despite variations in size of features imaged, Fig. 4(c,d) demonstrate the consistency with which spectra can be extracted from individual structures that provide reliable profiles to distinguish similar sample components. In particular, we can see clear spectral difference between PMMA with $\nu_2$ CH$_3$ vibration at 2950 cm$^{-1}$ and PS with aromatic CH stretching vibration at 3050 cm$^{-1}$ as samples that have been widely reported for coherent Raman system evaluation. Furthermore, while the ranges targeted here were selected...
Fig. 4. Spectral SRS imaging of pharmaceutical API powders and polymer microbeads. (a) High wavenumber SRS image of pharmaceutical APIs (Acetaminophen, (Acet) 2922-2938 cm\(^{-1}\) red; Ibuprofen, (Ibu) 2840-2856 cm\(^{-1}\) blue) within a 20× image FOV. Scale bar 50 µm. (b) Extracted high wavenumber and fingerprint spectra (µ ± σ) from individual regions of interest for structures in the SRS spectral stack. (c) Extracted spectra (µ ± σ) from \(n \geq 160\) individual polymer microbeads per type within (d) a 20× image FOV (magnified 5×) of 1-10 µm poly(methyl methacrylate), (PMMA, 2940-2958 cm\(^{-1}\) blue) and 3 µm polystyrene (PS, 3046-3064 cm\(^{-1}\), red) microbeads. Scale bar 20 µm. Dotted lines in (b) correspond to spectra extracted from the 10 ×10 pixel regions in (a) and in (c) to spectra extracted from the individual polymer structures marked in (d).

Based on the ability to capture multiple features from both samples, wider spectral ranges can be tuned or selected for mapping as desired.

To demonstrate the investigation of spectral components in a biological system utilizing spectral SRS tuning, we acquired \(XYZ\lambda\) datasets from skin structures in a nude mouse ear. Primarily capturing the lipid and protein components as CH stretching vibrations in the high wavenumber range, Fig. 5 represents a hyperstack of the spectral image data summed across the acquired spectral region where the intensity is encoded with color as a function of relative depth spanning 36 µm in 6 µm steps: superficial structures, in this case the sebaceous glands (SG), are encoded as blue-green and deeper structures, like subcutaneous fat (SCF), in red. The adipocytes (AD), which can manifest at multiple depths within the tissue, here appear as a mixture of violet and red depending upon the layer in which they were located. The spectral data depicted in Fig. 5(b) has been extracted from individual structures within the skin from a particular layer of the \(XYZ\lambda\) data with lineshapes colored accordingly. Consistent with previous reports utilizing Raman scattering techniques to investigate tissue lipid composition, the spectra from sebaceous glands have an
altered signature compared to the other two structures. This is likely a result of the different lipid composition of sebum, which contains increased saturated fatty acid and wax ester content versus that of epidermal adipose tissues [48]. While the extracted spectra are still largely consistent, multivariate decomposition methods and further investigations are possible given the spectral and spatial resolution of this approach [49] and may enable more detailed characterization of constituent lipid signals for specific tissue structures.

Fig. 5. Spectral SRS image of nude mouse ear skin. (a) Depth encoded projection of $XZ\lambda$ stack for the sum of acquired spectra. Each axial plane is separated by a 6 $\mu$m step size and color encoded for display using the “ice” look up table such that superficial sebaceous glands (SG) are encoded as blue-green, deep subcutaneous fat (SCF) as red, and adipocytes (AD), which are present in multiple layers of the hyperstack, as a mixture of violet and red depending upon the layer in which they were located. Scale bar 50 $\mu$m. (b) Spectra extracted from 26 pixel diameter regions of interest indicated in (a) depict spectral differences in the lipid profile of mouse skin structures. Spectra have been extracted from the hyperstack and color-encoded corresponding to the depth from individually masked tissue structures within the spectral SRS stack.

3.2. $S^4$RS of complex systems

To demonstrate the potential for large range, frequency shifted selective sparse spectral SRS ($S^4$RS) within dynamically changing specimens, we investigated the API uptake from a topically applied formulation. Here, the formulation consisted of a solution of Taz in PEG200, a common inactive ingredient, applied to the surface of an ex vivo nude mouse ear. Given the known average spectrum for Taz (Fig. 3(b)), the spectral SRS measured inactive ingredient (PEG200, Fig. 6(c)), and the skin stratifications (as shown in Fig. 5(b)), specific Raman vibrations of interest were selected based on a priori knowledge, enabling API visualization and localization within tissue while sparsely mapping each component of the complex drug-tissue system via $S^4$RS. Targeting $\nu$, CH$_2$ (2845 cm$^{-1}$) and $\nu_{as}$, CH$_2$ (2880 cm$^{-1}$) vibrations of both tissue lipids and PEG200, the $\beta$ CH$_2$ (1482 cm$^{-1}$) of PEG200 [50], and the $\nu$, C=C (1592 cm$^{-1}$) of Taz, $XYZ\lambda$ data was recorded at multiple depths per frequency over time. As seen in Fig. 6(a,b), the $\nu_{as}$, CH$_2$ data acquired from a single timepoint can be used to visualize structure while C=C and $\beta$ CH vibrations can be used to quantify both the API and inactive ingredient within the tissue as a function of space and time. Cutaneous pharmacokinetic data has known variability [15], which can be seen in Fig. 6(d). For ROI 1, we can see Taz concentration remains relatively flat in the stratum corneum after PEG200 permeates fairly quickly into the sebaceous gland. The possible
reason for the concentration of PEG200 increasing from time = 0 is that the formulation was removed after 10 minutes of application and there was no remaining PEG200 on top of the SC. On the other hand, ROI 2 displays a slow permeation of PEG200 from the stratum corneum into the sebaceous gland, which also coincidences with an increase in Taz within the sebaceous glands. While PEG200 alone has been suggested to stay on the surface of the skin [51], other reports in literature [52] have suggested that PEG200 may carry compounds (i.e., Taz). This can be seen in Fig. 6 in which there is a similarity between the compound localization (lipid-rich or lipid-poor) for the two compounds (i.e., higher lipid-rich concentration of PEG200 led to a higher concentration of Taz in the same ROI and skin stratification). With this methodology, we have the potential to understand mechanistic permeation of unlabelled compounds and how solvents play a role in localization.

While future work will investigate potential formulation effects on the penetration and permeation of topically applied APIs, the S\(^4\)RS approach enables rapid tuning across \(\sim 2450 \text{ cm}^{-1}\) between sequential frames to target specific spectral features with the spatiotemporal and spectral resolution to monitor dynamic processes like topical drug product administration. This is demonstrated in Visualization 2, which depicts live image acquisition within the Olympus Fluoview software for one such sample while programmatically triggering a frequency tuning sequence that alternates from 1592 cm\(^{-1}\) to 2880 cm\(^{-1}\) between consecutive frames. Note that banding occurs in several lines (each 2.118 ms) of the frame (each 1.087 s for 512\(\times\)512 pixels, 2 \(\mu\)s per pixel) when the FOPO output switches frequencies as directed. This is caused by slight asynchronization between frame acquisition and FOPO tuning as a result of user initialization for frequency tuning but does not occur when programmatically controlled during data acquisition. Furthermore, the Raman vibrations of interest can be freely tuned across the spectral range and visualized solely through defining discrete targets within the control program. This demonstration of rapid laser tuning across such a wide spectral range for SRS imaging without system modification cannot be achieved by any other reported coherent Raman imaging system to date. The simplicity and utility of the S\(^4\)RS system now provides the means by which to study dynamic processes such as topical drug delivery while targeting multiple distinct drug, formulation, and tissue components that cannot be characterized by peaks within a 300 cm\(^{-1}\) spectral window. Given the ease of spectral selection and the accessible tuning range of the S\(^4\)RS, careful selection of target vibrations for imaging must be considered: there is an inherent trade-off between spectral sampling and temporal resolution, which will become increasingly important for studies investigating multiple spatial locations, samples, and conditions concurrently.

### 3.3. Spectral decomposition and feature selection for optimized S\(^4\)RS

Given the rapid tuning performance for the S\(^4\)RS system, there is enormous potential to target complex dynamic biological, medical, and pharmaceutical samples, including those that lack single distinguishable features in their Raman spectra which are often probed in the silent region (i.e., deuterated compounds, alkynes, nitriles, etc.) [4,15,53]. Coinciding with this increased capacity for spectral sampling is the need to economically select frequencies that capture the significant and meaningful variations within the data without including spurious data or excess noise. The main tasks are thus (1) decomposition, to identify spectral profiles that discriminate salient targets, and (2) feature selection, by which a sparse, yet fully sufficient, subset of frequencies is selected for imaging. As demonstrated for the topical tazarotene solution applied in Fig. 6, some systems manifest obvious features for selection, especially when a priori information regarding some or all of the constituent spectra is available. Conversely, even systems with well characterized compositions may pose significant difficulties in selecting an optimal subset of features for sparse sampling. We chose to investigate this challenge with polymers that exhibit a high degree of spectral overlap, as depicted in Fig. 7(a-e). As can be seen in Fig. 7(b-d), individual wavenumber images selected per sample at spectral peaks of interest may
Fig. 6. SRS image of nude mouse ear skin along with topically applied API solution. (a) Representative depth-encoded sum intensity projection of XYZ T stack for tissue νas CH2 2880 cm−1 acquired over 2 hours following the topical application of API solution (50mM Taz in PEG200). The image is a sum intensity projection for the full experimental time-course for a single wavenumber tuning. Each axial plane is separated by a 12μm step size and color-encoded for display using the “ice” look up table relative to the skin surface (superficial stratum corneum are encoded as blue-green, sebaceous glands are encoded as blue or violet, adipocytes in magenta, and deep subcutaneous fat as red). Scale bar 50 μm. (b) The matched depth-encoded sum intensity projection of SRS retinoid νs C=C 1592 cm−1 from XYZ T data presented in (a). Comparing the matched depth-encoding colors in (a) and (b) indicates that the topically applied Taz primarily remains in the superficial stratum corneum of the ex vivo mouse skin for the duration of the experimental time-course and does not penetrate to deeper structures (minimal Taz signal below 24 μm). (c) Representative SRS spectra of PEG200 with and without 50mM Tazarotene confirms reported peaks for SRS time-course analysis (1470-1480 cm−1 for PEG200, 1592 cm−1 for Taz). (d) Flux profiles extracted from XYZ T data for both PEG200 and Taz in nude mouse ear tissue. Global intensity can further be separated into lipid-rich and lipid-poor for drug localization (line-type). Taz and PEG200 were not found to permeate deeper than the sebaceous glands. Two different ROIs are indicated by the line color, ROI 2 corresponds to image data in (a) and (b).
lack sufficient contrast to visualize or discriminate structures with distinct compositions such that even in the merged color image (Fig. 7(a)) generated from empirically selected spectral features for each of the components (Fig. 7(b-d)), crosstalk between components complicates differentiation. For this synthetic system, we were able to individually measure pure samples of each of the three components as well as acquire independent spectral SRS data from physical mixtures of two or three components (Fig. 7(e)).

The first step in determining optimal frequencies for S$^4$RS is to obtain spectra, either from a database or via spectral SRS, of sample constituents. Relying on spectral databases may pose additional complications as the relative intensities between SRS and spontaneous Raman scattering are not always consistent between features. This can also be observed in spontaneous Raman scattering literature when using different excitation frequencies (albeit due to differing excitation processes); however, the positions of peaks should be consistent between SRS and spontaneous Raman spectroscopy. If the constituents of the sample are unknown or are comprised of complex mixtures, as frequently encountered within cells and tissues, SRS spectra can be acquired and decomposed into distinct profiles to describe and differentiate sample components. Care should be taken to use an appropriate spectral sampling density to capture the relevant differences within the samples to optimize performance, a feature that can be easily tuned with the S$^4$RS system through programmatically selecting wavenumber tuning step sizes. Figure 7(e) depicts the pure components measured as well as the multivariate spectral decomposition performed via PCA, ICA, and MCR algorithms. While each approach is capable of extracting spectral components that differentiate image features, particular methods may be better suited as tools for sparse feature selection. PCA as an unsupervised technique may allow the user to select the ideal number of unique components within the spectral dataset after decomposition by examining the change in eigenvalue/variance explained by each principal component (potentially based on the elbow of the Scree plot). However, using PCA to extract distinct spectra for sparse feature selection is likely not ideal. While the individual PCs are mathematically orthogonal, the altered component profiles often contain negative intensities (which can also be observed using conventional ICA methods) that are not representative of measurable Raman spectral signatures. MCR, which has been investigated by several groups, requires the user to identify the number of components to extract yet may prove to be a more reliable algorithm to extract spectra that recapitulate the positive constrained true spectra present in the dataset. Spectral profiles decomposed from spectral SRS data that differ from underlying actual spectra may still enable the selection of a useful sparse subset of features and the visualization of sample differences; however, this requires further investigation beyond the initial demonstration provided here and is being actively pursued as an avenue of continued research. Furthermore, the altered component profiles may complicate the assignment of individual spectral features to characteristic vibrations of sample components and may hinder interpretation of the underlying sources of spectral contrast upon S$^4$RS imaging.

Here, individual structures have been identified and spectra have been extracted from the spectral SRS data, however, as previous reports have noted, spectral decomposition methods such as PCA, ICA, or MCR could be used for this step. Alternatively, approaches such as VCA or FSC$^3$ could be used to identify pixels within the spectral SRS data stack that represent distinct spectra. Each of these approaches are also be capable of identifying and extracting spectral signatures from pixels comprising a mixture of components [38]; however, obtained endmembers/vertices may contain some degree of spectral overlap depending upon the chosen algorithm. Regardless of the use of a priori information or experimentally derived profiles, decomposition of the spectra is a necessary step prior to the extraction of a sparse feature set for S$^4$RS.

The second step for optimized S$^4$RS imaging is the selection of the sparse subset of frequencies. Using the extracted spectra, or previously measured spectra for systems with known constituents as may be the case in many pharmacokinetic investigations, a supervised learning approach based
Fig. 7. Spectral SRS imaging of polymer particles. (a) Merged composite image of manually extracted spectral frames for (b) poly(ethyl methacrylate) (PEMA, blue in (a)), (c) poly(methyl methacrylate) (PMMA, red in (a)), and (d) poly(vinyl alcohol) (PVA, yellow in (a)) within a single 20× image FOV. (e) Examples of pure spectra (Control) obtained from unmixed samples along with spectral decomposition performance by principal component analysis (PCA), independent component analysis (ICA), and multivariate curve resolution (MCR). Dashed lines indicate zero intensity for components in PCA and ICA which allow negative signal contribution. (f) Sparse spectral feature extraction based on LASSO regression modeling identified nine frequency features capable of sample discrimination. Consistent predictive performance is obtained via ordinary least squares regression using the extracted sparse subset for sample discrimination.
on LASSO regression is performed. Feature selection is achieved by utilizing cross-validation criteria (AIC or BIC) to determine an appropriate sparse feature subset or with user-defined shrinkage term ($\alpha$), to include fewer or additional features as desired. Here, the LASSO model was trained with a set of spectral SRS data acquired from unmixed polymer samples with known labels. The algorithm identified nine of the original 150 frequencies (Fig. 7(f), top, gray bars) that could be modeled (coefficient weights depicted as black dots mapped against LASSO Coef. Score axis) for adequate polymer discrimination. Applying the resulting LASSO model to particles measured from independent physical mixtures ($n_{Total}=96$: $n_{PEMA}=59$, $n_{PMMA}=31$, $n_{PVA}=6$) achieved 100% accuracy in prediction of group membership. To validate the performance of this method, the subset of nine identified frequencies were then extracted from the original training and independent validation sets and an OLS regression was trained on the sparse data using the nine features from the control sample data to fit the regression model (Fig. 7(f), bottom, gray bars). In this case, the OLS regression on the sparse subset assigned different relative coefficient weights to particular features (black squares mapped onto OLS Coef. Score axis) when compared with the LASSO model. Despite the subtle differences in applied coefficient weights, the OLS prediction of the independent dataset from particle mixtures represented with only the nine features extracted by the LASSO model was again able to achieve 100% classification accuracy.

It is further worth noting that for this evaluation, we manually constrained the $\alpha$ parameter for the LASSO model to artificially include a larger number of features than suggested by AIC and BIC ($n=1$). Based on the single wavenumber images depicted in Fig. 7(b-d) some with low contrast that is not adequate to differentiate structures of differing composition, the $\alpha$ parameter was lowered to consider a greater number of spectral features for modeling and discrimination. As a result, the coefficient scores utilized in both the LASSO model and the OLS model on the sparse data for five of the nine features are nearly zero, indicating that these features do not contain significant predictive value and could potentially be omitted for increased sparsity of the model. Incorporating this information would lead to decreased data acquisition times and the potential for more densely sampled time-course data. Both are of great importance in biomedical imaging and, in particular, in the imaging and quantification of dynamic tissue pharmacokinetics. The data used here was derived from spectral SRS data for which regions of interest (ROIs) were defined for individual particles in both the pure training and physical mixture datasets and therefore there were no confounding background signals included in the model. Inclusion of a separate class of background spectra from other ROIs is not expected to significantly alter the performance of the LASSO approach, however, doing so may require the additional collection of frequencies to differentiate components from the background. Continued development of this approach, including the optimal choice of shrinkage parameters ($\alpha$) or the use of the more complex Elastic Net regression, may further improve performance of sparse feature selection in the context of complex biological systems with overlapping compositions and spectra; however, these are areas of active investigation that are beyond the scope of this proposed workflow.

3.4. General discussion

Label-free, nondestructive, and quantitative *in situ* imaging in biomedical samples is the ultimate goal to assess dynamic processes in cells and tissues. The advancement of SRS technologies that facilitate the selective measurement of multiple spectral targets to characterize complex samples represents significant progress towards the translation of coherent Raman imaging technologies towards clinical utility; many high quality and specific labels currently required to visualize compounds *in vitro* or *in vivo* within animal models may be either unavailable or face substantial regulatory challenges prior to human studies. The selective and rapid multi-window tuning capabilities of this S$^4$RS system will increase the capacity for label-free measurement of dynamic biological samples through targeted spectral imaging of native vibrations. Furthermore, the enabling FOPO technology employed here is compact, robust, and stable, making it well suited
for integration into clinically relevant systems capable of both CARS and SRS imaging. The approach presented here, which enables rapid chemical imaging through sparse spectral sampling, has the potential to deliver on the promise of coherent Raman imaging as a universal label-free tool in biology and medicine.

The narrowband S^4RS system presented here targets specific vibrational frequencies rather than utilizing a broadband approach as others have previously [54,55]. De la Cadena et al. demonstrated a multiplexed/hyperspectral broadband SRS system with application to plant cell walls. The authors were mostly limited to the HWN window (2800-3100 cm⁻¹) while having a 20 cm⁻¹ resolution. This application also required user intervention to achieve optimal temporal overlap as the wavenumber was changed. Czerwinski et al. was able to achieve a large spectral coverage from 800-3000 cm⁻¹ yet each pixel was acquired at 0.1-1 ms dwell time. Lin et al. utilized spectral focusing with ultrafast tuning to achieve fingerprint Raman imaging with a 200 cm⁻¹ spectral range and 10 cm⁻¹ spectral resolution in 1.8 s per image stack (200 ×200 µm² field of view) with the use of deep learning based denoising [38]. In contrast, our S^4RS approach is not restricted to a single window or limited spectral range, having the capability of acquiring hyperspectral images spanning 750-3200 cm⁻¹ with pixel dwell times on the orders of microseconds. Importantly, this approach can be rapid and highly specific to multiple species of interest by selectively tuning into only a user-defined list of narrowband spectral features to investigate dynamic samples with complex spectral signatures. Furthermore, based on the fingerprint spectral imaging reported in Fig. 3(b) and Visualization 1, the system is capable of differentiating spectral profiles with exquisite spectral resolution of ≤4 cm⁻¹ all visible within the same field of view. This high spectral resolution may result from the complex convolution between the laser excitation spectrum and the sample Raman spectrum, a hypothesis that will be further investigated to probe the limits of system performance.

There have been numerous investigations into the impact that inactive ingredients play in API delivery to the intended cutaneous site of action. However, most of these studies merely quantify the API and modify the formulations’ compositions of binary or ternary mixtures to provide insight into the inactive ingredients’ role in drug delivery. The S^4RS methodology presented here has the capability to individually quantify the API and inactive ingredients after topical application. This is an essential step toward a mechanistic understanding of how formulation composition ultimately impacts the API’s local biodistribution and cutaneous pharmacokinetics. While other methodologies provide bulk cutaneous pharmacokinetic data and how the formulation might modulate the bioavailability, S^4RS provides both visual and quantitative data to specifically show how this formulation ultimately modulates the API delivery. The capability to sparsely sample vibrational frequencies has the direct potential to enable quantification of the concentration-time profiles of the inactive ingredients and API(s) to correlate which inactive ingredients ultimately impact the biodistribution of a drug. As all drug products are developed with the intention of disease treatment, this S^4RS approach provides the ability to quantify API and inactive ingredient concentration over time as well as identify potential changes to the structure of diseased skin. Of course, an additional hurdle for true PK quantification of API concentrations using the S^4RS system is the need for calibration methods to convert relative or semi-quantitative measurements into absolute quantitative measurements which could be benchmarked against other methodologies. Additionally, this technology is not limited to only cutaneous pharmacokinetic investigations but may also be extended to the study of local target-site concentrations through the use of endoscopic procedures [56].

The S^4RS system has demonstrated the capability to rapidly and selectively tune between fingerprint, silent, and high wavenumber Raman spectral windows between consecutive frames during data acquisition, as well as the means to collect spectral profiles with variable, user-defined spectral resolution across the entire spectral range. Expanding the accessible spectrum may now facilitate investigation of imaging targets with distinct features, as well as those that can only be
characterized through a combination of features in the fingerprint and high wavenumber regions. Furthermore, the sparse and selective tuning capabilities may be particularly well suited for multiplex imaging of Raman labels [57] or mixtures of multiple components in complex biological and biomedical samples, all of which can be achieved with the all-electronic, programmable operation of the system for versatile sparse spectral sampling SRS.

It is worth noting that in this implementation of the S^4RS system, the manual time delay stage to overlap the pump and Stokes pulses prior to coupling into the microscope has been positioned for a single frequency and not adjusted further during the study. The bandwidth of the FOPO and the broad 7ps laser pulses facilitate spectral acquisition; however, compensating for the slight changes in time delay between pulses of the FOPO’s dual outputs should further improve coherent Raman scattering signals and can be implemented in order to enhance performance. The S^4RS system performance reported here demonstrated SNRs that are more than sufficient for numerous applications including those for pharmacokinetic imaging as presented in this work. For applications that may require improved SNR performance, alternative detection approaches that incorporate dual-balanced detection could be implemented in future studies based on previous developments for SRS imaging utilizing fiber laser sources [5]. The SNR values (3.4-4.9) achieved here are specifically calculated for acetaminophen, which is not a particularly strong Raman scatterer. The imaging of these samples was performed using 2 μs dwell time per pixel; rapid imaging at these rates is necessary to actively monitor numerous dynamic biological samples. Increased SNRs can also be achieved using longer integration or dwell times, or through frame averaging, but at the cost of temporal resolution. Therefore a careful balance is required in determining the necessary SNR for sample analysis with respect to the dynamics of the samples under investigation.

To enable quantitative SRS imaging, the excitation power delivered to the sample must be closely controlled and accounted for. The Picus Duo achieves rapid frequency tuning via a delay-free tuning mechanism, however provides non-continuous tuning and discrete output power with adjustment. By implementing power compensation between tuning, the performance of our current S^4RS system has the potential to acquire power compensated spectra in the Raman fingerprint window down to 750 cm\(^{-1}\), and may further improve the profiles of extracted spectra. The current limit for spectral acquisition will be constrained by adequate filter selection for SRG and the tuning range of the FOPO, but frame to frame switching of 750\(^{-1}\) to 3200 cm\(^{-1}\) is possible with current implementation, even without power compensation.

4. Conclusions

S^4RS is a versatile, yet simple approach for spectral SRS imaging of complex, dynamic samples based on the recent development of fiber laser technology. Spectral SRS tuning has been performed rapidly between the fingerprint and high wavenumber windows of the Raman spectrum (with the potential to access even greater tuning ranges) without the need for manual system adjustments. An approach for sparse spectral feature selection is proposed to capitalize on the rapid tuning of the S^4RS methodology that is compatible with both a priori and experimentally-derived Raman spectral signatures. Furthermore, while demonstrated as a laboratory benchtop microscope, the enabling laser technology is compact and robust, leading to the potential for this approach to be implemented in a portable system for clinical investigations. With continued development, potential access to the entire Raman spectral range with multi-window S^4RS offers a direct path to study dynamic biomedical systems such as cutaneous pharmacokinetics for a wide array of API formulations without labels or single distinctive spectral features.

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Data availability. Data underlying the results presented in this paper may be obtained from the authors upon request.

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