Chapter

Advanced Applications of 3D Raman Imaging in Biomedicine

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Abstract

Label-free, super-multiplexed Raman profiling of three-dimensional (3D) biological samples offers transformative potential across biomedical research and clinical diagnostics. Unlike conventional imaging methods, Raman spectral imaging enables simultaneous, preparation-free detection of a broad range of biomolecules, including nucleic acids, proteins, and diverse lipid subtypes in their native spatial context. However, the full utility of Raman spectroscopy has long been constrained by challenges in signal acquisition and spectral unmixing, which limit both molecular specificity and quantitative accuracy. Recent advances in reference-based computational unmixing techniques and spectral normalization now overcome these barriers, enabling not only improved quantification but also precise spatial mapping of biomolecular distributions in complex 3D systems. The accuracy of Raman-derived molecular maps can be validated by correlative immunostaining, which confirms the spatial fidelity of certain biomolecular patterns. A particularly promising application is the *in situ* characterization of the extracellular matrix (ECM), a dynamic microenvironment critical for tissue development, repair, and intercellular signaling. Traditional histological and molecular approaches often fail to preserve spatial integrity while detecting ECM-associated signaling molecules. In contrast, 3D Raman imaging uniquely captures both spatial and temporal heterogeneity of ECM components and secreted factors, offering mechanistic insights previously inaccessible. This advancement represents a major leap in the label-free, high-content analysis of live and fixed biological systems.

Keywords: 3D Raman imaging, ECM characterization, label-free imaging, live imaging, super-multiplexed imaging

1. Introduction

Monitoring the biomolecular composition of human cells and tissues is essential, as key physiological processes, such as tissue maturation, organ development, and disease progression, are often characterized by the emergence, disappearance, or fluctuation of specific biomolecules. Fundamental cellular activities, including differentiation, apoptosis, and proliferation, are closely associated with dynamic changes in molecular content and concentration. To capture these variations and assess changes in cellular density and

biomolecular profiles, a wide range of imaging techniques have been developed, each offering unique insights into the structural and functional states of biological systems.

A variety of advanced imaging modalities have been developed to explore the spatial and molecular organization of biological samples in three dimensions. While each method offers unique advantages, they also present specific limitations, particularly in the context of label-free, high-content, and non-destructive analysis of intact biological systems: Cryo-electron tomography (cryo-ET) provides high-resolution 3D imaging of cellular ultrastructure, but lacks the ability to differentiate molecular components and requires cryo-preservation [1]. Mass spectrometry imaging (MSI) offers label-free, high-specificity molecular detection but is limited to thin sections and 2D analysis [2]. Confocal fluorescence microscopy, commonly used for 3D tissue imaging, suffers from spectral overlap and requires labeling, which may alter cellular processes and obscure the natural state [3]. Light sheet fluorescence microscopy (LSFM) enables rapid volumetric imaging but also depends on fluorescent labels and requires optical clearing, limiting its multiplexing capabilities [4]. Expansion microscopy (ExM) provides nanoscale resolution but necessitates extensive sample preparation and labeling, making it incompatible with live-cell imaging [5]. Label-free infrared (IR) imaging offers chemical information but struggles with spatial resolution and water interference, particularly in thicker samples [6].

To characterize biological samples and monitor their compositional and structural changes, 3D confocal Raman spectroscopic imaging serves as a powerful tool. This label-free, inelastic light scattering optical technique enables the visualization of the spatial distribution of biomolecular structures and cellular morphology in both fixed and living tissues. Raman spectroscopy measures the characteristic vibrational modes of chemical bonds within various cellular components (e.g., nucleic acids, proteins, lipids, and cytochrome c), allowing distinct Raman spectra to be obtained for nearly all biomolecules. Compared to fluorescence imaging (~500 cm^{-1}), Raman imaging offers much narrower vibrational peaks (~10 cm^{-1}) and does not suffer from the "color barrier" issue, making it fundamentally advantageous for multiplexed imaging. In addition, Raman imaging benefits from the inherently low absorption cross-section of water, which stands in contrast to techniques such as infrared (IR) spectroscopy, where strong water absorption can obscure important spectral features [7]. This property gives Raman spectroscopy a significant advantage in the analysis of aqueous and hydrated biological samples, including live cells, tissues, and organoids. Moreover, Raman is uniquely compatible with confocal optical configurations, enabling subcellular spatial resolution and three-dimensional sectioning. This allows for the detailed mapping of spatial and biochemical heterogeneity at the single-cell level, making it a powerful tool for characterizing the pathophysiological microenvironment, cellular phenotypes, and molecular gradients in complex biological systems [8].

With a well-calibrated reference spectral library generated using liquid tissue phantoms and a standardized analytical method for spectral unmixing, 3D quantitative biomolecular profiles with subcellular resolution can be derived from Raman signals of human tissues or organoid samples [7]. This enables the simultaneous imaging and analysis of proteins, lipids, and metabolites. As a result, a comprehensive compositional and structural analysis of 3D biological systems can be achieved in a single multiplexed Raman scan, potentially targeting hundreds of biomolecules in samples up to half a millimeter thick, without the need for sample preparation. Importantly, living tissue organoids remain intact after Raman imaging, enabling continuous culture for further analysis [9].

Technique	Label-free	Resolution	Sample preparation	3D/2D	Key advantages	Key limitations
Cryo-Electron Tomography (cryo-ET)	No	Nanometer (~1–2 nm)	Cryo-preservation required	3D	High-resolution structural imaging of cellular ultrastructure and complexes	No chemical specificity; complex sample prep; low throughput
Mass spectrometry imaging (MSI)	Yes	Moderate (~10–50 μm)	Thin sections; destructive	2D	High molecular specificity; wide analyte coverage; label-free	Limited to 2D; requires thin samples; destructive; low resolution
Confocal fluorescence microscopy	No	High (~200 nm)	Fluorescent labeling required	3D	High-resolution optical sectioning; live-cell imaging	Label-dependent; spectral overlap limits multiplexing; perturbative
Light sheet fluorescence microscopy (LSFM)	No	High (~200 nm)	Fluorescent labeling required	3D	Fast volumetric imaging; minimal phototoxicity; suitable for large samples	Label-dependent; optical clearing required; multiplexing limits
Expansion microscopy (ExM)	No	Nanoscale (~20–50 nm)	Sample expansion, labeling required	3D	Achieves super-resolution with conventional microscopes; spatial fidelity	Requires extensive processing; labeling needed; not for live cells
Label-free infrared (IR) imaging	Yes	Moderate (~5–10 μm)	Minimal sample prep, no labeling	2D	Non-destructive; label-free chemical content analysis	Poor resolution; interference from water; challenges with thick samples
Raman spectroscopic imaging	Yes	Moderate (~250-350 nm)	Minimal sample prep, no labeling	3D	Label-free, non-destructive; multiplexed detection; live/fixed samples	Weak signal; fluorescence background; requires advanced unmixing technique [8]

Table 1.
Comparison of advanced imaging techniques for biological sample analysis.

Combined with Raman calibration bio-analytical techniques, Raman imaging overcomes the limitations of other biomolecular characterization methods shown in **Table 1**. In this chapter, we demonstrate the utility of Raman imaging by applying advanced spectral unmixing techniques to human intestinal organoids, human brain organoids, human breast cancer organoids, and real cartilage samples. Additionally, we highlight the application of Raman imaging in unraveling the composition of highly complex drug formulations, such as gel patches, showcasing its use in the field of generic drug reverse engineering.

2. Case studies: Application of Raman profiling in the field of biomedicine

2.1 Raman profiling of human intestinal organoid

Human intestinal organoids serve as a physiologically relevant 3D model for studying gut development, metabolism, barrier function, and disease. Using 3D confocal Raman spectroscopic imaging, we acquired volumetric biochemical maps of human intestinal organoids without any labeling or sample processing, as shown in **Figure 1** and Video 1, http://bit.ly/3J6jy59. Distinct Raman spectral signatures were assigned to Hyaluronan, Collagen, Laminin, Glycogen, Cholesterol, Saturated Lipid, Monounsaturated Lipid, and Unsaturated Lipid. Spectral unmixing, supported by a calibrated reference library, revealed zonal variation in lipid saturation, glycogen storage, ECM distribution, and the functional compartmentalization of the intestinal epithelium and its underlying matrix. These findings align well with known intestinal epithelial cell lineage hierarchies and validate Raman imaging as a powerful method for capturing the compositional architecture of the intestinal mucosa *in vitro*.

2.2 Raman imaging for quality control and drug analysis in human brain organoids

Human organoids represent a transformative model system for drug screening, disease modeling, and personalized therapy. However, ensuring the quality and reproducibility of organoid models remains a critical challenge. Conventional evaluation methods—such as immunostaining or biochemical assays—are typically invasive, endpoint-specific, and destructive, making them unsuitable for real-time, longitudinal monitoring of living samples. In this context, 3D Raman spectroscopic

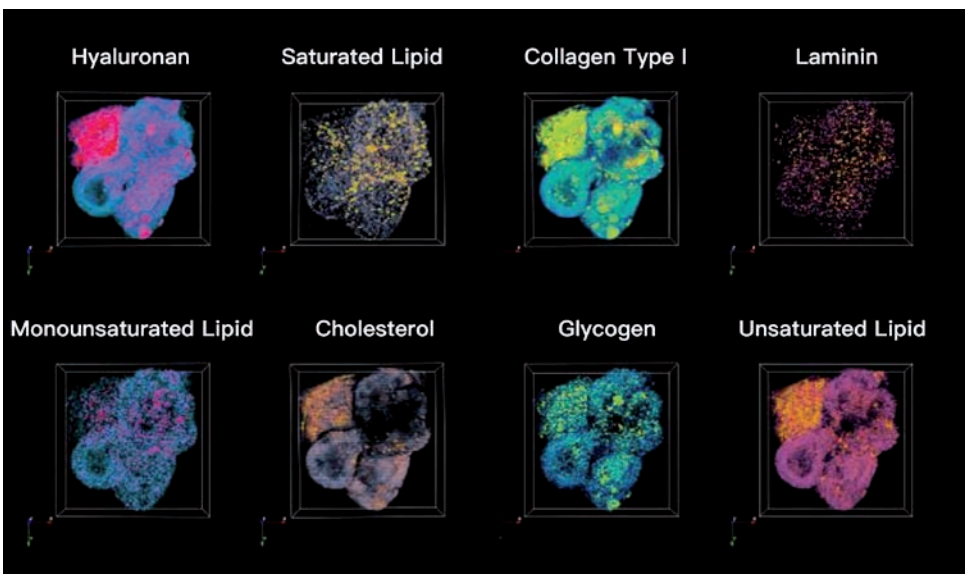

Figure 1.
Raman profiling of fixed human intestinal organoid. Figure reproduced with permission from SuperVision Medicine Co., Ltd.

imaging emerges as a powerful, non-invasive, and label-free technology uniquely suited for organoid quality control.

Due to its ability to detect intrinsic vibrational signatures of biomolecules, Raman imaging allows direct molecular characterization without any staining or labeling, and can be applied to both live and fixed samples. As a case study shown in **Figure 2**, we applied 3D Raman imaging to monitor fixed human brain organoids over a 10-day period. During storage, gradual evaporation of phosphate-buffered saline (PBS) resulted in the formation of PBS salt crystals within the organoid tissue. In **Figure 2**, Raman volumetric imaging at day 2, day 5, day 7, and day 10 enabled clear visualization of the 3D spatial distribution of PBS crystals, which would be difficult or impossible to detect using conventional histological or optical methods. However, since PBS has a well-characterized Raman spectral signature, it could be easily unmixed from the complex organoid background using a reference-based spectral deconvolution approach.

This example illustrates a key strength of Raman imaging: as long as a pure substance is available, its spectral fingerprint can be acquired and used to detect, localize, and quantify it within complex biological matrices. This principle extends to drug molecules and their metabolites, enabling Raman imaging to be used for *in situ* pharmacokinetic analysis [7], drug penetration assessment, and metabolite localization within organoids or tissues without the need for chemical labeling or destructive processing.

Thus, 3D Raman imaging not only solves the unmet need for non-destructive organoid quality control but also offers a unique platform for high-resolution drug distribution studies, batch-to-batch consistency verification, and longitudinal metabolic monitoring in organoid-based drug screening workflows. Its high-content, label-free nature makes it particularly suitable for personalized medicine, where maintaining the integrity and viability of patient-derived organoids is essential [8].

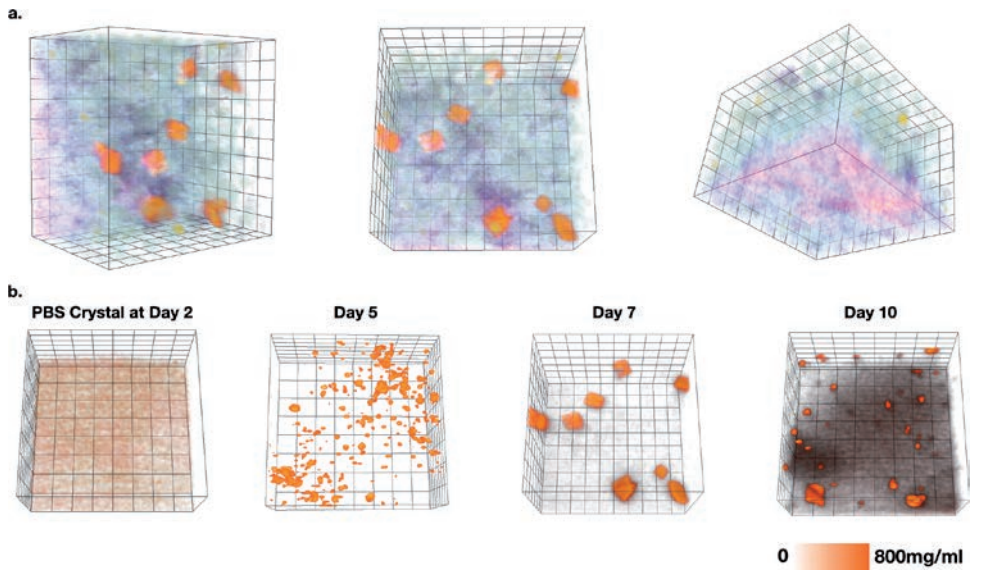

Figure 2.
Raman profiling of human brain organoids. (a) Brain organoid profiling at day 7. (b) PBS crystal formation within brain organoids at day 2, day 5, day 7, and day 10. Figure reproduced with permission from SuperVision Medicine Co., Ltd.

2.3 Raman profiling of human breast cancer orgnoid

Breast cancer organoids recapitulate the cellular and molecular heterogeneity of tumors and serve as a powerful platform for functional profiling of cancer subtypes [8]. Using super-multiplexed 3D Raman spectroscopic imaging, we conducted high-resolution, label-free molecular phenotyping of breast cancer organoids undergoing mesenchymal-to-epithelial transition (MET), as shown in **Figure 3** and Video 2, http://bit.ly/3J6jy59. Distinct Raman spectral signatures were identified for key extracellular matrix (ECM) components, including collagen, fibronectin, elastin, glycogen, and laminin, as well as for various lipid subtypes, such as saturated, monounsaturated, and polyunsaturated lipids. The spatial distribution and relative abundance of these biomolecules provided insights into both epithelial organization and stromal remodeling. These findings demonstrate the capability of Raman imaging to non-invasively distinguish molecular subtypes based on their biochemical composition and suggest its potential for tracking therapeutic responses with subcellular resolution.

2.4 Applications in living human cartilage tissue

Cartilage is a dense, ECM-rich tissue with complex biochemical gradients that are difficult to characterize with conventional methods. Raman spectroscopic imaging was used to quantify and spatially resolve the distribution of collagen types, proteoglycans, elastin, and mineral components in real human articular cartilage. Raman maps revealed zone-specific variation in collagen crosslinking and sulfated

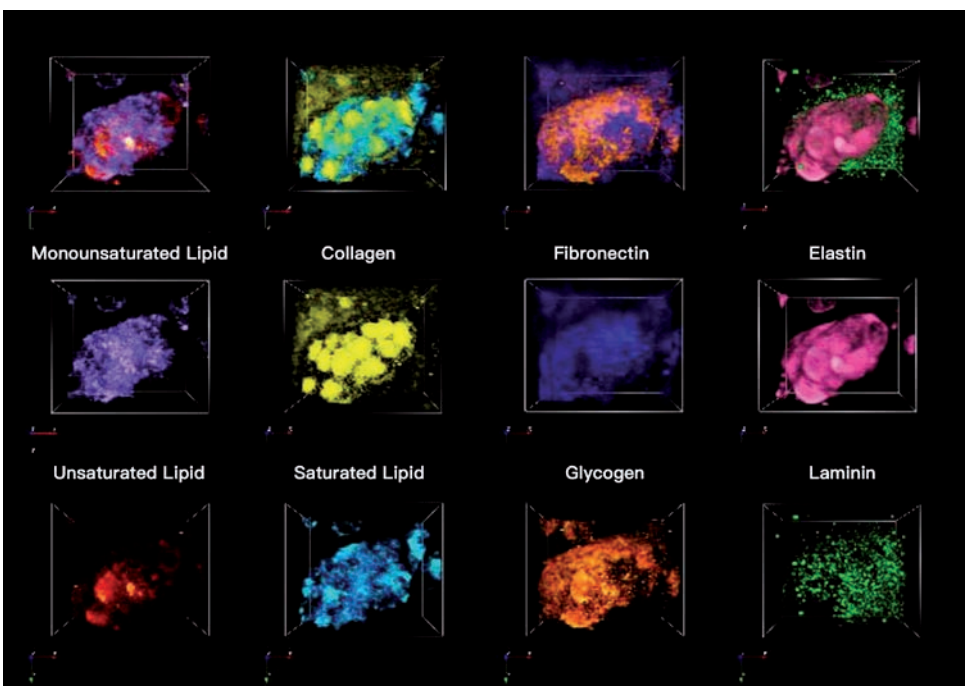

Figure 3.
Raman phenotyping of human breast cancer organoid. Figure reproduced with permission from SuperVision Medicine Co., Ltd.

glycosaminoglycans (sGAGs) from the superficial zone to the deep zone. These signatures correlate with mechanical gradients and structural integrity [9]. Importantly, Raman enabled non-destructive profiling of cartilage degeneration in osteoarthritic samples, identifying early molecular changes (e.g., collagen degradation and lipid infiltration) not detectable by histology alone. This label-free technique thus offers potential in early diagnosis and monitoring of joint disease. Raman imaging can also track the biomolecular changes during cartilage maturation. **Figure 4** shows the changes in the 3D distribution of aggrecan, collagen type, elastin, laminin, glycogen, and saturated lipid from human engineered cartilage tissues *in vitro* at weeks 3, 6, 9, and 15, *in vivo* at week 15, and the real human cartilage.

2.5 Reverse engineering of complex drug formulations

In pharmaceutical development, reverse engineering of complex formulations, such as drug-laden gels, nanocarriers, and transdermal patches, is essential for generic

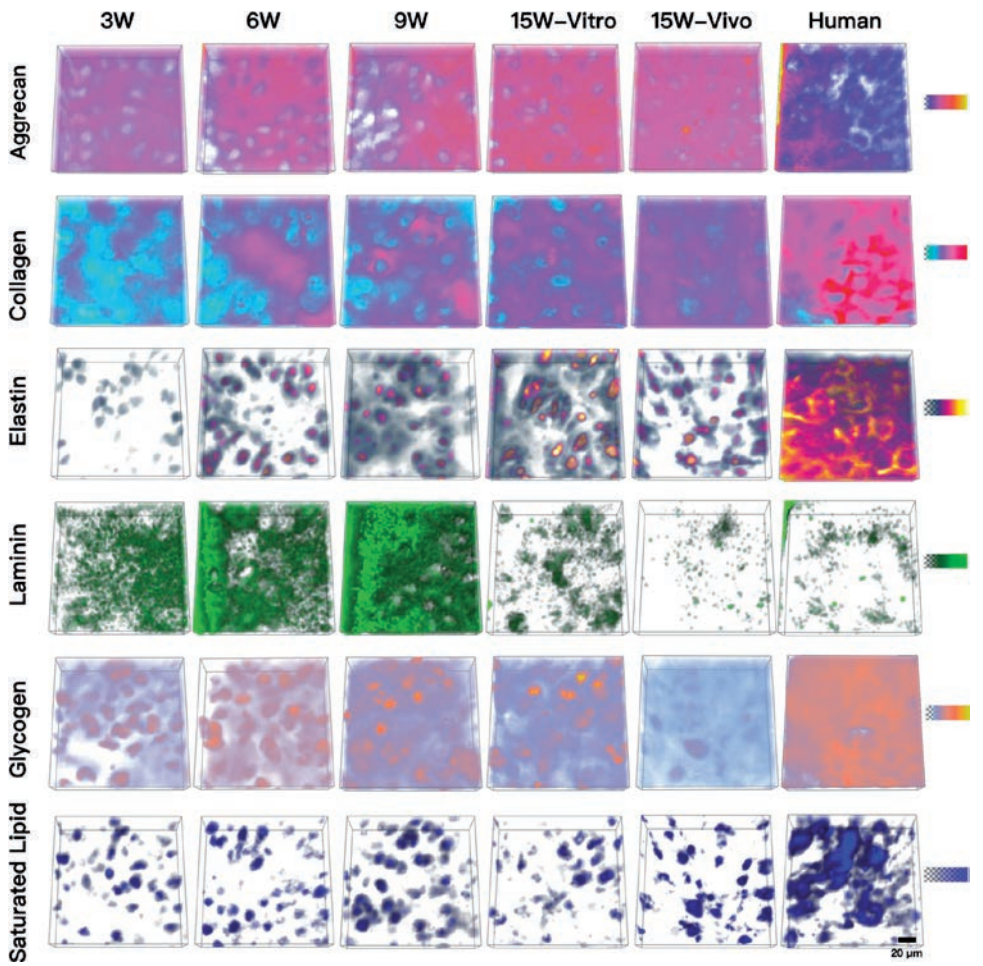

Figure 4.
Raman profiling of cartilage maturation process from week 3 to week 15. Figure reproduced with permission from SuperVision Medicine Co., Ltd.

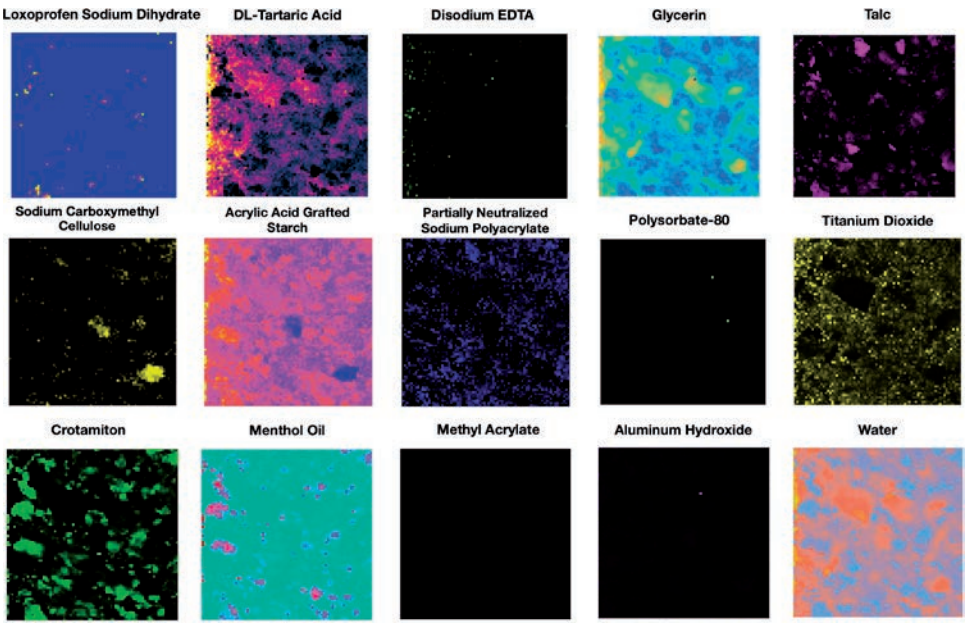

Figure 5.
Raman-based reverse engineering of a loxoprofen sodium gel patch. Figure reproduced with permission from SuperVision Medicine Co., Ltd.

drug development and regulatory approval. These systems often involve multilayered architectures and heterogeneous distribution of active pharmaceutical ingredients (APIs), excipients, and polymer matrices, making spatially resolved, component-level analysis critical.

Raman imaging, with its label-free, non-destructive nature and molecular specificity, offers a powerful tool to meet this need. We applied 2D Raman imaging to analyze highly complex gel patch formulations. By acquiring reference spectra from pure APIs, polymers, surfactants, and lipid carriers, and applying spectral unmixing techniques, we reconstructed the chemical architecture of the patches *in situ*, without the need for extraction or staining.

This enabled precise localization of APIs, detection of excipient crystallization, and identification of inhomogeneities in drug dispersion and polymer blending. Notably, Raman imaging preserved spatial integrity while revealing microstructural features inaccessible to conventional analytical methods. These capabilities make Raman spectroscopy particularly valuable for formulation optimization, stability assessment, and demonstrating bioequivalence in generic drug approval workflows. **Figure 5** presents the Raman-based reverse engineering of a loxoprofen sodium gel patch, demonstrating the capability of Raman spectroscopy to spectrally unmix and identify all major chemical constituents within the formulation. This analysis highlights the potential of Raman spectroscopy for non-destructive, label-free characterization of complex topical pharmaceutical preparations. The identified components and their respective functions in the gel patch are as follows:

1. Loxoprofen Sodium Dihydrate: The active pharmaceutical ingredient (API), a nonsteroidal anti-inflammatory drug (NSAID) used for analgesic and anti-inflammatory effects.

2. DL-Tartaric Acid: A pH adjuster and stabilizer, also used to enhance solubility.

3. Disodium EDTA: A chelating agent that binds divalent metal ions, improving formulation stability and preventing degradation.

4. Glycerin: A humectant that retains moisture and maintains the gel's softness and skin adhesion.

5. Talc: A filler and consistency enhancer; may also aid in moisture control.

6. Sodium Carboxymethyl Cellulose: A thickening agent that provides viscosity and helps form the gel matrix.

7. Acrylic Acid Grafted Starch: A bioadhesive and thickening polymer that supports gel structure and skin adherence.

8. Partially Neutralized Sodium Polyacrylate: A superabsorbent polymer that contributes to gel viscosity and moisture retention.

9. Polysorbate-80: A nonionic surfactant and emulsifier that stabilizes hydrophobic and hydrophilic components.

10. Titanium Dioxide: An opacifier and UV protector; also used for its whitening properties in topical formulations.

11. Crotamiton: An antipruritic agent that can enhance patient comfort.

12. Menthol Oil: Provides a cooling sensation and mild analgesic effect; also enhances skin penetration of the API.

13. Methyl Acrylate: A polymer precursor or monomer used in the matrix, typically present as a polymerized residue.

14. Aluminum Hydroxide: A pH buffering agent and mild astringent; may also contribute to stability.

15. Water: The primary solvent, serving as the carrier for dissolved and dispersed components.

This spectral deconvolution highlights the feasibility of label-free Raman imaging for comprehensive molecular analysis of complex pharmaceutical formulations. Such capability holds significant potential for applications in quality control, counterfeit drug detection, and formulation optimization. Moreover, this approach demonstrates the promise of Raman profiling in the characterization of creams, microspheres, and pellets—formulations that are inherently more complex than conventional solid tablets due to their multicomponent and heterogeneous nature.

3. From semi-quantitative to fully quantitative Raman imaging

For many years, Raman imaging of biological samples has been regarded as a semi-quantitative technique, offering insights into molecular identity and distribution,

but lacking a standardized method for absolute quantification. A significant breakthrough occurred in 2023, when the group led by Prof. Molly Stevens developed a robust strategy to transform spontaneous Raman imaging into a fully quantitative modality [7].

This advancement relies on the principle that Raman scattering intensity at each wavenumber is directly proportional to the molecular concentration of specific vibrational bonds. Thus, given a well-calibrated system and known reference spectra, it becomes possible to calculate the absolute abundance of multiple molecular species across a 3D biological volume. The Stevens group introduced a spectral normalization step by referencing each voxel's Raman spectrum to the intensity of the 3300 cm^{-1} peak, associated with water OH stretching. Given that water is ubiquitous and uniformly distributed in hydrated tissues, this internal normalization allows correction for depth-dependent scattering and acquisition noise. The key assumption here is that water content within each voxel remains relatively constant (e.g., ~5 fL), an assumption generally valid in osmotically balanced biological samples [7].

With this strategy, spontaneous Raman imaging can achieve voxel-level absolute quantification of proteins, lipids, nucleic acids, and other molecules in tissues and organoids. This method has been validated in liver organoids and mammary epithelial organoids models, with Raman-derived concentrations correlating with orthogonal biochemical assays and immunostaining [7, 8].

This approach circumvents key limitations of stimulated Raman scattering (SRS), which, although offering higher signal intensity, is limited to narrow spectral windows and cannot resolve complex overlapping signatures of similar biomolecules [10]. In contrast, spontaneous Raman can capture the full vibrational spectrum with high resolution (~1 cm^{-1}), enabling the separation of closely related protein or lipid species, such as Collagen Type I vs. Collagen Type II, or L-Alanine versus DL-Alanine. Enhanced techniques such as Surface-Enhanced Raman Spectroscopy (SERS) and Tip-Enhanced Raman Spectroscopy (TERS) are well-suited for the detection of specific nanoparticulate substances; however, their limited detection area and challenges in obtaining reproducible images render them less applicable to comprehensive biological imaging [11, 12]. These limitations, combined with their narrow spectral acquisition, largely explain why SERS and TERS have not been widely adopted for fully quantitative applications in complex biological systems.

Furthermore, integration of electron-multiplying CCD (EMCCD) detectors and real-time unmixing algorithms allows rapid acquisition of 0.01 to 0.05 seconds per spectrum, which is sufficient for high-throughput quantification in biological workflows [13]. These developments push Raman imaging from qualitative visualization toward a versatile platform for non-invasive molecular quantification in research and clinical applications.

4. Raman imaging for label-free *in situ* cell typing

Cell typing is foundational for understanding tissue architecture, immune responses, disease progression, and therapeutic targeting. Traditional methods such as fluorescence-activated cell sorting (FACS), immunohistochemistry (IHC), and single-cell RNA sequencing rely on labeling and often require tissue dissociation, which results in the loss of spatial context. In contrast, Raman imaging provides a label-free alternative capable of performing *in situ* cell typing directly within intact tissues and organoids, preserving spatial integrity and morphological context [13].

Unlike flow cytometry, where cells are analyzed in suspension and sorted based on fluorescence intensity, Raman imaging leverages intrinsic biochemical fingerprints of cells for classification [14]. After FACS isolation, label-free Raman reference spectra can be acquired from purified cell types, and these profiles can be used to deconvolve heterogeneous tissues using unmixing algorithms. This approach retains both biochemical and positional information.

4.1 Current challenges in cell typing

Conventional cell typing techniques face several constraints, as shown in **Table 2**:

1. *Label dependency*: Fluorescent or antibody-based labeling is required in flow cytometry and immunofluorescence, which can alter cellular physiology and is time-consuming [15].

2. *Multiplexing limitations*: Fluorescence-based platforms are generally limited to fewer than eight simultaneous targets due to spectral overlap and fluorescence bleed-through [16].

3. *Loss of spatial context*: Methods such as flow cytometry and single-cell RNA sequencing require dissociation of tissues, erasing the tissue architecture and microenvironmental context [3].

4. *Sample destruction*: Techniques like RNA-seq and mass cytometry (CyTOF) require cell lysis or fixation, preventing further analysis of the same sample [17].

5. *Subcellular resolution*: Most conventional methods cannot achieve subcellular localization, which is critical for understanding nuclear–cytoplasmic dynamics or intracellular biomolecular distributions [18].

6. *Marker discovery bottleneck*: Identification of novel or rare cell types is constrained by the need to define specific markers, which may be unknown or poorly characterized [11].

Feature	Flow cytometry	Label-free Raman imaging
Sample format	Cells in suspension	Cells/tissues *in situ*
Labels required	Yes (fluorescent)	No
Multiplexing	Limited (~4–8 markers)	Very high (>10 components)
Spatial context	No	Yes (3D + subcellular)
Cell sorting	Yes (FACS)	No (imaging only)
Destructive	Yes	No
Subcellular resolution	No	Yes
Marker discovery required	Yes	No
Cost and preparation	High	Low

Table 2.
Comparison between flow cytometry and Raman spectroscopy in cell typing.

4.2 Raman imaging as a label-free alternative

Raman spectroscopy provides a fundamentally different approach by detecting intrinsic vibrational signatures of biomolecules, such as lipids, proteins, nucleic acids, and metabolites, without the need for any labels or staining. Each cell type exhibits a unique Raman spectral fingerprint based on its biochemical composition, enabling label-free, non-destructive classification of multiple cell types simultaneously [8].

Raman imaging operates *in situ* and is compatible with three-dimensional biological systems such as tissues and organoids. This preserves tissue architecture and enables spatially resolved cell classification within their native microenvironment. Furthermore, Raman imaging is confocal-compatible, allowing subcellular resolution profiling of biomolecular distributions—a feature rarely achieved by conventional methods [7].

4.3 Complementarity with FACS for reference spectra acquisition

A synergistic workflow can be established by combining FACS and Raman imaging. After isolating pure cell populations *via* FACS, the cells can be imaged by Raman microscopy to generate reference spectral libraries. These reference spectra, devoid of fluorescent labels, can then be used to deconvolve complex tissue samples *via* spectral unmixing algorithms, enabling classification of heterogeneous populations without further labeling [19]. This approach transforms Raman imaging into a quantitative phenotyping tool that links well-characterized reference spectra with complex, intact biological tissues—offering new opportunities for personalized diagnostics, cancer subtype classification [8], and immunophenotyping.

4.4 From 2D to 3D cell typing

Unlike traditional immunofluorescence or histological staining, which are often restricted to 2D thin sections, Raman imaging supports full 3D molecular profiling. This allows for:

i. Visualization of cell–drug and cell–matrix interactions within tissue or organoids [7].

ii. Monitoring of functional states such as activation, senescence, or apoptosis based on spectral features [19].

iii. *In situ* tracking of dynamic transitions such as epithelial–mesenchymal transformation or immune activation [8].

iv. Such capability is especially valuable for studying tumor–immune niches, stem cell environments, and neural circuits, where spatial architecture influences function [8, 19].

4.5 True supermultiplexing and functional profiling

Fluorescence-based imaging is fundamentally constrained by the number of distinguishable dyes, but Raman imaging can distinguish 20 or more biomolecular targets simultaneously due to the narrow linewidth of Raman peaks (~10 cm^{-1})

and the broad spectral range (~400–3500 cm^{-1}) [8]. Cell types are identified not by specific surface markers, but by their composite molecular signature, allowing comprehensive phenotyping even in the absence of known biomarkers [7].

Moreover, Raman spectra reflect not just identity, but functional and metabolic state. For example, shifts in cytochrome c, lipid composition, or nucleic acid content can be used to infer: (i) cellular metabolism, (ii) proliferative status [20], (iii) apoptotic or stress responses, and (iv) Differentiation state [7, 19]. This enables functional cell typing beyond simple phenotypic labeling.

4.6 Operational simplicity and cost-effectiveness

Raman imaging is non-destructive, allowing the same sample to be reused for follow-up assays such as transcriptomics, proteomics, or pathology. Unlike immunolabeling protocols that require multiple washing, blocking, and incubation steps, Raman imaging: (i) requires no specialized reagents [9], (ii) has low running cost, (iii) offers real-time acquisition at the pixel level (typically 0.01–0.3 seconds per spectrum), and (iv) can be integrated into automated workflows for high-throughput analysis [13, 20].

Furthermore, since no chemical or physical alteration is applied to the sample, Raman imaging preserves true biological fidelity—a critical factor in drug screening, diagnostics, and longitudinal studies.

5. Limitations and outlook

Raman spectroscopic imaging offers unparalleled capabilities for label-free, super-multiplexed, and 3D molecular imaging. However, several limitations still hinder its broader adoption, particularly in high-throughput and clinical settings.

First, spontaneous Raman scattering generates inherently weak signals, requiring long acquisition times that limit real-time imaging. Stimulated Raman scattering (SRS) addresses this by enhancing signal intensity up to 10^4-fold, but at the cost of spectral breadth. SRS typically captures only a few Raman shifts per scan, reducing its ability to distinguish structurally similar biomolecules. Its technical complexity—demanding synchronized pulsed lasers, high-speed modulation, and custom optics—also increases cost and limits accessibility. Furthermore, the narrow spectral range of SRS constrains quantitative analysis due to fewer spectral variables for unmixing and curve fitting. In contrast, spontaneous Raman allows full-spectrum acquisition with high spectral resolution, enabling detailed molecular profiling. While slower for imaging, single-point spectra (0.01–0.5 s/pixel) are sufficient for real-time quantification, such as blood glucose monitoring. Hardware advances like electron-multiplying CCDs (EMCCDs) can reduce acquisition time, but their performance and cost vary widely, requiring careful selection for sensitive 3D imaging.

Second, autofluorescence from biological samples can obscure weak Raman signals. Near-infrared (785–1064 nm) excitation mitigates fluorescence but reduces Raman intensity, resolution, and detector sensitivity. Conversely, 532 nm excitation offers an optimal balance and is widely used due to strong signals, good resolution, and minimal phototoxicity. High-performance Raman systems with optimized optics can further suppress fluorescence, allowing strong signals from proteins, lipids, and nucleic acids. However, such systems are expensive (often $120,000–$800,000 USD) and require finely tuned configurations, limiting widespread deployment.

Third, the computational demands of spectral unmixing and quantification present a steep learning curve. Generating high-quality Raman images requires expertise across optics, biology, and data science, including custom algorithms for denoising, deconvolution, and quantification. This multidisciplinary barrier restricts high-level Raman imaging to a few expert groups worldwide. For example, the Stevens Lab has pioneered quantitative label-free Raman profiling in tissue engineering, while companies like SuperVision Medicine (China) offer professional Raman-based analysis services for biomedical research.

Despite these challenges, Raman imaging holds great promise. Its ability to perform super-multiplexed, label-free imaging without dyes or destructive processing makes it ideal for spatial metabolomics, early diagnostics, and label-free tissue classification. Emerging fiber-optic and miniaturized Raman systems are being developed for intraoperative use, enabling real-time tumor margin assessment. Ongoing efforts to standardize acquisition, build spectral libraries, and automate data analysis will enhance reproducibility and usability. Ultimately, Raman imaging is poised to become a powerful, non-destructive platform for personalized medicine, drug development, and clinical diagnostics.

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