



Chemical microscopy and image analysis for biomedical applications

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Chemical microscopy and image analysis for biomedical applications

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Chemical microscopy and image analysis for biomedical applications

A DISSERTATION PRESENTED

BY

YUXIAO WEI

TO

THE DIVISION OF MEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE SUBJECT OF

BIOLOGICAL AND BIOMEDICAL SCIENCES

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CAMBRIDGE, MASSACHUSETTS

MAY 2025

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Chemical microscopy and image analysis for biomedical applications

ABSTRACT

Chemical microscopy enables the identification and spatial analysis of chemical composition within a sample, offering powerful insights across a range of applications. This dissertation demonstrates the capability of chemical microscopy tools in diverse settings, from solid pharmaceutical materials to living organisms to human subjects in a clinical environment. The overarching principle guiding these studies is that, for simpler static samples, highly complex imaging techniques can be employed to extract rich chemical detail, whereas, for complex, dynamic biological systems, faster, more mobile tools, complemented by computational analysis, become essential.

The progression of this work begins with the application of high-resolution coherent Raman microscopy to analyze well-defined, abiotic samples, demonstrating its ability to rapidly visualize and quantify chemical components in solid pharmaceutical tablets. Extending this approach to biological systems, the technique is then applied to measure the uptake of cryoprotective agents in *Daphnia* in correlation with toxicity, a critical step towards improving the development of cryopreservation protocols. These studies illustrate how chemically specific imaging can enhance material characterization and biological assessment, while also highlighting the increasing challenges of applying sophisticated tools to living systems.

As the complexity of the sample increases, so do the demands on imaging methodology. In human subjects, where motion, heterogeneity, and physiological variability complicate data acquisition, a shift toward simpler, more mobile imaging modalities combined with computational approaches becomes essential. We use a cart-based multiphoton microscopy tool to characterize atopic dermatitis in humans *in vivo*, integrating imaging with spatial transcriptomics to improve our understanding of how RNA expression correlates with cellular level features. Further, the use of machine learning with these microscopy images enables disease classification and severity prediction, with the ultimate goal of replacing biopsies during longitudinal clinical studies of skin disease.

Through this progression from controlled, static samples to complex, dynamic systems, this dissertation illustrates both the power and the limitations of chemical microscopy across diverse applications. Ultimately, this work contributes to the advancement of chemical microscopy as a versatile, noninvasive tool for biomedical and translational research.

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Introduction

0.1 SIGNIFICANCE OF THIS THESIS

The ability to obtain spatially resolved chemical information is essential for advancements in both material and biomedical sciences. In fields ranging from pharmaceutical manufacturing to clinical diagnostics, understanding molecular composition *in situ* can improve quality control, optimize therapeutic strategies, and enhance disease characterization. Tra-

ditional chemical analysis methods, such as chromatography and mass spectrometry, provide excellent sensitivity but require extensive sample preparation and lack spatial resolution. Microscopy, on the other hand, offers critical structural insights but is traditionally limited in its ability to directly resolve chemical composition. Chemical microscopy bridges this gap by combining spatial and molecular specificity, enabling label-free, high-resolution imaging of both static materials and dynamic biological systems.

Despite its potential, the broader adoption of chemical microscopy in research, industry, and clinical practice has been hindered by challenges such as imaging speed, data complexity, equipment complexity, and cost. This dissertation advances the toolbox of chemical microscopy techniques through novel applications that demonstrate its versatility across a range of sample types. For well-defined, static samples, this work showcases how coherent Raman microscopy can rapidly analyze the spatial chemical composition of pharmaceutical tablets, offering a useful tool for manufacturing and quality control. In dynamic biological environments, chemical imaging is applied to quantify cryoprotective agent uptake in *Daphnia*, allowing for a more systematic method of cryopreservation protocol development. Finally, this work pushes chemical microscopy toward clinical applications by integrating multiphoton imaging with spatial transcriptomics and computational analysis to characterize atopic dermatitis *in vivo*, demonstrating its potential for noninvasive disease assessment.

By addressing key technical and analytical challenges such as speed, chemical specificity, and quantification, this dissertation moves chemical microscopy closer to practical implementation in real-world settings. It highlights how complex imaging techniques can be optimized for structured, controlled environments while emphasizing the need for faster,

more mobile solutions for *in vivo* applications. This work not only expands the utility of chemical microscopy across different scientific domains but also lays the groundwork for its broader adoption in research, industry, and clinical settings.

0.2 SPECIFIC AIMS

The overall goal of this dissertation is to demonstrate the capability of chemical microscopy tools in applications ranging from abiotic samples to living biological systems, with an emphasis on noninvasive, label-free imaging for translational applications. This work is guided by the principle that highly complex imaging techniques can be used for well-defined, static samples, while biological and clinical applications require faster, more mobile tools complemented by computational approaches. The specific aims of this dissertation are as follows:

1. **Visualize and quantify chemical components of pharmaceutical tablets using high-speed chemical imaging by Sparse Spectral Sampling Stimulated Raman Scattering (S₄RS) microscopy**
 - Demonstrate the ability of spectral decomposition methods to distinguish chemical components with overlapping spectra.
 - Identify chemical and spatial changes in tablet composition after exposure to changes in temperature and humidity.
2. **Visualize and quantify cryoprotective agent (CPA) uptake in *Daphnia* organisms using S₄RS microscopy.**
 - Establish label-free, high-resolution imaging protocol to immobilize organisms and quantify chemical uptake in various anatomical locations.

- Determine toxic levels of CPA concentrations in daphnia, specific to anatomical location and incubation time.
- Provide insights into optimizing cryopreservation protocols for biological specimens.

3. **Characterize atopic dermatitis in human subjects with advanced microscopy and immune profiling analysis.**

- Characterize atopic dermatitis using reflectance confocal, second harmonic generation, two photon excited fluorescence, fluorescence lifetime microscopy in the skin of human subjects.
- Characterize atopic dermatitis using spatial transcriptomics.
- Correlate spatial biomarker expression with cellular level image features on microscopy.

0.3 THESIS OUTLINE

This thesis is presented in six chapters. This chapter introduces the thesis, its significance, and specific aims. Chapter 2 presents the background information on tools used to obtain chemical and spatial information, particularly tools that retain spatial information, and presents the advantages and challenges related to these sets of tools. It provides a detailed explanation of the origins and implementation of each technique and their major applications, along with how their data have been analyzed.

Chapters 3-5 cover the original work conducted in this thesis along with the background related to each specific application of microscopy. Chapter 3 describes the application of

coherent Raman microscopy and spectral decomposition to rapidly analyze chemical components of a solid drug tablet, which can be valuable for pharmaceutical manufacturing and quality control. Chapter 4 describes the application of coherent Raman microscopy to quantify cryoprotective agent uptake in daphnia organisms, which can assist in developing cryopreservation protocols that promote long-term organism viability. Chapter 5 describes the application of advanced microscopy to characterize atopic dermatitis skin on human subjects *in vivo* along with correlative analysis to spatial transcriptomics. Chapter 6 presents a summary of the work, consideration of future directions in each application, and a discussion of the future potential of chemical microscopy in biomedical applications.

1

Background

Microscopy has played a fundamental role in advancing scientific understanding of both biological and non-biological materials, enabling visualization of structures far beyond the limit of human vision. Since the invention of the light microscope over four centuries ago, imaging technologies have evolved dramatically, allowing researchers to probe increasingly finer details with enhanced contrast mechanisms. Such advancements have been instru-

mental in fields ranging from biomedical research and clinical diagnostics to materials science and pharmaceutical development. Broadly, imaging techniques can be classified into non-destructive methods, which allow for the preservation of sample integrity and enable longitudinal studies, and destructive methods, which can provide detailed chemical and molecular information at the cost of sample viability.

Non-destructive imaging techniques, especially those based on optical microscopy, can provide spatially resolved information in a manner that preserves the dynamic and functional states of biological specimens and material systems. These range from conventional widefield microscopy to more sophisticated approaches such as confocal microscopy and label-free, chemically specific modalities such as coherent Raman microscopy.

While advances in optical microscopy have enabled label-free chemical and spatial characterization of samples *in situ*, its chemical specificity and quantification capabilities are still limited compared to many techniques that require macro-scale sample destruction. Methods such as nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) yield highly specific chemical information, while others such as single-cell RNA-sequencing and spatial transcriptomics (ST) provide detailed genetic insights. Not only are destructive methods crucial for biomedical research on their own, but have also been used to validate quantitative microscopy measurements of chemicals in cells and tissues *in situ*.^{24,25,26}

This chapter provides an overview of both non-destructive and destructive methods, briefly discussing their underlying physical principles, major technological advancements, and their most significant applications in biomedical research. Additionally, the integration of computational techniques, including spectral analysis, phasor analysis, and machine

learning, has become critical for interpreting imaging data. These approaches play a key role in extracting quantitative insights from microscopy, allowing for automated feature recognition, classification, and predictive modeling.

1.1 NON-DESTRUCTIVE IMAGING: MICROSCOPY METHODS AND CONTRAST MECHANISMS

1.1.1 WIDEFIELD MICROSCOPY

Widefield light microscopy serves as the foundation for modern optical imaging by establishing fundamental concepts such as image formation using visible light, contrast mechanisms, resolution limits, and magnification. It still remains widely today used due to its accessibility and straightforward implementation. In the simplest configuration, an illumination source emits light through an objective lens onto an area of a thin sample and the light is either passed through the sample to the other side, i.e., transmission, or reflected onto an ocular lens through which the image is viewed (Fig. 1.1A).²⁷

Traditional brightfield microscopy as described above enables visualization of structures that cause changes in light absorption and scattering. Light absorption by pigment or dyes in a sample leads to a decrease in the intensity of the light transmitted to the detector. Similarly, light scattering, which changes the path of propagation of light, will also decrease the intensity of light at the detector in the transmission orientation, but will increase the detected intensity in the reflected configuration (with the detector on the same side as the light source). These methods of contrast are often generated using broadband light sources in the visible range, such as gas arc lamps or LEDs. Brightfield microscopy has been widely adopted for histopathological examination of stained specimens,²⁸ quality control and polymer research in material science,²⁹ and pathogen identification in microbiology.³⁰

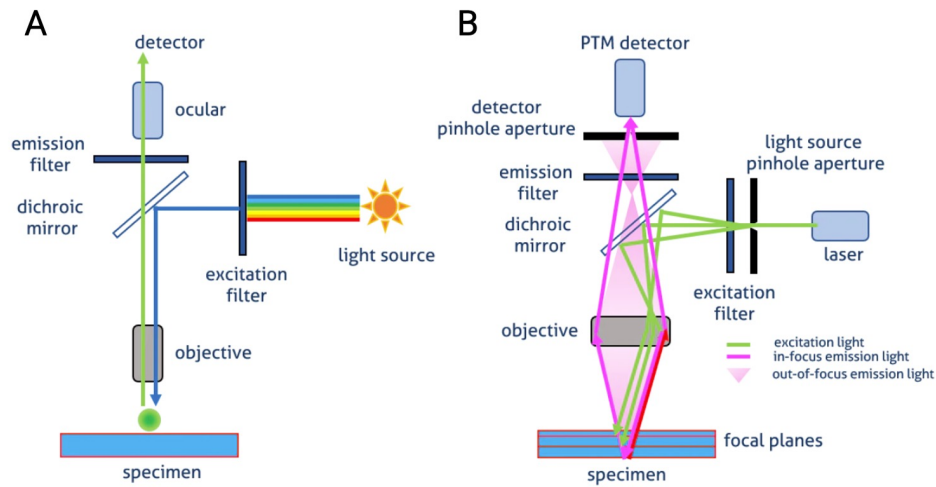


Figure 1.1: (A) Epi-illumination widefield microscopy setup. (B) Epi-illumination (reflectance) confocal microscopy setup. Adapted from Ref.¹

However, brightfield microscopy is inherently limited due to its low resolution and relatively low contrast, particularly when observing unstained samples²⁷.

One variation of brightfield microscopy, phase contrast microscopy, translates small variations in phase into a corresponding change in light amplitude, represented as image contrast.²⁷ This is helpful for visualizing very thin ($5\text{--}10\text{ }\mu\text{m}$) unstained specimens which exhibit little to no absorption or scattering in the visible spectrum.³¹ A related technique is differential interference contrast microscopy, which amplifies minute differences in specimen thickness gradients and refractive index to generate high contrast in cell boundaries such as plasma membranes, lipid bilayers, and mitochondria.^{32,27} Other mechanisms of generating contrast include darkfield illumination, which eliminates unscattered light to enhance contrast of thin unstained sample,³³ and polarized light microscopy, which emphasizes birefringent materials (materials with different refractive indices based on the polarization direction of light) such as muscle tissue and mitotic spindle.^{34,35}

Still, even with these modifications, a significant disadvantage of widefield light microscopy is that its techniques are hindered for thick specimen where material outside of the focal plane may strongly interfere with light transmission, producing a less informative image. This limitation has led to the development of more advanced optical techniques designed to isolate and enhance contrast at the focal plane and resolve finer structural and chemical details.

1.1.2 CONFOCAL MICROSCOPY

Confocal microscopy and reflectance confocal microscopy (RCM) offer substantial improvements over conventional widefield microscopy by employing a pinhole aperture to reject out-of-focus light, thereby enhancing optical sectioning and enabling three-dimensional reconstruction of thick samples (Fig. 1.1B).³⁶ In contrast to widefield microscopy where an entire imaging area is illuminated and captured at once, confocal microscopy uses its pinhole aperture to isolate a smaller focal spot. This means that to generate an image, the focus spot must be scanned over the area of interest such that data is collected point by point in x, y, and z. Confocal microscopy is commonly used for high-resolution imaging of immunohistochemistry (IHC)³⁷ and live imaging of organisms or cells.³⁸

Confocal microscopy can be implemented in the transmission or reflectance configuration. The RCM setup is modified from transmission confocal microscopy in that the same objective is used for light emission and detection, and only the reflected light is visualized, allowing for imaging the shallow surface of highly reflective or infinitely thick samples.³⁶ RCM has been adopted in materials science for surface topography analysis³⁹ and has been FDA approval in clinical dermatology for noninvasive skin imaging and cancer di-

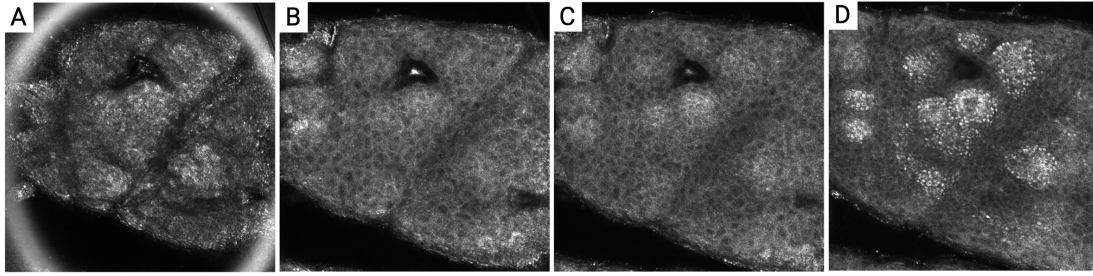


Figure 1.2: Representative RCM images of human skin with melanin pigment, acquired at depths corresponding to the (A) stratum corneum, (B) stratum granulosum, (C) stratum spinosum, and (D) stratum basale. The stratum corneum shows spots of high signal from keratin. The stratum granulosum and stratum spinosum demonstrate a "honeycomb pattern" of roughly hexagonal keratinocyte cell borders present in healthy skin. The stratum basale shows basal cells with high reflectivity due to their supranuclear melanin caps. Adapted from Ref. ²

agnosis. ⁴⁰ RCM contrast within the skin, as with any sample, comes from light scattering and absorption resulting from changes in refractive index. As such, tissue structures with distinctive refractive index transitions, such as keratin fibers, cell borders, and melanin, are more prominent in imaging (Fig. 1.2). ⁴¹ RCM has been studied for characterizing melanoma and nonmelanoma skin cancer for clinical use ^{42,43,44} and has also been applied to evaluate inflammatory skin diseases such as atopic dermatitis and psoriasis in academic research. ^{45,46,47} A large limitation of this modality is that inflammatory processes greater than 200 μm in depth cannot be examined by RCM due to low light penetration ⁴⁸. Additionally, for these and all other point scanning modalities mentioned heretofore, one clear limitation is speed.

1.1.3 FLUORESCENCE

Fluorescence imaging adds chemical selectivity as a separate contrast mechanism. In fluorescence, a molecule absorbs a photon and is promoted to an excited state before undergoing vibrational relaxation and then releasing a photon of lower energy as it relaxes to

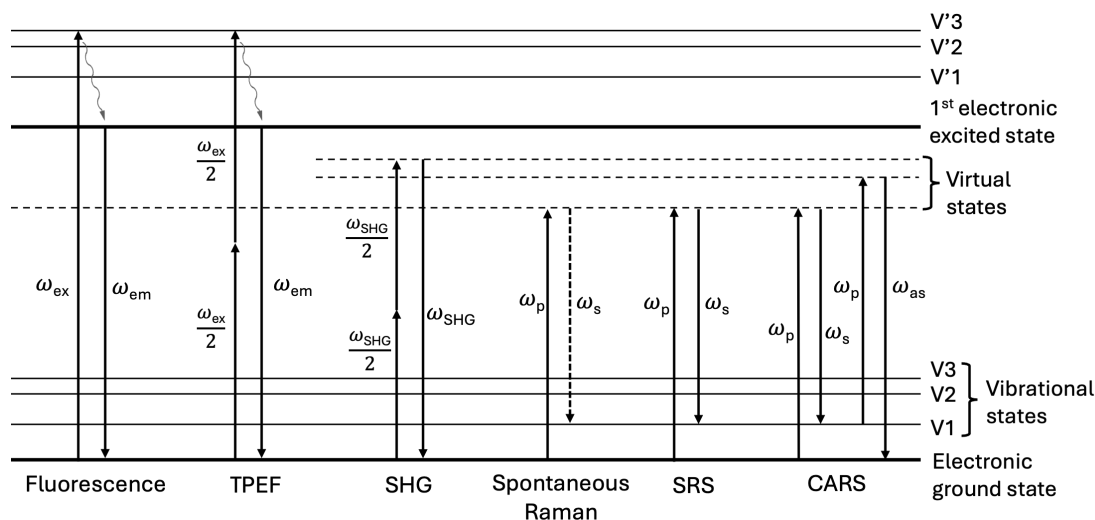


Figure 1.3: Energy diagram of fluorescence, two photon excited fluorescence (TPEF), second harmonic generation (SHG), spontaneous Raman, stimulated Raman scattering (SRS), and coherent anti-Stokes Raman scattering (CARS) interactions. Adapted from Ref.³

ground state (Fig. 1.3).⁴⁹ Fluorescent molecules are excited by different wavelengths of light and emit a range of wavelengths, which give them their individual excitation and emission spectra (Fig. 1.4). By employing fluorophores that emit light upon excitation at specific wavelengths, fluorescence microscopy provides highly specific molecular contrast that is distinct from light scattering and absorption. Fluorescence microscopy has been extensively utilized in live-cell imaging, molecular biology, and neurobiology for studying protein interactions through Förster resonance energy transfer (FRET),^{50,51} gene expression through fluorescence *in situ* hybridization (FISH),^{52,53} and cellular dynamics.⁵⁴

Certain endogenous biological molecules such as NADH/NADPH, flavins, and collagen, can exhibit autofluorescence, but these often generate weaker signals than externally introduced molecules used for fluorescent dyes or tags, such as rhodamine or green fluorescent protein (GFP). The reliance on exogenous fluorescent labels presents challenges,

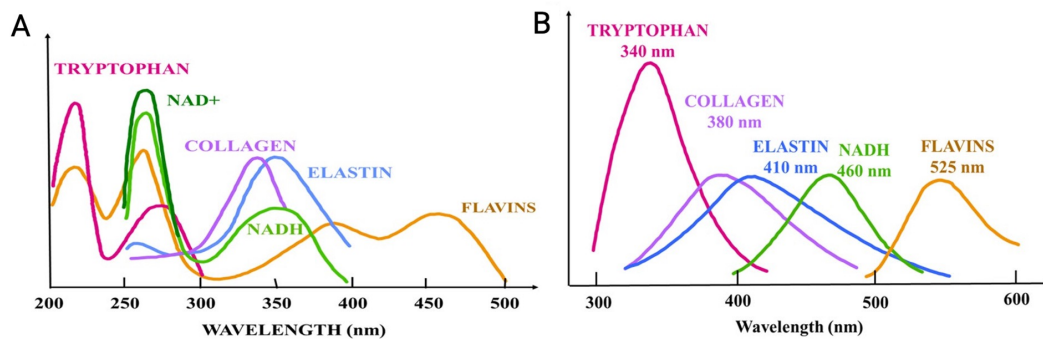


Figure 1.4: Fluorescence (A) absorption and (B) emission spectra of key biomolecules. Adapted from Ref.⁴

including photobleaching, cytotoxicity, and potential perturbation of native biological processes, restricting its applicability in certain *in vivo* and clinical settings.⁵⁵

FLUORESCENCE MICROSCOPY

Fluorescence microscopy is often performed using either widefield or confocal microscopes. For these setups, the simplest modification is to use a laser source or a narrowband filter applied to a broad source and add an emission filter which rejects the source wavelength and passes the fluorophores emission wavelengths.²⁷ As stated previously, widefield fluorescence microscopy illuminates the entire region of interest, enabling faster imaging at the risk of interference from fluorophores outside of the focal plane. On the other hand, confocal fluorescence microscopy enables optical sectioning in depth but requires a longer image acquisition time. There are also more complex setups for fluorescence imaging that allow for improvements in various imaging dimensions such as resolution and depth. The resolution of confocal fluorescence imaging is diffraction limited, which means the highest possible resolution is proportional to the wavelength of observable light and inversely pro-

portional to the numerical aperture: $d = \frac{1.22\lambda}{2NA}$ where d is the distance between two adjacent particles that can be perceived as separate, λ is the wavelength of illumination, and NA is the numerical aperture of the objective or illumination source, whichever is smaller.²⁷ In practice, for visible light, the lateral and axial resolution limits are around 200 nm and 600 nm, respectively.⁵⁶ One of the most successful techniques to overcome the diffraction limit is Stimulated Emission Depletion (STED) microscopy, which works by selective deactivation of fluorophores outside of the center of the excitation spot and can reach resolutions of 30 nm laterally and 100 nm axially.^{57,56}

TWO-PHOTON EXCITED FLUORESCENCE MICROSCOPY

A popular technique for improving penetration depth while maintaining high resolution is two-photon excited fluorescence (TPEF) microscopy. This is a nonlinear optical imaging modality which exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths.^{58,59} In TPEF, two photons of approximately half the energy needed for one-photon excitation can also excite a fluorophore in one quantum event. The two photons must be absorbed simultaneously, which requires a high peak flux of excitation photons generated by a femtosecond pulsed laser and only occurs at a very small focal spot. This also means that TPEF enables optical sectioning without the pinhole aperture required for confocal microscopy.^{58,59} Since most commonly used fluorophores have excitation spectra in the 400-500 nm range, lasers used to excite TPEF lie in the 700-1100 nm range and allows for an almost 2x increase in penetration depth.⁶⁰ Additionally, longer wavelengths are less likely to cause photobleaching because they carry less energy. As such, TPEF has commonly been used for live cell imaging and label-free *in vivo* and clinical imaging.⁶⁰

FLUORESCENCE LIFETIME IMAGING MICROSCOPY

Fluorescence lifetime imaging microscopy (FLIM)—often paired with single photon fluorescence microscopy or TPEF—provides additional functional contrast by measuring the temporal decay of fluorescence emission, which can be correlated with metabolic states and microenvironmental conditions and can also be used for multiplexing. One disadvantage of fluorescence microscopy or TPEF of autofluorophores is that they often have overlapping excitation and emission spectra, which can make it difficult to distinguish them from each other (Fig. 1.4). FLIM allows us to distinguish fluorophores with highly overlapping spectra based on their fluorescence lifetime (τ), which is the average time that a fluorophore remains in its excited state. During this period, the fluorescence intensity decreases to $1/e$ or 36.8% of its original value. FLIM also allows for detection of changes in the molecular environment as lifetimes of many molecules change in response to temperature and pH, as well as changes in molecular conformation. It has been applied often to study cellular metabolism *in vivo* through the endogenous fluorophores NAD(P)H and FAD because their bound and unbound states have different lifetimes and indicate usage of different metabolic pathways such as glycolysis, oxidative phosphorylation, and fatty acid oxidation.^{61,62}

Fluorescence lifetimes can range from picoseconds to nanoseconds, meaning that lifetime detection requires fast electronics coupled with efficient photon detectors. FLIM can be measured in the time or frequency domains. Typical time domain measurements use a short pulse of light relative to the lifetime of the sample, typically in the picosecond range, for excitation. Then, the exponential decay of fluorescent molecules is recorded directly via gated detection or pulse sampling, or using time-resolved electronics that bin photons

by their arrival times. Frequency domain measurements enable faster acquisition speed at the expense of photon count, so it is not often used to image autofluorophores. The most common implementation of FLIM uses a time domain fast electronic method called time-correlated single photon counting (TCSPC) due to its timing resolution and high SNR for weakly fluorescent samples. In TCSPC, the time between excitation and emission photons is measured to determine the time-of-arrival for each emission photon. These photons are detected across thousands of excitation pulses to build a histogram at each image pixel. The histogram distribution at each pixel can be exponentially or multi-exponentially fitted to determine the fluorescence lifetime(s) present.⁶¹

There are multiple methods for estimating fluorescence lifetime from FLIM data. Curve fitting methods determine the lifetime decay variables (α , τ) by fitting an estimated decay function and estimated or measured instrument response function (IRF) to experimental data. These methods include least square fitting, maximum likelihood estimation, global analysis, and Bayesian analysis. They are limited to use with time domain measurements because they rely on analyzing fluorescent decay as a function of time, and also require some prior assumptions, including the number of lifetime components, temporal offset of detected signals, and sources of background fluorescence.⁶¹

Fit-free methods for estimating lifetime parameters can be used with time domain or frequency domain data. The most common is the phasor approach, in which lifetime data is transformed into phasor space to visualize pixels with similar lifetime value:

$$g_{ij}(\omega) = \frac{\int_0^T I(t) \cdot \cos(n\omega t) dt}{\int_0^T I(t) dt} \quad (1.1)$$

$$s_{ij}(\omega) = \frac{\int_0^T I(t) \cdot \sin(n\omega t) dt}{\int_0^T I(t) dt} \quad (1.2)$$

in which $g_{ij}(\omega)$ and $s_{ij}(\omega)$ are the x and y coordinates of the phasor plot, n and ω are the harmonic frequency and the angular frequency of the excitation, respectively, and T is the repeat frequency of the acquisition.⁶³ Phasor analysis is instantaneous because it does not require an iterative fit procedure and is therefore useful for large FLIM datasets. All single exponential lifetimes will fall on a semicircle of radius $\frac{1}{2}$ and center $(\frac{1}{2}, 0)$, also called the universal circle. A short lifetime having a smaller phase will lie near the point $(1, 0)$, which corresponds to $\tau=0$, while a long lifetime will fall near the universal circle coordinates $(0, 0)$, which corresponds to $\tau=\infty$. Phasors are also linearly additive. As such, the phasor location of a mixture of two species will fall on a straight line joining the phasor locations of the two individual species on the universal circle (Fig. 1.5A). The relative contributions of the two components will be inversely proportional to the distance of the new phasor point from each of the individual species' points.

FLIM has been used to understand molecular features of biological systems and changes due to disease progression or drug treatment. To gain a better understanding of cutaneous pharmacokinetics, Jeong et. al., used FLIM with phasor analysis to visualize topical minocycline and tazarotene distribution within human skin (Fig. 1.5B).⁶ FLIM has been used to investigate changes in human skin disease *in vivo*, showing changes in cell metabolism in lesional skin of subjects with atopic dermatitis (Fig. 1.5C).^{64,7} Other groups have applied FLIM to identify differences in autofluorescence lifetime between normal, low-grade, and high-grade precancerous epithelia in the hamster cheek pouch *in vivo*,⁶⁵ and reveal distinct neuron and glial cell responses post-injury in 3D brain tissue models.⁶⁶

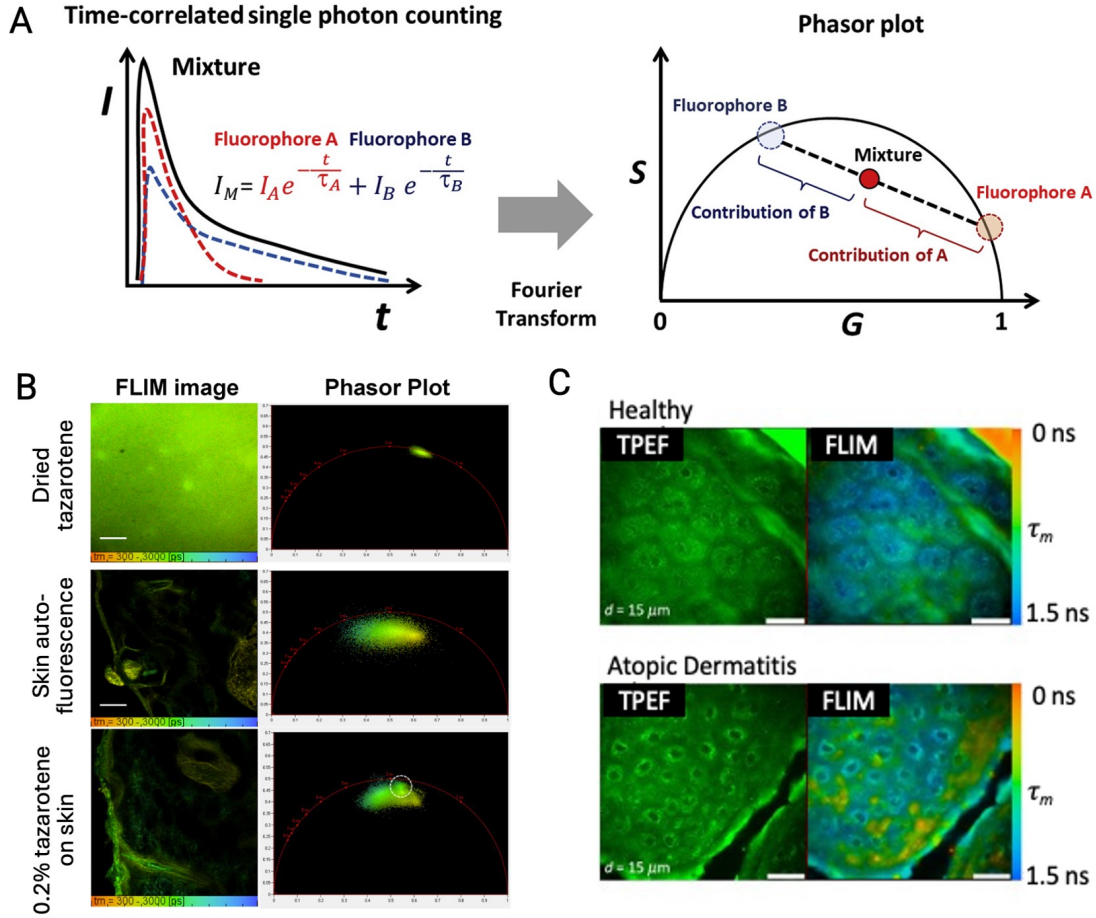


Figure 1.5: (A) Phasor analysis of two single-lifetime components τ_A and τ_B . Schematic of time-correlated single-photon counting fluorescence lifetime exponential decay traces (left) and the phasor plots of two fluorophores and their mixture (right). The contributions of two fluorophores to the mixture pixels can be determined by computing the distance between the pixel's phasor and two references phasor cluster. Adapted from Ref. ⁵ (B) FLIM images and phasor plots of dried tazarotene, skin autofluorescence (axial section), and skin with 0.2% tazarotene applied after 24 hours. The white circle indicates the phasor cluster region associated with tazarotene uptake. Adapted from Ref. ⁶ (C) *in vivo en face* TPEF and FLIM images showing a decrease in the fluorescence lifetime of keratinocytes in humans with atopic dermatitis compared with healthy skin. Adapted from Ref. ⁷.

Despite the abundant information that FLIM provides, the technique is limited by cost, speed, and signal quality in living organisms. Sophisticated instrumentation is required for fast counting, which is more expensive than traditional fluorescence microscopy. Image acquisition is also slower because more photons are needed for accurate lifetime estimations, making it more susceptible to motion artifacts from breathing and heartbeats in live imaging. SNR is often low for *in vivo* applications, particularly at imaging depths greater than 200 μm , and analysis can be time intensive and requires specific expertise.⁶¹ As the technology progresses, instrumentation improvements can advance the capabilities of FLIM, for example, providing detectors with shorter dead time. But even now, computational improvements can compensate for certain limitations, for example, we can use machine learning techniques to improve SNR such that we can use a shorter acquisition time and image at deeper depths. These techniques will be discussed in the "Machine learning for image analysis" subsection.

1.1.4 SHG AND RAMAN IMAGING

Other optical techniques have been developed for label-free high-resolution, chemically-specific imaging. Generating contrast via exogenous agents may compromise sample integrity and affect experimental outcomes. Whether the label is a genetically expressed fluorescent protein, conjugated fluorescent tag, quantum dot, or nanoparticle, the presence of an exogenous agent can alter the environment to an extent that the results may be inaccurate.⁵⁵ In one instance, if we want to longitudinally monitor the concentration of a diffusive small molecule within an organism, tagging the molecule with a fluorescent marker could change the diffusion constant of the molecule and alter the observed concentration.

Still, in other settings such as imaging chemicals or tissues *in situ*, or humans in clinical settings, it may not be possible to apply exogenous labels. Thus, the ability to generate intrinsic contrast from endogenous molecules and structures is highly desirable.

SECOND HARMONIC GENERATION MICROSCOPY

Second-harmonic generation (SHG) microscopy is a coherent second-order nonlinear imaging modality often used for *in vivo* and *in vitro* investigations into cancer and disease. SHG generates intrinsic contrast from non-centrosymmetric structures (lacking a center of symmetry) such as type I and II collagen, myosin, and crystalline materials, making it useful in connective tissue imaging and material science applications.⁶⁷ In SHG, two lower energy photons are up-converted to exactly twice the incident frequency of the excitation laser (Fig. 1.3). This occurs only when two photons of the same frequency interact within a non-centrosymmetric material, and generates highly specific signal with sharp contrast.⁵⁸ SHG arises from induced polarization rather than absorption, which leads to substantially reduced photobleaching and phototoxicity compared to fluorescence methods.

SHG has been used to differentiate between healthy and diseased tissues because structural changes that alter the helical pitch angle and/or interchain crosslinking will also alter the physical assembly of collagen fibrils and fibers, which yield different signatures when viewed in SHG.^{68,8} Another form of contrast arises as SHG intensity depends on the level of phase matching between the incident and SHG photons. If the sample is phase-matched, as with uniaxial doubling crystals, then the phases of the SHG and incident photon are exactly matched: $\Delta k = k(2\omega) - 2k(\omega)$. Collagen in real tissues has inherent randomness and dispersion which results in a distribution of nonzero Δk values. Along with

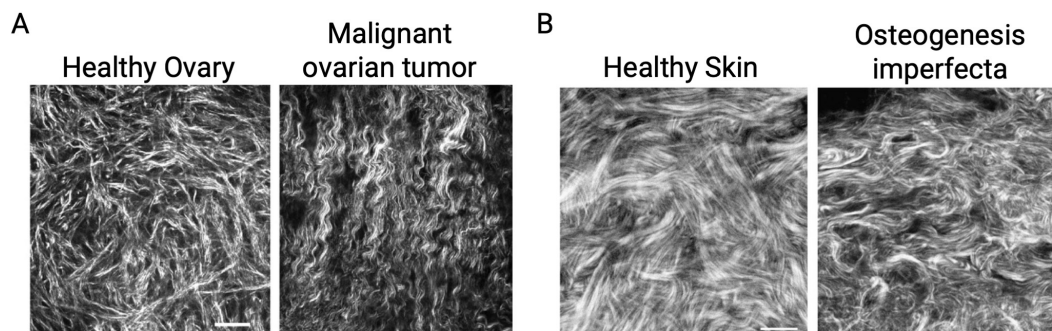


Figure 1.6: (A) SHG images of normal ovary and malignant tumor arising within an ovary. Collagen fibers are "cross-hatched" in the normal, whereas they form highly regular helical structures in the tumor specimens. Adapted from Ref.⁸. (B) SHG images of collagen in the dermis of wild type and osteogenesis imperfecta (oim) mouse models. In comparison to wild type, oim tissue demonstrates less dense packing of collagen fibers. Adapted from Ref.⁹. Scale bars=25 μ m.

imperfect phase matching, SHG emission from tissues has a distribution of emitted forward and backward components. As such, the SHG intensity scales with the level of phase mismatch, which is caused by the regularity of the fibril/fiber assembly, and which is often different between healthy and diseased tissues.⁶⁹ For example, it has been shown that bones with osteogenesis imperfecta have greater phase mismatch and lower SHG intensity compared to healthy tissues.⁹ SHG has also been able to differentiate other connective tissue disorders such as Marfans syndrome,⁷⁰ Ehler-Danlos syndrome,⁷¹ as well as dermal photoaging, corneal disorders, breast cancer, ovarian cancer, and skin cancer.⁶⁷

The greatest limitation of SHG is that the endogenous contrast agents that generate SHG signal are limited to types I and II collagen and acto-myosin complexes in tissues. Microtubule assemblies in live cells can also yield low levels of signal.⁶⁷ Other limitations of SHG are shared with other nonlinear optical techniques and include slow acquisition speed and limited penetration depth to a few hundred microns.

COHERENT RAMAN MICROSCOPY

Among the most promising advances in label-free chemical imaging are coherent Raman techniques, including coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) microscopy. These methods exploit intrinsic molecular vibrations to provide chemically specific contrast, enabling the rapid visualization of molecular distributions within biological tissues and complex materials.

RAMAN SCATTERING CARS and SRS utilize the Raman scattering effect, which represents another form of intrinsic contrast distinct from elastic scattering and absorption, fluorescence, or SHG (Fig. 1.3). Spontaneous Raman scattering is an inelastic scattering of photons which occurs when the energy of incoming light changes as it scatters off of a sample. In one scenario, the incident photon excites the molecules in the sample to a higher energy virtual state, then relaxes down to a higher energy vibrational state. The photon transfers energy to the molecule so the scattered photon has a lower energy (and longer wavelength), which is called Stokes Raman scattering. In another scenario, when the molecule starts in the higher energy vibrational state and is excited to the virtual state by the incident photon, and it relaxes back down to a lower energy level, the scattered photon will have a higher energy (shorter wavelength) than the incident photon. This is called anti-Stokes Raman scattering.

RAMAN SPECTROSCOPY AND CONFOCAL RAMAN MICROSCOPY Raman spectroscopy is an analytical technique based on the Raman effect which excites a sample using a monochromatic light source and collects the Raman spectrum or the Raman shift (the intensity of

scattered light as a function of its frequency difference from the incident photons). This process creates a unique fingerprint used for sample identification as Raman shifts are functions of the specific chemical bond vibrations within the sample. Raman scattering requires a change in the polarizability of the chemical bond during a vibrational motion. As such, molecules with bonds that have a strong dipole moment, high bond strength, and small bond length will have weaker Raman signals.⁷² Raman spectroscopy is often used in chemistry to identify molecules and study chemical bonding and intramolecular bonds.⁷³ It is also used in the biopharmaceutical industry to identify active pharmaceutical ingredients (APIs) and their polymorphic forms.⁷⁴ Within biology and medicine, Raman spectroscopy can provide noninvasive and nondestructive measurements for *in vivo* and *ex vivo* tissues and cells.⁷⁵ Raman spectroscopy is a point-based method, but can be scanned for imaging, aka confocal Raman microscopy (CRM).

STIMULATED RAMAN IMAGING While CRM enables highly specific chemical analysis of molecular vibration modes with spatial context, the spontaneous Raman scattering effect is too weak to allow image acquisition speeds sufficient for real-time monitoring of dynamic events. The invention of pulsed lasers with high peak power facilitated the discovery of coherent Raman scattering (CRS) phenomena to enhance sensitivity and offer much faster image acquisition capabilities.^{76,77} CRS imaging is typically implemented as coherent anti-Stokes Raman scattering (CARS) or stimulated Raman scattering (SRS) microscopy, where both techniques utilize synchronized pulsed lasers to coherently excite vibrational modes within a sample. When the energy difference between the two pulsed lasers matches the vibrational modes' energy of the sample molecule, CARS and SRS can be detected within the sample (Fig. 1.3). CARS is detected via the generation of photons at the anti-

Stokes frequency, while SRS detects the energy transfer from the higher frequency beam (pump) to the lower frequency beam (Stokes), detected either through stimulated Raman loss (SRL) or stimulated Raman gain (SRG), respectively. CARS is technically easier to implement and has seen widespread use in cell and tissue imaging.^{78,79} However, in contrast to SRS, CARS is a parametric process and thus suffers from a significant non-resonant background and spectral distortion.⁸⁰ Additionally, SRS signal intensity scales linearly with the concentration of Raman scatterers, which enables quantitative analysis.⁸¹ As such SRS is preferred over CARS in a variety of applications, including pharmacokinetic studies of dermal drugs^{82,83,84,85} as well as quantification of drug uptake, metabolism, and protein concentrations in cells.^{86,87,82}

In SRS, highly efficient scattering occurs such that energy of the incident pump beam is converted into the Stokes frequency and allows for 4 to 5 magnitudes of gain in efficiency compared to spontaneous Raman scattering.⁵⁸ The physical phenomena can be understood intuitively through an energy level diagram (Fig. 1.3). In SRS, two beams, pump (ω_p) and Stokes (ω_s) incident on the sample generate stimulated emission when the energy difference $\Delta\omega = \omega_p - \omega_s$ matches a particular molecular vibration in the sample. Stimulated emission can be thought of as the excitation of a molecule from the ground state to a virtual state by the pump photon with the emission of an additional Stokes photon as the molecule spontaneously falls to a vibrational state. The incident Stokes beam ensures a higher initial number of photons in ω_s , which enhances the stimulated emission process and increases the rate of ω_s photon emission.⁸⁸ The SRS interaction can be measured through the loss of photons in ω_p (stimulated Raman loss, SRL) or the gain of photons in ω_s (stimulated Raman gain, SRG). A clear and practical difference between spontaneous

Raman and CRS is that while spontaneous Raman uses one laser to probe all vibrational bands, CRS typically uses two lasers, whose frequencies are tuned to match a specific vibrational mode to achieve much more efficient signal generation.

Initial SRS microscopy was reported using a femtosecond amplified laser, split into a narrowband femtosecond pulse and a broadband white light pulse for excitation, and multiplex detection with a photodiode array.⁸⁹ However, the amplified laser system was unsuitable for imaging biological specimens due to high peak powers causing sample damage and low repetition rates limiting image acquisition speed. The first practical SRS microscope for biomedical imaging was developed using a high-repetition rate (76 MHz) laser with four orders of magnitude lower peak power, modulation transfer, and lock-in detection.⁹⁰ In a modulation transfer scheme, either one of the Stokes or pump beams is modulated (SRG at the pump wavelength or SRL on the Stokes wavelength, respectively) such that the generated SRS signal is also modulated at the chosen frequency. Lock-in amplification at the frequency of Stokes (or pump) modulation is critical to detect the small SRL (or SRG) signals over laser noise, enabling signal detection at four orders of magnitude more sensitive than the previous multiplex detection scheme.

Most current SRS microscopy systems employ dual-color picosecond or femtosecond laser sources in the near-infrared region, a laser scanning microscope, and a photodetector with lock-in amplification (Fig. 1.7). The laser source can be a free-space optical parametric oscillator (OPO) system, which offers automatic pulse synchronization and high spectral resolution at the expense of high cost and necessary cooling systems, or an all-fiber laser, which can be less expensive and easier to maintain but often provides less power and has higher laser noise. Single-frequency SRS microscopes are often operated with two pi-

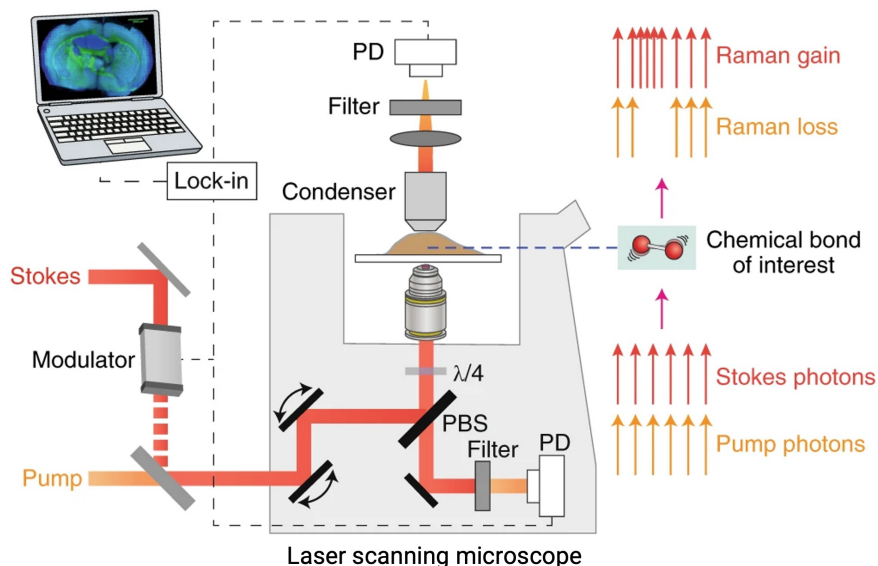


Figure 1.7: SRS microscopy setup. In this configuration, the Stokes beam is modulated before pump and Stokes beams are combined and focused onto the sample. SRS signal (SRL or SRG) can be detected in either transmitted or reflected directions. Adapted from Ref.¹⁰

co-second sources or two spectrally focused femtosecond beams. Spectral or hyperspectral SRS can be implemented with two tunable picosecond lasers^{21,84} or with two femtosecond sources with linear chirping or pulse shaping,^{91,92,93,94,95} along with other techniques.^{96,97}

Either the pump or Stokes beam is modulated at megahertz frequency using an acousto-optic modulator (AOM) or electro-optical modulator (EOM). Pump and Stokes beams are then combined, fed into a home-built or commercial laser scanning microscope, and focused onto the sample, typically using an objective with a high numerical aperture (NA). The pump and Stokes beams must be spatially aligned (propagating in the same direction and overlapped) and temporally aligned (path-length matched). In the transmission-detection scheme, the SRS signal propagated in the forward direction is collected through a condenser, optically filtered to remove the modulated excitation beam, and detected by a

photodiode. In an epi-detection scheme, backscattered SRS signal is collected through the focusing objective. A polarization beam splitter placed just before the objective is typically used to pass the linearly polarized excitation light and reflect the polarization-scrambled backscattered light to the photodiode.^{98,99,99,92,100} In either case, the electrical signal from the photodiode is sent to a lock-in amplifier which extracts the modulated signal from the noisy raw signal. The extracted electrical signal, corresponding to the SRS signal, is then read and output for image formation.

As mentioned previously, SRS typically probes one vibrational mode at a time. For studies with multiple chemical components such as drug tablet analysis, it is often more desired to probe multiple vibrational modes to detect multiple APIs or API and excipients and account for background signals. Imaging by probing multiple vibrational modes is called hyperspectral imaging and has been executed in several ways. Frequency tuning is the simplest implementation of hyperspectral SRS and is achieved by obtaining a series of SRS images while changing the frequency of either the pump or the Stokes beam.^{90,101,83,21} The speed of this method is directly limited by the speed required to tune the beam and the number of frequencies measured. Another method is spectral focusing,^{102,93,103} in which time delay is tuned between two chirped broadband pulses. Yet another implementation is through multiplexing wavelengths,^{91,104,105} where multiple SRS wavelengths are simultaneously excited by narrowband Stokes pulses and broadband pump pulses and spectral decomposition is used to generate individual chemical maps. Spectral decomposition is performed by algorithms such as multivariate curve resolution (MCR), alternating least squares (ALS), non negative least squares (NNLS), and vertex component analysis (VCA), among others.^{21,93,106} Other variations of hyperspectral SRS include the use of more than two colors

by time-gating, adding single color fields, or with multicolor broadband sources.^{95,94,107,97,96} Of note, hyperspectral SRS imaging is crucial for proper calibration when performing quantitative concentration calculations due to laser noise and effects from light absorption and scattering throughout the field of view.¹⁰⁸

1.2 DESTRUCTIVE TECHNIQUES FOR OBTAINING CHEMICAL AND MOLECULAR INFORMATION

Destructive analytical techniques can often provide more detailed chemical and molecular information compared to microscopy techniques by breaking down or altering the sample. These methods are essential for understanding molecular composition and chemical interactions in systems such as tissues, cells, and pharmaceutical formulations, particularly deeper within the sample than can be reached with microscopy. For the purpose of this dissertation, destructive techniques are defined as techniques that require isolation of the sample from its biological or pharmaceutical context, even if the sample stays intact during the measurement itself.

Destructive methods established for quantitative, chemical, or molecular characterization are often used to provide ground-truth measurements for validating optical microscopy methods. For example, gas chromatography and mass spectrometry have been used to validate CARS and SRS for lipid imaging in cells and tissues.^{109,110} Nuclear magnetic resonance (NMR) spectroscopy has also been used to confirm chemical specificity in SRS and FLIM in live-cell imaging studies.^{111,112} Additionally, immunohistochemistry has been used to validate protein localization detected in FLIM and Raman-based molecular imaging.^{113,114,115}

More recently, microscopy has been paired with bulk, single-cell, and spatial transcriptomics to relate morphology to molecular state in brain injury^{116,66} and various cancers.^{117,118} However, these studies utilize microscopy images of tissue models or *ex vivo* stained histology slides, which remove the sample from its existing context and limit the ability to track the same area longitudinally. To our knowledge, no studies have attempted to connect *in vivo* structural and chemical images to transcriptomics. In this background section, a brief introduction is provided on select destructive techniques applied in this thesis to validate or expand the capabilities of advanced optical microscopy.

1.2.1 NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique used to determine the molecular structure, dynamics, and chemical environment of compounds. It is based on the principle that atomic nuclei with an odd number of protons or neutrons (e.g., ^1H , ^{13}C , ^{31}P) possess an intrinsic magnetic moment. When subjected to a strong external magnetic field, these nuclei align with or against the field, creating discrete energy states. By applying a radiofrequency pulse, the nuclei are excited to higher energy states, and as they relax back to equilibrium, they emit characteristic electromagnetic signals. These signals are detected and translated into an NMR spectrum, which provides detailed information about molecular structure, chemical environment, and dynamic interactions.¹¹⁹

NMR plays a vital role in pharmaceutical research and development by enabling precise molecular characterization, though its utility is limited by the need for sample extraction and lack of spatial resolution. In pharmaceuticals, NMR can be used for evaluating drug formulations, elucidating the structure of active pharmaceutical ingredients (APIs), and study-

ing interactions between drugs and biological targets.¹¹⁹ In pharmaceutical quality control, it can be applied to confirm the identity of the compound, detect impurities, and unravel counterfeit drugs.^{120,121} Though it can accurately characterize the molecular structure of a sample, it requires removal of the sample from its native environment and does not preserve spatial information, limiting its application in *in situ* studies and in-line monitoring. Although *in vivo* NMR and magnetic resonance spectroscopy have been demonstrated, especially in neurological and metabolic research, they remain specialized and less commonly used due to technical and sensitivity limitations.

1.2.2 RNA SEQUENCING - BULK, SINGLE-CELL, AND SPATIAL

Bulk RNA sequencing (bulk RNA-seq) is a high-throughput molecular technique that provides global gene expression profiles via total RNA extraction and sequencing from a cell population. This method allows for the quantification of transcriptomic changes under different conditions, making it an essential tool in disease research, drug development, and personalized medicine. While highly informative, bulk RNA-seq averages gene expression across thousands to millions of cells, potentially masking heterogeneity and rare cell populations.^{122,123}

Single-cell RNA sequencing (scRNA-seq) enables the analysis of gene expression at the single-cell level, revealing cellular heterogeneity and identifying distinct cell subpopulations within complex tissues. scRNA-seq has been transformative in cancer research, developmental biology, and immunology, enabling the discovery of rare cell types, mapping of differentiation trajectories, and characterization of immune responses. However, since cells must be dissociated prior to sequencing, spatial relationships and tissue archi-

texture are lost, necessitating the development of complementary spatial transcriptomic approaches.^{122,124}

One of the most advanced spatial molecular techniques is spatial transcriptomics (ST), which enables micron-level resolution mapping of gene expression within the spatial context of intact tissue sections. This method integrates molecular sequencing with spatially defined barcoding techniques to retain the positional context of RNA transcripts. By combining RNA-seq with spatial imaging, ST allows for the visualization of gene expression patterns across tissue architectures.

The field has rapidly expanded since its inception, with multiple platforms offering varied resolutions, throughput, and molecular sensitivity.^{11,125} Among the most widely used is the 10x Genomics Visium platform, which captures mRNA on an array of barcoded targets (55 μm in diameter), allowing transcriptome-wide profiling across large tissue sections (Fig. 1.8B). While Visium provides high transcript capture efficiency and full-length mRNA sequencing, its resolution is limited, with each capture region typically encompassing multiple cells.¹¹ To address this shortcoming, a newer iteration of the platform, Visium HD, increases spatial resolution to 8 μm (binned from 2 μm squares) through a denser array of barcoded capture areas, pushing the technology toward near-single-cell resolution while maintaining whole-transcriptome coverage.¹²⁶ However, it still relies on tissue sectioning and ultimately does not achieve the true subcellular resolution provided by some other platforms.

Slide-seq and its successor Slide-seqV2 offer higher resolution than traditional Visium by using randomly deposited barcoded beads on a surface to capture mRNA at $\sim 10 \mu\text{m}$ resolution (Fig. 1.8C). Slide-seq's strength lies in its resolution and relatively low cost,

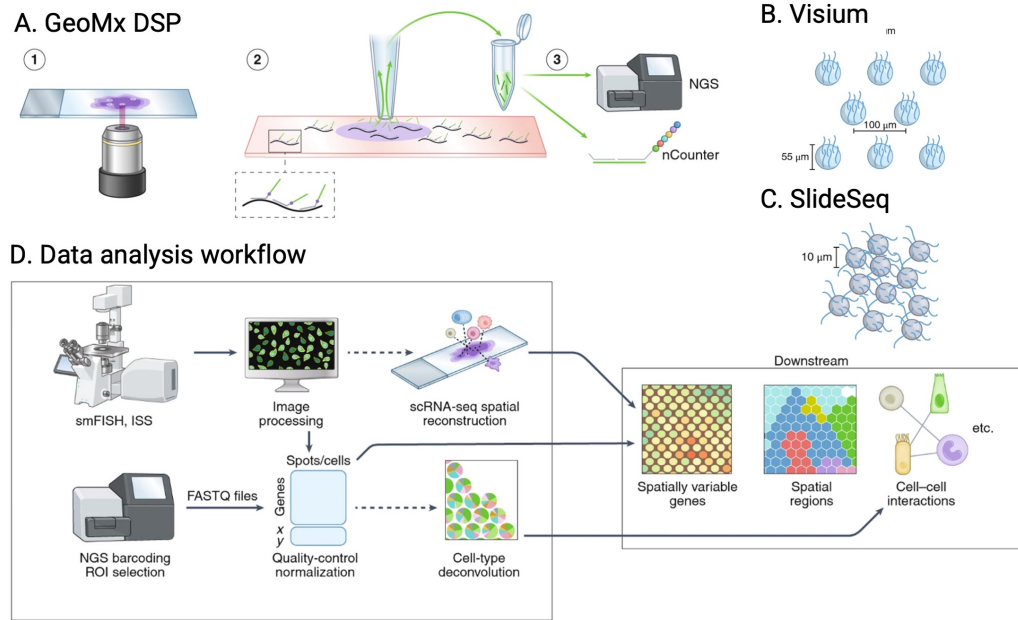


Figure 1.8: (A) GeoMX DSP allows for optical region of interest (ROI) selection by shining a UV light on ROIs to release photo-cleavable gene barcodes for quantification with nCounter or next gen sequencing (NGS). The purple circle in step 2 is the UV-illuminated ROI. (B) Visium spots are arranged in a hexagonal grid, 100 μm apart center to center and 55 μm in diameter. (C) In SlideSeq, barcoded beads 10 μm in diameter are spread in a single layer on a slide. (D) Data-analysis workflow: upstream analysis is technology specific and includes FASTQ file processing, quality control of the gene count matrix, and data normalizing for NGS-based technologies such as Visium and SlideSeq. Non spatial scRNA-seq data can be integrated by deconvolving cell types from spatial technologies. Downstream analysis includes finding spatially variable genes, transcriptionally defined spatial regions, and cell-cell interactions. Adapted from Ref.¹¹

though it requires complex tissue-bead alignment and tends to suffer from lower transcript capture efficiency compared to Visium or other array-based methods.^{11,127} On the other hand, NanoString's GeoMx Digital Spatial Profiler (DSP) uses oligo-tagged probes to hybridize with preselected transcripts or proteins within user-defined regions of interest (ROIs), offering high sensitivity and flexibility in region selection, but at the cost of whole-transcriptome coverage and spatial granularity (Fig. 1.8A).^{128,129} GeoMx is useful for targeted analysis in clinical FFPE samples and for integrating protein and RNA measurements. NanoString's CosMx Spatial Molecular Imager, in contrast, enables subcellular resolution with multiplexed single-molecule detection, typically targeting hundreds to thousands of genes. It provides up to 50 nm spatial resolution but requires prior selection of gene panels, up to 1000 genes, and is more limited in area coverage.¹³⁰

In terms of applications, regardless of the specific platform, spatial transcriptomics has enabled important discoveries through the discovery of spatially variable genes, genetic heterogeneity in healthy and disease tissues, and cell-cell interactions (Fig. 1.8D). In oncology, it has been used to identify spatially distinct tumor niches and immune microenvironments, revealing mechanisms of immune evasion and therapeutic resistance.^{131,132} In neuroscience, spatial transcriptomics has mapped transcriptional differences across brain regions and layers, contributing to a deeper understanding of neural diversity and disease-associated gene expression.^{133,134} In infectious disease and immunology, the spatial profiling of tissues affected by SARS-CoV-2 has uncovered localized inflammatory responses and immune cell interactions not observable by dissociative methods.^{135,136}

While spatial transcriptomics has extended our ability to explore gene expression within intact tissues, it is resource intensive and requires invasive sampling. All current implemen-

tations require tissue sampling, such as biopsies or surgical resections, limiting their applicability for longitudinal studies as well as sensitive or inaccessible tissues. In addition, spatial transcriptomics requires high sequencing depth, specialized reagents, and complex workflows that can take days to weeks from sample preparation to data analysis. Importantly, because the tissue is removed and processed, the same location cannot be sampled repeatedly, making it impossible to observe dynamic changes in gene expression over time within the same tissue region.

Given these limitations, it would be transformative to develop noninvasive approaches that could partially approximate the spatial and molecular information provided by ST. One promising strategy is to explore correlations between spatial transcriptomic data and cellular-level *in vivo* microscopy. If consistent relationships can be identified between optical features and the underlying gene expression patterns, we could infer molecular states *in vivo* without excising tissues. Such an approach may unite molecular profiling and functional imaging, opening up the possibility of real-time, longitudinal monitoring of tissue health and disease at the molecular level.

1.3 MACHINE LEARNING IN IMAGE ANALYSIS

Machine learning has improved the speed and quality of image analysis by automating feature extraction, pattern recognition, and predictive modeling. In microscopy, machine learning has been applied across biomedical research, clinical diagnostics, and high-throughput imaging. Traditional microscopy techniques generate large volumes of high-dimensional data, often requiring manual annotation, extensive preprocessing, and computationally expensive analysis. ML methods have significantly improved microscopy-based image analysis

by enabling better denoising, segmentation, and classification, reducing the dependence on manual labor while increasing reproducibility and scalability.

1.3.1 BASICS OF NEURAL NETWORKS:

Neural networks are a type of machine learning model designed to extract patterns from data, and are inspired by the structure and function of biological neural networks. They learn to map inputs to outputs through a series of transformations defined by layers of interconnected nodes, or neurons. A simple feed-forward network consists of layers of neurons organized into 1) an input layer, which accept the raw data, 2) hidden layers, which perform intermediate computations and extract features, and 3) an output layer, which produces the final prediction or output, such as a class label, a continuous value, or array (Fig. 1.9A). Each neuron computes a weighted sum of its inputs, adds a bias term, and passes the result through an activation function. Mathematically, the operation at a single neuron can be expressed as

$$z = \sum_i w_i x_i + b, \quad a = \varphi(z) \quad (1.3)$$

where x_i are the inputs, w_i are the learnable weights, b is a bias term, $\varphi(z)$ is an activation function (e.g., ReLU, sigmoid, tanh), and a is the neuron's output. By stacking many neurons and layers, the network can learn increasingly abstract representations of data.¹³⁷

During learning and optimization, a model is provided with input-output pairs and learns to minimize a loss function that quantifies the error between its predictions and the true labels. Some common approaches to training are gradient-based optimization via back-propagation and stochastic gradient descent (SGD). This approach consists of iterative

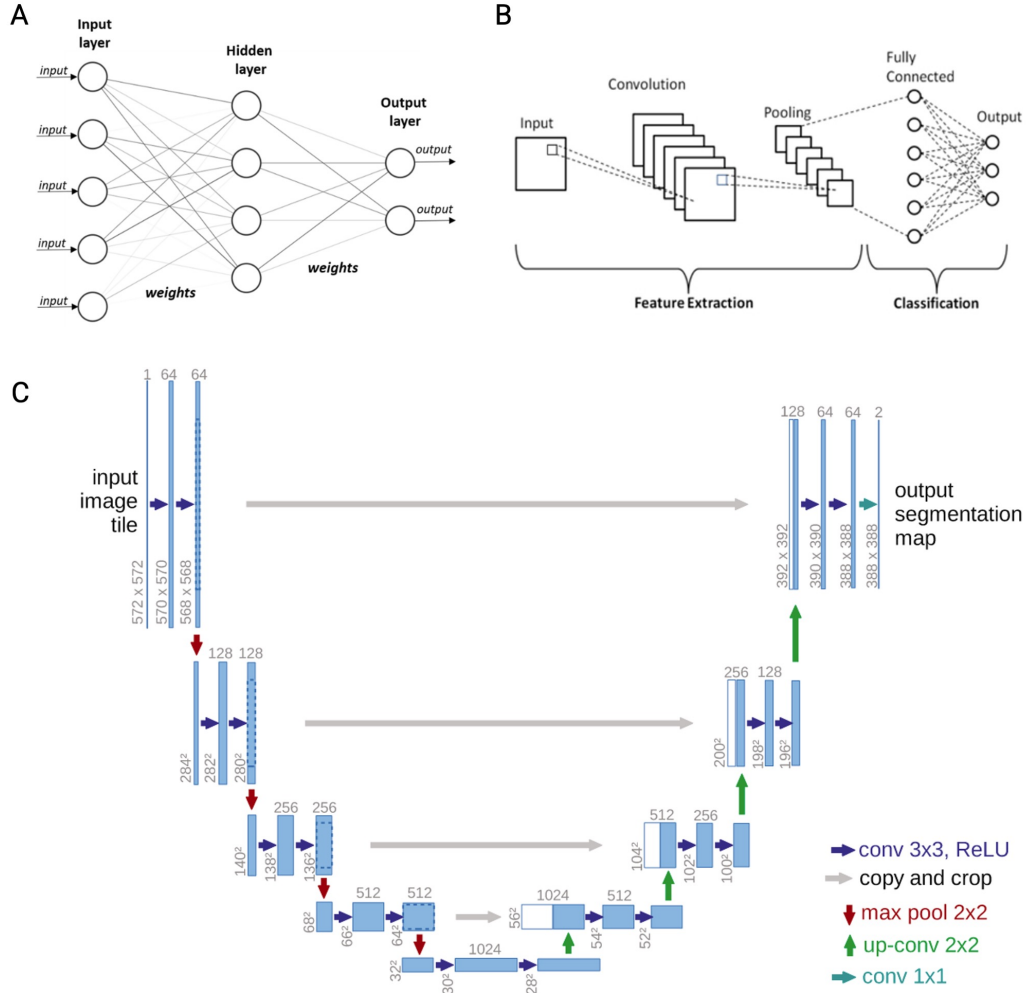


Figure 1.9: (A) The simple architecture of a three layer feed-forward neural network. Adapted from ¹² (B) Block diagram of a CNN with input, convolution, pooling, fully-connected, and output layers. Adapted from ¹³ (C) U-net architecture (example for 32x32 pixels in the lowest resolution). Each blue box corresponds to a multi-channel feature map. The number of channels is denoted on top of the box. The z-y-size is provided at the lower left edge of the box. White boxes represent copied feature maps. Arrows denote different operation. Adapted from Ref. ¹⁴

cycles of forward pass, loss computation, backward pass (backpropagation) and parameter update. In the forward pass, data is propagated through the network and its layers and neurons to produce an output. During loss computation, the difference between the predicted output and the true label is quantified using a loss function. Classification tasks use loss functions such as cross-entropy, while regression tasks use functions such as mean squared error. In the backward pass, the gradient of the loss with respect to each parameter is computed using the chain rule. Finally, in parameter update, gradients are used to update the weights and biases in a direction that reduces loss.¹³⁷

Most image-based machine learning models are based on convolutional neural networks (CNNs), which are a class of models designed to learn spatial hierarchies of features through local connectivity and parameter sharing, making them especially useful for image classification, object detection, and segmentation. They consist of three main types of layers: 1) convolutional layers, which apply filters (kernels) with learned weights across an input image, 2) activation functions, which enable the model to learn complex (linear or nonlinear) relationships within the data, and 3) pooling layers, which reduce spatial resolution and summarize features in localized regions, often by taking the average or maximum value in a small region (Fig. 1.9B). As the images pass through deeper layers of the model, the CNN encodes more complex and abstract patterns, from vertical or horizontal edges in early layers to shapes and whole objects in deeper layers. The output of the final convolutional layers is typically flattened and passed to fully connected layers for classification or regression tasks.^{137,138}

A specific type of CNN is a U-Net, which is designed for tasks that require an image output, such as segmentation or denoising. The U-Net architecture includes an encoder

and decoder (Fig. 1.9C). The encoder, or downsampling path, uses convolutional and pooling layers to shrink the image and extract deeper features like a traditional CNN. In deeper layers, the network captures the semantic information in the original image but loses spatial detail. In the decoder, or upsampling path, the encoded features maps are upsampled to recover the original image resolution. At each upsampling stage, the feature maps from the encoder are concatenated via skip connections, allowing the decoder to reuse low-level spatial information that was lost during downsampling, while keeping the semantic information extracted from the encoder.¹⁴ For segmentation tasks, the final layer is often a 1×1 convolution that maps the output to the desired number of classes for segmentation. For denoising tasks, the final layer is a 1×1 convolution that maps the output to an image that is the size of the input. U-Nets are trained with pixel-wise loss functions, most commonly binary cross-entropy, categorical cross-entropy for segmentation, or mean squared error for denoising.

1.3.2 DENOISING

Denoising represents a fundamental preprocessing step in microscopy image analysis, as many imaging modalities suffer from noise due to low photon counts, scattering, motion artifacts, and optical aberrations. The need for denoising is crucial in *in vivo* microscopy and label-free optical techniques, where achieving high-quality images often requires trade-offs between imaging speed, laser power, and resolution. Traditional noise reduction techniques, such as Gaussian filtering and wavelet denoising, are limited in their ability to preserve fine structural details while suppressing noise. Machine learning-based denoising methods have addressed these limitations by learning complex noise patterns and recon-

structing high-fidelity images from corrupted inputs.

Deep learning approaches to denoising can be broadly categorized into supervised and unsupervised methods. Supervised denoising requires paired clean and noisy images for training, allowing the model to learn a direct mapping between degraded and high-quality images such that when given a low-quality image, it can accurately predict a higher quality image that retains and sharpens important structural features (Fig. 1.10).¹⁵ U-Nets have been widely used for this purpose due to their ability to preserve spatial details while enhancing signal-to-noise ratios. The difficulty with a supervised approach is that high-quality images are often unavailable or lack sufficient numbers for training.

Unsupervised denoising techniques, such as Noise2Noise, Noise2Void, and Noise2Self, eliminate the need for paired training data by leveraging self-supervised learning principles and statistical assumptions. Noise2Noise demonstrates that effective denoising can be obtained by only using pairs of independently corrupted images of the same underlying signal.¹³⁹ Intuitively, while both images are noisy, the underlying object is the same. A neural network trained to predict many pairs of artificially noisy images learns the consistent structure present in both images and ignores noise, so long as the noise is statistically independent and additive. Noise2Void and Noise2Self require only single noisy images rather than pairs by masking and predicting pixels or sections of the images, and taking advantage of the assumption that in a structured image, pixel values can be predicted from its surrounding context.^{140,141} Approaches like these are particularly valuable in biomedical microscopy applications where image fidelity is low and ground truth images are unobtainable.

In our study involving *in vivo* human skin imaging, our FLIM images had low photon counts (less than ten photons per pixel) due to limitations on acquisition time, so we ap-

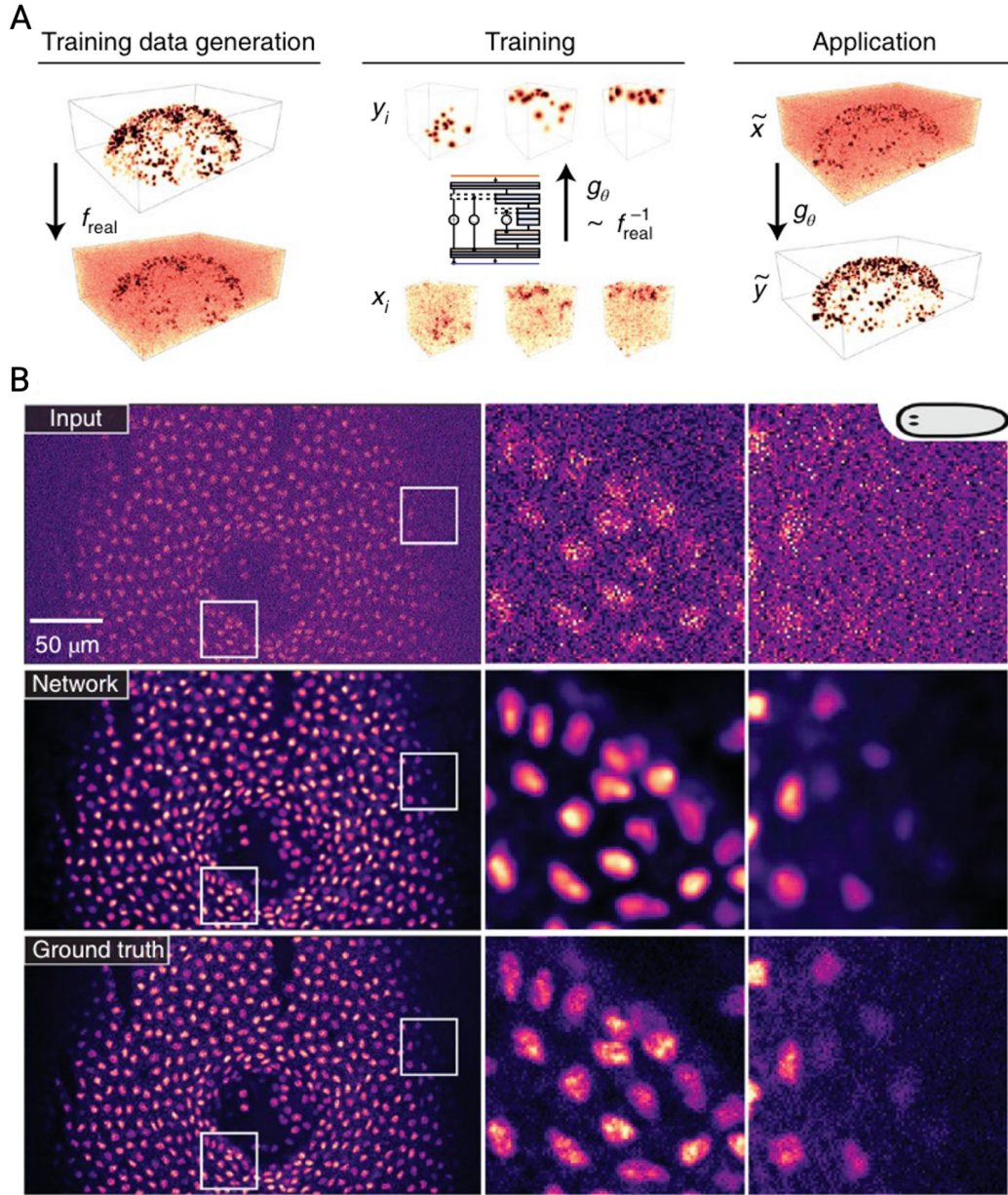


Figure 1.10: (A) Overview of a supervised denoising pipeline: content-aware image restoration (CARE) for image denoising. Pairs (x_i, y_i) of registered high- and low-SNR volumes are acquired at the microscope. A convolutional neural network is trained to restore y_i from x_i . The trained CARE network can then be applied to previously unseen low-SNR images $\tilde{x}$, yielding $\tilde{y}$ (B) Input data and restorations for nucleus-stained (RedDot1) flatworm (*S. mediterranea*). Shown are a single image-plane of a raw input stack (top row), the output of the network prediction (second row), and the high-SNR gold standard/ground truth (bottom row). Adapted from Ref. ¹⁵

plied DeepFLR, a self-supervised model specifically for FLIM signal restoration.¹⁴² DeepFLR is designed to restore FLIM signal in photon-starved conditions, and is based on similar concepts as Noise2Noise. First, FLIM data are split into even and odd number time bins. The model is then given the task of minimizing the L2 loss between the even and odd bins. Even though both have low photon counts, since the lifetime property is independent of the total fluorescent intensity, the model can learn the conditional probability distribution shared between the even and odd number time frames and predict a "restored" FLIM images with more photons and accurate fluorescence lifetimes.

1.3.3 CELL SEGMENTATION

Cell segmentation, i.e., the identification and delineation of cells in microscopy images, is a foundational task in biological image analysis to quantify cellular structural features and interactions. Specifically, accurate segmentation enables a wide range of downstream analyses, such as measuring cell morphology, tracking cell dynamics, and quantifying fluorescence intensity. However, microscopy images often present challenges such as variable contrast, overlapping cells, and diverse cell morphologies, making classical image processing techniques, e.g., thresholding, watershed segmentation, insufficient because they lack granularity and nuance. To address these challenges, the field has increasingly turned to machine learning-based approaches, which offer improved accuracy and adaptability across a variety of imaging modalities.

Several tools have emerged for cell segmentation, including StarDist, and Cellpose. StarDist has been adopted for its speed and excellent performance on well-separated, roughly circular nuclei. However, its star-convex assumption limits its applicability to more com-

plex cell morphologies or cytoplasmic segmentation tasks, where cells may have irregular or elongated shapes.¹⁴³ A more generalist and increasingly popular tool is Cellpose, which is a deep learning model trained to perform instance segmentation on a broad range of cell types, tissue structures, and imaging modalities (Fig. 1.11).^{16,144,145} Unlike most models that require training on data from a specific domain, Cellpose and its successors, Cellpose2.0 and Cellpose3.0, were designed, intentionally, to generalize a wide variety of biological images, including fluorescence and brightfield microscopy. One of its key innovations is that it does not segment cells by directly classifying pixels as belonging to cell interiors or boundaries. Instead, it predicts two outputs for each pixel: a flow field that points toward the center of the nearest cell, and a probability map indicating whether the pixel belongs to any cell at all.¹⁶

The underlying architecture of Cellpose is based on a modified U-Net. Like U-Net, Cellpose uses an encoder-decoder structure with skip connections that allow the network to combine coarse semantic information with fine spatial details. However, Cellpose extends this architecture by adding two output "heads": one for predicting the spatial gradients (or flow fields) and one for predicting the probability map. During inference, the flow fields are integrated to simulate how pixels "move" toward the center of a cell (Fig. 1.11A-F), allowing the model to group pixels into coherent cell instances, even when cells are closely packed or irregularly shaped.¹⁶

Cellpose was trained on a large and diverse dataset consisting of over 70,000 manually annotated cell images, drawn from more than 30 imaging conditions, including both 2D and 3D microscopy, fluorescent and brightfield images, and tissue as well as cultured cell samples (Fig. 1.11G). This diversity in training data contributes to its strong generalization

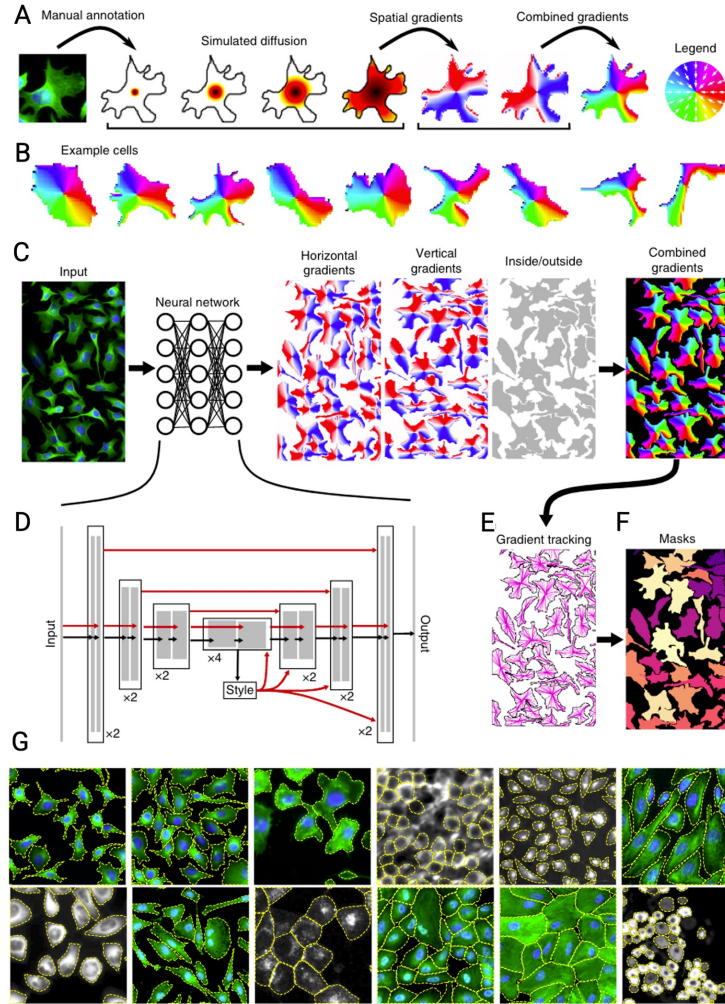


Figure 1.11: Cellpose model architecture and examples (A) Procedure for transforming manually annotated masks into a vector flow representation that can be predicted by a neural network. A simulated diffusion process starting from the center of the mask is used to derive spatial gradients that point toward the center of the cell, potentially indirectly around corners. The x and y gradients are combined in a single direction from 0° to 360°. (B) Example spatial gradients for cells from the training dataset. (C) A neural network is trained to predict the horizontal and vertical gradients, as well as whether a pixel belongs to any cell. The three predicted maps are combined into a gradient vector field. (D) The details of the neural network that contains a standard backbone U-net3 to downsample and then upsample the feature maps, with skip connections between layers of the same size and global skip connections from the image styles, computed at the lowest resolution, to all successive computations. (E) At test time, the predicted gradient vector fields are used to construct a dynamical system with fixed points whose basins of attraction represent the predicted masks. Informally, every pixel ‘tracks the gradients’ toward their eventual fixed point. (F) All the pixels that converge to the same fixed point are assigned to the same mask. (G) Example Cellpose segmentation for 12 test images. The masks predicted by Cellpose are shown with dotted yellow lines. Adapted from Ref.¹⁶

performance. Importantly, Cellpose2.0 and Cellpose3.0 can be fine-tuned to individual datasets with relatively few examples using a simple python-based graphical user interface GUI, enabling customization to specific cell types or imaging setups. ¹⁴⁴

2

Quantitative analysis of drug tablet aging by fast hyperspectral SRS microscopy

This chapter is a slightly modified version of "Quantitative analysis of drug tablet aging by fast hyper-spectral stimulated Raman scattering microscopy" published in The Analyst and has been reproduced here with the permission of the copyright holder.¹⁴⁶

PHARMACEUTICAL DEVELOPMENT OF SOLID-STATE FORMULATIONS requires testing active pharmaceutical ingredients (API) and excipients for uniformity and stability. Solid-state properties such as component distribution and grain size are crucial factors that influence dissolution profile, which greatly affect drug efficacy and toxicity, and can only be analyzed spatially by chemical imaging (CI) techniques. Current CI techniques such as near infrared microscopy and confocal Raman spectroscopy are capable of high chemical and spatial resolution but cannot achieve measurement speeds necessary for integration into the pharmaceutical production and quality assurance processes. To fill this gap, we demonstrate fast chemical imaging by epi-detected sparse spectral sampling stimulated Raman scattering to quantify API and excipient degradation and distribution.

2.1 INTRODUCTION

Oral solid dosage formulations account for over 60% of existing drugs and almost 50% of novel drug approvals between 2018 and 2022.^{147,148,149,150,151,152} They are efficient, cost-effective to manufacture, shelf stable, and easy to administer.¹⁵³ The development of a solid drug formulation is a complex process, however, involving the mixture of an active pharmaceutical ingredient (API) and inactive ingredients (excipients) which are typically mixed as powders and compressed into tablet form.¹⁵⁴ Manufacturers must ensure consistency in particle size, polymorphism, dosage, and dissolution profile during quality control as changes in these qualities could lead to toxicity or decreased efficacy.^{155,156,157,158} With the establishment of the US Food and Drug Administration's (FDA) Process Analytical Technology (PAT) initiative, a framework for innovative pharmaceutical development, manufacturing, and quality assurance, development of new tools for understanding and

controlling the manufacturing process are critical.¹⁵⁹

In the past few decades, chemical imaging (CI) has emerged as a crucial method for understanding the chemical and physical properties of pharmaceutical tablets.^{20,106,160} CI combines spectroscopy for identification of API and excipients with microscopy for visualizing component distribution, particle size, and chemical stability. These attributes can greatly influence the dissolution rate and effectiveness of an oral tablet. For example, API agglomeration can lead to tablets with higher potency than expected,^{161,162} and is particularly important for poorly soluble compounds such as APIs in BCS classes II and IV, while heterogeneous distribution of a common lubricant such as magnesium stearate (Mgst) can lead to changes in tablet tensile strength required to withstand handling.^{163,164,165} Identification of agglomeration, heterogeneity, and chemical and physical stability through CI is useful for diagnosing production issues, refining tablet manufacturing, and achieving therapeutic efficacy.

The most commonly applied CI methods for pharmaceutical process monitoring and quality assurance are near infrared (NIR) and confocal Raman imaging,^{166,20,106} both of which are based on vibrational spectroscopy. In both modalities, the chemical composition of a sample at various spatial positions is examined by measuring the spectrum of light emitted after excitation with a high-intensity laser light. Spectral emission at each point is dependent on the interaction of the excitation light with vibrations from the chemical bonds and the secondary structure present at that spatial location. NIR offers high speed and greater depths of penetration with relatively low spatial resolution while confocal Raman imaging can provide high spatial resolution at up to around 50 μm depth at the expense of long acquisition times (Fig. 2.1).^{20,167,168,17} Additionally, while Raman is generally

Current methods for Chemical Imaging

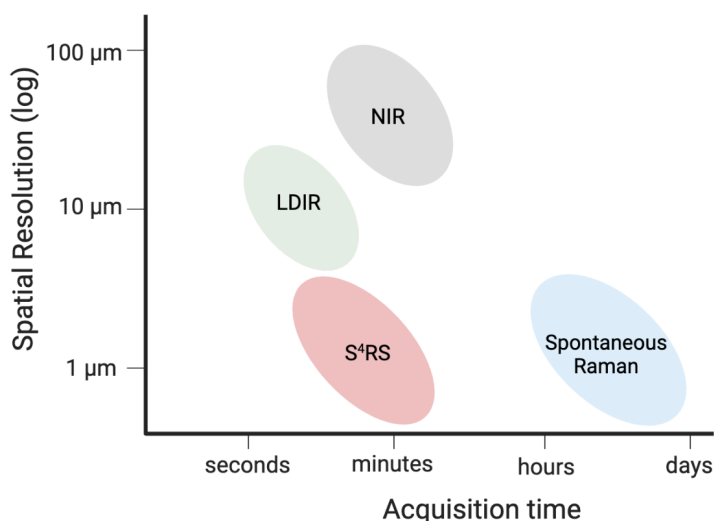


Figure 2.1: Comparison of spatial resolution and acquisition times of CI techniques. Note that acquisition time depends on many factors such as image area, pixel count, pixel dwell time, and averaging. This illustration is based on approximated typical use for pharmaceutical tablet imaging among representative papers.^{17,18,19,20,21} NIR: near infrared; LDIR: laser direct infrared; S⁴RS: sparse spectral sampling stimulated Raman scattering.

preferred for high resolution and high molecular specificity CI, the presence of background fluorescence limits its application for imaging many formulations, including common excipients such as microcrystalline cellulose (MCC).¹⁶⁹ Recently, laser direct infrared imaging (LDIR) has gained traction for chemical imaging of tablets within seconds at high spectral resolution and spatial resolution up to 10 μm.^{18,19} This method couples a quantum cascade laser (QCL) with an infrared microscope to obtain signal at specific spectral bands, greatly reducing imaging time compared to NIR. However, it still fails to identify small API domains particularly at low concentrations when compared to Raman imaging.¹⁸

Given the current methods of CI and their drawbacks, there is a clear need for a new CI tool with fast acquisition time, low background, high resolution, and high chemical sensi-

tivity, particularly for low concentration APIs. We propose sparse spectral sampling stimulated Raman scattering (S⁴RS), an extension of stimulated Raman scattering (SRS) microscopy, for this purpose. As a coherent Raman scattering (CRS) microscopy technique, it generates significantly increased signal compared to confocal Raman imaging, allowing for acquisition speed improvement by orders of magnitude while maintaining chemical specificity and submicron resolution.⁷⁹ CRS is typically applied in two modalities: coherent anti-Stokes Raman scattering (CARS) or SRS, both of which use a pump beam and a Stokes beam to enhance the excitation of Raman-active molecular vibrations in a sample. CARS signal is detected as the intensity of the anti-Stokes frequency which is generated from the interaction of the pump and Stokes beams in the specimen.^{77,170} In contrast, the SRS process involves a transfer of energy from the pump to the Stokes frequency and can be measured through stimulated Raman gain (SRG, the increase in Stokes intensity), or stimulated Raman loss (SRL, the decrease in pump intensity).^{171,101} SRS spectra are typically similar to spontaneous Raman spectra while CARS spectra are distorted due to non-resonant background and four-wave mixing.^{80,171} As such SRS is often preferred for high contrast imaging of multiple chemical components and for quantitative chemical imaging. Even so, CARS has previously been applied for real-time solid-state drug imaging to monitor drug dissolution,^{172,173,174,175} while SRS has been demonstrated for mapping API distribution,⁹⁹ probing API polymorphism,¹⁷⁶ and detecting microscale chemical reactions in tablets.¹⁰⁰

Commercial drug tablets often contain API and excipients with overlapping spectra and may require hyperspectral SRS to distinguish each component. One of the greatest challenges for hyperspectral SRS is acquiring chemical specific information at an imaging

speed compatible with pharmaceutical assessment or dynamic process monitoring (e.g. dissolution). Frequency tuning is the simplest implementation of hyperspectral SRS and is achieved by obtaining a series of SRS images while changing the frequency of either the pump or the Stokes beam.^{90,101,83} The speed of this method is directly limited by the speed required to tune the beam and the number of frequencies measured. Other methods include spectral focusing,^{102,93,103} in which time delay is tuned between two chirped broadband pulses, and multiplexing wavelengths,^{91,104,105} where multiple SRS wavelengths are simultaneously excited by narrowband Stokes pulses and broadband pump pulses. Still, these techniques and other proposed variations^{95,94,107,97,96} are unable to tune between the fingerprint and high wavenumber regions without manual adjustments, and have not been applied for epi-detected SRS.

Recently, our group has demonstrated multi-window S⁴RS which is a version of hyperspectral SRS using frequency tuning and allows for automatic wavelength switching between Raman shifts across the fingerprint (700-1800 cm⁻¹), silent (2000-2400 cm⁻¹), and high wavenumber (2700-3200 cm⁻¹) spectral windows in less than 5 ms with < 4 cm⁻¹ spectral resolution.²¹ This method is enabled by a rapidly tunable dual-output all-fiber optical parametric oscillator.¹⁷⁷ The ability to rapidly tune to specific wavenumbers across multiple spectral windows enables us to select a sparse set of wavenumbers to spectrally distinguish multiple chemical components while maintaining high imaging speeds. We have previously applied S⁴RS for monitoring spatiotemporal dynamics of topical drug delivery in mouse skin.^{21,84} Here, we demonstrate the utility of S⁴RS to achieve hyperspectral unmixing of components with high spectral overlap with rapid acquisition of multiple fields of view, along with full tablet cross-sectional imaging. We conduct quantitative analysis of

API and excipient distributions to uncover spatially heterogeneous chemical instability at stress conditions. Additionally, we develop a 3D printed tablet holder to standardize and facilitate tablet preprocessing for efficient integration into quality control processes.

2.2 EXPERIMENTAL

2.2.1 RAW MATERIALS

Compressed tablets made in the same batch were obtained from UNION Therapeutics. Each tablet contained 10 mg naproxen along with four excipients, hydroxypropyl methylcellulose (HPMC), lactose monohydrate, magnesium stearate (Mgst), and anhydrous silica. Silica contributed less than 0.5% of the tablet by weight and did not display a Raman peak between $1300\text{--}3600\text{ cm}^{-1}$ so it was excluded in component analyses. All tablets were also coated with Opadry® II film coating. API and excipients were separately purchased in powder form (PHR1040, H9262, PHR1024, PHR1363, Sigma Aldrich, USA) for obtaining SRS and spontaneous Raman reference spectra. Groups of four tablets each were randomly assigned to be placed in control conditions at 20°C and 55% relative humidity (RH), or stress conditions at 60°C and 10% RH for 3 weeks, 70°C and 10% RH for 6 weeks, or 37°C and 94% RH for 6 weeks.

2.2.2 SAMPLE PREPARATION

The tablets were coated with a pigmented film coating which can present a problem for SRS imaging. We developed a protocol for obtaining a flat cross section for imaging the tablet at various depths (Fig. 2.2). Film coated tablets were fitted into custom reusable flexible 3D printed tablet holders made to slot into a 3D printed chuck for a Leica Ultrami-

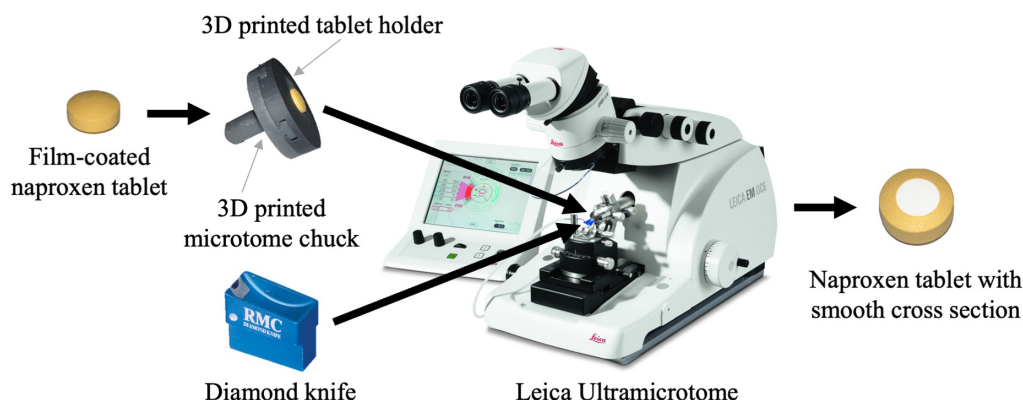


Figure 2.2: Protocol for obtaining a drug tablet cross section. A tablet holder is 3D printed using flexible resin such that it fits snugly around the tablet and has two slots to slide onto the microtome chuck. The chuck is 3D printed using hard resin such that one side fits into the slots of the tablet holder and the other side fits into the Leica Ultramicrotome. A diamond knife is used for precise tablet sectioning.

icrotome (EM UC7, Leica Microsystems GmbH, Germany). The tablets were sectioned at 500 μm steps using a diamond knife. For imaging, the flat surface of the tablet was placed on a glass cover slip which was then placed on the microscope stage. This technique can be adjusted to fit tablets of a range of shapes and sizes without damaging the tablet by 3D printing alternate custom reusable tablet holders.

2.2.3 RAMAN SPECTROSCOPY

Raman spectroscopy for spectral point acquisition was conducted on a LabRAM HR Evolution Confocal Raman Microscope (Horiba Scientific, Japan) with a 20x 0.4 NA objective, CCD detector, 532 nm laser excitation source, and 600 g/mm grating. Drug tablets were placed on a kinetic stage and imaged directly on its cross section while powders were placed on microscope slides wrapped in aluminum foil to avoid background signal. Spec-

tra were obtained with 2 sec. acquisition time and 2 accumulations. All obtained spectra underwent polynomial-fitted baseline correction within the LabSpec 6 software (Horiba Scientific, Japan) and normalization in our analysis code in Python.

2.2.4 SRS AND S⁴RS IMAGING

The SRS microscopy setup (Fig. 2.3) was modified from that described in Pence et. al.²¹ for epi-direction SRS imaging. The system uses a dual output rapidly tunable all-fiber optical parametric oscillator (FOPO) light source (Picus Duo, Refined Laser Systems, Germany), capable of generating synchronized pump pulses (tunable from 780 to 980 nm) and Stokes pulses (tunable between 1020 to 1060 nm) at a 40.5 MHz repetition rate with 7 ps duration.¹⁷⁷ The pump beam was modulated at half of the repetition rate for generating SRS contrast and both beams were passed through half-wave plates (HWP) and overlapped in space and time (Dio2-R980-25x36 980 nm dichroic beamsplitter, Semrock, USA) before coupling into a commercially available inverted microscope (IX3, Olympus, Japan) equipped with a confocal scanner (FV3000, Olympus, Japan). Epi-SRS imaging was performed by relaying the overlapped pump and Stokes pulses to the back focal plane of a 20x 0.8 NA microscopy objective (UPLXAPO20X, Olympus, Japan). A polarizing cube beam-splitter (PBS255, Thorlabs, USA) was installed in a custom 3D-printed cube assembly for the microscope to reflect s-polarized excitation light to the objective and transmit p-polarized scattered epi signal to be filtered by three long pass filters (FELH1000, Thorlabs, USA; ET1000LP, Chroma, USA; LPO2-980RU, Semrock, USA) and onto a NIR optimized photodiode and lock-in amplifier (SRS detector set, 1064 optimized, APE GmbH, Germany).

SRS spectral images of pure API and excipient powders were recorded in the 1350-1450 cm^{-1} and 2700-3100 cm^{-1} spectral ranges (2 cm^{-1} spectral steps) based on visual inspection of peak locations for the components. Reference spectra for pure components were obtained by averaging over 1000 spectra from pixels in regions of images containing the component of interest. Based on locations of unique peaks and overlap between the reference spectra of all four API and excipients, twenty wavenumbers were manually chosen for sparse sampling for S⁴RS: 1350, 1360, 1394, 1420, 1440, 2700, 2800, 2846, 2856, 2880, 2892, 2894, 2896, 2922, 2946, 2954, 2988, 3050, 3080, 3098 cm^{-1} . Subsequent tablet imaging was conducted at these twenty spectral bands.

All individual images were obtained with 2 μs pixel dwell time and 1024 x 1024 pixels at 0.48 μm per pixel for a 489 x 489 μm field of view (FOV). Full spectra SRS images of pure powder components were acquired in 10 minutes while the S⁴RS images were obtained in under 1 minute for each z-step. Larger area images were generated using the Olympus FV3000 multi-area time-lapse software module with stitching function. Tablets were set to be imaged at 5 depths at 10 μm z-steps to achieve a depth of 40 μm to account for the slight roughness of the tablet cross sectional surface. In tablets stored in high humidity, swelling and loosening of the tablet led to increased roughness of the surface after sectioning, requiring imaging to a depth of 110 μm at 10 μm z-steps to obtain full signal across a FOV.

2.2.5 NMR

Proton nuclear magnetic resonance (¹H-NMR) spectroscopy was employed to investigate the chemical composition of solid drug tablets. ¹H-NMR experiments were conducted using a Bruker 300 MHz NMR magnet equipped with a suitable probe (Bruker, USA). The

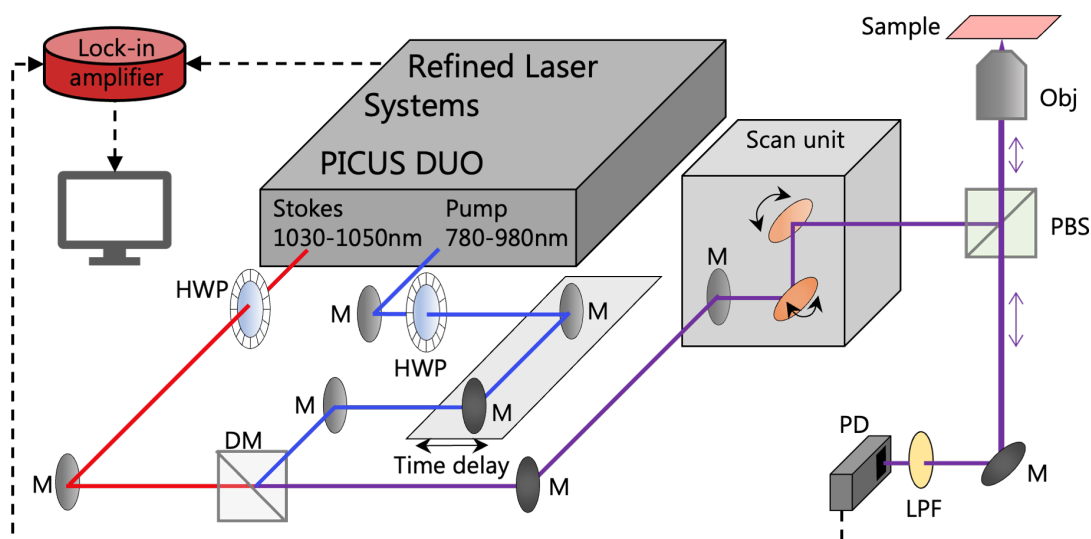


Figure 2.3: Schematic of the epi-detected SRS microscopy system. HWP: adjustable half wave plate, M: mirror, DM: dichroic mirror, PBS: polarizing beam-splitter, Obj: Objective, LPF: long pass filter, PD: photodetector.

solid drug tablets were carefully scraped to obtain representative samples, and the samples were dissolved in deuterium oxide (D_2O , 99.9% purity, Sigma Aldrich, USA) to ensure efficient proton exchange and minimize interference from the solvent. A zg30 sequence with $13 \mu s$ pulse duration, 1 s relaxation delay, and 30° pulse was employed for data acquisition. All experiments were performed at room temperature. Each sample was subjected to 60 acquisitions to enhance signal-to-noise ratio. The acquired NMR data were then subjected to Fourier transformation, baseline correction, and phase correction using the Bruker Topspin software to improve spectral quality for visual comparison.

2.2.6 IMAGE ANALYSIS

Tablet components were resolved by performing non-negative least squares (NNLS) on each pixel's normalized SRS or S^4 RS spectrum against reference spectra obtained from

pure components (including a background spectrum to account for laser power differences between wavenumbers) to generate abundance maps for each component (Fig. A.1).^{93,100} Within each component abundance map, each pixel value represents the likelihood that that pixel can be attributed to the specified component. Composite abundance maps are used to visualize qualitative differences between images and ensure correct component attribution. Thresholding was applied to these abundance maps to attribute each pixel to a single component for subsequent quantitative analysis.

SURFACE AREA CONTRIBUTION

Percent surface area contribution for each component was calculated by summing the number of pixels attributed to the specified component divided by total pixels attributed to all components (not including pixels attributed to background).

GRAIN AND CLUSTER SIZE

Grain sizes were obtained by detecting the contours of each component, calculating the number of pixels within each contour, and scaling by a factor of $0.48\ \mu\text{m}$ per pixel. For HPMC, there appeared to be several grains with cavities that resulted in artificial splitting by the thresholding algorithm. As such, we chose to cluster HPMC contours within $10\ \mu\text{m}$ of each other in groups of three or more to obtain a more accurate estimate of HPMC grain size.

NAPROXEN DENSITY RATIO (NDR)

NDR is a measure of the density of naproxen pixels within a defined radius of an excipient. NDR was calculated by taking the density of naproxen pixels in the area between the contour of an excipient grain and the dilated contour of the same excipient grain. The number of pixels of contour dilation is converted to a radius in μm . The naproxen pixel density in that area is divided by the overall naproxen pixel density in the field of view (FOV) such that this ratio approaches 1 as distance from excipient increases. This final ratio is the NDR.

2.3 RESULTS AND DISCUSSION

2.3.1 SRS AND S⁴RS IMAGING

SRS microscopy is expected to have nearly identical spectral features to spontaneous Raman aside from slight peak broadening due to laser pulse duration and bandwidth.^{170,171} We confirmed that confocal spontaneous Raman and SRS spectra show similar peaks using our setup and for our four chemical components as pure powders: naproxen, HPMC, lactose monohydrate, and Mgst (Fig. 2.4A). Naproxen has distinct peaks at 1402 cm^{-1} and 3080 cm^{-1} (corresponding to previous spectra,¹⁷⁸) but the three excipients demonstrate highly overlapping bands in the high wavenumber region. This degree of overlap poses a challenge for coherent Raman microscopy, which is typically performed at a specific band for each component of interest. In this case, full or partial spectra must be obtained, and spectral decomposition can be performed to determine the relative contribution of each pure component to a sample of interest. We obtained SRS images of powder mixes and

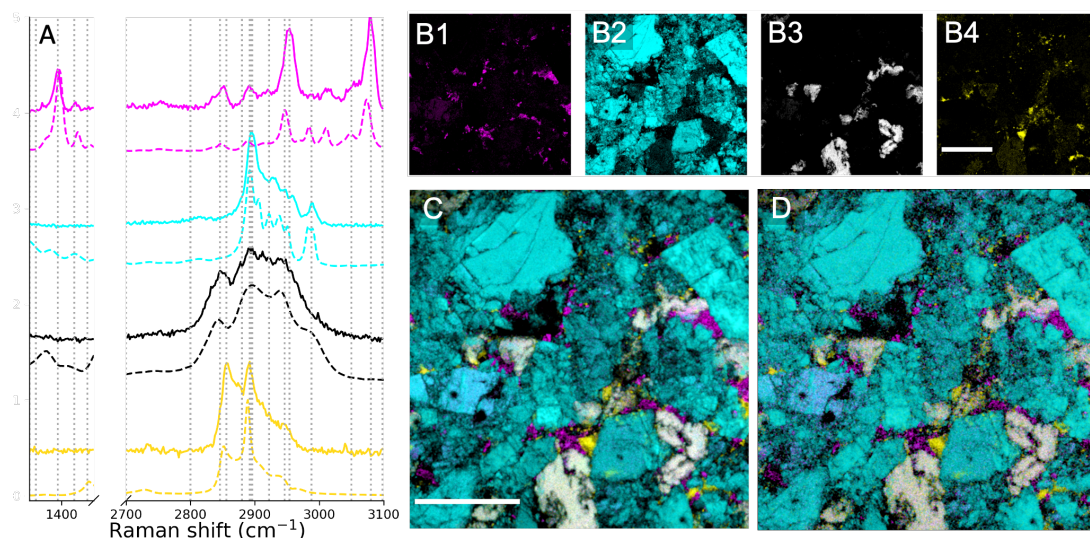


Figure 2.4: SRS and S⁴RS imaging of API and excipients in a naproxen tablet cross section. (A) SRS (solid) and spontaneous Raman (dotted) spectra of pure naproxen (magenta), lactose (cyan), HPMC (black), and Mgst (yellow). (B1-B4) Pseudo-color abundance maps of naproxen (magenta), lactose (cyan), HPMC (white), and Mgst (yellow), respectively, obtained from NNLS spectral unmixing of images taken from 1350-1450 cm⁻¹ and 2700-3100 cm⁻¹ at 2 cm⁻¹ steps. Black color in these images represents background not attributed to any API or excipient. These abundance maps can be added to generate (C), a composite abundance map with the same corresponding colors. (D) Composite abundance map generated from NNLS spectral unmixing of sparse spectra with 20 Raman shifts corresponding to dashed lines in (A), with the same corresponding colors as (B1-B4). Scale bars are 100 μm. Def.: deformation. Str.: stretching.

drug tablets from 1350-1450 cm⁻¹ and 2700-3100 cm⁻¹ at 2 cm⁻¹ steps (10 min. acquisition) and performed spectral decomposition using non-negative least squares (NNLS) to obtain abundance maps for each component (Fig. 2.4B1-B4). These individual abundance maps are combined into four color channels to generate a composite abundance map for easier visual analysis (Fig. 2.4C).

Based on the spectral shapes and peak positions, 20 Raman bands were chosen for S⁴RS imaging (1 min. acquisition), corresponding to the naproxen CH₃ symmetrical deformation band at 1394, naproxen aromatic CH stretching band at 3080, lactose CH₂ stretching band at 2894, HPMC CH₂ stretching band at 2846, and stearate CH₂ stretching bands

at 2856 and 2892 wavenumbers, among others (Table A.1).^{179,180,181,178} Imaging at these sparse bands with spectral decomposition generated abundance maps comparable to those obtained from full spectral imaging at 2 cm^{-1} steps (Fig. 2.4C, 2.4D) with a 10-fold decrease in acquisition time. Both composite abundance maps show a majority of the cross-sectional surface containing large grains of lactose (cyan), followed by grains of HPMC (white) with a wrinkled appearance, and much smaller grains and lower composition of naproxen (magenta) and Mgst (yellow) filling in the crevices of the larger grained components. The pixel size of SRS and S⁴RS images is $0.48\text{ }\mu\text{m}$, which allows for detection of these small grains and low concentrations of API and excipients, as shown by the clear representation of naproxen (3.3% w/w) and Mgst (1% w/w) in these images. Dark areas are attributed to background and indicate areas of surface roughness following microtome sectioning. These dark areas are reduced in subsequent images by imaging at multiple z-steps.

2.3.2 ACCELERATED AGING CONDITIONS

Next, we sectioned and performed S⁴RS imaging of naproxen tablets stored in four different accelerated aging conditions: 1) $20^{\circ}\text{C}/55\%\text{RH}$ room/control conditions, 2) $60^{\circ}\text{C}/10\%\text{RH}/3$ weeks, 3) $70^{\circ}\text{C}/10\%\text{RH}/6$ weeks, and 4) $37^{\circ}\text{C}/94\%\text{RH}/6$ weeks (Fig. 2.5). Conditions 1-3 were chosen within the range of Accelerated Predictive Stability (APS) study conditions for testing long-term stability of pharmaceutical products,^{182,183} while condition 4 was selected as an extreme stress condition. All tablets used in this experiment were produced in the same batch, and as such, each group of tablets was assumed to have similar chemical composition and physical distribution of API and excipients.

Initial visual inspection showed that the tablets stored in $37^{\circ}\text{C}/94\%\text{RH}/6$ weeks had

swollen in size and that the film coating had become loosely attached to the tablet core. Microtome sectioning of these tablets also produced surfaces that were rougher than tablets aged in other conditions. As such, these required imaging to a depth of 110 μm at 10 μm steps to obtain full signal across a field of view (FOV), while the control and high heat tablets required a depth stack of just 40 μm at 10 μm steps.

2.3.3 QUANTITATIVE IMAGE COMPARISONS

Thresholding was performed on the abundance maps to generate a binary map for each pure component (Fig. 2.5B) for quantitative comparisons of surface area contribution, grain sizes, and API distribution. Nine 489 x 489 μm FOVs were taken from each tablet for two to four tablets in each aging condition. The surface area contribution of each component was calculated by dividing the number of pixels attributed to one component by the number of pixels attributed to all components in a FOV. Noticeably, the tablets aged in 37°C/94%RH/6 weeks show increased surface area contributions from naproxen (Fig. 2.6A) and HPMC (Fig. 2.6B) and decreased lactose contribution (Fig. 2.6C) compared to the formulations stored at room temperature or in high heat. Further, the average grain sizes of naproxen (Fig. 2.6E) and HPMC (Fig. 2.6F) are increased in the tablets aged in high humidity compared to those of tablets aged at room temperature or high heat. These changes indicate physical instability of the tablets at high temperature and humidity. Specifically, we see increased surface area contribution along with increased grain size of the API, naproxen, at high humidity, which will likely lead to changes in dissolution profile. Additionally, HPMC, which is crucial for maintaining the formulation's extended-release profile, has increased surface area contribution, and enlarged grain sizes in tablets stored at

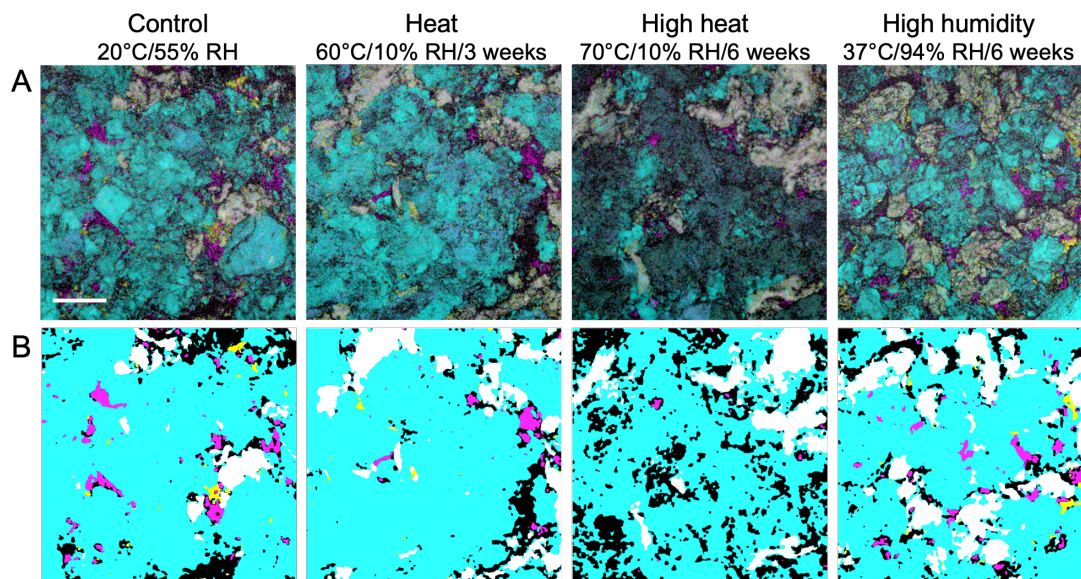


Figure 2.5: S^4RS composite abundance maps (A) and thresholding images (B) of tablet cross-sections from different accelerated aging conditions. Images are color coded as follows: naproxen (magenta), lactose (cyan), HPMC (white), and Mgst (yellow). Black represents background not attributed to any API or excipient. Scale bar is 100 μm .

high humidity, indicative of swelling and further alterations to dissolution.

Interestingly, the surface area contribution of Mgst in formulations stored at 70°C/10%RH/6 weeks was almost nonexistent and significantly lower than all other conditions (Fig. 2.6D). This is indicative of chemical instability, specifically degradation. Though the difference in grain size was not statistically significant due to the low number of grains, we can see that average Mgst grain size trends lower in tablets stored at 70°C (Fig. 2.6H).

An observation of note from the composite abundance maps is that naproxen often tends to associate with or cluster near grains of HPMC. Since HPMC plays an important role in the extended release of naproxen, changes in the proximity of these components are likely to affect the tablet's dissolution profile and naproxen bioavailability. To determine if our aging conditions affect the distribution of naproxen with regards to lactose and

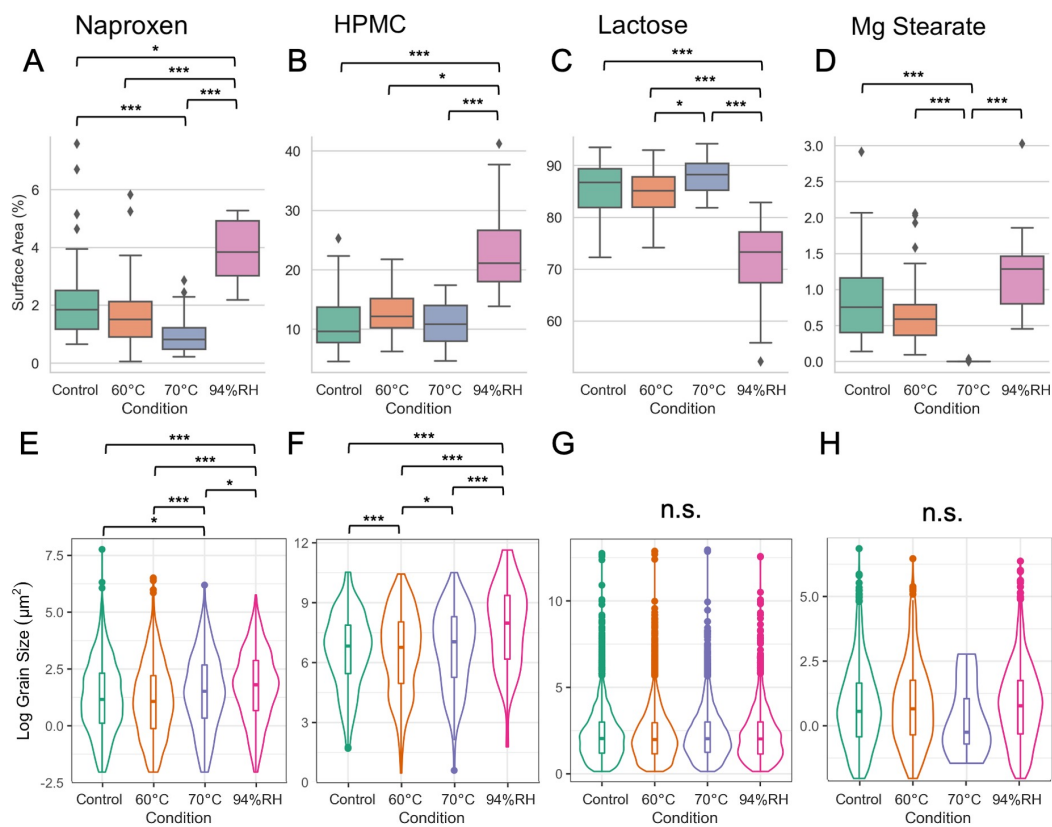


Figure 2.6: Quantitative comparisons of surface area (A-D) and grain size (E-H) for each chemical component within naproxen tablets stored in accelerated aging and stress conditions. Surface area was calculated as the percent contribution of each component to the total area in a $489 \times 489 \mu\text{m}$ FOV from which signal was obtained. Grain sizes were calculated from $1.4 \times 1.4 \text{ mm}$ areas with 9 stitched FOVs in order to capture large grains. Statistical significance: * $p < 0.01$, *** $p < 0.0001$ determined by Wilcoxon test with Holm adjustment.

HPMC, we quantified naproxen density ratio (NDR), a measure of naproxen's proximity to excipient grains at a given distance away from a grain contour. We found that NDR peaks above 1.5 at 11-15 μm away from HPMC grains in all tablets, but that there was a significant difference between the NDR slope in tablets aged at room conditions and those aged in high humidity (Fig. 2.7, left). Accordingly, the NDR for lactose in tablets aged at 37°C/94%RH was higher than that of tablets aged in room conditions (Fig. 2.7, right), indicating more even distribution of naproxen throughout the cross section, but did not increase above 1.0 in any of the tablets, which demonstrates the lack of naproxen clustering around lactose in contrast to HPMC.

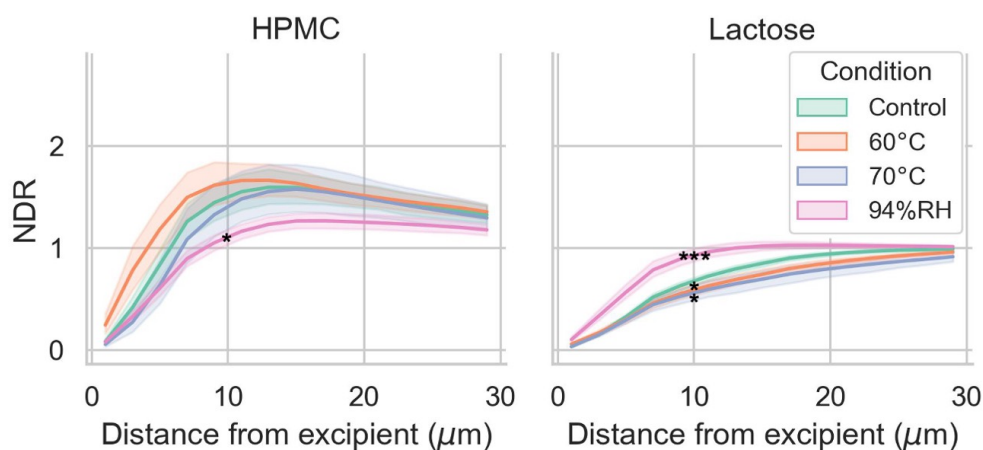


Figure 2.7: Differences in Naproxen Density Ratio (NDR) for excipients HPMC and lactose in tablets stored in accelerated aging and stress conditions. Statistical significance (* $p < 0.01$, *** $p < 0.0001$) is based on the NDR slope up to 10 μm from generalized linear model analysis.

2.3.4 HETEROGENEOUS DISTRIBUTION OF CHEMICAL INSTABILITY

SRS imaging of tablets stored in high heat (70°C/10%RH/6 weeks) showed decreased signal intensity for lactose in a heterogeneous distribution, as well as an absence of Mgst signal (Fig. 2.5). To explore the cause of this signal change in lactose-attributed areas, we obtained full SRS spectra (1350-1450 cm^{-1} and 2700-3100 cm^{-1} at 2 cm^{-1} steps) of areas of lactose with low signal and found a peak at 2932 cm^{-1} instead of the expected peak at 2894 cm^{-1} (Fig. 2.8A). Spontaneous Raman spectroscopy confirmed this spectral shift, and additionally showed a decrease in the OH stretch signal between 3000 cm^{-1} and 3600 cm^{-1} (Fig. 2.8A). The spectral change from a 2894 cm^{-1} peak to a 2932 cm^{-1} peak indicates a loss of aliphatic CH_2 with a gain in CH , CH_3 , or a different CH_2 group, while the decrease in OH vibrational signal indicates a loss of OH groups. Both spectroscopic changes represent lactose degradation due to prolonged heating at 70°C since they were not seen in tablets aged in 60°C/10%RH/3 weeks. Further investigation by ^1H -NMR spectroscopy demonstrated the emergence of a peak at 3.725 ppm (Fig. 2.8B) not seen in tablets aged in any other conditions. Taken together, we posit that one potential thermal degradation mechanism could be the change of lactose to lactulose, shown to be catalyzed by heat treatment and alkaline conditions,^{184,185,186} followed by lactulose degradation with formic acid as a byproduct.¹⁸⁷ The lactose isomerization process leads to the conversion of one of the 6-atom sugar monomers to a 5-atom ring that generates a hydroxymethyl group without a change in the total number of hydroxyl groups, which can result in the appearance of the extra CH_2 peak in SRS and NMR. Formic acid production can then occur by a hydroxymethyl group elimination from lactulose, which explains the loss of OH groups seen in SRS.

Interestingly, no SRS or spontaneous Raman spectral shifts were seen for pure lactose powder aged in 70°C, which suggests that the environment of the compressed tablet may induce the degradation. Mgst has been shown to elevate microenvironmental pH¹⁸⁸ and to act as a proton acceptor in salt disproportionation reactions.¹⁰⁰ Indeed, we see that areas of the tablet with lactose degradation (Fig. 2.8C, dark blue) shows little trace of Mgst (Fig. 2.8C, yellow), which is indicative of Mgst consumption or of a reaction that alters the spectrum of Mgst such that our SRS sparse sampling is unable to identify it. Chemical imaging of an entire cross-sectional surface at a depth of 2 mm in a control tablet and a tablet stored at 70°C revealed lactose chemical instability with spatial heterogeneity, where degradation occurred in the periphery while the lactose in the tablet center was stable. We believe this heterogeneity occurs as a result of the degradation spreading internally over time, and with longer exposure to heat conditions, the center of the tablet would also be impacted. The entire cross-sectional image, however, was useful in identifying small areas of chemical heterogeneity, such as the agglomeration of Mgst in the periphery for potential investigation into mechanisms of chemical reaction with lactose.

2.3.5 DISCUSSION

Spatially mapping API and excipients in compacted pharmaceutical products poses challenges for many chemical imaging methods, especially for analysis of dynamic changes in composition or distribution. Here, we report the development of a high-speed S⁴RS imaging capable of mapping API and excipients in solid drug tablets. We have demonstrated this protocol with chemical components that have highly overlapping Raman spectra in the high wavenumber region. This method generates images at less than 1 μ m resolution to de-

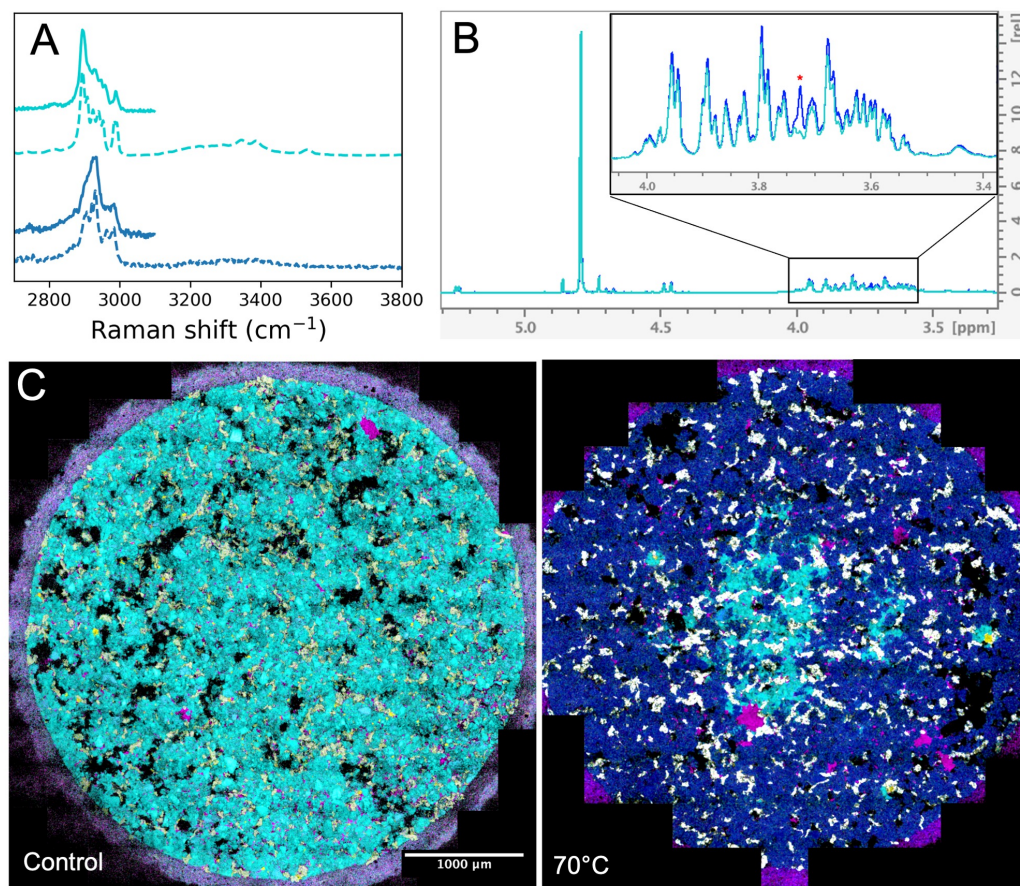


Figure 2.8: Lactose degradation in tablets aged in 70°C/10 %RH for 6 weeks demonstrated by (A) SRS and spontaneous Raman microscopy, (B) NMR spectroscopy, and (C) S⁴RS microscopy. (A) SRS (solid) and spontaneous Raman (dotted) spectra of lactose from control tablets (cyan) and lactose from tablets aged in 70°C (blue) obtained in situ. The difference spectra (black) between control tablets and tablets aged in 70°C are also shown. (B) NMR spectra of lactose from control tablets (cyan) and lactose from tablets aged in 70°C (blue), scraped from the imaged cross sections in (C). (C) S⁴RS composite abundance maps of the full cross sections of a control tablet and a tablet stored at 70°C. The abundance map of the 70°C tablet was generated by performing NNLS using SRS reference spectra from degraded lactose (as shown in (A)) in addition to pure components. Images are color coded as follows: naproxen (magenta), lactose (cyan), degraded lactose (blue), HPMC (white), and Mgst (yellow).

tect Mgst and naproxen with small grain sizes and for 20 spectral samples at a 0.5 x 0.5 mm FOV, we are able to acquire images in less than 1 minute per z-step. This method enables the visualization of spatially heterogeneous chemical instability and quantification of surface area contribution, grain sizes, and API distribution to uncover physical instability due to storage in various aging conditions.

One disadvantage of epi-collected SRS in combination with the small axial resolution inherent to SRS is that it requires a smooth surface, which is difficult to obtain in a compressed tablet except on the compressed surface. Here, we demonstrate a method using simple 3D printed components fitted to the tablet and to an ultramicrotome for generating a cross sectional surface with minimal roughness. We can capture chemical signal from over 80% of an entire tablet cross section by imaging up to just 40 μm in depth in a majority of tablets.

There remain limitations to using S⁴RS for chemical imaging of pharmaceutical tablets. Chemical changes identified from S⁴RS must be confirmed with other methods such as spontaneous Raman spectroscopy, NMR, or MALDI-TOF. Though it is possible and convenient to obtain a full spectra SRS image after sparse sampling, our current SRS system is limited to the range 800 cm^{-1} to 3100 cm^{-1} . In this study, we performed spontaneous Raman spectroscopy and NMR to confirm the chemical reaction occurring with lactose in our drug tablets initially identified with S⁴RS. Another constraint on the use of SRS and S⁴RS for pharmaceutical applications is that the APIs and excipients of interest must have strong Raman signal, typically compounds containing nitriles or alkynes and aromatic compounds, among others. Lastly, identifying sparse sampled Raman shifts and performing spectral decomposition using NNLS requires prior knowledge of the Raman spectra of

each component. Though this is technically feasible in the pharmaceutical production process, it does require extra time. Obtaining spectra of pure components may not always be possible in other applications such as for counterfeit drug identification. Other techniques such as MCR-ALS (multivariate curve resolution - alternating least squares) or PCA (principal component analysis) can be employed for spectral decomposition without the need for reference spectra, though these techniques are particularly useful for formulations with fewer components or components that exhibit less spectral overlap.

2.4 CONCLUSION

Our implementation of S⁴RS with a tunable dual-output fiber laser and sample processing for cross-sectional imaging represents a feasible method for chemical and spatial analysis of solid-state drug tablets at a faster speed at higher resolution compared to existing technologies. Importantly, the ability to "skip" between target bands without collecting spectral information between the bands allows S⁴RS to collect high quality hyperspectral data rapidly. We have demonstrated the identification and mapping of API and excipients within naproxen tablets containing small grains of API. The samples were placed in select high stress conditions designed to investigate the effect of extreme temperature and humidity conditions on the physical and chemical stability of the formulations, which may reasonably occur during transport of the tablets across the world, even if storage and usage occur according to manufacturer specifications at the origin and destination. We revealed that stress conditions resulted in spatially heterogeneous chemical degradation from high heat and excipient grain swelling from high humidity, which are likely to lead to changes in dissolution rate and even toxicity. It is worth noting that the high humidity conditions

tested are not too dissimilar from the temperatures and humidities found in the homes of many areas of the world, in particular in austere conditions for some of the most vulnerable populations. This makes the study of tablet compositional changes under both normal and extreme conditions important for key medicines, including those to treat pain and infection.

Integrating S⁴RS chemical imaging into drug tablet manufacturing may be particularly valuable for guiding the design of tablet composition, assessing root causes of changes in tablet performance, setting storage condition limits, and deciding packaging requirements. S⁴RS could serve an important role in tablet production, which may be particularly important not only for brand and generic medications, but additionally for supplements that are far less widely studied. This tool can be a powerful addition to the pharmaceutical development, quality control, and quality assurance processes.

3

SRS microscopy enables quantification of cryoprotective agent in *Daphnia magna*

3.1 INTRODUCTION

Advancements in clinical and engineering technologies have created a growing need for the cryopreservation of living systems. This includes storage of cell therapies for patient treat-

ment, tissues for biological dressings and trauma care, organs for life-saving transplantation, and whole organisms for biodiversity preservation, among others.^{189,190,191,192,193,194,195,196,197} Despite the promise of cryopreservation technologies, a major barrier to developing successful preservation protocols is cryoprotectant or cryoprotective agent (CPA) toxicity. CPAs such as DMSO, glycerol, and ethylene glycol are added to biological specimens prior to cooling to minimize damage from ice crystallization. Although necessary, CPAs are toxic at required concentrations (2-8 M).¹⁹⁸

CPA toxicity has been considered the single most limiting factor in the field of cryobiology.^{199,189,200} A major challenge to the optimization of CPA loading is our inability to reliably quantify CPA uptake. As CPA toxicity increases over time, the key is to identify CPA loading parameters that minimize the duration of exposure while enabling maximal uptake. In other words, if exposure is too brief, insufficient CPA concentrations will accumulate, resulting in lethal ice crystallization. If exposure is too long, specimens will succumb to CPA-induced cytotoxicity. The development of cryopreservation protocols is thus largely empirical, where CPA loading (concentration and duration) and cooling rates are iteratively optimized, a process that can require several years. Quantifying CPA uptake and spatial distribution is especially critical for whole organism cryopreservation, particularly in complex biological specimens such as mosquito larvae and *Daphnia*. These organisms represent key targets for the development of effective preservation protocols for biodiversity and stock preservation. The high degree of cellular differentiation and the presence of different organ systems may lead to the compartmentalization of CPAs. Furthermore, unlike cells and cell clusters, these organisms often lack a typical shrink-swell response and instead exhibit active osmoregulation during CPA exposure.

Previous approaches to study CPA permeation kinetics have used mathematical modeling,^{201,202} NMR,^{203,204} wide-field microscopy,²⁰⁵ and Raman spectroscopy.²⁰⁶ Mathematical modeling can give general approximations of diffusion kinetics but becomes complicated and less accurate when considering CPA cocktails and cell or tissue compartments. While NMR is highly chemically specific and can quantify CPA diffusion, sample processing is destructive and thus cannot capture the temporal or spatial dynamics of CPA permeation. Wide-field microscopy is fast enough to capture the dynamic osmotic changes occurring in cells during CPA permeation but cannot directly visualize CPAs, and studies have shown that cellular permeation occurs much earlier than the shrink-swell response.²⁰⁷ Like NMR, Raman spectroscopy provides the chemical specificity required to track CPAs and has the benefit of being non-destructive; however, its acquisition time is an order of magnitude greater than CPA permeation dynamics. Coherent anti-Stokes Raman scattering (CARS) was developed as a Raman-based imaging method that improved acquisition speed, but its non-resonant background decreases chemical specificity,¹⁷⁰ which limits the ability to image multiple CPAs and to quantify chemical concentrations.

Stimulated Raman scattering (SRS) microscopy is a Raman-based technique that has been demonstrated for imaging chemical transport in cells and tissues in the last two decades^{208,209,84,83} but it has not yet been applied for imaging chemical transport in small organisms or for quantifying CPA permeation. SRS can provide submicron resolution and signal intensity scales linearly with concentration. SRS also delivers improved chemical specificity and spectral interpretability compared to CARS due to a lack of non-resonant signal. As such SRS microscopy is a promising technique for quantifying CPA kinetics during the dynamic permeation process. An SRS method called normalized Raman imaging (NoRI) was re-

cently developed for measuring absolute mass concentrations of protein and lipids in cells of living and fixed tissue samples.¹⁰⁸ NoRI computationally removes the heterogeneous effects of light scattering in an image through normalization by the sum of the chemical components present in the image, including water. In this study, we apply a modified NoRI method to quantify the absolute concentration of CPA within different anatomical regions of *Daphnia* at different incubation times and concentrations.

Daphnia are chosen as a proof-of-concept model for SRS-based CPA quantification due to their complexity and transparency in visible light wavelengths. Additionally, while cryopreservation protocols have been successfully developed for certain cells, embryos, and tissues, the cryopreservation of complex organs and living organisms remains challenging. *Daphnia* contain complex tissues and organs, including a heart which pumps an open blood system, antenna which function for sensory and locomotion, and a digestive system with a mouth, esophagus, gut, and anus. To our knowledge, quantification of CPA kinetics in cells and organisms in a spatial manner has never been accomplished. Characterization of CPA kinetics in different *Daphnia* tissues and organs can inform cryopreservation protocol development for other small organisms and other complex tissues. DMSO is a common CPA used for cryopreservation of cells and embryos; however, its Raman spectrum demonstrates peaks in the high wavenumber range that overlap with lipids and proteins.²¹⁰ A simple alternative is DMSO-d₆, which can be assumed to have similar kinetics to DMSO due to its similar molecular mass and structure, and has a unique deuterium peak in the silent region that does not overlap with Raman bands of living systems.

3.2 EXPERIMENTAL

3.2.1 *DAPHNIA MAGNA* MAINTENANCE AND STANDARD REARING CONDITIONS

Daphnia magna stocks (IL-MI-8 heat-tolerant clone - provided by Dr. Leonid Peshkin, Department of Systems Biology, Harvard Medical School) were maintained in two transparent culture tanks with a total volume of 2 and 10 liters each filled with 1.5 and 4 liters respectively of 100% natural spring water (Poland Spring, Maine, USA). All cultures were maintained at room temperature (20-22°) and fed weekly with green algae food source (Carolina Biological Supply 14-2337) at a concentration of 0.05 mL per mL of spring water. The air-flow in the tanks was maintained using an air pump (Marina 11135) connected to an air stone (Top Fin 5289732). Water maintenance was performed every other week by replacing half of the total volume, with a complete water change conducted every other month.

One day prior to experimentation, 20-30 *Daphnia* ranging from 0.5 mm to 1.5 mm in size were collected and transferred to a container containing 100 mL of spring water. To facilitate gut clearance and improve imaging quality, no food was provided during this period.

3.2.2 CRYOPROTECTANT SOLUTIONS

All CPA solutions are made with the stock solution DMSO-d₆ (1.03424, Sigma Aldrich, USA) in spring water. Solutions ranging from 0.01M to 2M DMSO-d₆ are made for calibration images, while solutions of 1M and 2M DMSO-d₆ are additionally made for *Daphnia* incubation. These are concentrations of DMSO used in plant and animal cryopreservation and are chosen for their applicability.

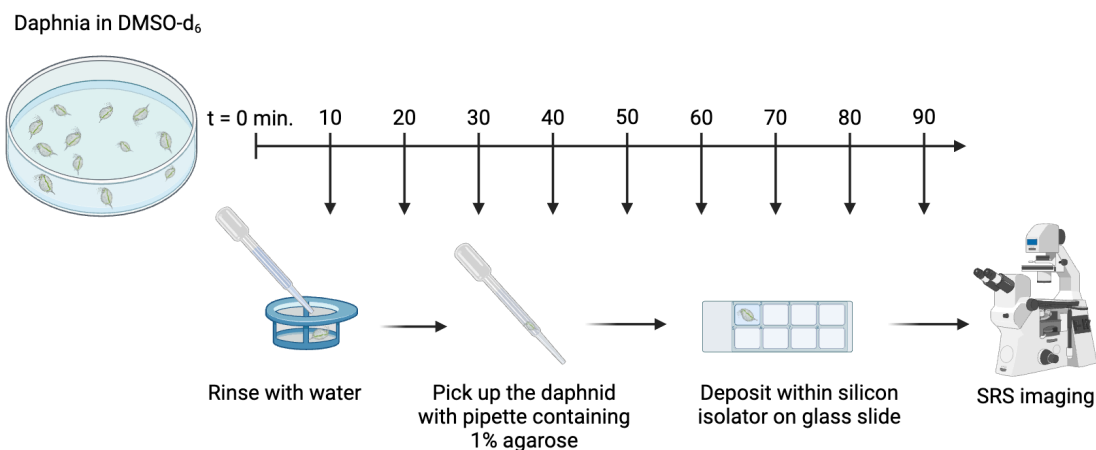


Figure 3.1: Protocol for *Daphnia* incubation, washing, and immobilization prior to SRS imaging. (Created with BioRender.com)

3.2.3 *DAPHNIA* PREPARATION FOR IMAGING

Daphnia in the size range 0.5 to 1.5 mm are starved for 24 hours and placed in petri dishes with 1M or 2M DMSO-d₆ at room temperature for incubation (Figure 3.1). At each time point, every 10 minutes, one daphnid is removed from DMSO-d₆ with a transfer pipette, rinsed with spring water in a cell strainer, and dried on a Kimwipe placed under the cell strainer. The daphnid is picked up with a transfer pipette containing one drop of 1% low melting point agarose (NuSieve GTG, Cambrex Bio Science Rockland, Inc., USA) that has been melted and cooled to around 40°C and deposited onto a glass slide within a CultureWell reuseable gasket (Grace Bio-Labs, Oregon, USA) and covered with a glass cover slip. The slide is placed on ice for 30 seconds to solidify the agarose and immobilize the daphnid before being placed onto the microscope stage for SRS imaging.

3.2.4 SRS INSTRUMENTATION

The SRS microscopy setup (Figure 3.2A) is similar to that described in Pence et al. The system uses a dual output rapidly tunable all fiber optical parametric oscillator (FOPO) light source (Picus Duo, Refined Laser Systems, Germany), capable of generating synchronized pump pulses (tunable from 780 to 980 nm) and Stokes pulses (tunable between 1020 to 1060 nm) at a 40.5 MHz repetition rate with 7 ps duration.¹⁷⁷ The pump beam is modulated at half of the repetition rate and both beams are passed through half wave plates (HWP) and overlapped in space and time (Dio2-R980-25 x 36 980 nm dichroic beamsplitter, Semrock, USA) before coupling into a commercially available inverted microscopy (IX3, Olympus, Japan). The overlapped pump and Stokes pulses are transmitted to the back focal plane of a 10X 0.4 NA microscopy objective (UPLXAPO10X, Olympus, Japan). The emitted light from the sample is passed through the microscope condenser, filtered for the Stokes pulse using a dichroic mirror and long pass filters (DMSP1000R and FELH1000, Thorlabs, USA) and detected using an in-house built SRS detector²¹¹ connected to a lock-in-amplifier (Moku:Lab, Liquid Instruments, USA). The demodulated SRS signal is amplified by a gain block, connected to an analog-to-digital converter, and read into the Olympus software. The imaging system is programmed to run via a Python script and Olympus RDK, which enables automation of switching to specific Raman shift wavenumbers.²¹

3.2.5 SRS IMAGING

Spectral images of DMSO-d₆, spring water, and *Daphnia* anatomical locations are recorded from 700 to 3220 cm⁻¹ at 5 cm⁻¹ spectral steps for reference spectra. All SRS images of

Daphnia during the experiment are obtained at 1950, 2140, 2855, 2935, and 3220 cm^{-1} for non-resonant background, DMSO-d₆, lipid, protein, and water, respectively.

All individual images are obtained with 2 μs pixel dwell time such that the acquisition time for a single 1024 x 1024 resolution SRS image is around 2 seconds. We use a 10X objective to capture most daphnids in one frame, obtaining a pixel resolution of 1.243 μm and a field of view (FOV) of 1.27 x 1.27 mm. Z-stacks of *Daphnia* are taken at 20 μm z-steps up to 400 μm in depth to capture anatomical structures of interest. Image volumes with all five Raman bands are acquired in 3 to 7 minutes depending on the number of z-steps required.

3.2.6 IMAGE ANALYSIS AND NoRI APPLICATION

SRS images at the DMSO-d₆ and H₂O Raman bands (2140 and 3220 cm^{-1} , respectively) are preprocessed by subtracting the background offset and laser noise measured at the non-resonant Raman band (1950 cm^{-1}). They are then divided by the flat field correction mask of the matching Raman band. Flat-field correction masks are generated by measuring the intensity profile of 1M DMSO-d₆ at the 2140 cm^{-1} band and the profile of spring water at the 3220 cm^{-1} band. An SRS intensity image at the z-plane of highest intensity is smoothed by 2D polynomial fitting and normalized by dividing with the maximum intensity. The mask is applied to all z planes in the sample data.

In the original implementation of NoRI, BSA, DOPC, and water were used as the calibration samples for protein, lipid, and water.¹⁰⁸ In our implementation, the chemicals of interest are DMSO-d₆ and water, so we use 1M DMSO-d₆ and spring water as calibration samples. We obtain the 2x2 spectral decomposition matrix, **M**, that maps **S**, the SRS inten-

sities of the calibration samples at the deuterium and H₂O Raman bands to **C**, the DMSO-d₆ and water concentration of the calibration samples in the same manner as detailed in Ref¹⁰⁸ (Appendix A).

Concentration standards of DMSO-d₆ (0.01, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 M) are made by diluting the DMSO-d₆ stock solution in spring water. DMSO-d₆ dilutions in spring water are used for *Daphnia* incubation because that the solution is less toxic to *Daphnia* and is also used for cryopreservation studies, therefore, we also use spring water dilutions to test our calibration.

Because the *Daphnia* are often still alive within the agarose, there may be some movement during imaging at the different Raman bands. So instead of applying the spectral decomposition matrix to our sample images in a pixel-by-pixel manner, anatomical regions of interest (ROI) are first manually segmented for the DMSO-d₆ and H₂O Raman bands at the z-plane with the best focus, making sure to avoid regions containing lipid droplets and high levels of protein, using the lipid (2855 cm⁻¹) and protein (2935 cm⁻¹) band images as reference. The average intensities of each anatomical ROI are measured to obtain a matrix, **a**, containing the average SRS intensities of each anatomical ROI at the deuterium and H₂O Raman bands, representing each image volume. The spectral decomposition matrix, **M**, is then multiplied with **a** to obtain **d**, the linearly decomposed chemical contribution of DMSO-d₆ and water to each anatomical ROI. The DMSO-d₆ and H₂O rows of matrix **d** are summed to obtain the attenuation values for each anatomical ROI. We divide **d** by the attenuation values to obtain the absolute average concentration (v/v) of DMSO-d₆ and water within each anatomical ROI, multiply by density and divide by molar mass to obtain concentration in molarity (Appendix A).

3.3 RESULTS AND DISCUSSION

3.3.1 SRS CHARACTERIZATION OF *DAPHNIA* AND DMSO-D₆

The Raman spectrum of DMSO-d₆ and various anatomical regions of daphnids are visualized using our SRS microscopy system (Figure 3.2A and B). Anatomical regions of interest for CPA sequestration or clearance include the heart, hemocoel, and carapace (Figure 3.2D and E). While the gut is also of interest, we find that it often consists of processed algae food that has broad and variable SRS background signal, which makes it impossible to obtain reliable SRS signal of CPA. We also visualize other regions to avoid for CPA quantification, such as areas with significant light scattering or absorption, including the pigmented compound eye, the pigmented ocellus, and the hairy thoracic limbs (Figure 3.2C). CPA quantification is also difficult in regions with many lipid droplets (Figure 3.2E). *Daphnia* store spherical lipid droplets in fat cells in the hemocoel and are typically reduced during starvation.²¹² Therefore, to reduce the number of lipid droplets and background signal in the gut, we starve *Daphnia* for 24 hours prior to CPA incubation and SRS imaging.

The Raman peaks for DMSO-d₆ and water are located at 2140 and 3220 cm⁻¹, originating from the C-D and the O-H stretching modes, respectively. Though the literature value of the H₂O peak is around 3420 cm⁻¹, we are limited by the tuning range of our laser and still obtain good signal for water at 3220 cm⁻¹. These peaks are distinct from the spectrum of *Daphnia* anatomical regions in the wavenumber range 1900 to 3220 cm⁻¹. We assume that, as with most biological samples, water, proteins, and lipids account for nearly 100% of the chemical composition of *Daphnia* tissues.²¹³ Outside of the gut contents, non-reproductive *Daphnia* are primarily composed of 1) carapace, made of chitin which

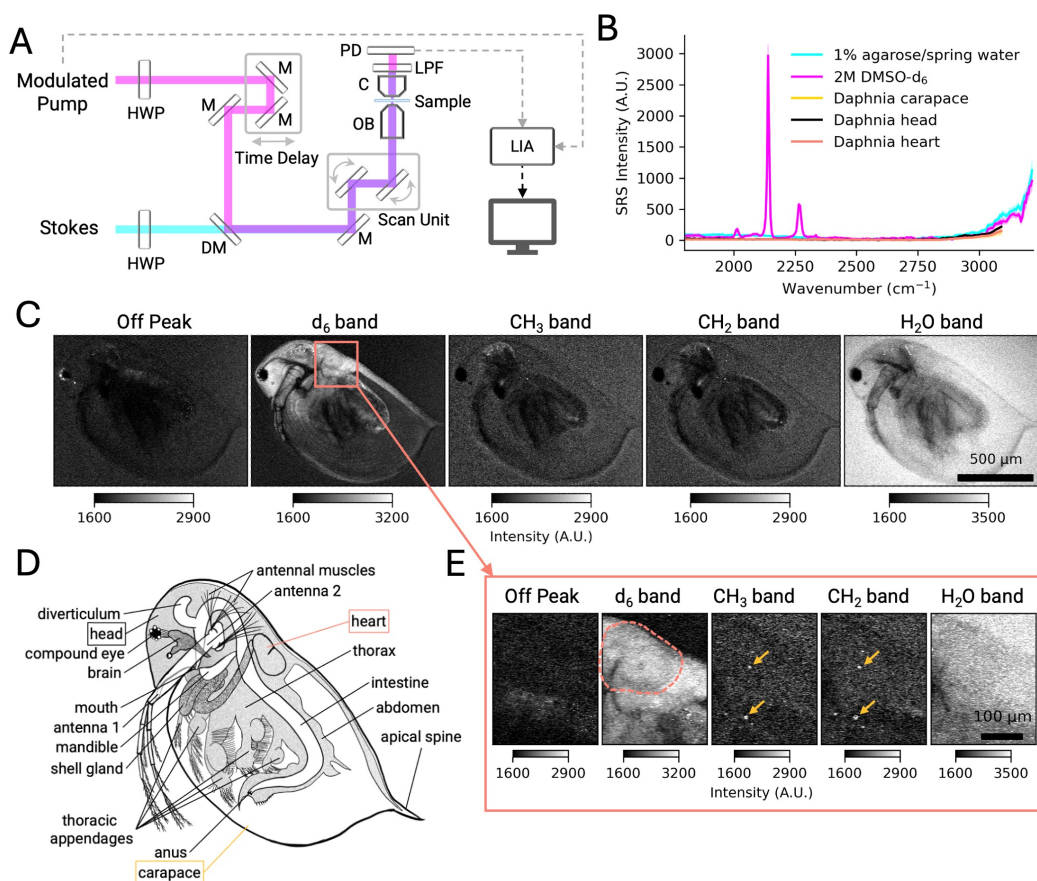


Figure 3.2: SRS imaging of DMSO- d_6 and *Daphnia*. (A) Schematic of the stimulated Raman scattering microscope. (B) SRS spectra of DMSO- d_6 , water, and select *Daphnia* anatomical locations: carapace, head, and heart. (C) Representative maximum projection SRS images of a daphnid organism at the off peak (1950 cm^{-1}), d_6 (2140 cm^{-1}), CH_3 (2835 cm^{-1}), CH_2 (2955 cm^{-1}), and H_2O (3220 cm^{-1}) bands after incubation in DMSO- d_6 . (D) *Daphnia* diagram with labeled anatomy (redrawn from Refs ^{22,23}). (E) Close-up of the *Daphnia* heart showing the heart outline (dotted outline) in the d_6 band and lipid droplets (arrows) in the CH_3 and CH_2 bands. HWP: adjustable half wave plate, M: mirror, DM: dichroic mirror, OB: objective, C: condenser, LPF: long pass filter, LIA: lock-in amplifier, PD: photodetector.

has very low signal in the Raman protein band, and 2) hemocoel, the body cavity filled with hemolymph, made of mostly water with low levels of salts, proteins, and other organic constituents,^{2,2} and occasionally containing lipid droplets. Our Raman spectra of the *Daphnia* anatomy support these assumptions (Figure 3.2B, C, and E).

3.3.2 SRS DETECTS AND QUANTIFIES CPA CONCENTRATION INCREASE IN *DAPHNIA*

Though SRS signal intensity scales linearly with sample concentration, heterogeneous signal attenuation in complex or thick samples has previously limited the application of SRS for accurate quantification of chemicals in small organisms and tissues *in situ*. Recently, Oh et. al.¹⁰⁸ reported on normalized Raman imaging (NoRI), a method of obtaining absolute concentrations of proteins and lipids in cells by normalizing away the intensity variations caused by sample light scattering. We apply NoRI for obtaining absolute concentration of CPA within *Daphnia* after incubation in different concentrations of CPA over time.

First, we image a titration series of DMSO-d₆ solutions to demonstrate the linearity of NoRI concentration measurements and accuracy of the absolute concentration calculation for DMSO-d₆ in spring water (Figure 3.3). The NoRI concentration measurements show excellent agreement with the actual concentration of solutions, despite saturation artifact in the SRS image of 2 M DMSO-d₆ at the deuterium band after flat-field correction.

Next, we image *Daphnia* that were incubated in 1 M or 2 M DMSO-d₆ over a period of 90 minutes. Because each image volume requires 3 to 7 minutes to acquire, the *Daphnia* must be immobilized in agarose during imaging and a different daphnid must be extracted for each time point. SRS images of *Daphnia* at the deuterium band, focused on the head,

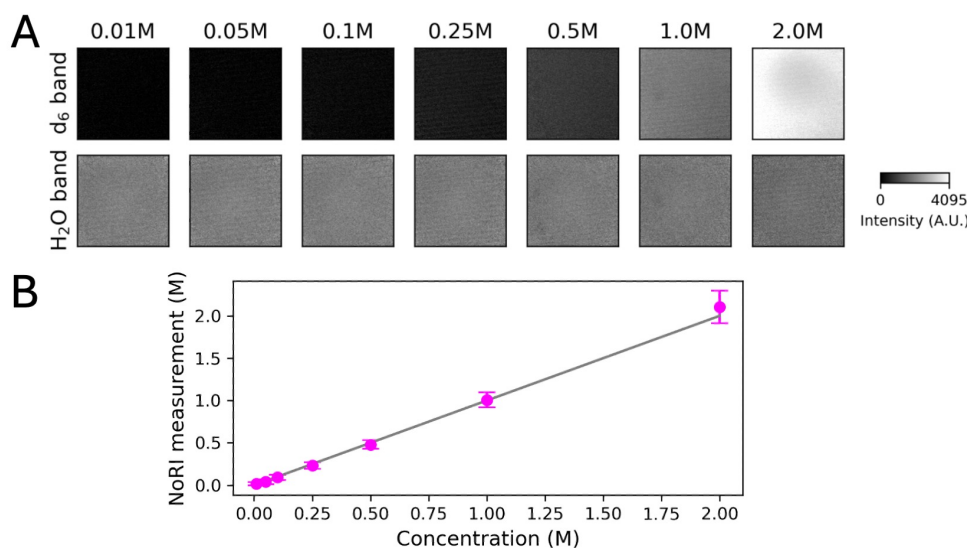


Figure 3.3: (A) Flat-corrected SRS images and (B) NoRI measurements of DMSO-d₆ concentration standard solutions. Despite some saturation artifact at 2M concentration, NoRI measurements show good agreement with the actual concentration. Error bars indicate standard deviations.

show a clear increase in DMSO-d₆ concentration over time for *Daphnia* incubated in both 1 M and 2 M DMSO-d₆, with a greater increase for *Daphnia* incubated in 2 M DMSO-d₆ (Figure 3.4A, B). NoRI measurements also demonstrate increasing DMSO-d₆ concentrations in the heart over time, up to the concentration of the incubation solution at 90 minutes for both 1 M and 2 M incubations (Figure 3.4C). Interestingly, the hemolymph in the carapace had consistently lower concentrations of DMSO-d₆ compared to the heart. One potential explanation is that the carapace has less retention capability and greater diffusion with the surrounding agarose. In the hemal system of a daphnid, contractions of the heart force hemolymph anteriorly into the hemocoel of the head from which it flows posteriorly into three hemocoelic channels, two of which serve each side of the carapace, which is the chief gas exchange surface.²¹⁴ This may also explain why the concentration of DMSO-d₆

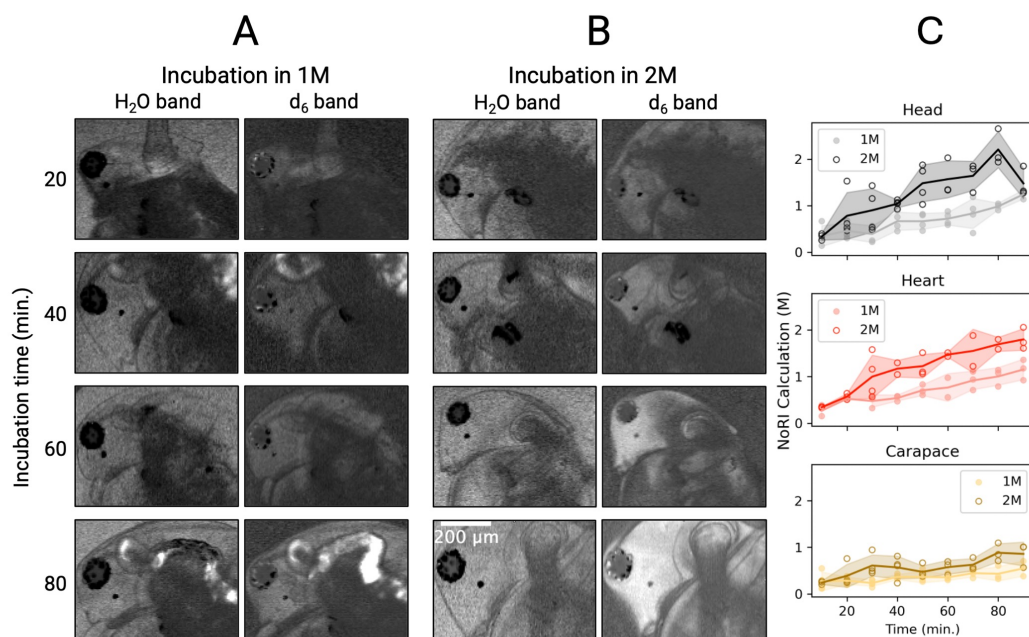


Figure 3.4: DMSO-d₆ concentration within *Daphnia* increases gradually over 90 minutes. (A, B) Representative raw SRS images of *Daphnia* at the H₂O and d₆ bands, incubated in (A) 1M and (B) 2M DMSO-d₆ for different durations. Images are selected for a z depth for best focus on the head of the daphnid. (C) NoRI calculations of DMSO-d₆ molarity at different *Daphnia* anatomical locations over 90 minutes.

in the hemolymph within the head increases consistently when *Daphnia* are incubated in 1 M solution, but peaks at 80 minutes for *Daphnia* incubated in 2 M DMSO-d₆. DMSO-d₆ toxicity may lead to paralysis, heartbeat cessation, and death of the organism in 2 M solution. When the heart is continually beating, the stored DMSO-d₆ concentration can be maintained throughout the organism, with lower concentrations only in the carapace. As the heart stops, the gradient between the hemolymph in the head and heart increases and the head becomes more equilibrated with the carapace.

The DMSO-d₆ concentration is also measured just outside of the organism to detect any significant diffusion of DMSO-d₆ out of the organism during imaging. Some DMSO-d₆ is

present in the surrounding agarose, and is maintained below 0.2 M. This may account for some diffusion of DMSO-d₆ out of the carapace.

3.4 CONCLUSION AND FUTURE STEPS

In this study, we demonstrated the utility of SRS as a chemical imaging tool for quantifying CPA concentrations within live biological specimens, using *Daphnia magna* as a model organism. Our results show that SRS microscopy can distinctly resolve the chemical signature of DMSO-d₆ within the complex whole *Daphnia* organism, enabling spatial and temporal mapping of CPA uptake *in vivo*. We observed a clear increase in DMSO-d₆ concentration within *Daphnia* over a 90-minute incubation period for both 1 M and 2 M solutions, confirming the capability of SRS to monitor CPA accumulation at concentrations relevant for cryopreservation. These findings represent a step towards understanding the kinetics of CPA diffusion in live organisms and provide a foundation for future studies correlating internal CPA concentrations with toxicity and survival. We are currently conducting survival assays to establish these correlations between internal CPA concentrations and CPA toxicity for *Daphnia*, which will be critical for informing the development of safe and effective cryopreservation protocols.

Importantly, this work serves as a proof-of-concept for the broader application of SRS microscopy in dynamic, longitudinal studies of CPA behavior in living systems. While this study required removing and immobilizing organisms at discrete time points, the next phase of this research will involve the design of a microfluidic platform to facilitate continuous *in situ* monitoring. This system will allow precise control of CPA exposure while maintaining organism viability and positioning, enabling higher temporal resolution and

potentially revealing rapid osmoregulatory responses.

Looking forward, the methodology developed here opens the door to multi-CPA imaging, where SRS can differentiate between agents based on their unique vibrational peaks. This will allow comparative studies of CPA diffusion rates, anatomical distribution, and cellular permeability, ultimately supporting the rational design of cryopreservation protocols tailored to organismal physiology and specific preservation goals.

4

Connecting *in vivo* microscopy features and RNA expression in atopic dermatitis

4.1 INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin condition that affects 3-5% of adults and 20% of children globally.^{215,216} Its pathophysiology is multifactorial, involving genetic

predisposition, compromised skin barrier integrity, and immune system dysregulation. Clinically, AD is characterized by pruritus (itchiness), erythema (redness), lichenification (thickening and roughening), and xerosis (dry skin), yet there are large variations in AD features by ethnicity, age, and time course.^{217,218,219} The current standard of care is based on visual assessment by expert clinicians, who carry out differential diagnoses and scoring of skin lesions to determine disease severity and response to treatment. The selection of therapeutic approaches is typically determined as part of the diagnostic process, with the efficacy of topical and systemic agents assessed by the clinician in follow up visits, and via patient-reported outcomes. While systemic immunosuppression is the ultimate treatment for the most severe conditions, adverse drug events and insufficient therapeutic response remain challenging.

Clinical evaluations can be augmented using advanced tissue analysis methods, including genomic, proteomic, and cytokine profiling. Still, these methods require biopsies which limit their value for longitudinal assessments, often not allowing for parallel evaluation with multiple techniques, in addition to the obstacle of tissue collection from patients. Histology, immunofluorescence, and similar pathology tools can provide a direct assessment of tissue structure and molecular state, but also require invasive biopsy. Furthermore, these imaging approaches only provide static visualization via 2D cross-sections at a single point in time, which can be limiting when considering the dynamic and complex 3D structure of the skin. An ideal approach for studying skin inflammatory conditions would enable noninvasive and objective lesion measurement, allowing the acquisition of 3D imaging information linked to cutaneous pathophysiology.

Advanced microscopy methods have been translated to mobile, clinical instruments that

can be used directly on patients to capture a range of parameters associated with skin morphology and physiology.^{7,64} Imaging information can include tomography of skin chemical components (e.g., collagen structure and content), structural information (e.g., cell shape and size, epidermal shape and thickness, papillary dermis structure), and cellular metabolic state. This functional information can be acquired noninvasively, enabling serial imaging over time to capture the evolving inflammatory status as well as the potential to assess tissue response to therapeutic intervention.

This *in situ* imaging of skin can be accomplished using a combination of imaging techniques that take advantage of distinct optical interactions within the tissue. Reflectance confocal microscopy (RCM) provides detailed structural tomographic imaging of skin structures with intrinsic contrast based on differences in reflection and backscattering.^{220,220,40,48} Two photon excited fluorescence (TPEF) microscopy provides detailed metabolic information from fluorophores in the skin such as NADH.^{221,222,223,4} Second harmonic generation (SHG) microscopy reveals type I and II collagen content and structure.⁶⁷ Fluorescence lifetime imaging microscopy (FLIM) via TPEF describes the lifetimes of fluorescent molecules that can be used to quantify NADH redox state and map melanin within the skin.^{64,223,224,225,222,65} All these techniques can be powered by a single laser system for real-time, co-registered chemical-spatial imaging of skin on systems such as the MPTcompact.^{226,227}

The objective of this study is to evaluate how well noninvasive multimodal imaging can capture differences in disease states and perform the first demonstration of whether this imaging can infer transcriptomic changes in atopic dermatitis through structural and metabolic cellular features. We also demonstrate how the combination of *in vivo* microscopy

and spatial transcriptomics can help us understand how changes in RNA expression correlate with cellular-level image features and metabolic function in AD.

4.2 METHODS

4.2.1 STUDY SUBJECTS

Research performed in this study complies with all relevant ethical regulations. The study was approved by the institutional review board and all patients gave written informed consent. The study cohort included 10 patients with AD and 10 healthy control subjects, who were recruited between January 2023 and September 2024 at Massachusetts General Hospital (Boston, Massachusetts). Inclusion criteria for all subjects included age 18-40 years and Fitzpatrick skin type I-III. AD patients had a clinical diagnosis of AD and mild to moderate AD at the time of inclusion. Patients did not use systemic immunosuppressive or immunomodulating biologic drugs within 24 weeks, any topical or systemic janus kinase (JAK) or tyrosine kinase 2 (TYK2) inhibitor within 12 weeks, any systemic or immunomodulating small-molecule drugs within 4 weeks, or any topical corticosteroids or calcineurin inhibitors on their arm within 2 weeks prior to their study visit. All subjects did not use any sunscreen on the arm within 72 hours or any moisturizers on the arm within 24 hours of their study visit.

4.2.2 CLINICAL SEVERITY ASSESSMENT

Patients with AD underwent a clinical assessment for local lesion severity based on criteria in the Eczema Area and Severity Index. We call our local index the Lesional Eczema Severity (LES) score. This score is the sum of scores for erythema/redness (E), edema/papulation

(T), scratching/excoriation (S), and lichenification (L) rated on a scale from 0 (none) to 3 (severe), using a standard visual scoring guide (Appendix A.4 and A.5). A separate score for self-reported pruritus was also collected, rated on a scale from 0 (no itching) to 3 (bothersome itching that disturbs sleep or normal activity).

4.2.3 MULTIMODAL CLINICAL IMAGING

We used the MPTcompact, a certified CE-marked multiphoton microscope from JenLab (JenLab GmbH, Jena, Germany) described in Ref.²²⁷. Briefly, it is a cart-based imaging system capable of obtaining reflectance confocal microscopy (RCM), second harmonic generation (SHG), two photon excited fluorescence (TPEF), and fluorescence lifetime (FLIM) images with one excitation source such that they are fully spatially and temporally overlapped. It has an articulating scanhead for secure attachment to the imaging area of interest. The system contains a femtosecond fiber laser emitting at 780 nm with pulse width < 90 fs, repetition rate 80 MHz, and power ranging 2-50 mW. The bandwidths for fluorescence and SHG detection are 417 to 650 nm and 381 to 394 nm, respectively. The system employs a 40X NA 1.3 objective. Three separate photomultipliers (PMTs) provide intensity images for RCM, SHG, and TPEF. One PMT is read out via time-correlated single-photon counting to calculate fluorescence lifetime values. This setup allows us to non-invasively image human skin *in vivo* to create high-resolution RCM, SHG, autofluorescence, and fluorescence lifetime images up to a penetration depth of 150 μm . SHG imaging captures collagen structure and organization, while an excitation wavelength of 780 nm and emission bandwidth of 415 to 650 nm collects autofluorescence signal from NAD(P)H, FAD, keratin, melanin, collagen, and elastin that has the potential to be distin-

guished using fluorescence lifetime analysis.

Images were collected at $250 \times 250 \mu\text{m}$ laterally and $100 \mu\text{m}$ depth at $5 \mu\text{m}$ Z-steps for each image volume. Laser power remained constant at 50 mW. Each Z-slice had an exposure time of 6 seconds for a total of 3 minutes acquisition time per image volume. For each healthy volunteer, images were acquired at four to five regions of interest (ROIs) containing three to six areas of interest (AOIs) each for a total of twelve to thirty image volumes of healthy skin per subject (Appendix A.6). For each patient with AD, images were acquired at two lesional ROIs and two nonlesional ROIs containing three to six AOIs each for a total of six to twelve image volumes of lesional skin and six to twelve image volumes of nonlesional skin per subject (Appendix A.6).

4.2.4 IMAGE ANALYSIS

EPIDERMAL THICKNESS

For this study, imaging was limited to a depth of $100 \mu\text{m}$ to limit acquisition time per volume and maximize SNR in all z-frames, so not every image volume reaches the dermis, particularly where epidermal thickening is significant. Where the dermis appeared within the image volume, epidermal thickness was determined using a 3D automatic segmentation method modified from Ref. ²²⁸. Since SHG signal is present only where collagen is present, SHG can be considered a proxy indicator for the dermis. However, due to noise and crosstalk from TPEF in the SHG channel, we cannot perform simple thresholding to determine the presence of dermis. To be certain that SHG signal indicates the true dermis, for each image volume, we calculate a z-profile from the gradient of the average SHG signal at each z-frame. We then apply an empirical threshold on that gradient profile that

ensures a true rise in SHG signal that is representative of entry into the dermis. The z-depth at which the gradient reaches the gradient threshold is the minimum depth at which we can obtain markers for the dermis. If SHG signal in an image volume does not increase quickly enough or the absolute intensity of the SHG channel does not increase to an absolute threshold, we conclude that the depth of the dermis at every pixel is greater than 100 μm . For image volumes that reach the dermis, Otsu thresholds are applied to both the SHG and TPEF channels to construct markers for the dermis and epidermis, respectively. Priority is given to SHG markers when conflicts occur because SHG signal is only present in the dermis, but while TPEF signal is mostly present in the epidermis, it may also be generated from elastin in the dermis. In this way, markers are mutually exclusive for epidermis or dermis. Marker propagation is performed using a fast watershed algorithm and merged using a graph cut algorithm. The final result of the segmentation is a 3D volume containing an epidermal layer and a dermal layer. The percent of epidermal thickness less than 100 μm ($\text{ET} < 100 \mu\text{m}$) per volume is calculated from the fraction of xy-positions in a 3D volume that contain part of the epidermal layer. The mean epidermal thickness calculated from one image volume is the average of the dermal depth at every xy-position in the image volume.

CELL FEATURES

Cells were segmented using a custom Cellpose3.0 model fine-tuned with tens of manually annotated images from our dataset.^{16,144,145} Z-frames from each image volume containing clear cell nuclei and borders were manually selected for cell segmentation. Frames that were purely stratum corneum, stratum basale, or dermis were excluded. Average cell area for each z-frame was determined by averaging the pixel areas of each segmented cell

in the frame and converting to μm^2 . Average cell diameter for each z-frame was determined by assuming circularity for each cell and calculating a diameter from the average area: $d = 2\sqrt{\frac{A}{\pi}}$. Distance between cells could not be calculated automatically because not every cell was successfully segmented due to low SNR or out-of-plane cells. Instead, distance between cells was calculated by manually drawing lines through at least four neighboring segmented cells within a frame. Three to six lines were drawn per frame and the distances between neighboring cells along the line were averaged to obtain an average cell distance metric in μm .

FLUORESCENCE LIFETIME

FLIM analysis was performed using custom Python code implementing the phasor approach. The modulation frequency (f) set to the first harmonic frequency of 80 MHz, and the angular modulation frequency (ω) was defined by the relation $\omega = 2\pi f$. Phasor coordinates (G and S) were calculated from the fluorescence decay signal ($F(t)$) as follows:

$$G = \frac{\int F(t) \cos(\omega t) dt}{\int F(t) dt}, \quad S = \frac{\int F(t) \sin(\omega t) dt}{\int F(t) dt} \quad (4.1)$$

Phasor coordinate maps (G and S) with spatial dimensions of 512×512 pixels were extracted from each AOI in the lifetime image stack ($251 \times 512 \times 512$; t, x, y). Subsequently, fluorescence lifetime (τ) maps were calculated from G and S maps as follows

$$\tau = \frac{S/G}{\omega} \quad (4.2)$$

Mean values of G, S, and τ were computed for each AOI with and without cell segmenta-

tion masks.

The pixel-wise fraction of free NADH ($f_{\text{NADH-free}}$) and bound NADH ($f_{\text{NADH-bound}}$) was derived as follows:

$$f_{\text{NADH-free}} = \frac{|\tau_{\text{NADH-bound}} - \tau_{\text{skin}}|}{\tau_{\text{NADH-bound}} - \tau_{\text{NADH-free}}} \quad (4.3)$$

$$f_{\text{NADH-bound}} = 1 - f_{\text{NADH-free}} \quad (4.4)$$

where $\tau_{\text{NADH-bound}}$ represents the fluorescence lifetime of bound NADH from reference calibration, while $\tau_{\text{NADH-free}}$ corresponds to the fluorescence lifetime of free NADH from reference calibration. The reference fluorescence lifetime of bound NADH was measured from a standard solution prepared using the Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (Cayman Chemical, Catalog No. 601170) and had a fluorescence lifetime of approximately 2.35 ns. The reference fluorescence lifetime of free NADH was measured from a prepared solution of β -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (Sigma Aldrich, Catalog No. N8129), yielding a lifetime of approximately 0.43 ns.

4.2.5 BIOPSY SAMPLE COLLECTION

Lesional, nonlesional AD, and healthy skin punch biopsies were obtained under IRB-approved protocols. Nonlesional biopsies were obtained either perilesionally (> 2 cm away from active lesions) or contralaterally. Biopsied sites showed no evidence of infection. All biopsied samples and controls were taken from the dorsal and ventral arms. All biopsies were immediately processed for standard formalin-fixed paraffin-embedded (FFPE) proce-

ture.

BIOPSY AND MICROSCOPY ALIGNMENT

All regions for biopsy were marked with a punch biopsy tool and waterproof ink marker prior to imaging or biopsy. After coupling the targeted skin region to the imaging scan-head using an *in vivo* adapter provided by JenLab, the xy-boundaries of biopsy marker were identified through TPEF and all AOI image volumes were obtained within the xy-boundaries of the biopsy marker. As such, all biopsy-microscopy matched ROIs were obtained from the same 3 mm diameter.

4.2.6 SPATIAL TRANSCRIPTOMICS

FFPE samples were sectioned at 5 μ m thickness and placed onto hydrogel slides for deparaffinization, hematoxylin and eosin (H&E) staining, and imaging. Slides were processed using the Visium HD Spatial Gene Expression Reagent and User Guide (CG000685). All twenty individual libraries were pooled together into a single final sample, which was sequenced using an Illumina Novaseq X Plus platform. Sequencing was carried out using three 1.5B-read, 100-cycle flow cell cartridges under paired-end, dual-index conditions.

Spatial gene expression sequencing data and tissue images were processed using the 10X Space Ranger pipeline (v. 3.1.2) to generate count matrices and spatial metadata for each sample. Outputs from multiple samples were merged into a single Seurat object for downstream analysis. Spots with fewer than 10 total transcripts or zero detected genes were excluded. Additionally, only spots with nonempty tissue annotations were retained.

The filtered dataset was normalized and variance-stabilized using the SCTransform

method (Seurat v. 5.1.0). To correct for sample-specific effects, Harmony integration was performed. Principal component analysis was conducted using 30 components, followed by dimensionality reduction with UMAP for visualization.

Differential gene expression analysis was performed between segmented epidermis in tissue states. Comparisons included lesional skin (LS) versus non-lesional skin (NL), LS versus healthy control (HC), and NL versus HC. Only genes expressed in at least 10% of cells in either group and with a log₂ fold change greater than 0.25 were tested.

4.3 RESULTS AND DISCUSSION

4.3.1 DEMOGRAPHICS AND CLINICAL CHARACTERISTICS OF RECRUITED SUBJECTS

In our study cohort, the lesions evaluated in our AD group were characterized by a mean Local Eczema Score (LES) of 3.8 (Table 4.1). This local assessment is based on the reliable and well-known Eczema Area and Severity Index (EASI), which includes a 0 to 3 grading for each criteria of erythema, edema/papulation, scratching/excoriation, and lichenification.^{229,230} In the EASI assessment, each body region (head/neck, trunk, upper extremities, and lower extremities) is given an area score of 0 to 6 and a constant multiplier based on the anatomy. However, since the biopsy and imaging in this study only occur in a 3 mm diameter region on AD lesions in the upper extremities, a local assessment would be more informative for correlations with clinical severity. As such, we generated the LES, which uses the same four criteria on a 0 to 3 scale, but without an area score or multiplier, and is only associated with each specific lesion we image and biopsy (Appendix A.4).

Demographic characteristics of each group are reported in Table 4.1

	HC (n=9)	AD (n=10)
Age (y)	25.2 ± 3.2	24.9 ± 5.0
Female sex (n)	6	7
Fitzpatrick skin type	2.3 ± 0.5	2.4 ± 0.5
BMI (kg/m ²)	23.7 ± 3.1	25.7 ± 4.6
Race (n)		
Asian/Pacific Islander	2	4
Black/African American	0	1
White	7	5
LES score of imaged lesions	–	3.8 ± 1.7

Table 4.1: Demographic and Clinical Characteristics of healthy control (HC) and atopic dermatitis (AD) Groups. Abbreviations: LES, Local Eczema Severity

4.3.2 STRUCTURAL MICROSCOPY FEATURES CORRELATE WITH DISEASE GROUP AND CLINICAL SEVERITY

Multiphoton imaging provides the unique ability to capture detailed structural differences *in vivo* across varying disease states in AD, including healthy, nonlesional, and lesional skin. By combining TPEF and SHG signals, we are able to visualize both cellular and extracellular architecture in a depth-resolved manner. In these studies, TPEF in epidermal cells primarily arises from endogenous fluorophores such as NADH, a metabolic cofactor localized largely to the mitochondria of keratinocytes, providing contrast that reflects both structure and metabolic activity.

Qualitatively, structural differences are evident between healthy and lesional skin in the TPEF images (Fig. 4.1A). In the SC, healthy skin appears evenly keratinized, while lesional skin shows clear evidence of spots of uneven keratinization, suggestive of impaired terminal differentiation (Fig. 4.1A, 5 μ m). At the level of the stratum granulosum (SG), where

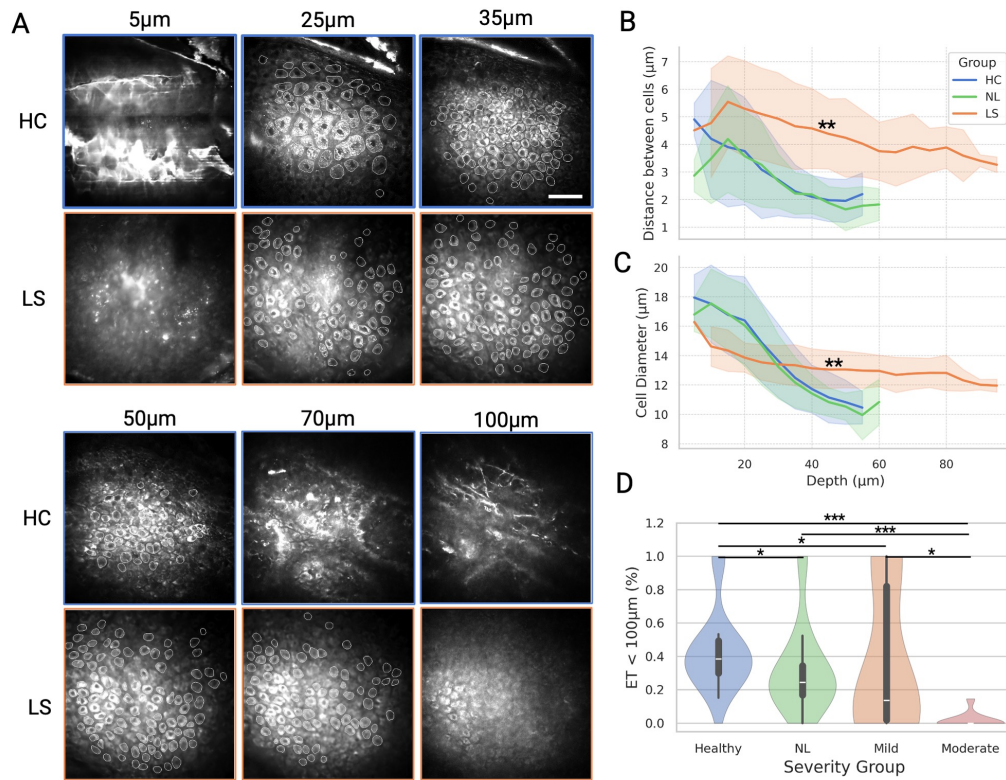


Figure 4.1: Cellular structural differences in skin biopsies from healthy control (HC), lesional (LS), and nonlesional (NL) groups. (A) Representative TPEF images and Cellpose segmentation of corresponding depth frames in a healthy and lesional image volume. Scale bar is 50 μm. Plots showing (B) distance between keratinocytes and (C) the size of keratinocytes change differently as a function of depth between groups. Shaded lines represent standard deviation. Asterisks ** indicate significant differences ($P < 0.01$ for the main and interaction effects in the linear portions of data, between 15 and 55 μm depth) Linear mixed effects with holm correction was used to assess differences between groups. (D) Comparison of how much of the epidermis was visualized at less than 100 μm in depth in each image volume (measured as % of the image area) from healthy, NL, mild AD, and moderate AD lesions. Asterisks *, *** over bars indicate significant differences ($P < 0.05$, 0.0001, respectively) between severity groups using pairwise nested mixed effects analysis with holm correction after ordered quantile normalization transformation. Abbreviations: ET, epidermal thickness

we typically observe large keratinocytes in healthy skin, lesional samples exhibit smaller keratinocytes that are more widely spaced (Fig. 4.1A, 25 μm). This increased intercellular distance is likely due to intercellular edema, a feature also observed in histological sections of biopsies from AD subjects.²³¹ Furthermore, in healthy epidermis, keratinocytes decrease in size with depth—from around 25 μm through 70 μm —as the tissue transitions from the stratum spinosum (SS) and stratum basale (SB) toward the dermal-epidermal junction (DEJ). In contrast, keratinocyte size in lesional skin remains relatively constant around 14 μm in diameter across depth, indicating a disruption in normal epidermal differentiation (Fig. 4.1A, C). In healthy skin, the dermis is typically present at 100 μm , evident from the appearance of long, thin strands of fibrillar elastin structures visualized via TPEF. However, in lesional samples, we are still within the SS at this depth, reflecting an overall epidermal thickening in affected skin.

Quantitative analysis further validates these qualitative observations. Using Cellpose, a generalist deep learning-based cell segmentation tool,¹⁶ we were able to segment keratinocytes in the SG and SS layers based on TPEF signal alone. This segmentation enabled the measurement of intercellular distance between neighboring cells, which was found to be significantly greater in lesional skin compared to both healthy control and nonlesional skin (Fig. 4.1B).

Cell size also showed significant differences across disease groups. In the SG, approximately above 30 μm depth, keratinocytes in lesional skin were significantly smaller than those in both healthy and nonlesional skin. However, at deeper levels (past 40 μm), due to the thickening of the SS, keratinocytes in lesional skin were larger than those in the SS and stratum basale (SB) of healthy and nonlesional samples.

Due to the epidermal thickening in AD past 100 μm , we often could not directly measure the full ET in lesional samples, as the DEJ extended beyond the 100 μm imaging limit. As an alternative, we measured the percentage of the field of view where the epidermis was located within the top 100 μm . This measurement was derived using a combined TPEF and SHG-based segmentation approach involving watershed and graph cut algorithms.²²⁸

We compared this percent-based ET metric between healthy, nonlesional, mild AD, and moderate AD groups. We divided the range of LES scores into mild (1-4), moderate (5-8) and severe (9-12) as a way to assess severity correlations in the data, resulting in six subjects in the mild group, four in the moderate group, and none in the severe group. There was a significant difference in ET between nearly all groups, except between nonlesional and mild lesions (Fig. 4.1D). Notably, moderate AD lesions consistently had no dermis present at less than 100 μm depth, confirming significant thickening. Interestingly, nonlesional skin had significantly thicker epidermis than healthy skin (15% less ET < 100 μm on average), even in the absence of visible clinical inflammation. This suggests that subclinical structural changes persist in areas of nonlesional skin. The lack of significant difference between nonlesional and mild lesional skin may be attributed to high variance in the mild group (0.18 compared to 0.06, 0.06, and 0.02 in the HC, NL, and mild groups, respectively), reflecting either biological variability or transitional tissue states.

4.3.3 FLIM MEASUREMENTS SHOW DIFFERENCES IN AD METABOLISM

FLIM is a label-free tool for probing cellular metabolism *in vivo*. In biological tissues, fluorescence lifetime serves as a proxy for metabolic state, particularly through measurements of NADH, a key metabolic coenzyme. In our study, we utilized FLIM in conjunction with

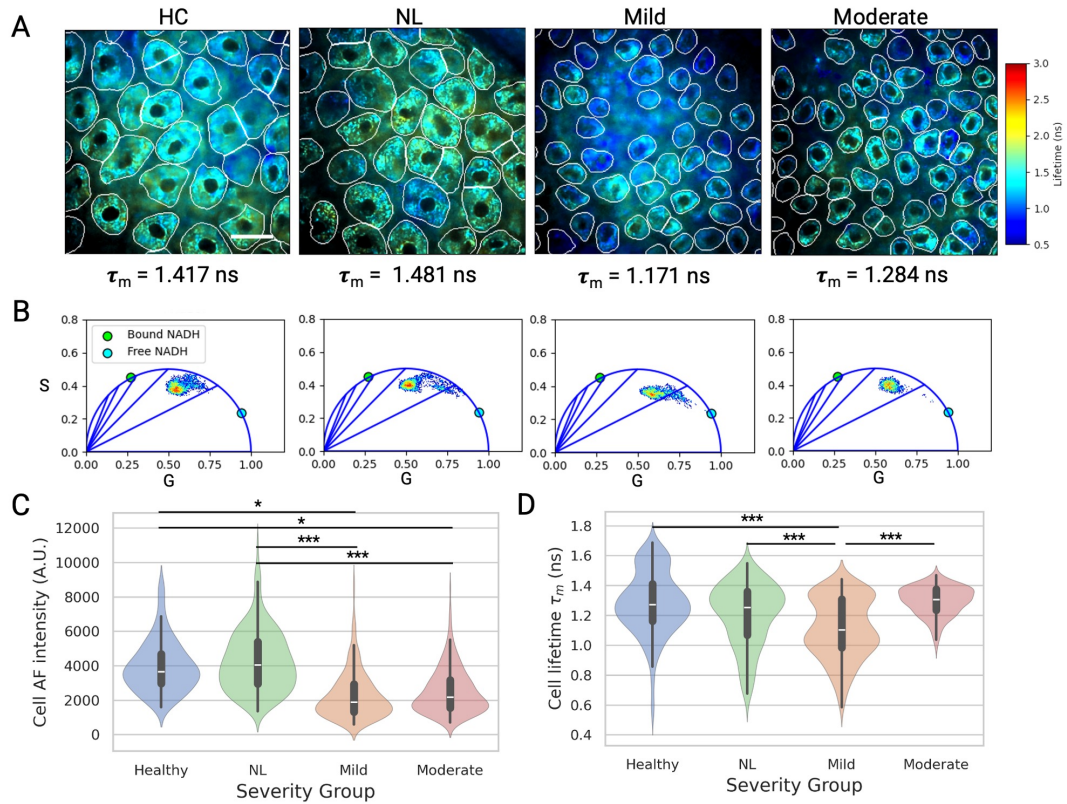


Figure 4.2: Comparison of fluorescence lifetime and intensity of Stratum granulosum (SG) and Stratum spinosum (SS) cells in healthy, non-lesional, mild AD, and moderate AD biopsies. (A) Images of TPEF intensity overlaid with color-coded fluorescence lifetime (τ_m) values. The average τ_m value is displayed below each frame. Scale bar is 25 μ m. (B) Phasor plots of all pixels of the FLIM image showing the lifetime distribution between free and bound NADH with 5 x 5 pixel spatial binning. Comparison of the fluorescence (C) intensity and (D) lifetime within segmented SS and SG cells between disease groups. Asterisks *, *** over bars indicate significant differences ($P < 0.05$, 0.0001, respectively) between severity groups using pairwise nested mixed effects analysis with holm correction.

TPEF at 780 nm excitation and 415–650 nm emission detection, a range that captures the autofluorescence of several endogenous fluorophores in skin, including keratin, NADH, FAD, melanin, collagen, and elastin. However, keratin is largely localized to the stratum corneum, melanin is concentrated in the basal layers and dermal-epidermal junction, and collagen and elastin contribute to signal only in the dermis. As such, based on the depth-resolved distribution of these fluorophores, the dominant fluorophore in the SG and SS keratinocytes is NADH. Additionally, FAD has a lower fluorescence quantum yield, particularly when excited at 780 nm, and is typically found in one to five times lower concentrations than NADH, making it a minor contributor to the TPEF signal under our imaging conditions.^{232,221,233} Thus, the TPEF and FLIM signals obtained from SG and SS layers can be interpreted as predominantly reflective of NADH dynamics.

TPEF intensity provides a measurement of the total NADH pool - including both free and protein-bound forms - while FLIM allows us to differentiate between these two pools based on their distinct fluorescence lifetimes. In our reference measurements, we confirmed literature-consistent lifetimes for free NADH (0.43 ns) and bound NADH (2.35 ns) solutions.^{225,234,223} Given the need for short acquisition times *in vivo* (6 seconds per frame), photon counts in the FLIM images were low, which presented a challenge for accurate lifetime estimation. To address this, we employed DeepFLR, a deep learning-based model trained to denoise FLIM data under photon-starved conditions.¹⁴² This approach enabled us to recover reliable lifetime values and conduct robust phasor analysis, despite low photon counts.

To ensure our analysis was focused on keratinocytes within the SG and SS layers, we included only image frames in which the average cell diameter exceeded 12 μm —consistent

with suprabasal epidermal cell sizes—and excluded frames with high melanin signal (dominated by a short lifetime of around 0.1 ns), which can distort phasor coordinates.²²⁴ Specifically, we omitted any frames with phasor coordinates falling outside the physiological range for NADH, using a cutoff of $G > 0.941$ or $S < 0.236$, corresponding to the region of the phasor plot with shorter lifetimes than free NADH. After applying these criterion, 52.3% or 781 image frames were included for analysis.

Qualitatively, we observed that the average fluorescence lifetimes were longer in healthy control compared to lesional samples, consistent with previous literature (Fig. 4.2A).^{222,7} Notably, mild AD skin displayed the shortest average fluorescence lifetime ($\tau_m = 1.13$ ns, compared to 1.31, 1.23, and 1.30 ns for HC, NL, and moderate AD, respectively), suggesting a higher fraction of free NADH and a shift toward glycolytic metabolism. Phasor analysis supported this observation: the distribution of phasor points in mild AD samples was shifted toward the free NADH region along the line connecting bound and free NADH phasors (Fig. 4.2B). This pattern is consistent with a metabolic shift favoring glycolysis, a hallmark of proliferative or stressed cells.^{235,236,237,238}

To complement the phasor lifetime analysis, we quantified the absolute TPEF intensity of segmented keratinocytes in the SG and SS layers. Both mild and moderate AD samples showed significantly lower fluorescence intensity compared to healthy and nonlesional skin, indicating a reduced total NADH pool and, consequently, an overall decrease in metabolic activity (Fig. 4.2C). It is important to note that data from four healthy control subjects and one AD subject were excluded from intensity analysis due to a technical error during acquisition (PMT offset setting), which affected fluorescence gain and would have biased comparisons. These subjects were not excluded from fluorescence lifetime analysis as lifetime

estimates are not significantly affected by absolute intensity, particularly after DeepFLR restoration.

Quantitative comparison of fluorescence lifetimes between groups revealed a significant reduction in mean lifetime for the mild AD group compared to all others (Fig. 4.2D). Interestingly, the violin plots of average lifetimes in mild AD samples displayed a bimodal distribution, suggesting the presence of two distinct populations of keratinocytes with differing metabolic profiles. One possible interpretation is that during this transitional disease stage, glycolysis and TCA cycle pathways remain active, but oxidative phosphorylation is impaired, leading to accumulation of free NADH that results in shorter lifetimes. In contrast, moderate AD may reflect a state of more globally dysfunctional metabolism, with all major pathways impaired and a reduced overall NADH pool, resulting in neither strong accumulation of free NADH nor long lifetimes associated with oxidative phosphorylation. These findings provide new insights into the metabolic heterogeneity of keratinocytes in AD, suggesting that even within the same disease category, different regions of the epidermis may be engaged in distinct metabolic states. Future work involving pathway enrichment analysis of matched transcriptomic data may reveal whether these observed metabolic shifts are associated with alterations in specific pathways such as glycolysis, TCA cycle activity, or mitochondrial function, and whether these differences are predictive of disease severity or progression.

4.3.4 EPIDERMAL DEGS CORRELATE WITH DISEASE STATE

To explore the transcriptional changes associated with atopic dermatitis (AD) across disease states, we performed spatial transcriptomic profiling on biopsies from healthy controls

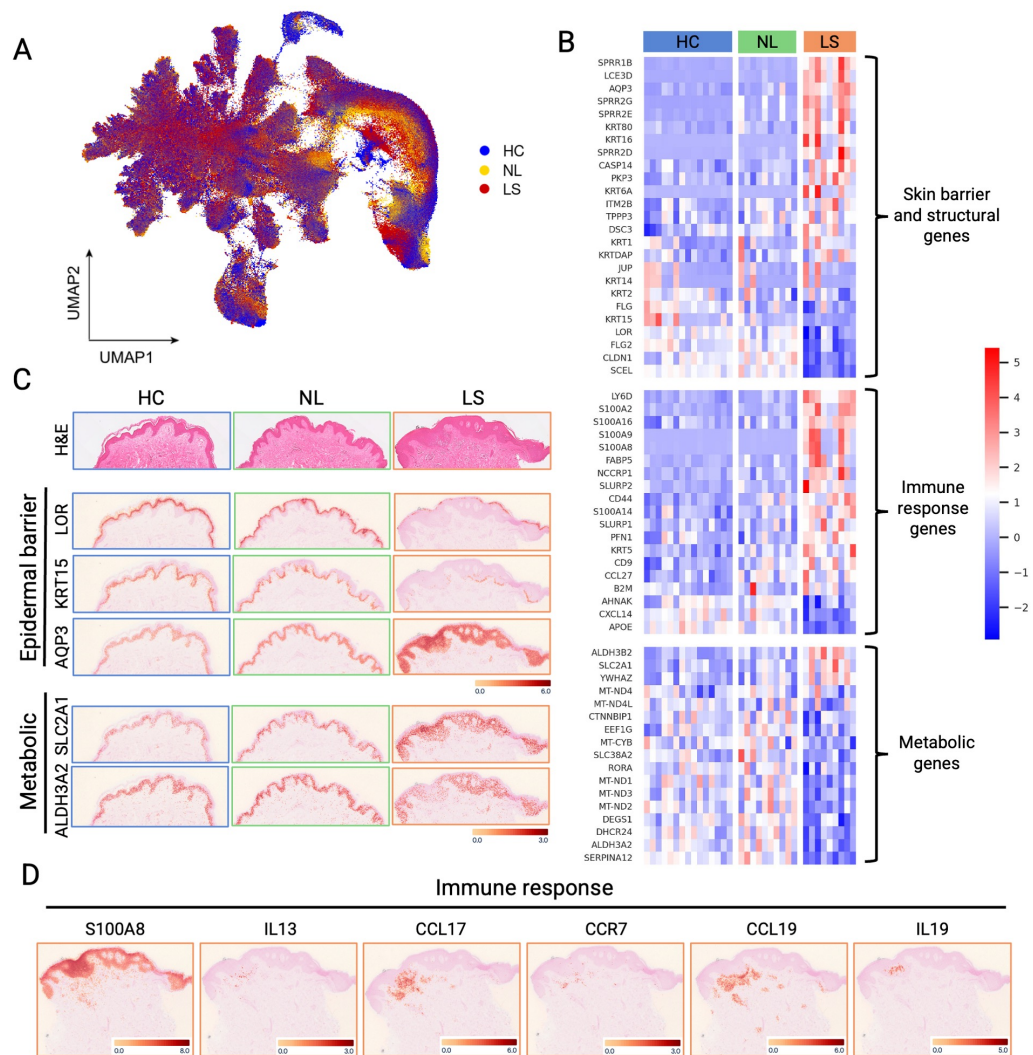


Figure 4.3: Spatial transcriptomic profile of AD. (A) UMAP (Uniform Manifold Approximation and Projection) plot showing the distribution of spots within healthy control (HC), AD nonlesional (NL), and AD lesional (LS) skin biopsies. (B) Heatmap showing a combination of known AD immune response genes and select differentially expressed genes (DEGs) between groups (HC/NL/LS). Colorbar represents z-scaled log2-transformed counts. (C) H&E and spatial feature plots of select epidermal barrier and metabolic DEGs in the epidermis shown on a biopsy from each group. Colorbars represent log2 expression. (D) Spatial feature plots of select immune response genes shown on a lesional biopsy with leukocytic infiltrate. Colorbars represent log2 expression.

(HC), nonlesional (NL), and lesional (LS) skin. Visualization of the transcriptomic landscape using Uniform Manifold Approximation and Projection (UMAP) revealed several areas of separation between spots derived from healthy, nonlesional, and lesional samples (Fig. 1.8A). Although we have not yet performed formal cell type clustering or annotation, the distribution of spots in the UMAP suggests that transcriptional profiles vary meaningfully between disease states.

To focus our analysis on the region most relevant to our paired microscopy data, we initially limited DEG analysis to epidermal regions, which we segmented on each 3 mm biopsy using H&E histology. The epidermis provides the clearest TPEF imaging signal and includes the keratinocyte layers where AD-driven barrier dysfunction and metabolic dysregulation are most apparent. Using the following criteria for differential gene expression analysis: expression in at least 10% of spots in either group and a $\log_2$ fold change > 0.25 , we identified 72 DEGs between LS and HC, 80 DEGs between LS and NL, and 15 DEGs between NL and HC, yielding a total of 88 unique genes. These DEGs broadly fell into three major categories: barrier/structural genes, immune response genes, and metabolic genes (Fig. 1.8B).

Among the barrier and structural genes, we observed upregulation of *SPRR1B*, *SPRR2G*, *SPRR2E*, and *LCE3D* in lesional skin (Fig. 1.8B). These genes are known components of the cornified envelope in terminally differentiating keratinocytes and are typically elevated in hyperproliferative or reparative epidermis.^{239,240,241} *AQP3*, a gene encoding an aquaglyceroporin involved in water transport, was also upregulated in LS samples (and to a lesser extent, in NL skin, although not statistically significant in our analysis) (Fig. 1.8B, C). Elevated *AQP3* expression is known to contribute to increased transepidermal water loss, a

hallmark of AD.^{242,243} KRT6 and KRT16, keratin genes activated during epidermal hyperproliferation, were likewise upregulated in LS skin (Fig. 1.8B).²⁴⁴

Conversely, we observed significant downregulation of classic barrier genes in lesional skin, including FLG, FLG2, LOR, and CLDN1 (Fig. 1.8B, C). These genes play critical roles in stratum corneum formation and tight junction integrity. Loss-of-function mutations in FLG and its related genes are well-established genetic risk factors for AD and other barrier-associated dermatoses.^{245,246} KRT15, a type I keratin typically expressed in non-activated basal keratinocytes, was also significantly downregulated in LS samples and, notably, in NL skin (Fig. 1.8B, C). KRT15 is known to be downregulated in hyperproliferating epidermis and maintains a hyperplastic phenotype.^{247,248} Its decrease in NL skin suggests a subclinical activation state or pre-lesional remodeling that does not yet manifest visibly.

Within the category of metabolic genes, we observed significant upregulation of ALDH3B2, SLC2A1 (GLUT1), and YWHAZ (14-3-3 zeta) in LS epidermis (Fig. 1.8B, C). ALDH3B2 is involved in fatty aldehyde detoxification, indicating potential compensatory lipid processing under inflammatory stress.²⁴⁹ SLC2A1, a constitutively active glucose transporter, may reflect increased glucose demand during keratinocyte hyperproliferation or stress. It is known to be highly expressed in keratinocytes and activated T lymphocytes^{250,251} and has also been correlated with psoriasis severity, increased epidermal thickness, and inflammatory cell density.²⁵² YWHAZ, a multifunctional adaptor protein, is known to influence keratinocyte migration and negatively regulate apoptosis in cancer cells.^{253,254,255} It also has known roles in insulin signaling and melanogenesis, hinting at broader metabolic remodeling.²⁵⁶ Interestingly, several mitochondrial genes, particularly components of the

electron transport chain, including MT-NDs, were downregulated in LS skin (Fig. 1.8B). In contrast, MT-CYB and MT-ND₃ were upregulated in NL skin compared to HC, but decreased again in LS skin, suggesting dynamic mitochondrial regulation during disease progression. We also found decreased expression of ALDH₃A₂ and SERPINA₁₂ in LS skin, which may reflect downregulation of lipid oxidation and gluconeogenesis, respectively.^{257,258} Spatial expression of ALDH₃A₂ was especially reduced in leukocyte-rich regions, implying local metabolic suppression in inflamed microenvironments (Fig. 1.8C). DHCR₂₄ is known to protect cells from oxidative stress by reducing caspase 3 activity during apoptosis induced by oxidative stress.^{259,260} It was differentially expressed gene between LS and NL groups was found to be negatively correlated with lesion severity, suggesting a deficiency in the protection mechanism correlating with AD lesions (Fig. 1.8C, 4.4A).

In terms of immune-related genes, we observed significant upregulation of S100A family members, including S100A₈ and S100A₉, specifically in lesional skin (Fig. 1.8B, D). These calcium-binding proteins are part of the epidermal stress and inflammation response, and their expression is closely tied to disruptions in calcium gradients, which regulate keratinocyte differentiation.²⁶¹ SLURP₂, another gene involved in inhibiting apoptosis, was also upregulated in LS samples, further supporting a model of dysregulated keratinocyte turnover.²⁶² Additionally, NCCRP₁, a gene linked to cell proliferation, was significantly increased, reinforcing the transcriptomic evidence of hyperproliferation in the lesional epidermis (Fig. 1.8B).²⁶³

Classic Th₂/Th₂₂-associated cytokines such as IL₄, IL₁₃, and IL₂₂, were not detected as DEGs in the epidermis-specific analysis, likely because their expression is restricted to infiltrating immune cells in the dermis, beyond the resolution of our current segmentation.

However, localized inspection of a lesional biopsy with an area of leukocyte infiltration revealed low-level IL13 expression, along with stronger expression of its downstream effector gene, CCL17, in surrounding spots, consistent with previous literature (Fig. 1.8D).²⁶⁴ Similarly, CCL19 and its receptor CCR7, which regulate dendritic cell and T cell trafficking, were upregulated in the same region (Fig. 1.8D).²⁶⁵ We also observed expression of IL19, a cytokine classically associated with the IL-23/IL-17 axis in psoriasis, potentially suggesting an endotype with overlap between AD and psoriasis features.^{266,218,219}

4.3.5 IMAGING FEATURES CORRELATE WITH DEGs

To investigate the relationship between epidermal gene expression, clinical phenotype, and quantitative imaging features, we performed a correlation analysis using Spearman correlation coefficients across all AD subjects. The results were visualized as a heatmap, with genes grouped by functional categories: barrier/structural, immune response, and metabolic (Fig. 4.4A). We observed that many barrier and immune-related genes showed meaningful correlations with clinical severity scores, particularly with specific subcomponents of the composite severity scale. Notably, while a number of genes were correlated with overall severity, several genes showed stronger correlations with the erythema score specifically. Itch severity, as reported by patients, correlated strongly with a number of inflammatory and barrier-associated DEGs, suggesting that pruritus may be a strong predictor of underlying epidermal barrier dysfunction and inflammation. In contrast, correlations with the scratching/excoriation score were generally weaker across all gene categories.

Correlations between demographic traits and gene expression were generally weaker than those observed with clinical severity or imaging features (Fig. 4.4A). Though skin

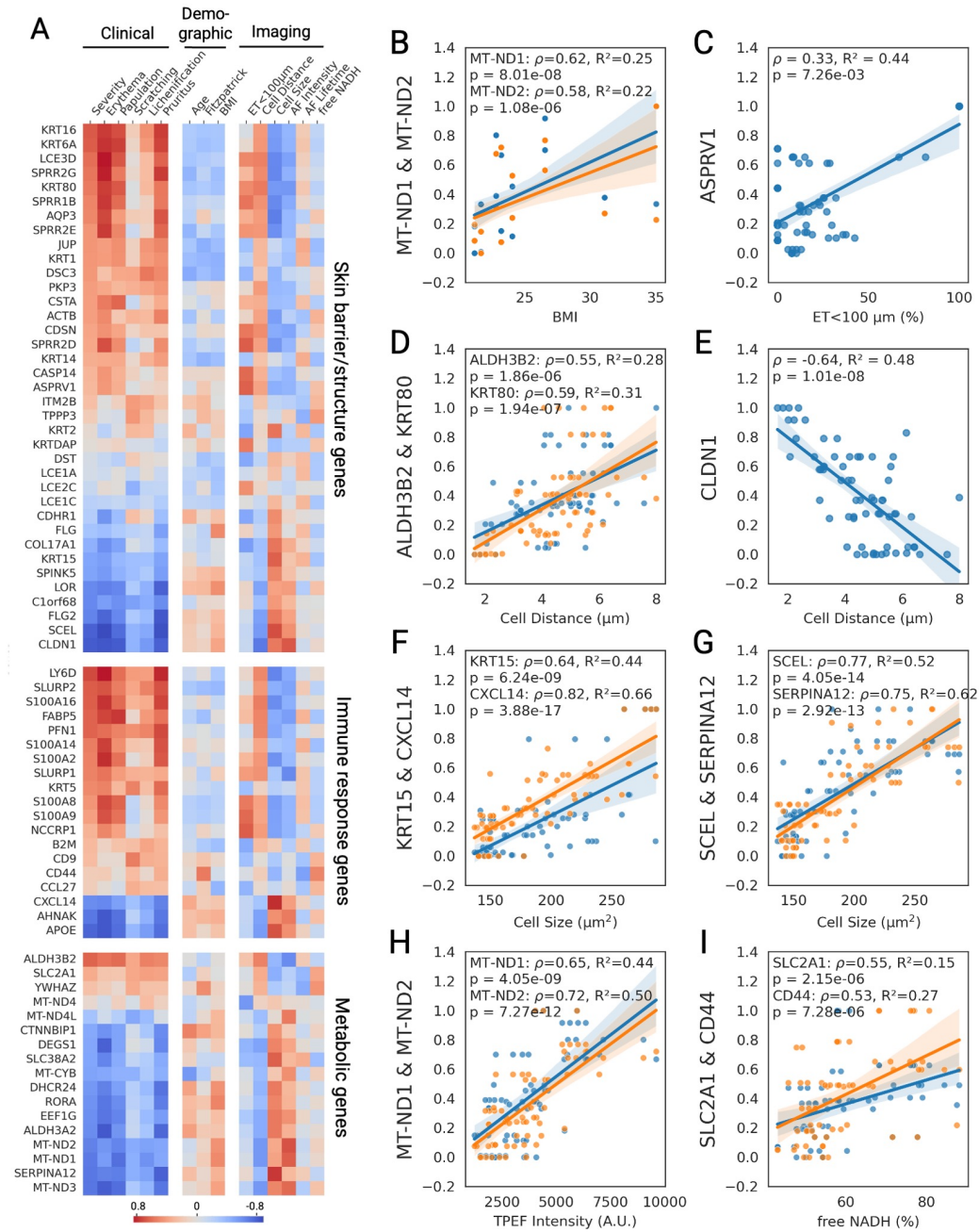


Figure 4.4: Correlations between epidermal DEG expression, demographic traits, and imaging measurements within NL and LS groups. (A) Heatmap of Spearman correlations between epidermal DEGs and clinical assessments, demographic traits, and quantitative imaging measurements. Colorbar represents Spearman coefficient. Scatter plots showing closer examinations of normalized gene expression and (B) BMI, (C) epidermal thickness, (D, E) distance between cells, (F, G) cell size, (H) TPEF intensity, and (I) free NADH. Spearman correlation coefficient (ρ), coefficient of determination (R^2), and p-values (p) are displayed for each correlation.

changes can occur with age, our inclusion criteria (18-40 years) and age range of recruited AD subjects (21-38 years) provided relatively consistent gene expression and skin structure. Similarly, Fitzpatrick skin type had minimal impact on the expression of the DEGs, most of which are unrelated to pigmentation, with the possible exception of YWHAZ, a gene with known roles in melanogenesis.²⁵⁶ However, our AD cohort only included Fitzpatrick skin types II and III, which limits our correlation analysis. BMI, which might be expected to influence expression of metabolic genes, did show moderate positive correlations with several genes, including RORA, MT-ND1, and MT-ND2. RORA (Fig. 4.4A, B), a transcription factor associated with immune regulation and metabolic activity, is known to be upregulated in adipose tissue macrophages in the context of obesity.^{267,268} The MT-ND genes encode components of complex I of the mitochondrial electron transport chain (ETC).^{269,270} Interestingly, the positive correlation between BMI and MT-ND expression in our dataset suggests that oxidative metabolism may be upregulated at baseline in normal-to-overweight individuals, even though oxidative phosphorylation is typically impaired in obesity-associated metabolic dysfunction.

We identified strong associations between quantitative microscopy-derived imaging features and gene expression (Fig. 4.4A). A particularly notable correlation involved our imaging-based proxy for epidermal thickness, defined as the percentage of the imaged region with epidermis confined to $<100\ \mu\text{m}$ in depth. This measure was negatively correlated with ASPRV1 expression, meaning that ASPRV1 was more highly expressed in samples with thinner epidermis (Fig. 4.4A, C). ASPRV1 encodes SASPase, a protease critical for the processing of proflaggrin into flaggrin, a key component of the epidermal barrier. This finding corroborates previous studies showing that SASPase and flaggrin are both down-

regulated in AD, particularly in thickened, hyperplastic epidermis.^{271,272}

We also examined the correlation between intercellular distance (a marker of epidermal edema) and gene expression. ALDH3B2 and KRT80 were both positively correlated with increased cell spacing (Fig. 4.4A, D), suggesting that these genes may be associated with barrier dysfunction and inflammation, even if not directly causative of edema. ALDH3B2 plays a role in lipid aldehyde detoxification,²⁴⁹ and KRT80 is an intermediate filament protein expressed in keratinocytes,²⁷³ though neither is known to regulate extracellular fluid dynamics. In contrast, CLDN1, which encodes claudin-1, a core tight junction protein, was negatively correlated with cell spacing (Fig. 4.4A, E), consistent with its well-established role in regulating paracellular permeability.^{274,275} Downregulation of CLDN1 compromises the skin barrier and promotes inflammatory cell infiltration and intercellular fluid accumulation, resulting in increased edema and greater spacing between keratinocytes—phenotypes well captured by our microscopy measurements.

Keratinocyte size was another imaging-derived metric that showed meaningful correlations with gene expression. Genes including KRT15, CXCL14, SCEL, and SERPINA12 were all positively correlated with average cell size in the SG and SS (Fig. 4.4A, F, G), which is more closely aligned with the nonlesional phenotype. KRT15, as discussed previously, is a basal keratin not associated with activation, and its loss in AD lesions is part of the hyperproliferative phenotype. CXCL14, a chemokine involved in immune surveillance and keratinocyte migration, is known to be expressed in healthy epidermis and may play a role in maintaining normal cell architecture.^{276,277,278} SCEL (scellin) is a structural protein that stabilizes the cornified envelope,^{279,280} and SERPINA12, a serine protease inhibitor with metabolic functions, has also been implicated in epidermal differentiation and may con-

tribute to maintaining cellular morphology.²⁵⁸

Correlations between metabolic imaging features and gene expression were among the most biologically consistent. TPEF intensity, which reflects the size of the NADH pool and overall metabolic activity, was strongly positively correlated ($\rho > 0.5$) with several genes involved in oxidative phosphorylation, particularly MT-ND1 and MT-ND2 (Fig. 4.4A, H). These are core components of mitochondrial complex I and are critical for electron transport chain function.^{269,270} Moderate positive correlations were also observed with other mitochondrial genes including MT-ND3, MT-ND4L, and MT-CYB, indicating that TPEF intensity is a reliable optical marker of ETC activity in keratinocytes (Fig. 4.4A). The level of free NADH, a proxy for glycolytic shift, showed strong positive correlations with SLC2A1 (GLUT1) and CD44 (Fig. 4.4A, I). The former is a glucose transporter that facilitates glycolytic metabolism,^{250,251} while the latter is a cell surface receptor involved in multiple signaling pathways, including those regulating metabolic reprogramming in epithelial cells. CD44 has been shown to promote glycolysis in cancer cells^{281,282} and may play a similar role in keratinocyte metabolism in AD. The positive association between CD44 expression and free NADH levels further supports the presence of a metabolic shift toward glycolysis in lesional epidermis, as observed in our FLIM data.

Together, these findings highlight the rich interplay between structural and metabolic features captured by *in vivo* microscopy and underlying gene expression in the epidermis. Correlations between DEGs and imaging measurements validate the utility of microscopy-derived features as noninvasive biomarkers of disease state, and suggest that changes in gene expression—particularly in barrier integrity, immune response, and metabolic pathways—manifest in quantifiable alterations in tissue structure and function. These relationships

underscore the potential for integrating imaging and transcriptomic data to develop a more comprehensive understanding of AD pathophysiology and to identify molecular drivers of key phenotypes observed *in vivo*.

4.4 CONCLUSION AND FUTURE STEPS

In this study, so far, we have demonstrated how *in vivo* multiphoton microscopy, combined with spatial transcriptomics, can provide a detailed, multimodal understanding of the epidermal landscape in AD. Through high-resolution imaging of healthy, nonlesional, and lesional skin, we identified distinct structural and metabolic features that correlate with both clinical severity and molecular changes in the tissue. These features—such as epidermal thickening, altered keratinocyte morphology, intercellular spacing, and NADH-based metabolic shifts—reflect the underlying pathological processes that define the progression from healthy to diseased skin.

Multiphoton imaging revealed that lesional skin exhibits hallmark structural abnormalities, including increased intercellular spacing due to edema and a loss of normal epidermal stratification, with these alterations becoming more pronounced with increasing disease severity. Fluorescence lifetime measurements further revealed that metabolic reprogramming is already evident in mild AD, with a shift toward glycolysis and accumulation of free NADH, suggesting early-stage mitochondrial dysfunction.

Spatial transcriptomic analysis of the epidermis corroborated these findings at the molecular level. We identified 88 unique differentially expressed genes (DEGs) across disease states, encompassing genes involved in barrier formation, immune response, and metabolism. Lesional skin showed increased expression of hyperproliferation- and stress-associated

genes, such as SPRRs, KRT6/16, AQP3, and S100A family members, alongside downregulation of key barrier genes including FLG, FLG2, LOR, and CLDN1. Importantly, several skin barrier and immune-related genes exhibited altered expression even in nonlesional skin, supporting the concept of subclinical disease in uninvolved areas of AD patients.

By correlating gene expression with both clinical assessments and quantitative imaging features, we uncovered biologically meaningful relationships, particularly in cell metabolism. These findings validate the relevance of imaging-based biomarkers and emphasize the close coupling between molecular dysfunction and cellular- and tissue-level phenotypes in AD. By linking structural and functional imaging features with transcriptional changes in the epidermis, we gain new insights into barrier dysfunction, metabolic reprogramming, and inflammation in AD. This work brings us closer to leveraging *in vivo* microscopy for the development of image-based biomarkers capable of real-time functional assessment of skin disease. Such advancements have important implications for noninvasive monitoring of disease progression in clinical trials, enhancing diagnostic precision in clinical practice, and informing more personalized treatment strategies for patients with skin disorders.

As a next step in the transcriptomic analysis, we plan to perform single-cell deconvolution of the spatial transcriptomics data to better resolve the cell type-specific transcriptional programs within the epidermis and underlying dermis. This will be followed by pathway enrichment analysis and ligand-receptor interaction modeling, allowing us to identify not only which genes are differentially expressed, but also which biological pathways are activated or suppressed across different regions and disease states. By correlating these pathway-level insights with quantitative imaging features, we aim to expand our understanding beyond the current DEG-based analysis and determine how specific signaling and metabolic

pathways align with structural and functional imaging readouts. In particular, this will help clarify which metabolic pathways are driving the changes observed with FLIM, especially in mild versus severe AD, and how these metabolic shifts relate to disease progression. Furthermore, mapping inflammatory pathway activation and determining which cell types are initiating, responding to, or regulating immune signaling within spatially defined regions will offer a more complete picture of tissue-level dynamics in AD. These integrative analyses will provide essential insight into the functional relevance of imaging biomarkers, and guide the future development of noninvasive, real-time assessments of skin disease activity.

Additionally, we aim to develop a deep learning-based framework capable of predicting disease severity and the enrichment of specific inflammatory pathways directly from *in vivo* microscopy images. As an initial step, we have trained 2D and 3D convolutional neural networks (CNNs) to classify samples by disease state (healthy control, nonlesional AD, lesional AD) and will do so for clinical severity scores. Building on this, and informed by our spatial transcriptomic findings on AD endotypes and pathway enrichment, we plan to extend the model to predict the expression levels of individual biomarkers or clusters of genes associated with specific inflammatory axes (e.g., Th2, Th17, Th22). By learning complex, high-dimensional representations of the imaging data, CNNs may capture latent features that reflect underlying biological processes not fully captured by our current quantitative metrics of structure or metabolism. These models have the potential to uncover subtle morphological or functional patterns that correlate with immune activation and tissue remodeling. Furthermore, we have already acquired multiphoton imaging and matched biopsy data from cohorts of patients with psoriasis and vitiligo, and we plan to apply similar integrative analyses to those datasets. This will allow us to explore the generalizability

of imaging-transcriptomic relationships across inflammatory skin diseases, ultimately advancing the use of noninvasive optical imaging as a tool for mechanistic investigation and clinical decision support in dermatology.

5

Conclusion

This thesis has explored how scientific and analytical advancements in chemical microscopy have real-world applications ranging from structured, controlled systems to complex, living environments. Across three thematically related projects, this work has addressed the diverse challenges that arise when applying chemically specific optical imaging to pharmaceuticals, cryobiology, and dermatology. The unifying goal has been to optimize and adapt

advanced imaging tools—not only for scientific discovery but also for translational impact. Together, these studies demonstrate how chemical microscopy, when coupled with tailored analytical techniques such as spectral decomposition and machine learning, can evolve into a versatile platform capable of informing clinicians and researchers in healthcare, biotechnology, and pharmaceutical sciences.

In Chapter 2, we developed a rapid and quantitative method to characterize chemical changes in pharmaceutical tablets using SRS microscopy. This project addressed the need for nondestructive, high-throughput, inline quality control in pharmaceutical manufacturing, offering a label-free technique to assess active ingredient distribution and degradation. By applying fast spectral tuning and spectral unmixing, we were able to map compositional changes at less than one minute per 0.25 sq mm area and at submicron resolution, showing the potential for SRS to replace slower, labor-intensive methods such as NIR and Raman spectroscopy. This work highlights how complex imaging methods can be particularly effective in structured, static samples when paired with rigorous quantitative analysis.

In Chapter 3, we extended chemical microscopy to biological systems, quantifying cryoprotective agent uptake in live aquatic organisms. Here, the challenge was to adapt a high-resolution, chemically-specific imaging tool to a dynamic, living sample, while obtaining absolute concentration measurements. Using NoRI, we were able to measure intracellular concentrations of cryoprotectants and assess their distribution across *Daphnia* organisms. In the future, correlating these measurements with toxicity data may help improve cryopreservation protocols, which have applications in preserving transgenic stocks, assisting in reproduction, and preserving organs for transplantation. This chapter emphasized the need for spatially resolved chemical data in biological contexts and underscored the adaptability

of SRS when properly optimized.

In Chapter 4, we pushed chemical microscopy to its most complex application—*in vivo* human imaging—and pioneered its integration with spatial transcriptomics. Using multiphoton imaging, including SHG, TPEF, and FLIM, we identified structural and metabolic features of skin in healthy and diseased states. These features were associated with disease severity and correlated with gene expression patterns in the epidermis. We emphasized how barrier and metabolic gene expression correlated with noninvasive measurements of epidermal thickness ($ET < 100\ \mu\text{m}$), edema (cell distance), disordered keratinocyte maturation (cell size), and overall shifts in metabolic activity (TPEF intensity, fluorescence lifetime, and fraction of free NADH). The use of machine learning for cell segmentation and image denoising allowed us to bridge the gap between *in vivo* optical imaging and molecular data. This work represents a step toward real-time, noninvasive, and non-destructive clinical assessment of disease and paves the way for future tools in personalized medicine.

Across all three chapters, a central theme emerges: highly detailed, complex imaging techniques excel in controlled environments, but advanced microscopy coupled with ML and analytical advancements could serve a persistent need in living systems and clinical settings. Each project used a combination of modern imaging techniques to collect a more complete dataset describing the scientific or clinical problem space. Combined with advanced analysis methods, e.g., spectral unmixing, matrix decomposition, denoising, and/or segmentation, we were able to convert this abundant raw data into meaningful information that may be used to improve medical research and patient outcomes.

Each project offers promising future directions. The pharmaceutical application could evolve into real-time, in-line monitoring tools for manufacturing. In fact, our group is now

collaborating with a pharmaceutical equipment company and a pharmaceutical manufacturer to develop an automated system for SRS monitoring. The cryopreservation study sets the stage for optimization of protocols across species and organ types, potentially extending to clinical cryobiology. Our collaborators are currently conducting survival assays to corroborate our CPA quantification data and further the work with spheroids a subsequent next model. Lastly, our *in vivo* dermatology work lays the foundation for noninvasive, image-guided disease monitoring and the development of machine learning models for predicting molecular phenotypes from standalone microscopy images. The next steps for this work are to further analyze the ST data and explore the psoriasis and vitiligo cohort results.

Underlying all of this work is a personal motivation rooted in my training and goals as a physician-scientist. My deep interest in improving patient care with novel technologies has driven my belief that optical imaging methods—when thoughtfully applied and rigorously analyzed—can transform how we approach pharmaceutical development, biological preservation, and clinical care. Whether we are ensuring the quality of life-saving medications, improving the preservation of biological materials for transplantation and research, or enabling real-time, personalized decisions in clinical dermatology, the future of medicine will increasingly rely on technologies like those explored in this thesis.



Supplementary Material

A.1 QUANTITATIVE ANALYSIS OF DRUG TABLET AGING BY FAST HYPERSPECTRAL SRS
MICROSCOPY

Raman Band (cm^{-1})	Probable molecular assignment
1350	background
1360	background
1394	naproxen CH_2 bending
1420	background
1440	background
2700	background
2800	background
2846	HPMC CH_2 asymmetric stretching
2856	Moist CH_2 stretching
2880	HPMC CH stretching
2892	lactose CH_2 stretching, HPMC CH_2 stretching, Moist CH_2 stretching
2894	lactose CH_2 stretching, HPMC CH_2 stretching, Moist CH_2 stretching
2896	lactose CH_2 stretching, HPMC CH_2 stretching, Moist CH_2 stretching
2922	lactose CH stretching, HPMC CH_2 symmetric stretching
2946	naproxen CH_2 stretching, HPMC CH_2 symmetric stretching
2954	naproxen CH_2 stretching, HPMC CH_2 symmetric stretching
2980	lactose CH stretching
3050	background
3080	naproxen aromatic CH stretching
3098	background

Table A.1: Probable molecular structure assignments for each of 20 manually selected sparse wavenumbers.

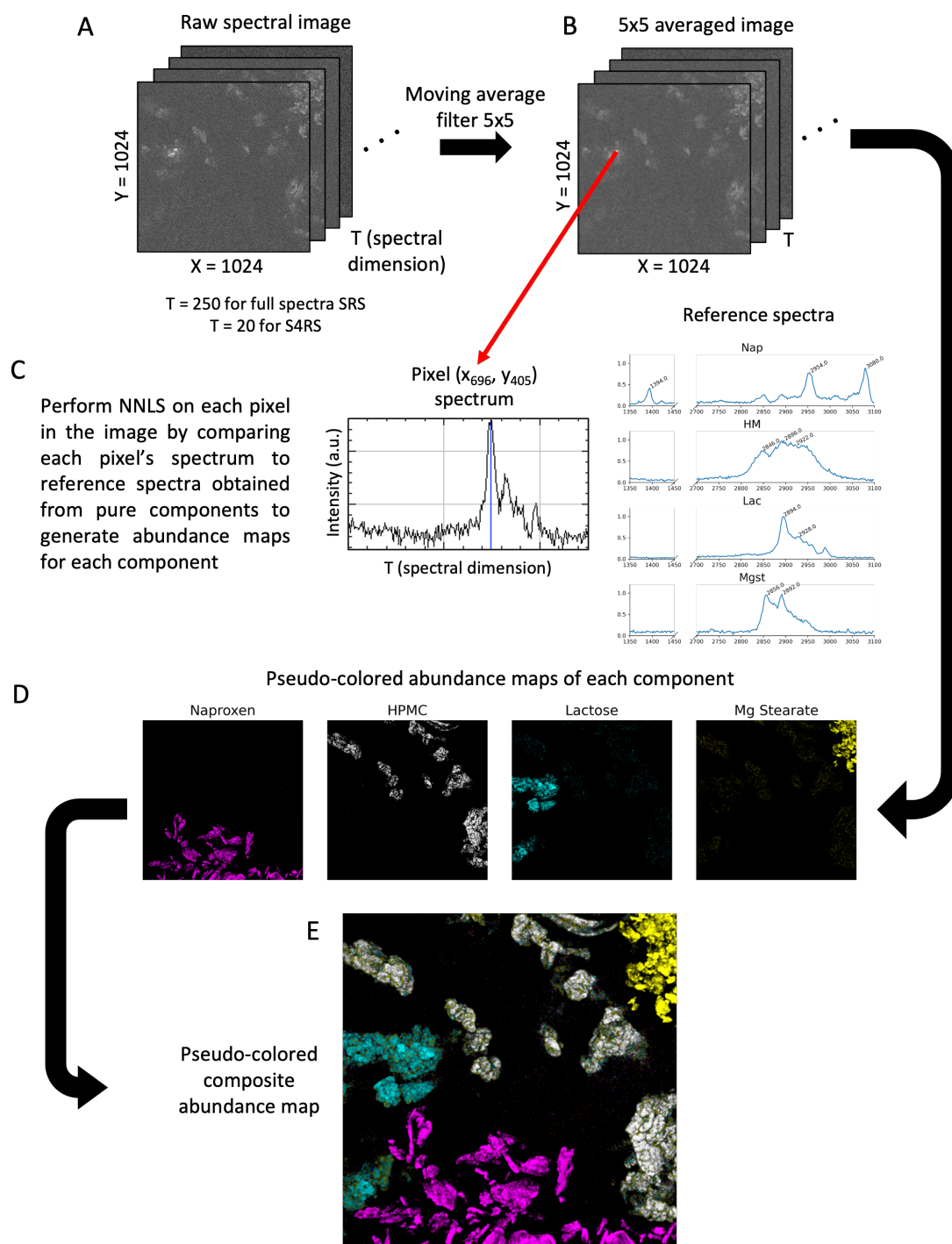


Figure A.1: Spectral unmixing methodology, demonstrated with a known spatial mix of API and excipient powders.

A.2 SRS MICROSCOPY ENABLES QUANTIFICATION OF CRYOPROTECTIVE AGENT IN *DAPH-*
NIA MAGNA

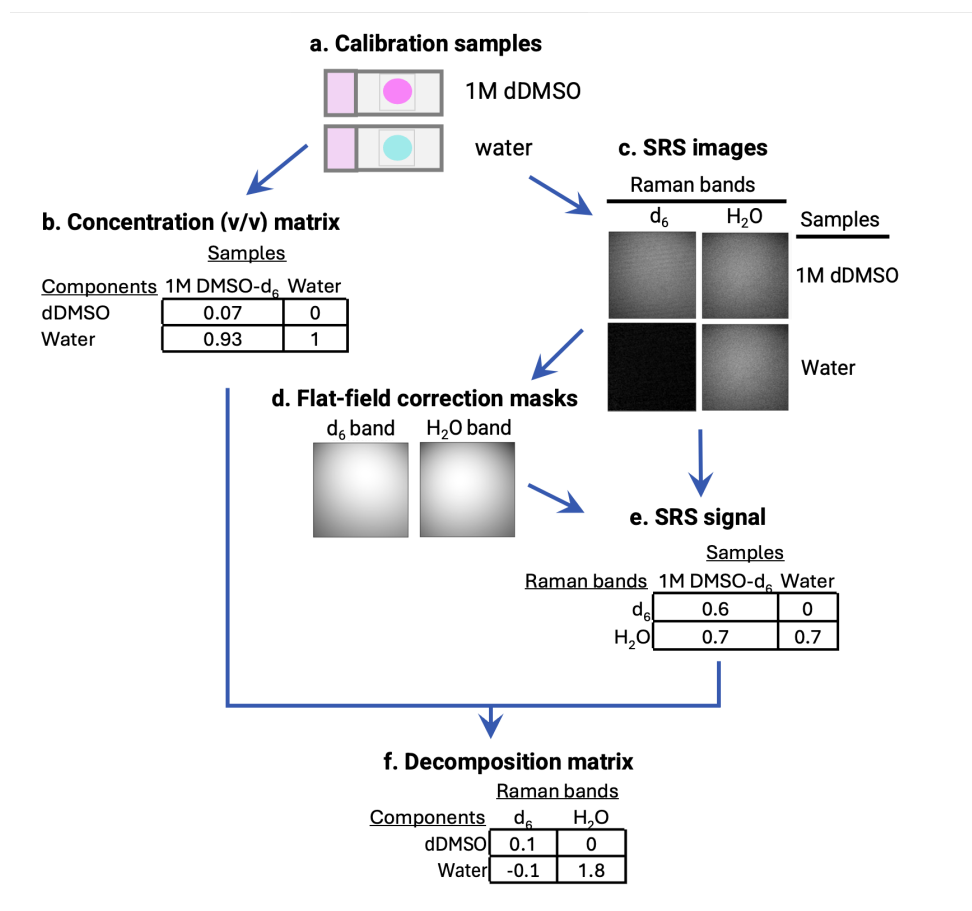


Figure A.2: Flow chart of calibration steps.

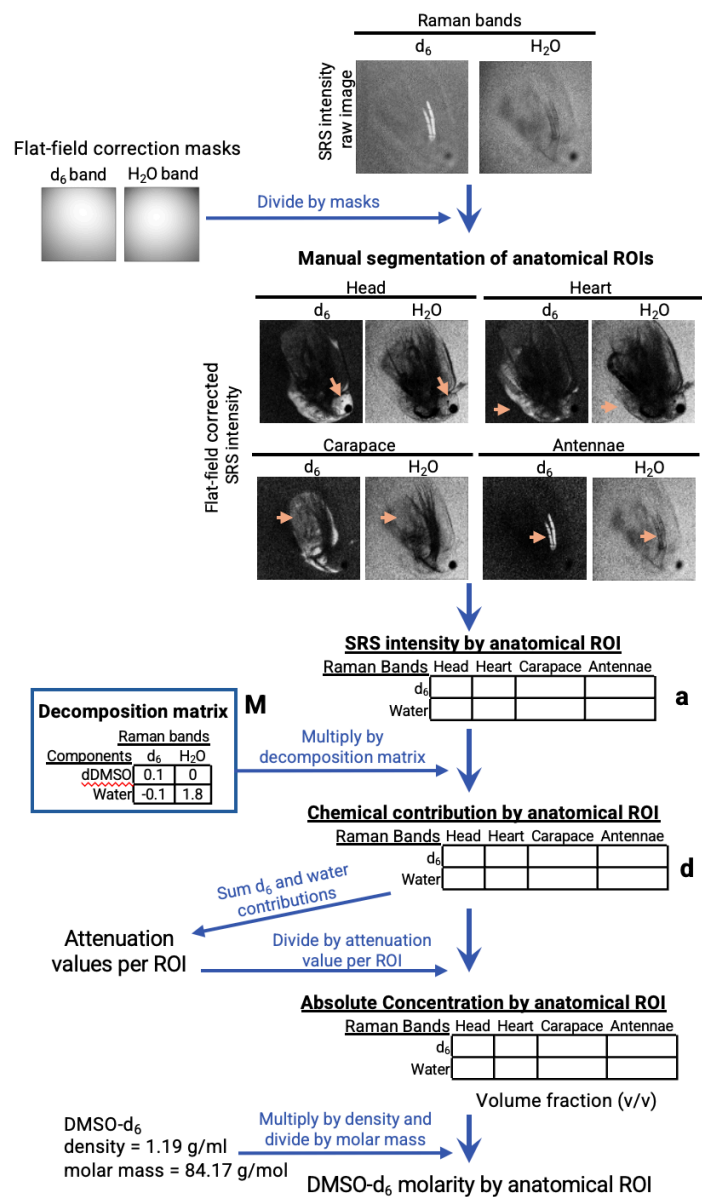


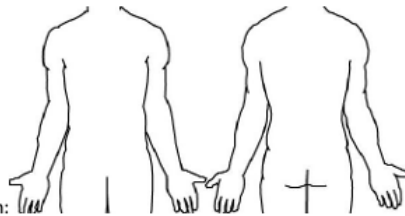
Figure A.3: Flow chart of sample data processing steps.

A.3 CONNECTING *IN VIVO* MICROSCOPY FEATURES AND RNA EXPRESSION IN ATOPIC DERMATITIS

Local/Lesional Eczema Severity (LES)

Complete one form for each lesion of interest

Lesion 1



Mark location of lesion:

Table 1: Local Lesion Scoring Table		
Eczema characteristics	Rating Scores	
		Score
Erythema/Redness (E)	0 = none	
	1 = slight	
Edema/ Papulation (T)	2 = moderate	
	3 = severe	
Scratching/ Excoriation (S)		
Lichenification (L)		
LES score (E+T+S+L)=		

Patient-reported pruritus:

0 = no itching

1 = occasional, slight itching

2 = constant or intermittent itching with no sleep disturbance

3 = bothersome itching that disturbs sleep or normal activity

Size of Lesion(must be 25mm x 25mm):

Figure A.4: Local/Lesional Eczema severity scoring guide.

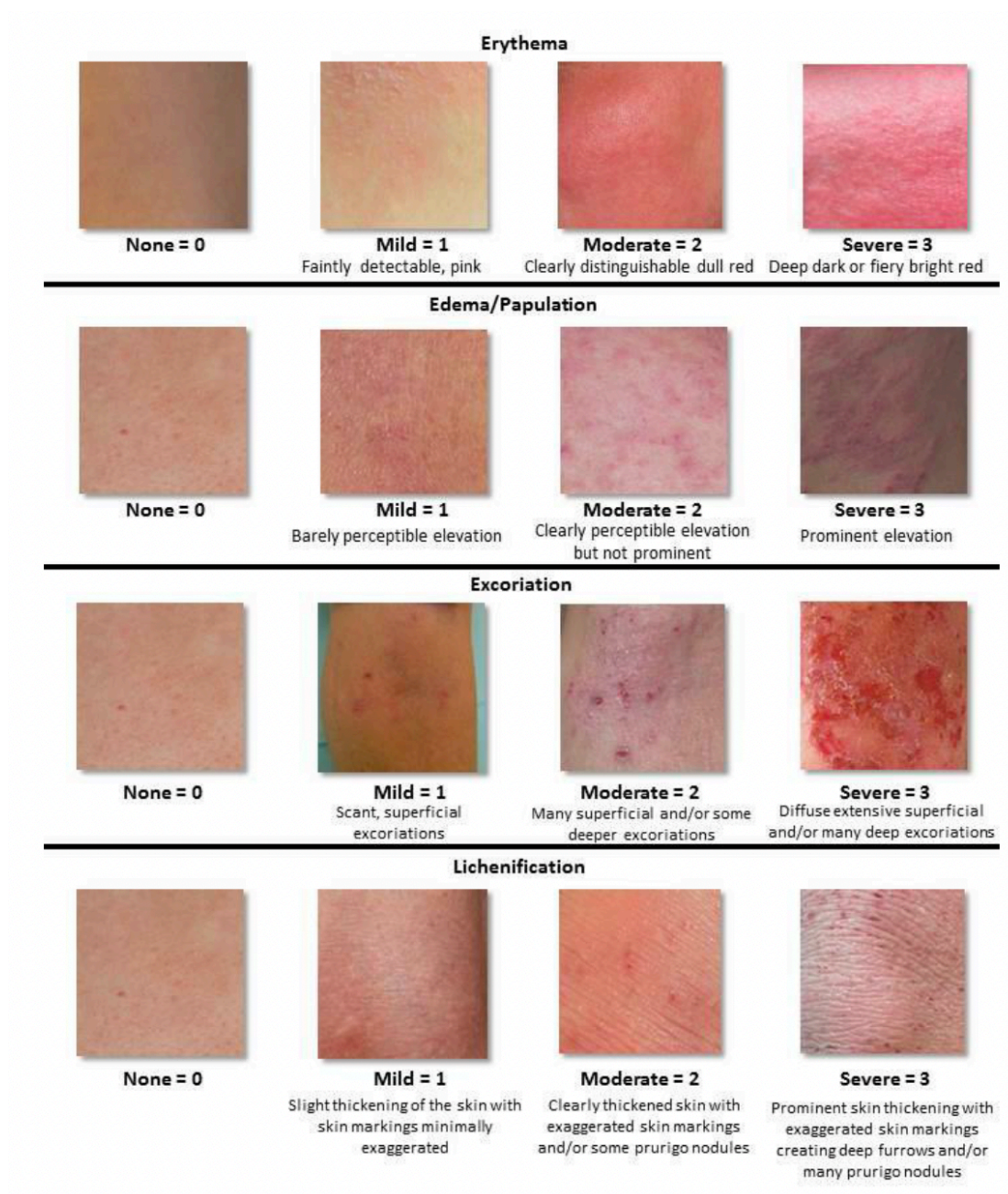


Figure A.5: Images for standardized scoring of eczema characteristics by physicians.

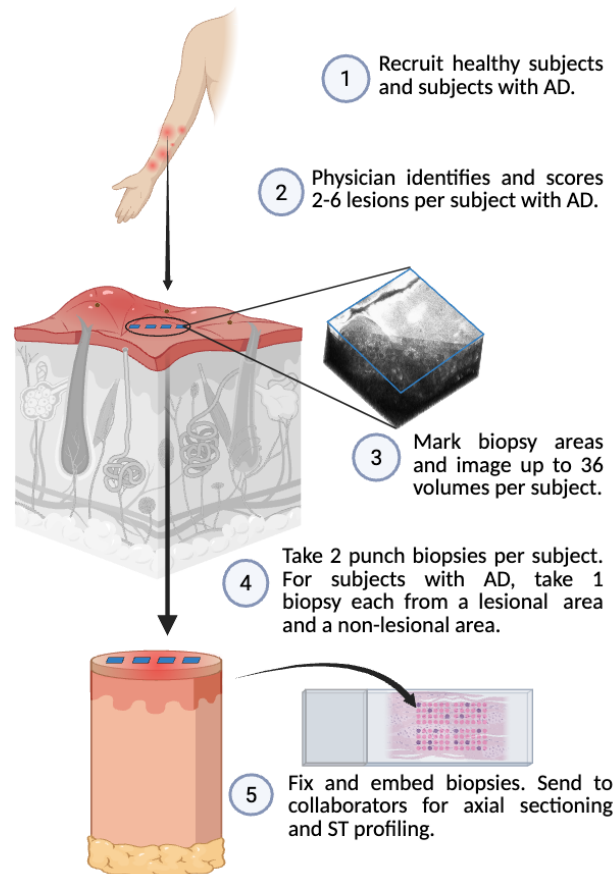


Figure A.6: Local Eczema Scoring (LES) sheet used in clinical AD assessments

References

- [1] I. Proteintech Group, “IF imaging: Widefield versus confocal microscopy.” <https://www.ptglab.com/news/blog/if-imaging-widefield-versus-confocal-microscopy/>.
- [2] I. Lboukili, G. Stamatias, and X. Descombes, “Automating reflectance confocal microscopy image analysis for dermatological research: A review,” *Journal of Biomedical Optics*, vol. 27, no. 07, 2022.
- [3] S. N. Murthy, *Topical Products and Dermal Drug Delivery*. 2025.
- [4] L. Shi, L. Lu, G. Harvey, T. Harvey, A. Rodríguez-Contreras, and R. R. Alfano, “Label-Free Fluorescence Spectroscopy for Detecting Key Biomolecules in Brain Tissue from a Mouse Model of Alzheimer’s Disease,” *Scientific Reports*, vol. 7, no. 1, p. 2599, 2017.
- [5] A.-M. Pena, X. Chen, I. J. Pence, T. Bornschlöggl, S. Jeong, S. Grégoire, G. S. Luenigo, P. Hallegot, P. Obeidy, A. Feizpour, K. F. Chan, and C. L. Evans, “Imaging and quantifying drug delivery in skin – Part 2: Fluorescence and vibrational spectroscopic imaging methods,” *Advanced Drug Delivery Reviews*, vol. 153, pp. 147–168, 2020.
- [6] S. Jeong, D. A. Greenfield, M. Hermsmeier, A. Yamamoto, X. Chen, K. F. Chan, and C. L. Evans, “Time-resolved fluorescence microscopy with phasor analysis for visualizing multicomponent topical drug distribution within human skin,” *Scientific Reports*, vol. 10, no. 1, p. 5360, 2020.
- [7] K. König, H. G. Breunig, A. Batista, A. Schindele, M. Zieger, and M. Kaatz, “Translation of two-photon microscopy to the clinic: Multimodal multiphoton CARS tomography of in vivo human skin,” *Journal of Biomedical Optics*, vol. 25, no. 01, 2020.

- [8] O. Nadiarnykh, R. B. LaComb, M. A. Brewer, and P. J. Campagnola, "Alterations of the extracellular matrix in ovarian cancer studied by Second Harmonic Generation imaging microscopy," *BMC Cancer*, vol. 10, no. 1, p. 94, 2010.
- [9] R. LaComb, O. Nadiarnykh, and P. J. Campagnola, "Quantitative Second Harmonic Generation Imaging of the Diseased State Osteogenesis Imperfecta: Experiment and Simulation," *Biophysical Journal*, vol. 94, no. 11, pp. 4504–4514, 2008.
- [10] F. Hu, L. Shi, and W. Min, "Biological imaging of chemical bonds by stimulated Raman scattering microscopy," *Nature Methods*, vol. 16, no. 9, pp. 830–842, 2019.
- [11] L. Moses and L. Pachter, "Museum of spatial transcriptomics," *Nature Methods*, vol. 19, no. 5, pp. 534–546, 2022.
- [12] A. Drewek-Ossowicka, M. Pietrolaj, and J. Rumiński, "A survey of neural networks usage for intrusion detection systems," *Journal of Ambient Intelligence and Humanized Computing*, vol. 12, no. 1, pp. 497–514, 2021.
- [13] V. H. Phung and E. J. Rhee, "A Deep Learning Approach for Classification of Cloud Image Patches on Small Datasets," vol. 16, no. 3, pp. 173–178, 2018.
- [14] O. Ronneberger, P. Fischer, and T. Brox, "U-Net: Convolutional Networks for Biomedical Image Segmentation," in *Medical Image Computing and Computer-Assisted Intervention – MICCAI 2015* (N. Navab, J. Hornegger, W. M. Wells, and A. F. Frangi, eds.), (Cham), pp. 234–241, Springer International Publishing, 2015.
- [15] M. Weigert, U. Schmidt, T. Boothe, A. Müller, A. Dibrov, A. Jain, B. Wilhelm, D. Schmidt, C. Broaddus, S. Culley, M. Rocha-Martins, F. Segovia-Miranda, C. Norden, R. Henriques, M. Zerial, M. Solimena, J. Rink, P. Tomancak, L. Royer, F. Jug, and E. W. Myers, "Content-aware image restoration: Pushing the limits of fluorescence microscopy," *Nature Methods*, vol. 15, no. 12, pp. 1090–1097, 2018.
- [16] C. Stringer, T. Wang, M. Michaelos, and M. Pachitariu, "Cellpose: A generalist algorithm for cellular segmentation," *Nature Methods*, vol. 18, no. 1, pp. 100–106, 2021.
- [17] H. Carruthers, D. Clark, F. Clarke, K. Faulds, and D. Graham, "Comparison of Raman and Near-Infrared Chemical Mapping for the Analysis of Pharmaceutical Tablets," *Applied Spectroscopy*, vol. 75, no. 2, pp. 178–188, 2021.

- [18] H. Carruthers, D. Clark, F. C. Clarke, K. Faulds, and D. Graham, "Evaluation of laser direct infrared imaging for rapid analysis of pharmaceutical tablets," *Analytical Methods: Advancing Methods and Applications*, vol. 14, no. 19, pp. 1862–1871, 2022.
- [19] P. Y. Sacré, M. Alaoui Mansouri, C. De Bleye, L. Coïc, Ph. Hubert, and E. Ziemons, "Evaluation of distributional homogeneity of pharmaceutical formulation using laser direct infrared imaging," *International Journal of Pharmaceutics*, vol. 612, p. 121373, 2022.
- [20] A. V. Ewing and S. G. Kazarian, "Recent advances in the applications of vibrational spectroscopic imaging and mapping to pharmaceutical formulations," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 197, pp. 10–29, 2018.
- [21] I. J. Pence, B. A. Kuzma, M. Brinkmann, T. Hellwig, and C. L. Evans, "Multi-window sparse spectral sampling stimulated Raman scattering microscopy," *Biomedical Optics Express*, vol. 12, no. 10, pp. 6095–6114, 2021.
- [22] D. Ebert, "Introduction to Daphnia Biology," in *Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]*, National Center for Biotechnology Information (US), 2005.
- [23] R. Fox, "Invertebrate Anatomy OnLine: Daphnia magna." <https://lanwebs.lander.edu/faculty/rsfox/invertebrates/daphnia.html>, 2006.
- [24] L. Rima, C. Berchtold, S. Arnold, A. Fränkl, R. Sütterlin, G. Dernick, G. Schlöterbeck, and T. Braun, "Single and few cell analysis for correlative light microscopy, metabolomics, and targeted proteomics," *Lab on a Chip*, vol. 24, no. 18, pp. 4321–4332, 2024.
- [25] O. Chuchuen, M. H. Henderson, C. Sykes, M. S. Kim, A. D. M. Kashuba, and D. F. Katz, "Quantitative Analysis of Microbicide Concentrations in Fluids, Gels and Tissues Using Confocal Raman Spectroscopy," *PLOS ONE*, vol. 8, no. 12, p. e85124, 2013.
- [26] T. Shimada, D. Kawakami, A. Fujita, R. Yamamoto, S. Hara, K. Ito, I. Mizushima, S. Kitajima, Y. Iwata, N. Sakai, M. Kawano, T. Wada, and Y. Sai, "Validation of an automated sample preparation module directly connected to LC-MS/MS (CLAM-LC-MS/MS system) and comparison with conventional immunoassays for quantitation of tacrolimus and cyclosporin A in a clinical setting," *Journal of Pharmaceutical Health Care and Sciences*, vol. 10, no. 1, p. 5, 2024.

- [27] M. W. Davidson and M. Abramowitz, "Optical Microscopy," in *Encyclopedia of Imaging Science and Technology*, John Wiley & Sons, Ltd, 2002.
- [28] K. S. Suvarna, C. Layton, and J. D. Bancroft, *Bancroft's Theory and Practice of Histological Techniques E-Book*. Elsevier Health Sciences, 2018.
- [29] M. C. Tanzi, S. Farè, and G. Candiani, *Foundations of Biomaterials Engineering*. Academic Press, 2019.
- [30] R. Franco-Duarte, L. Černáková, S. Kadam, K. S. Kaushik, B. Salehi, A. Bevilacqua, M. R. Corbo, H. Antolak, K. Dybka-Stępień, M. Leszczewicz, S. Relison Tintino, V. C. Alexandrino de Souza, J. Sharifi-Rad, H. D. Melo Coutinho, N. Martins, and C. F. Rodrigues, "Advances in Chemical and Biological Methods to Identify Microorganisms—From Past to Present," *Microorganisms*, vol. 7, no. 5, p. 130, 2019.
- [31] A. H. Bennet, H. Jupnik, H. Osterberg, O. W. Richards, and A. V. Baez, "Phase Microscopy Principles and Applications," *American Journal of Physics*, vol. 20, no. 5, pp. 318–319, 1952.
- [32] W. Lang, "Nomarski differential interference-contrast microscopy," 1968.
- [33] P. F. Gao, G. Lei, and C. Z. Huang, "Dark-Field Microscopy: Recent Advances in Accurate Analysis and Emerging Applications," *Analytical Chemistry*, vol. 93, no. 11, pp. 4707–4726, 2021.
- [34] M. Wolman, "Polarized light microscopy as a tool of diagnostic pathology,," *Journal of Histochemistry & Cytochemistry*, vol. 23, no. 1, pp. 21–50, 1975.
- [35] M. Koike-Tani, T. Tani, S. B. Mehta, A. Verma, and R. Oldenbourg, "Polarized light microscopy in reproductive and developmental biology," *Molecular Reproduction and Development*, vol. 82, no. 7-8, pp. 548–562, 2015.
- [36] P. Davidovits and M. D. Egger, "Scanning Laser Microscope," *Nature*, vol. 223, no. 5208, pp. 831–831, 1969.
- [37] A. Wheeler, "Confocal Microscopy," in *Immunohistochemistry and Immunocytochemistry*, ch. 7, pp. 169–198, John Wiley & Sons, Ltd, 2017.
- [38] E. C. Jensen, "Overview of Live-Cell Imaging: Requirements and Methods Used," *The Anatomical Record*, vol. 296, no. 1, pp. 1–8, 2013.

- [39] H.-J. Jordan, M. Wegner, and H. Tiziani, “Highly accurate non-contact characterization of engineering surfaces using confocal microscopy,” *Measurement Science and Technology*, vol. 9, no. 7, pp. 1142–1151, 1998.
- [40] A. Levine and O. Markowitz, “Introduction to reflectance confocal microscopy and its use in clinical practice,” *JAAD Case Reports*, vol. 4, no. 10, pp. 1014–1023, 2018.
- [41] N. F. Braghiroli, S. Sugerik, L. A. R. de Freitas, M. Oliviero, and H. Rabinovitz, “The skin through reflectance confocal microscopy — Historical background, technical principles, and its correlation with histopathology,” *Anais Brasileiros de Dermatologia*, vol. 97, no. 6, pp. 697–703, 2022.
- [42] V. Ahlgrimm-Siess, M. Laimer, H. S. Rabinovitz, M. Oliviero, R. Hofmann-Wellenhof, A. A. Marghoob, and A. Scope, “Confocal Microscopy in Skin Cancer,” *Current Dermatology Reports*, vol. 7, no. 2, pp. 105–118, 2018.
- [43] F. Farnetani, A. Scope, R. P. Braun, S. Gonzalez, P. Guitera, J. Malvehy, M. Manfredini, A. A. Marghoob, E. Moscarella, M. Oliviero, S. Puig, H. S. Rabinovitz, I. Stanganelli, C. Longo, C. Malagoli, M. Vinceti, and G. Pellacani, “Skin Cancer Diagnosis With Reflectance Confocal Microscopy: Reproducibility of Feature Recognition and Accuracy of Diagnosis,” *JAMA Dermatology*, vol. 151, no. 10, pp. 1075–1080, 2015.
- [44] S. Borsari, R. Pampera, A. Lallas, A. Kyrgidis, E. Moscarella, E. Benati, M. Raucci, G. Pellacani, I. Zalaudek, G. Argenziano, and C. Longo, “Clinical Indications for Use of Reflectance Confocal Microscopy for Skin Cancer Diagnosis,” *JAMA Dermatology*, vol. 152, no. 10, pp. 1093–1098, 2016.
- [45] E. A. Csuka, S. C. Ward, C. Ekelem, D. A. Csuka, M. Ardigò, and N. A. Mesinkovska, “Reflectance Confocal Microscopy, Optical Coherence Tomography, and Multiphoton Microscopy in Inflammatory Skin Disease Diagnosis,” *Lasers in Surgery and Medicine*, vol. 53, no. 6, pp. 776–797, 2021.
- [46] S. Astner, S. González, and E. Gonzalez, “Noninvasive Evaluation of Allergic and Irritant Contact Dermatitis by In Vivo Reflectance Confocal Microscopy,” *DERM*, vol. 17, no. 4, pp. 182–191, 2006.
- [47] L. Hoogedoorn, M. Peppelman, P. van de Kerkhof, P. van Erp, and M. Gerritsen, “The value of in vivo reflectance confocal microscopy in the diagnosis and monitoring of inflammatory and infectious skin diseases: A systematic review,” *British Journal of Dermatology*, vol. 172, no. 5, pp. 1222–1248, 2015.

- [48] N. Shahriari, J. M. Grant-Kels, H. Rabinovitz, M. Oliviero, and A. Scope, “Reflectance confocal microscopy: Principles, basic terminology, clinical indications, limitations, and practical considerations,” *Journal of the American Academy of Dermatology*, vol. 84, no. 1, pp. 1–14, 2021.
- [49] J. W. Lichtman and J.-A. Conchello, “Fluorescence microscopy,” *Nature Methods*, vol. 2, no. 12, pp. 910–919, 2005.
- [50] A. K. Kenworthy, “Imaging Protein-Protein Interactions Using Fluorescence Resonance Energy Transfer Microscopy,” *Methods*, vol. 24, no. 3, pp. 289–296, 2001.
- [51] B. Wu, K. D. Piatkevich, T. Lionnet, R. H. Singer, and V. V. Verkhusha, “Modern fluorescent proteins and imaging technologies to study gene expression, nuclear localization, and dynamics,” *Current Opinion in Cell Biology*, vol. 23, no. 3, pp. 310–317, 2011.
- [52] J. M. Levsky and R. H. Singer, “Fluorescence in situ hybridization: Past, present and future,” *Journal of Cell Science*, vol. 116, no. 14, pp. 2833–2838, 2003.
- [53] C. Xia, H. P. Babcock, J. R. Moffitt, and X. Zhuang, “Multiplexed detection of RNA using MERFISH and branched DNA amplification,” *Scientific Reports*, vol. 9, no. 1, p. 7721, 2019.
- [54] T. H. Kim and M. J. Schnitzer, “Fluorescence imaging of large-scale neural ensemble dynamics,” *Cell*, vol. 185, no. 1, pp. 9–41, 2022.
- [55] M. J. García, A. Kamaid, and L. Malacrida, “Label-free fluorescence microscopy: Revisiting the opportunities with autofluorescent molecules and harmonic generations as biosensors and biomarkers for quantitative biology,” *Biophysical Reviews*, vol. 15, no. 4, pp. 709–719, 2023.
- [56] G. Vicidomini, P. Bianchini, and A. Diaspro, “STED super-resolved microscopy,” *Nature Methods*, vol. 15, no. 3, pp. 173–182, 2018.
- [57] S. W. Hell and J. Wichmann, “Breaking the diffraction resolution limit by stimulated emission: Stimulated-emission-depletion fluorescence microscopy,” *Optics Letters*, vol. 19, no. 11, pp. 780–782, 1994.
- [58] R. W. Boyd, *Nonlinear Optics*. San Diego: Academic Press is an imprint of Elsevier, 4 ed., 2019.

- [59] W. Denk, J. H. Strickler, and W. W. Webb, “Two-Photon Laser Scanning Fluorescence Microscopy,” *Science*, vol. 248, no. 4951, p. 73, 1990.
- [60] D. R. Miller, J. W. Jarrett, A. M. Hassan, and A. K. Dunn, “Deep tissue imaging with multiphoton fluorescence microscopy,” *Current Opinion in Biomedical Engineering*, vol. 4, pp. 32–39, 2017.
- [61] R. Datta, T. M. Heaster, J. T. Sharick, A. A. Gillette, and M. C. Skala, “Fluorescence lifetime imaging microscopy: Fundamentals and advances in instrumentation, analysis, and applications,” *Journal of Biomedical Optics*, vol. 25, no. 07, p. 1, 2020.
- [62] B. Torrado, B. Pannunzio, L. Malacrida, and M. A. Digman, “Fluorescence lifetime imaging microscopy,” *Nature Reviews Methods Primers*, vol. 4, no. 1, pp. 1–23, 2024.
- [63] S. Ranjit, L. Malacrida, D. M. Jameson, and E. Gratton, “Fit-free analysis of fluorescence lifetime imaging data using the phasor approach,” *Nature Protocols*, vol. 13, no. 9, pp. 1979–2004, 2018.
- [64] K. König, “Review: Clinical in vivo multiphoton FLIM tomography,” *Methods and Applications in Fluorescence*, vol. 8, no. 3, 2020.
- [65] M. C. Skala, K. M. Riching, D. K. Bird, A. Gendron-Fitzpatrick, J. Eickhoff, K. W. Eliceiri, P. J. Keely, and N. Ramanujam, “In vivo multiphoton fluorescence lifetime imaging of protein-bound and free nicotinamide adenine dinucleotide in normal and precancerous epithelia,” *Journal of Biomedical Optics*, vol. 12, no. 2, p. 024014, 2007.
- [66] Y. Zhang, M. Savvidou, V. Liaudanskaya, P. Singh, Y. Fu, A. Nasreen, M. Coe, M. Kelly, D. Snapper, C. Wagner, J. Gill, A. Symes, A. Patra, D. L. Kaplan, A. Beheshti, and I. Georgakoudi, “Synergistic label-free fluorescence imaging and miRNA studies reveal dynamic human neuron-glia metabolic interactions following injury,” *Science Advances*, vol. 10, no. 50, p. eadp1980, 2024.
- [67] P. Campagnola, “Second harmonic generation imaging microscopy: Applications to diseases diagnostics,” *Analytical Chemistry*, vol. 83, no. 9, pp. 3224–3231, 2011.
- [68] E. Brown, T. McKee, E. diTomaso, A. Pluen, B. Seed, Y. Boucher, and R. K. Jain, “Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation,” *Nature Medicine*, vol. 9, no. 6, pp. 796–800, 2003.

- [69] X. Chen, O. Nadiarynkh, S. Plotnikov, and P. J. Campagnola, "Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure," *Nature Protocols*, vol. 7, no. 4, pp. 654–669, 2012.
- [70] J. Z. Cui, A. Y. Tehrani, K. A. Jett, P. Bernatchez, C. van Breemen, and M. Esfandiari, "Quantification of aortic and cutaneous elastin and collagen morphology in Marfan syndrome by multiphoton microscopy," *Journal of Structural Biology*, vol. 187, no. 3, pp. 242–253, 2014.
- [71] N. Kiss, D. Haluszka, K. Lőrincz, E. Kuroli, J. Hársing, B. Mayer, S. Kárpáti, G. Fekete, R. Szipőcs, N. Wikonkál, and M. Medvecz, "Ex vivo nonlinear microscopy imaging of Ehlers–Danlos syndrome-affected skin," *Archives of Dermatological Research*, vol. 310, no. 5, pp. 463–473, 2018.
- [72] E. Smith and G. Dent, *Modern Raman Spectroscopy: A Practical Approach*. John Wiley & Sons, 2019.
- [73] K. Kneipp, H. Kneipp, I. Itzkan, R. R. Dasari, and M. S. Feld, "Ultrasensitive Chemical Analysis by Raman Spectroscopy," *Chemical Reviews*, vol. 99, no. 10, pp. 2957–2976, 1999.
- [74] Y. Roggo, K. Degardin, and P. Margot, "Identification of pharmaceutical tablets by Raman spectroscopy and chemometrics," *Talanta*, vol. 81, no. 3, pp. 988–995, 2010.
- [75] Z. Movasaghi, R. , Shazza, and I. U. and Rehman, "Raman Spectroscopy of Biological Tissues," *Applied Spectroscopy Reviews*, vol. 42, no. 5, pp. 493–541, 2007.
- [76] G. Eckhardt, R. W. Hellwarth, F. J. McClung, S. E. Schwarz, D. Weiner, and E. J. Woodbury, "Stimulated Raman Scattering From Organic Liquids," *Physical Review Letters*, vol. 9, no. 11, pp. 455–457, 1962.
- [77] A. Zumbusch, G. R. Holtom, and X. S. Xie, "Three-Dimensional Vibrational Imaging by Coherent Anti-Stokes Raman Scattering," *Physical Review Letters*, vol. 82, no. 20, pp. 4142–4145, 1999.
- [78] C. L. Evans and X. S. Xie, "Coherent Anti-Stokes Raman Scattering Microscopy: Chemical Imaging for Biology and Medicine," *Annual Review of Analytical Chemistry*, vol. 1, no. 1, pp. 883–909, 2008.

- [79] C. Zhang, D. Zhang, and J.-X. Cheng, "Coherent Raman Scattering Microscopy in Biology and Medicine," *Annual review of biomedical engineering*, vol. 17, pp. 415–445, 2015.
- [80] H. Lotem, R. T. Lynch, and N. Bloembergen, "Interference between Raman resonances in four-wave difference mixing," *Physical Review A*, vol. 14, no. 5, pp. 1748–1755, 1976.
- [81] B. Manifold and D. Fu, "Quantitative Stimulated Raman Scattering Microscopy: Promises and Pitfalls," *Annual Review of Analytical Chemistry*, vol. 15, no. 1, pp. 269–289, 2022.
- [82] K. Sepp, M. Lee, M. T. J. Bluntzer, G. V. Helgason, A. N. Hulme, and V. G. Brunton, "Utilizing Stimulated Raman Scattering Microscopy To Study Intracellular Distribution of Label-Free Ponatinib in Live Cells," *Journal of Medicinal Chemistry*, vol. 63, no. 5, pp. 2028–2034, 2020.
- [83] A. Feizpour, T. Marstrand, L. Bastholm, S. Eirefelt, and C. L. Evans, "Label-Free Quantification of Pharmacokinetics in Skin with Stimulated Raman Scattering Microscopy and Deep Learning," *Journal of Investigative Dermatology*, vol. 141, no. 2, pp. 395–403, 2021.
- [84] B. A. Kuzma, D. Tu, A. Goss, F. Iliopoulos, J. B. Slade, A. Wiatrowski, A. Feizpour, and C. L. Evans, "Instantaneous topical drug quantification using a 3D printed microfluidic device and coherent Raman imaging," *OpenNano*, vol. 12, p. 100151, 2023.
- [85] B. A. Kuzma, I. J. Pence, D. A. Greenfield, A. Ho, and C. L. Evans, "Visualizing and quantifying antimicrobial drug distribution in tissue," *Advanced Drug Delivery Reviews*, vol. 177, p. 113942, 2021.
- [86] W. Hong, C. W. Karanja, N. S. Abutaleb, W. Younis, X. Zhang, M. N. Seleem, and J.-X. Cheng, "Antibiotic Susceptibility Determination within One Cell Cycle at Single-Bacterium Level by Stimulated Raman Metabolic Imaging," *Analytical Chemistry*, vol. 90, no. 6, pp. 3737–3743, 2018.
- [87] D. Fu, J. Zhou, W. S. Zhu, P. W. Manley, Y. K. Wang, T. Hood, A. Wylie, and X. S. Xie, "Imaging the intracellular distribution of tyrosine kinase inhibitors in living cells with quantitative hyperspectral stimulated Raman scattering," *Nature Chemistry*, vol. 6, no. 7, pp. 614–622, 2014.

- [88] R. C. Prince, R. R. Frontiera, and E. O. Potma, "Stimulated Raman Scattering: From Bulk to Nano," *Chemical Reviews*, vol. 117, no. 7, pp. 5070–5094, 2017.
- [89] E. Ploetz, S. Laimgruber, S. Berner, W. Zinth, and P. Gilch, "Femtosecond stimulated Raman microscopy," *Applied Physics B*, vol. 87, no. 3, pp. 389–393, 2007.
- [90] C. W. Freudiger, W. Min, B. G. Saar, S. Lu, G. R. Holtom, C. He, J. C. Tsai, J. X. Kang, and X. S. Xie, "Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy," *Science*, vol. 322, no. 5909, pp. 1857–1861, 2008.
- [91] C.-S. Liao, M. N. Slipchenko, P. Wang, J. Li, S.-Y. Lee, R. A. Oglesbee, and J.-X. Cheng, "Microsecond scale vibrational spectroscopic imaging by multiplex stimulated Raman scattering microscopy," *Light: Science & Applications*, vol. 4, no. 3, pp. e265–e265, 2015.
- [92] K. Bae, W. Zheng, K. Lin, S. W. Lim, Y. K. Chong, C. Tang, N. K. King, C. B. Ti Ang, and Z. Huang, "Epi-Detected Hyperspectral Stimulated Raman Scattering Microscopy for Label-Free Molecular Subtyping of Glioblastomas," *Analytical Chemistry*, vol. 90, no. 17, 2018.
- [93] D. Fu, G. Holtom, C. Freudiger, X. Zhang, and X. S. Xie, "Hyperspectral Imaging with Stimulated Raman Scattering by Chirped Femtosecond Lasers," *The Journal of Physical Chemistry B*, vol. 117, no. 16, pp. 4634–4640, 2013.
- [94] A. Francis, K. Berry, Y. Chen, B. Figueroa, and D. Fu, "Label-free pathology by spectrally sliced femtosecond stimulated Raman scattering (SRS) microscopy," *PLOS ONE*, vol. 12, no. 5, p. e0178750, 2017.
- [95] Y. Ozeki, W. Umemura, K. Sumimura, N. Nishizawa, K. Fukui, and K. Itoh, "Stimulated Raman hyperspectral imaging based on spectral filtering of broadband fiber laser pulses," *Optics Letters*, vol. 37, no. 3, pp. 431–433, 2012.
- [96] S. Karpf, M. Eibl, W. Wieser, T. Klein, and R. Huber, "A Time-Encoded Technique for fibre-based hyperspectral broadband stimulated Raman microscopy," *Nature Communications*, vol. 6, no. 1, p. 6784, 2015.
- [97] B. Figueroa, W. Fu, T. Nguyen, K. Shin, B. Manifold, F. Wise, and D. Fu, "Broadband hyperspectral stimulated Raman scattering microscopy with a parabolic fiber amplifier source," *Biomedical Optics Express*, vol. 9, no. 12, pp. 6116–6131, 2018.

- [98] P. Wang, M. N. Slipchenko, B. Zhou, R. Pinal, and J. X. Cheng, "Mechanisms of epi-detected stimulated raman scattering microscopy," *IEEE Journal on Selected Topics in Quantum Electronics*, vol. 18, no. 1, 2012.
- [99] M. N. Slipchenko, H. Chen, D. R. Ely, Y. Jung, M. T. Carvajal, and J. X. Cheng, "Vibrational imaging of tablets by epi-detected stimulated Raman scattering microscopy," *Analyst*, vol. 135, no. 10, pp. 2613–2619, 2010.
- [100] B. Figueroa, T. Nguyen, S. Sotthivirat, W. Xu, T. Rhodes, M. S. Lamm, R. L. Smith, C. T. John, Y. Su, and D. Fu, "Detecting and Quantifying Microscale Chemical Reactions in Pharmaceutical Tablets by Stimulated Raman Scattering Microscopy," *Analytical Chemistry*, vol. 91, no. 10, pp. 6894–6901, 2019.
- [101] C. W. Freudiger, W. Yang, G. R. Holtom, N. Peyghambarian, X. S. Xie, and K. Q. Kieu, "Stimulated Raman scattering microscopy with a robust fibre laser source," *Nature Photonics*, vol. 8, no. 2, pp. 153–159, 2014.
- [102] E. R. Andresen, P. Berto, and H. Rigneault, "Stimulated Raman scattering microscopy by spectral focusing and fiber-generated soliton as Stokes pulse," *Optics Letters*, vol. 36, no. 13, p. 2387, 2011.
- [103] B. Liu, P. Wang, J. I. Kim, D. Zhang, Y. Xia, C. Chapple, and J.-X. Cheng, "Vibrational Fingerprint Mapping Reveals Spatial Distribution of Functional Groups of Lignin in Plant Cell Wall," *Analytical Chemistry*, vol. 87, no. 18, pp. 9436–9442, 2015.
- [104] F. Saltarelli, V. Kumar, D. Viola, F. Crisafi, F. Preda, G. Cerullo, and D. Polli, "Broadband stimulated Raman scattering spectroscopy by a photonic time stretcher," *Optics Express*, vol. 24, no. 19, pp. 21264–21275, 2016.
- [105] C.-S. Liao, P. Wang, P. Wang, J. Li, H. J. Lee, G. Eakins, and J.-X. Cheng, "Spectrometer-free vibrational imaging by retrieving stimulated Raman signal from highly scattered photons," *Science Advances*, vol. 1, no. 9, p. e1500738, 2015.
- [106] P. Y. Sacré, C. De Bleye, P. F. Chavez, L. Netchacovitch, Ph. Hubert, and E. Ziemons, "Data processing of vibrational chemical imaging for pharmaceutical applications," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 101, pp. 123–140, 2014.
- [107] Y. Otsuka, K. Makara, S. Satoh, H. Hashimoto, and Y. Ozeki, "On-line visualization of multicolor chemical images with stimulated Raman scattering spectral microscopy," *Analyst*, vol. 140, no. 9, pp. 2984–2987, 2015.

- [108] S. Oh, C. Lee, W. Yang, A. Li, A. Mukherjee, M. Basan, C. Ran, W. Yin, C. J. Tabin, D. Fu, X. S. Xie, and M. W. Kirschner, “Protein and lipid mass concentration measurement in tissues by stimulated Raman scattering microscopy,” *Proceedings of the National Academy of Sciences*, vol. 119, no. 17, p. e2117938119, 2022.
- [109] T. T. Le, H. M. Duren, M. N. Slipchenko, C.-D. Hu, and J.-X. Cheng, “Label-free quantitative analysis of lipid metabolism in living *Caenorhabditis elegans*[S],” *Journal of Lipid Research*, vol. 51, no. 3, pp. 672–677, 2010.
- [110] F. X. Xu, G. N. Ioannou, S. P. Lee, C. Savard, C. L. Horn, and D. Fu, “Discrimination of lipid composition and cellular localization in human liver tissues by stimulated Raman scattering microscopy,” *Journal of Biomedical Optics*, vol. 29, no. 1, p. 016008, 2024.
- [111] L. Wei, F. Hu, Y. Shen, Z. Chen, Y. Yu, C.-C. Lin, M. C. Wang, and W. Min, “Live-cell imaging of alkyne-tagged small biomolecules by stimulated Raman scattering,” *Nature methods*, vol. 11, no. 4, pp. 410–412, 2014.
- [112] M. Li, Y. Chen, W. He, and Z. Guo, “Fluorescence and Lifetime Imaging of Endoplasmic Reticulum Polarity Change During Ferroptosis,” *Chemistry – A European Journal*, vol. 30, no. 35, p. e202401285, 2024.
- [113] M. Kröger, J. Scheffel, E. A. Shirshin, J. Schleusener, M. C. Meinke, J. Lademann, M. Maurer, and M. E. Darvin, “Label-free imaging of M1 and M2 macrophage phenotypes in the human dermis in vivo using two-photon excited FLIM,” *eLife*, vol. 11, p. e72819, 2022.
- [114] F.-K. Lu, S. Basu, V. Igras, M. P. Hoang, M. Ji, D. Fu, G. R. Holtom, V. A. Neel, C. W. Freudiger, D. E. Fisher, and X. S. Xie, “Label-free DNA imaging in vivo with stimulated Raman scattering microscopy,” *Proceedings of the National Academy of Sciences*, vol. 112, no. 37, pp. 11624–11629, 2015.
- [115] M. Ji, S. Lewis, S. Camelo-Piragua, S. H. Ramkisson, M. Snuderl, S. Venneti, A. Fisher-Hubbard, M. Garrard, D. Fu, A. C. Wang, J. A. Heth, C. O. Maher, N. Sanai, T. D. Johnson, C. W. Freudiger, O. Sagher, X. S. Xie, and D. A. Orringer, “Detection of human brain tumor infiltration with quantitative stimulated Raman scattering microscopy,” *Science translational medicine*, vol. 7, no. 309, p. 309ra163, 2015.
- [116] P. Androvic, M. Schifferer, K. Perez Anderson, L. Cantuti-Castelvetri, H. Jiang, H. Ji, L. Liu, G. Gouna, S. A. Berghoff, S. Besson-Girard, J. Knoferle, M. Simons,

- and O. Gokce, “Spatial Transcriptomics-correlated Electron Microscopy maps transcriptional and ultrastructural responses to brain injury,” *Nature Communications*, vol. 14, no. 1, p. 4115, 2023.
- [117] B. He, L. Bergenstråhle, L. Stenbeck, A. Abid, A. Andersson, Å. Borg, J. Maaskola, J. Lundeberg, and J. Zou, “Integrating spatial gene expression and breast tumour morphology via deep learning,” *Nature Biomedical Engineering*, vol. 4, no. 8, pp. 827–834, 2020.
- [118] R. J. Chen, M. Y. Lu, J. Wang, D. F. Williamson, S. J. Rodig, N. I. Lindeman, and F. Mahmood, “Pathomic Fusion: An Integrated Framework for Fusing Histopathology and Genomic Features for Cancer Diagnosis and Prognosis,” *IEEE Transactions on Medical Imaging*, vol. 41, no. 4, pp. 757–770, 2022.
- [119] U. Holzgrabe, I. Wawer, and B. Diehl, *NMR Spectroscopy in Drug Development and Analysis*. John Wiley & Sons, 1999.
- [120] R. Martino, M. Malet-Martino, V. Gilard, and S. Balayssac, “Counterfeit drugs: Analytical techniques for their identification,” *Analytical and Bioanalytical Chemistry*, vol. 398, no. 1, pp. 77–92, 2010.
- [121] R. M. Maggio, N. L. Calvo, S. E. Vignaduzzo, and T. S. Kaufman, “Pharmaceutical impurities and degradation products: Uses and applications of NMR techniques,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 101, pp. 102–122, 2014.
- [122] X. Li and C. Y. Wang, “From bulk, single-cell to spatial RNA sequencing,” *International Journal of Oral Science*, vol. 13, no. 1, 2021.
- [123] U. Nagalakshmi, Z. Wang, K. Waern, C. Shou, D. Raha, M. Gerstein, and M. Snyder, “The Transcriptional Landscape of the Yeast Genome Defined by RNA Sequencing,” *Science*, vol. 320, no. 5881, pp. 1344–1349, 2008.
- [124] F. Tang, C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B. B. Tuch, A. Siddiqui, K. Lao, and M. A. Surani, “mRNA-Seq whole-transcriptome analysis of a single cell,” *Nature Methods*, vol. 6, no. 5, pp. 377–382, 2009.
- [125] P. L. Ståhl, F. Salmén, S. Vickovic, A. Lundmark, J. F. Navarro, J. Magnusson, S. Giacomello, M. Asp, J. O. Westholm, M. Huss, A. Mollbrink, S. Linnarsson, S. Codeluppi, Å. Borg, F. Pontén, P. I. Costea, P. Sahlén, J. Mulder, O. Bergmann, J. Lundeberg, and J. Frisén, “Visualization and analysis of gene expression in tissue sections by spatial transcriptomics,” *Science*, vol. 353, no. 6294, pp. 78–82, 2016.

- [126] M. F. Oliveira, J. P. Romero, M. Chung, S. Williams, A. D. Gottscho, A. Gupta, S. E. Pilipauskas, S. Mohabbat, N. Raman, D. Sukovich, D. Patterson, V. H. D. Team, and S. E. B. Taylor, “Characterization of immune cell populations in the tumor microenvironment of colorectal cancer using high definition spatial profiling,” 2024.
- [127] R. R. Stickels, E. Murray, P. Kumar, J. Li, J. L. Marshall, D. J. Di Bella, P. Arlotta, E. Z. Macosko, and F. Chen, “Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-seqV2,” *Nature Biotechnology*, vol. 39, no. 3, pp. 313–319, 2021.
- [128] S. Hernandez, R. Lazcano, A. Serrano, S. Powell, L. Kostousov, J. Mehta, K. Khan, W. Lu, and L. M. Solis, “Challenges and Opportunities for Immunoprofiling Using a Spatial High-Plex Technology: The NanoString GeoMx® Digital Spatial Profiler,” *Frontiers in Oncology*, vol. 12, 2022.
- [129] D. R. Zollinger, S. E. Lingle, K. Sorg, J. M. Beechem, and C. R. Merritt, “GeoMx™ RNA Assay: High Multiplex, Digital, Spatial Analysis of RNA in FFPE Tissue,” in *In Situ Hybridization Protocols* (B. S. Nielsen and J. Jones, eds.), pp. 331–345, New York, NY: Springer US, 2020.
- [130] D. P. Cook, K. B. Jensen, K. Wise, M. J. Roach, F. S. Dezem, N. K. Ryan, M. Zamojski, I. S. Vlachos, S. R. V. Knott, L. M. Butler, J. L. Wrana, N. E. Banovich, J. T. Plummer, and L. G. Martelotto, “A Comparative Analysis of Imaging-Based Spatial Transcriptomics Platforms,” 2023.
- [131] Y. Ren, Z. Huang, L. Zhou, P. Xiao, J. Song, P. He, C. Xie, R. Zhou, M. Li, X. Dong, Q. Mao, C. You, J. Xu, Y. Liu, Z. Lan, T. Zhang, Q. Gan, Y. Yang, T. Chen, B. Huang, X. Yang, A. Xiao, Y. Ou, Z. Su, L. Chen, Y. Zhang, Y. Ju, Y. Zhang, and Y. Wang, “Spatial transcriptomics reveals niche-specific enrichment and vulnerabilities of radial glial stem-like cells in malignant gliomas,” *Nature Communications*, vol. 14, no. 1, p. 1028, 2023.
- [132] W. Liu, A. Puri, D. Fu, L. Chen, C. Wang, M. Kellis, and J. Yang, “Dissecting the tumor microenvironment in response to immune checkpoint inhibitors via single-cell and spatial transcriptomics,” *Clinical & Experimental Metastasis*, vol. 41, no. 4, pp. 313–332, 2024.
- [133] M. Piwecka, N. Rajewsky, and A. Rybak-Wolf, “Single-cell and spatial transcriptomics: Deciphering brain complexity in health and disease,” *Nature Reviews Neurology*, vol. 19, no. 6, pp. 346–362, 2023.

- [134] C. Ortiz, M. Carlén, and K. Meletis, “Spatial Transcriptomics: Molecular Maps of the Mammalian Brain,” *Annual Review of Neuroscience*, vol. 44, no. Volume 44, 2021, pp. 547–562, 2021.
- [135] N. Desai, A. Neyaz, A. Szabolcs, A. R. Shih, J. H. Chen, V. Thapar, L. T. Nieman, A. Solovyov, A. Mehta, D. J. Lieb, A. S. Kulkarni, C. Jaicks, K. H. Xu, M. J. Raabe, C. J. Pinto, D. Juric, I. Chebib, R. B. Colvin, A. Y. Kim, R. Monroe, S. E. Warren, P. Danaher, J. W. Reeves, J. Gong, E. H. Rueckert, B. D. Greenbaum, N. Hacohen, S. M. Lagana, M. N. Rivera, L. M. Sholl, J. R. Stone, D. T. Ting, and V. Deshpande, “Temporal and spatial heterogeneity of host response to SARS-CoV-2 pulmonary infection,” *Nature Communications*, vol. 11, no. 1, p. 6319, 2020.
- [136] D. Butler, C. Mozsary, C. Meydan, J. Foox, J. Rosiene, A. Shaiber, D. Danko, E. Afshinnkoo, M. MacKay, F. J. Sedlazeck, N. A. Ivanov, M. Sierra, D. Pohle, M. Zietz, U. Gisladdottir, V. Ramlall, E. T. Sholle, E. J. Schenck, C. D. Westover, C. Hassan, K. Ryon, B. Young, C. Bhattacharya, D. L. Ng, A. C. Granados, Y. A. Santos, V. Servellita, S. Federman, P. Ruggiero, A. Functammasan, C.-S. Chin, N. M. Pearson, B. W. Langhorst, N. A. Tanner, Y. Kim, J. W. Reeves, T. D. Het-her, S. E. Warren, M. Bailey, J. Gawrys, D. Meleshko, D. Xu, M. Couto-Rodriguez, D. Nagy-Szakal, J. Barrows, H. Wells, N. B. O’Hara, J. A. Rosenfeld, Y. Chen, P. A. D. Steel, A. J. Shemesh, J. Xiang, J. Thierry-Mieg, D. Thierry-Mieg, A. Ift-ner, D. Bezdan, E. Sanchez, T. R. Champion, J. Sipley, L. Cong, A. Craney, P. Velu, A. M. Melnick, S. Shapira, I. Hajirasouliha, A. Borczuk, T. Iftner, M. Salvatore, M. Loda, L. F. Westblade, M. Cushing, S. Wu, S. Levy, C. Chiu, R. E. Schwartz, N. Tatonetti, H. Rennert, M. Imielinski, and C. E. Mason, “Shotgun transcriptome, spatial omics, and isothermal profiling of SARS-CoV-2 infection reveals unique host responses, viral diversification, and drug interactions,” *Nature Communications*, vol. 12, no. 1, p. 1660, 2021.
- [137] I. Goodfellow, Y. Bengio, and A. Courville, *Deep Learning*. MIT Press, 2016.
- [138] Y. LeCun, Y. Bengio, and T. B. Laboratories, “Convolutional Networks for Images, Speech, and Time-Series,” 1995.
- [139] J. Lehtinen, J. Munkberg, J. Hasselgren, S. Laine, T. Karras, M. Aittala, and T. Aila, “Noise2Noise: Learning Image Restoration without Clean Data,” 2018.
- [140] A. Krull, T.-O. Buchholz, and F. Jug, “Noise2Void - Learning Denoising From Sin-
gle Noisy Images,” in *2019 IEEE/CVF Conference on Computer Vision and Pattern
Recognition (CVPR)*, (Long Beach, CA, USA), pp. 2124–2132, IEEE, 2019.

- [141] J. Batson and L. Royer, “Noise2Self: Blind Denoising by Self-Supervision,” 2019.
- [142] J. Shi, K. K. D. Tan, A. Ho, J. E. Sorrells, R. R. Iyer, E. J. Chaney, D. R. S. Jr, M. Marjanovic, and S. A. Boppart, “DeepFLR: A self-supervised deep learning framework for robust FLIM signal restoration under photon-starved conditions,” in *Multiphoton Microscopy in the Biomedical Sciences XXV*, vol. PC13324, p. PC1332416, SPIE, 2025.
- [143] U. Schmidt, M. Weigert, C. Broaddus, and G. Myers, “Cell Detection with Star-Convex Polygons,” in *Medical Image Computing and Computer Assisted Intervention – MICCAI 2018* (A. F. Frangi, J. A. Schnabel, C. Davatzikos, C. Alberola-López, and G. Fichtinger, eds.), (Cham), pp. 265–273, Springer International Publishing, 2018.
- [144] M. Pachitariu and C. Stringer, “Cellpose 2.0: How to train your own model,” *Nature Methods*, vol. 19, no. 12, pp. 1634–1641, 2022.
- [145] C. Stringer and M. Pachitariu, “Cellpose3: One-click image restoration for improved cellular segmentation,” *Nature Methods*, vol. 22, no. 3, pp. 592–599, 2025.
- [146] Y. Wei, I. J. Pence, A. Wiatrowski, J. B. Slade, and C. L. Evans, “Quantitative analysis of drug tablet aging by fast hyper-spectral stimulated Raman scattering microscopy,” *The Analyst*, vol. 149, no. 5, pp. 1436–1446, 2024.
- [147] D. G. Brown and H. J. Wobst, “A Decade of FDA-Approved Drugs (2010–2019): Trends and Future Directions,” *Journal of Medicinal Chemistry*, vol. 64, no. 5, pp. 2312–2338, 2021.
- [148] U.S. Food and Drug Administration, “New Drug Therapy Approvals 2022,” 2022.
- [149] U.S. Food and Drug Administration, “New Drug Therapy Approvals 2021,” 2021.
- [150] U.S. Food and Drug Administration, “New Drug Therapy Approvals 2020,” 2020.
- [151] U.S. Food and Drug Administration, “New Drug Therapy Approvals 2019,” 2019.
- [152] U.S. Food and Drug Administration, “New Drug Therapy Approvals 2018,” 2018.
- [153] H. Zhong, G. Chan, Y. Hu, H. Hu, and D. Ouyang, “A Comprehensive Map of FDA-Approved Pharmaceutical Products,” *Pharmaceutics*, vol. 10, no. 4, p. 263, 2018.

- [154] L. L. Augsburger and S. W. Hoag, *Pharmaceutical Dosage Forms - Tablets*. CRC press, 3rd ed., 2008.
- [155] G. L. Amidon, H. Lennernäs, V. P. Shah, and J. R. Crison, "A Theoretical Basis for a Biopharmaceutic Drug Classification: The Correlation of in Vitro Drug Product Dissolution and in Vivo Bioavailability," *Pharmaceutical Research*, vol. 12, no. 3, pp. 413–420, 1995.
- [156] B. Y. Shekunov, P. Chattopadhyay, H. H. Y. Tong, and A. H. L. Chow, "Particle size analysis in pharmaceuticals: Principles, methods and applications," *Pharmaceutical Research*, vol. 24, no. 2, pp. 203–227, 2007.
- [157] A. J. Hlinak, K. Kuriyan, K. R. Morris, G. V. Reklaitis, and P. K. Basu, "Understanding critical material properties for solid dosage form design," *Journal of Pharmaceutical Innovation*, vol. 1, no. 1, pp. 12–17, 2006.
- [158] O. o. R. Affairs, "Guide to Inspection of Oral Solid Dosage Forms: Pre/Post Approval Issues for Development and Validation," tech. rep., U.S. Food and Drug Administration, 1994.
- [159] U.S. Food and Drug Administration, "Process Analytical Technology FDA Guidance," 2004.
- [160] G. P. S. Smith, C. M. McGoverin, S. J. Fraser, and K. C. Gordon, "Raman imaging of drug delivery systems," *Advanced Drug Delivery Reviews*, vol. 89, pp. 21–41, 2015.
- [161] R. Saunders, "The Effect of Particle Agglomeration in Pharmaceutical Preparations," *Journal of the Royal Statistical Society Series D: The Statistician*, vol. 40, no. 1, pp. 77–86, 1991.
- [162] W. J. Thiel, L. T. Nguyen, and F. J. Sberna, "Content uniformity of microdose tablets (dosage 1 Mg-10 mg) produced by fluid bed granulation of interactive mixtures," *Journal of Pharmacy and Pharmacology*, vol. 38, no. 5, pp. 335–343, 1986.
- [163] H. Kato, K. Kimura, S. Izumi, K. Nakamichi, K. Danjo, and H. Sunada, "The effect of magnesium stearate particle size on tablet properties and tableting characteristics of granules prepared with standard formulation," *Journal of Drug Delivery Science and Technology*, vol. 15, no. 6, pp. 475–480, 2005.
- [164] J. Li and Y. Wu, "Lubricants in Pharmaceutical Solid Dosage Forms," *Lubricants*, vol. 2, no. 1, pp. 21–43, 2014.

- [165] S. Lakio, B. Vajna, I. Farkas, H. Salokangas, G. Marosi, and J. Yliruusi, "Challenges in Detecting Magnesium Stearate Distribution in Tablets," *AAPS PharmSciTech*, vol. 14, no. 1, pp. 435–444, 2013.
- [166] A. A. Gowen, C. P. O'Donnell, P. J. Cullen, and S. E. J. Bell, "Recent applications of Chemical Imaging to pharmaceutical process monitoring and quality control," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 69, no. 1, pp. 10–22, 2008.
- [167] J. M. Amigo and C. Ravn, "Direct quantification and distribution assessment of major and minor components in pharmaceutical tablets by NIR-chemical imaging," *European Journal of Pharmaceutical Sciences*, vol. 37, no. 2, pp. 76–82, 2009.
- [168] N. Everall, "Depth Profiling with Confocal Raman Microscopy, Part II," *Spectroscopy*, vol. 19, no. 11, p. 22, 2004.
- [169] S. Kumar, Z. Nagy, G. V. Reklaitis, and M. Gonzalez, "Considerations in Raman Spectroscopy for Real-Time API Concentration Measurement at Tablet Press Feed Frame," *Journal of Spectroscopy*, vol. 2023, p. e8631288, 2023.
- [170] J.-X. Cheng and X. S. Xie, "Coherent Anti-Stokes Raman Scattering Microscopy: Instrumentation, Theory, and Applications," *The Journal of Physical Chemistry B*, vol. 108, no. 3, pp. 827–840, 2004.
- [171] "Stimulated Raman Scattering Microscopy," in *Stimulated Raman Scattering Microscopy* (J.-X. Cheng, W. Min, Y. Ozeki, and D. Polli, eds.), pp. i–ii, Elsevier, 2022.
- [172] M. Jurna, M. Windbergs, C. J. Strachan, L. Hartsuiker, C. Otto, P. Kleinebudde, J. L. Herek, and H. L. Offerhaus, "COHERENT ANTI-STOKES RAMAN SCATTERING MICROSCOPY TO MONITOR DRUG DISSOLUTION IN DIFFERENT ORAL PHARMACEUTICAL TABLETS," *Journal of Innovative Optical Health Sciences*, vol. 02, no. 01, pp. 37–43, 2009.
- [173] M. Windbergs, M. Jurna, H. L. Offerhaus, J. L. Herek, P. Kleinebudde, and C. J. Strachan, "Chemical imaging of oral solid dosage forms and changes upon dissolution using coherent anti-stokes raman scattering microscopy," *Analytical Chemistry*, vol. 81, no. 6, pp. 2085–2091, 2009.
- [174] A. Fussell, E. Garbacik, H. Offerhaus, P. Kleinebudde, and C. Strachan, "In situ dissolution analysis using coherent anti-Stokes Raman scattering (CARS) and hyperspectral CARS microscopy," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 85, no. 3, Part B, pp. 1141–1147, 2013.

- [175] J. G. Porquez and A. D. Slepko, "Application of spectral-focusing-CARS microscopy to pharmaceutical sample analysis," *AIP Advances*, vol. 8, no. 9, p. 095213, 2018.
- [176] B. Sarri, R. Canonge, X. Audier, V. Lavastre, G. Pénarier, J. Alie, and H. Rigneault, "Discriminating polymorph distributions in pharmaceutical tablets using stimulated Raman scattering microscopy," *Journal of Raman Spectroscopy*, vol. 50, no. 12, pp. 1896–1904, 2019.
- [177] M. Brinkmann, A. Fast, T. Hellwig, I. Pence, C. L. Evans, and C. Fallnich, "Portable all-fiber dual-output widely tunable light source for coherent Raman imaging," *Biomedical Optics Express*, vol. 10, no. 9, pp. 4437–4449, 2019.
- [178] R. S. Saji, J. C. Prasana, S. Muthu, J. George, T. K. Kuruvilla, and B. R. Raajaraman, "Spectroscopic and quantum computational study on naproxen sodium," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 226, p. 117614, 2020.
- [179] I. Fleming and D. Williams, "Infrared and Raman Spectra," in *Spectroscopic Methods in Organic Chemistry* (I. Fleming and D. Williams, eds.), pp. 85–121, Cham: Springer International Publishing, 2019.
- [180] L. S. Taylor and F. W. Langkilde, "Evaluation of solid-state forms present in tablets by Raman spectroscopy," *Journal of Pharmaceutical Sciences*, vol. 89, no. 10, pp. 1342–1353, 2000.
- [181] F. Adar, "Characterizing Modified Celluloses Using Raman Spectroscopy," *Spectroscopy*, vol. 31, no. 11, pp. 22–27, 2016.
- [182] O. González-González, I. O. Ramirez, B. I. Ramirez, P. O'Connell, M. P. Ballesteros, J. J. Torrado, and D. R. Serrano, "Drug Stability: ICH versus Accelerated Predictive Stability Studies," *Pharmaceutics*, vol. 14, no. 11, p. 2324, 2022.
- [183] K. C. Waterman, A. J. Carella, M. J. Gumkowski, P. Lukulay, B. C. MacDonald, M. C. Roy, and S. L. Shamblin, "Improved Protocol and Data Analysis for Accelerated Shelf-Life Estimation of Solid Dosage Forms," *Pharmaceutical Research*, vol. 24, no. 4, pp. 780–790, 2007.
- [184] E. Rentschler, K. Schuh, M. Krewinkel, C. Baur, W. Claaßen, S. Meyer, B. Kuschel, T. Stressler, and L. Fischer, "Enzymatic production of lactulose and epilactose in milk," *Journal of Dairy Science*, vol. 98, no. 10, pp. 6767–6775, 2015.

- [185] R. Schuster-Wolff-Bühning, L. Fischer, and J. Hinrichs, "Production and physiological action of the disaccharide lactulose," *International Dairy Journal*, vol. 20, no. 11, pp. 731–741, 2010.
- [186] F. Zokaee, T. Kaghazchi, A. Zare, and M. Soleimani, "Isomerization of lactose to lactulose—study and comparison of three catalytic systems," *Process Biochemistry*, vol. 37, no. 6, pp. 629–635, 2002.
- [187] I. Gould, "The Formation of Volatile Acids in Milk by High-Temperature Heat Treatment," *Journal of Dairy Science*, vol. 28, no. 5, pp. 379–386, 1945.
- [188] C. T. John, W. Xu, L. K. Lupton, and P. A. Harmon, "Formulating Weakly Basic HCl Salts: Relative Ability of Common Excipients to Induce Disproportionation and the Unique Deleterious Effects of Magnesium Stearate," *Pharmaceutical Research*, vol. 30, no. 6, pp. 1628–1641, 2013.
- [189] G. M. Fahy, B. Wowk, and J. Wu, "Cryopreservation of complex systems: The missing link in the regenerative medicine supply chain," *Rejuvenation Research*, vol. 9, no. 2, pp. 279–291, 2006.
- [190] C. J. Hunt, "Cryopreservation of Human Stem Cells for Clinical Application: A Review," *Transfusion Medicine and Hemotherapy*, vol. 38, no. 2, pp. 107–123, 2011.
- [191] S. H. Day, D. A. Nicoll-Griffith, and J. M. Silva, "Cryopreservation of Rat and Human Liver Slices by Rapid Freezing," *Cryobiology*, vol. 38, no. 2, pp. 154–159, 1999.
- [192] M. Kuwayama, "Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method," *Theriogenology*, vol. 67, no. 1, pp. 73–80, 2007.
- [193] K. P. Katayama, J. Stehlik, M. Kuwayama, O. Kato, and E. Stehlik, "High survival rate of vitrified human oocytes results in clinical pregnancy," *Fertility and Sterility*, vol. 80, no. 1, pp. 223–224, 2003.
- [194] J. K. Lewis, J. C. Bischof, I. Braslavsky, K. G. M. Brockbank, G. M. Fahy, B. J. Fuller, Y. Rabin, A. Tocchio, E. J. Woods, B. G. Wowk, J. P. Acker, and S. Giwa, "The Grand Challenges of Organ Banking: Proceedings from the first global summit on complex tissue cryopreservation," *Cryobiology*, vol. 72, no. 2, pp. 169–182, 2016.

- [195] S. Giwa, J. K. Lewis, L. Alvarez, R. Langer, A. E. Roth, G. M. Church, J. F. Markmann, D. H. Sachs, A. Chandraker, J. A. Wertheim, M. Rothblatt, E. S. Boyden, E. Eidbo, W. P. A. Lee, B. Pomahac, G. Brandacher, D. M. Weinstock, G. Elliott, D. Nelson, J. P. Acker, K. Uygun, B. Schmalz, B. P. Weegman, A. Tocchio, G. M. Fahy, K. B. Storey, B. Rubinsky, J. Bischof, J. A. W. Elliott, T. K. Woodruff, G. J. Morris, U. Demirci, K. G. M. Brockbank, E. J. Woods, R. N. Ben, J. G. Baust, D. Gao, B. Fuller, Y. Rabin, D. C. Kravitz, M. J. Taylor, and M. Toner, “The promise of organ and tissue preservation to transform medicine,” *Nature Biotechnology*, vol. 35, no. 6, pp. 530–542, 2017.
- [196] P. Mazur, K. W. Cole, J. W. Hall, P. D. Schreuders, and A. P. Mahowald, “Cryobiological Preservation of *Drosophila* Embryos,” *Science*, vol. 258, no. 5090, pp. 1932–1935, 1992.
- [197] J. E. Nesbitt, A. Swei, C. Hunt, E. M. Dotson, M. Toner, and R. D. Sandlin, “Cryoprotectant toxicity and hypothermic sensitivity among *Anopheles* larvae,” *Cryobiology*, vol. 99, pp. 106–113, 2021.
- [198] G. Marcantonini, D. Bartolini, L. Zatini, S. Costa, M. Passerini, M. Rende, G. Luca, G. Basta, G. Murdolo, R. Calafiore, and F. Galli, “Natural Cryoprotective and Cytoprotective Agents in Cryopreservation: A Focus on Melatonin,” *Molecules*, vol. 27, no. 10, p. 3254, 2022.
- [199] E. A. Szurek and A. Eroglu, “Comparison and Avoidance of Toxicity of Penetrating Cryoprotectants,” *PLOS ONE*, vol. 6, no. 11, p. e27604, 2011.
- [200] B. P. Best, “Cryoprotectant Toxicity: Facts, Issues, and Questions,” *Rejuvenation Research*, vol. 18, no. 5, pp. 422–436, 2015.
- [201] R. M. Warner and A. Z. Higgins, “Mathematical modeling of protectant transport in tissues,” *Methods in molecular biology (Clifton, N.J.)*, vol. 2180, pp. 173–188, 2021.
- [202] J. D. Benson, A. Z. Higgins, K. Desai, and A. Eroglu, “A toxicity cost function approach to optimal CPA equilibration in tissues,” *Cryobiology*, vol. 80, pp. 144–155, 2018.
- [203] M. Hagedorn, E. W. Hsu, U. Pilatus, D. E. Wildt, W. R. Rall, and S. J. Blackband, “Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmental biological system,” *Proceedings of the National Academy of Sciences*, vol. 93, no. 15, pp. 7454–7459, 1996.

- [204] H. Newton, J. Fisher, J. R. P. Arnold, D. E. Pegg, M. J. Faddy, and R. G. Gosden, "Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation," *Human Reproduction*, vol. 13, no. 2, pp. 376–380, 1998.
- [205] M. Gallardo, J. Saenz, and R. Risco, "Human oocytes and zygotes are ready for ultra-fast vitrification after 2 minutes of exposure to standard CPA solutions," *Scientific Reports*, vol. 9, no. 1, p. 15986, 2019.
- [206] T. Louwagie, M. Wagner, R. Li, G. Yu, A. Petersen, and A. Hubel, "Characterizing cellular membrane partitioning of DMSO using low-temperature Raman spectroscopy," *Frontiers in Molecular Biosciences*, vol. 10, 2023.
- [207] F. M. Samuels, K. C. Pearce, S. Soderlund, D. G. Stich, R. Bonnart, G. M. Volk, and N. E. Levinger, "Direct observation of common cryoprotectant permeation into rice callus by CARS microscopy," *Cell Reports Physical Science*, vol. 4, no. 7, p. 101469, 2023.
- [208] W. Mao, H.-T. D. Bui, W. Cho, and H. S. Yoo, "Spectroscopic techniques for monitoring stem cell and organoid proliferation in 3D environments for therapeutic development," *Advanced Drug Delivery Reviews*, vol. 201, p. 115074, 2023.
- [209] M. Wei, L. Shi, Y. Shen, Z. Zhao, A. Guzman, L. J. Kaufman, L. Wei, and W. Min, "Volumetric chemical imaging by clearing-enhanced stimulated Raman scattering microscopy," *Proceedings of the National Academy of Sciences*, vol. 116, no. 14, pp. 6608–6617, 2019.
- [210] W. N. Martens, R. L. Frost, J. Kristof, and J. Theo Klopogge, "Raman spectroscopy of dimethyl sulphoxide and deuterated dimethyl sulphoxide at 298 and 77 K," *Journal of Raman Spectroscopy*, vol. 33, no. 2, pp. 84–91, 2002.
- [211] N. S. Lemberger, K. Wallmeier, and C. Fallnich, "High-bandwidth noise-reduced loss-corrected autobalanced detection," *Optics Express*, vol. 33, no. 1, pp. 62–74, 2025.
- [212] R. Jordão, J. Casas, G. Fabrias, B. Campos, B. Piña, M. F. Lemos, A. M. Soares, R. Tauler, and C. Barata, "Obesogens beyond Vertebrates: Lipid Perturbation by Tributyltin in the Crustacean *Daphnia magna*," *Environmental Health Perspectives*, vol. 123, no. 8, pp. 813–819, 2015.
- [213] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, "The Chemical Components of a Cell," in *Molecular Biology of the Cell. 4th Edition*, Garland Science, 2002.

- [214] E. E. Ruppert, R. S. Fox, and R. D. Barnes, *Invertebrate Zoology: A Functional Evolutionary Approach*. Thomson-Brooks/Cole, 2004.
- [215] M. I. Asher, S. Montefort, B. Björkstén, C. K. Lai, D. P. Strachan, S. K. Weiland, and H. Williams, “Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys,” *The Lancet*, vol. 368, no. 9537, pp. 733–743, 2006.
- [216] S. Barbarot, S. Auziere, A. Gadkari, G. Girolomoni, L. Puig, E. L. Simpson, D. J. Margolis, M. de Bruin-Weller, and L. Eckert, “Epidemiology of atopic dermatitis in adults: Results from an international survey,” *Allergy*, vol. 73, no. 6, pp. 1284–1293, 2018.
- [217] Y. W. Yew, J. P. Thyssen, and J. I. Silverberg, “A systematic review and meta-analysis of the regional and age-related differences in atopic dermatitis clinical characteristics,” *Journal of the American Academy of Dermatology*, vol. 80, no. 2, pp. 390–401, 2019.
- [218] A. Lefèvre-Utile, M. Saichi, P. Oláh, M. Delord, B. Homey, V. Soumelis, and MAARS consortium, “Transcriptome-based identification of novel endotypes in adult atopic dermatitis,” *Allergy*, vol. 77, no. 5, pp. 1486–1498, 2022.
- [219] Y. Tokura and S. Hayano, “Subtypes of atopic dermatitis: From phenotype to endotype,” *Allergology International*, vol. 71, no. 1, pp. 14–24, 2022.
- [220] M. Ardigo, C. Cota, E. Berardesca, and S. González, “Concordance between in vivo reflectance confocal microscopy and histology in the evaluation of plaque psoriasis,” *Journal of the European Academy of Dermatology and Venereology*, vol. 23, no. 6, pp. 660–667, 2009.
- [221] J. V. Rocheleau, W. S. Head, and D. W. Piston, “Quantitative NAD(P)H/Flavoprotein Autofluorescence Imaging Reveals Metabolic Mechanisms of Pancreatic Islet Pyruvate Response*,” *Journal of Biological Chemistry*, vol. 279, no. 30, pp. 31780–31787, 2004.
- [222] V. Huck, C. Gorzelanny, K. Thomas, V. Getova, V. Niemeyer, K. Zens, T. R. Unnerstall, J. S. Feger, M. A. Fallah, D. Metze, S. Ständer, T. A. Luger, K. Koenig, C. Mess, and S. W. Schneider, “From morphology to biochemical state - Intravital multiphoton fluorescence lifetime imaging of inflamed human skin,” *Scientific Reports*, vol. 6, 2016.

- [223] T. S. Blacker, Z. F. Mann, J. E. Gale, M. Ziegler, A. J. Bain, G. Szabadkai, and M. R. Duchon, “Separating NADH and NADPH fluorescence in live cells and tissues using FLIM,” *Nature Communications*, vol. 5, 2014.
- [224] A.-M. Pena, E. Decenci re, S. Brizion, P. Sextius, S. Koudoro, T. Baldeweck, and E. Tancre de-Bohin, “In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses,” *Scientific Reports*, vol. 12, no. 1, p. 1642, 2022.
- [225] M. Y. Berezin and S. Achilefu, “Fluorescence lifetime measurements and biological imaging,” *Chemical Reviews*, vol. 110, no. 5, pp. 2641–2684, 2010.
- [226] K. K nig, A. M. Gon alves Batista, M. Zieger, M. Kaatz, H. H n le, C. Fink, and H. G. Breunig, “Clinical multimodal multiphoton tomography of pigmented skin lesions with an ultracompact femtosecond fiber laser,” in *Photonics in Dermatology and Plastic Surgery 2020* (B. Choi and H. Zeng, eds.), (San Francisco, United States), p. 12, SPIE, 2020.
- [227] K. K nig, D. Pankin, A. Paudel, H. H n le, J. K. Winkler, M. Zieger, and M. Kaatz, “Skin cancer detection with a compact multimodal fiber laser multiphoton FLIM tomograph,” in *Multiphoton Microscopy in the Biomedical Sciences XXI*, vol. 11648, p. 116480A, SPIE, 2021.
- [228] E. Decenci re, E. Tancre de-Bohin, P. Dokl dal, S. Koudoro, A.-M. Pena, and T. Baldeweck, “Automatic 3D segmentation of multiphoton images: A key step for the quantification of human skin,” *Skin Research and Technology*, vol. 19, no. 2, pp. 115–124, 2013.
- [229] J. M. Hanifin, M. Thurston, M. Omoto, R. Cherill, S. J. Tofte, M. Graeber, and T. E. Evaluator Group, “The eczema area and severity index (EASI): Assessment of reliability in atopic dermatitis,” *Experimental Dermatology*, vol. 10, no. 1, pp. 11–18, 2001.
- [230] J. M. Hanifin, W. Baghoomian, E. Grinich, Y. A. Leshem, M. Jacobson, and E. L. Simpson, “The Eczema Area and Severity Index—A Practical Guide,” *Dermatitis*, vol. 33, no. 3, pp. 187–192, 2022.
- [231] A. K. F. Tong and M. C. Mihm, “The pathology of atopic dermatitis,” *Clinical Reviews in Allergy*, vol. 4, no. 1, pp. 27–42, 1986.

- [232] S. Huang, A. A. Heikal, and W. W. Webb, "Two-Photon Fluorescence Spectroscopy and Microscopy of NAD(P)H and Flavoprotein," *Biophysical Journal*, vol. 82, no. 5, pp. 2811–2825, 2002.
- [233] G. H. Patterson, S. M. Knobel, P. Arkhammar, O. Thastrup, and D. W. Piston, "Separation of the glucose-stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet β cells," *Proceedings of the National Academy of Sciences*, vol. 97, no. 10, pp. 5203–5207, 2000.
- [234] A. Song, N. Zhao, D. C. Hilpert, C. Perry, J. A. Baur, D. C. Wallace, and P. M. Schaefer, "Visualizing subcellular changes in the NAD(H) pool size versus redox state using fluorescence lifetime imaging microscopy of NADH," *Communications Biology*, vol. 7, no. 1, pp. 1–13, 2024.
- [235] K. Brand, "Aerobic Glycolysis by Proliferating Cells: Protection against Oxidative Stress at the Expense of Energy Yield," *Journal of Bioenergetics and Biomembranes*, vol. 29, no. 4, pp. 355–364, 1997.
- [236] V. Masutin, C. Kersch, and S. Schmitz-Spanke, "A systematic review: Metabolomics-based identification of altered metabolites and pathways in the skin caused by internal and external factors," *Experimental Dermatology*, vol. 31, no. 5, pp. 700–714, 2022.
- [237] J. LI, J. XING, F. LU, W. CHANG, N. LIANG, J. LI, Y. WANG, X. LI, X. ZHAO, R. HOU, M. MAN, G. YIN, X. LI, and K. ZHANG, "Psoriatic Dermal-derived Mesenchymal Stem Cells Reduce Keratinocyte Junctions, and Increase Glycolysis," *Acta Dermato-Venereologica*, vol. 100, no. 8, p. 5737, 2020.
- [238] S. Y. Lunt and M. G. V. Heiden, "Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation," *Annual Review of Cell and Developmental Biology*, vol. 27, no. Volume 27, 2011, pp. 441–464, 2011.
- [239] S. Gibbs, R. Fijneman, J. Wiegant, A. G. van Kessel, P. van De Putte, and C. Backendorf, "Molecular characterization and evolution of the SPRR family of keratinocyte differentiation markers encoding small proline-rich proteins," *Genomics*, vol. 16, no. 3, pp. 630–637, 1993.
- [240] K. W. Marvin, M. D. George, W. Fujimoto, N. A. Saunders, S. H. Bernacki, and A. M. Jetten, "Cornifin, a cross-linked envelope precursor in keratinocytes that is down-regulated by retinoids," *Proceedings of the National Academy of Sciences*, vol. 89, no. 22, pp. 11026–11030, 1992.

- [241] D. Marshall, M. J. Hardman, K. M. Nield, and C. Byrne, "Differentially expressed late constituents of the epidermal cornified envelope," *Proceedings of the National Academy of Sciences*, vol. 98, no. 23, pp. 13031–13036, 2001.
- [242] K. Ishibashi, S. Sasaki, F. Saito, T. Ikeuchi, and F. Marumo, "Structure and Chromosomal Localization of a Human Water Channel (AQP3) Gene," *Genomics*, vol. 27, no. 2, pp. 352–354, 1995.
- [243] M. Olsson, A. Broberg, M. Jernäs, L. Carlsson, M. Rudemo, M. Suurküla, P.-A. Svensson, and M. Benson, "Increased expression of aquaporin 3 in atopic eczema," *Allergy*, vol. 61, no. 9, pp. 1132–1137, 2006.
- [244] X. Zhang, M. Yin, and L.-j. Zhang, "Keratin 6, 16 and 17—Critical Barrier Alarmin Molecules in Skin Wounds and Psoriasis," *Cells*, vol. 8, no. 8, p. 807, 2019.
- [245] S. Q. Gan, O. W. McBride, W. W. Idler, N. Markova, and P. M. Steinert, "Organization, structure, and polymorphisms of the human profilaggrin gene," *Biochemistry*, vol. 29, no. 40, pp. 9432–9440, 1990.
- [246] G. M. O'Regan, A. Sandilands, W. H. I. McLean, and A. D. Irvine, "Filaggrin in atopic dermatitis," *Journal of Allergy and Clinical Immunology*, vol. 122, no. 4, pp. 689–693, 2008.
- [247] A. Waseem, Y. Alam, A. Lalli, B. Dogan, N. Tidman, P. Purkis, S. Jackson, M. Machesney, and I. M. Leigh, "Keratin 15 Expression in Stratified Epithelia: Downregulation in Activated Keratinocytes," *Journal of Investigative Dermatology*, vol. 112, no. 3, pp. 362–369, 1999.
- [248] S. Werner, S. Werner, and B. Munz, "Suppression of Keratin 15 Expression by Transforming Growth Factor β *in Vitro* and by Cutaneous Injury *in Vivo*," *Experimental Cell Research*, vol. 254, no. 1, pp. 80–90, 2000.
- [249] A. Ahmed Laskar and H. and Younus, "Aldehyde toxicity and metabolism: The role of aldehyde dehydrogenases in detoxification, drug resistance and carcinogenesis," *Drug Metabolism Reviews*, vol. 51, no. 1, pp. 42–64, 2019.
- [250] M. Mueckler, C. Caruso, S. A. Baldwin, M. Panico, I. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish, "Sequence and Structure of a Human Glucose Transporter," *Science*, vol. 229, no. 4717, pp. 941–945, 1985.

- [251] Z. Zhang, Z. Zi, E. E. Lee, J. Zhao, D. C. Contreras, A. P. South, E. D. Abel, B. F. Chong, T. Vandergriff, G. A. Hosler, P. E. Scherer, M. Mettlen, J. C. Rathmell, R. J. DeBerardinis, and R. C. Wang, "Differential glucose requirement in skin homeostasis and injury identifies a therapeutic target for psoriasis," *Nature Medicine*, vol. 24, no. 5, pp. 617–627, 2018.
- [252] A. A.-H. Hodeib, Y. M.-H. Neinaa, S. S. Zakaria, and H. A.-S. Alshenawy, "Glucose transporter-1 (GLUT -1) expression in psoriasis: Correlation with disease severity," *International Journal of Dermatology*, vol. 57, no. 8, pp. 943–951, 2018.
- [253] M. Wagner, H. Hintner, J. W. Bauer, and K. Onder, "Gene expression analysis of an epidermolysis bullosa simplex Dowling-Meara cell line by subtractive hybridization: Recapitulation of cellular differentiation, migration and wound healing," *Experimental Dermatology*, vol. 21, no. 2, pp. 111–117, 2012.
- [254] K. Kligys, J. N. Claiborne, P. J. DeBiase, S. B. Hopkinson, Y. Wu, K. Mizuno, and J. C. R. Jones, "The Slingshot Family of Phosphatases Mediates Rac1 Regulation of Cofilin Phosphorylation, Laminin-332 Organization, and Motility Behavior of Keratinocytes *," *Journal of Biological Chemistry*, vol. 282, no. 44, pp. 32520–32528, 2007.
- [255] Y. Nishimura, S. Komatsu, D. Ichikawa, H. Nagata, S. Hirajima, H. Takeshita, T. Kawaguchi, T. Arita, H. Konishi, K. Kashimoto, A. Shiozaki, H. Fujiwara, K. Okamoto, H. Tsuda, and E. Otsuji, "Overexpression of YWHAZ relates to tumor cell proliferation and malignant outcome of gastric carcinoma," *British Journal of Cancer*, vol. 108, no. 6, pp. 1324–1331, 2013.
- [256] S.-J. Yin, J.-R. Lee, M.-J. Hahn, J.-M. Yang, G.-Y. Qian, and Y.-D. Park, "Tyrosinase-mediated melanogenesis in melanoma cells: Array comparative genome hybridization integrating proteomics and bioinformatics studies," *International Journal of Biological Macromolecules*, vol. 170, pp. 150–163, 2021.
- [257] W. B. Rizzo and G. Carney, "Sjögren-Larsson syndrome: Diversity of mutations and polymorphisms in the fatty aldehyde dehydrogenase gene (ALDH3A2)," *Human Mutation*, vol. 26, no. 1, pp. 1–10, 2005.
- [258] D. Ulbricht, J. Pippel, S. Schultz, R. Meier, N. Sträter, and J. T. Heiker, "A unique serpin P1/ glutamate and a conserved β -sheet C arginine are key residues for activity, protease recognition and stability of serpinA12 (vaspin)," *Biochemical Journal*, vol. 470, no. 3, pp. 357–367, 2015.

- [259] K. Kuehnle, C. , Arames, K. , Roland E., L. , Paola, B. , Susanna, P. , Alessandro, R. , Francesca, R. , Monica, K. , Luka, H. , Frank L., N. , Roger M., and M. H. and Mohajeri, "Prosurvival Effect of DHCR24/Seladin-1 in Acute and Chronic Responses to Oxidative Stress," *Molecular and Cellular Biology*, vol. 28, no. 2, pp. 539–550, 2008.
- [260] D. Di Stasi, V. Vallacchi, V. Campi, T. Ranzani, M. Daniotti, E. Chiodini, S. Fiorentini, I. Greeve, A. Prinetti, L. Rivoltini, M. A. Pierotti, and M. Rodolfo, "DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis," *International Journal of Cancer*, vol. 115, no. 2, pp. 224–230, 2005.
- [261] D. D. Bikle, A. Ratnam, T. Mauro, J. Harris, and S. Pillai, "Changes in calcium responsiveness and handling during keratinocyte differentiation. Potential role of the calcium receptor," *The Journal of Clinical Investigation*, vol. 97, no. 4, pp. 1085–1093, 1996.
- [262] Y. Moriwaki, K. Yoshikawa, H. Fukuda, Y. X. Fujii, H. Misawa, and K. Kawashima, "Immune system expression of SLURP-1 and SLURP-2, two endogenous nicotinic acetylcholine receptor ligands," *Life Sciences*, vol. 80, no. 24, pp. 2365–2368, 2007.
- [263] H. Kallio, M. Tolvanen, J. Jänis, P.-w. Pan, E. Laurila, A. Kallioniemi, S. Kilpinen, V. J. Tuominen, J. Isola, J. Valjakka, S. Pastorekova, J. Pastorek, and S. Parkkila, "Characterization of Non-Specific Cytotoxic Cell Receptor Protein 1: A New Member of the Lectin-Type Subfamily of F-Box Proteins," *PLOS ONE*, vol. 6, no. 11, p. e27152, 2011.
- [264] A. Schäbitz, C. Hillig, M. Mubarak, M. Jargosch, A. Farnoud, E. Scala, N. Kurzen, A. C. Pilz, N. Bhalla, J. Thomas, M. Stahle, T. Biedermann, C. B. Schmidt-Weber, F. Theis, N. Garzorz-Stark, K. Eyerich, M. P. Menden, and S. Eyerich, "Spatial transcriptomics landscape of lesions from non-communicable inflammatory skin diseases," *Nature Communications*, vol. 13, no. 1, p. 7729, 2022.
- [265] Y. Zeng, X. Nie, and Y. Deng, "CCL19 promotes TNF-alpha/IFN-gamma-induced production of cytokines by targeting CCR7/NF-κB signalling in HaCaT cells," *Folia Histochemica Et Cytobiologica*, vol. 62, no. 4, pp. 203–211, 2024.
- [266] J. Zhou, G. Liang, L. Liu, S. Feng, Z. Zheng, Y. Wu, X. Chen, X. Li, L. Wang, L. Wang, and Z. Song, "Single-cell RNA-seq reveals abnormal differentiation of

- keratinocytes and increased inflammatory differentiated keratinocytes in atopic dermatitis,” *Journal of the European Academy of Dermatology and Venereology*, vol. 37, no. 11, pp. 2336–2348, 2023.
- [267] E. Hams, J. Roberts, R. Bermingham, A. E. Hogan, D. O’Shea, L. O’Neill, and P. G. Fallon, “Role for Retinoic Acid-Related Orphan Receptor Alpha (ROR α) Expressing Macrophages in Diet-Induced Obesity,” *Frontiers in Immunology*, vol. 11, 2020.
- [268] H. S. Kang, K. Okamoto, Y. Takeda, J. Y. Beak, K. Gerrish, C. D. Bortner, L. M. DeGraff, T. Wada, W. Xie, and A. M. Jetten, “Transcriptional profiling reveals a role for ROR α in regulating gene expression in obesity-associated inflammation and hepatic steatosis,” *Physiological Genomics*, vol. 43, no. 13, pp. 818–828, 2011.
- [269] S. C. Lim, J. Hroudová, N. J. Van Bergen, M. I. G. L. Sanchez, I. A. Trounce, and M. McKenzie, “Loss of mitochondrial DNA-encoded protein ND1 results in disruption of complex I biogenesis during early stages of assembly,” *The FASEB Journal*, vol. 30, no. 6, pp. 2236–2248, 2016.
- [270] T. Danhelovska, H. Kolarova, J. Zeman, H. Hansikova, M. Vaneckova, L. Lambert, V. Kucerova-Vidrova, K. Berankova, T. Honzik, and M. Tesarova, “Multisystem mitochondrial diseases due to mutations in mtDNA-encoded subunits of complex I,” *BMC Pediatrics*, vol. 20, no. 1, p. 41, 2020.
- [271] T. Matsui, K. Miyamoto, A. Kubo, H. Kawasaki, T. Ebihara, K. Hata, S. Tanahashi, S. Ichinose, I. Imoto, J. Inazawa, J. Kudoh, and M. Amagai, “SASPase regulates stratum corneum hydration through profilaggrin-to-filaggrin processing,” *EMBO Molecular Medicine*, vol. 3, no. 6, pp. 320–333, 2011.
- [272] V. Moosbrugger-Martinz, C. Leprince, M.-C. Méchin, M. Simon, S. Blunder, R. Gruber, and S. Dubrac, “Revisiting the Roles of Filaggrin in Atopic Dermatitis,” *International Journal of Molecular Sciences*, vol. 23, no. 10, p. 5318, 2022.
- [273] L. Langbein, L. Eckhart, M. A. Rogers, S. Praetzel-Wunder, and J. Schweizer, “Against the Rules: Human Keratin K80: TWO FUNCTIONAL ALTERNATIVE SPLICE VARIANTS, K80 AND K80.1, WITH SPECIAL CELLULAR LOCALIZATION IN A WIDE RANGE OF EPITHELIA *,” *Journal of Biological Chemistry*, vol. 285, no. 47, pp. 36909–36921, 2010.
- [274] N. Kirschner and J. M. Brandner, “Barriers and more: Functions of tight junction proteins in the skin,” *Annals of the New York Academy of Sciences*, vol. 1257, no. 1, pp. 158–166, 2012.

- [275] S. Asad, M. Winge, C.-F. Wahlgren, K. Bilcha, M. Nordenskjöld, F. Taylan, and M. Bradley, "The tight junction gene Claudin-1 is associated with atopic dermatitis among Ethiopians," *Journal of the European Academy of Dermatology and Venereology*, vol. 30, no. 11, pp. 1939–1941, 2016.
- [276] T. Hasegawa, Z. Feng, Z. Yan, K. H. Ngo, J. Hosoi, and S. Demehri, "Reduction in Human Epidermal Langerhans Cells with Age Is Associated with Decline in CXCL14-Mediated Recruitment of CD14⁺ Monocytes," *Journal of Investigative Dermatology*, vol. 140, no. 7, pp. 1327–1334, 2020.
- [277] P. Schaerli, K. Willmann, L. M. Ebert, A. Walz, and B. Moser, "Cutaneous CXCL14 Targets Blood Precursors to Epidermal Niches for Langerhans Cell Differentiation," *Immunity*, vol. 23, no. 3, pp. 331–342, 2005.
- [278] C. Maerki, S. Meuter, M. Liebi, K. Mühlemann, M. J. Frederick, N. Yawalkar, B. Moser, and M. Wolf, "Potent and Broad-Spectrum Antimicrobial Activity of CXCL14 Suggests an Immediate Role in Skin Infections," *The Journal of Immunology*, vol. 182, no. 1, pp. 507–514, 2009.
- [279] M.-F. Champlaud, H. P. Baden, M. Koch, W. Jin, R. E. Burgeson, and A. Viel, "Gene Characterization of Sciellin (SCEL) and Protein Localization in Vertebrate Epithelia Displaying Barrier Properties," *Genomics*, vol. 70, no. 2, pp. 264–268, 2000.
- [280] H. P. Baden, M.-F. Champlaud, J. P. Sundberg, and A. Viel, "Targeted deletion of the sciellin gene resulted in normal development and maturation," *genesis*, vol. 42, no. 4, pp. 219–228, 2005.
- [281] K. Nam, S. Oh, and I. Shin, "Ablation of CD44 induces glycolysis-to-oxidative phosphorylation transition via modulation of the c-Src–Akt–LKB1–AMPK α pathway," *Biochemical Journal*, vol. 473, no. 19, pp. 3013–3030, 2016.
- [282] M. Shatirishvili, A. S. Burk, C. M. Franz, G. Pace, T. Kastilan, K. Breuhahn, E. Hinterseer, A. Dierich, L. Bakiri, E. F. Wagner, H. Ponta, T. N. Hartmann, M. Tanaka, and V. Orian-Rousseau, "Epidermal-specific deletion of CD44 reveals a function in keratinocytes in response to mechanical stress," *Cell Death & Disease*, vol. 7, no. 11, pp. e2461–e2461, 2016.