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High-throughput single-cell imaging and sorting by stimulated Raman scattering microscopy and laser-induced ejection

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HIGH-THROUGHPUT SINGLE-CELL IMAGING AND SORTING BY STIMULATED RAMAN SCATTERING MICROSCOPY AND LASER-INDUCED EJECTION

by

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ABSTRACT

Single-cell bio-analytical techniques play a pivotal role in contemporary biological and biomedical research. Among current high-throughput single-cell imaging methods, coherent Raman imaging offers both high bio-compatibility and high-throughput information-rich capabilities, offering insights into cellular composition, dynamics, and function. Coherent Raman imaging finds its value in diverse applications, including live cell dynamic imaging, high-throughput drug screening, fast antimicrobial susceptibility testing, etc. In this thesis, I first present a deep learning algorithm to solve the inverse problem of getting a chemically labeled image from a single-shot femtosecond stimulated Raman scattering (SRS) image. This method allows high-speed, high-throughput tracking of lipid droplet dynamics and drug response in live cells. Second, I provide image-based single-cell analysis in an engineered Escherichia coli
(E. coli) population, confirming the chemical composition and subcellular structure organization of individual engineered E. coli cells. Additionally, I unveil metabolon formation in engineered E. coli by high-speed spectroscopic SRS and two-photon fluorescence imaging.

Lastly, I present stimulated Raman-activated cell ejection (S-RACE) by integrating high-throughput SRS imaging, in situ image decomposition, and high-precision laser-induced cell ejection. I demonstrate the automatic imaging-identification-sorting workflow in S-RACE and advance its compatibility with versatile samples ranging from polymer particles, single live bacteria/fungus, and tissue sections.

Collectively, these efforts demonstrate the valuable capability of SRS in high-throughput single-cell imaging and sorting, opening opportunities for a wide range of biological and biomedical applications.
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4.2 Limonene-producing E. coli strains and GC-MS measurements. (a) Metabolic pathway from Acetyl-CoA to limonene in E. coli. Enzymes and some of the reaction intermediates necessary for the production of limonene are shown. AtoB: acetoacetyl-CoA synthase; HMGS: HMG-CoA synthase; HMGR: MG-CoA reductase; MK: Mevalonate Kinase; PMK: PhosphoMevalonate Kinase; PMD: PhosphoMevalonate Decarboxylase; IPP: isopentenyl pyrophosphate; idi: isopentenyl diphosphate isomerase; DMAPP: dimenthylallyl pyrophosphate; GPPS: Geranyl PyroPhosphate Synthase; GPP: geranyl pyrophosphate; LS: limonene synthase. (b) Genetic design of limonene pathway for strains with a two-plasmid system. (c) GC-MS measurements of microbial production of limonene (BW25113 strain with a two-plasmid system). Error bar: std.

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4.17 Real-time imaging and sorting of cells in culture and in tissue environment with a real-time precision opto-control (RPOC) system. (a) Real-time imaging-sorting of 1 µm polymer beads. Top to bottom: SRS image (2950 cm\(^{-1}\)) before sorting; active pixels; SRS image after sorting. (b) Real-time imaging sorting of live *S. cerevisiae*. Top to bottom: SRS image (2940 cm\(^{-1}\)) of live *S. cerevisiae* before sorting; active pixels; SRS image after sorting; culturing result of sorted *S. cerevisiae*. (c)-(d) Real-time imaging-sorting of rat brain tissue (thickness 5 µm). (c): SRS image at 2940 cm\(^{-1}\). (d): SRS image at 2850 cm\(^{-1}\).

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## List of Abbreviations

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<tr>
<td>AOM</td>
<td>Acousto-optic modulator</td>
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<tr>
<td>BODIPY</td>
<td>Boron-dipyrromethene</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CRS</td>
<td>Coherent Raman scattering</td>
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<td>DM</td>
<td>Dichroic mirror</td>
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<td>DMAPP</td>
<td>Dimenylallyl pyrophosphate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FT</td>
<td>Fourier-transform</td>
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<td>GPP</td>
<td>Geranyl pyrophosphate</td>
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<td>GT</td>
<td>Glycerol trioleate</td>
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<td>HWP</td>
<td>Half-wave plate</td>
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<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
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<td>IPTG</td>
<td>Isopropyl $\beta$-d-1-thiogalactopyranoside</td>
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<td>LD</td>
<td>Lipid droplet</td>
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<tr>
<td>LIA</td>
<td>Lock-in amplifier</td>
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<tr>
<td>LS</td>
<td>Limonene synthase</td>
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<tr>
<td>MDC</td>
<td>Monodansycladaverine</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymer chain reaction</td>
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<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<td>PS</td>
<td>Polystyrene</td>
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<tr>
<td>QWP</td>
<td>Quarter-wave plate</td>
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<td>RACE</td>
<td>Raman activated cell ejection</td>
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<td>RACS</td>
<td>Raman-activated cell sorting</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SRS</td>
<td>Stimulated Raman scattering</td>
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<td>UV</td>
<td>Ultraviolet</td>
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Chapter 1

INTRODUCTIONS

1.1 Motivation and overview

Heterogeneity is a fundamental attribute of cellular systems, providing important biological functions and containing meaningful information [1]. Various biological applications are enabled by exploiting cellular heterogeneity, like sorting super producers for renewable biofuel production, identifying unknowable or uncultivable cells by their functions, and detecting cancerous cells for diagnosis, to name a few. Ensemble measurements such as those obtained through powerful biological imaging tools like magnetic resonance imaging (MRI), computerized tomography (CT), and positron emission tomography (PET), offer valuable insights into phenotypic and functional changes at the organ or tissue level [2]. Omics technologies, on the other hand, aim to reveal the relationship between genotype and phenotype [3]. Yet, these techniques often provide population-averaged results, simplifying the complex cellular population and primarily reflecting dominant biological mechanisms. It is crucial to recognize that results derived from ensemble averages may not accurately represent subpopulations of cells, potentially leading to inaccurate models of cellular states. Recent refinements in mass spectrometry (MS) have led to single-cell proteomics and single-cell metabolomics but are not applicable to live cells [4]. To better understand cellular heterogeneity and gain insights into single-cell states and functions, single-cell analysis and manipulation tools are crucial.

Microscopic imaging offers an intermediate layer to integrate omics-data with spa-
tial information at different levels [2, 5, 3]. With the advancements in all facets of microscopic imaging, various sorting contrasts have been explored to sort single cells. For physical properties like morphology, deformability, and electric changes, cells can be sorted without labeling. For other cellular biological characteristics, affinity methods could be applied, e.g. fluorescent or magnetic labels, and then activate cell sorting based on protein expression level [6]. Aside from cell sorting contrast, different cell sorting schematics have been applied, including microfluidics, laser capture microdissection, limiting dilution, etc. depending on the nature of the sample and the analysis to be performed on the cells [7]. In the following paragraphs, I will introduce more of the current technologies for single-cell analysis with a focus on single-cell chemical imaging, and discuss the current challenges for high-throughput single-cell analysis.

1.2 Technologies for single-cell imaging

1.2.1 Overview

Single-cell analysis offers opportunities to unveil the single-cell states and functions of a heterogeneous population. There are multiple perspectives to consider when choosing single-cell analysis tools, including information content, throughput, specificity, sensitivity, cell integrity, etc. [7, 8]

For information content, single-cell analysis can provide characterization of genomics, transcriptomics, proteomics, metabolomics, etc. Multiple modalities have been explored, ranging from single-cell genome sequencing, single-cell mass spectroscopy, and single-cell imaging.

In the introductory sections of this thesis, my primary emphasis will be single-cell imaging, with a specific focus on chemical imaging.

Chemical imaging has multiple modes: optical absorption, emission (fluorescence), and scattering (Raman). Fluorescence imaging has been a powerful tool in biological
study since its first discovery. However, it suffers from cytotoxicity and potential perturbation of cellular functions. For small molecules, it may be challenging to have specific probes and the probe may significantly perturb the biological function. Label-free imaging methods like transmission, phase contrast, and differential interference contrast offer refractive index distribution and morphological features. Yet, these features may not be directly linked with cellular states or biological functions. Recently, \textit{in silico} labelling methods have been reported, with the capability to predict fluorescent labels in unlabeled cell images [9, 10], while these methods also have potential limitations in the number of multiplexed fluorescent labels.

1.2.2 Spontaneous Raman spectroscopy

Among all the label-free imaging modalities, Raman spectroscopy offers chemical content information at a single-cell level with high biocompatibility and high chemical specificity.

Spontaneous Raman was first discovered by Dr. C. V. Raman and coworkers in the 1920s [11]. This technique detects the inelastic photon scattering induced by the vibrational modes of molecules. By capturing the energy shift of the laser after interacting with the molecules, the chemical content of the sample could be decomposed. Single-cell Raman spectrum can provide over 1000 Raman bands, characterizing cellular nucleic acid [12], protein [13], lipids [14], etc. The molecular fingerprint further provides insights into the physiological states, phenotypes, and genotypes of the cell [15]. Raman spectroscopy, with its capacity for precise biochemical analysis at the individual live cell level, has proven to be a powerful tool in biological studies and clinical diagnosis. However, due to its intrinsically small cross-section (in the range of $10^{-23}$ to $10^{-30} \text{ cm}^2$) [16], a typical integration time for Raman spectroscopy of a biological sample ranges from 15 to 60 seconds to obtain a sufficient signal-to-noise ratio [17].
Recent research progress in enhancing the signal of spontaneous Raman spectroscopy includes resonance Raman spectroscopy, coherent Raman scattering, and surface-enhanced Raman scattering. In resonance Raman spectroscopy, the frequency of the excitation laser beam is tuned to be close to an electronic transition, thus the vibrational modes associated with the particular transition will be greatly enhanced. However, the requirement for wavelength matching of the excitation laser limits its applicability. For surface-enhanced Raman scattering, nano-scaled metal structures were applied, and the signal can be enhanced by $10^6$ to $10^{14}$ orders. However, the need to co-culture the cells with nanoparticles or substrate restricts its applications.

### 1.2.3 Coherent Raman scattering microscopy

Thanks to the advancement of laser technology, these difficulties have been overcome by advancements in coherent Raman scattering (CRS), which is based on stimulated Raman scattering (SRS) or coherent anti-Stokes Raman scattering (CARS) since their first demonstrated about 40 years ago [18, 19]. With better laser sources, detectors, and signal-processing techniques, both CARS and SRS have greatly evolved and exhibit broad applications in biological research. Instead of one excitation laser in spontaneous Raman, CRS utilizes two laser beams termed pump ($\omega_p$) and Stokes ($\omega_S$). When the energy difference between these two laser beams ($\omega_p - \omega_S$) matches a particular molecular vibrational frequency $\Omega$, the Raman loss/gain in the SRS process or the new frequency in the CARS process could be detected. For CARS, the new frequency at $(\omega_p - \omega_S) + \omega_p$ was usually detected by a photomultiplier tube. For SRS, the stimulated Raman gain (SRG) appears at $\omega_S$ and the stimulated Raman loss (SRL) appears at $\omega_p$. Using a photodiode detector, the SRS signal can be detected and then extracted by a demodulator. Specifically, the intensity of the pump or Stokes is modulated at a megahertz level, and a lock-in amplifier is used to extract the small variation induced by SRL or SRG. Compared to CARS, SRS
does not contain nonlinear background and has a linear dependence on the molecular concentration [20].

Early SRS microscopy utilizes laser beams with fixed frequency and allows high-speed imaging of a single Raman band [21, 22]. It achieves video rate imaging but lacks spectral selectivity. With the aid of spectral focusing, hyperspectral SRS imaging is achieved and provides more informative spectral signatures [20, 23]. In the spectral focusing scheme, two broadband (femtosecond) laser beams are chirped by a dispersive medium, and a time delay is used to scan the spectrum. However, the frame-by-frame imaging scheme limits imaging speed and it is inapplicable to dynamic live samples. Fast delay line tuning was later implemented by resonant mirror [24].

By harnessing the sparsity of the hyperspectral image stack, the sub-sampling method is introduced to break the tradeoff between imaging speed and spectral selectivity. Berto et al. combined a digital micromirror device with broadband SRS and applied multiplexed Hadamard spectral basis with compressive sensing detection [25]. In the work of Lin et al., the samples were scanned by sparse points in Lissajous trajectories, and then the subsampled image stack was decomposed into chemical concentration maps [26]. Another work utilized a spatial light modulator to select the dominant spectral bands in SRS imaging. In addition to these aforementioned subsampling strategies, the recent resurgence of deep neural networks (DNNs) also shows great potential in enhancing SRS imaging performance, e.g. reducing potential photodamage [27], and increasing chemical imaging speed [28].

There have been extensive applications using SRS microscopy for cell imaging. The most widely used spectral regions include C-H (2800-3100 cm\(^{-1}\)), silent region (1800-2700 cm\(^{-1}\)), and fingerprint region. The strong SRS signal in the C-H region provides a detailed characterization of C-H rich biomolecules, e.g. lipids, protein,
and collagen [14, 13]. By taking advantage of the large Raman cross-section of lipids, Freudiger et al. used the SRS signal at 3015 cm\(^{-1}\) to visualize fatty acid uptake in live cells [29]. In vivo quantification of lipids by SRS imaging also enabled novel genetic regulators of fat storage [30]. Wang et al. imaged live Caenorhabditis elegans in the fingerprint region and obtained quantification of lipid metabolism after decomposing the hyperspectral image stack [31]. High-throughput SRS imaging and single-cell analysis recently revealed the metabolism reprogramming from glucose and glycolysis dependent to fatty acid uptake and beta-oxidation dependent in cisplatin-resistant cells [32]. Other uses of SRS in deciphering the chemical composition of single cells include drug uptake and distribution. Fu et al. showed the enrichment of imatinib and nilotinib drugs in single BaF3 cells using hyperspectral SRS microscopy [33]. Also, antifungal drug distribution in single fungal cells was visualized by polarization-sensitive SRS imaging, resolving the drug assembly in the cell membrane [34]. Using a deuterated analog of the general anesthetic propofol, the dynamics of propofol binding and its interaction with neuron membrane were visualized for the first time. Except from lipid and small-molecule drugs, other functional molecules investigated by SRS include nucleic acid [35], acetylcholine [36], retinoids [37], and squalene [38].

In conjunction with stable isotope labeling, SRS provides rapid single-cell antibiotic susceptibility testing (AST) by quantitatively tracking the incorporation of glucose-d\(_7\) or D\(_2\)O into biomass in live bacteria [39, 40]. Carbon dynamics of storage organelles in Euglena gracilis were also tracked via 13C isotope labeling and SRS microscopy [41]. Taking advantage of the fluorescence capability of SRS microscopy, identity analysis can be achieved by fluorescence in situ hybridization (FISH) complementing the functional analysis provided by SRS imaging.

Thanks to the high dimensionality of SRS imaging, morphological features, and subcellular organelle structure could be quantitatively evaluated in a high-throughput
manner. Huang et al. developed multiplex SRS imaging cytometry and achieved high-content single-cell analysis with more than 260 features from single-cell and sub-cellular organelles [42]. By integrating SRS and fluorescence microscopy, Shou demonstrated multiplex time-lapse imaging and image-based cytometry [43]. In a recent report, Tan et al. present high-content single-cell metabolism analysis by investigating the sparsity of the spectral domain and achieving simultaneous mapping of five major biomolecules [44]. Except for single-cell analysis, SRS imaging-based histology can also be successfully applied in a pathology workflow [45]. Thanks to the recent resurgence of convolutional neural networks, it is possible to directly learn histologic features from SRS images, paving the way for AI-assisted diagnosis [46, 47, 48]. Assisted by laser capture microdissection, histologically resolved multiomics was realized by precisely ejecting the regions of interest in SRS images of human oral squamous cell carcinoma and quantifying gene expression [49].

In summary, SRS imaging offers high biocompatibility and high imaging speed. The information provided by SRS imaging includes chemical composition, functional activity, and morphological and subcellular organelle features.

1.3 Technologies for single-cell isolation

1.3.1 Overview

Alongside single-cell imaging, single-cell sorting serves as an indispensable tool for achieving a more comprehensive analysis of the heterogeneity within a “seemingly identical” cell population. Among all the cell sorting contrasts discussed in the last chapter, the following paragraphs will mainly focus on Raman-activated cell sorting (RACS).
1.3.2 Raman-activated cell sorting

Taking advantage of its high biocompatibility and high chemical specificity, a series of spontaneous Raman-activated cell sorting platforms have been developed. These platforms sort single cells based on the phenotypic characteristics provided by the Raman signatures. Thanks to its label-free nature, cells can be differentiated without exogenous labels. After cell sorting, additional analytic methods, including genomics, transcriptomics, proteomics, etc. could be applied to further characterize the identity, functionality, and composition of the cells of interest.

Based on the cell sorting techniques utilized, RACS can be characterized into 4 main categories: Raman-activated cell ejection (RACE), Raman-activated microfluidic sorting (RAMS), and trapping-based Raman-activated cell sorting [50, 51]. For RAMS, Raman spectra are collected while the cells are in motion. While the cells are static in RACE and trapping-based sorting schemes.

Microfluidic systems emerged in the 1980s and can handle small volumes of liquid precisely. RAMS integrates Raman spectroscopy and microfluidic equipment and accomplishes the separation of single cells based on Raman signature. Another advantage offered by RAMS is the high cell viability maintained by the fluidic environment, which is vital for downstream single-cell culture. The RAMS device is typically composed of a flow channel made of polydimethylsiloxane (PDMS) and glass subassemblies. The cells are loaded to the device opening and driven by the pressure of a flow pump. Since the 2000s, there have been lots of efforts to improve the throughput, sensitivity, and specificity of the RAMS systems. Wang et al. reported Raman-activated droplet sorting of single microalgal cells with a throughput of 260 cells per minute, which is the highest in the current reported full spectrum RACS system [52, 53].

For cell trapping-based Raman-activated cell sorting, the cells are trapped from the flow stream for Raman measurements and these platforms achieve flexible signal
integration time compared to non-trapping schemes. Current cell trapping techniques include laser tweezers and dielectrophores. Optical tweezers generate optical potential gradients that could be utilized to manipulate single cells in a non-contacting way. Manual sorting of the cells in a microcapillary with optical tweezers only offers very low throughput [54, 55]. In 2019, Lee reported an automated Raman-based platform for sorting live cells with an optical tweezer. Vertical focusing was employed for a higher capture rate and to minimize the background signal from the PDSM chamber. This automatic platform achieved high-precision classification (98.3% ± 1.7%) and a throughput 3.3-8.3 cell/min. Using periodical positive dielectrophoresis induced deterministic lateral displacement (pDEP-DLD) force, fast-moving cells can be trapped for efficient Raman measurement [51]. This work achieved a throughput of 30-2700 events per minute for nonresonance and resonance Raman bands and demonstrated applications including single microorganisms and single cancer cells.

When handling the cell samples from the natural environment, microfluidics-based sorting techniques require suspension and dilution that may result in cell loss and interference with the original cell states. Different from the techniques mentioned above, RACE handles samples that are directly deposited on a coverslip with a laser-absorbing coating. It is based on laser-induced forward transfer (LIFT) which is widely used in material transfer and bioprinting [56]. In SRACE, after acquiring Raman spectra of individual cells, a nanosecond laser acts on the target cell location. The coating then absorbs the laser energy and provides forward momentum for the cells to be sorted and received [50]. RACE was first demonstrated by Wang et al. in 2013. In 2018, Jing introduced an “all-in-one” device integrating multiple functions and successfully linking the phenotype and genotypes of carbon-fixing bacteria at a single-cell level. A recent improvement provided by Liang et al. used 3-layer LIFT systems and achieved successful cell recovery after RACE [57]. Nonetheless, the
throughput of RACE is still low around 2-3 cells per minute [58].

RACS has found wide applications in microbiology for isolating functional individuals from a community. It has been illustrated to differentiate antibiotic-resistant bacteria [59, 60, 61] or microbes that possess specific pathways in a complex environment such as the human gut [60], or natural ecosystems [58, 59, 62].

Despite the advantages of its biocompatibility and high chemical specificity, spontaneous Raman-activated cell sorting suffers from the relatively low throughput due to its intrinsically small cross-section.

1.3.3 Coherent Raman-activated cell sorting

Advancements in coherent Raman-activated cell sorting have been reported recently, taking advantage of signal enhancement in coherent Raman scattering. The first generation of coherent Raman flow cytometry only provides a single-cell CARS spectrum per cell and throughput of 100 events per second (eps) [63]. Fourier-transform CARS flow cytometry reported by Kinegawa et al. obtained broadband Raman spectrum in the fingerprint region and achieved throughput of around 2000 eps [64]. Further coupling of the FT-CARS platform and a cell sorter was recently demonstrated by Lindley et al. achieved a throughput of 50 cells per second. Apart from CARS flow cytometry, SRS flow cytometry was first demonstrated by Zhang et al. with a 32-channel photodiode array [65]. In 2020, Nitta et al. reported the integration of the SRS imaging microscope, real-time image processor, and a cell sorter [66]. This platform achieved the real-time imaging and sorting of single cells. However, these coherent Raman-activated cell sorting mainly focuses on microfluidic techniques. It achieved high imaging and sorting speed but encountered challenges in handling small cells (<3 μm) [66]. Besides, the unstable flow caused by the debris and/or bubbles in the microfluidic chamber might perturb laser focusing and reduce the Raman signal from the cell [67].
In summary, RACS has demonstrated wide applications in biological and biomedical studies but with limitations in the low throughput. Coherent Raman-activated cell sorting enables single-sorting with a throughput of up to 100 eps but suffers from the intrinsic limitations of microfluidic design. In the future, new regimes are needed to achieve better cell sorting performance with high throughput, high specificity, and high cell viability.

1.4 Dissertation objectives

In this dissertation, I explore the methodologies to advance the application of SRS microscopy in high-throughput single-cell analysis.

In Chapter 2, two SRS imaging schemes were discussed. One is hyperspectral SRS imaging with spectral focusing. Another is single-frame SRS imaging with non-chirped femtosecond pulses. To leverage the strengths of both optical setups, I proposed and developed a deep learning model, breaking the trade-offs between imaging speed, chemical specificity, and signal-to-noise ratio. In achieving this goal, I collected about 1000 hyperspectral images of cancer cells as the training set. I developed a spatial-spectral hyperspectral image segmentation method to generate maps of subcellular organelles as the ground truth. Four subcellular structures can be successfully segmented with this proposed method, including lipid droplet, endoplasmic reticulum, nucleus, and cytoplasm, confirmed with fluorescence imaging. For the deep learning model, I used dense-net architectures as the base structure. With this pre-trained model and test-time augmentation, subcellular organelle maps were successfully predicted from single-shot femtosecond SRS images, and speed was improved by about 2 orders of magnitude compared to conventional frame-by-frame hyperspectral imaging. To further demonstrate the utility of this method, SRS imaging of lipid droplet dynamics in live cells was performed. In addition, high-throughput
drug response analysis was achieved, where 400 images can be collected in less than 10 minutes with four subcellular structures classified. Collectively, this method solved the inverse problem of getting a chemically labeled image from a single-shot femtosecond SRS image, demonstrating how computational methods can augment the capabilities of SRS microscopy.

In Chapter 3, I discuss the challenges in profiling the metabolic-engineered microorganisms in renewable biofuel applications. To address these challenges in limonene-producing \textit{E. coli} samples, I utilized SRS microscopy and image-based single-cell analysis. First, I found the signature Raman band in limonene-producing \textit{E. coli} cells. Second, I demonstrated the polygon scanner-based SRS achieved higher sensitivity in limonene detection. Third, SRS imaging successfully visualized limonene-rich aggregates in live limonene-producing \textit{E. coli} cells. A distinct subpopulation of producers and non-producers was also observed. With spectral unmixing and image segmentation, limonene content was quantified at the subcellular level. By establishing a pipeline to extract the morphological features of each cell, asymmetric aggregate localization was also evaluated. Last but not least, the synthetic function of the limonene-rich aggregates was revealed by tagging the limonene synthase with green fluorescent protein (GFP) and imaging with two-photon fluorescence along with SRS. Collectively, SRS imaging provided direct visualization of the chemical composition and synthetic function of the limonene-rich aggregates inside engineered \textit{E. coli}. Further integration of SRS microscopy with cell sorting and recultivation will be discussed in Chapter 3.

In Chapter 4, I investigate the compatibility of laser-induced cell ejection with SRS microscopy for SRS-activated cell sorting. The common challenges in microfluidics-based cell sorting include handling small cells and perturbation of imaging focus due to unstable flow. Laser-induced cell ejection overcomes these challenges by adopting a
specialized coverslip with a laser-absorbing coating. In this chapter, I first introduce
the optimization of laser-absorbing coating and TiO2 shows good performance in
both SRS imaging and ejection. Second, I constructed the ejection module and inte-
grated it with SRS microscope. The platform is termed stimulated Raman-activated
cell ejection (S-RACE). With acousto-optic modulator as a fast pulse picker, each
ejection operation takes about 8 ms. For a mixture of 1.0-µm polymer beads, S-
RACE achieved a high yield of approximately 95%, a high purity level of around
98%, with a throughput of approximately 14 events per second (eps). In addition, I
demonstrated fast identification and sorting of lipid-rich \textit{R. glutinis} cells from a yeast
cell mixture and confirmed the result by quantitative PCR amplification. A notable
advantage of our platform is the live cell sorting capability. Furthermore, by har-
nessing a comparator circuit for communication between imaging and laser ejection,
real-time SRS-guided sorting is demonstrated on a variety of samples including poly-
mer beads, live cells, and regions of interest in a brain tissue slice. Collectively, the
reported S-RACE platform opens exciting opportunities for a wide range of single-cell
applications in biology and medicine.
Chapter 2

CHEMICAL IMAGING BY DENSE-NET LEARNING OF FEMTOSECOND STIMULATED RAMAN SCATTERING

The work presented in this chapter was published in the Journal of Physical Chemistry Letters [28]. Reprinted with permission from ACS publications.

Hyperspectral stimulated Raman scattering (SRS) by spectral focusing can generate label-free chemical images through temporal scanning of chirped femtosecond pulses. Yet, pulse chirping decreases the pulse peak power and temporal scanning increases the acquisition time, resulting in a much slower imaging speed compared to single-frame SRS using femtosecond pulses. In this paper, we present a deep learning algorithm to solve the inverse problem of getting a chemically labeled image from a single-frame femtosecond SRS image. Our DenseNet-based learning method, termed as DeepChem, achieves high-speed chemical imaging with a large signal level. Speed is improved by 2 orders of magnitude with four subcellular components (lipid droplet, endoplasmic reticulum, nuclei, cytoplasm) classified in MIA PaCa-2 cells and other cell types that were not used for training. Lipid droplet dynamics and cellular response to dithiothreitol in live MIA PaCa-2 cells are demonstrated using this computationally multiplex method.
2.1 Introduction

Vibrational spectroscopic imaging of cellular and tissue structures is opening a new window for cell biology research and clinical diagnosis [68, 69, 70]. Stimulated Raman scattering (SRS) microscopy is a vibrational imaging technique with label-free chemical specificity [29, 71]. SRS microscopy has been implemented with both picosecond and femtosecond pulses, respectively, allowing the single-color and hyperspectral imaging capabilities [20, 72]. Despite these advances, the trade-off between speed, signal-to-noise ratio (SNR), and spectroscopic bandwidth prevents its broader application in biology and biomedicine. Using picosecond pulse trains, video-rate SRS imaging was realized via a fast lock-in amplifier [22]. SNR was tenfold increased using femtosecond pulse excitation because of the integration over a spectral window compared to picosecond pulse excitation [21]. Although single-shot femtosecond SRS imaging permits real-time skin imaging in live mice and cellular metabolism quantification [73], it lacks spectroscopic information and thus cannot discriminate chemical components with overlapping Raman signatures. Spectral focusing provides an efficient method for femtosecond pulse-based hyperspectral SRS (hSRS) measurements by linear chirping of pump and Stokes pulses [74, 23]. However, the speed of time-delay scanning for parallel detection of several Raman bands is often limited by the motorized translational stage due to the waiting time for communication and stabilization [24]. Improved frequency tuning via a galvanometer mirror has reached a speed of seconds per stack [24, 75]. Nevertheless, just as illustrated in Fig. 2-1, tens to hundreds of times more measurements are needed for an SRS stack than a single-frame image and limits further improvement of its imaging speed. Multiplex SRS enabled single spectrum recording within several microseconds by wavelength-division or modulation-division [76, 77, 78]. These multiplex designs, however, either came with deterioration in SNR or loss of spectral selectivity. Collectively, the lim-
limitations of each modality highlight a need to fill the gap between the low spectral resolution in femtosecond pulse excitation and the low speed in hyperspectral measurement, aiming at a high-speed, high-SNR, hyperspectral SRS imaging method.

In parallel with instrumentation development, computational approaches have been applied to boost the speed and/or SNR in SRS microscopy. With prior knowledge of the low-rankness of SRS images, sparse sampling methods improved the SRS imaging speed with linear models [79, 25, 26]. Thanks to the recent resurgence of deep neural networks (DNNs), image interpretation and translation problems can be resolved via direct learning of the underlying image mapping relation [80]. A recently reported U-Net DNNs-based algorithm for SRS image denoising shows applicability to reduce potential photodamage and enable deep tissue imaging [27]. Other applications of DNNs to optical microscopy include image translation [9, 10], denoising [27, 81], super-resolution [82], cross-modality image fusion [83], focus correction [84], and transmission correction [85], largely in the fluorescence microscopy field.
Figure 2.1: Schematic of SRS imaging and workflow of DeepChem training and prediction. (a) Schematic of two SRS imaging schemes in the 3D domain of time, Raman shift, and intensity. Left: hyperspectral SRS imaging with linearly chirped pulses. Right: single-frame SRS imaging with non-chirped femtosecond pulses. (b) The workflow of DeepChem training and prediction. The training set consists of pairs of spectrally summed hyperspectral SRS images and their corresponding subcellular organelle maps from Phasor-MRF. After training, DeepChem is capable of predicting subcellular organelle maps based on a single-frame femtosecond SRS image. MRF: Markov Random Field.
In this work, we tackle the trade-off between spectral specificity, speed, and SNR by learning the correlation between spectral and spatial features to derive chemical maps from a high-speed femtosecond SRS image. Specifically, we deploy a customized DNN model, namely a DenseNet-based neural network architecture [86, 87]. DenseNet has significant advantages over conventional DNNs such as U-Net in computer vision problems, including semantic segmentation [88]. By introducing connections between each layer, DenseNet has achieved advanced performance, such as the alleviation of gradient vanishing, reduction of the number of parameters, and encouraging feature reuse [86]. We term our DenseNet-based DNN as DeepChem. The pairs of spectrally summed hSRS images of MIA PaCa-2 cells and the spatially segmented subcellular organelle maps are used for training DeepChem. Then the well-trained network is capable of generating subcellular organelle maps using femtosecond SRS images (Fig. 2·1b). Based on this method, the needed frame number is reduced to one while the chemical selectivity of four subcellular components (lipid droplet, endoplasmic reticulum, nuclei, and cytoplasm) is preserved. Thus, the trade-off between high SNR, chemical selectivity, and speed qualities is broken, without additional need for fluorescent labels, parameter estimation, or hardware design.

2.2 Experimental design and methods

2.2.1 Optical setup for SRS microscope

In this work, SRS images were acquired using a homebuilt SRS microscope as in [21] (Fig. 2·2). The laser is a Spectra-Physics Insight DeepSee. The center wavelengths of the tunable beam (pump) and the fixed beam (Stokes) are 798 nm and 1040 nm, respectively. As we note in the main text, two SRS imaging schemes were applied. One is hyperspectral SRS imaging with spectral focusing where both the pump and Stokes were chirped with high-dispersion glass (SF57, 90 cm in length), and the hSRS
images were collected via spectral scanning controlled by a motorized translational stage. Another is single-frame SRS imaging with non-chirped femtosecond pulses. In both schemes, the dwell time is set at 10 µs to maintain a moderate imaging speed with good SNR. The microscope is equipped with a 60x water immersion objective (NA = 1.2, UPlanApo/IR, Olympus) and oil condenser (NA = 1.4, U-AAC, Olympus). The schematic of these two imaging schemes in the time, Ramam shift, and intensity domain is illustrated in Fig. 2·1a.

**Figure 2·2:** Optical setup for SRS microscope. Dashed lines denote the light path with non-chirped femtosecond pulses. AOM, acousto-optic modulator; GV, galvanometer mirror; Obj, objective; C, condenser; F, filter; PD, photodiode; LIA, lock-in amplifier. 4f system before the Objective contains a pair of lens (Φ1 = 150 mm, Φ2 = 150 mm).
2.2.2 Phasor-MRF for hyperspectral image segmentation

For the collected hSRS images, we implemented a hyperspectral image segmentation method based on Phasor analysis and Markov Random Field (Phasor-MRF) to incorporate both spectral and spatial features in segmenting the subcellular organelle maps. This framework is inspired by the spectral-spatial hyperspectral image segmentation methods used in the remote sensing community [89, 90]. By fusing the reciprocal spectral and spatial domains, better segmentation quality is achieved with less human operation time compared to the conventional phasor analysis in ImageJ [91].

The initial subcellular organelle label map is produced by manual phasor segmentation [92]. Dimension reduction is applied via Principle Components Analysis (PCA). In the following maximum a posteriori (MAP) step, two penalty terms \( U(x) \) and \( U(y|x) \) are calculated for each pixel as follows [90].

\[
U(x) = \sum_{c \in C} V_c(x_i, y_i) \quad (2.1)
\]

\[
V_c(x_i, y_i) = \frac{1}{2} (1 - I_{x_i, y_i}) \quad (2.2)
\]

\[
U(y|x) = \sum_i \left( \frac{(y_i - \mu_{x_i})^2}{2\sigma_i^2} + \frac{1}{2\sigma_i^2} \right) \quad (2.3)
\]

In equations 2.1-2.7, \( x_i \) is the class label; \( y_i \) is the spectrum after dimension reduction; \( C \) is the set of all possible cliques; \( V_c(x_i, y_i) \) is the clique potential; \( I_{x_i, y_i} \) is the Ising function. \( U(x) \) denotes the penalty term that encourages spatial smoothness. \( U(y|x) \) measures the fit within each label class. Thus, the MAP step can be written as a
minimization optimization problem.

\[
\hat{x} = \arg \min_{x \in X} U(x) + U(y|x)
\] (2.4)

\(\mu_x\) and \(\sigma_x\) in equation 2.5-2.7 are the parameters of each label class (assumed as Gaussian distribution) and updated in the Expectation-Maximization (EM) below, where the posterior \(q^{(k)}(j|y_i)\) is obtained in the MAP step above.

\[
\mu_l^{(k+1)} = \frac{\sum_i q^{(k)}(j|y_i)y_i}{\sum_i q^{(k)}(j|y_i)}
\] (2.5)

\[
\sigma_l^{2(k+1)} = \frac{\sum_i q^{(k)}(j|y_i)(y_i - \mu_l^{(k+1)})}{\sum_i q^{(k)}(j|y_i)}
\] (2.6)

The above procedures are repeated until it reaches error convergence. Phasor-MRF largely reduces laborious labeling work and improves labeling accuracy. One sample image is shown in Fig. 2·3.

(a) Spectrally summed hyperspectral SRS (b) Manual phasor label (c) Phasor-MRF

**Figure 2·3:** Phasor-MRF performance. (a) Spectrally summed hyperspectral SRS image of MIA PaCa-2 cells. (b) Spatially separated subcellular map from manual phasor segmentation. (c) Spatially separated subcellular component map from Phasor-MRF. Bkg, LD, ER, Nuclear, Cyto are abbreviations for background, lipid droplet, endoplasmic reticulum, and cytoplasm.
In practice, we updated $\mu_i$ and $\sigma_i$ per row rather than per pixel for higher calculation efficiency. Besides, we applied two parameters $\alpha$ and $\beta$ as row vectors to equation 2.2.2 to refine the two penalty terms (equation 2.7). In most cases, fine-tuning $\alpha$ and $\beta$ is not needed and can be fixed at [1111]. While for some datasets, we use $\beta = [110.951]$ to accommodate different experiment conditions.

$$\hat{x} = \arg\min_{x \in X} \alpha U(x) + \beta U(y|x)$$  \hspace{1cm} (2.7)

Comparing the subcellular organelle maps from Phasor-MRF and fluorescent images in Fig. 2.4, it becomes evident that the Phasor-MRF method is a reliable subcellular organelle segmentation.

Figure 2.4: Comparison between the subcellular component maps from Phasor-MRF and fluorescent images. (a) The averaged spectrum of different subcellular components (averaged across 55 images). ER is the abbreviation for endoplasmic reticulum. (b) Comparison between subcellular component maps from Phasor-MRF and fluorescence images. Top, second, and third rows show MIA PaCa-2 cells labeled with BODIPY, ER tracker, and PI, respectively.
2.2.3 Comparison between spectrally summed hyperspectral SRS and femtosecond SRS

We show that DeepChem is trained on pairs of spectrally summed hyperspectral SRS (hSRS) images and their corresponding subcellular component maps. Then the model is tested on single-frame femtosecond SRS. Thus it is of interest whether the single-frame femtosecond SRS image can be approximated by the spectrally summed hSRS image. To validate the equivalence between these two imaging schemes, we first define the electrical field of both pump and Stokes beams after chirping, as equations below.

\[ E_p = E_{0p} e^{\left(-\frac{(t - t_0)^2}{\tau_G^2}\right)} e^{i(\omega_S + \beta t) t} \]  \hspace{1cm} (2.8)

\[ E_S = E_{0S} e^{\left(-\frac{(t - t_0)^2}{\tau_G^2}\right)} e^{i(\omega_S + \beta(t - t_0))(t - t_0)} \]  \hspace{1cm} (2.9)

\[ E_p \] and \[ E_S \] are the optical field amplitudes of, the Stokes and pump beam, respectively. \[ E_{0p} \] and \[ E_{0s} \] are the amplitude of the pump and Stokes fields. \( \omega_p \) and \( \omega_S \) are central frequencies of the pump and Stokes fields. \( \tau_G, t_0, \) and \( \beta \) represent the pulse duration, inter-pulse delay, and chirp of the propagating pulses. Then, the signal intensity of the pump beam stimulated Raman loss (SRL) is written as in the equation below.

\[ \delta I = \propto -I_p I_S \propto |E_p^2||E_S^2| = E_{0p} E_{0s} e^{\left(-\frac{t^2}{\tau_G^2}\right)} e^{\left(-\frac{(t - t_0)^2}{\tau_G^2}\right)} \]  \hspace{1cm} (2.10)

Thus, \( hSRS(t_n) \) denoting the signal at each frame of hSRS can be represented as the equation below.

\[ hSRS(t_n) \propto \int_k P_S(\omega_S(t, k)) P_p(\omega_p(t, k)) \]  \hspace{1cm} (2.11)
After discretization, it becomes

\[ hSRS(t_n) \propto \sum_{k \in K} P_S(\omega_S(t_n, k))P_p(\omega_p(t_n, k)) \]  

(2.12)

Here, \( P_S(\omega_S(t_n, k)) \) and \( P_p(\omega_p(t_n, k)) \) are input power of the Stokes and pump beam after chirping respectively at time \( t \) and overlapping frame \( k \), and \( k \) in \( K = \{ k | \omega_p(t_n, k) - \omega_S(t_n, k) = \delta \omega(t_n) \} \). A detailed illustration is shown in Fig. 2.5.

**Figure 2.5:** Illustration of pulse chirping and vibrational mode excitation at each frame of hyperspectral SRS stack. All pump pulses (blue) and Stokes pulses (yellow) are in Gaussian shape.

Thus the spectrally summed hyperspectral SRS and femtosecond SRS can be expressed in the equation below. \( P_p(\omega_p0) \) and \( P_s(\omega_s0) \) denote the power for pump and Stokes at central wavelength after chirping.

\[ \int_t hSRS(t_n) \propto \int_t \sum_{k \in K} P_S(\omega_S(t, k))P_p(\omega_p(t, k)) \]  

(2.13)

Similarly, denoting signal intensity for femtosecond SRS can be expressed as:

\[ fsSRS \propto \sum_{t_1 \ldots t_n} P_S(\omega_n) \sum_{t_1 \ldots t_n} P_p(\omega_n) \]  

(2.14)
The spectral full width half maximum (HWFM) for both pump and Stokes beams are 7 nm. After being linearly chirped by several SF57 rods (90 cm in length for the Stokes beam, 75 cm in length for the pump beam), pulse durations of the pump and the Stokes beam were stretched to 1.3 and 0.8 ps. Based on these calculations, the left side of equation 2.14 can be simplified to \(205.2717P_p(\omega_{p0})P_s(\omega_{s0})\). Because both the signal of femtosecond SRS and the signal of the spectrally summed hSRS can be expressed proportionally to the product of \(P_p(\omega_{p0})\) and \(P_s(\omega_{s0})\), the difference between these two can be written as proportional to \(1.7371 \cdot 10^{-6}P_p(\omega_{p0})P_s(\omega_{s0})\). It is negligible compared to the signal \(fsSRS\). Then it justifies our training-testing scheme where the training pairs of spectrally summed hSRS images and subcellular component maps enable prediction of femtosecond SRS images. we also compared the prediction results from femtosecond SRS, spectrally summed hSRS, and Phasor-MRF in Fig. 2·6c-e. The high similarity between the prediction results from femtosecond SRS and spectrally summed hSRS (Fig. 2·6c-d) further verifies that femtosecond SRS can be reliably approximated by spectrally summed hSRS. Thus it justifies our training-testing scheme where the training pairs of spectrally summed hSRS images and subcellular component maps enable prediction of femtosecond SRS images.
Figure 2.6: Comparison between spectrally summed hyperspectral SRS images and single-frame femtosecond SRS images. The two rows are two sets of example images of MIA PaCa-2 cells. (a) Femtosecond SRS. (b) Spectrally summed hSRS. (c) Phasor-MRF results from hSRS. (d) Prediction results from femtosecond SRS. (e) Prediction results from spectrally summed hSRS. Bkg, LD, ER, and Cyto are abbreviations for background, lipid droplet, endoplasmic reticulum, and cytoplasm.

2.2.4 Training set generation and neural network design

To generate the training set, we applied normalization for each image to accommodate different experimental conditions. Following the normalization, we applied image augmentation, such as image rotation and transpose, which was used to generate a training set with 4000 images, each with 128x128 pixels. DeepChem is constructed with repeated dense blocks consisting of several densely-connected convolution blocks. Each convolution block consists of one convolutional layer followed by a batch normalization layer and a ReLU activation layer. DeepChem employs an exponentially decaying learning rate with an Adam optimizer and a batch size of 2. Different learning rate initialization is applied for each segmentation class. The neural network is
implemented in Keras framework on a single GPU (GEFORCE 2080Ti). The detailed architecture can be found in Fig. 2.7. Test-time augmentation is then applied towards a better segmentation accuracy as [93]. For each image in the testing set, the prediction ensemble of multiple transformed versions of this image is first calculated using the pre-trained network. Accordingly, we get the final prediction result \( \hat{Y} = E(Y|X) = \sum_{1}^{N} y_{n}/N \). \( y_{n} \) denotes the probability map of each instance in the prediction ensemble, and \( X \) denotes the input image for inference.
Figure 2-7: DeepChem architecture. Curved blue lines are connections between different layers. BN: batch normalization; CONV: 2D convolution; CONV Trans: 2D convolution transpose; Avg Pooling: 2D average pooling; Trans Down: transition down; Trans Up: transition up; Conc: concatenate

2.3 Results

2.3.1 Predicted subcellular organelle maps from spectrally summed hSRS images and single-frame femtosecond SRS images

Predicted subcellular organelle maps using spectrally summed hSRS show clear biological details and high structural similarity to ground truth images (Fig. 2-8a). The
confusion matrix in Fig. 2.8b shows good prediction accuracy (high intensities cluster in the diagonals). Specifically, DeepChem has a 0.787 F1 score for nuclei segmentation, better than other subcellular organelle segmentation methods using fluorescence images (0.7) [94]. For the other three classes (lipid droplets, ER, cytoplasm), our method has F1 score of 0.645, 0.805, 0.789. In addition, our method is greatly simplified and easier to use compared with networks deploying multi-scale branches with different z-depth inputs [9, 10].

\[
F_1 = \frac{2 \cdot \text{precision} \cdot \text{recall}}{\text{precision} + \text{recall}}
\]  

(2.15)

\[
\text{precision} = \frac{\text{TruePositive}}{\text{TruePositive} + \text{FalsePositive}}
\]  

(2.16)

\[
\text{recall} = \frac{\text{TruePositive}}{\text{TruePositive} + \text{FalseNegative}}
\]  

(2.17)
Figure 2-8: Predicted subcellular organelle maps from spectrally summed hSRS images and single-frame femtosecond SRS images. (a) Predicted subcellular organelle maps from spectrally summed hSRS images. (b) Confusion matrix of subcellular component classes. Top: normalized by column; bottom: normalized by row. LD, Bkg, ER, and Cyto are abbreviations for lipid droplet, background, endoplasmic reticulum, and cytoplasm. (c) Predicted subcellular organelle maps from single-frame femtosecond SRS images. Scale bar: 10 µm
Fig. 2·8c and Fig. 2·9 show predicted subcellular organelle maps from femtosecond SRS images including those cell types (OVCAR-5 and HPDE-6) that never appeared in the training set. It means the neural network “has learned” to provide reliable results from new input of different modalities and different cell types. This result demonstrates the generalization ability of our method and heralds potential in functional cell imaging with high speed and chemical selectivity. In terms of the speed performance, a hSRS stack (256×256×100, FOV 64×64 µm²) takes 110 s, while a single shot femtosecond SRS image with the same size takes 1 to 2 s. This dramatic speed improvement allows real-time imaging of living cells.
Figure 2·9: Comparison between predicted subcellular component maps from single-frame femtosecond SRS images and fluorescence images. Top, second, and third rows show MIA PaCa-2 cells labeled with BODIPY, ER tracker, and PI, respectively. ER is the abbreviation for endoplasmic reticulum.

Fig. 2·10 shows the advantage of this DenseNet-based learning method over brightness thresholding, by comparing these two methods using the femtosecond SRS image. Compared to the Phasor-MRF results and DeepChem prediction results in Fig. 2·10b–c, brightness thresholding (Fig. 2·10d) only leverages information from the intensity domain and the threshold is subject to different experimental conditions. Furthermore, a single threshold can not guarantee consistent segmentation in one image because of the cell-to-cell variation. Thus, brightness thresholding requires much more human intervention and usually results in unstable subcellular component seg-
mentation maps. For example, nuclei and cytoplasm have similar intensity distribution and it is very hard to segment these two subcellular components using a single threshold. Instead of an intensity-only linear scheme, this DenseNet-based learning method extracts comprehensive information from both the intensity and the morphological features based on a nonlinear optimization training process. After training, the sophisticated network finally outputs more robust segmentation results with high accuracy.

![Figure 2.10](image)

**Figure 2.10:** Comparison between brightness thresholding and DeepChem prediction results using single-frame femtosecond SRS images. (a) Femtosecond SRS. (b) Phasor-MRF results from hSRS (the spectrally summed hSRS is in Fig. 2.6b). (c) Prediction results from femtosecond SRS. (d) Subcellular component map of femtosecond SRS by brightness thresholding. Bkg, LD, ER, and Cyto are abbreviations for background, lipid droplet, endoplasmic reticulum, and cytoplasm.

### 2.3.2 Lipid droplet tracking in live MIA PaCa-2 cells by femtosecond SRS and DeepChem

To demonstrate the advantage of our DenseNet-based method over single-frame femtosecond SRS imaging, we show lipid droplet tracking with paralleled ER labels in live MIA PaCa-2 cells using high-speed high-sensitivity femtosecond pulses excitation. Previous research has shown the correlation between the spatial-temporal dynamics...
of lipid droplets and cellular lipid metabolism [95]. However, the previous method using single-frame femtosecond SRS was unable to detect other subcellular organelles simultaneously. A traditional frame-by-frame SRS imaging system can provide multiple subcellular organelles simultaneously, but the speed is around one hundred times slower than our method. In our experiment, we imaged live MIA PaCa-2 cells with a temporal resolution of 1.5 seconds per frame for about 2 min. Then, DeepChem predicted lipid droplet maps from the collected femtosecond SRS images. These maps were then analyzed by the particle-tracking plugin in ImageJ [96]. Two sample images are shown in Fig. 2·11a. To quantify the lipid droplet dynamics, we define two parameters: traveled distance (the cumulative position displacement between consecutive frames) and distance to the origin (the displacement of the current position to the origin of the movement). Using the two parameters defined above, less active lipid droplet movement is observed in cells treated with 0.5 mM Dithiothreitol (DTT) for 1 hour (Fig. 2·11b). DTT is a strong reducing agent that can break down disulfide bond formation and thus lead to ER stress in minutes37. Lipid droplet has been shown as a functional organelle connected to the ER lumen. Thus, our observation implies that the enlarged ER lumen is more likely to trap the lipid droplets and limit their movement [97]. Besides lipid metabolism, DeepChem-based femtosecond SRS has the hyperspectral competence to address the rising interest in understanding organelle interaction and cooperation from different aspects, including morphology and functionality.
Figure 2-11: Lipid droplet tracking in live MIA PaCa-2 cells by femtosecond SRS and DeepChem. (a) Trajectories of lipid droplets (solid lines) and contours of the ER regions (dashed green lines). Scale bar: 5 µm. (b) Quantification of lipid droplet dynamics. The insets in the right column show corresponding plots in the range of 75 to 125 seconds.
2.3.3 Quantification of cellular response to DTT in live MIA PaCa-2 cells

Another strong desire in biomedical applications is large-scale imaging with single-cell resolution. The strengths of our method in resolving this need are motion artifact suppression and quantitative evaluation of each population. To illustrate these strengths, 400 images (64×64 µm² each) were captured in 10 minutes for both the control and DTT-treated group (0.5mM DTT, 1-hour incubation) (Fig. 2·12a,b). We then quantify the cellular response by normalized ER total intensity and ER area ratio, defined in the caption of Fig. 2·13. In the DTT-treated group, ER total intensity (2·13a) and ER area ratio (Fig. 2·13b) are both increased compared to the control group, indicating an expansion of ER lumen induced by DTT. The hyperspectral capability of our method enables this large-scale high-speed imaging with high SNR displaying great advantage over the conventional frame-by-frame SRS imaging system. In contrast to the flow setting where the sample cannot be retrieved, our image cytometry method can perform continuous imaging of the same field of view. Potential applications for this method include SRS-enabled cell sorting.
Figure 2-12: Large-area femtosecond SRS images from the control (a) and the DTT-treated group (b). The insets show areas framed with white boxes. Scale bar: 10 µm. Green lines denote the contour of the ER region predicted. Red lines denote the cell contour analyzed by CellProfiler.
Figure 2.13: Quantification of cellular response to DTT in live MIA PaCa-2 cells. (a-b) Histogram showing the distribution of the normalized ER total intensity and ER area ratio from the two groups. ER area ratio = ER area/Cell area; normalized ER total intensity = ER area × ER intensity/Cell area. ER intensity is defined by the averaged SRS signal in the ER region given the linear relationship between SRS signal intensity and the concentration of resonant molecules.

2.4 Discussion and conclusion

We note that in our manuscript, the subcellular components ER, nucleus, cytoplasm, and lipid droplets can be differentiated based on the intensity of femtosecond SRS images. This condition implies that machine learning is not a magic; it pushes the limit in one domain by leveraging information in another domain. Nevertheless, compared to the threshold approach, machine learning ensures higher accuracy through a sophisticated network. Fig. 2.10 compares the prediction results between brightness thresholding and DeepChem, showing that brightness thresholding based on a simple linear scheme suffers from different experimental conditions and cell-to-cell variation. The advantage of this DenseNet-based learning method is that it trains
a nonlinear network utilizing the information of both the intensity and the morphological features in the training process, and after training, it outputs much more robust segmentation results with high accuracy. In conclusion, we demonstrated a high-speed multiplex chemical imaging method by DenseNet-based learning of femtosecond SRS images. This method is capable of revealing rapid cellular dynamics, including lipid droplet movement and cellular response to DTT, an inducer of ER stress. High speed and chemical selectivity provided by this method offer various possibilities, and potential applications including large-area tissue segment imaging and deciphering cell metabolism. An optimized algorithm may be applied to enable massive parallel visualization of subcellular organelles in the future. Based on rapid developments in both SRS microscopy and deep learning methods, we foresee a more integrated computational SRS microscope providing advanced imaging schemes for a comprehensive understanding of biology and materials.
Chapter 3

SINGLE-CELL ANALYSIS OF METABOLIC-ENGINEERED E. COLI

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Monitoring biosynthesis activity at single-cell level is key to metabolic engineering but is still difficult to achieve in a label-free manner. Using hyperspectral stimulated Raman scattering imaging in the 670–900 cm$^{-1}$ region, localized limonene synthesis are visualized inside engineered Escherichia coli. The colocalization of limonene and GFP-fused limonene synthase is confirmed by co-registered stimulated Raman scattering and two-photon fluorescence images. The finding suggests a limonene synthesis metabolon with a polar distribution inside the cells. This finding expands the knowledge of de novo limonene biosynthesis in engineered bacteria and highlights the potential of SRS chemical imaging in metabolic engineering research.

3.1 Introduction

A metabolon is a supramolecular complex of sequential metabolic enzymes [99, 100]. Metabolons provide multiple advantages for enhanced product flux, and protection from toxic metabolic intermediates [101, 102]. Moreover, a few examples of metabolons such as glucosome and purinosome have been reported [103, 104, 105]. Advanced
imaging techniques such as gas cluster ion beam secondary ion mass Spectrometry (GCIB-SIMS), super-resolution fluorescence microscopy, and fluorescence resonance energy transfer (FRET) microscopy have been employed to image metabolons, e.g. purinosomes in eukaryotic cells [106, 107, 108]. However, the investigation of metabolons in a single bacterial cell remains extremely challenging, especially when the metabolites involved are small molecules for which fluorescent reporters are not available.

Limonene is a type of cyclic monoterpenes that widely exist in plants. Both limonene and its derivatives have diverse applications in the food industry and pharmaceuticals [109, 110, 111]. Despite increasing demand, the supply of limonene, mainly produced from food processing, is unstable [112]. Empowered by the latest advancements in genetic engineering and metabolic engineering, microbial fermentation facilitating the conversion of limonene from low-value materials like glucose has been a promising approach for the bulk and stable production of limonene [113, 114]. However, cell-to-cell variation of biochemical synthesis hinders the maximum yield and the lack of detailed single-cell level characterization is one of the bottlenecks to solve the problem. Recently, spontaneous Raman has been applied to study metabolite production in engineered fungi or bacteria [115, 116]. Due to the small Raman cross-sections of biological samples, the speed of spontaneous Raman is limited, and it usually requires tens of milliseconds to seconds dwell time. This long acquisition time hinders the further utility of spontaneous Raman in biological applications.

Employing a two-laser system (a pump beam and a Stokes beam), stimulated Raman scattering (SRS) offers accelerated imaging speed compared to spontaneous Raman [70, 117, 19]. The rapid identification of Raman signals offered by SRS paves the way for single-cell imaging with live-cell compatibility, which is beyond the reach of mass spectrometry [81, 37, 118]. Compared to fluorescence imaging, SRS has the
potential to detect molecules that do not have commercially available fluorescent probes. Here, we report direct visualization of localized limonene synthesis inside single engineered *Escherichia coli* by hyperspectral stimulated Raman scattering (hSRS). Compared to previous study of limonene biosynthesis detection by single-color SRS [119, 120], hSRS allows simultaneous quantification of multiple chemicals inside one specimen by recording a Raman spectrum at each pixel. Intracellular compositions of various biological specimens have been studied using hSRS, ranging from tissues [121, 122], to cancerous cells [123, 124], to bacteria [34, 125]. Many of these applications employ the C-H stretching region (2800 – 3100 cm\(^{-1}\)) for its strong SRS signal or the C-D stretching region (2070 – 2300 cm\(^{-1}\)) for selective imaging of Raman tags. However, both C-H region and C-D band in the silent region lack specificity in detecting small-molecule synthesis because of Raman signal contributions from other intracellular biomacromolecules. In comparison, the fingerprint region (600-1800 cm\(^{-1}\)) takes advantage of the rich spectral features of limonene [126], making hSRS in the fingerprint region an ideal tool to study limonene biosynthesis at the single-cell level.

In this study, we harness polygon-scanner-based hSRS to map the de novo synthesized limonene distribution inside *E. coli* cells. We first verify that limonene is synthesized from engineered *E. coli* using gas chromatography-mass spectrometry (GC-MS). To investigate subcellular limonene distribution, L1-regularized least squares fitting was used for hSRS image stack in 670 – 900 cm\(^{-1}\) region, enabling selective mapping of limonene and proteins inside individual *E. coli* cells. The chemical content and spatial distribution of the limonene-rich aggregates were then characterized by single-cell and single-aggregate segmentation. In addition, two-photon fluorescence (TPF) imaging of GFP-fused limonene synthase supports the colocalization of limonene with limonene synthase. Together, our data support the possible existence of metabolons
in engineered *E. coli*. The result also highlights the potential of hSRS imaging in metabolic engineering through multiplexed biomolecular analysis at the sub-cellular level.

### 3.2 Experimental design and methods

#### 3.2.1 Sample preparation

We harnessed the heterogeneous mevalonate pathway to produce limonene in *E. coli* (Fig. 3·1a).[34] Isoprenoid precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are products of the mevalonate pathway derived from acetyl-CoA, one of the key node molecules in *E. coli* central metabolism. A geranyl pyrophosphate synthase (GPPS) converts IPP and DMAPP to geranyl pyrophosphate (GPP) which is a universal precursor of terpenes and a limonene synthase (LS) specifically converts GPP to limonene. For easy genetic modification, we distributed necessary enzymes in two plasmids: one plasmid encodes the mevalonate pathway and the other encodes GPPS and LS which are translationally fused (denoted as a two-plasmid system, Fig. 3·1b) [127]. *E. coli* strain BW25113 was used as the host. Limonene production is induced by a small molecule isopropyl β-d-1-thiogalactopyranoside (IPTG). IPTG is a molecular reagent that induces expression where the gene is under the control of the lac repressor.

The *E. coli* strains in this study were derived from the BW25113 and DH1 strains. These wild-type strains were transformed with plasmids expressing the heterologous pathways for limonene production. For the two-plasmid system, the limonene synthesis pathway is split among two plasmids. First, the mevalonate pathway was expressed using plasmid pJBEI-3085 from Taek Soon Lee (Addgene #87950). Second, GPPS was expressed from a plasmid derived from pJBEI-3933 [128], but where we replaced pinene synthase with limonene synthase using golden gate cloning. pJBEI-3933 was
a gift from Jay D. Keasling. For the one-plasmid system, the transformed plasmid was pJBEI-6409 from Taek Soon Lee (Addgene #47048) or variants of this plasmid.

**Figure 3-1:** Limonene-producing *E. coli* strains and GC-MS measurements. (a) Metabolic pathway from Acetyl-CoA to limonene in *E. coli*. Enzymes and some of the reaction intermediates necessary for the production of limonene are shown. AtoB: acetoacetyl-CoA synthase; HMGS: HMG-CoA synthase; HMGR: MG-CoA reductase; MK: Mevalonate Kinase; PMK: PhosphoMevalonate Kinase; PMD: PhosphoMevalonate Decarboxylase; IPP: isopentenyl pyrophosphate; idi: isopentenyl diphosphate isomerase; DMAPP: dimethylallyl pyrophosphate; GPPS: Geranyl Pyrophosphate Synthase; GPP: geranyl pyrophosphate; LS: limonene synthase. (b) Genetic design of limonene pathway for strains with a two-plasmid system. (c) GC-MS measurements of microbial production of limonene (BW25113 strain with a two-plasmid system). Error bar: std.

Cell cultures were inoculated in Luria Bertani (LB) medium with appropriate antibiotics for plasmid maintenance. For two-plasmid system, chloramphenicol (25 µg/mL) and carbenicillin (100 µg/mL) were applied. For one-plasmid system, chloramphenicol (25 µg/mL) was applied. On the following day, the cell culture was refreshed in M9 media (3 mL). M9 salts are composed of MgSO4 (2mM), CaCl2 (100 µM), casamino acids (0.2%), thiamine (340 mg/L), and is supplemented with glucose (20 g/L) and appropriate antibiotics. Limonene was induced by isopropyl β-d-1-thiogalactopyranoside (IPTG) when OD600 (optical density at 600 nm) reached
0.8. The induction level was 100 µM for two-plasmid strains and 50 µM for one-plasmid strains if not otherwise specified. SRS images were taken after about 72 hours for two-plasmid strains and 24 hours for one-plasmid strains (except for the one-plasmid BW25113 strain in Fig. 3·7, which was imaged after 24 hours). For SRS imaging of cells grown in M9 culture medium, 2-4 µL of cell culture was placed on a poly-l-lysine coated coverslip and sandwiched with another coverslip on the top. For SRS imaging of cells grown on M9 media agarose pads, 3 µL of cell culture was placed on an agarose pad (3%) and sandwiched with another coverslip.

We then used gas chromatography–mass spectrometry (GC-MS) to confirm the successful limonene synthesis in these engineered strains (Fig. 3·1c). The GC-MS analysis was conducted using an Agilent GC-MS 6890N equipped with an MS detector for up to 800 m/z. Helium was used as a carrier gas at a constant flow rate of 1 ml/min in an Agilent 222-5532LTM column. The inlet temperature was set to 300°C. The oven temperature was held at 50°C for 30 seconds, ramped up to 150°C at a rate of 25°C/min, and then further ramped to 250°C at a rate of 40°C/min. The results were analyzed using the MSD Productivity ChemStation (E.02.02.1431). An internal standard of α-pinene was used as a reference to calculate limonene concentrations.

### 3.2.2 Optical setup for ultrafast-tuning spectroscopic SRS and two-photon fluorescence microscope

SRS images were acquired using a lab-built SRS microscope (Fig. 3·2) previously reported.[22] A polygon scanner (Lincoln SA24, Cambridge Technology) scanned the Stokes beam onto a blazed grating (GR50-0310, Thorlabs). As a reflective wedge, the grating introduces a continuous temporal delay between the pump and the retroreflected Stokes beam. Both the pump and Stokes beams were chirped with high-dispersion glass (SF57, 90 cm in length for the Stokes beam and 75 cm in length for the pump beam). For all experiments, the power on the sample was 20 mW for 966
nm, 14 mW for 800 nm, and 75 mW for 1040 nm, if not otherwise noted. The microscope was equipped with a 60x water immersion objective (NA = 1.2, UPlan-Apo/IR, Olympus). The SRS signal was then captured by a photodiode with a custom-built resonant circuit and extracted by a lock-in amplifier (UHFLI, Zurich Instrument).

**Figure 3-2:** Schematic diagram of ultrafast spectroscopic SRS. AOM, acousto-optic modulator; C, condenser; F, filter; GM, galvo mirror; HWP, half-wave plate; L, lens; LIA, lock-in amplifier; OBJ, objective; PBS, polarizing beam splitter; PD, photodiode; PS, polygon scanner; QWP, quarter-wave plate.
The same setup was used for two-photon fluorescence imaging with 20 mW for 966 nm. After filtering the excitation beam following the interaction with the sample, a photomultiplier tube (H7422-40, Hamamatsu) was used to measure the two-photon fluorescence signal.

### 3.2.3 Single-cell chemical content evaluation by SRS images

For each hyperspectral SRS image stack captured, a pixel-wise spectral unmixing was performed by the built-in MATLAB function LASSO (Least absolute shrinkage and selection operator). For every single pixel, LASSO decomposes its spectrum into the combination of several pure chemicals: \( D = CS + E \). \( D \) is the detected signal \( (D \in \mathbb{R}^{(1\cdot N_\lambda)}) \). \( C \) is the decomposed concentration \( (C \in \mathbb{R}^{(1\cdot k)}) \). \( S \) is the measured spectral profiles of pure chemicals \( (S \in \mathbb{R}^{(k\cdot N_\lambda)}) \). \( E \) is the residual term. \( N_\lambda \) and \( k \) are the number of frames and the number of pure components, respectively. To get the optimal solution for this least-square fitting problem, L1-norm regularization was applied and the optimization problem is formulated as:

\[
\hat{C} = \arg \min \left( \frac{1}{2} \| D - CS \|_2^2 + \| \lambda \|_1 \right).
\]

\( \lambda \) is a positive real number that denotes the regularizer penalty level. The same \( \lambda \) was chosen for a set of data recorded in the same imaging and digitizing conditions. Three spectral profiles were chosen as \( S \) including pure limonene, wild-type strain, and background (of each SRS image stack). The averaged Raman spectrum from wild-type was used as a cell background signal as it does neither show limonene content in GC-MS measurement nor Raman peak unique to limonene in SRS spectra. The averaged spectrum of the non-cell area of each SRS image stack was used as a reference to eliminate the background contribution from measurement.

For the hyperspectral SRS images, cell and aggregate segmentation was performed using the pixel classification + object classification workflow in ilastik [129]. For cells grown into the clustered colony, an additional step of manual correction from
Schnitzcells was applied in MATLAB to ensure high segmentation accuracy [130]. Using these single-cell segmentation maps, quantification of single-cell features was performed by home-built MATLAB (MathWorks) scripts. Five categories of cell features were quantified: cell feature (area, major axis length), aggregate feature (area, major axis length), intracellular location of aggregate (distance from the aggregate centroid to the furthest cell pole), SRS image feature (SRS intensity at limonene peak 760 cm\(^{-1}\), spectrally summed SRS intensity at C-H and fingerprint window), chemical profile (limonene channel, cell body channel). Spectra of cells/aggregates were represented as mean with a shaded error bar (std). Wilcoxon rank sum test was applied to compare the statistical significance between different cell/aggregate populations.

The workflow of single-cell and single-aggregate analysis is shown below.

**Figure 3-3:** Single-cell and single-aggregate analysis pipeline. Schematic representation of single-cell and single-aggregate analysis. The strain shown in this figure is *E. coli* DH1 one-plasmid strain.

For quantification of colocalization of limonene and limonene synthase, Mander’s colocalization coefficient was defined as \( \sum_i Lim_{i,\text{colocal}} / \sum_i Lim_i \), where \( Lim_{i,\text{colocal}} = Lim_i \) if \( LimSyn \geq \text{threshold} \), and \( Lim_{i,\text{colocal}} = 0 \) if \( LimSyn < \text{threshold} \).
3.3 Results

3.3.1 Hyperspectral SRS imaging unveils chemical aggregates inside engineered limonene-producing *E. coli*

To visualize limonene biosynthesis at the single-cell level, we utilized both Raman spectroscopy and SRS microscopy. Raman spectroscopy was used to determine the vibrational signatures of limonene and its precursors (Fig. 3-4a,b). Pure limonene, glycerol trioleate (GT), and bovine serum albumin (BSA) were measured using a LabRAM HR800 confocal Raman microscopy. Spectra of geraniol, prenol, and isoprenol from [131] were used to present the spectra of geranyl diphosphate (GPP), dimethylallyl diphosphate (DMAPP), and isopentenyl diphosphate (IPP), which are the intermediates in the limonene synthesis pathway. Limonene exhibits strong Raman signals in the high wavenumber C-H stretching region (2800 to 3100 cm$^{-1}$), but this region lacks limonene specificity as C-H bonds are the most abundant functional groups in the cell. In the fingerprint region (600 to 1800 cm$^{-1}$), limonene shows several signature peaks. We chose the peak at 760 cm$^{-1}$ considering the high intensity of limonene and the relatively low background contributed by other biomolecules in the 670 to 900 cm$^{-1}$ region. This unique limonene peak stems from a combination of ring and CH2 vibration in limonene [132]. We also measured the spectra of GT and BSA to confirm that neither protein nor fatty acids inside the cells do not have a significant contribution to the 760 cm$^{-1}$ Raman peak. 3.4 Discussion and outlook
Figure 3-4: Spontaneous Raman spectra. (a) Spontaneous Raman spectra of limonene, geraniol, prenol, isoprenol, GT (glycerol trioleate), and BSA (bovine serum albumin) in the fingerprint window (600-1800 cm\(^{-1}\)). Spectra of geraniol, prenol, and isoprenol represent the spectra of precursors in limonene synthesis pathway (GPP, DMAPP, IPP). (b) Spontaneous Raman spectra of limonene, geraniol, prenol, isoprenol, GT, and BSA in the C-H window. The spectra of GT and BSA were included to show that neither protein nor fatty acids inside the cells have a significant contribution to the 760 cm\(^{-1}\) Raman peak. Red arrows denote peaks at Raman shift 760 cm\(^{-1}\) (limonene), 774 cm\(^{-1}\) (prenol), and 767 cm\(^{-1}\) (isoprenol).

Next, hSRS imaging was utilized to characterize the spatial distribution of limonene inside single engineered \textit{E. coli} cells. We employed an ultrafast delay-tuning hSRS scheme\[22\] shown in Fig. 3-2. Briefly, polygon-scanner-based SRS collects image stacks in a “\(\lambda\)-x-y” manner and provides an acquisition speed of down to 20 \(\mu\)s per spectrum with a 10 cm\(^{-1}\) resolution in the fingerprint region.\[22\] In this study, each single spectrum in the SRS stack takes 120 \(\mu\)s, which improved the spectral fidelity and eliminated low-frequency noise compared to the conventional motorized-stage-based hSRS scheme. It thus provides great advantages for differentiating limonene from other biomolecules inside cells. Fig. 3-5 shows the linearity and sensitivity of limonene detection at the 760 cm\(^{-1}\) Raman peak. The detection limit is found to be 21 mM at a speed of 0.89 seconds per frame of 200x200 pixels. Compared to the conventional frame-by-frame scheme, the use of the polygon scanner increased the hSRS imaging speed by about 5 times at the same signal-to-noise ratio (SNR) (Fig. 3-6).
Figure 3.5: SRS spectra of limonene solution in dodecane with different concentrations. (a) SRS spectra of limonene at different concentrations. Acquisition speed: 0.89 seconds per frame, 128 frames per image stack. (b) Linear dependence of SRS intensity (at 760 cm\(^{-1}\)) on limonene concentration. The error bars show the noise level on the images (Standard deviation of 10 batches. Each batch is composed of 3x3 pixels).
Figure 3-6: Comparison between polygon-scanning and frame-by-frame SRS. (a) Single frame SRS at 760 cm\(^{-1}\) of 20x diluted limonene acquired in polygon-scanning SRS. Image contrast: [0 4414]. Scale bar: 10 µm. (b) Single frame SRS at 760 cm\(^{-1}\) of 20x diluted limonene acquired in frame-by-frame SRS. This image was acquired using the same pump and Stokes power as polygon-scanning scheme. Image contrast: [0 1.135]. Scale bar: 20 µm. (c) Spectra of 20x diluted limonene from the squared area in (a) and (b). Blue: spectrum collected by polygon-scanning system; orange: spectrum collected by frame-by-frame system. The different signal scales in these two imaging schemes were due to different data acquisition strategies. (d) Linear dependence of SRS intensity (at 760 cm\(^{-1}\)) on limonene concentration in frame-by-frame scheme. These image stacks (200x200x128) were acquired using the same pump and Stokes as polygon-scanning scheme. The detection limit is 30.0 mM.

By hSRS imaging, intracellular aggregates were observed in the 670-900 cm\(^{-1}\) region for the limonene-producing strains, as indicated by arrows in Fig. 3-7a. In contrast, the control strains without the heterologous limonene synthesis pathway did not exhibit obvious intracellular aggregates. Fig. 3-4b shows the fingerprint Raman
spectra of the aggregates and the rest of the cell. Specifically, the Raman peak at 760 cm$^{-1}$ confirms the presence of limonene inside these aggregates. The spectrum of the rest of the cell, lacking the 760 cm$^{-1}$ Raman peak, is comparable to the spectrum of the wild-type strain (Wilcoxon rank sum test, Fig. 3.7c). As a comparison, hSRS imaging in the C-H region was also performed. Intracellular aggregates were also observed in the limonene-producing strain but not in the control strain (Fig. 3.7b). As opposed to the fingerprint region, the spectra in the C-H region do not distinguish the aggregate from the rest of the cell, as the C-H region does not have enough specificity for limonene detection (Fig. 3.7c,d). Besides, in the C-H region, the spectra of aggregates resemble the spectrum of BSA but not limonene, indicating the coexistence of proteins with the locally aggregated limonene. Because of the low solubility of limonene and the intermediates in water, these proteins might offer protection to hydrophobic molecules against the hydrophilic environment of the cytoplasm. Further testing of the chemical composition and potential functions of these intracellular aggregates is shown in the following sections.
Figure 3.7: Hyperspectral SRS imaging unveils chemical aggregates inside limonene-producing E. coli. (a) Spectrally summed hSRS images of BW25113 strain (fingerprint window). Left: wild-type; Right: limonene-producing strain. White arrows denote the position of the intracellular aggregates. (b) Average of the normalized spectra of the wild-type strain, aggregate of limonene-producing strain, and non-aggregating part of limonene-producing strain. Red arrow marks 760 cm\(^{-1}\) Raman peak. Shaded error bar: std. (c) Scatter plot of the Raman intensity at 760 cm\(^{-1}\) per region of the normalized spectra in (b). The solid green, blue, and red lines were averaged by 68 cells, 33 cells, 4 aggregates respectively. NS, nonsignificant, \(P > 0.05\). *, \(10^{-5} < P < 0.05\) (Wilcoxon rank sum test). (d) Spectrally summed hSRS images of BW25113 strain (C-H window). Left: wild-type; Right: limonene-producing strain. (e) Average of the raw spectra of the wild-type strain, aggregates of limonene-producing strain, and non-aggregating part of limonene-producing strain. Red arrow marks 2930 cm\(^{-1}\) Raman peak. (f) Average of the normalized spectra of wild-type strain, aggregates of limonene-producing strain, and non-aggregating part of limonene-producing strain. Red arrow marks 2930 cm\(^{-1}\) Raman peak. Shaded error bar: std.
3.3.2 Pixel-wise spectral unmixing reveals the chemical composition of the intracellular aggregates

To extract the limonene distribution map from the hyperspectral stack, we applied the least absolute shrinkage and selection operator (LASSO) [133]. The effectiveness of LASSO in unmixing hSRS images in the fingerprint region has been tested on various biological samples [81]. Compared to least squares fitting, LASSO introduces a pixel-wise sparsity constraint. It hypothesizes that each pixel of our hyperspectral image is dominated by sparsity components. This feature makes LASSO ideal for our study because limonene is localized in the intracellular aggregates and the rest of the cell (the non-aggregating part) has the same spectral profile as the control strain (Fig. 3·7b). Therefore, using the reference spectra for pure limonene, wild-type strain, and background (of each hSRS image stack), chemical maps of three channels can be generated, denoted as limonene, cell body, and background.

By LASSO analysis of the fingerprint hSRS data (Fig. 3·8a), we generated concentration maps of limonene (Fig. 3·8b). Locally concentrated limonene was found in engineered strains but not in the wild-type. The cell body channel was similar across all samples (Fig. 3·8c). By single-cell and single-aggregate segmentation (Fig. 3·3, details in the Methods section), segmentation maps of two types of cell regions were generated: aggregates and the rest of the cell. By averaging the limonene intensity of both regions, the limonene amount of each aggregate and the rest of the cell is plotted (Fig. 3·8d). This result indicates the limonene intensity of aggregate depends on IPTG concentration, which is qualitatively correlated to GC-MS measurements (Fig. 3·1c). Importantly, SRS imaging and LASSO analysis quantitatively show that the produced limonene is locally concentrated and not spread over the whole cell.
Figure 3-8: Chemical composition analysis of limonene-producing *E. coli* strains. (a) Spectrally summed hSRS images (670-900 cm\(^{-1}\)). Left: wild-type; middle: Limonene-producing strain with 0.1 mM IPTG; right: Limonene-producing strain with 0.5 mM IPTG. (b) Chemical map of limonene. Localized limonene distribution is observed in limonene-producing strain. Cyan lines: contour of each single cell. White lines: contour of each single aggregate. (c) Chemical map of cell body. (d) Scatter plot of limonene intensity in aggregates and rest of the cell. Each dot represents the averaged limonene intensity in a single aggregate.
To verify that the SRS-mapped limonene indeed originates from de novo synthesis, we tested *E. coli* strains with incomplete synthesis pathways in addition to the wild-type strain and the strain with the complete limonene synthesis pathway. Specifically, we tested three incomplete pathways: \( \delta \text{GPPS} \) without GPPS but having all other enzymes, \( \delta \text{LS} \) without LS but having all other enzymes, and GPPS-LS only having GPPS and LS but none of the upstream genes in the mevalonate pathway. Based on hSRS images in the 670-900 cm\(^{-1}\) spectral region (Fig. 3·9a), the concentration map of limonene was produced via LASSO analysis (Fig. 3·9b). A high abundance of limonene was only observed in the strain with a full limonene synthesis pathway, in the form of aggregates. By applying single-aggregate segmentation to SRS images (C-H region, Fig. 3·9c) of the same specimens, limonene-channel intensities in aggregate and the rest of the cell are plotted in Fig. 3·9d. Using a threshold sweeping method, 1.1 was set as the limonene intensity threshold (red dashed line in Fig. 3·9d) to distinguish the limonene-rich aggregates. Using this threshold, the percentage of cells with limonene-rich aggregates is 16.9% in the strain with a complete pathway. Further tests with a different construct design are shown in the following section.
3.3.3 Localized limonene synthesis is confirmed in one-plasmid limonene-producing strains

To confirm the evidence of localized limonene aggregate formation, engineered strains with a one-plasmid version of the limonene production pathway were tested as a different construct design (denoted as one-plasmid system, Fig. 3·10a). As production is impacted by the strain background, we tested E. coli strain DH1 in addition to BW25113. These one-plasmid strains show higher limonene production in GC-MS measurements than the two-plasmid design in BW25113 (Fig. 3·11a,b). To further demonstrate the in situ live-cell imaging compatibility of our imaging system, we
directly grew the cells on a thin layer of agarose gel pads after IPTG induction (method). With growth medium and air exchange provided by the agarose pad [134], colonies composed of a single layer of cells form on top of the agarose pad. After culturing for 24 hours, the cells growing on the agarose pad were directly imaged using SRS. Using this protocol, we tested the *E. coli* DH1 strain with one-plasmid, and we were also able to observe intracellular aggregates in both the C-H and the 670-900 cm\(^{-1}\) region (Fig. 3·10b,c). Limonene concentration maps were produced using LASSO analysis (Fig. 3·10d), showing that limonene is localized and enriched in these aggregates. By segmenting these bright spots from the rest of the cell, fingerprint spectra of the aggregates were extracted and exhibited a distinct Raman peak at 760 cm\(^{-1}\) unique to limonene (Fig. 3·10e). Similar results were obtained with another host strain (Fig. 3·11c-f).

HSRS imaging further allowed us to study the intracellular location of these aggregates as well as cell-to-cell variation. By extracting the central line along the major axis of each aggregate-containing cell, the position of the aggregate was determined as the pixel with the maximal intensity along the central line (Fig. 3·10f). The scatter plot of the relative location of the aggregate (defined as \(d/L\)) is shown in Fig. 3·10g, and 48.5% of aggregates resided between 70% to 90% of the cell length for DH1 strains. We also found that only 18.9% of the cells contain aggregates (Fig. 3·10h).
Figure 3.10: Characterization of limonene production in one-plasmid limonene-producing *E. coli*. (a) Genetic design for strains with one-plasmid system. (b-c) Spectrally summed hSRS images of DH1 one-plasmid strain. (d) Chemical maps of limonene channel of the same field of view in (b). Cyan lines: contour of each single cell. White lines: contour of each single aggregate. (e) Averaged spectra of aggregates and rest of the cell (670 – 900 cm⁻¹). Shaded error bar: std. (f) Diagram of a cell with an intracellular aggregate. (g) Violin plot with values of d/L for DH1 one-plasmid strain. (h) Histogram of aggregate counts per cell for DH1 one-plasmid strain.

Similar results were observed in the two-plasmid design in BW25113 strain (Fig. 3.11), which has lower limonene production in GC-MS measurements compared to the one-plasmid strains in Fig. 3.13.
Figure 3.11: Characterization of limonene production in limonene-producing E. coli (a) GC-MS measurement of microbial production of limonene in BW25113 one-plasmid strain. Error bar: std. (b) GC-MS measurement of microbial production of limonene in DH1 one-plasmid strain. Error bar: std. (c-d) Spectrally summed SRS images of BW25113 one-plasmid strain. (e) Chemical map of limonene of the same field of view in (c-d). Cyan lines: contour of each single cell. White lines: contour of each single aggregate. (f) Averaged spectra (670 – 900 cm$^{-1}$) of aggregate and rest of the cell. The spectrum of the aggregate shows the distinct Raman peak at 760 cm$^{-1}$ unique to limonene. Shaded error bar: std. (g) Violin plot with values of d/L for BW25113 one-plasmid strain. 56.3% of aggregates resided between 70. (h) Histogram of aggregate counts per cell for BW25113 one-plasmid strain.
Cell-to-cell variation in limonene biosynthesis was also quantified using the average and total limonene content per aggregate (Fig. 3·12). Interestingly, a larger aggregate tends to be accompanied by higher average limonene content (Fig. 3·12a). Moreover, the scatter plot of aggregate size versus average limonene intensity was bounded by a constant upper threshold and an asymptotic lower threshold. In addition, by comparing the spectra of these aggregates and limonene solution in dodecane, the local concentration of limonene was estimated to be around tens to one hundred mM (Fig. 3·5). Further evidence revealing the biosynthetic function of these proteins is shown in the next section.
Figure 3-12: Characterization of aggregate formation in one-plasmid limonene-producing E. coli. (a) Scatter plot of area per aggregate versus average limonene content per aggregate. (b) Histogram of average limonene content per aggregate. Both of the histograms are fitted to a Gaussian distribution and DH1 has significantly higher limonene concentration per aggregate (BW25113 one-plasmid: 173 ± 100, 5th percentile 24, 95th percentile 361; DH1 one-plasmid: 224 ± 12, 5th percentile 60, 95th percentile 393; arbitrary units; p = 0.0014, Wilcoxon rank-sum test). (c) Histogram of total limonene content per aggregate. Both of the histograms are fitted to a log-normal distribution. (BW25113 one-plasmid: 5th percentile 156, 95th percentile 13620; DH1 one-plasmid: 5th percentile 60, 95th percentile 18178; arbitrary units).

3.3.4 Intracellular limonene is co-localized with limonene synthase

To further validate our observation, simultaneous imaging of both limonene and LS, the final enzyme in the pathway that synthesizes it, was carried out. For this purpose, green fluorescence protein (GFP) was translationally fused to LS. Notably, limonene was observed in the strain with GFP-LS fusion (Fig. 3-13a,b). Co-registered images of SRS and two-photon fluorescence of both BW25113 with two-plasmid system and DH1 with one-plasmid system are shown in Fig. 3-13c,d, and Fig. 3-13f,g, respectively.
Limonene maps generated from SRS images are overlaid with the contour of the aggregates in the fluorescence channel, exhibiting the colocalization of limonene with GFP-fused LS (Fig. 3·13e,h). Co-occurrence between limonene and limonene synthase was quantified by Mander’s colocalization coefficient (MCC) [135]. MCC is defined as the summed limonene intensity of the colocalized pixels over the summed limonene intensity of all pixels (detailed definition in the Methods section). Higher MCC values indicate a higher colocalization level. BW25113 with two-plasmid system (Fig. 3·13e) has an MCC of 0.79, and DH1 with one-plasmid system (Fig. 3·13h) has an MCC of 0.71. This quantification confirms that these aggregates encompass both enzyme and limonene, with the potential function of limonene synthesis and storage. Together, our observation of colocalization of limonene and its synthesis enzyme verifies the formation of limonene synthesis metabolon inside the engineered \textit{E. coli} strains.
Figure 3.13: SRS and two-photon fluorescence (TPF) imaging unveil co-localization of limonene and limonene synthase. (a) GC-MS measurement of microbial production of limonene in BW25113 two-plasmid strain. Error bar: std. (b) GC-MS measurement of microbial production of limonene in DH1 one-plasmid strain. Error bar: std. (c) Spectrally summed hSRS images of BW25113 two-plasmid strain (670-900 cm\(^{-1}\) region). (d) Two-photon fluorescence image of the same FOV in (a). (e) Limonene map of the same FOV in (a-b). White lines: contour of each single cell. Green lines: contour of each single aggregate by two-photon fluorescence image. (f-h) The same order as (a-c). DH1 one-plasmid strain.

3.4 Discussion and conclusion

Limonene is a high-value chemical with various applications in the industry. A thorough understanding of the limonene biosynthesis process in the host microorganism
is desired to guide metabolic engineering and potentially enhance the limonene yield. Our study here revealed the formation of limonene-rich aggregates in engineered *E. coli* strains with a limonene synthesis pathway. Quantification of the chemical content of these aggregates was achieved by spectral unmixing of hSRS images of spectral region 670 – 900 cm$^{-1}$. Furthermore, taking advantage of simultaneous hSRS and TPF imaging, colocalization of limonene and limonene synthase was observed in the engineered *E. coli*. Two biological systems (one-plasmid DH1 strain and two-plasmid BW25113 strain) were tested and both exhibited aggregates in variants with a full limonene pathway. These data offer direct evidence of metabolite channeling and metabolon formation in these engineered strains.

Intracellular organization achieved through compartmentalization is key to cell metabolism. Consisting of sequential enzymes and metabolites, metabolon offers intracellular spatial organization in both eukaryotic [136, 137] and prokaryotic cells [138], bringing multiple advantages in terms of biosynthesis efficiency, cell growth, and survival [99, 139, 140]. Both natural and engineered systems could take advantage of cellular localization [141, 142]. Benefiting from the multimodal capabilities of our imaging system, this study suggests that these intracellular aggregates are limonene synthesis metabolons. First, limonene was shown enriched inside aggregates. In the DH1 one-plasmid strain, limonene concentration of aggregates is around tens to one hundred mM, which is one to two order magnitude higher than the limonene concentration measured by GC-MS (Fig. 3·10e, Fig. 3·5a). Moreover, larger aggregates were accompanied by higher limonene intensities, indicating the continuous limonene deposition in these regions (Fig. 3·12a). Second, proteins were found existing inside the limonene-rich aggregates by comparing the C-H stretching spectra of aggregates with a standard protein sample BSA (Fig. 3·4e,f). Considering the hydrophobicity of limonene and its precursors, these proteins may offer protection of these metabolites
from the hydrophilic environment.[5] Another advantage of these coexisting proteins is preventing the diffusion of reactive or potentially toxic intermediates, which potentially increases the reaction efficiency and reduces the metabolic cross-talk [143]. Last, the enrichment of GFP-fused limonene synthase inside these aggregates suggests active limonene synthesis at these spots (Fig. 3·13). This provides substantial evidence of the synthetic function of the proteins coexisting with limonene. Collectively, these data confirmed both the chemical composition and synthetic function of these intracellular aggregates, documenting a new case of metabolon formation in limonene-producing E. coli.

HSRS imaging also offers an interesting insight into the cell-to-cell variation of these limonene-producing cells. For all the strains with the full limonene synthesis pathway in this study, heterogeneity in terms of intracellular localization and limonene content were observed. Most of the cells contain zero or one aggregate, suggesting the sparsity of the high-producers in the whole population (Fig. 3·10h, Fig. 3·11h). The histogram of average limonene content per aggregate showed about ten times variation (Fig. 3·12b) within an isogenic population for both BW25113 and DH1 one-plasmid strains. We are also able to observe the spatial bias where aggregates were formed. Asymmetric cell partitioning might be one possible reason for the observed single-cell heterogeneity and the pole-biased accumulation in these limonene-producing E. coli (Fig. 3·10f,g, Fig. 3·11g) [144, 145].

We would also note a few limitations of our method in characterizing the proposed limonene synthesis metabolon. First, we assumed that at each single pixel, the chemical composition is sparse. By using three standard reference spectra: pure limonene, the wild-type strain, and the background (of each hSRS image stack), the potential Raman signal contribution from the precursors was forced to go to the cell body channel. To discriminate limonene and three precursors (IPP, DMAPP, and
GPP), a broadband coherent Raman imaging system could be applied [146, 147]. By collecting a wider Raman spectral window, more metabolites could be quantified simultaneously, providing direct evidence of metabolite channeling [148]. Second, limited by the limonene detection sensitivity (21 mM) of our hSRS imaging system, limonene distribution in the non-aggregating area of the cell was not ruled out. Although most limonene is expected to exist in an aggregate form because of its low solubility in a hydrophilic environment, limonene efflux [149] is still of special interest and could be achieved by surface-enhanced Raman scattering [150]. Third, time-lapse imaging of the limonene-producing *E. coli* was not achieved in this study due to laser toxicity [151]. Machine learning-based methods offer a potential way to mitigate laser toxicity by overcoming tradeoffs between imaging speed, sensitivity, and specificity [28, 152]. In summary, our study provides direct visualization of the chemical composition and synthetic function of the limonene-rich aggregates inside engineered *E. coli*. This evidence of channeled limonene and its colocalization with limonene synthase is an important addition to our knowledge of the spatial control of limonene biosynthesis in engineered *E. coli*, and can offer conceivable guidance on metabolic engineering designs. Further investigations of localized limonene biosynthesis may include broadband coherent Raman imaging and time-lapse SRS imaging of live *E. coli* cells. With the live-cell imaging capacity, integration of our imaging platform with downstream cell sorting [153, 57] is expected.
Chapter 4

**SINGLE-CELL SORTING BY STIMULATED RAMAN-ACTIVATED EJECTION**

The work presented in this chapter is in preparation of a submitted manuscript.

Single-cell sorting is essential to explore cellular heterogeneity in biology and medicine. Recently developed Raman-activated cell sorting (RACS) circumvents the limitations of fluorescence-activated cell sorting, such as the cytotoxicity of labels. However, the sorting throughputs of all forms of RACS are limited by the intrinsically small cross-section of spontaneous Raman scattering. Here, we report a stimulated Raman-activated cell ejection (S-RACE) platform that enables high-throughput single-cell sorting based on high-resolution multi-channel stimulated Raman chemical imaging, in situ image decomposition, and laser-induced cell ejection. The performance of this platform was illustrated by sorting a mixture of 1-µm polymer beads, where 95% yield, 98% purity, and 14 events per second throughput were achieved. Notably, our platform allows live cell ejection, allowing for the growth of single colonies of bacteria and fungi after sorting. To further illustrate the chemical selectivity, lipid-rich (Rhodotorula glutinis) cells were successfully sorted from a mixture with (Saccharomyces cerevisiae), confirmed by downstream quantitative PCR. Furthermore, by integrating a closed-loop feedback control circuit to the system, we realized real-time single-cell imaging and sorting, and applied this method to precisely eject regions of interest from a rat brain tissue section. The reported S-RACE platform
opens exciting opportunities for a wide range of single-cell applications in biology and medicine.

4.1 Introduction

Cell sorting is indispensable for characterizing a heterogeneous cell population from various perspectives, such as chemical, structural, and genomic analyses [154, 1, 155]. Current cell sorting techniques include flow cytometry, laser microdissection, cell picking, and microfluidics [7, 6]. Among this array of techniques, fluorescent and magnetic labeling are commonly used for sorting target cells, whereas the exogenous labels may potentially induce cytotoxicity and disrupt cellular functions. Additional challenges such as the lack of specific labels and susceptibility to photobleaching limit the utility of labeling-based cell sorting. In contrast, label-free cell sorting methods rely on cellular properties like morphology, deformability, and chemical content. However, the morphological and mechanical attributes may not exhibit a direct correlation with biological states, thus reducing the sorting specificity [156]. For example, quantitative phase imaging could map the volumetric distribution of the refractive index but is difficult to identify specific cells [157, 158](7, 8).

Raman spectroscopy possesses the capacity to surpass the constraints faced by the above regimes. By detecting inelastic photon scattering [159], Raman spectroscopy could characterize the endogenous chemical content of single cells and is capable of probing cell metabolic activity [160]. By integrating Raman spectroscopy with cell sorting methodologies, including flow [161, 162], optical tweezer [54, 153], dielectrophoresis [163], and cell ejection [164, 57], a plurality of biomedical applications were achieved [50]. Raman-activated cell sorting (RACS) has found extensive use in microbiology for isolating functional individuals from a community. It has been illustrated to differentiate antibiotic-resistant [165, 60, 61] or functional microbes that
possess specific pathways in a complex environment such as the human gut [60] or natural ecosystems [59, 58, 62]. Other cell types like mammalian and fungal cells can also be characterized and sorted using RACS [166, 53, 167]. Among the RACS techniques, Raman-activated cell ejection (RACE) has been proven especially powerful in high-precision sorting of smaller cells. RACE is based on laser-induced forward transfer, a method widely used in material transfer [56]. In RACE, the specimens are first placed on coverslips with a laser-absorbing coating material. A pulsed laser then acts on the target location to ablate the coating, providing forward momentum to eject the cells to the collector for downstream analysis [50], for example, linking single-cell phenotype and genotype of microorganisms sampled from the natural environment [58]. Despite its versatility, current RACS methods have a low throughput due to the small cross-section of spontaneous Raman scattering. Typical integration time for a single Raman measurement ranges from 15 and 60 seconds per spectrum for biological samples to obtain a sufficient signal-to-noise ratio [17].

Here, we demonstrate stimulated Raman-activated ejection (S-RACE) to achieve automated high-throughput cell sorting. In coherent Raman microscopy, 10^4-10^5 signal enhancement can be achieved compared to spontaneous Raman scattering [70, 69]. Coherent Raman microscopy employs two pulsed laser beams to probe coherent vibrations in a sample. Both stimulated Raman scattering (SRS) and coherent anti-Stokes Raman scattering (CARS) have been combined with microfluidics for high-throughput cell imaging [168, 65, 64, 169, 42]. Recently, two coherent Raman-activated cell sorting studies were reported, one in the C-H stretching region [66] and the other in the fingerprint region (400-1800 cm\(^{-1}\)) by an FT-CARS spectrometer [170]. Despite the high throughput, these microfluidic-based methods encounter challenges in handling smaller cells (<3 \(\mu\)m) [66], and unstable flow caused by bubbles and/or debris in the microfluidic channel [67](39). Apart from cell detection and sorting, SRS microdis-
section and sequencing was recently reported for in situ laser microdissection of tissue slices and downstream DNA and RNA sequencing [49].

Our S-RACE platform integrates multicolor SRS imaging, online image processing, and a lab-built ejection module to enable high-throughput image-based cell sorting. This is the first demonstration of single-cell ejection guided by coherent Raman microscopy. We achieved a high yield of approximately 95% and a high purity level of around 98% for a mixture of 1.0-µm polymer beads, with a throughput of approximately 14 events per second (eps). Additionally, we demonstrated fast identification and sorting of lipid-rich *R. glutinis* cells from a mixture with *S. cerevisiae* and confirmed the result by quantitative PCR amplification of the ITS2 region. A notable feature of our platform is its live cell sorting capability, a pivotal component for isolating and purifying cells with specific functions while ensuring their viability. Successful cell recovery was observed for both bacteria (*Escherichia coli*) and fungus (*Saccharomyces cerevisiae*, *Candida albicans*). Furthermore, by harnessing a comparator circuit for communication between imaging and laser ejection, we achieved real-time SRS-guided sorting of single polymer beads, live cells, and regions of interest in a brain tissue slice. The S-RACE platform promises various biomedical applications, including metabolic engineering [98], precise diagnosis [17], and bacterial therapies [171].

### 4.2 Experimental design and methods

#### 4.2.1 Sample preparation

**Polymer bead mixture:** Polystyrene (PS) microbeads with red fluorescence and poly (methyl methacrylate) (PMMA) microbeads with 1.0 µm diameter were mixed with a 2:1 ratio in deionized water. The mixture was dropped onto a coverslip with TiO$_2$ coating and then air-dried prior to the S-RACE experiments.
**E. coli sample:** The *E. coli* strain with GFP label was from the Mary Dunlop lab at Boston University, which harbored a plasmid containing a constitutive promoter-driven superfolder GFP. The cells were first recovered from -80°C on a Trypticase Soy Agar (TSA) plate for 37°C overnight. Then the TSA plate was stored at 4°C for future use. On the experiment day, a single colony was scrapped from the TSA plate and suspended in a culture tube with 2 ml TSB medium. The suspended cells were cultured at 37°C with shaking at 200 RPM for 4 hours.

**S. cerevisiae and C. albicans sample:** The *S. cerevisiae* strain was from the Ahmad (Mo) Khalil lab at Boston University. For both *S. cerevisiae* and *C. albicans* cells, the cells were first recovered from -80°C on a Yeast Peptone Dextrose (YPD) plate for 30°C overnight. Then the YPD plate was stored at 4°C for future use. One day before the experiment, a single colony was scrapped from the YPD plate and suspended in a culture tube with 2 ml YPD medium. The suspended cells were cultured at 30°C with shaking at 200 RPM for 4-6 hours.

**R. glutinis sample:** The *R. glutinis* strain was from the Agricultural Research Service Culture Collection (NRRL). The cells were first recovered from -80°C on a medium No. 6 plate for 30°C overnight. Then the YPD plate was stored at 4°C for future use. A two-phase growing protocol was adapted to promote lipid production. For phase one: a single colony was scrapped from the YPD plate and suspended in a culture tube with 2 ml YPD medium. The cells were cultured at 30°C with agitation at 200 RPM for 24 to 48 hours. For phase two: 100-200 µl of cell culture from phase one was mixed with 2 ml of medium No. 6 supplemented with 3% glucose. The cells were cultured at 30°C with shaking at 200 RPM for 96 hours. Medium No. 6 is composed of dextrose 10 g/L, yeast extract 3 g/L, peptone 5 g/L, malt extract 3 g/L.

**Tissue sections:** The SJSA-1 tumor tissue and rat brain tissue were fixed with formaldehyde fixative overnight. The tissue section was then transferred to a con-
tainer with 30% sucrose in 1X PBS at 4°C. After the tissue sank, it was removed from the liquid and embedded in the OCT compound. The tissue sample was then placed in a -80°C freezer until fully frozen. The frozen tissue sample was then sectioned to 5 µm with a cryostat machine (cm1950, Leica).

4.2.2 Optical setup for S-RACE

SRS images were acquired using a lab-built SRS microscope (Fig. 4·1). Briefly, a femtosecond laser source (InSight DeepSee, Spectra-Physics) was used for SRS excitation. The laser output two femtosecond pulse trains used for the pump (tunable wavelength) and Stokes (fixed wavelength at 1045 nm), respectively. Both the pump and Stokes beams were chirped for hyperspectral imaging through spectral focusing with high-dispersion glass (SF57, 90 cm in length for the Stokes beam and 75 cm in length for the pump beam). An acousto-optic modulator (522c, Isomet) was used to modulate the Stokes beam at 2.5 MHz. A translation stage (Zaber Technologies) was used to scan the interpulse delay between the pump and the Stokes beams, thus the excitation frequency. The combined pump and Stokes beam were directed to a microscope frame by a 2D galvo mirror (GVS002, Thorlabs). The microscope was equipped with a 60X water immersion objective (NA = 1.2, UPlan-Apo/IR, Olympus) or a 40X water immersion objective (NA = 0.8, LUMPLFLN, Olympus). The SRS signal was then captured by a photodiode with a custom-built resonant circuit and extracted by a lock-in amplifier (UHFLI, Zurich Instrument). For the polymer beads sample, the power on the sample was 14 mW for 800 nm and 25-40 mW for 1040 nm. For E. coli/S. cerevisiae/C. albicans sample, the power on the sample was 14 mW for 800 nm and 50 mW for 1040 nm. For R. glutinis, the power on the sample was 14 mW for 802 nm and 50 mW for 1040 nm.

For microparticle/cell ejection, a 532-nm laser (ALPHALAS, pulse width 0.89 ns) was collinearly combined with the pump and Stokes before the galvo mirror. The
A 532-nm laser was operated at a 1.7 A current with a repetitive rate of 4 kHz. For automated cell sorting, an acousto-optic modulator (522C-2, Isomet) was used as a pulse picker. A function generator (DG1022Z, Rigol) was used to trigger AOM (3 kHz, burst mode). The AOM modulation frequency matched the repetition rate of the 532-nm laser. The 532-nm laser was combined with the pump and Stokes beams before the 2D galvo mirror with a 650-nm long pass filter.

**Figure 4.1:** Optical setup for S-RACE. AOM: acousto-optic modulator. DMSP: short pass dichroic mirror. DMLP: long pass dichroic mirror. 2D GM: 2D galvo mirror. L: lens. OBJ: objective. COND: condenser. F: filter. PD: Photodiode. LIA: lock-in amplifier.

### 4.2.3 Automatic imaging-sorting

A customized program was developed in LabVIEW that seamlessly integrates SRS imaging, target detection, and single-pulse ejection functions. For single-color SRS images, objects are detected by first generating the object mask and then calculating the centroid of each object. For multi-color SRS images, an additional least square fitting step was executed, allowing for object classification based on their spectral
features. For each targeted object, 2D galvo mirrors were employed to precisely position the green laser on the object. Subsequently, an acousto-optic modulator (AOM) was activated to project the green laser onto the coating. The targeted object was then pushed away from the ejection coverslip and collected by the collector. By moving the sample stage, multiple FOVs were stitched together to achieve high throughput.

Before each day’s experiment, one registration step was performed. This step aligns the coordinates of the SRS image with the position of the green laser.

4.2.4 Preparation of the ejection module

Coverslips with TiO$_2$ (Titanium dioxide) coating were used for cell ejection. The TiO$_2$ coating absorbs the 532-nm laser pulse and forms an ejecting force by nanosecond laser irradiation. Coverslips with TiO$_2$ coating were prepared by magnetron sputtering (3.00-inch diameter Angstorm Science ONYX-3 Mag II cathode) with a TiO$_2$ target (purity 99.99%, QA13-11200, Angstorm Engineering Inc.). The sputtering time for coverslips used in this work is 2 minutes if not otherwise specified. For live *E. coli* and *S. cerevisiae* sorting, a thin agarose layer was added on top of TiO$_2$ by dropping 5 µl 1% agarose and squeezing with another coverslip. This agarose layer was 6 µm (measured under a microscope). For the receiver, a normal coverslip with PDMS (polydimethylsiloxane, thickness 200 µm) spacer was used for the polymer beads sample. For imaging GFP labeled *E. coli* in the receiver, a standard coverslip with an agarose gel layer (1%) was used as a receiver and was also with a PDMS spacer (thickness 200 µm). For recultivating GFP labeled *E. coli* and *S. cerevisiae*, a petri dish with an agarose gel layer (2% agarose in YPD medium for *S. cerevisiae*, 2% agarose in TSB for *E. coli*).
4.2.5 Quantitative PCR

For sorted *R. glutinis*, DNA was first extracted using a protocol adapted from a previous study (52). In the final step, DNA in each collection well was diluted to 50 µl. Each qPCR well contained 7.5 µl DNA template, 10 µl PowerTrack SYBR Green Mix, 1 µl forward primer and 1 µl reverse primer (final concentration 1 µM), and 0.5 µl yellow buffer. A 96-well plate with all reagents was then sent to a qPCR machine (StepOne Plus RT-PCR, Applied Biosystems). The qPCR run started at 95°C for 2 minutes, then ran for 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. After the qPCR run, a melt curve was measured at 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds. The primer used in this study was from a previous study (53). The forward ITS2 primer was 5’-GCATCGATGAAGAACGCAGC-3’. The reverse ITS2 primer was 5’-TCCTCCGCTTATTGATATGC-3’. The primers were from Integrated DNA Technologies, Inc.

4.2.6 Real-time precision opto-control:

We then seamlessly integrated a real-time precision opto-control (RPOC) system with our S-RACE platform. The RPOC utilized a comparator circuit box is from The Chi Zhang lab at Purdue University. The comparator box was operated in manual selection mode. The selection threshold was set by turning the knob on the box. The TTL signal output was sent to AOM installed in the optical path of the 532 nm laser. For the TTL signal <0.8 V, the AOM was off. For the TTL signal >2.7V, the AOM was ON and coupled a 532 nm laser into the optical path. Before the experiment, the 532 nm laser was first aligned with the SRS lasers for high-precision sorting.
4.3 Results

4.3.1 Development of S-RACE platform

Our S-RACE system includes a multispectral SRS microscope and a laser ejection module (Fig. 4·2a). The SRS microscope was described in our previous work (41). Detailed setup can be found in Fig. 4·1. A multicolor SRS stack was collected by scanning the interpulse delay between the spectrally chirped pump and Stokes beams. The ejection module (Fig. 4·2b) consists of a 532-nm 1.0-ns pulsed laser, an ejection coverslip, and a collector assembled in a sandwich-like manner. The ejection coverslip is coated with a thin layer of titanium dioxide (TiO$_2$) as the dynamic release layer [172]. For the polymer microparticle mixture sorting, the sample is first loaded onto the coverslip with TiO$_2$ coating, followed by an air-drying step in preparation for ejection. During ejection, the TiO$_2$ coating absorbs energy from the 532-nm laser and generates gas pressure pushing the targeted microparticle away. The “ejected” microparticle is then collected by the bottom collector, which is fabricated by coating a layer of polydimethylsiloxane (PDMS) on a standard coverslip. For live cell sorting, an additional thin layer of agarose gel is introduced on the TiO$_2$ coating, mitigating potential mechanical damage during the ejection process (18). In addition, a complementary layer of agarose is introduced at the bottom of the collector to safeguard the cells from any impact during the landing process [57, 173].
Figure 4·2: Limonene-producing *E. coli* strains and GC-MS measurements. (a) Metabolic pathway from Acetyl-CoA to limonene in *E. coli*. Enzymes and some of the reaction intermediates necessary for the production of limonene are shown. AtoB: acetoacetyl-CoA synthase; HMGS: HMG-CoA synthase; HMGR: MG-CoA reductase; MK: Mevalonate Kinase; PMK: PhosphoMevalonate Kinase; PMD: PhosphoMevalonate Decarboxylase; idi: isopentenyl pyrophosphate; IPP: isopentenyl pyrophosphate isomerase; DMAPP: dimethylallyl pyrophosphate; GPPS: Geranyl Pyrophosphate Synthase; GPP: geranyl pyrophosphate; LS: limonene synthase. (b) Genetic design of limonene pathway for strains with a two-plasmid system. (c) GC-MS measurements of microbial production of limonene (BW25113 strain with a two-plasmid system). Error bar: std.

Metals such as gold, metal oxides, and polymer materials have been used as the dynamic release layer in laser-induced forward transfer (LIFT) systems for cell ejection or tissue dissection (43). Among various coatings tested in this study, TiO$_2$ showed the best performance in both SRS imaging and laser ejection (Fig. 4·3). The TiO$_2$-coated coverslip (150 µm thick) is fabricated by magnetron sputtering to 4-nm thickness for 2-minute sputtering time. Fig. 4·4a shows that TiO$_2$ coating has minimal background in SRS imaging and no interference with the SRS signal of polymer beads. In contrast to TiO$_2$ coating, commonly used Au-coated coverslips contributed...
a significant thermal background to the SRS image (Fig. 4·4b). The UV-Vis spectrum of the TiO$_2$-coated coverslip shows an absorbance peak at 532 nm (Fig. 4·4d), which facilitates the laser ejection. To characterize the spatial resolution of ejection, bead clusters dried on the TiO$_2$-coated coverslip were used as a test bed (Fig. 4·4E). For the 60X objective, the ejection spot diameter is $4.20 \pm 0.74 \mu m$. For the 40X objective and underfilled objective back aperture, the ejection spot size is $6.19 \pm 1.00 \mu m$. The ejection spot is larger than the optical resolution, probably due to the domino effect of ejection. The test was performed with a minimum laser energy guaranteeing a successful ejection without photodamage. Specifically, the laser power used was lower than 1.5 mW before the objective, and the energy of the laser pulse was less than 1 µJ. We further studied the impact of the axial focal position on the ejection efficiency (Fig. 4·4f). It was found that maximal efficacy was reached when the objective was focused on the TiO$_2$ coating, which is about 1 micron above the beads.
Figure 4-3: Optimization of laser absorbing coating in terms of SRS imaging and ejection. (a) Photo of coverslips with different types of coating. (b) Different types of coating. a,b,d,e: fabricated at BU Optoelectronic Processing Facility, parameters as denoted in the table; c, purchased from sigma (serial number 643254-12EA).

<table>
<thead>
<tr>
<th>No.</th>
<th>Au (nm)</th>
<th>TiO₂ (min)</th>
<th>Ti</th>
<th>SRS</th>
<th>Ejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>Strong background</td>
<td>Good</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>Strong background</td>
<td>Good</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>0</td>
<td>Unknown</td>
<td>Strong background</td>
<td>Damage</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>
Figure 4.4: Characterization of the LIFT system used in this work. (a) TiO$_2$ coating (2 min sputtering time) has minimal background in SRS image. Top: SRS image of polymer beads mixture (PS 2 µm, PMMA 3 µm); Bottom: SRS spectra of beads marked in the top image (bottom inset shows the background level). (b) Au coating (Au 1 nm, TiO$_2$ 2 min sputtering time) leads to a high background in SRS image. Top: SRS image of polymer beads mixture (PS 2 µm, PMMA 3 µm); Bottom: SRS spectra of beads marked in the left image. (c) Photo of standard coverslip without coating (left) and TiO$_2$ coated coverslip (right) placed on top of a Boston University logo. (d) Absorption of TiO$_2$ coated coverslip (2 min sputtering time). The black dotted line denotes 532 nm. (e) Demonstration of spatial resolution by microbead cluster (1 µm polymer beads dried on TiO$_2$ coated coverslip). The red circle shows the equivalent diameter of the ejection spots. Left: configuration 1 (60X objective). Right: configuration 2 (40X objective, underfilled back aperture of objective). (f) Impact of laser focal position on microbead ejection (1 µm polymer beads).
The workflow of S-RACE is shown in Fig. 4.2c. For each field of view (FOV), SRS images are first collected at 10 µs pixel dwell time. An SRS stack of 300 by 300 pixels and 4 wavenumbers takes 6 seconds. Subsequently, microparticles/cells within the FOV are identified by spectral analysis. For the discrimination of target objects in the context of 4-color SRS image involving two types of polymer microbeads, the target detection step consumes 0.2 seconds. For each targeted microparticle/cell, 2D galvo mirrors were employed to precisely position the green laser on the object. An acousto-optic modulator (AOM) was then activated to emit the 532-nm, 4-kHz laser onto the coating. The targeted object was then pushed away from the ejection coverslip and received by the collector coverslip. With AOM as a fast pulse picker, single-pulse ejection is achieved and each ejection takes 8 ms, which is sufficient to stabilize 2D galvo mirrors pinpoint and compensate for laser repetition rate. Multiple FOVs were stitched to a larger FOV by moving the sample stage. After sorting all the targeted cells, downstream phenotypic and/or genomic analysis, e.g., sequencing and proteomics, could be applied to the cells in the collector. For live cell ejection and cultivation, an agarose dish is used as a collector. After incubating the collector with sorted live cells at an appropriate temperature (30°C for fungus and 37°C for bacteria), single colonies can be observed and recovered after 24 to 48 hours, as illustrated in the following sections.

**4.3.2 S-RACE performance evaluated with mixtures of polymer beads**

We tested S-RACE performance using a polymer bead mixture (polystyrene (PS), with red fluorescence; poly (methyl methacrylate) (PMMA), without fluorescence; both 1.0 µm in diameter). Four wavenumbers (2860, 2905, 2950, and 2994 cm⁻¹) representing the Raman signatures of PS (2905 cm⁻¹) and PMMA (2950 cm⁻¹) were selected (Fig. 4.5a). All the beads were classified into two types (PS or PMMA) based on the workflow in Fig. 4.5b, and PS beads were targeted for sorting. This
polymer beads test was performed with a laser excitation from the top configuration (Fig. 4·4e), which features a small focus size.

![Figure 4·5: Target selection in polymer bead mixture. (a) SRS spectra of PS and PMMA beads. Light blue lines represent selected wavenumbers used in Fig. 4·6 (2860, 2905, 2950, and 2994 cm\(^{-1}\)). (b) Steps of single-bead detection used in Fig. 4·6b.](image)

A composite image of two-color SRS before ejection is shown in Fig. 4·6a. The composite SRS image with classified beads color-labelled is shown in Fig. 4·6b. To further enhance sorting purity, PS beads (the targeted class) that have neighboring PMMA beads located closer than 2.5 µm (identified as “clustered” PS) were excluded from sorting. The post-ejection identity of the beads is visualized in Fig. 4·6c. In the zoom-in image of Fig. 4·6b, two white arrows highlight PMMA beads situated adjacent to a PS bead. Both PMMA beads were retained following the ejection of all the targeted PS beads. Averaged spectra of beads before and after ejection were shown in Fig. 4·6d, confirming the beads classification. During experiments, we observed bright spots remaining on the coverslip after ejection (Fig. 4·6c). The spectra of these bright spots (Fig. 4·7a)) were distinguishable from those of PS or PMMA (Fig. 4·5a), indicating that these bright spots might have been caused by the deformations in the TiO\(_2\) coating [174, 175].
Figure 4·6: Evaluation of S-RACE performance by polymer microbead mixtures (polystyrene (PS), with red fluorescence; poly(methyl methacrylate) (PMMA), without fluorescence; both 1.0 µm in diameter). (a) 4-color SRS image before ejection. (b) Beads identified based on (a). White arrows in zoom-in image mark the detected 2 PS beads which excluded from ejection. (c) Beads identify after ejection. (d) Multicolor SRS of polymer beads before and after ejection. Shaded error bar: standard deviation. (e)-(f) Quantification of ejection performance.

The quantification of ejection performance is presented in Fig. 4·6e-f. Our S-RACE achieved 95% yield, 98% purity, and 14 events per second (eps). Given that the PS beads possessed red fluorescence, we were able to quantify the PS beads in the collector using wide-field fluorescence (Fig. 4·7b). This analysis revealed the presence of 292 PS beads in the collector, with a collection rate of 55%. Experiments
conducted using different bead exclusion criteria demonstrated that stricter exclusion criteria resulted in higher purity (Fig. 4-8).

**Figure 4-7:** Confirmation of successful polymer beads ejection. (a) Multicolor SRS of burning spots in Fig 2c. (b) Wide-field fluorescence image of the collector bottom. Red dots represent the collected red fluorescence-labeled PS beads after sorting.

<table>
<thead>
<tr>
<th></th>
<th>PS</th>
<th>PMMA</th>
<th>Yield</th>
<th>Purity</th>
<th>Throughput</th>
</tr>
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<tbody>
<tr>
<td><strong>Before</strong></td>
<td>563 (targeted)</td>
<td>273</td>
<td>95.2%</td>
<td>98.0%</td>
<td>13.9 eps</td>
</tr>
<tr>
<td></td>
<td>108 (clustered)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After</strong></td>
<td>135</td>
<td>262</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>exclude PS beads if they are close to PMMA beads (d&lt;2.5 um)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Before</strong></td>
<td>220 (targeted)</td>
<td>72</td>
<td>88.6%</td>
<td>93.8%</td>
<td>11.0 eps</td>
</tr>
<tr>
<td></td>
<td>17 (clustered)</td>
<td></td>
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<tr>
<td><strong>After</strong></td>
<td>42</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>exclude PS beads if they are close to PMMA beads (d&lt;1.5 um)</strong></td>
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<td></td>
<td></td>
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<tr>
<td><strong>Before</strong></td>
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<td>388</td>
<td>90.5%</td>
<td>76.9%</td>
<td>6.7 eps</td>
</tr>
<tr>
<td><strong>After</strong></td>
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**Figure 4-8:** Evaluation of S-RACE performance by polymer beads mixture ejection using different bead exclusion conditions.
4.3.3 S-RACE is applicable to live cells

To validate the biocompatibility of our S-RACE platform in sorting single live cells, we conducted tests on *S. cerevisiae*, *C. albicans*, and *E. coli*, representing both eukaryotic and prokaryotic cells. Hydrogels, polymers, and aqueous media layers have been reported in printing viable cells [57, 176]. In this study, to protect live cells from heat and mechanical damage process, we adapted the protocols from Liang [57] and Hong [39] to prepare an agarose layer on TiO$_2$ coating. The thickness of the agarose gel is $6.25 \pm 0.95 \text{m}$ based on estimation under a microscope. As shown in Fig. 4·9a-b, individual *S. cerevisiae* cells on the agarose gel were identified based on bright-field imaging. The image of the agarose layer after ejection is shown in Fig. 4·9c. The agarose plate with the sorted cells was then sent to a 30°C incubator, and 27 colonies were observed after 44 hours (Fig. 4·9d). The recovery rate of single *S. cerevisiae* cell ejection was 31%. The control group, in which non-cell areas were ejected, did not lead to any colony growth on the receiving agar plate. Similar tests were performed on *C. albicans* (Fig. 4·9e-h) and GFP-labeled *E. coli* (Fig. 4·9i-l). Cultivation recovery rates were 23% and 21% for *C. albicans*, and *E. coli*, respectively. Recultivated GFP-labeled *E. coli* colonies were observed (Fig. 4·9l). These results established the coating condition for live cell ejection.
Figure 4·9: Live cell ejection (S. cerevisiae, C. albicans, and E.coli). (a)-(d) Bright-field image guided sorting of S. cerevisiae (cultivation recovery rate 31%). (e)-(h) Bright-field image guided sorting of C. albicans (cultivation recovery rate 23%). (i)-(l) Bright-field image guided sorting of GFP-labelled E. coli (cultivation recovery rate 21%).

To assess the impact of SRS laser radiation on cell viability during the S-RACE of live cells, time-lapse imaging was performed to visualize E. coli growth after SRS laser radiation (Fig. 4·10). Three laser radiation levels were tested (No SRS imaging. SRS imaging at 0 min with power: pump beam 24 mW, Stokes beam 50 mW; stepsize: 0.15 µm/pixel; 10 frames. SRS imaging at 70 min with power: pump beam 24 mW, Stokes beam 100 mW; stepsize: 0.15 µm/pixel; 10 frames.). The medium radiation was more stringent compared to the experimental condition. E. coli cells were dropped onto a 1% agarose pad after sampling from liquid culture, and sandwiched with a top coverslip. The cells were kept in an enclosed incubator stabilized at 30°C. For all three laser radiation levels, cell growth and colony formation were observed. By
fitting the growth curve of the cell colony areas $S$ (µm²). The growth rates, $\mu$ (h⁻¹) can be calculated as $\mu = \ln\left(\frac{S_t}{S_0}\right)/(t-\lambda)$, where $\lambda$ is the lag time (h). The growth rate $\mu$ was not significantly different across the three radiation levels. This result confirms the biocompatibility of the SRS laser radiation.

![Figure 4.10](image)

**Figure 4.10:** Safe SRS imaging condition has minimal impact on *E. coli* colony growth. Figures in each row represent the time-lapse of *E. coli* colony growing under a specific SRS imaging condition.

We then performed S-RACE of two types of live cells: *S. cerevisiae* (Fig. 4.12a-f) and GFP-labeled *E. coli* (Fig. 4.12g-l). Fig. 4.12a shows the schematic of the ejection module. Fig. 4.12b shows SRS spectra in the C-H stretching region of the cells. Individual *S. cerevisiae* cells were identified based on single-frame SRS images at 2935 cm⁻¹ (signal-to-background ratio 4, Fig. 4.12c-d). SRS image after ejection is shown in Fig. 4.12e. Bright spots in Fig. 4.12e were probably caused by the deformed agarose layer and/or deformed TiO₂ coating. The spectra of bright spots after ejection are shown in Fig. 4.11, different from the spectra of cells or the image background. Recultivated cells were transferred to culture tubes after 40 hours of cultivation on an agarose plate. The culture medium with ejected cells became turbid, indicating
successful cell growth, whereas the medium of the control group remained clear. For GFP-labeled *E. coli*, the collector used was composed of a thin agar layer (60 µm) and a standard coverslip. This design enables the visualization of sorted cells in the collector. Fig. 4·12i-k shows the image before and after ejection. After cell ejection, a wide-field fluorescence image of the sorted GFP-labeled *E. coli* in the collector confirmed that most of the cells remained in good shape (Fig. 4·12l).

**Figure 4·11:** S-RACE and recovery of live cells (*S. cerevisiae* and GFP labeled *E. coli*). (a) Schematic of the ejection module used for *S. cerevisiae* ejection. (b) SRS spectra of *S. cerevisiae* and background. (c) single-color SRS image of *S. cerevisiae* before ejection. (d) Cell identified based on (c). (e) Single-color SRS image of the same FOV as D after ejection. (f) Picture of culture tube after 48 hours. (g) Schematic of the ejection module used for *E. coli* ejection. (h) SRS spectra of *E. coli* and background. Shaded error bar: standard deviation. (i) Single-color SRS image of *E. coli* before ejection. (j) Cell identified based on (i). (k) Single-color SRS image of the same FOV as J after ejection. (l) Wide-field fluorescent image of collected *E. coli* on the bottom agar.
4.3.4 S-RACE of lipid-rich *R. glutinis* from cell mixture followed by qPCR identification

To show the utility of our S-RACE platform to sort target cells from a mixture, we tested lipid-rich *Rhodotorula glutinis* (*R. glutinis*) cells mixed with *S. cerevisiae*. *R. glutinis* has been identified as oleaginous yeast and can contain up to 70% lipids in its dry-weight biomass [177, 178]. In contrast, *S. cerevisiae* only has 6% lipids in its biomass [177]. Lipid-rich yeasts including *R. glutinis* have been valuable models for sustainable biofuel production. Using multicolor SRS, the lipid content in individual yeast cells can be quantified. Lipid-rich intracellular aggregates found in the SRS image had a spectrum similar to that of glycerol trioleate (Fig. 4·13a-b). While the cell body part has a similar spectrum as peptone, a standard protein sample (Fig. 4·13b), the lipid channel after background subtraction in Fig. 4·13c showed the SRS signal at 2851 cm\(^{-1}\) is contributed by lipid [14].

Fig. 4·13d shows the spectral summation of a 5-color SRS image of a mixture of *R. glutinis* and *S. cerevisiae* (ratio 1:1). The lipid-rich cells were subsequently
identified from the lipid channel (Fig. 4·13e-f). The cells were sorted with the bottom configuration in Fig. 4·4e. After sorting the lipid-rich cells, the lipid channel has a much reduced intensity (Fig. 4·13g), which confirms the successful ejection of lipid-rich cells.

**Figure 4·13:** S-RACE of lipid-rich *R. glutinis* from mixture with *S. cerevisiae* and qPCR identification. (a) Spectrally summed hyperspectral SRS of *R. glutinis*. (b) SRS Spectra of cell body (yellow solid line) and lipid droplet (green solid line), with two standard samples (glycerol trioleate: green dashed line; peptone: yellow dashed line). The pink shaded region denotes the signature peak of lipid. (c) Left: definition of lipid and background intensity. Right: lipid channel of the same FOV in (a). Lipid channel presents SRS signal at around 2850 cm$^{-1}$ with linear background subtracted. (d) Spectrally summed 5-color SRS image of *R. glutinis* and *S. cerevisiae* mixture before ejection. (e) Lipid channel of the same FOV in (d). (f) Location of lipid-rich cells found by automatic program based on lipid channel in (e). (g) Lipid channel after ejection.

The sorted cells were collected by a customized collector made with coverslip and PDMS (Fig. 4·14a). Four independent trials were performed (presented as a-d in Fig. 4·14a). Each well received sorted cells from 8-12 SRS FOVs. The throughput is
11.9 cells per second.

To confirm the identity of the sorted cells are *R. glutinis* we targeted, quantitative polymerase chain reaction (qPCR) was performed on the collected cells. The second internal transcribed spacer (ITS2) in the nuclear ribosomal DNA was used as the target sequence. The workflow of qPCR amplification preparation is shown in Fig. 4.14a: after DNA extraction [179], the supernatant containing resuspended DNA was used for qPCR, using primers from a previous study [180]. Each well produced 3 replicates in qPCR amplification, and each replicate contained 16% of the total DNA content in this well. The amplifications of the collected cell contents present a
peak of around 81.3°C in the melt curve (Fig. 4·14i), consistent with the amplification results of *R. glutinis* pure culture without ambiguous peaks (Fig. 4·14b). We would also like to note that the cell number per well estimated from qPCR amplification is lower than the number of ejection due to the cell loss during the transfer process and the presence of larger cells that necessitate multiple ejections (Fig. 4·14c-d). In conclusion, these results demonstrate that S-RACE successfully sorted specific cell populations based on their phenotype/functions.
Figure 4.15: qPCR assay. (a)-(b) Amplification plot and melt curve from pure culture of *R. glutinis* and *S. cerevisiae*. (c) Test sensitivity of qPCR assay for detection of ITS2 region in *R. glutinis*. The gray lines denote the DNA extracted from serial dilution of *R. glutinis* pure culture. The numbers in the legend denote the estimated number of cells in the qPCR reaction well. The qPCR amplification plots of the 4 trials in Fig. 4.14 are also shown using the same color scheme. (d) Linear fitting of CT (cycle threshold) and log(1/#cell) of serial dilution samples. The 4 trials in Fig. 4.14a (well a-d) were also plotted with #cell estimated from the linear fitting. The estimated cell number for well a-d are 91, 286, 162, 354. The number of ejection for each small well ranges from 1100 to 1700. Cell number is lower than the number of ejection due to the cell loss during the transfer process and the presence of larger cells that necessitate multiple ejections.

4.3.5 S-RACE with opto-control allows for real-time sorting of cells in culture and regions of interest in a tissue slice

Seamlessly integrating a real-time precision opto-control (RPOC) system with our S-RACE platform yielded a real-time cell sorting approach. The RPOC utilizes a closed-loop feedback control circuit for laser manipulation with a fast response of
sub-microsecond [181]. This innovation enables imaging-identifying-sorting to occur within a single pixel during laser scanning, bringing opportunities for higher precision and efficiency. The concept of real-time imaging sorting is illustrated in Fig. 4-16a. The front panel of the comparator circuit is shown in Fig. 4-16b. During laser scanning, the SRS signal carrying chemical information from the sample was sent to the comparator circuit. For an SRS signal higher than the preset threshold, the comparator circuit will command AOM to rapidly couple the 532 nm laser, which subsequently ejects the targeted object residing in the current pixel. Limited by the repetition rate of 532 nm laser (16.6 kHz), 70 µs dwell time was applied.

**Figure 4-16:** Real-time imaging and sorting of cells in culture and in tissue environment with a real-time precision opto-control (RPOC) system. (a) An illustration of real-time imaging-sorting with RPOC technology. (b) Front panel of the comparator circuit box with ports used in this study. (c) Real-time imaging-sorting of 1 µm polymer beads. Top to bottom: SRS image (2950 cm\(^{-1}\)) before sorting; active pixels; SRS image after sorting. (d) Real-time imaging sorting of live *S. cerevisiae*. Top to bottom: SRS image (2940 cm\(^{-1}\)) of live *S. cerevisiae* before sorting; active pixels; SRS image after sorting; culturing result of sorted *S. cerevisiae*. (e)-(f) Real-time imaging-sorting of rat brain tissue (thickness 5 µm). (e): SRS image at 2940 cm\(^{-1}\). (f): SRS image at 2850 cm\(^{-1}\).

To show the utility of this real-time imaging-sorting regime, we sorted 1-µm polymer beads, single cells, and tissue sections based on their SRS images. For polymer beads, the SRS image (2950 cm\(^{-1}\)) before sorting is shown in Fig. 4-17a (top). Active pixels were set by thresholding the SRS image, and each polymer bead contained 4-6
active pixels for best sorting performance. Most of the polymer beads were successfully sorted based on their SRS intensity. For live *S. cerevisiae*, SRS images before and after real-time imaging sorting are shown in Fig. 4·17b. After 48 hours, six *S. cerevisiae* colonies were observed in the petri dish with sorted cells and no colony growth was observed in the control group.
Figure 4.17: Real-time imaging and sorting of cells in culture and in tissue environment with a real-time precision opto-control (RPOC) system. (a) Real-time imaging-sorting of 1 µm polymer beads. Top to bottom: SRS image (2950 cm$^{-1}$) before sorting; active pixels; SRS image after sorting. (b) Real-time imaging sorting of live *S. cerevisiae*. Top to bottom: SRS image (2940 cm$^{-1}$) of live *S. cerevisiae* before sorting; active pixels; SRS image after sorting; culturing result of sorted *S. cerevisiae*. (c)-(d) Real-time imaging-sorting of rat brain tissue (thickness 5 µm). (c): SRS image at 2940 cm$^{-1}$. (d): SRS image at 2850 cm$^{-1}$.

We further tested the applicability of S-RACE to tissue sections. We prepared a
cryosectioned rat brain tissue and ablated multiple regions of interest (ROIs) based on SRS images at 2850 cm\(^{-1}\) (Fig. 4·17c) and 2940 cm\(^{-1}\) (Fig. 4·17d). Raman bands centered around 2850 and 2940 cm\(^{-1}\) are representative of cellular lipids and proteins and provide contrast in molecular signatures and morphological features of the cryosectioned tissues. Successful tissue ablation was confirmed by SRS spectra (Fig. 4·17e), where the ejected spots showed relatively low intensities, while the unimpacted area showed a typical protein-rich Raman spectrum. In the future, instead of single-color SRS, lipid and protein contrast could be combined for tissue ablation with the aid of two comparator circuits. In addition to rat brain tissue, we also tested a bone cancer tissue slice, where 3 FOVs were imaged, featuring protein-rich, vessel, and collagen-rich regions (Fig. 4·18). Ejection targets were manually selected, and successful ejections were confirmed by the SRS spectrum after ejection. The size of the ejection spots was quantified. For protein-rich bone cancer tissue, the spot size was 10.98 ± 2.24 µm. The collagen-rich region of bone cancer tissue had a smaller ejection size probably because of the higher rigidity of collagen compared to the protein-rich region. These results highlight the potential of S-RACE as an integrative tool for spatial multiomics measurements across diverse samples ranging from single cells to tissue sections.
Figure 4.18: S-RACE of an SJSA-1 tumor tissue section (thickness 5 µm). (a) Spectral summation of hyperspectral SRS images of SJSA-1 tissue section before and after ejection. Average ejection spot size: 10.98±2.24 µm (SJSA-1 protein-rich), 7.10±1.35 µm (SJSA-1 collagen-rich). Single-cell ejection was made on single red blood cells. (b) Spectra of 3 regions of interest marked by dashed circles in (a).
4.4 Discussion and conclusion

Towards the goal of high-throughput label-free single-cell sorting, we reported in this work a stimulated Raman-activated cell sorting system, termed stimulated Raman-activated cell ejection (S-RACE). This platform integrates a multispectral SRS microscope, a laser ejection module, and an online image processing framework. Successful sorting of various samples was demonstrated, including a 1.0-µm polymer beads mixture, bacteria, fungi, and tissue sections. These results illustrate high-throughput sorting of single particles or cells based on their Raman signatures, with precision and minimal impact on the microenvironment around the targeted particle or cell. S-RACE achieves a throughput of approximately 14 eps on a mixture of polymer beads with 4-color SRS imaging and single-pulse ejection. For 5-color SRS-activated cell sorting, the throughput is approximately 12 eps. Successful qPCR amplification was demonstrated with ejection numbers ranging from 1000-1700, where the ejection can be completed in less than 3 minutes. Considering the sample preparation time, the entire process can be completed in less than 10 minutes. The potential for multiplexed high-throughput sorting is also on the horizon, as different species or cells can be sorted into different wells in the collector for subsequent phenotypic and/or genotypic analyses. The sorting purity has been enhanced by addressing the domino effect of ejection, which can be mitigated by calculating inter-object distances and excluding clustering objects. High sorting yield was achieved for microparticles, microorganisms, and tissue sections, thanks to the high success rate of ejection and the automatic target detection. Importantly, S-RACE possesses the capability for live cell imaging and sorting, a feat that has long been pursued in Raman-activated cell ejection but remains challenging due to mechanical stress, thermal, and dehydration damage [182]. In our study, a thin layer of agarose gel was added to the TiO₂-coated coverslip to reduce cell damage from the pushing force and heating. This thin and
uniform agar layer is crucial to maximize the success rate of cell ejection while maintaining cell viability. A customized receiver with an agarose gel layer was used to protect cells during the landing process. Successful cultivation after ejection for three microorganism species (*S. cerevisiae*, *C. albicans*, *E. coli*) was shown. For *E. coli*, a similar recovery rate as the previous report (22%) (18) was achieved. We note that there is still room for improvement in the S-RACE platform. First, LIFT-based cell sorting typically necessitates an air layer to separate the original sample on the top from the sorted cells in the bottom collector. However, this practice contradicts the prerequisites of SRS imaging and consequently attenuates the SRS signal quality. Besides, non-Raman background, e.g. cross-phase modulation, engenders some spurious effects. Removal of the non-resonant background in the SRS image will significantly improve the sensitivity and signal fidelity. The exploration of alternative modalities like frequency-modulation SRS [183], stimulated Raman photothermal microscopy [184] and mid-infrared photothermal microscopy [185, 186] holds promise owing to their high detection sensitivity. The challenges associated with the refractive index mismatch between the sample and the air layer can also be mitigated through the use of photothermal detection regimes. Second, the image dwell time in the current real-time imaging-sorting regime is limited by the relatively low repetition rate of the ejection laser. A higher throughput could be achieved by incorporating a nanosecond laser with a higher laser repetition rate. Furthermore, the use of a hardware-based spectral processor in the module could enhance specificity through multiplex imaging. We envision that S-RACE would benefit multiple biological applications that were previously challenging or impractical with conventional fluorescence-based or flow-based sorting technologies. First, a synergistic integration of S-RACE with advanced genome, epigenome, and transcriptome sequencing technologies will provide insights into the link between phenotype and genotype at the single-cell level. Sec-
ond, unlike microfluidics, S-RACE is applicable to tissue samples. Harnessing its high-throughput capacity and advanced image recognition capabilities, discrimination and further genetic analysis of distinct ROIs based on both Raman signatures and morphological features can be achieved. Third, the majority of microbial organisms are still regarded as "dark matter" and waiting to be revealed [187]. In tandem with genomic analysis, S-RACE can be a potent tool for discovering unknown species without the need for microorganism cultivation. For example, complex samples from environmental soil could be directly placed onto a TiO$_2$-coated coverslip and sorted based on specific phenotypes for single-cell genomics analysis. Tissue samples like sectioned gut tissues could also be studied by S-RACE to gain insights into microbiota activities and the interactions between gut and microbiota while retaining the spatial architecture of the microenvironment. Lastly, it can enable the selection of cells for in vivo cell therapies, including stem cells and chimeric antigen receptor T (CAR-T) cells for personalized medicine. In summary, by integrating an SRS microscope, online image processing, and a cell ejection module, we have developed an automated, high-throughput S-RACE platform for precise single-cell sorting. This platform is compatible with a wide range of samples, spanning from small microorganisms to tissue sections. We expect that S-RACE will open avenues across many biological and biomedical applications.
Chapter 5

SUMMARY AND OUTLOOK

Gaining insight into how individual cells respond to environmental changes continues to be a central challenge in biology, necessitating the use of single-cell analysis. When selecting single-cell analysis tools, there are multiple perspectives to consider, including information content, throughput, specificity, sensitivity, cell integrity, etc. This dissertation has explored the methodologies to advance the application of SRS microscopy in high-throughput single-cell analysis.

In chapter two, I proposed and developed a deep learning model to bridge the trade-offs between imaging speed, chemical specificity, and signal-to-noise ratio. This model leverages the spatial-spectral correlation in hyperspectral SRS images to predict chemical maps from high-speed single-shot femtosecond SRS images. Specifically, it can differentiate four subcellular components, including lipid droplet, endoplasmic reticulum, and cytoplasm. This method not only unveils rapid cellular dynamics but also facilitates large-scale tissue section imaging and multiplex subcellular component analysis. It is important to mention that the network performance is dependent on the labels in the training data. In the future, transfer learning may enable prediction of new subcellular components from a limited data set or image data set from other modalities. The various functionalities of deep learning tools can augment information content derived from SRS imaging and ultimately enhance imaging performance.

Except throughput, another crucial perspective to consider is information dimensionality. There are two primary strategies to enhance the information extracted...
from the images: one involves optimizing feature extraction [42], and the other entails incorporating vibrational/fluorescent tags for multi-modality imaging [43]. In chapter three, I employed polygon scanner-based SRS microscopy to image metabolic-engineered *E. coli* cells for limonene production. Limonene and its derivatives are important biofuels and microbial production offers a renewable carbon source. Within the population of limonene-producing *E. coli* cells, distinct subpopulations of producers and non-producers were observed. By segmenting the cells and subcellular structures, morphological features were extracted. Spectral unmixing was then employed to discern the chemical content at the subcellular level, thereby confirming the presence of limonene-rich aggregates within the engineered *E. coli*. To further validate the synthetic function of the limonene-rich aggregates, I utilized a dual-modality imaging of SRS and two-photon fluorescence. Collectively, SRS imaging with single-cell analysis facilitated the direct visualization of the limonene-rich aggregates inside the engineered *E. coli*.

To further explore the heterogeneity that serves a biological function and contains meaningful information, the process of cell sorting is indispensable. After cell sorting, further characterization like genomics, transcriptomics, proteomics, and more can be applied, enriching the depth and breadth of information content. In chapter 4, I introduce the integration of laser-induced cell ejection with SRS microscopy for Stimulated Raman-activated Cell Ejection (S-RACE). In contrast to microfluidics-based CRS-activated cell sorting, S-RACE provides stable performance for small cells (≤3 μm). S-RACE allows label-free chemical imaging guided cell sorting through multispectral SRS imaging, on-the-fly image analysis, and laser-induced cell ejection. Versatile applications of S-RACE to a wide range of samples, such as polymer particles, single-live bacteria, single-live fungus, and tissue sections. For a mixture of polymer beads, S-RACE achieved high yield (95%), and high purity (98%), with a
throughput of approximately 14 events per second (eps). Furthermore, by integrating a closed-loop feedback control circuit into the system, real-time single-cell imaging and sorting were realized and applied to precisely eject regions of interest from a rat brain tissue section. Towards further development, we expect the high-throughput imaging-sorting will benefit multiple biological applications that were previously impractical or challenging with conventional fluorescence-activated or microfluidics-based sorting technologies.
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