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Multimodal techniques and strategies for chemical and metabolic imaging at the single-cell level

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Single-cell chemical and metabolic imaging technologies provide unprecedented insights into individual cell dynamics, advancing our understanding of cellular processes, molecular interactions, and metabolic activities. Advances in fluorescence, Raman, optoacoustic (photoacoustic), or mass spectrometry methods have paved the way to characterize metabolites, signaling molecules, and other moieties within individual cells. These modalities can also lead to single-cell imaging capabilities by targeting endogenous cell contrast or by employing exogenous contrast generation techniques, including contrast agents that target specific cell structure or function. In this review, we present key developments, summarize recent applications in single-cell interrogation and imaging, and illustrate their advantages, limitations, and outlook.

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Introduction

Single-cell imaging (SCI) is critical in biological sciences and aims to increase our understanding of cell development, function, aging, and disease progression. Optical microscopy has been the classical approach to visualizing single cells, and when aided by fluorescent proteins or targeted agents with specificity to cellular structures and functions, it can enable the study of intricate cellular processes and cellular dysregulation throughout the course of pathological conditions and responses to therapeutic interventions. Different optical methods are considered for SCI, including fluorescence imaging, optoacoustic (OA) (photoacoustic) imaging, Raman spectroscopic imaging, and mass spectroscopy imaging (MSI). With appropriate implementation, such methods can visualize individual cells or reach abilities to resolve the distribution of specific molecules within cells and the cell microenvironment [1]. With different advantages, disadvantages, and performance characteristics, careful selection of one or more modalities in hybrid mode is required depending on the application considered.

Critical differentiating factors of the technology employed relate to the resolution and sensitivity achieved and to whether the method operates with the use of external labels (e.g. fluorescent agents), or whether it exploits an intrinsic contrast mechanism based on the integration of the interrogating energy with an endogenous cellular moiety, that is, label-free operation. Imaging performance may also depend on the intricacies of biological sample preparation, the contrast-to-background ratio, and the signal-to-noise ratio achieved. When using labels, imaging results also depend on the physicochemical and biodistribution characteristics of the label selected. Additional factors to consider for selecting a technique for SCI include the compatibility of the selected SCI approach and probes with living cells, acquisition times required and throughput, the complexity of sample preparation, or the necessity to employ multimodal operation that integrates different imaging techniques to yield a more complete information profile [2]. In this review, we highlight state-of-the-art SCI techniques and discuss their applications, advantages, limitations, and outlook (Figure 1).

Fluorescence microscopy

Conventional phase-contrast microscopy enhances the contrast of colorless and transparent cells and specimens by exploiting differences in refractive index in different parts of the specimen, thus allowing visualization of cells, their migration, cell division, and other such cellular functions. However, phase-contrast microscopy is limited by a lack of molecular specificity and, therefore, cannot visualize specific chemical and metabolic processes within single cells. For molecular-level imaging applications, fluorescence microscopy is widely exploited using fluorescent proteins or probes that selectively bind to specific targets. Because of its

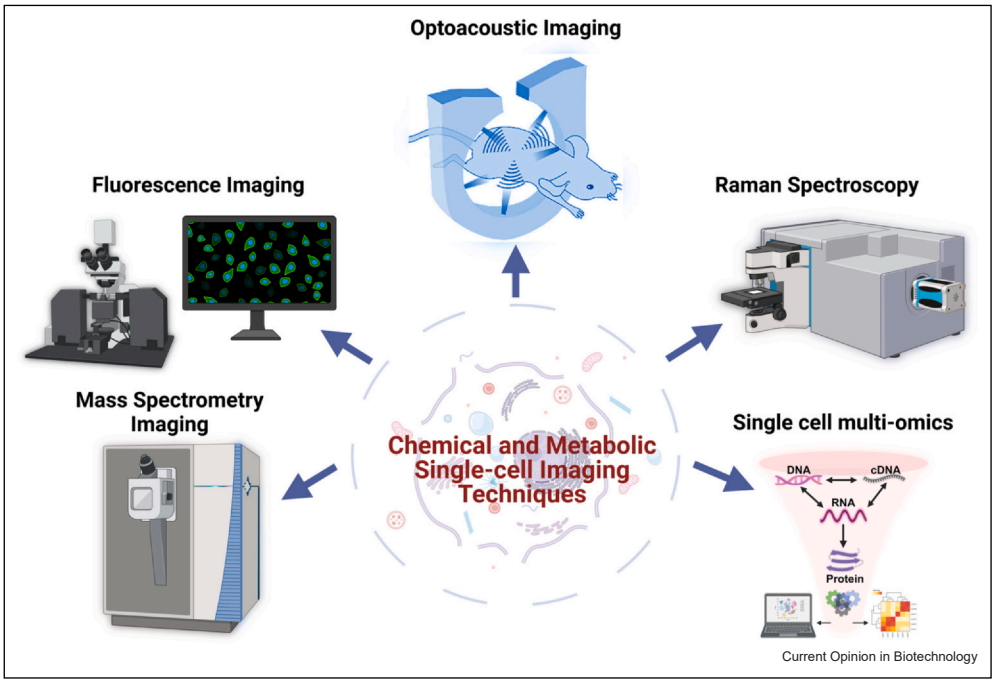
submicrometer resolution, fluorescence microscopy is suited for single-cell-level observation of cellular processes, metabolic activity, and functional characteristics in real time. Common applications of fluorescence SCI include the analyses of the spatial distribution, concentrations, and dynamics of cellular components such as DNA, RNA, proteins, lipids, ions, and metabolites [3–6]. Various genetically employed fluorescent proteins or exogenously administered fluorescent agents based on antibody conjugates, ion-sensitive probes, and metabolite-specific probes are used in biological and medical research [7–11]. The choice of imaging probe depends on the user's requirements, as imaging probes vary in their target specificities, multiplexing capabilities, *in vitro* and *in vivo* compatibilities, and suitability for dynamic imaging.

In addition to tracking fluorescent intensities, Förster resonance energy transfer or fluorescence resonance energy transfer (FRET) [12] has been developed for sensing biochemical activity. FRET exploits the transfer of light energy from a donor fluorophore, in a certain wavelength range, to an acceptor fluorophore that emits at a shifted longer wavelength range. Energy transfer occurs within a 1- to 10-nanometer radius through dipole–dipole coupling. Another fluorescence-based technique is fluorescence recovery after photobleaching, which allows for studying protein dynamics in individual cells by selectively bleaching a small region using a high-intensity laser pulse [13]. The recovery of fluorescence intensity in the bleached region occurs over time due to the diffusion or movement of unbleached fluorescent molecules from the surrounding areas. This fluorescence recovery allows the visualization of molecular dynamics and processes (e.g. turnover, mobility, interactions, and recovery kinetics) within the cells.

Fluorescence lifetime imaging microscopy is another method developed to sample intracellular processes. It senses changes in the temporal emission profile of fluorescence molecules due to the molecular environment where the fluorochrome is immersed. In particular, the technique measures the duration for which a fluorescent molecule remains at the excited state before returning to the ground state. Since sensing is not based on intensity measurements but on the timing characteristics of the fluorescence de-excitation process, the method is not sensitive to fluorochrome biodistribution and has also been used as a reliable method to read FRET. Application examples include the study of the metabolic response to glucose in living human Langerhans islets, spatial-metabolic changes in 3D breast cancer spheroids, or plasma membrane dynamics [14–19].

Currently, the fluorescence imaging techniques with the highest resolution fall under the domain of fluorescence super-resolution microscopy (SRM). These

Figure 1



Five techniques used in single-cell chemical and metabolic imaging. Each of these techniques will be briefly discussed in this review. Created with BioRender.com.

techniques, which include stimulated emission depletion (STED) microscopy, photoactivated localization microscopy, stochastic optical reconstruction microscopy (STORM), and single-molecule localization microscopy, achieve resolutions below the diffraction limit, allowing for unprecedented spatial resolutions (~50 nm) sufficient to image subcellular compartments in single cells [20]. While all these techniques allow for the visualization of fluorescent targets, integrating SRM with click-expansion microscopy (ExM) can provide more detailed information on the chemical composition of subcellular components and the biodistribution of molecules such as lipids, glycans, DNA, and RNA in single cells and tissue samples. In this approach, ExM physically magnifies the samples by severalfold, and click labeling enables multicolor imaging (Figure 2a,b) [7,21,22]. To further increase the imaging resolution, Shi et al. [23] developed a multimodal approach, which they termed molecule anchorable gel-enabled nanoscale imaging of fluorescence and stimulated Raman scattering microscopy (MAGNIFIERS). MAGNIFIERS integrates ExM with stimulated Raman scattering (SRS) microscopy resulting in ~7.2-fold tissue expansion, which allows imaging of protein, DNA, and lipids in small extracellular vesicles (EVs) with a lateral and axial resolution of ~41 nm and ~194 nm, respectively (Figure 2c). However, all ExM techniques require the fixation of tissues and are not suitable for *in vivo* imaging. In a recent study, to overcome the alterations in regular cell function due to the use of fluorescent tags and a lack of significance to the human context, Bai et al. [24] used a single-cell metabolic imaging platform called an optical photothermal infrared (OPTIR) microscope in combination with azide-tagged infrared (IR) probes. This approach enabled the direct imaging of lipid metabolism with submicrometer resolution and high specificity in various human-derived 2D and 3D culture systems (Figure 2d).

In summary, fluorescence imaging can image single cells with sufficiently high resolution, contrast, and sensitivity for the visualization of cellular structures and molecular interactions. Despite these advantages, the effective use of fluorescence SCI requires careful consideration of its inherent limitations, including photobleaching and phototoxicity due to the prolonged exposure of laser light and limited penetration depth, which restricts the imaging of structures deep within thick samples in their native form. Improving multifunctional probes for label-retention (LR) ExM and adopting appropriate tissue fixation methods can prevent the loss of fluorescence intensity and enhance resolution [25]. Additionally, integrating fluorescence imaging with techniques like optoacoustic imaging (OAI) and SRS enables deep tissue imaging for cellular structures.

Optoacoustic imaging

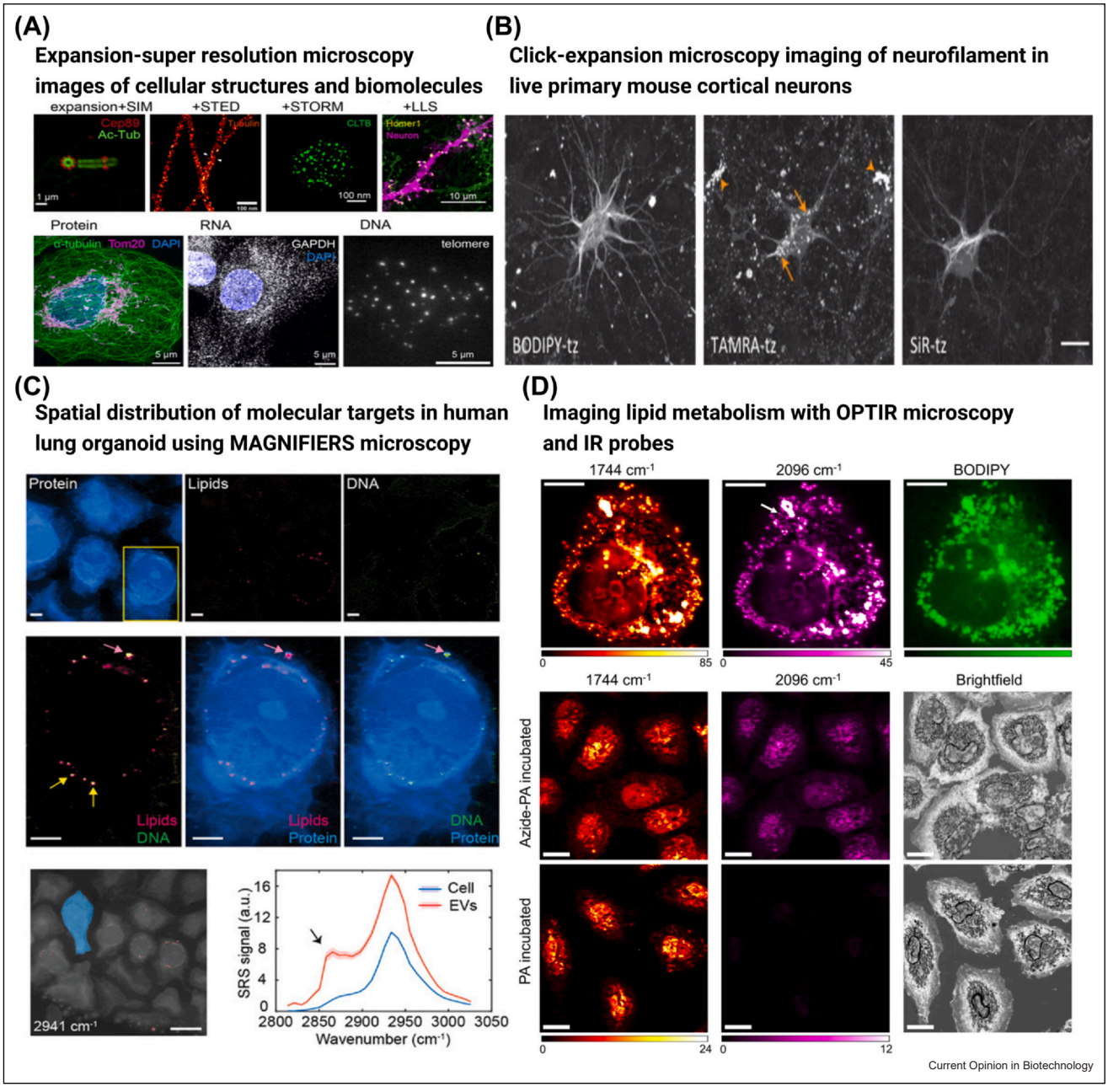
In OAI, also termed photoacoustic imaging, short pulses of laser light are used to irradiate biological samples. The absorption of the laser pulses by endogenous chromophores (e.g. hemoglobin, melanin, and lipids) or exogenous contrast agents in the biological sample causes rapid thermal expansion, resulting in the generation of acoustic waves. These waves are then detected and converted into electric signals by an ultrasound transducer. The acquired signal is processed and used to reconstruct detailed images of internal structures based on the distribution of optical absorption in the tissue [26,27]. OAI offers spatial resolutions of up to 5 μm and can visualize structures at depths of up to 3–9 mm, which is challenging for traditional optical imaging methods [28,29]. Single-cell OA microscopy can achieve throughputs of approximately 12 000 cells per hour without altering the metabolism of the cells while maintaining sufficient diffraction-limited lateral resolution, which is challenging to achieve with traditional electrical and fluorescence methods individually [30].

Various OAI techniques have been developed for subcellular chemical and metabolic imaging in tissue, offering label-free detection of biomolecules with high signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR). This is difficult to achieve with other techniques and is limited to certain molecules even when achieved by techniques such as mid-IR spectroscopy or SRS microscopy [31]. For example, mid-infrared optoacoustic microscopy (MiROM), which is based on biomolecule-specific vibrational transitions, is a novel technique for label-free live-cell metabolic imaging and can monitor carbohydrates, lipids, and proteins on a subcellular level. In MiROM, mid-IR excitation is combined with OA detection, producing images with high SNR and CNR from the simultaneous acquisition of OA signals and biomolecular vibrations. MiROM also offers a high detection limit of metabolites in single cells, with a lower risk of phototoxicity (Figure 3a,b) [32].

Another notable approach for single-cell OAI is photoacoustic flow cytometry (PAFC). PAFC allows noninvasive, continuous assessment of circulating cells in blood vessels and lymphatic vessels *in vivo* [33]. Figure 3c showcases the application of this novel PAFC method to examine embolus or clot formation in the bloodstream of tumor mouse models [34]. PAFC has also been used in the evaluation of melanoma metastasis by monitoring the melanin content in circulating tumor cells [35].

Other new developments in single-cell OAI focus on enhancing the compactness, stability, and speed of OAI to broaden its applicability in SCI and biomedical research. Of these, a notable new development is a fiber-laser-based modality called an optical resolution photoacoustic microscope (OR-PAM). OR-PAM has a laser

Figure 2


(A)
Expansion-super resolution microscopy images of cellular structures and biomolecules
expansion+SIM
+STED
+STORM
+LLS
Cep89
Ac-Tub
Tubulin
CLTB
Homer1
Neuron
1 μm
100 nm
10 μm
Protein
RNA
DNA
α-tubulin Tom20 DAPI
GAPDH
DAPI
telomere
5 μm
(B)
Click-expansion microscopy imaging of neurofilament in live primary mouse cortical neurons
BODIPY-tz
TAMRA-tz
SiR-tz
(C)
Spatial distribution of molecular targets in human lung organoid using MAGNIFIERS microscopy
Protein
Lipids
DNA
Lipids
DNA
Lipids
Protein
DNA
Protein
2941 cm-1
SRS signal (a.u.)
16
12
8
4
0
2800 2850 2900 2950 3000 3050
Wavenumber (cm-1)
Cell
EVs
(D)
Imaging lipid metabolism with OPTIR microscopy and IR probes
1744 cm-1
2096 cm-1
BODIPY
0
85
0
45
1744 cm-1
2096 cm-1
Brightfield
Azide-PA incubated
PA incubated
0
24
0
12
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State-of-the-art multimodal fluorescence imaging techniques used in SCI. **(a)** Representative images of cellular organelles and biomolecules to highlight the effective resolution of ExM using different super-resolution microscopes. Top row from left to right**:** ultra-ExM structured illumination microscopy (SIM) image of cilia (lateral resolution: 30 nm), ExSTED image of microtubules (10 nm lateral and 50 nm axial resolution), LR-ExSTORM image of clathrin-coated pits in a HeLa cell (5 nm lateral and axial resolution), expansion lattice light-sheet (ExLLS) image of neurons (lateral resolution ~70 nm). Bottom row from left to right: LR-ExM confocal image of microtubules, mitochondria, and DNA in a U2OS cell; expansion fluorescent *in situ* hybridization (ExFISH) confocal image of a HeLa cell labeled with GAPDH, DNA oligos, and DAPI; ExM image of a telomere in chromatin of an IMCD3 cell (Bottom row: lateral resolution ~70 nm). **(b)** Genetic code expansion, click labeling, and confocal scanning microscope imaging of neurofilaments (NFL) in live primary mouse cortical neurons (MCNs). BODIPY-tz, TAMRA-tz, or SiR-tz stained MCNs expressing various click-labeled NFL tags ($NFL^{K363TAG}$-FLAG, NFM, and NES PylRS/$tRNA_{CUA}^{Pyl}$). In the TAMRA-tz panel, the background staining of lysosomes is highlighted in both $NFL^{K363TAG}$-expressing neurons (arrows) and in nontransfected neurons (arrowheads). Z-stack images are shown as maximum intensity projections. Scale bars: 20 μm. **(c)** Label-free nanoscale imaging of chemical compositions in a human lung organoid using MAGNIFIERS. Top row**:** spectrally unmixed C-H channels for protein (left), lipids (middle), and DNA (right). Middle row from left to right, two-color overlay images of zoomed-in areas outlined by the yellow box. Arrows indicate EVs containing lipids and DNA. Images were collected using a 1.05 NA objective (4.5-fold expansion). Scale bar: 2 μm. Bottom: SRS spectral analysis of the cell area (blue) and EV (red) in the human lung organoid (left) scale bar 10 μm and background-subtracted SRS spectra (right). Arrow indicates a side peak at ~2865 cm^{-1} contributing from the CH_2 signal of lipids in small EVs. **(d)** Lipid metabolism was monitored using OPTIR microscopy and IR probes. Representative total lipids (1744 cm^{-1}), newly synthesized lipids (2096 cm^{-1}), and BODIPY imaging from a single cell (top). OPTIR images at 1744 cm^{-1} and 2096 cm^{-1} of human neuroglioma H4 cells after incubation in azide-palmitic acid (PA) and PA-containing media, along with corresponding brightfield images (bottom). Scale bar: 20 μm. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole; IMCD3 cells, Inner medulla collecting duct 3 cells; NFM, Neurofilament medium chain; NES: Nuclear export signal; BODIPY, Fluorinated Boron-Dipyrromethene.

focal zone, which has a diameter comparable to that of a single cell and can reach a resolution of ~5 μm. This technique provided high contrast when used to identify melanin within a single melanoma cell passing through a glass microtube *in vitro* (Figure 3d) [36]. OR-PAM shows potential for applications in both preclinical and clinical cancer research [36].

To further improve the resolution of OAI at the single-cell level, subwavelength-resolution photoacoustic microscopy (SW-PAM) was developed by combining OAI and optical objective lenses of varying refractive indices, providing an optical resolution as high as 220 nm at 532 nm wavelength [37]. When SW-PAM was applied to the *in vivo* imaging of a nude mouse ear, it was able to visualize blood vessels, capillaries, and red blood cells (Figure 3e) [37]. SW-PAM could potentially function as an *in vivo* flow cytometer to enumerate red blood cells, assess blood flow velocity in capillaries, and monitor conditions such as sickle cell disease [37].

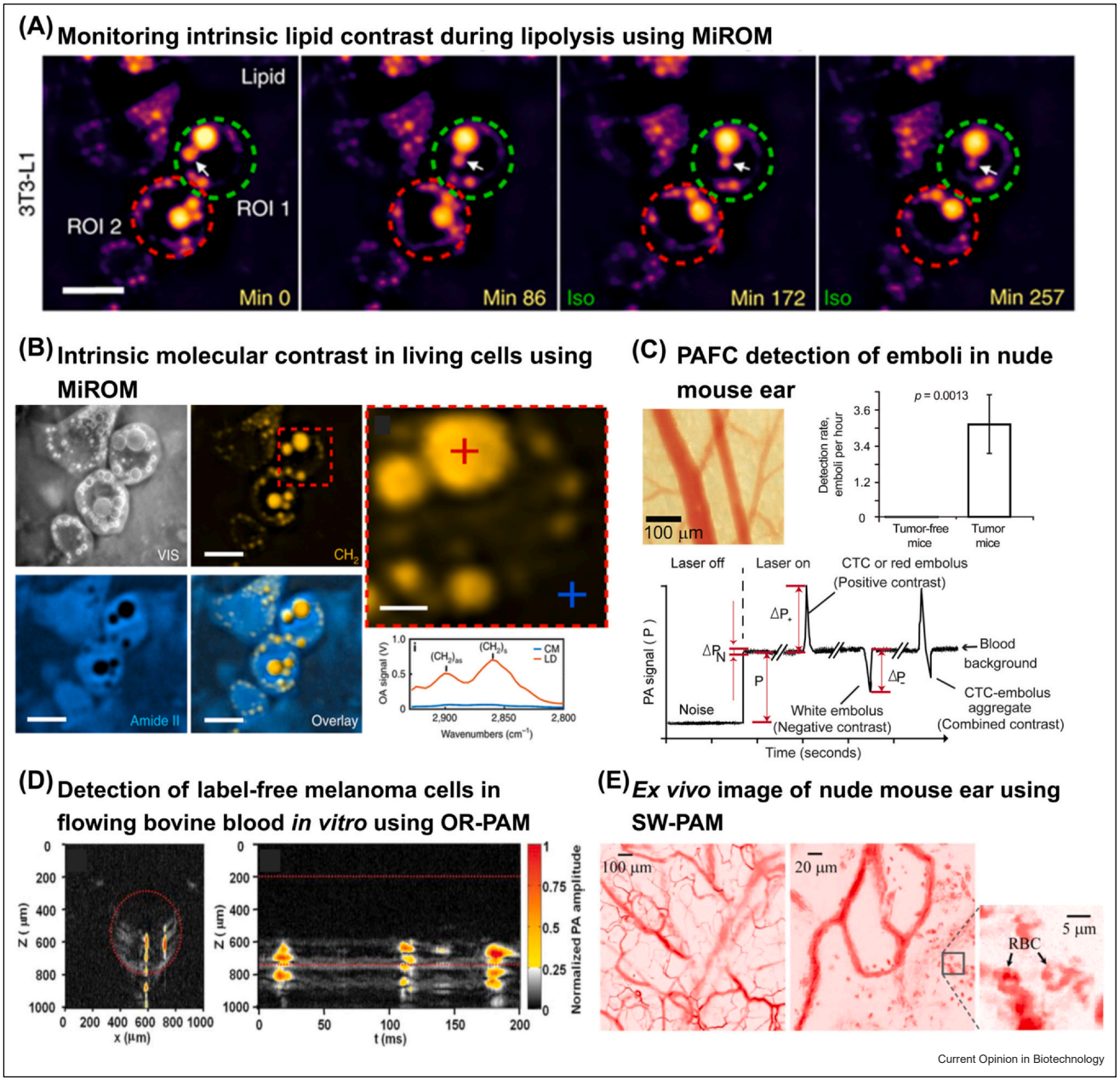
OAI techniques can provide noninvasive, real-time, label-free imaging with deep tissue penetration along with functional multimodal imaging of molecular and cellular structure within a single cell with high sensitivity and specificity. However, while OAI has been shown to achieve submicrometer resolution *in vivo* in tissues [37], molecular specificity at the single-cell level without the use of tags remains an area of ongoing development. Also, spectra can be difficult to analyze due to optical absorption by multiple biomolecules at the same wavelengths because of spectral overlap, limiting the amount of chemical information that can be gained. Research to address these challenges is proceeding at a rapid pace, and promising developments are underway in contrast agents, machine learning tools, and rapid data processing to improve the capabilities of OAI.

Raman spectroscopic imaging

Raman imaging is based on the scattering of monochromatic light by molecules, which results in the formation of a unique vibrational spectrum that reflects the molecular structure and composition of the targeted cells. When used in SCI, Raman imaging provides insights into cellular biochemistry, metabolism, and disease processes without perturbing cell physiology [38]. Many different Raman imaging techniques have been developed, each with specific applications, which are reviewed in detail by Wang et al. [39] and Liu et al. [40]. In this section, we highlight some of the most recent advancements in single-cell Raman imaging.

Metabolic profiling in SCI, whether through MSI-based omics approaches or through fluorescence-based imaging, presents numerous challenges. Often, metabolic profiling techniques require bulk measurements consisting of millions of cells, which obscure the metabolic variations present in individual cells in heterogeneous cell environments. Moreover, the existence of broad overlapping fluorescence emission spectra impedes the accurate resolution of specific metabolite species. To address these challenges, hyperspectral SRS (hSRS) imaging was developed, using pulse shaping and spectral multiplexing to achieve an image in less than one second with spectral coverage exceeding 200 cm^{-1} and a spectral resolution of less than 10 cm^{-1} [41]. In a recent study, Tan et al. [42] applied hSRS imaging in combination with a hyperspectral image unmixing method called least absolute shrinkage and selection operator (LASSO). The combined approach was named high-content hSRS (h^2SRS) imaging. In its first reported use, h^2SRS facilitated the simultaneous mapping of five key biomolecules at the single-cell level: proteins, carbohydrates, fatty acids, cholesterol, and nucleic acids (Figure 4a) [42].

Figure 3


(A) Monitoring intrinsic lipid contrast during lipolysis using MiROM
Lipid
3T3-L1
ROI 2
ROI 1
Min 0
Min 86
Iso
Min 172
Iso
Min 257
(B) Intrinsic molecular contrast in living cells using MiROM
VIS
CH2
Amide II
Overlay
OA signal (V)
(CH2)as
(CH2)s
CM
LD
Wavenumbers (cm−1)
(C) PAFC detection of emboli in nude mouse ear
100 μm
p = 0.0013
Detection rate, emboli per hour
Tumor-free mice
Tumor mice
Laser off
Laser on
CTC or red embolus
(Positive contrast)
ΔP+
ΔPN
P
ΔP-
Blood background
CTC-embolus aggregate
(Combined contrast)
PA signal (P)
Noise
White embolus
(Negative contrast)
Time (seconds)
(D) Detection of label-free melanoma cells in flowing bovine blood in vitro using OR-PAM
Z (μm)
x (μm)
t (ms)
Normalized PA amplitude
(E) Ex vivo image of nude mouse ear using SW-PAM
100 μm
20 μm
5 μm
RBC
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State-of-the-art multimodal OAI techniques used in SCI. **(a)** Representative MiROM micrographs demonstrating the monitoring of induced lipolysis in differentiated 3T3-L1 adipocytes. Two regions of interest (ROIs) enclosing individual adipocytes are indicated by a green dashed circle for ROI 1 and a red dashed circle for ROI 2. The white arrow traces the process of lipid droplet remodeling in a single adipocyte within ROI 1. Time and the presence of isoproterenol (ISO) are noted at the bottom corners of each frame. Scale bars: 40 μm. **(b)** MiROM micrographs of 3T3-L1 cells showcasing endogenous lipid contrast (CH_2 vibration) and protein contrast (amide II). An enlarged view of a single adipocyte is highlighted by a dashed red square, with two spots (red cross: lipid droplet and blue cross: culture medium) earmarked for spectral analysis and fine-tuning of the imaging wavelength. OA spectra in the CH vibrational region for the specified spots are presented. Scale bars: 40 μm. **(c)** *In vivo* real-time PAFC detection of emboli in tumor-induced nude mouse ear. A representative OA image of a mouse ear showing blood capillaries and veins (upper left). The bar graph represents the detection rate of white emboli in 16 melanoma-bearing mice and 14 tumor-free mice (upper right). The rate of emboli detection in melanoma-bearing mice was 0,51 ± 0,18 emboli/10 min. Label-free detection of emboli using PA signals for positive (CTC or red embolus), negative (white embolus), and combined (CTC-embolus aggregate) contrast is shown at the bottom. **(d)** OR-PAM detection of label-free melanoma cells in flowing bovine blood *in vitro*. B-scan OA image showing two melanoma cells at x = 588 and 720 μm. M-mode OA image tracking three melanoma cells acquired through the center of a glass microtube. **(e)** *Ex vivo* image of a nude mouse ear using SW-PAM showing microvasculature at single-cell resolution. From left to right: SW-PAM image of a nude mouse ear with clearly visible blood vessels and capillaries; SW-PAM image of mouse ear; zoomed-in SW-PAM image of mouse ear where red blood cells (RBCs) can be identified. The magnified image shows the biconcave structure of RBCs.
(a) Adapted from Pleitez et al. [32]. **(b)** Adapted from Pleitez et al. [32]. **(c)** Adapted from Juratli et al. [34] under a CC-BY 4.0 license. **(d)** Adapted with permission from Wang et al. [36]. **(e)** Adapted with permission from Zhang et al. [37] © Optical Society of America.

To enhance the sensitivity and speed of hSRS, Ge et al. [43] developed a multimodal approach, which combines hSRS with single-cell isotope probing and two-photon fluorescence *in situ* hybridization (FISH). This approach, known as SRS-FISH, enabled high-throughput analysis of single-cell metabolism in the human gut microbiome with imaging speeds ranging from 10 to 100 ms per cell, surpassing the capabilities of previous state-of-the-art methods by twofold to threefold [43].

The abovementioned developments have served to greatly improve the capabilities of Raman spectroscopic imaging in the SCI space. Despite these improvements, Raman imaging is still associated with considerable challenges. To unmix Raman spectra, prior knowledge of chemical composition is required. Furthermore, Raman techniques have limited penetration depth, making deep tissue imaging difficult if not impossible. Combining Raman imaging with techniques such as OAI and MSI in a multimodal sensing concept can help in overcoming these obstacles [44].

Mass spectrometry imaging

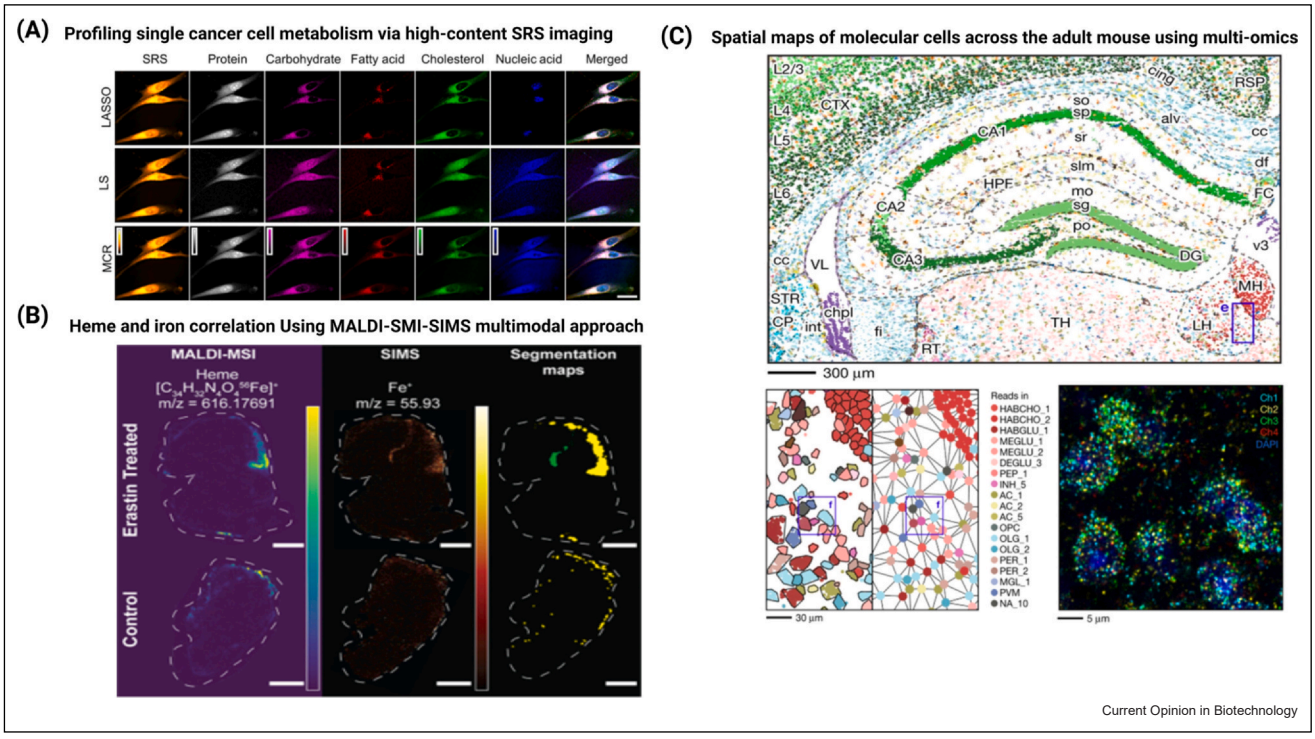
Mass spectrometry is a chemical analysis technique that separates ionized molecules from a sample based on their mass-to-charge ratio, creating a mass spectrum. MSI can map the distribution of chemicals across a sample surface by generating an image where each pixel is one mass spectrum. In SCI, MSI can be used to analyze the molecular composition of a single cell, providing high-resolution information about the subcellular distribution of metabolites and biomolecules [45]. MSI stands out as a potent technology for single-cell analysis due to its remarkable sensitivity, selectivity, and capability to simultaneously monitor multiple chemicals and molecules of interest. MSI-based methods applied in SCI are reviewed in detail by Xu et al. [45], Wang et al. [46], Yao et al. [47], and Yin et al. [48].

This section will highlight recent multimodal approaches used to study the spatial distribution of molecules in the same tissue [49–51]. Gorman et al. [49] combined matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) with secondary ion mass spectrometry (SIMS) to analyze changes in spatial lipidomics and metal compositions in the tissue of a mouse model of ovarian cancer, and to examine the effect of a therapeutic approach for ovarian cancer treatment (Figure 4b).

To enhance the spatial resolution and broad molecular coverage of MSI, and to study lipid metabolism, Lv et al. [50] combined a segmented temperature controller (STC) with a modified desorption electrospray ionization (DESI) method. The resulting method, named STC-DESI, was able to detect low-abundance small metabolites and neutral lipids with a remarkable spatial resolution (20 μm) in brain tissue. In another approach, Dunne et al. [51] were able to study the metabolic changes in the extracellular microenvironment and cellular composition simultaneously contributing to the tissue pathology in a disease by combining and testing different antibody-mediated single-cell spatial omics techniques such as immunohistopathology staining, Geomax, and imaging mass cytometry combined with MALDI-MSI.

Despite these unprecedented advancements, MSI is associated with several critical challenges. One common challenge is that MSI approaches are not quantitative on their own; the units of collected data are 'counts', which are affected by the rate at which different molecules ionize. Using standards of known concentrations can help quantify MSI data, such as in the study by Vandenbosch et al. [52], where a stable isotope analog was used as an internal standard to quantify lipids in brain tissue using both positive and negative ion MALDI. This approach also provided an approximately fourfold increase in mass resolution compared with time-of-flight and Orbitrap mass spectrometers.

Figure 4



State-of-the-art Raman spectroscopy, mass spectroscopy, and spatial multiomics techniques used in SCI. **(a)** Single cancer cell metabolic profiling by using hSRS spectroscopy. hSRS images representing mapped proteins, carbohydrates, fatty acids, cholesterol, and nucleic acids, as well as the merged image of metabolites for U87 brain tumor cells using different hyperspectral image unmixing methods. The application of LASSO unmixing significantly enhanced the accuracy of chemical mapping for nucleic acids, cholesterol, and carbohydrates compared with multivariate curve resolution (MCR) or least squares (LS) fitting in U87 cells. Each channel has the same contrast and shares a color bar. The ranges of color bars are 0 to 150, 0 to 8, 0 to 3, 0 to 5, 0 to 2, and 0 to 0.6, respectively. Scale bar: 20 µm. **(b)** Detection of heme and iron (Fe) in an ovarian cancer mouse model as proof of concept a MALDI-MSI-SIMS multimodal approach. Using this approach, Heme $[C_{34}H_{32}N_4O_4{}^{56}Fe]^+$ was detected using MALDI-MSI and compared with the Fe^+ detected by SIMS in ovarian tissue from an ovarian cancer mouse model. The segmentation maps show the distributions of heme and Fe^+ (yellow) and the distribution of Fe^+ alone (green). Scale bars are 2 mm. **(c)** Spatial maps of molecularly defined cell types across the adult mouse brain using a multi-omics technique called STARmap PLUS. A zoomed-in view of a brain tissue slice where each dot represents a DNA amplicon generated from an RNA molecule, color-coded by its cell-type identity. Brain region abbreviations are based on the Allen Mouse Brain Reference Atlas (top). A zoomed-in view of the habenula region with cell boundaries outlined and a mesh graph of physically neighboring cells connected by edges (bottom left). A representative fluorescent image of the habenula region highlighted from the first cycle of sequencing by litigation with error reduction. Each dot represents an amplicon (bottom right).
(a) Adapted from Tan et al. [42] under a CC-BY-NC 4.0 license. **(b)** Adapted with permission from Gorman et al. [49] Copyright 2023 American Chemical Society. **(c)** Adapted with permission from Shi et al. [56] under a CC-BY license.

MSI's position as a combined chemical analysis and imaging technique also presents additional challenges. Although MSI can achieve high sensitivities, the sensitivity of MSI is dependent on the abundance of the analyte, resulting in lower SNRs and higher errors when measuring low-abundance molecules. Also, to date, there has yet to be an MSI technique that can examine living cells, as MSI requires samples to be fixed and possibly embedded in resin before analysis. Other challenges include balancing spatial resolution with the speed of analysis and ensuring reproducible sample preparation. These techniques also typically generate large amounts of data, needing advanced processing, data analysis tools, and training even when imaging single cells. Therefore, standard protocols and calibrated methods should be developed to ensure reliable and reproducible results.

Other multimodal methods

This section briefly discusses other advanced molecular biology methods for comprehensive single-cell analysis. Although these techniques do not traditionally generate images, they can be used in conjunction with conventional imaging methods to provide insights into cellular heterogeneity and molecular interactions at the single-cell level.

Individual omics methodologies such as MSI are unable to tag specific cells and continually monitor their

Table 1

Summary table comparing the recent developments in various SCI techniques.

Imaging techniques	Technical complexity	Quantitative capability	Scalability	Throughput	Resource requirements	Costs
Fluorescence microscopy	[c] Advanced modalities require complex components and staining agents	[c] Sensitive detection of probed molecules	[c] Dependent on the imaging system and application	[c] Throughput depends on the scanning mechanism	Specialized equipment and fluorescence labels	[c] Suitable for routine application, advanced systems are expensive
OAI	[c] Involves advanced lasers and signal acquisition technologies	[c] Great contrast, measures physiological parameters	[c] Complex setup and analyses	[c] Volumetric acquisition with enhanced depth affects the throughput	Acoustic detection system, laser source, data processing tools	[c] Emerging technique with specialized instrumentation and moderate cost
Raman spectroscopy	[c] Requires knowledge of spectral analyses	[c] Specific information about molecules	[c] Time-consuming analyses	[c] Lower throughput due to higher sample concentration requirements	Spectrometer, laser source, and variable sample preparation protocols	[c] Portable benchtop systems for laboratories are affordable
MSI	[d] Complex data interpretation and analyses	[e] Exceptional specificity and sensitivity	[c] Multiplexing and parallel processing of samples	[b] Variable throughput dependent on MSI technique	Controlled environment and expensive instrumentation	[e] High cost of system, operations, and maintenance
Single-cell multi-omics	[e] Complex workflow and data analyses	[e] Simultaneous multi-omics analyses	[d] Time-intensive sample preparation	[c] Parallel processing of molecules	Computational infrastructure and automation for sample preparation	[e] A high throughput system requires higher investments
In-cell NMR	[e] Complex data interpretation and analyses	[d] Details at the molecular level in live cells	[b] Data from a specific target only	[a] Low due to the complexity of sample preparation and data analysis	Large magnets, expertise in sample preparation	[d] Shared facilities are usually preferred due to the high cost

[a] Very low.
[b] Low.
[c] Moderate.
[d] High.
[e] Very high.

metabolism in tissues. To address these limitations, recent research has focused on single-cell multiomics techniques, also referred to as multimodal omics [53]. These techniques involve the simultaneous examination of various molecular datasets using multiple techniques, which encompass genomics, transcriptomics, proteomics, metabolomics, and epigenomics. Multimodal omics play a crucial role in elucidating the pathogenesis of diseases involving somatic genetic alterations, such as cancer, Alzheimer's, and Parkinson's disease [54–57]. In a recent study, Hu et al. [55] developed an approach (scSpaMet) incorporating a three-dimensional spatially-resolved metabolic profiling framework for untargeted spatial metabolomic analysis and imaging mass cytometry for targeted multiplexed protein imaging. In combination with MSI, scSpaMet was also used to profile the migration of individual immune and cancer cells in human lung cancer tissues and into tonsil tissue at submicron resolution. In another multi-omics approach, Shi et al. [56] used a new spatial gene mapping method combined with histological staining (STARmap PLUS) and single-cell RNA sequencing to spatially resolve the transcriptomic profile of murine central nervous system cells at molecular resolution (Figure 4c).

Despite the immense potential of multimodal omics approaches, several limitations remain. These limitations include limited throughput, sample loss at various stages (e.g. sample preparation, fractionation, and sorting), difficulty in achieving high spatial resolution while maintaining high sensitivity and specificity, and complicated data analyses [57]. Improving sequencing technologies and multiplexed imaging methods, standardizing sample preparation protocols, and developing sophisticated computational tools and algorithms for data integration will facilitate the wider application of multi-omics techniques in biomedical research.

Other multimodal approaches, such as in-cell nuclear magnetic resonance (NMR) spectroscopy, are used to investigate chemical and metabolic processes at the atomic level in living cells [58]. In a recent study, Ikari et al. [59] combined ^{19}F nanoprobes, used for site-specific labeling of the membrane protein H-Ras, with in-cell NMR. In a separate study, the time-dependent cellular distribution of the H-Ras protein was first confirmed by fluorescence imaging after labeling H-Ras with Alexa-488. The combination of NMR spectroscopy and fluorescence imaging not only improved on the low specificity of in-cell NMR spectra but also enabled the structural examination of H-Ras within cells.

NMR spectroscopy is a noninvasive and nondestructive technique that offers unique advantages when combined with chemical and metabolic imaging techniques, as it allows for the acquisition of quantitative and structural information from target molecules. However, analyzing and interpreting NMR spectra can be challenging due to interference from overlapping biomolecular signals. Also, NMR instruments are expensive, limiting the system's availability.

A comparative analysis is summarized in Table 1, considering the recent developments in the context of technical complexity, quantitative capability, scalability and throughput, resource requirements, and costs associated with the advanced state-of-the-art techniques in SCI.

Conclusion and outlook

Single-cell chemical and metabolic imaging has benefited from advancements in technology, particularly the development of methodologies that achieve higher spatial and temporal resolution, speed, and data analysis. Cutting-edge technologies, including single-cell fluorescence imaging, advanced Raman spectroscopy, combined multimodal OAI, MSI, and NMR spectroscopy, contribute to a more detailed understanding of cellular composition and dynamics. While no individual technique is flawless, combining multiple techniques can help mitigate their respective limitations to allow simultaneous assessment of various aspects of the cellular microenvironment. These multimodal techniques can enable the examination of cellular and extracellular interactions within a single tissue [49,51], or the pairing of single-cell expression analysis with the measurement of the tissue's physical properties [60]. Such techniques ultimately enhance spatiotemporal resolution and enable deep tissue imaging at the individual cell level [24,32]. Also, the development of computational tools for data analysis and interpretation plays a crucial role in extracting meaningful insights from the complex datasets generated in SCI studies [1]. Overall, the development of multimodal approaches holds promise for unlocking deeper insights into cellular heterogeneity, functional dynamics, and metabolic processes at the single-cell level.

CRediT authorship contribution statement

Ajay Kesharwani: Conceptualization, Visualization, Writing – review & editing. **Vipul Gujrati:** Supervision, Writing – review & editing.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

Authors declare no conflict of interest.

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