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REVIEW ARTICLE

Single cell metabolism: current and future trends

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Abstract

Single cell metabolomics is an emerging and rapidly developing field that complements developments in single cell analysis by genomics and proteomics. Major goals include mapping and quantifying the metabolome in sufficient detail to provide useful information about cellular function in highly heterogeneous systems such as tissue, ultimately with spatial resolution at the individual cell level. The chemical diversity and dynamic range of metabolites poses particular challenges for detection, identification and quantification. In this review we discuss both significant technical issues of measurement and interpretation, and progress toward addressing them, with recent examples from diverse biological systems. We provide a framework for further directions aimed at improving workflow and robustness so that such analyses may become commonly applied, especially in combination with metabolic imaging and single cell transcriptomics and proteomics.

Keywords Single cell metabolism · Spatial metabolomics · Metabolic imaging

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1 Introduction

The Precision Medicine Task Group of the Metabolomics Society and MANA held a virtual Workshop on Single Cell Metabolomics on Friday February 26, 2021 (Kaddurah-Daouk etal., 2021). This summary overview article builds on the presentations and discussions of that meeting, to highlight the current state of the art, and where further effort is needed to enhance metabolic coverage and spatial resolution and address issues of data quality and reliability. Numerous general and specific questions were asked; some of them are addressed directly in the following Discussion, and others remain open questions for the future. Of major interest were the technologies, software and techniques available and being developed for the field. Of special interest for the future is how reliability and quality assurance of the results can be assured, such that they are both reproducible and reflect in-vivo metabolism. In particular we address or highlight the following points that were raised.

1. What is the impact of sorting dissociated cells on metabolism (Llufrio et al., 2018), and what are the technical and biological consequences? See Sect. 2.2
2. What are the technical and biological consequences of fixed cell versus live cell metabolism? See Sects. 2.2
   (a) How can we expand these technologies to infectious diseases?
   (b) How do we enable compatibility with biohazardous processing pipelines? This is important for clinical applications as well, to be able to handle human material under Universal Precautions. See Sect. 2.1
3. How will we deal with cellular heterogeneity? Will the error be small enough to be able to determine minor metabolic chances in a single cell as it undergoes malignant transformation? See Sects. 2.1, 2.3, 2.4
   How will subcellular heterogeneity (cf. compartmentation) be addressed? See Sects. 2.3, 2.4
4. How do we ensure quality management?
(a) How to assign confidence of metabolite annotations? See Sect. 2.5
(b) How to include proper blanks, controls and performance measures? See Sect. 2.8
(c) How can we ensure throughput and robustness, to enable sufficient numbers for adequate statistical analyses?
5. What statistical tools are available and what is needed? Is it appropriate to use the same statistics tools as single cell transcriptomics or flow cytometry for single cell metabolomics? See Sect: 2.7.

1.1 What is single-cell (sc) metabolomics?

The metabolome is often (circularly) defined as the net content of metabolites in a cell, tissue or system (Beger, et al., 2016; Oliver et al., 1998). Metabolites may be defined as the consequences of metabolism, i.e. the transformation of one molecule into another via enzyme-catalyzed biochemical reactions. As such most molecules in a system are metabolites, either endogenous, or exogenous, and logically include the macromolecules built by anabolic processes from smaller, activated precursors, using energy derived by catabolic oxidation of a variety of substrates (which are metabolites). If metabolomics comprises the determination of the parts list and the quantitative analysis of the metabolome, then sc metabolomics are the same analyses carried out at the single cell level, which requires the ability to determine the metabolome of individual cells. Further, the cellular metabolome reflects the biochemical activity of each cell in its particular environment. In order to cover the cellular metabolome, technologies developed for analyzing small molecules should be combined with those designed for macromolecules, i.e. transcriptomics, proteomics and glycomics (Baccin et al., 2019; Kearney et al., 2021; Lundberg and Borner, 2019; Minoshima et al., 2019; Zilionis et al., 2019).

1.2 Why is sc metabolism/omics important?

Measuring the metabolites in a single cell is inherently more difficult than measuring the metabolites in a larger sample, and so the first and overarching question is why make such an effort to make such measurements? If a particular biological question cannot be adequately addressed by analysis of bulk samples, then additional or different approaches are necessary. Where heterogeneity of cellular interactions is fundamental to the biological problem, single-cell analyses become warranted. Tissues in particular are heterogeneous both at the level of the diversity of types of cells present (which have intrinsically different metabolic activities related to their function determined by expression profile) and positional variability of the same cell type within tissue due to cell–cell interactions, different nutrient supply within the tissue determined by proximity to capillaries and metabolic demands of neighboring cells (Cassidy et al., 2015; Hensley et al., 2016) (cf Fig. 1). As several exemplars, many diseases progress depending on differences between cells; some tissues such as the brain function because of differences in connections and chemistries between adjacent cells, and embryogenesis involves metabolic changes between cells. If a sufficient number of individual cells is analyzed in detail, then it is possible to generate a population profile of the metabolic properties, whereas a bulk sample analyzed as

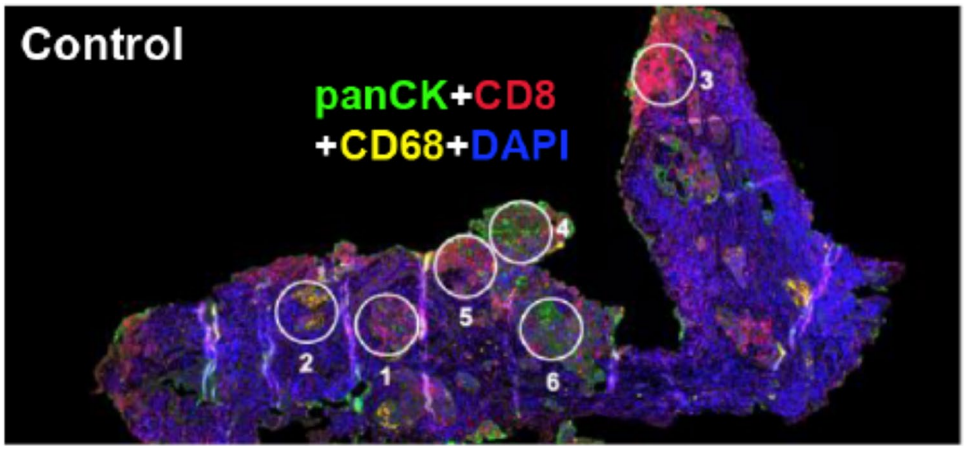

Fig. 1 Thin tissue slice of lung cancer- Digital Signal Processing. Adjacent fresh slices were incubated with ^{2}H glucose + ^{13}C, ^{15} N Gln and analyzed by NMR and ultra-high resolution mass spectrometry. This slice (10 m thickness) was stained for cancer (panCK), CD8 (T cells), CD68 (macrophages) expressing cells and DAPI (localizing nuclei). Circled regions (100 m diameter) were further analyzed for the protein expression level of 58 different markers of cancer and immune cell functional states using oligonucleotide-barcoded antibodies using the NanoString Digital Spatial Profiling system. H&E stained sections showed the presence of other cell types including fibroblasts and endothelial cells. From Fig. 4C of Fan et al. (Fan et al., 2021) under creative commons https://creativecommons.org/licenses/by/4.0/

a whole can be rather difficult to decompose in the absence of spatial information or the specific origin of the averaged levels of various metabolites (Fan et al., 2021). However, regional analysis of sequential thinly sectioned tissue slices can provide valuable spatially resolved metabolic information (Dean et al., 2021) (Fan et al., 2020; Hossain et al., 2020) (Quinn etal., 2020) whereas in situ sc-metabolomics can provide greater detail.

Even knowledge of the chemical variability between so-called identical cells requires SC measurements. Thus single-cell analysis provides information about the state and potentially, the activity of different cell types within a sample, and if spatial information is retained, how activities vary according to position-dependent interactions among cells and nutrient exchange.

As cells can vary greatly in their volumes, connections, and contents, the best approaches to use may be cell type specific. For example, some individual cells for much of their biological lifetime make few long term cell–cell interactions, the most obvious being circulating cells of various kinds (different leukocytes, circulating tumor cells (CTCs) for example). Indeed, CTCs are rare cells (Haber and Velculescu, 2014; Keller and Pantel 2019; Zhong et al., 2020), and they are not all necessarily functionally the same, depending also on their interactions with other tumor cells or with circulating immune cells-only sc level analysis can truly discover the variability within an individual subject. Once these cells infiltrate tissues, their state may change dramatically with a concomitant change of function and thus metabolic activity. Measuring populations of CTCs is thus less desirable than measuring them individually.

At the other extreme, a microliter (1 mm^3) of a mammalian brain will contain hundreds of thousands of cells tightly entwined with each other, with the predominant cell types including astrocytes, oligodendrocytes, microglia, and a host of distinct neuronal cell types, and within a cell type, different (activity-dependent) connections underlie functions such as memory. Understanding such a process requires single cell resolution. Both types of applications are driving the development and many of the initial applications of sc metabolomics.

Current applications of sc-MS are revealing highly heterogenous drug concentrations between cells (Newman et al., 2017; Pan et al., 2014; 2019). Assessing drug uptake and drug metabolism on an individual cell level is and will be extremely valuable to generate a comprehensive understanding of mechanisms of treatment failure and selection of drug resistance.

Another field in which sc metabolomic analyses will be important is in the field of infectious diseases, either to study individual rare dormant or drug tolerant microorganisms (*e.g.* (Mikolajczak et al., 2015; Sanchez-Valdez et al., 2018), or to distinguish between effects of infection on pathogen-containing cells versus bystander effects on adjacent uninfected cells versus matched cells from an uninfected animal (Liu et al., 2021; Nguyen et al., 2022).

In the context of solid tumors, there is tremendous interest in understanding the intratumoral heterogeneity, i.e. the identity of cells within the tumor microenvironment (cf Fig. 1), since heterogeneity is thought to be responsible for drug resistance and the evolution of tumors in time towards novel clones via mutational acquisition (Greaves and Maley

2012; Temko et al., 2018). In addition, the ability to identify single cancer cells within the normal tissue would benefit surgeons tremendously in determining tumor margins more precisely.

As with all single-cell 'omics methods, the goals of sc metabolomics are first to identify and quantify the metabolic content of individual cells, in each cell type while maintaining spatial information. This then defines the metabolic phenotype, or functional status of the cells which may be impacted by various factors such as age, genetics, and environmental perturbations such as pathologies, or drug treatment. Such information may also provide the basis for more detailed mechanistic analyses, especially when combined with single-cell transcriptomics and proteomics.

Where metabolism differs from transcriptomics and proteomics is in the tracing of pathways that occur in different compartments, such that the same metabolite can occur at different concentrations within different compartments of the cell, which with some exceptions (Chen et al., 2016) is generally not captured by extraction techniques.

2 Discussion

2.1 Current state of the art- what is possible with existing technology

Single-cell metabolomics is a rapidly evolving field with a number of recent examples (Guo et al., 2021; Liu et al., 2021; Seydel, 2021a; Steinbeck et al., 2020; Taylor et al., 2021). However, because of the scale, there are differences in what information is obtained at the individual cell level. For example, rather than the thousands of metabolites quantified in bulk samples, tens to hundreds of the most abundant metabolites are typically identified in sc experiments. Thus the advantages of single-cell resolution need to outweigh the experimental complexity and issues related to limited depth of metabolomic coverage. Perhaps not surprisingly, there have been several divergent approaches for obtaining single cell measurements. Several of the major approaches covered here include downscaling separations via capillary electrophoresis mass spectrometry, direct single cell mass spectrometry and mass spectrometry imaging at the single cell level. Current work on single-cell metabolism has focused mainly on endogenous cellular heterogeneity and cancer (*e.g.* (Bensen et al., 2021; Lombard-Banek et al. 2021; Rappez et al. 2021a; Tian et al. 2021)). In all cases, the metabolites measured reflect cellular content and are distinct from the metabolome determined from biofluids for example. In contrast, there has been limited work in the context of heterogeneity in response to infection. Specifically, most pathogens will only colonize a subset of available cells. Bulk analysis will conflate the metabolome of infected cells with adjacent bystander (uninfected) cells. In contrast, single-cell analyses will enable differentiation of pathogen-containing versus uninfected cells. Applying existing technology to infection models either requires BSL-1 level infectious agents that can safely be handled in the analytical laboratory (Samarah et al. 2020a; 2020b; Stopka et al. 2021), or a method to inactivate pathogens prior to analytical analysis, some of which are already compatible with existing data acquisition methods (e.g. heat fixation (Cazares et al., 2015; Wang et al., 2021a, 2021b), glutaraldehyde fixation (Li et al., 2021). Recent work has demonstrated that single-cell metabolism in the context of infection is feasible and provides valuable insight into infection processes, while also enabling direct validation that observed heterogeneity is biologically mediated rather than an analysis artefact (Nguyen et al., 2022).

2.2 Experimental design and sample preparation

MS-based metabolomics measurements can be divided into the procedures and protocols related to sampling and sample preparation, the spectrometry/spectroscopy-based measurement and finally, data analyses. The sample preparation for single-cell metabolomics is distinct from larger scale metabolomics measurements. As illustrated in Fig. 2, there are in principle several ways in which single-cell metabolism can sample cells including:

(i) Capturing and analyzing individual free cells, such as circulating cells in the plasma, including individual immune cells from peripheral blood or lymph or rare circulating tumor cells (Abouleila et al., 2019; Haber and Velculescu 2014; Keller and Pantel 2019; Zhong et al., 2020).
(ii) Selecting specific cells based on markers via manual isolation or automated cell sorting: Assaying populations of individual cells after dissociating tissue into single cells and sorting;
(iii) Measuring metabolites in tissue slices at the single cell spatial resolution level.

Many of these approaches have been used for scSeq genomic/transcriptomic methods (Al-Sabah et al., 2020; Asp et al. 2019; Baccin et al., 2019; Close et al. 2020; Kalb et al., 2019; Kyrochristos et al., 2019; Yang et al., 2019; Zilionis et al., 2019) (Bartoschek et al., 2018; Cohen et al., 2018; Luecken and Theis, 2019; Neal et al., 2018; Nguyen et al., 2018), as well as proteomics (Liu et al., 2019; Lundberg and Borner, 2019; Mistry et al., 2019). Generally, metabolomics lags these DNA and RNA based technologies (Ali et al., 2019a, 2019b; Gilmore et al., 2019; Liu et al., 2019; Misra, 2020; Neumann et al., 2019a, 2019b; Seth Nanda et al.,

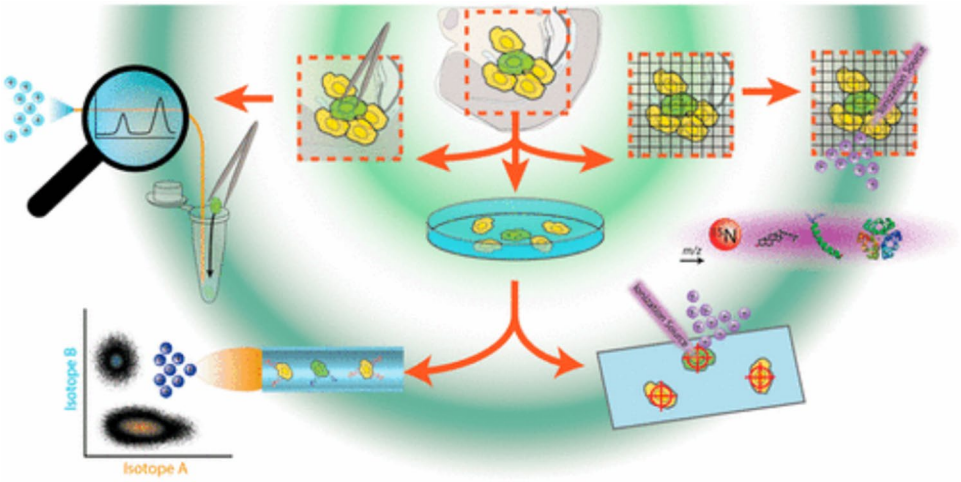

Fig. 2 Schematic outline of the approaches used for isolating and preparing cells for single cell measurements. The workflow includes tissue sectioning and mounting for imaging native distributions of analytes, isolating specific cells, and cell dissociation. Adapted from the American Chemical Society (J. Am. Chem. Soc. 2017, 139, 11, 3920–3929). (Comi et al., 2017). https://pubs.acs.org/doi/full/10.1021/jacs.6b12822 further permissions related to the material excerpted should be directed to the ACS

2019; Thiele et al., 2019), though considerable progress is being made. (Seydel, 2021b), (Duncan et al., 2019).

For example, at one extreme, one can select specific cells in a living tissue such as a brain slice, and characterize their electrical activity (and cell type) via the patch clamp method; briefly, this involves patching onto a cell with a pulled pipette so that the electrical activity can be measured; this pipette can also be used to withdraw some of the cytoplasm from the cell soma and introduce it into a characterization method such as CE-MS; in this way, electrical activity and metabolomics can be performed using the same living cell and the sampling does not even kill the cell (Aerts et al., 2014) This is a serial sampling approach (one cell at a time) but is information rich.

At the other extreme, the Sweedler group has published a series of articles where they take a tissue and disperse the cells across a slide, label select cells with a probe and determine the cell positions on the slide (Comi et al.,). They use the positions to determine the spatial coordinates to acquire data using a range of MALDI or SIMS MS-based approaches for direct MS acquisitions. As it only takes a second or so to probe each cell, they can acquire spectra on tens of thousands of individual cells. This has been used for rodent brain cells, islets cells and other animal models (Do et al., 2018a, 2018b; Jansson et al., 2016; Neumann et al., 2019a, 2019b). While the approach is high throughput, the number of metabolites and lipids measured is reduced but still provides enough data to differentiate similar cell types; as material is left behind after the MALDI or SIMS measurement, they are able to assay the same spot using other approaches including other types of MS (Comi et al., 2017a, 2017b, 2017c; Neumann et al., 2019a, 2019b).

The metabolome of a cell can change rapidly to mitigate stress and perform necessary tasks based on changes in their chemical microenvironment (Llufrio et al., 2018). To measure the resting metabolome of an individual cell, the sample preparation method needs to conserve it without introducing additional stress. There are in principle two strategies: either keep the cell alive in an optimal environment or snap freeze it. For analysis with MALDI or SIMS cells are preferably snap frozen while the currently most popular strategy is to keep cells alive for ambient sampling and ionization (Lanekoff et al., 2022). Ambient sampling followed by electrospray ionization mass spectrometry does not require any particular sample preparation (Fig. 3). Instead, metabolites are extracted direct from the cell using a solvent. An organic solvent will directly lyse the cell and enable metabolomics from intact individual cells using a plethora of creative strategies for cells in suspension or cells adhered to a substrate (Fig. 4). For subcellular sampling, a dual channel pipette with a continuously flowing solvent can be inserted into the cell's interior to extract the material for direct transfer to the mass spectrometer inlet (Pan et al., 2014). Alternatively, a micropipette can be inserted into the cell and withdraw material from the cytosol and subcellular compartments for subsequent transfer for mass spectrometric analysis without liquid extraction (Mizuno et al., 2008a, 2008b; Pan et al., 2020). The choice of sampling approach will be highly dependent on the experimental design and research question.

2.3 Metabolic imaging at single cell spatial resolution

2.3.1 Mass spectrometry-based metabolic imaging

Imaging mass spectrometry (IMS) enables cellular metabolite analysis while preserving the spatial architecture of the specimen of interest and has significant potential to be impactful in measuring metabolic processes at single- and subcellular resolutions. Since the inception of laser desorption ionization (LDI) by Koichi Tanaka (Tanaka et al., 1988), through the advances by Michael Karas and Franz

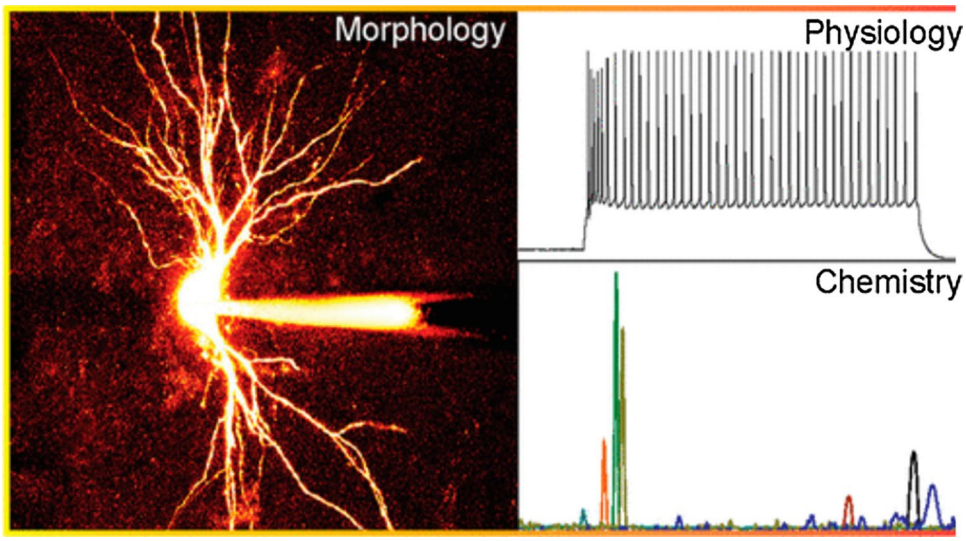

Fig. 3 Sampling a living cell with patch clamp pipette, enabling physiology, morphology and capillary electrophoresis mass spectrometry-based metabolomics on the same cell. Adapted from the American Chemical Society (Aerts et al., 2014). Further permissions related to this material should be directed to the ACS

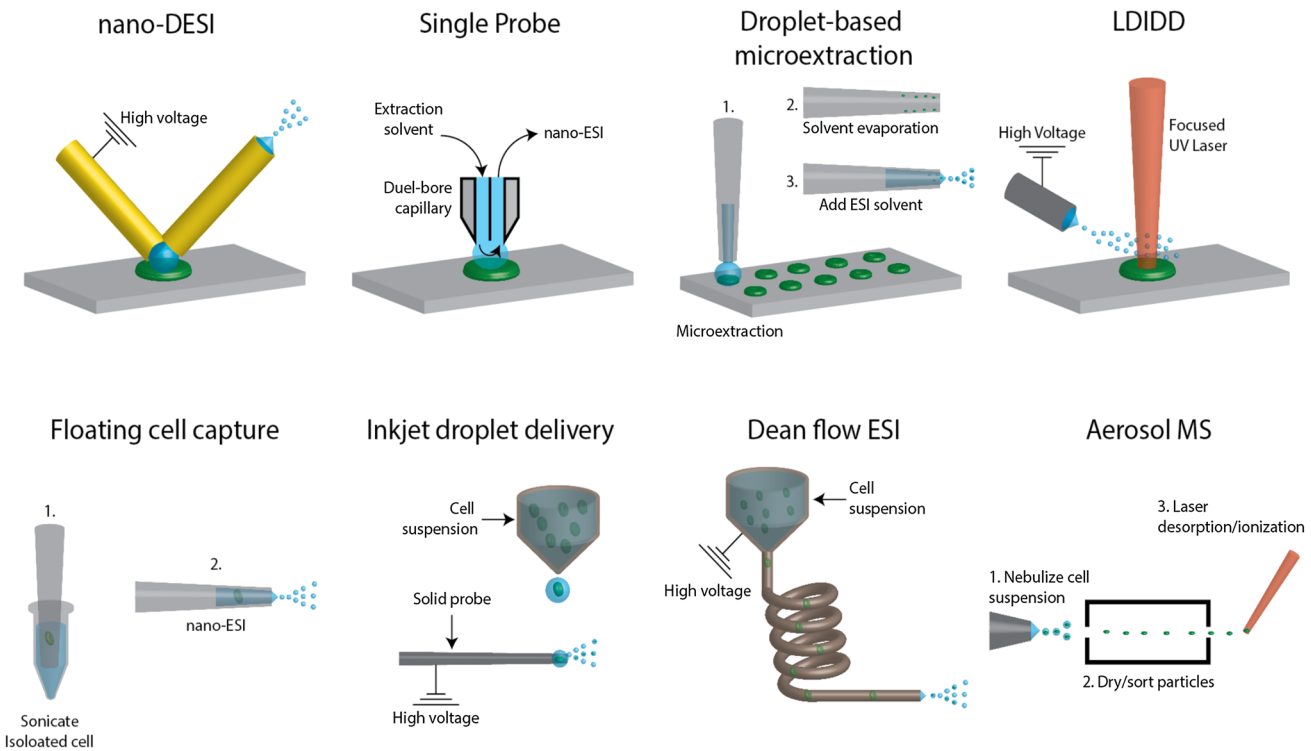

Fig. 4 Schematics of some strategies for ambient ionization techniques for single-cell metabolomics. *DESI* Desorption Electrospray Ionization; *LDIDD* Laser Desorption/Ionization Droplet Delivery; *ESI* electrospray ionization. Reproduced from Duncan et al. (Duncan et al., 2019) with permissionfrom the Royal Society of Chemistry

Hillenkamp (Hillenkamp et al., 1991; Karas et al., 1985), who demonstrated that small molecules could be used as a matrix to assist laser desorption ionization (MALDI), the analytical focus of these laser-based ionization methods has focused on molecules with higher mass-to-charge (*m/z*) ratio (e.g., peptides, polymers, and proteins). Although the amino acids tryptophan and alanine, along with another small-molecule (Vitamin B12, cobalamin), were among the first analytes ionized and analyzed by Karas and Hillenkamp using MALDI, the sensitivity for detection of molecules with low *m/z* (70–500 Da) (Hillenkamp et al., 1991; Karas et al., 1985), such as metabolites involved in central metabolism has been limited. In large part this is attributable to spectral interference of the matrix compounds required

for ionization or other ion-suppressive compounds (such as lipids) in the low *m/z* range. Recent advances in analytical chemistry and instrumentation have facilitated the development of MALDI-IMS-based methods on cryosectioned specimens while achieving spatially resolved metabolite measurements at 5–100 µm lateral resolution with significantly higher metabolic coverage.

High spatial resolution MS imaging is beginning to show some promise beyond protein analysis, including with stable isotope tracers (Wang et al., 2022). MS imaging using MALDI -FT MS is especially well suited analyzing for surface protein glycosylation, with spatial resolution determined by the laser spot size which is typically in the range 10–50 m, close to individual cellular resolution (Hawkinson and Sun, 2022; Powers et al., 2014) Using longer wavelength lasers (in the near IR), more labile sugars such as sialic acid were preserved in paraffin embedded blocks (Pace et al., 2022). As indicated in Fig. 4, there are several techniques for ionization under ambient conditions in addition to MALDI-based methods. In addition to MALDI, direct Laser Ablation Electrospray Ionization (LAESI) using near IR lasers has been used for metabolite imaging (Nemes et al., 2008; Shrestha et al., 2011) (Roman et al., 2018) which typically use a large spot size (≈200 µm) though more recent work using fiber optics greatly increased resolution to the single cell level (Kiss and Hopfgartner, 2016).

2.3.2 Iso-imaging

Whereas most metabolomics analyses focus on measuring absolute or quasi steady-state abundances of metabolites, additional information about pathway utilization and flux analysis can be obtained by stable isotope tracing either in vitro or in vivo (DeBerardinis et al., 2007; Le et al., 2012; Sellers et al., 2015) (Niedenfuhr et al., 2015). To date, this approach has been limited to bulk samples, but spatial information would greatly enhance the information retrieval and modeling.

Infusions of stable-isotope-labeled nutrients have been coupled to MALDI-IMS ("Iso-imaging") to determine spatial metabolic activity (Wang et al., 2022). Coupling stable-isotope tracing to MALDI-IMS avoids several problems commonly associated with making metabolic measurements using MALDI-IMS. First, spatial differences in tissue composition result in differential ion suppression across the same tissue, a situation analogous to the '[tissue-] matrix effect' in conventional liquid-chromatography coupled to mass spectrometry. Therefore, without isotope labeling, it is difficult to know whether observed metabolite changes across a tissue are biologically meaningful or the result of regional ion suppression. Second, stable-isotope labeling patterns can be compared to those obtained by LCMS and offer advantages for compound validation if isotopologue distributions match between methods.

Isotopically enriched metabolites can also be isolated and fragmented by MS2, and fragmentation patterns can be compared across regions or tissues or with other mass-spectrometry modalities. Third, stable-isotope tracing enables the potential of performing additional calculations to either determine relative nutrient contribution or perform a more rigorous flux analysis (Niedenfuhr et al., 2015) (Bartman et al., 2021; Selivanov et al., 2020). Although stable-isotope labeling approaches can add additional information and analytical rigor to MALDI-IMS experiments, unlabeled experiments that assess regional metabolite or lipid levels have also demonstrated utility in various settings. Combining MALDI-IMS data obtained in isotope-labeled or -unlabeled studies with other existing and emerging 'spatial-omics' methods promises to address critical biological questions surrounding regional nutrient use.

2.3.3 The future of MALDI-IMS

Important advances in MALDI-IMS technology and accompanying analytical chemistry-based strategies will facilitate the successful implementation of MALDI for broad coverage of the metabolome at the single-cell level. Atmospheric pressure (AP) MALDI has recently exhibited 1.4 µm lateral spatial resolution (Kompauer et al., 2017). The application of this technology for small molecules is still hindered by the general sensitivity for molecules in the low *m/z* range. Improving metabolite sensitivity to sufficient levels will likely result from strategies that will enhance ionization or reduce ion suppression from interfering compounds (Yang et al., 2018), along with hardware advances that better select small molecules in the gas phase. Metabolome coverage using any single matrix is relatively incomplete compared to conventional chromatography-based methods. The further development of different matrices (Qiao and Lissel, 2021), including reactive matrices (matrices that act as derivatizing agents and absorb the UV laser pulse essential for MALDI) (Shariatgorji et al., 2019) and those matrices that may be compatible with mixed-mode (positive–negative switching) may improve the metabolome coverage and throughput. The relative speed at which data are acquired will be crucial as lateral resolution decreases. Fourier-transform Ion Cyclotron Resonance (FT-ICR)-based mass spectrometers provide higher mass accuracy, which is essential for distinguishing isotope labeling patterns but is relatively slow compared to Time-of-flight (TOF) based mass-spectrometry. Without chromatographic separation, molecule identification is challenging, and isobaric (molecules with the same mass) are indecipherable. Ion mobility, the process of separating ions in the gas phase based on their collisional cross-section (CCS) with a carrier gas, would theoretically enhance

sensitivity and aid in identifying molecules based on an added time dimension (similar in principle to chromatography). While ion-mobility has been particularly useful for peptides and lipids, it is unclear whether the utility will be extended to enhance small-molecule metabolites. For certain application, targeted imaging by MALDI or DESI by tandem quadrupole MS can identify predefined metabolites of interest, albeit not at single cell resolution (Lamont et al., 2018, 2021). Breakthroughs in any of these areas could lead to significant improvements in making spatially-resolved metabolite measurements.

2.3.4 Optical imaging

Optical imaging techniques have been explored to quantify tumor cell metabolism non-destructively at cellular level resolution by using endogenous contrast or appropriate probes (Madonna et al. 2019b; Walsh et al., 2014; Zhu et al., 2019, 2018a, 2018b). For example, two endogenous fluorophores, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) have been explored (Zhu et al., 2014) to report the reduction–oxidation (redox) state in the electron transport chain of cancer cells (Hou et al., 2016). Multi-photon microscopes have been explored for cellular level optical redox ratio imaging of in vitro cancer cells (Hou et al., 2016) and tumor organoids (Walsh et al., 2014) for cancer research. These cutting-edge microscopes quantify the auto-fluorescence of both NADH and FAD, and then provide an indirect measurement of the balance between glycolysis and OXPHOS by looking at the ratio of the two. Although it is an indirect measure of redox balance, it has been firmly validated with Seahorse assays in cancer research studies (Hou et al., 2016). Being able to simultaneously quantify both glycolysis and mitochondrial metabolism directly and explicitly would further facilitate cancer research and translational applications. To achieve this capability, several metabolic probes have been explored for intracellular metabolic measurements. The 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) has been used in cancer cells to report glucose uptake, similar to the clinically available FDG-PET (Yamada et al., 2007; Zhu et al., 2018). TMRE has been utilized to quantify mitochondrial membrane potential (MMP) to study OXPHOS (Perry et al., 2011; Zhu et al., 2017). BODIPY has been explored to report fatty acid uptake in cancer cells (Megan C Madonna 2021). All of these three key metabolic parameters are associated with human cancer therapy resistance development (Tang et al., 2018). When these metabolic probes were used, a labeling procedure was required but it was non-destructive and lasted less than 1 h (Madonna et al., 2019a). Optical metabolic imaging enables repeatable non-destructive measurement on various cancer models and it provides spatial information, while these techniques have poor coverage which is limited by usable metabolic probes. However, an increasing number of fluorescence labeled metabolites are becoming available, substantially enhancing the metabolic coverage of optical microscopy (Benson et al., 2019; Hong et al., 2021).

Redox biochemistry, specifically the determination of $NAD(P)^+$, NAD(P)H and their ratios, in different compartments within live cells is an important area of research that has seen the development of numerous fluorescent probes that bind the free nucleotides and can be imaged by confocal microscopy. These studies enable the redox responses to experimental conditions in different cell types to be determined as a function of time (Bilan and Belousov, 2017; Cambronne et al., 2016; Hu et al., 1800; Sallin et al., 2018; Steinbeck et al., 2020; Zhao and Yang, 2016). For example, although the total dinucleotide concentration is of the order 0.5 mM in mammalian cells, the concentrations and redox ratios are significantly different in the cytoplasm versus the mitochondria, and indeed, the free concentrations are much lower than the total amounts, owing to binding to the large number of dehydrogenases present. This also leads to NAD^+/NADH and NADPH/$NADP^+$ ratios quite different from the ratios of the totals. Typically, the free NAD^+/NADH ratio in the cytoplasm is 500–1500 (Christensenet al., 2014) compared with < 10 in mitochondria (Williamson et al., 1967). In contrast, the free NADPH/$NADP^+$ ratios are in the range 20–80 (cytoplasm) and ca. twofold higher in mitochondria, whereas the free NAD^+ concentration is lower in the cytoplasm (ca. 100 M) than in the mitochondria (Sallin et al., 2018).

2.3.5 Raman imaging spectroscopy

Owing to recent progress in the diode lasers and notch filters, the study of biological systems became possible in the form of Raman Imaging Microscopy. Focusing the laser through the microscope allows the collection of Raman spectra for each pixel and subsequent movement of the stage permits the acquisition of spectra in a new position (Shen et al., 2019). This approach provides a chemical and biochemical map of the entire cell without the need to remove it from the cellular milieu. Therefore, the technique is non-destructive, non-invasive, and can accept a wide range of samples from bulk to microscopic, from solids to liquids or gasses. The differences in the intensity and positioning of peaks in the spectra are attributed to the compositional changes between the different cells or organelles. The variation in the intensity of these peaks is due to the variation in the amount of lipids, proteins and DNA contents present in that location of the cell. In the past, the lack of methodology has hampered the use of Raman in biological samples. Recently, the development of a single organelle approach for the quantification of

DNA, proteins, RNA, and lipids has opened a new way to study spatial metabolism (Lita et al., 2019, 2021; Pliss et al., 2021). With the introduction of a deconvolution method and the use of external markers, it is now possible to accurately quantify biomolecular components such as total DNA, RNA, lipids, and proteins in organelles of live cells. Lipids could be characterized using the lipid unsaturation parameter (LSU) which describes the ratio of double bonds to single bonds; the TCP parameter which describes the ratio of trans/cis in the lipid isomer; phosphatidylcholine sphingomyelin (PC); cholesterol and cholesteryl ester (CL); and triglycerides (TG). In addition, the spatial resolution of 500 nm positions this technique at the frontiers of metabolic analysis. Since the Raman microscope is not able to visualize organelles and distinguish one organelle from another, as some other methods can, Figs. 5 and 6, show how Lita et al. (Lita et al., 2021) circumvented this. They labeled one organelle with a fluorescent dye, and with the help of a fluorescence filter they visualize the desired organelle is in the field of view. Once they have the organelle identified, they can start recording the metabolic information that is intrinsically hidden in the Raman spectra. Lita et al., (Lita et al., 2021) applied this approach to gliomas cell that contains either $IDH1^{wt}$ or $IDH1^{mut}$ and identified a unique lipid profile associated with $IDH1^{mut}$, which could be exploited for therapeutic purposes.

Another way to image metabolites is to use a special type of Raman spectroscopy named Stimulated Raman Spectroscopy (SRS) as done by Zhang et al. (Zhang et al., 2019). Contrary to the spontaneous Raman, this method uses two synchronized pulsed lasers, and the difference frequency between the lasers, accelerates the transitions of molecular vibrations that match that difference. By this method the signal can be enhanced up to 10^8 fold, therefore allowing detection of low intensity signals.

This approach was used in conjunction with deuterated glucose to image the incorporation of glucose into proteins, lipids, DNA, and glycogen in tissues of live animals and cells. The use of deuterium allows for specific pathways to be visualized as more enriched in glucose-derived than others, while the spatial resolution of the technique allows for tracing of this isotope in different types of cells or organelles.

While these efforts are pushing the limit of current detection with Raman spectroscopy both in terms of metabolite identification as well as signal sensitivity, more developments are needed for this method to increase the number of metabolites that it can detect. In the future, the use of Raman spectroscopy in combination with mass spectrometry such as Nano ESI could increase the potential of subcellular metabolomics (Ali et al., 2019a, 2019b). Combining with sc proteomics and transcriptomics and the limited Stable Isotope Resolved Metabolomics (SIRM) information obtainable (see above) (Lima et al., 2022) and improved computational modeling will make the in situ metabolic imaging more powerful.

2.4 Technical bottlenecks and new approaches

There are several technical bottlenecks for deep metabolic analysis at single cell resolution (see for example (Shrestha, 2020)), that are being addressed with new approaches as discussed below.

2.4.1 Spatial dispersion and sensitivity

Nuclear magnetic resonance (NMR) and high-performance liquid chromatography mass spectrometry (HPLC–MS) are the "gold standards" for metabolite identification. However, the low sensitivity of NMR means that comparatively

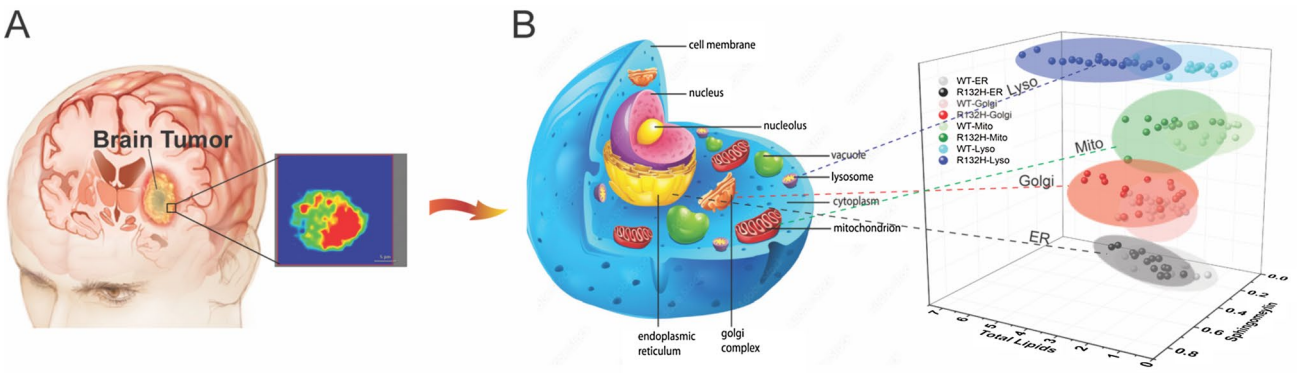

Fig. 5 Overview of organelle level metabolic characterization of cells. Using the Raman Microscope, a single cell can be selected from the tumor microenvironment (**A**) and a more detailed image of organelle inside this cell can be obtained (**B**). Using our methodology, different classes of lipids in one organelle in different cell types can be quantified. Each organelle measurement is displayed with a different color (lysosomes in blue, mitochondria in green, Golgi apparatus in red and Endoplasmic reticulum in black. R132H is an active site mutation in isocitrate dehydrogenase, very prevalent in lower grade gliomas. From (Lita et al., 2021) with permission

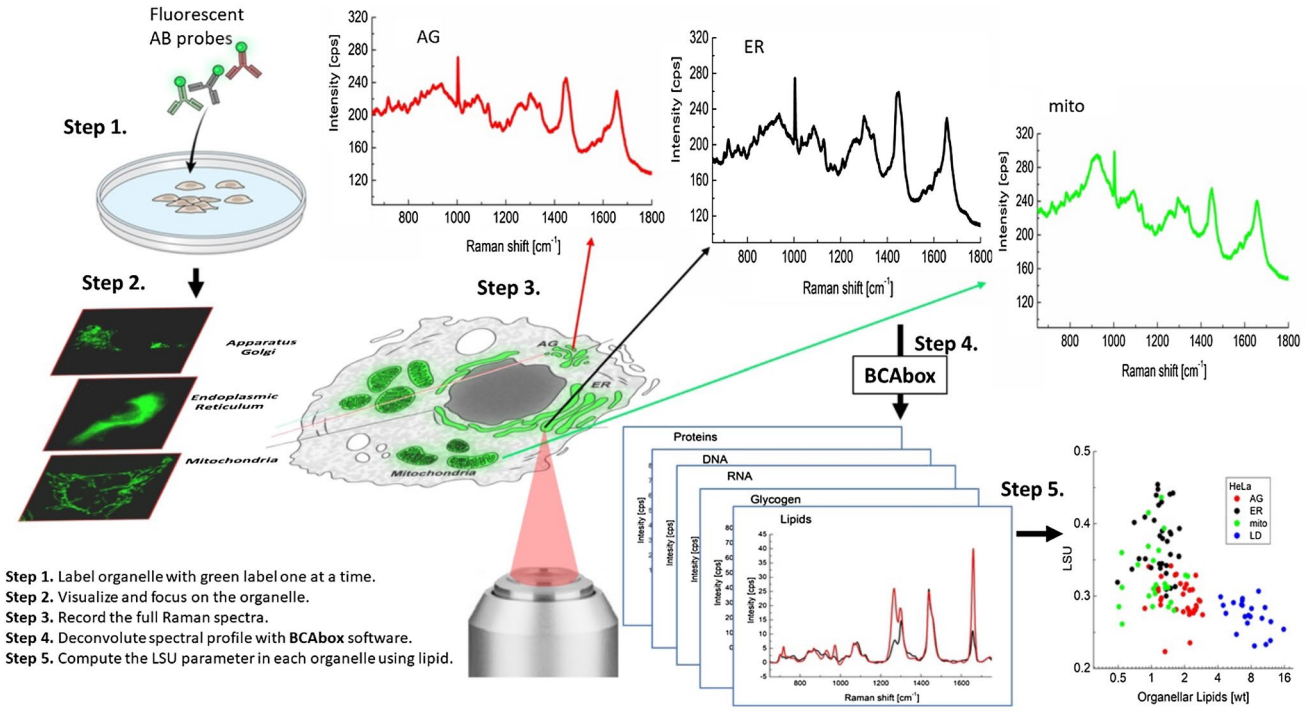

Fig. 6 Protocol for Raman-based quantification of lipids at the organelle level. In Step 1, a green, fluorescent label is applied to the cells. In Step 2, organelles are localized and focused under microscope using this tag. In Step 3 and 4, Raman spectra are collected and BCAbox algorithm is applied to extract lipid profiles. In the last step, the unsaturation of lipid (LSU) is computed based upon the area ratio of 1655 cm^{-1} to 1443 cm^{-1}. Via the same procedure, TCP parameter, which characterize trans/cis C=C bonds ratio in lipid species can be obtained from the intensities at 1666 cm^{-1} and 1655 $cm.^{-1}$. AG, Golgi apparatus, LD, lipid droplets. With permission from (Lita et al., 2019)

large amounts of compound are needed, such that Magnetic Resonance Imaging (MRI) cannot achieve single cell or near single cell resolution. Sensitivity enhancement via dynamic nuclear polarization (DNP) for example allows for localized kinetic analysis of a few reactions, but is still limited to mm^3 sized volumes (Ehrhardt et al., 2022). The great advantage of MRI is that the imaging is in vivo and can be carried out at greater depth than optical imaging (Ahn et al., 2019; Dang et al., 2019). Mass spectrometry is a far more sensitive technique but since it consumes material the sensitivity is inversely proportional to the square of the probed area, and being destructive, does not readily lend itself to kinetic studies at the same sampling point. From an analytical perspective, this creates what can be termed the "*Metabolite Uncertainty Principle*" where the more certain we are about a metabolite's identity, the less certain we are about its localization. Many of the pioneering efforts over the last decades including sample preparation for spatial dispersion or enhancements in sensitivity for imaging have focused on escaping this frustrating confine. The evolution of these techniques is illustrated in Fig. 7 with the width of each evolutionary line indicating the approximate number of instruments in the field and the color of each label categorizing the certainty of metabolite identity from gold (fully identified, level 1) through silver (putative annotation, levels 2,3) to bronze (class of metabolite, level 4) according to the classifications in to bronze (class of metabolite, level 4) according to the classifications in (Blaženović et al., 2018; Sumner et al., 2007).

Innovation has been driven by the needs to increase sensitivity and simultaneously reduce the probe size. In electrospray MS this has been achieved extracting material with a fine capillary as a probe guided by a stereo microscope followed by nanospray ionization (Masujima, 2009; Mizuno et al., 2014). Whilst not an imaging method per se, it is possible to precisely target locations at the organelle scale and from live cells (Fujii et al., 2015; Mizuno et al., 2008a, 2008b). Laser capture micro-dissection (LCMD) may also be combined with electrospray and is increasingly used to give multi-omic information with spatial localization. Desorption electrospray ionization (DESI) (Takats et al., 2004) uses an electrospray as the probe with MS analysis of analytes captured in secondary droplets. This has the advantage of no sample preparation requirement and gentle electrospray ionization enabling improved confidence in metabolite identification but with a spatial resolution of above 50 µm (Gilmore et al., 2019).

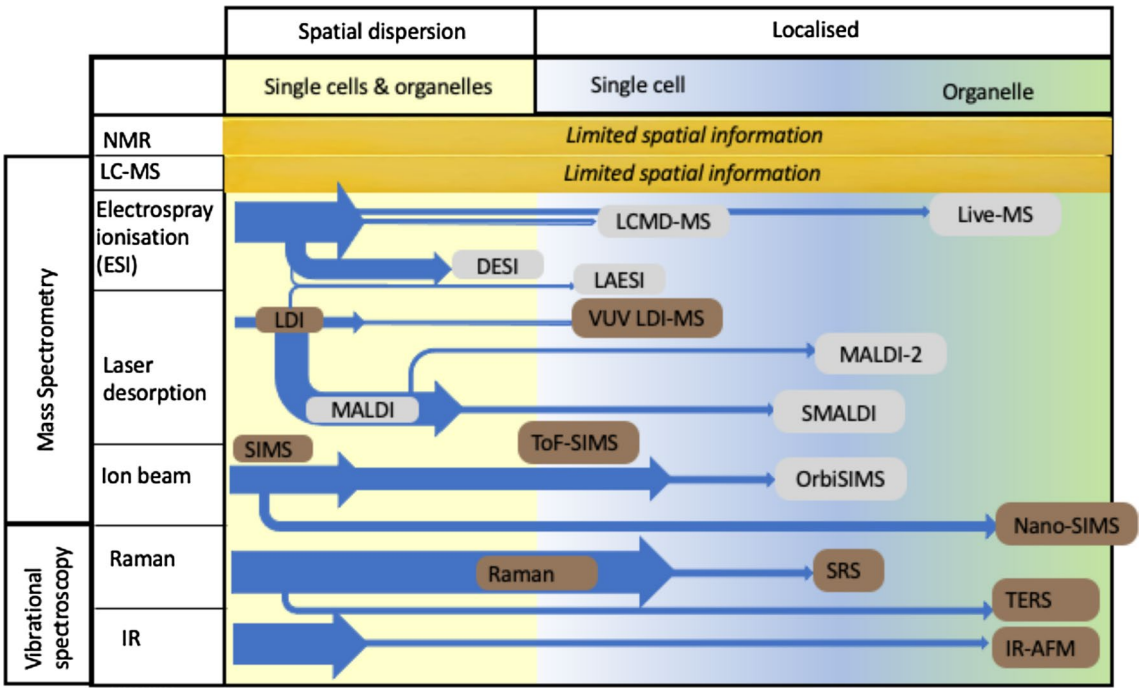

Fig. 7 Analytical techniques for metabolite identification and their evolution towards single cell metabolomics using either spatial dispersion methodologies or by imaging. The width of each evolutionary line indicates the approximate number of instruments in the field (see key) and the color categorizes the certainty of metabolite identify from gold (full identification), silver (annotated) to bronze (class of metabolite, e.g. not specifically identified). LDI Laser Desorption Ionization; LAESI ablation electrospray ionization; SIMS secondary ion mass spectrometry; TERS tip-enhanced Raman spectroscopy

In the desorption mass spectrometries, the probability of a neutral molecule being ionized is low (typically $< 1 \times 10^{-3}$) so that much effort has gone into increasing this probability through combining with a laser to post-ionize neutral compounds. The conversion of a neutral molecule in the gas phase to an ion requires an energy of around 6–8 eV (579–769 kJ mol^{-1}) so photons in the UV and vacuum UV (120–210 nm) are needed. Niehaus et al. developed a MALDI-2 instrument where a second 266 nm laser was used to post-ionize neutral molecules enabling a resolution of 600 nm to be achieved (Niehaus et al., 2019) and Walker et al. used VUV post-ionization femtosecond laser desorption/ionization mass spectrometry (LDI-MS) to image the distribution of lipids in pancreas tissue (Walker et al., 2018).

Reducing the uncertainty of metabolite identify requires high-performance mass spectrometers which includes high *m/z* resolving power to separate confounding peaks, high mass accuracy for searching databases of calculated or known mass peaks and MS/MS capabilities for structural identification. These attributes, generally, require Fourier Transform (FT)-MS using magnetic trapping (e.g. FT-ICR) or electrostatic trapping (e.g. Orbitrap). Sub-cellular imaging with both MALDI and SIMS in combination with Orbitrap Mass Spectrometry detection has been achieved in SMALDI (Kompauer et al., 2017), OrbiSIMS (Passarelli et al., 2017) and MALDI-2 (Niehaus et al., 2019). Analysis of samples in their native state is another important technological driver. Cryogenic sample preparation is also an important direction, for example Cryo-OrbiSIMS where the sophisticated sample preparation methods developed for cryo-SEM are utilized (Zhang et al., 2020).

Raman and IR optical methods have the advantage of operating directly in ambient conditions. Similarly to MS, there have been tremendous advances over the last decades to boost signal intensities using non-linear methods. For example, Stimulated Raman Spectroscopy (SRS) is able to achieve high-speed imaging (usually at a selected frequency rather than broadband) with a spatial resolution of 400 nm and an axial resolution of < 1 µm. The spatial resolution of Raman and IR spectroscopies is substantially increased when combined with electric field enhancement caused by the sharp tip of an Atomic Force Microscopy (AFM probe) in Tip Enhanced Raman Spectroscopy (TERS) and IR-AFM, respectively.

2.4.2 Sensitivity

Sensitivity and dynamic range are a significant issue for metabolomics. Metabolite concentrations in cells (averaged over the entire cell) vary widely from abundant molecules

such as ATP in the mM range, to amino acids and dinucleotides in the sub millimolar range, to low µM for many intermediates, and in the nM range for some signaling molecules, a range of more than 6 orders of magnitude. For a cell of total volume 0.5–5 pL, this range is equivalent to zmol to fmol amounts. However, modern mass spectrometers can easily measure sub fmol to amol quantities, and the more abundant metabolites (100 M to mM) are accessible by confocal light microscopy on live cells (see Sect. 2.3.4). Many biological questions can be addressed even with moderate metabolic coverage, especially when multiple modalities are engaged on the system as a whole. The fast progress in the field attests that attomole to femtomole levels provides sufficient coverage to address may biochemical questions, and there have even been examples of individual organelle MS (Rubakhin et al., 2000) (and see above).

2.4.3 Microfluidics

The small number of single cells presents another issue for metabolomics. As mentioned above, there is a broad range of metabolite concentrations in a given cell; however, the typical mammalian cell volume is in the picolitre scale. Therefore, controlling the sampled volume throughout the analytical workflow and preventing sample loss is essential to maintain the ability to detect the less abundant, but highly relevant molecules such as signaling molecules. As it stands, traditional sample preparation and separation techniques are ill equipped to handle such small volumes. However, techniques that inherently constraint the sample volume in a microfluidics format by means of slugs or droplets are much more suited to the task. Furthermore, separation techniques that can be incorporated into microfluidic chips such as capillary electrophoresis, or that occur after the sample introduction to the MS (ion mobility) can improve confidence in metabolite identification, in contrast to methods that rely on MS measurements alone. The work done by Li et al. (Li et al., 2016) highlights the potential of microfluidics in single cell analysis. Their work showcased an automated microfluidic platform that lyses cells using nanoelectrodes followed by a separation step using electrophoresis, and subsequent introduction into the MS instrument via nanospray.

Controlling perturbations of the cells' microenvironment and the metabolic response to them is an important variable of any metabolomic experiment. This is especially so when targeting relatively rare cells such as circulating tumor cells, where the generated data are highly sensitive to fluctuations and outliers due to the low number of cells measured, which makes it difficult to discern the source of variation (e.g. heterogeneity, or perturbations of microenvironment). Therefore, minimizing the perturbations to the microenvironment, as well as rapid quenching of metabolic reactions ensure that the variations seen in the metabolome representative to the true cellular state, and not in response to experimental conditions. Controlling perturbations is especially important in methods where the cell is sampled while it is still alive as is the case in capillary microsampling techniques. Considering that metabolic reactions occur in seconds (Tung et al., 2017b), quenching the sampled cells by a cold mixture of organic solvent and buffer (Reichard and Asosingh 2019) is required prior to further cell manipulation and sample preparation to ensure that the measured metabolites represent the normal cellular state as much as possible (Nhu et al., 2020; Tung et al., 2017a).

2.5 Metabolite identification

As already described, NMR is insufficiently sensitive to detect and analyze even high abundance metabolites at the single cell level, though is important for determining metabolites in bulk samples, which may support conclusions based on high resolution experiments. The main source of information is mass spectrometry which with sufficient resolution can return accurate masses from which molecular formulae can be determined (Higashi, 2011; Lane et al., 2009). This does not discriminate among structural isomers, for which tandem MS at ultrahigh resolution (preferably with LC retention time) is the gold standard (Sun et al., 2021), but may not always be technically possible. In many cases, analysis of an additional bulk sample is still employed to assist peak assignment; the greater spectral intensity enables better fragmentation data leading to improved analyte identification. Increasing mass spectrometer sensitivity can be expected to help with tandem MS of lower abundance an analytes. Other (orthogonal) biochemical information can help in deciding plausible identification.

Stereochemical information will be lacking with most LC–MS/MS-based annotation practices, which may matter for chiral drug metabolites, although there are recent examples of probing the stereochemistry of compounds in individual cells using CE-MS coupled to ion mobility MS (Mast et al., 2021) and this is expected to become more common with the growth of ion mobility enhanced spectrometers. Even with MS/MS, annotation may not always be possible, either due to lack of diverse enough reference libraries combined with persistent (though improving) limitations of computational structure prediction approaches, or insufficiently informative MS/MS spectra (for example for glycerophosphocholines in positive ion mode, where often only fragments from the head group are observed and there is a dearth of informative fragments from the fatty acid tails).

Optical/microscopy methods provide high resolution metabolic imaging on cell models (Madonna et al., 2019a, 2019b; Shah et al., 2014; Shah et al., 2017), organoid model (Gil et al., 2021; Walsh et al., 2014) or even tumor slices without extraction. However, it is challenging to use the

technique for absolute amounts quantification as most of the existing optical techniques are semi-quantitative or ratiometric (Gil et al., 2021) based. It is possible to quantify the molecular amounts using optical techniques, while rigorous calibration procedures are typically needed.

2.6 Metabolite quantification

Quantification of metabolites in single cells with mass spectrometry is analytically challenging but desirable from several perspectives. In particular, quantification will enable the direct comparison of the abundance of individual metabolites in the cell despite their different ionization efficiency and detectability. Furthermore, quantification will allow for calculation of significance and fold changes between cells and groups of cells that will otherwise be highly dependent on the sensitivity of individual metabolites. For liquid extraction approaches, internal standards are included in the extraction and ionization solvent to measure the detected concentration of individual metabolites with one point calibration (Bergman and Lanekoff, 2017). The internal standards are typically isotopically labeled to minimize overlap with endogenous signals. Another approach is to perform relative quantification using the ratio of two similar metabolites and assuming that they ionize similarly (Bergman and Lanekoff, 2017). Although this does not provide a quantitative measure, it allows for direct comparison between the metabolome of individual cells. Absolute quantification remains a significant challenge in metabolomics.

2.7 Statistical tools-what is available and what is needed?

While the statistical analysis of single-cell metabolomics remains in its infancy, single cell transcriptomics have matured to the point where sophisticated statistical tools for high-dimensional data can be routinely applied as part of standard data processing workflows (Luecken and Theis, 2019). This is largely facilitated by the widespread adoption among the scRNA community of mature open-source data analysis packages written in modern scripting languages, such as ScanPy (Wolf et al., 2018) for Python, its extension SquidPy for spatial omics (Palla et al., 2022), and Seurat (Hao et al., 2021) for R. Such tools can also be applied to MS-based single-cell metabolomics data once mass spectra are converted to intensities of annotated metabolites. Rappez et al. (Rappez et al., 2021b) used ScanPy to perform statistical analyses on a dataset of 740 metabolites from 29,738 hepatocytes, including cell-to-cell normalization, nonlinear batch correction, UMAP dimensionality reduction (Becht et al., 2019), Leiden clustering, and pseudotime trajectory analysis (Trapnell et al., 2014). This revealed three clusters of cells corresponding to homeostasis, steatosis, and an intermediate metabolic state, for which marker metabolites were identified via hypothesis tests of differential expression. The ScanPy package was also used (Castro et al., 2021b) to carry out clustering and differential expression testing for mass spectra of single organelles, and by Yuan et al. (Yuan et al., 2021) for differential expression analysis, dimensionality reduction, and clustering of mass spectra of single hepatocyte nuclei in-situ.

However, single-cell metabolomics data differs from other modalities in ways that may present obstacles to further cross-application of existing statistical methods. First, MS, optical, and vibrational metabolomics data are natively continuous-valued signals, whereas sequencing data is count-valued and often sparse. The meanings of zeroes also differ between transcriptomics and MS-based metabolomics: while the technical versus biological origins of sparsity in scRNA have been the subject of considerable debate (Jiang et al., 2022; Svensson, 2020), metabolite peaks can fail to be detected in mass spectra for computational reasons: e.g. if they insufficiently exceed background spectral noise during peak detection (Alexandrov, 2012), or are mis-annotated due to mass drift (La Rocca et al., 2021). While such events may be digitally recorded as zeroes, they are better understood and modelled as a form of random missingness (Do et al., 2018a, 2018b).

Methods based upon MS imaging of single cells also risk co-sampling of neighboring cells:

although Rappez et al. (Rappez et al., 2021b) indicate this minimally affected their analysis of cultured hepatocytes, this may bias estimates of spatial autocorrelation of metabolites in tissues where cells are tightly packed (and irregularly shaped) relative to the MS spatial resolution. Finally, a significant promise of single-cell omics is its potential (through sufficiently large sample sizes) to infer regulatory relationships de-novo between biomolecules. Many algorithms toward this end have been developed for single-cell RNA data (Aibar et al., 2017; Chan et al., 2017; Pratapa et al., 2020), as well as for mass cytometry (Sachs et al., 2005; Wang et al., 2019). While in principle such methods could also be applied toward learning metabolic networks from data, we caution these may not be robust to sources of (co-) variation specific to MS-based single-cell metabolomics, such as in-source fragmentation (Dolatmoradi et al., 2021), region-dependent ion suppression (Taylor et al., 2018), or global metabolic shifts arising from cell manipulation (Llufrio et al., 2018): these may induce correlations between metabolites that are primarily technical rather than biological in origin.

A goal at times is to determine cell heterogeneity within a population of cells and to understand what biochemical species drive this heterogeneity. While sc MS can determine mass markers that drive cell differences, the gold standard for determining cell type remains immunohistochemistry

with established markers; Neumann et al. (Neumann et al., 2019a, 2019b) performed single cell MALDI MS and then followed up on the same cells using immunohistochemical staining via GFAP and neurofilament antibodies and thus were able to correlate both datasets and determine the differences in lipids between astrocytes and neurons (Neumann et al., 2019a, 2019b) The lipid markers discovered later have been shown to be consistent with data obtained on a distinct platform (Neumann et al., 2019a, 2019b). Using these large datasets of thousands of individual rodent brain single cell MALDI MS spectra, Xie et al. created a new statistical pipeline based on interpretable machine learning. Using these same datasets, they were able to classify cells based on their original location (e.g., hippocampus and cerebellum) and found they could classify cells 96% of the time (Xie et al., 2020). This does not necessarily mean that the remaining 4% of the cells are wrongly classified, but perhaps up to 4% of the cells do not have features that allow such classification. Additional studies use larger datasets such as 150,000 human brain cells (Bhaduri et al., 2021).These approaches have recently been adapted to work with single organelle MS measurements (Castro et al., 2021a).

2.8 QA/QC- how to include proper blanks, controls and performance measures?

2.8.1 The goals of QA/QC

A scientific measurement must be reliable enough that it enables robust conclusions to be drawn from the experiment. Quality management (QM) is the process of ensuring such reliability. It is divided into quality assurance and quality control. Quality assurance describes the procedures that are undertaken in the laboratory to have a reasonable confidence that quality standards will be met. Quality control describes the points in the process where specific measurements are recorded to demonstrate that the quality level has been achieved (Barwick, 2020). QM gives the researcher confidence in their biological results and enables experimental results to be reproducible in other laboratories. As such, it is an essential part of good scientific practice. However, the exact requirements for QM will be method specific. Given the novelty of much of the latest single cell technologies, QM is still very much an active area of development in this field. Ultimately, being able to reproduce the results in a different independent biological cohort, preferably in a different laboratory is the ultimate quality assurance test, and is improved still further where a second alternative method can be applied e.g. optical imaging (see above) with Seahorse assays (Little et al., 2020; Wu et al., 2007) although this is not available to most studies.

QM has some particular challenges in single cell metabolomics, not only because of new technology, but also because, by its definition, it is difficult to conduct proper tests of reproducibility on single cell data. Traditional methods in metabolomics for measuring repeatability or reproducibility across an experiment by identifying a suitable quality control sample for regular measurement is more challenging in SC metabolomics, especially given that small sample volumes often preclude replicate measurements on the same samples. However, several of the other QA practices can be taken direct from more traditional metabolomics. Readers are directed to some seminal and some more recent reviews on this e.g. (Beger et al., 2019; Broadhurst et al., 2018; Sumner et al., 2007).

All experiments should have a clear, easy to follow experimental protocol. This should highlight not only the important steps in the procedure, but also which steps have been previously shown to be most critical for quality and reproducibility. This reduces the risk that new technologies are only successful in the hands of a particular scientist. While written protocols are still most useful in the lab, supplementary video protocols are often a clearer way of sharing new technologies. Standard experimental practice such as randomizing the order in which samples are analyzed (and the order of analysis of rastering patterns in the case of metabolic imaging), are also to be strongly recommended.

As for any metabolomics experiments, high quality reagents, especially solvents are required. Before starting an analysis, an instrument should be calibrated, and demonstrated to be properly working with an appropriate system suitability test (SST). While manufacturers often provide SST reagents to use for such a purpose, some laboratories prefer to use their own, more complex mixtures. This enables a much better overview of the complete scan range of interest. Both calibration and the SST results can be used as points of quality control. As such, their results should be recorded (Broadhurst etal., 2018).

Appropriate blank samples should also be used, both to demonstrate that the instrument is free from contamination before starting, and from carryover midway through experimentation, and the software must be set up to identify impurities from the sample preparation and instrumental artefacts as extensive manual curation is impractical.

In some technologies such as optical microscopy, a background fluorescence needs to be measured as a starting point (Sun and Zhu 2021). For mass spectrometry and similar methods, process blanks (i.e. solvent mixtures that have followed the exact processing steps of the samples) enable identification and removal or concentration adjustment of peaks that appear to come from the processing steps themselves.

Since single cell analyses are likely to be subject to the same intra and inter-batch effects seen in more traditional metabolomics analysis, this should be monitored where possible. This has typically been achieved with some form of

representative quality control (QC) sample which can be repeatably analyzed across and between batches. There are already proven batch effects in most currently used methods for single cell analysis (Balluff et al., 2021; Rappez et al., 2021b). The ideal QC sample is a pooled sample consisting of aliquots of all, or a representative cohort, of the samples to be analyzed. This mimics better the matrix (and therefore the matrix effects) of the samples themselves and should also enable detection of a very similar range of metabolites. For obvious reasons, such a QC sample is difficult to achieve in most single cell analyses with the exception of some techniques which extract cell contents before analysis. Alternatives to a traditional QC have included the analysis of technical replicates (Choi et al., 2021; Nemes et al., 2012; Onjiko et al., 2016), as well as several of the approaches already described above including the use of larger cellular populations to obtain robust results, the measurement of the cells using more than one platform and monitoring reproducibility and precision of internal standards.

Some methods have additional requirements for QM, such as addressing the effects of variation in slice or matrix thickness on MALDI results for example. For many technologies, such considerations still need to be defined and appropriate solutions found.

So far, the discussion has concentrated on technical validation; that an instrument is carrying out reliable and reproducible measurements. However, biological degradation as a result of the process itself needs also to be considered. As with other metabolomics measurements, single cell metabolomics typically requires multiple steps to process samples before the point of an analysis. Some methods also take many hours (e.g. MALDI imaging). Both may lead to post-harvesting changes being measured as part of the metabolome. Currently, little research has been published on how these processes may themselves be affecting the measurements. Some methods lend themselves better to testing this hypothesis than others e.g. analyzing monocultures of cells before and after FACs sorting may enable an estimate of how much the FACS process changes the metabolome. Degradation, which occurs over the time course of an analysis, is easier to measure, either in technical replicates (Nemes et al., 2012) of samples, or by analyzing the trend of analyses over a single slide in the case of MALDI imaging. Some recent developments such as fluidic force microscopy (Guillaume-Gentil et al., 2017) claim to circumvent this problem by extracting intracellular fluid directly from living cells in a two-step process. Obviously, short lived metabolites (e.g., nitric oxide) would be difficult to measure with all of these approaches.

Many single cell metabolomics methods rely on relative differences between groups of cells. However, as the technologies move toward true quantification, quantitative methods need themselves to be validated. Existing guidelines from the EMA and FDA will probably require some adaptation to allow for the unique challenges of sc metabolomics.

3 Future directions: prospects for integration with large scale 'omics'

Major advances have been made in metabolic analyses of individual cells that provide biological mechanisms and complement the information available from metabolomics studies of biofluids. Nevertheless, there remain several technological issues. In addition to numerous technical developments to increase sensitivity and reliability of identification and quantification discussed above, it is desirable to integrate metabolic information with protein and gene expression data. As changes in metabolite abundances can have more than one origin (altered synthesis routes and/or altered utilization routes), for mechanistic purposes (and overall confidence), it may be important to determine the levels of the relevant enzyme isoforms (enzyme catalyzed rates are usually proportional to enzyme concentration), and any post translational modifications that may impact the intrinsic enzymic activity independent of the expression level. If enzyme abundances are important determinants of the fluxes and metabolite levels, then transcriptomics can determine whether this is transcriptionally controlled versus post translationally (protein degradation). The metabolite levels corresponding to enzyme activities (or groups of enzymes) may also shed light on the possible importance of non-covalent enzyme modifications of intrinsic activity (cf. allosteric interactions). There have been attempts to link, for example, metabolomics information with transcriptomics (Al-Sabah et al., 2020; Baccin et al., 2019; Fan, 2012; Zhang et al., 2013), such as using the confirmation from isotope tracer methods to interrogate RNAseq data sets with metabolic modeling (Fan et al., 2019). As it is now possible to apply metabolomics methods at the single cell level, at least for relatively abundant metabolites, as well as proteomics and near single cell resolution spatially resolved transcriptomics (e.g. the Visium system from 10X Genomics and single cell resolution via CosMx from NanoString) major issues are corresponding coverage of the different omics levels, and to what extent these 'omics can be acquired on the same cells, or at least similar cells from (for example) adjacent regions of tissue. Different workflows might be envisaged, such that tissues are first interrogated by non-destructive imaging modalities prior to fixing, staining and subsequent proteomic/transcriptomic processing. Incorporating metabolic tracer information even for a few metabolites greatly increases the information content in the context of interacting metabolic pathways.

To do justice to the very large amounts of quantitative data that such multiomics approaches imply, a major growth

area will be in the construction of detailed integrative models of cells in complex (tissue) environments, incorporating large data bases (Lorenzi et al., 2021; Muraro et al., 2016; Robinson et al., 2020; Thul and Lindskog 2018; Wang et al., 2021a, 2021b; Wishart et al., 2022) and informatics approaches to enhancing overall throughput.

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Declarations

Competing interests The authors declare no competing interests.

References

Abouleila, Y., Onidani, K., Ali, A., Shoji, H., Kawai, T., Lim, C. T., Kumar, V., Okaya, S., Kato, K., Hiyama, E., Yanagida, T., Masujima, T., Shimizu, Y., & Honda, K. (2019). Live single cell mass spectrometry reveals cancer-specific metabolic profiles of circulating tumor cells. *Cancer Science, 110*, 697–706.

Aerts, J. T., Louis, K. R., Crandall, S. R., Govindaiah, G., Cox, C. L., & Sweedler, J. V. (2014). Patch clamp electrophysiology and capillary electrophoresis-mass spectrometry metabolomics for single cell characterization. *Analytical Chemistry, 86*, 3203–3208.

Ahn, C., Hwang, B., Nam, K., Jin, H., Woo, T., & Park, J.-H. (2019). Overcoming the penetration depth limit in optical microscopy: Adaptive optics and wavefront shaping. *Kournal of Innovative Optical Health Sciences, 12*, 1930002.

Aibar, S., Gonzalez-Blas, C. B., Moerman, T., Van, A. H. T., Imrichova, H., Hulselmans, G., Rambow, F., Marine, J. C., Geurts, P., Aerts, J., van den Oord, J., Atak, Z. K., Wouters, J., & Aerts, S. (2017). SCENIC: single-cell regulatory network inference and clustering. *Nature Methods, 14*, 1083.

Alexandrov, T. (2012). MALDI imaging mass spectrometry: statistical data analysis and current computational challenges. *BMC Bioinformatics*. https://doi.org/10.1186/1471-2105-13-s16-s11

Ali, A., Abouleila, Y., Shimizu, Y., Hiyama, E., Emara, S., Mashaghi, A., & Hankemeier, T. (2019a). Single-cell metabolomics by mass spectrometry: Advances, challenges, and future applications. *Trac-Trends in Analytical Chemistry*. https://doi.org/10.1016/j.trac.2019.02.033

Ali, A., Abouleila, Y., Shimizu, Y., Hiyama, E., Watanabe, T. M., Yanagida, T., & Germond, A. (2019b). Single-cell screening of tamoxifen abundance and effect using mass spectrometry and raman-spectroscopy. *Analytical Chemistry, 19*, 2710–2718.

Al-Sabah, J., Baccin, C., & Haas, S. (2020). Single-cell and spatial transcriptomics approaches of the bone marrow microenvironment. *Current Opinion in Oncology, 32*, 146–153.

Asp, M., Giacomello, S., Larsson, L., Wu, C., Furth, D., Qian, X., Wardell, E., Custodio, J., Reimegard, J., Salmen, F., Osterholm, C., Stahl, P. L., Sundstrom, E., Akesson, E., Bergmann, O., Bienko, M., Mansson-Broberg, A., Nilsson, M., Sylven, C., & Lundeberg, J. (2019). A spatiotemporal organ-wide gene expression and cell atlas of the developing human heart. *Cell, 179*, 1647.

Baccin, C., Al-Sabah, J., Velten, L., Helbling, P. M., Grunschlager, F., Hernandez-Malmierca, P., Nombela-Arrieta, C., Steinmetz, L. M., Trumpp, A., & Haas, S. (2019). Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. *Nature Cell Biology, 22*, 38–48.

Balluff, B., Hopf, C., Siegel, T. P., Grabsch, H. I., & Heeren, R. M. A. (2021). Batch effects in MALDI mass spectrometry imaging. *Journal of the American Society for Mass Spectrometry, 32*, 628–635.

Bartman, C. R., Shen, Y., Lee, W. D., TeSlaa, T., Jankowski, C. S. R., Wang, L., Yang, L., Roichman, A., Bhatt, V., Lan, T., Hu, Z., Xing, X., Lu, W., Guo, J. Y., & Rabinowitz, J. D. (2021). Slow TCA flux implies low ATP production in tumors. *BioRxiv*. https://doi.org/10.1101/2021.10.04.463108

Bartoschek, M., Oskolkov, N., Bocci, M., Lovrot, J., Larsson, C., Sommarin, M., Madsen, C. D., Lindgren, D., Pekar, G., Karlsson, G., Ringner, M., Bergh, J., Bjorklund, A., & Pietras, K. (2018). Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. *Nature Communications, 9*, 5150.

Barwick, V.J. (2020) Eurachem—A Focus for Analytical Chemistry in Europe. *Proceedings of The Virtual Eurachem Workshop 2020—Quality Assurance for Analytical Laboratories in the University Curriculum 55,* 19

Becht, E., McInnes, L., Healy, J., Dutertre, C. A., Kwok, I. W. H., Ng, L. G., Ginhoux, F., & Newell, E. W. (2019). Dimensionality reduction for visualizing single-cell data using UMAP. *Nature Biotechnology, 37*, 38.

Beger, R. D., Dunn, W., Schmidt, M. A., Gross, S. S., Kirwan, J. A., Cascante, M., Brennan, L., Wishart, D. S., Oresic, M., Hankemeier, T., Broadhurst, D. I., Lane, A. N., Suhre, K., Kastenmüller, G., Sumner, S. J., Thiele, I., Fiehn, O., & Kaddurahdaouk, R. (2016). Metabolomics enables precision medicine—"A white paper, community perspective." *Metabolomics, 12*, 149.

Beger, R. D., Dunn, W. B., Bandukwala, A., Bethan, B., Broadhurst, D., Clish, C. B., Dasari, S., Derr, L., Evans, A., Fischer, S., Flynn, T., Hartung, T., Herrington, D., Higashi, R., Hsu, P. C., Jones, C., Kachman, M., Karuso, H., Kruppa, G., … Zanetti, K. A. (2019). Towards quality assurance and quality control in untargeted metabolomics studies. *Metabolomics*. https://doi.org/10.1007/s11306-018-1460-7

Bensen, R. C., Standke, S. J., Colby, D. H., Kothapalli, N. R., LeMcClain, A. T., Patten, M. A., Tripathi, A., Heinlen, J. E., Yang, Z., & Burgett, A. W. G. (2021). Single cell mass spectrometry quantification of anticancer drugs: proof of concept in cancer patients. *ACS Pharmacol Transl Sci, 4*, 96–100.

Benson, S., Fernandez, A., Barth, N. D., de Moliner, F., Horrocks, M. H., Herrington, C. S., Abad, J. L., Delgado, A., Kelly, L., Chang, Z., Feng, Y., Nishiura, M., Hori, Y., Kikuchi, K., & Vendrell, M. (2019). SCOTfluors: small, conjugatable, orthogonal, and tunable fluorophores for in vivo imaging of cell metabolism. *Angewandte Chemie International Edition, 58*, 6911–6915.

Bergman, H.-M., & Lanekoff, I. (2017). Profiling and quantifying endogenous molecules in single cells using nano-DESI MS. *The Analyst, 142*, 3639–3647.

Bhaduri, A., Neumann, E. K., Kriegstein, A. R., & Sweedler, J. V. (2021). Identification of lipid heterogeneity and diversity in the developing human brain. *JACS Au, 12*, 2261–2270.

Bilan, D. S., & Belousov, V. V. (2017). New tools for redox biology: From imaging to manipulation. *Free Radical Biology and Medicine, 109*, 167–188.

Blaženović, I., Kind, T., Ji, J., & Fiehn, O. (2018). Software tools and approaches for compound identification of LC-MS/MS data in metabolomics. *Metabolites*. https://doi.org/10.3390/metabo8020031

Broadhurst, D., Goodacre, R., Reinke, S. N., Kuligowski, J., Wilson, I. D., Lewis, M. R., & Dunn, W. B. (2018). Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. *Metabolomics, 14*, 72.

Cambronne, X. A., Stewart, M. L., Kim, D., Jones-Brunette, A. M., Morgan, R. K., Farrens, D. L., Cohen, M. S., & Goodman, R. H. (2016). Biosensor reveals multiple sources for mitochondrial NAD(+). *Science, 352*, 1474–1477.

Cassidy, J. W., Caldas, C., & Bruna, A. (2015). Maintaining tumor heterogeneity in patient-derived tumor xenografts. *Cancer Research, 75*, 2963–2968.

Castro, D. C., Xie, Y. R., Rubakhin, S. S., Romanova, E. V., & Sweedler, J. V. (2021a). Image-guided MALDI mass spectrometry for high-throughput single-organelle characterization. *Nature Methods, 18*, 1233.

Castro, D. C., Xie, Y. R., Rubakhin, S. S., Romanova, E. V., & Sweedler, J. V. (2021b). Image-guided MALDI mass spectrometry for high-throughput single-organelle characterization. *Nature Methods, 18*, 1233–1238.

Cazares, L. H., Van Tongeren, S. A., Costantino, J., Kenny, T., Garza, N. L., Donnelly, G., Lane, D., Panchal, R. G., & Bavari, S. (2015). Heat fixation inactivates viral and bacterial pathogens and is compatible with downstream MALDI mass spectrometry tissue imaging. *BMC Microbiology, 15*, 101.

Chan, T. E., Stumpf, M. P. H., & Babtie, A. C. (2017). Gene regulatory network inference from single-cell data using multivariate information measures. *Cell Systems, 5*, 251.

Chen, W. W., Freinkman, E., Wang, T., Birsoy, K., & Sabatini, D. M. (2016). Absolute quantification of matrix metabolites reveals the dynamics of mitochondrial metabolism. *Cell, 166*, 1324–1337.

Choi, S. B., Munoz-Llancao, P., Manzini, M. C., & Nemes, P. (2021). Data-dependent acquisition ladder for capillary electrophoresis mass spectrometry-based ultrasensitive (Neuro)proteomics. *Analytical Chemistry, 93*, 15964–15972.

Christensen, C. E., Karlsson, M., Winther, J. R., Jensen, P. R., & Lerche, M. H. (2014). Non-invasive in-cell determination of free cytosolic NAD(+)/NADH ratios using hyperpolarized glucose show large variations in metabolic phenotypes. *Journal of Biological Chemistry, 289*, 2344–2352.

Close, H. J., Stead, L. F., Nsengimana, J., Reilly, K. A., Droop, A., Wurdak, H., Mathew, R. K., Corns, R., Newton-Bishop, J., Melcher, A. A., Short, S. C., Cook, G. P., & Wilson, E. B. (2019). Expression profiling of single cells and patient cohorts identifies multiple immunosuppressive pathways and an altered NK cell phenotype in glioblastoma. *Clinical and Experimental Immunology, 114*, 146.

Cohen, M., Giladi, A., Gorki, A.-D., Solodkin, D. G., Zada, M., Hladik, A., Miklosi, A., Salame, T.-M., Halpern, K. B., David, E., Itzkovitz, S., Harkany, T., Knapp, S., & Amit, I. (2018). Lung single-cell signaling interaction map reveals basophil role in macrophage imprinting. *Cell, 175*, 1031.

Comi, T. J., Do, T. D., Rubakhin, S. S., & Sweedler, J. V. (2017a). Categorizing cells on the basis of their chemical profiles: progress in single-cell mass spectrometry. *Journal of the American Chemical Society, 139*, 11.

Comi, T. J., Makurath, M. A., Philip, M. C., Rubakhin, S. S., & Sweedler, J. V. (2017b). MALDI MS guided liquid microjunction extraction for capillary electrophoresis-electrospray ionization MS analysis of single pancreatic islet cells. *Analytical Chemistry, 89*, 7765–7772.

Comi, T. J., Neumann, E. K., Do, T. D., & Sweedler, J. V. (2017c). microMS: A Python platform for image-guided mass spectrometry profiling. *Journal of the American Society for Mass Spectrometry, 28*, 1919–1928.

Dang, X., Bardhan, N. M., Qi, J., Gu, L., Eze, N. A., Lin, C.-W., Kataria, S., Hammond, P. T., & Belcher, A. M. (2019). Deep-tissue optical imaging of near cellular-sized features. *Scientific Reports, 9*, 3873.

Dean, D. A., Gautham, G., Siqueira-Neto, J. L., McKerrow, J. H., Dorrestein, P. C., & McCall, L. I. (2021). Spatial metabolomics identifies localized chemical changes in heart tissue during chronic cardiac Chagas Disease. *PLoS Neglected Tropical Diseases, 15*, e0009819.

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., & Thompson, C. B. (2007). Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 19345–19350.

Do, K. T., Wahl, S., Raffler, J., Molnos, S., Laimighofer, M., Adamski, J., Suhre, K., Strauch, K., Peters, A., Gieger, C., Langenberg, C., Stewart, I. D., Theis, F. J., Grallert, H., Kastenmuller, G., & Krumsiek, J. (2018a). Characterization of missing values in untargeted MS-based metabolomics data and evaluation of missing data handling strategies. *Metabolomics*. https://doi.org/10.1007/s11306-018-1420-2

Do, T. D., Ellis, J. F., Neumann, E. K., Comi, T. J., Tillmaand, E. G., Lenhart, A. E., Rubakhin, S. S., & Sweedler, J. V. (2018b). Optically guided single cell mass spectrometry of rat dorsal root ganglia to profile lipids, peptides and proteins. *ChemPhysChem, 19*, 1180–1191.

Dolatmoradi, M., Samarah, L. Z., & Vertes, A. (2021). Single-cell metabolomics by mass spectrometry: Opportunities and challenges. *Analysis & Sensing, 2*, e202100032.

Duncan, K. D., Fyrestam, J., & Lanekoff, I. (2019). Advances in mass spectrometry based single-cell metabolomics. *The Analyst, 144*, 782–793.

Ehrhardt, M. J., Gallagher, F. A., McLean, M. A., & Schönlieb, C.-B. (2022). Enhancing the spatial resolution of hyperpolarized carbon-13 MRI of human brain metabolism using structure guidance. *Magnetic Resononance Medicine, 87*, 1301–1312.

Fan, T. W. (2012). Metabolomics-edited transcriptomics analysis (META). In T. W. Fan, A. N. Lane, & R. M. Higashi (Eds.), *Handbook of metabolomics* (Vol. 17, pp. 439–480). Humana.

Fan, T. W. M., Bruntz, R. C., Yang, Y., Song, H., Chernyavskaya, Y., Deng, P., Zhang, Y., Shah, P. P., Beverly, L. J., Qi, Z., Mahan, A. L., Higashi, M., Dang, C. V., & Lane, A. N. (2019). De novo synthesis of serine and glycine fuels purine nucleotide biosynthesis in human lung cancer tissues. *Journal of Biological Chemistry, 294*, 13464–13477.

Fan, T.W.-M., Higashi, R. M., Chernyavskaya, Y., & Lane, A. N. (2020). Resolving metabolic heterogeneity in experimental models of the tumor microenvironment from a stable isotope resolved metabolomics perspective. *Metabolites, 10*, 249.

Fan, T. W. M., Higashi, R. M., Song, H., Daneshmandi, S., Mahan, A. L., Purdom, M. S., Bocklage, T. J., Pittman, T. A., He, D. H., Wang, C., & Lane, A. N. (2021). Innate immune activation by checkpoint inhibition in human patient-derived lung cancer tissues. *eLife, 10*, e69578.

Fujii, T., Matsuda, S., Tejedor, M. L., Esaki, T., Sakane, I., Mizuno, H., Tsuyama, N., & Masujima, T. (2015). Direct metabolomics for plant cells by live single-cell mass spectrometry. *Nature Protocols, 10*, 1445–1456.

Gil, D. A., Deming, D., & Skala, M. C. (2021). Patient-derived cancer organoid tracking with wide-field one-photon redox imaging to assess treatment response. *Journal of Biomedical Optics*. https://doi.org/10.1117/1.Jbo.26.3.036005

Gilmore, I. S., Heiles, S., & Pieterse, C. L. (2019). Metabolic imaging at the single-cell scale: Recent advances in mass spectrometry imaging. In P. W. Bohn & J. E. Pemberton (Eds.), *Annual review of analytical chemistry* (Vol. 12, pp. 201–224). Annual Review.

Greaves, M., & Maley, C. C. (2012). Clonal evolution in cancer. *Nature, 481*, 306–313.

Guillaume-Gentil, O., Rey, T., Kiefer, P., Ibanez, A. J., Steinhoff, R., Bronnimann, R., Dorwling-Carter, L., Zambelli, T., Zenobi, R., & Vorholt, J. A. (2017). Single-cell mass spectrometry of metabolites extracted from live cells by fluidic force microscopy. *Analytical Chemistry, 89*, 5017–5023.

Guo, S. H., Zhang, C., & Le, A. (2021). The limitless applications of single-cell metabolomics. *Current Opinion in Biotechnology, 71*, 115–122.

Haber, D. A., & Velculescu, V. E. (2014). Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. *Cancer Discovery, 4*, 650–661.

Hao, Y. H., Hao, S., Andersen-Nissen, E., Mauck, W. M., Zheng, S. W., Butler, A., Lee, M. J., Wilk, A. J., Darby, C., Zager, M., Hoffman, P., Stoeckius, M., Papalexi, E., Mimitou, E. P., Jain, J., Srivastava, A., Stuart, T., Fleming, L. M., Yeung, B., … Satija, R. (2021). Integrated analysis of multimodal single-cell data. *Cell, 184*, 3573.

Hawkinson, T. R., & Sun, R. C. (2022). Matrix-assisted laser desorption/ionization mass spectrometry imaging of glycogen in situ. *Methods in Molecular Biology (clifton, NJ), 2437*, 215–228.

Hensley, C. T., Faubert, B., Yuan, Q., Lev-Cohain, N., Jin, E., Kim, J., Jiang, L., Ko, B., Skelton, R., Loudat, L., Wodzak, M., Klimko, C., McMillan, E., Butt, Y., Ni, M., Oliver, D., Orrealba, J., Malloy, C. R., Kernstine, K., … DeBerardinis, R. J. (2016). Metabolic heterogeneity in human lung tumors. *Cell, 164*, 681–694.

Higashi, R. M. (2011). Structural mass spectrometry for metabolomics. In T. W. Fan, R. M. Higashi, & A. N. Lane (Eds.), *Handbook of metabolomics methods*. Humana Press.

Hillenkamp, F., Karas, M., Beavis, R. C., & Chait, B. T. (1991). Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Analytical Chemistry, 63*, 1193A-1203A.

Hong, S., Pawel, G. T., & Pei, R. (2021). Recent progress in developing fluorescent probes for imaging cell metabolites. *Biomedical Materials, 16*, 044108.

Hossain, E., Khanam, S., Dean, D. A., Wu, C., Lostracco-Johnson, S., Thomas, D., Kane, S. S., Parab, A. R., Flores, K., Katemauswa, M., Gosmanov, C., Hayes, S. E., Zhang, Y., & Li, D. (2020). Mapping of host-parasite-microbiome interactions reveals metabolic determinants of tropism and tolerance in Chagas disease. *Science Advances, 6*, eeaz2015.

Hou, J., Wright, H. J., Chan, N., Tran, R., Razorenova, O. V., Potma, E. O., & Tromberg, B. J. (2016). Correlating two-photon excited fluorescence imaging of breast cancer cellular redox state with seahorse flux analysis of normalized cellular oxygen consumption. *Journal of Biomedial Optics, 21*, 60503.

Hu, Q., Wu, D., Walker, M., Wang, P., Tian, R., & Wang, W. (1800). Genetically encoded biosensors for evaluating NAD+/NADH ratio in cytosolic and mitochondrial compartments. *Cell Reports Methods*. https://doi.org/10.1016/j.crmeth.2021.100116

Jansson, E. T., Comi, T. J., Rubakhin, S. S., & Sweedler, J. V. (2016). Single cell peptide heterogeneity of rat islets of langerhans. Figshare. https://doi.org/10.1021/acschembio.6b00602.s002

Jiang, R. C., Sun, T. Y., Song, D. Y., & Li, J. J. (2022). Statistics or biology: the zero-inflation controversy about scRNA-seq data. *Genome Biology*. https://doi.org/10.1186/s13059-022-02601-5

Kaddurah-Daouk, R., Kirwan, J., Larion, M. and Lane, A.N. (2021) Sc Metabolism Workshop Report. Metabonews.

Kalb, D. M., Adikari, S. H., Hong-Geller, E., & Werner, J. H. (2019). Single-cell correlations of mRNA and protein content in a human monocytic cell line after LPS stimulation. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0215602

Karas, M., Bachmann, D., & Hillenkamp, F. (1985). Influence of the wavelength in high-irradiance ultraviolet-laser desorption mass-spectrometry of organic-molecules. *Analytical Chemistry, 57*, 2935–2939.

Kearney, C. J., Vervoort, S. J., Ramsbottom, K. M., Todorovski, I., Lelliott, E. J., Zethoven, M., Pijpers, L., Martin, B. P., Semple, T., Martelotto, L., Trapani, J. A., Parish, I. A., Scott, N. E., Oliaro, J., & Johnstone, R. W. (2021). SUGAR-seq enables simultaneous detection of glycans, epitopes, and the transcriptome in single cells. *Science Advances*. https://doi.org/10.1126/sciadv.abe3610

Keller, L., & Pantel, K. (2019). Unravelling tumour heterogeneity by single-cell profiling of circulating tumour cells. *Nature Reviews Cancer, 19*, 553–567.

Kiss, A., & Hopfgartner, G. (2016). Laser-based methods for the analysis of low molecular weight compounds in biological matrices. *Methods, 104*, 142–153.

Kompauer, M., Heiles, S., & Spengler, B. (2017). Atmospheric pressure MALDI mass spectrometry imaging of tissues and cells at 1.4-mu m lateral resolution. *Nature Methods, 14*, 90–96.

Kyrochristos, I. D., Ziogas, D. E., Goussia, A., Glantzounis, G. K., & Roukos, D. H. (2019). Bulk and single-cell next-generation sequencing: individualizing treatment for colorectal cancer. *Cancers*. https://doi.org/10.3390/cancers11111809

La Rocca, R., Kune, C., Tiquet, M., Stuart, L., Eppe, G., Alexandrov, T., De Pauw, E., & Quinton, L. (2021). Adaptive Pixel Mass Recalibration for Mass Spectrometry Imaging Based on Locally Endogenous Biological Signals. *Analytical Chemistry, 93*, 4066–4074.

Lamont, L., Eijkel, G. B., Jones, E. A., Flinders, B., Ellis, S. R., Siegel, T. P., Heeren, R. M. A., & Vreeken, R. J. (2018). Targeted drug and metabolite imaging: Desorption electrospray ionization combined with triple quadrupole mass spectrometry. *Analytical Chemistry, 90*, 13229–13235.

Lamont, L., Hadavi, D., Viehmann, B., Flinders, B., Heeren, R. M. A., Vreeken, R. J., & Siegel, T. P. (2021). Quantitative mass spectrometry imaging of drugs and metabolites: A multiplatform comparison. *Analytical and Bioanalytical Chemistry, 413*, 2279–2791.

Lane, A. N., Fan, T.W.-M., Xie, X., Moseley, H. N., & Higashi, R. M. (2009). Stable isotope analysis of lipid biosynthesis by high resolution mass spectrometry and NMR. *Analytica Chimica Acta, 651*, 201–208.

Lane, A. N., Higashi, R. M., & Fan, T. W. M. (2020). Metabolic reprogramming in tumors: Contributions of the tumor microenvironment. *Genes & Diseases, 7*, 185–198.

Lanekoff, I., Sharma, V. V., & Marques, C. (2022). Single-cell metabolomics: Where are we and where are we going? *Current Opinion in Biotechnology, 75*, 102693–102693.

Le, A., Lane, A. N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C. J., Slusher, B. S., Zhang, H., Zimmerman, L. J., Liebler, D. C., Slebos, R. J., Lorkiewicz, P. K., Higashi, R. M., Fan, T. W., & Dang, C. V. (2012). Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metabolism, 15*, 110–121.

Li, X., Zhao, S., Hu, H., & Yi-Ming Liu, Y.-M. (2016). A microchip electrophoresis-mass spectrometric platform with double cell lysis nano-electrodes for automated single cell analysis. *Journal of Chromatography A, 1451*, 156–163.

Li, Z., Cheng, S., Lin, Q., Cao, W., Yang, J., Zhang, M., Shen, A., Zhang, W., Xia, Y., Ma, X., & Ouyang, Z. (2021). Single-cell

lipidomics with high structural specificity by mass spectrometry. *Nature Communications, 12*, 2869.
Lima, C., Muhamadali, H., & Goodacre, R. (2022). Simultaneous raman and infrared spectroscopy of stable isotope labelled *Escherichia Coli*. *Sensors, 22*, 3928.
Lita, A., Kuzmin, A. N., Pliss, A., Baev, A., Rzhevskii, A., Gilbert, M. R., Larion, M., & Prasad, P. N. (2019). Toward single-organelle lipidomics in live cells. *Analytical Chemistry, 91*, 11380–11387.
Lita, A., Pliss, A., Kuzmin, A., Yamasaki, T., Zhang, L., Dowdy, T., Burks, C., de Val, N., Celiku, O., Ruiz-Rodado, V., Nicoli, E.-R., Kruhlak, M., Andresson, T., Das, S., Yang, C., Schmitt, R., Herold-Mende, C., Gilbert, M. R., Prasad, P. N., & Larion, M. (2021). IDH1 mutations induce organelle defects via dysregulated phospholipids. *Nature Communications, 12*, 614.
Little, A. C., Kovalenko, I., Goo, L. E., Hong, H. S., Kerk, S. A., Yates, J. A., Purohit, V., Lombard, D. B., Merajver, S. D., & Lyssiotis, C. A. (2020). High-content fluorescence imaging with the metabolic flux assay reveals insights into mitochondrial properties and functions. *Communications Biology, 3*, 271.
Liu, R. M., & Yang, Z. B. (2021). Single cell metabolomics using mass spectrometry: Techniques and data analysis. *Analytica Chimica Acta, 1143*, 124–134.
Liu, Y., Chen, X., Zhang, Y., & Liu, J. (2019). Advancing single-cell proteomics and metabolomics with microfluidic technologies. *The Analyst, 144*, 846–858.
Liu, Z., Ulrich vonBargen, R., & McCall, L.-I. (2021). Central role of metabolism in Trypanosoma cruzi tropism and Chagas disease pathogenesis. *Current Opinion in Microbiology, 63*, 204–209.
Llufrio, E. M., Wang, L., Naser, F. J., & Patti, G. J. (2018). Sorting cells alters their redox state and cellular metabolome. *Redox Biology, 16*, 381–387.
Lombard-Banek, C., Li, J., Portero, E. P., Onjiko, R. M., Singer, C. D., Plotnick, D. O., Al Shabeeb, R. Q., & Nemes, P. (2021). In vivo subcellular mass spectrometry enables proteo-metabolomic single-cell systems biology in a chordate embryo developing to a normally behaving tadpole (X. laevis)*. *Angewandte Chemie (international Ed. in English), 60*, 12852–12858.
Lorenzi, L., Chiu, H. S., Cobos, F. A., Gross, S., Volders, P. J., Cannoodt, R., Nuytens, J., Vanderheyden, K., Anckaert, J., Lefever, S., Tay, A. P., de Bony, E. J., Trypsteen, W., Gysens, F., Vromman, M., Goovaerts, T., Hansen, T. B., Kuersten, S., Nijs, N., … Mestdagh, P. (2021). The RNA Atlas expands the catalog of human non-coding RNAs. *Nature Biotechnology, 39*, 1453.
Luecken, M. D., & Theis, F. J. (2019). Current best practices in single-cell RNA-seq analysis: A tutorial. *Molecular Systems Biology*. https://doi.org/10.15252/msb.20188746
Lundberg, E., & Borner, G. H. H. (2019). Spatial proteomics: A powerful discovery tool for cell biology. *Nature Reviews Molecular Cell Biology, 20*, 285–302.
Madonna, M. C., Fox, D. B., Crouch, B. T., Lee, J., Zhu, C., Martinez, A. F., Alvarez, J. V., & Ramanujam, N. (2019a). Optical imaging of glucose uptake and mitochondrial membrane potential to characterize Her2 breast tumor metabolic phenotypes. *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.Mcr-18-0618
Madonna, M. C., Fox, D. B., Crouch, B. T., Lee, J., Zhu, C., Martinez, A. F., Alvarez, J. V., & Ramanujam, N. (2019b). Optical imaging of glucose uptake and mitochondrial membrane potential to characterize Her2 breast tumor metabolic phenotypes. *Molecular Cancer Research, 17*, 1545–1555.
Madonna, M. C., Duer, J. E., Lee, J. V., Williams, J., Avsaroglu, B., Zhu, C., Deutsch, R., Wang, R., Crouch, B. T., Hirschey, M. D., Goga, A., & Ramanujam, N. (2021). In vivo optical metabolic imaging of long-chain fatty acid uptake in orthotopic models of triple-negative breast cancer. *Cancers (basel), 13*, 20.
Mast, D. H., Liao, H.-W., Romanova, E. V., & Sweedler, J. V. (2021). Analysis of peptide stereochemistry in single cells by capillary electrophoresis-trapped ion mobility spectrometry mass spectrometry. *Analytical Chemistry, 93*, 6205–6213.
Masujima, T. (2009). Live single-cell mass spectrometry. *Analytical Sciences, 25*, 953–960.
Mikolajczak, S. A., Vaughan, A. M., Kangwanrangsan, N., Roobsoong, W., Fishbaugher, M., Yimamnuaychok, N., Rezakhani, N., Lakshmanan, V., Singh, N., Kaushansky, A., Camargo, N., Baldwin, M., Lindner, S. E., Adams, J. H., Sattabongkot, J., & Kappe, S. H. I. (2015). Plasmodium vivax liver stage development and hypnozoite persistence in human liver-chimeric mice. *Cell Host & Microbe, 17*, 526–535.
Minoshima, F., Ozaki, H., Odaka, H., & Tateno, H. (2021). Integrated analysis of glycan and RNA in single cells. *iScience, 24*, 102882.
Misra, B. B. (2020). Open-Source Software Tools, Databases, and Resources for Single-Cell and Single-Cell-Type Metabolomics. *Methods in Molecular Biology (clifton, N.J.), 2064*, 191–217.
Mistry, A. M., Greenplate, A. R., Ihrie, R. A., & Irish, J. M. (2019). Beyond the message: Advantages of snapshot proteomics with single-cell mass cytometry in solid tumors. *Febs Journal, 286*, 1523–1539.
Mizuno, H., Tsuyama, N., Date, S., Takanori Harada, T., & Masujima, T. (2008a). Live single-cell metabolomics of tryptophan and histidine metabolites in a rat basophil leukemia cell. *Analyt. Sciences, 24*, 1525–1527.
Mizuno, H., Tsuyama, N., Harada, T., & Masujima, T. (2008b). Live single-cell video-mass spectrometry for cellular and subcellular molecular detection and cell classification. *Journal of Mass Spectrometry, 43*, 1692–1700.
Mizuno, H., Tsuyama, N., & Masujima, T. (2014). Live single-cell mass spectrometry for organelle metabolomics. *Bunseki Kagaku, 63*, 477–484.
Muraro, M. J., Dharmadhikari, G., Grun, D., Groen, N., Dielen, T., Jansen, E., van Gurp, L., Engelse, M. A., Carlotti, F., de Koning, E. J. P., & van Oudenaarden, A. (2016). A single-cell transcriptome atlas of the human pancreas. *Cell Systems, 3*, 385.
Neal, J. T., Li, X., Zhu, J., Giangarra, V., Grzeskowiak, C. L., Ju, J., Liu, I. H., Chiou, S.-H., Salahudeen, A. A., Smith, A. R., Deutsch, B. C., Liao, L., Zemek, A. J., Zhao, F., Karlsson, K., Schultz, L. M., Metzner, T. J., Nadauld, L. D., Tseng, Y.-Y., … Kuo, C. J. (2018). Organoid modeling of the tumor immune microenvironment. *Cell, 175*, 1972.
Nemes, P., Barton, A. A., Li, Y., & Vertes, A. (2008). Ambient molecular imaging and depth profiling of live tissue by infrared laser ablation electrospray ionization mass spectrometry. *Analytical Chemistry, 80*, 4575–4582.
Nemes, P., Knolhoff, A. M., Rubakhin, S. S., & Sweedler, J. V. (2012). Single-cell metabolomics: changes in the metabolome of freshly isolated and cultured neurons. *Acs Chemical Neuroscience, 3*, 782–792.
Neumann, E. K., Comi, T. J., Rubakhin, S. S., & Sweedler, J. V. (2019a). Lipid heterogeneity between astrocytes and neurons revealed by single-cell MALDI-MS combined with immunocytochemical classification. *Angewandte Chemie-International Edition, 58*, 5910–5914.
Neumann, E. K., Ellis, J. F., Triplett, A. E., Rubakhin, S. S., & Sweedler, J. V. (2019b). Lipid analysis of 30 000 individual rodent cerebellar cells using high-resolution mass spectrometry. *Analytical Chemistry, 91*, 7871–7878.
Newman, C. F., Havelund, R., Passarelli, M. K., Marshall, P. S., Francis, I., West, A., Alexander, M. R., Gilmore, I. S., & Dollery, C. T. (2017). Intracellular drug uptake—a comparison of single cell measurements using ToF-SIMS imaging and quantification from cell populations with LC/MS/MS. *Analytical Chemistry, 89*, 11944–11953.

Nguyen, T. D., Lan, Y., Kane, S. S., Haffner, J. J., Liu, R., McCall, L. I., Yang, Z. (2022). Single-cell mass spectrometry enables insight into heterogeneity in infectious disease. *Analytical Chemistry, 94*(30), 10567–10572. https://doi.org/10.1021/acs.analchem.2c02279.

Nguyen, Q. H., Pervolarakis, N., Blake, K., Ma, D., Davis, R. T., James, N., Phung, A. T., Willey, E., Kumar, R., Jabart, E., Driver, I., Rock, J., Goga, A., Khan, S. A., Lawson, D. A., Werb, Z., & Kessenbrock, K. (2018). Profiling human breast epithelial cells using single cell RNA sequencing identifies cell diversity. *Nature Communications, 9*, 2028.

Niedenfuhr, S., Wiechert, W., & Noh, K. (2015). How to measure metabolic fluxes: A taxonomic guide for (13)C fluxomics. *Current Opinion in Biotechnology, 34*, 82–90.

Niehaus, M., Soltwisch, J., Belov, M. E., & Dreisewerd, K. (2019). Transmission-mode MALDI-2 mass spectrometry imaging of cells and tissues at subcellular resolution. *Nature Methods, 16*, 925.

Oliver, S. G., Winson, M. K., Kell, D. B., & Baganz, F. (1998). Systematic functional analysis of the yeast genome. *Trends in Biotechnology, 16*, 373–376.

Onjiko, R. M., Morris, S. E., Moody, S. A., & Nemes, P. (2016). Single-cell mass spectrometry with multi-solvent extraction identifies metabolic differences between left and right blastomeres in the 8-cell frog (Xenopus) embryo. *The Analyst, 141*, 3648–3656.

Pace, C. L., Angel, P. M., Drake, R. R., & Muddiman, D. C. (2022). Mass spectrometry imaging of N-linked glycans in a formalin-fixed paraffin-embedded human prostate by infrared matrix-assisted laser desorption electrospray ionization. *Journal of Proteome Research, 21*, 243–249.

Palla, G., Spitzer, H., Klein, M., Fischer, D., Schaar, A. C., Kuemmerle, L. B., Rybakov, S., Ibarra, I. L., Holmberg, O., Virshup, I., Lotfollahi, M., Richter, S., & Theis, F. J. (2022). Squidpy: a scalable framework for spatial omics analysis. *Nature Methods, 19*, 171.

Pan, N., Rao, W., Kothapalli, N. R., Liu, R., Burgett, A. W. G., & Yang, Z. (2014). The single-probe: A miniaturized multifunctional device for single cell mass spectrometry analysis. *Analytical Chemistry, 86*, 9376–9380.

Pan, N., Standke, S. J., Kothapalli, N. R., Sun, M., Bensen, R. C., Burgett, A. W. G., & Yang, Z. (2019). Quantification of drug molecules in live single cells using the single-probe mass spectrometry technique. *Analytical Chemistry, 91*, 9018–9024.

Pan, N., Rao, W., & Yang, Z. (2020). Single-probe mass spectrometry analysis of metabolites in single cells. In B. Shrestha (Ed.), *Single cell metabolism: Methods and protocols methods in molecular biology* (Vol. 2064, pp. 61–71). Humana.

Passarelli, M. K., Pirkl, A., Moellers, R., Grinfeld, D., Kollmer, F., Havelund, R., Newman, C. F., Marshall, P. S., Arlinghaus, H., Alexander, M. R., West, A., Horning, S., Niehuis, E., Makarov, A., Dollery, C. T., & Gilmore, I. S. (2017). The 3D OrbiSIMS-label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. *Nature Methods, 14*, 1175.

Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., & Gelbard, H. A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *BioTechniques, 50*, 98.

Pliss, A., Kuzmin, A. N., Lita, A., Kumar, R., Celiku, O., Atilla-Gokcumen, G. E., Gokcumen, O., Chandra, D., Larion, M., & Prasad, P. N. (2021). A single-organelle optical omics platform for cell science and biomarker discovery. *Analytical Chemistry, 93*, 8281–8290.

Powers, T. W., Neely, B. A., Shao, Y., Tang, H., Troyer, D. A., Mehta, A. S., Haab, B. B., & Drake, R. R. (2014). MALDI imaging mass spectrometry profiling of n-glycans in formalin-fixed paraffin embedded clinical tissue blocks and tissue microarrays. *PLoS ONE, 9*, e106255.

Pratapa, A., Jalihal, A. P., Law, J. N., Bharadwaj, A., & Murali, T. M. (2020). Benchmarking algorithms for gene regulatory network inference from single-cell transcriptomic data. *Nature Methods, 17*, 147.

Qiao, Z., & Lissel, F. (2021). MALDI matrices for the analysis of low molecular weight compounds: rational design, challenges and perspectives. *Chemistry-an Asian Journal, 16*, 868–878.

Quinn, R. A., Melnik, A. V., Vrbanac, A., Fu, T., Patras, K. A., Christy, M. P., Bodai, Z., Belda-Ferre, P., Tripathi, A., Chung, L. K., Downes, M., Welch, R. D., Quinn, M., Humphrey, G., Panitchpakdi, M., Weldon, K. C., Aksenov, A., da Silva, R., Avila-Pacheco, J., ... Dorrestein, P. C. (2020). Global chemical effects of the microbiome include new bile-acid conjugations. *Nature, 579*, 123–129.

Rappez, L., Stadler, M., Triana, S., Gathungu, R. M., Ovchinnikova, K., Phapale, P., Heikenwalder, M., & Alexandrov, T. (2021a). SpaceM reveals metabolic states of single cells. *Nature Methods, 18*, 799.

Rappez, L., Stadler, M., Triana, S., Gathungu, R. M., Ovchinnikova, K., Phapale, P., Heikenwalder, M., & Alexandrov, T. (2021b). SpaceM reveals metabolic states of single cells. *Nature Methods, 18*, 799–805.

Reichard, A., & Asosingh, K. (2019). Best practices for preparing a single cell suspension from solid tissues for flow cytometry. *Cytometry Part A, 95A*, 219–226.

Robinson, J. L., Kocabas, P., Wang, H., Cholley, P. E., Cook, D., Nilsson, A., Anton, M., Ferreira, R., Domenzain, I., Billa, V., Limeta, A., Hedin, A., Gustafsson, J., Kerkhoven, E. J., Svensson, L. T., Palsson, B. O., Mardinoglu, A., Hansson, L., Uhlen, M., & Nielsen, J. (2020). An atlas of human metabolism. *Science Signaling*. https://doi.org/10.1126/scisignal.aaz1482

Roman, J. K., Walsh, C. M., Oh, J., Dana, C. E., Hong, S., Jo, K. D., Alleyne, M., Miljkovic, N., & Cropek, D. M. (2018). Spatially resolved chemical analysis of cicada wings using laser-ablation electrospray ionization (LAESI) imaging mass spectrometry (IMS). *Analytical and Bioanalytical Chemistry, 410*, 1911–1921.

Rubakhin, S. S., Garden, R. W., Fuller, R. R., & Sweedler, J. V. (2000). Measuring the peptides in individual organelles with mass spectrometry. *Nature Biotechnology, 18*, 172–175.

Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D. A., & Nolan, G. P. (2005). Causal protein-signaling networks derived from multiparameter single-cell data. *Science, 308*, 523–529.

Sallin, O., Reymond, L., Gondrand, C., Raith, F., Koch, B., & Johnsson, K. (2018). Semisynthetic biosensors for mapping cellular concentrations of nicotinamide adenine dinucleotides. *eLife, 7*, e32638.

Samarah, L. Z., Khattar, R., Tran, T. H., Stopka, S. A., Brantner, C. A., Parlanti, P., Veličković, D., Shaw, J. B., Agtuca, B. J., Stacey, G., Paša-Tolić, L., Tolić, N., Anderton, C. R., & Vertes, A. (2020a). Single-cell metabolic profiling: metabolite formulas from isotopic fine structures in heterogeneous plant cell populations. *Analytical Chemistry, 92*, 7289–7298.

Samarah, L. Z., Tran, T. H., Stacey, G., & Vertes, A. (2020b). In vivo chemical analysis of plant sap from the xylem and single parenchymal cells by capillary microsampling electrospray ionization mass spectrometry. *Analytical Chemistry, 92*, 7299–7306.

Sanchez-Valdez, F. J., Padilla, A., Wang, W., Orr, D., & Tarleton, R. L. (2018). Spontaneous dormancy protects Trypanosoma cruzi during extended drug exposure. *eLife*. https://doi.org/10.7554/eLife.34039

Selivanov, V. A., Marin, S., Tarragó-Celada, J., Lane, A. N., Higashi, R. M., Fan, T.W.-M., de Ataur, P., & Cascante, M. (2020). Software supporting a workflow of quantitative dynamic flux maps

estimation in central metabolism from sirm experimental data. *Methods in Molecular Biology, 2088*, 271–298.
Sellers, K., Fox, M. P., Bousamra, M., 2nd., Slone, S. P., Higashi, R. M., Miller, D. M., Wang, Y., Yan, J., Yuneva, M. O., Deshpande, R., Lane, A. N., & Fan, T. W. (2015). Pyruvate carboxylase is critical for non-small-cell lung cancer proliferation. *The Journal of Clinical Investigation, 125*, 687–698.
Seth Nanda, C., Venkateswaran, S. V., Patani, N., & Yuneva, M. (2019). Defining a metabolic landscape of tumours: genome meets metabolism. *British Journal of Cancer*. https://doi.org/10.1038/s41416-019-0663-7
Seydel, C. (2021a). Single-cell metabolomics hits its stride. *Nature Methods, 18*, 1452–1456.
Seydel, C. (2021b). Single-cell metabolomics hits its stride. *Nature Methods, 18*, 1452–1456.
Shah, A. T., Beckler, M. D., Walsh, A. J., Jones, W. P., Pohlmann, P. R., & Skala, M. C. (2014). Optical metabolic imaging of treatment response in human head and neck squamous cell carcinoma. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0090746
Shah, A. T., Heaster, T. M., & Skala, M. C. (2017). Metabolic imaging of head and neck cancer organoids. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0170415
Shariatgorji, M., Nilsson, A., Fridjonsdottir, E., Vallianatou, T., Kallback, P., Katan, L., Savmarker, J., Mantas, I., Zhang, X., Bezard, E., Svenningsson, P., Odell, L. R., & Andren, P. E. (2019). Comprehensive mapping of neurotransmitter networks by MALDI-MS imaging. *Nature Methods, 16*, 1021.
Shen, Y., Hu, F., & Min, W. (2019). Raman imaging of small biomolecules. *Annual Review of Biophysics, 48*, 347–369.
Shrestha, B. (2020). Ten Major Future Challenges in Single-Cell Metabolomics. In B. Shrestha (Ed.), *Single Cell Metabolism Methods in Molecular Biology* (Vol. 2064, pp. 219–223). Springer.
Shrestha, B., Patt, J. M., & Vertes, A. (2011). In situ cell-by-cell imaging and analysis of small cell populations by mass spectrometry. *Analytical Chemistry, 83*, 2947–2955.
Steinbeck, J., Fuchs, P., Negroni, Y. L., Elsasser, M., Lichtenauer, S., Stockdreher, Y., Feitosa-Araujo, E., Kroll, J. B., Niemeier, J.-O., Humberg, C., Smith, E. N., Mai, M., Nunes-Nesi, A., Meyer, A. J., Zottini, M., Morgan, B., Wagner, S., & Schwarzlander, M. (2020). In vivo NADH/NAD+ biosensing reveals the dynamics of cytosolic redox metabolism in plants. *The Plant Cell, 32*, 3324–3345.
Stopka, S. A., Wood, E. A., Khattar, R., Agtuca, B. J., Abdelmoula, W. M., Agar, N. Y. R., Stacey, G., & Vertes, A. (2021). High-throughput analysis of tissue-embedded single cells by mass spectrometry with bimodal imaging and object recognition. *Analytical Chemistry, 93*, 9677–9687.
Sumner, L. W., Amberg, A., Barrett, D., Beger, R., Beale, M. H., Daykin, C., Fan, T.W.-M., Fiehn, O., Goodacre, R., Griffin, J. L., Hardy, N., Higashi, R. M., Kopka, J., Lindon, J. C., Lane, A. N., Marriott, P., Nicholls, A. W., Reily, M. D., & Viant, M. (2007). Proposed minimum reporting standards for chemical analysis. *Metabolomics, 3*, 211–221.
Sun, T., & Zhu, C. (2021). Empirical method for rapid quantification of intrinsic fluorescence signals of key metabolic probes from optical spectra measured on tissue-mimicking turbid medium. *Journal of Biomedical Optics, 26*, 045001.
Sun, Q., Fan, T.W.-M., Lane, A. N., & Higashi, R. M. (2021). Ion chromatography-ultra high-resolution MS1/MS2 method for stable isotope-resolved metabolomics (SIRM) reconstruction of metabolic networks. *Analytical Chemistry, 93*, 2749–2757.
Svensson, V. (2020). Droplet scRNA-seq is not zero-inflated. *Nature Biotechnology, 38*, 147–150.
Takats, Z., Wiseman, J. M., Gologan, B., & Cooks, R. G. (2004). Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science, 306*, 471–473.
Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., Yoshida, T., & Matsuo, T. (1988). Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry, 2*, 151–153.
Tang, L., Wei, F., Wu, Y., He, Y., Shi, L., Xiong, F., Gong, Z., Guo, C., Li, X., Deng, H., Cao, K., Zhou, M., Xiang, B., Li, X., Li, Y., Li, G., Xiong, W., & Zeng, Z. (2018). Role of metabolism in cancer cell radioresistance and radiosensitization methods. *Journal of Experimental & Clinical Cancer Research, 37*, 87.
Taylor, A. J., Dexter, A., & Bunch, J. (2018). Exploring ion suppression in mass spectrometry imaging of a heterogeneous tissue. *Analytical Chemistry, 90*, 5637–5645.
Taylor, M. J., Lukowski, J. K., & Anderton, C. R. (2021). Spatially resolved mass spectrometry at the single cell: Recent innovations in proteomics and metabolomics. *Journal of the American Society for Mass Spectrometry, 32*, 872–894.
Temko, D., Tomlinson, I. P. M., Severini, S., Schuster-Böckler, B., & Graham, T. A. (2018). The effects of mutational processes and selection on driver mutations across cancer types. *Nature Communications, 9*, 1857.
Thiele, C., Wunderling, K., & Leyendecker, P. (2019). Multiplexed and single cell tracing of lipid metabolism. *Nature Methods, 16*, 1123.
Thul, P. J., & Lindskog, C. (2018). The human protein atlas: A spatial map of the human proteome. *Protein Science, 27*, 233–244.
Tian, H., Sparvero, L. J., Anthonymuthu, T. S., Sun, W.-Y., Amoscato, A. A., He, R.-R., Bayır, H., Kagan, V. E., & Winograd, N. (2021). Successive high-resolution (H2O)n-GCIB and C60-SIMS imaging integrates multi-omics in different cell types in breast cancer tissue. *Analytical Chemistry, 93*, 8143–8151.
Tran, H. T. N., Ang, K. S., Chevrier, M., Zhang, X., Lee, N. Y. S., Goh, M., & Chen, J. (2020). A benchmark of batch-effect correction methods for single-cell RNA sequencing data. *Genome Biology*. https://doi.org/10.1186/s13059-019-1850-9
Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S. Q., Morse, M., Lennon, N. J., Livak, K. J., Mikkelsen, T. S., & Rinn, J. L. (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature Biotechnology, 32*, 381-U251.
Tung, P., Blischak, J., Hsiao, C., Knowles, D. A., Burnett, J. E., Pritchard, J. K., & Gilad, Y. (2017a). Batch effects and the effective design of single-cell gene expression studies. *Science and Reports, 7*, 39921.
Tung, P.-Y., Blischak, J. D., Hsiao, C. J., Knowles, D. A., Burnett, J. E., Pritchard, J. K., & Gilad, Y. (2017b). Batch effects and the effective design of single-cell gene expression studies. *Scientific Reports*. https://doi.org/10.1038/srep39921
Tweeddale, H., Notley-McRobb, L., & Ferenci, T. (1998). Effect of slow growth on metabolism of Escherichia coli, as revealed by global metabolite pool ("metabolome") analysis. *Journal of Bacteriology, 180*, 5109–5116.
Walker, A. V., Gelb, L. D., Barry, G. E., Subanajouy, P., Poudel, A., Hara, M., Veryovkin, I. V., Bell, G. I., & Hanley, L. (2018). Femtosecond laser desorption ionization mass spectrometry imaging and multivariate analysis of lipids in pancreatic tissue. *Biointerphases*. https://doi.org/10.1116/1.5016301
Walsh, A. J., Cook, R. S., Sanders, M. E., Aurisicchio, L., Ciliberto, G., Arteaga, C. L., & Skala, M. C. (2014). Quantitative optical imaging of primary tumor organoid metabolism predicts drug response in breast cancer. *Cancer Research, 74*, 5184–5194.
Wang, S., Lin, J. R., Sontag, E. D., & Sorger, P. K. (2019). Inferring reaction network structure from single-cell, multiplex data, using

toric systems theory. *Plos Computational Biology*. https://doi.org/10.1371/journal.pcbi.1007311

Wang, H., Robinson, J. L., Kocabas, P., Gustafsson, J., Anton, M., Cholley, P. E., Huang, S., Gobom, J., Svensson, T., Uhlen, M., Zetterberg, H., & Nielsen, J. (2021a). Genome-scale metabolic network reconstruction of model animals as a platform for translational research. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.2102344118

Wang, N., Sarathy, J. P., Zimmerman, M., Kaya, F., Wang, H., Dartois, V., & Carter, C. L. (2021b). On-slide heat sterilization enables mass spectrometry imaging of tissue infected with high-threat pathogens outside of biocontainment: a study directed at mycobacterium tuberculosis. *Journal of the American Society for Mass Spectrometry, 32*, 2664–2674.

Wang, L., Xing, X., Zeng, X., Jackson, S. R., TeSlaa, T., Yang, L., McReynolds, M., Li, X., Wolff, J., Rabinowitz, J. R., & SM., D. (2022). Spatially resolved stable-isotope tracing reveals regional metabolic activity. *Nature Methods, 19*, 223–230.

Williamson, D. H., Lund, P., & Krebs, H. A. (1967). The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *The Biochemical Journal, 103*, 514–527.

Wishart, D. S., Guo, A., Oler, E., Wang, F., Anjum, A., Peters, H., Dizon, R., Sayeeda, Z., Tian, S., Lee, B. L., Berjanskii, M., Mah, R., Yamamoto, M., Jovel, J., Torres-Calzada, C., Hiebert-Giesbrecht, M., Lui, V. W., Varshavi, D., Varshavi, D., … Gautam, V. (2022). HMDB 5.0: The human metabolome database for 2022. *Nucleic Acids Research, 50*, D622–D631.

Wolf, F. A., Angerer, P., & Theis, F. J. (2018). SCANPY: large-scale single-cell gene expression data analysis. *Genome Biology*. https://doi.org/10.1186/s13059-017-1382-0

Wu, M., Neilson, A., Swift, A. L., Moran, R., Tamagnine, J., Parslow, D., Armistead, S., Lemire, K., Orrell, J., Teich, J., Chomicz, S., & Ferrick, D. A. (2007). Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *American Journal of Physiology. Cell Physiology, 292*, C125-136.

Xie, Y. R., Castro, D. C., Bell, S. E., Rubakhin, S. S., & Sweedler, J. V. (2020). Single-cell classification using mass spectrometry through interpretable machine learning. *Analytical Chemistry, 13*, 9338–9347.

Yamada, K., Saito, M., Matsuoka, H., & Inagaki, N. (2007). A real-time method of imaging glucose uptake in single, living mammalian cells. *Nature Protocols, 2*, 753–762.

Yang, H., Ji, W., Guan, M., Li, S., Zhang, Y., Zhao, Z., & Mao, L. (2018). Organic washes of tissue sections for comprehensive analysis of small molecule metabolites by MALDI MS imaging of rat brain following status epilepticus. *Metabolomics*. https://doi.org/10.1007/s11306-018-1348-6

Yang, L., George, J., & Wang, J. (2019). Deep profiling of cellular heterogeneity by emerging single-cell proteomic technologies. *Proteomics*. https://doi.org/10.1002/pmic.201900226

Yuan, Z. Y., Zhou, Q. M., Cai, L. S., Pan, L., Sun, W. L., Qumu, S. W., Yu, S., Feng, J. X., Zhao, H. S., Zheng, Y. C., Shi, M. L., Li, S., Chen, Y., Zhang, X. R., & Zhang, M. Q. (2021). SEAM is a spatial single nuclear metabolomics method for dissecting tissue microenvironment. *Nature Methods, 18*, 1223.

Zhang, G., He, P. J., Tan, H. S., Budhu, A., Gaedcke, J., Ghadimi, B. M., Ried, T., Yfantis, H. G., Lee, D. H., Maitra, A., Hanna, N., Alexander, H. R., & Hussain, S. P. (2013). Integration of metabolomics and transcriptomics revealed a fatty acid network exerting growth inhibitory effects in human pancreatic cancer. *Clinical Cancer Research, 19*, 4983–4993.

Zhang, L., Shi, L., Shen, Y., Miao, Y., Wei, M., Qian, N., Liu, Y., & Min, W. (2019). Spectral tracing of deuterium for imaging glucose metabolism. *Nature Biomedical Engineering, 3*, 402–413.

Zhang, J. T., Brown, J., Scurr, D. J., Bullen, A., MacLellan-Gibson, K., Williams, P., Alexander, M. R., Hardie, K. R., Gilmore, I. S., & Rakowska, P. D. (2020). Cryo-OrbiSIMS for 3D molecular imaging of a bacterial biofilm in its native state. *Analytical Chemistry, 92*, 9008–9015.

Zhao, Y., & Yang, Y. (2016). Real-time and high-throughput analysis of mitochondrial metabolic states in living cells using genetically encoded NAD(+)/NADH sensors. *Free Radical Biology and Medicine, 100*, 43–52.

Zhong, X., Zhang, H., Zhu, Y., Liang, Y., Yuan, Z., Li, J., Li, J., Li, X., Jia, Y., He, T., Zhu, J., Sun, Y., Jiang, W., Zhang, H., Wang, C., & Ke, Z. (2020). Circulating tumor cells in cancer patients: developments and clinical applications for immunotherapy. *Molecular Cancer*. https://doi.org/10.1186/s12943-020-1141-9

Zhu, C., Chen, S., Chui, C. H., Tan, B. K., & Liu, Q. (2014). Early prediction of skin viability using visible diffuse reflectance spectroscopy and autofluorescence spectroscopy. *Plastic and Reconstructive Surgery, 134*, 240e–247e.

Zhu, C. G., Martinez, A. F., Martin, H. L., Li, M., Crouch, B. T., Carlson, D. A., Haystead, T. A. J., & Ramanujam, N. (2017). Near-simultaneous intravital microscopy of glucose uptake and mitochondrial membrane potential, key endpoints that reflect major metabolic axes in cancer. *Scientific Reports*. https://doi.org/10.1038/s41598-017-14226-x

Zhu, C., Li, M., Vincent, T., Martin, H. L., Crouch, B. T., Martinez, A. F., Madonna, M. C., Palmer, G. M., Dewhirst, M. W., & Ramanujam, N. (2018a). Simultaneous in vivo optical quantification of key metabolic and vascular endpoints reveals tumor metabolic diversity in murine breast tumor models. *Journal of Biophotonics*. https://doi.org/10.1002/jbio.201800372

Zhu, C., Martin, H. L., Crouch, B. T., Martinez, A. F., Li, M., Palmer, G. M., Dewhirst, M. W., & Ramanujam, N. (2018b). Near-simultaneous quantification of glucose uptake, mitochondrial membrane potential, and vascular parameters in murine flank tumors using quantitative diffuse reflectance and fluorescence spectroscopy. *Biomedical Optics Express, 9*, 3399–3412.

Zhu, C., Li, M., Vincent, T., Martin, H. L., Crouch, B. T., Martinez, A. F., Madonna, M. C., Palmer, G. M., Dewhirst, M. W., & Ramanujam, N. (2019). Simultaneous in vivo optical quantification of key metabolic and vascular endpoints reveals tumor metabolic diversity in murine breast tumor models. *Journal of Biophotonics, 12*, e201800372.

Zilionis, R., Engblom, C., Pfirschke, C., Savova, V., Zemmour, D., Saatcioglu, H. D., Krishnan, I., Maroni, G., Meyerovitz, C. V., Kerwin, C. M., Choi, S., Richards, W. G., De Rienzo, A., Tenen, D. G., Bueno, R., Levantini, E., Pittet, M. J., & Klein, A. M. (2019). Single-cell transcriptomics of human and mouse lung cancers reveals conserved myeloid populations across individuals and species. *Immunity, 50*, 1317.