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Achieving a deeper understanding of drug metabolism and responses using single cell technologies

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Running Title: Single cell technologies for pharmacology

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Abbreviations: CE, capillary electrophoresis; CE-LIF, capillary electrophoresis coupled with laser-induce fluorescence detection; CODEX, codetection by indexing; CyTOF, mass cytometry; DESI, desorption electrospray ionization; HRMS, high-resolution mass spectrometry; LAESI, laser ablation electrospray ionization; LC-MS, liquid chromatography mass spectrometry; LDI, laser desorption/ionization; MALDI, matrix-assisted laser desorption/ionization; MIBI, multiplexed ion beam imaging; mPOP, minimal proteomic sample preparation; MSI, mass spectrometry imaging; nanoPOTs, nanodroplet processing in one pot for trace samples; NATs, N-acetyltransferases; P450s, cytochromes P450; SCoPE-MS/SCoPE2 – single-cell proteomics by mass spectrometry; scRNA-seq, single-cell RNA sequencing; SIMS, secondary ion mass spectrometry; SULTs, sulfotransferases; UGTs, UDP-glucuronosyltransferases

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Abstract

Recent advancements in single-cell technologies have enabled detection of RNA, proteins, metabolites, and xenobiotics in individual cells, and the application of these technologies has the potential to transform pharmacological research. Single-cell data has already resulted in the development of human and model species cell atlases, identifying different cell-types within a tissue, further facilitating the characterization of tumor heterogeneity and providing insight into treatment resistance. Research discussed in this review demonstrates that distinct cell populations express drug metabolizing enzymes to different extents, indicating there may be variability in drug metabolism not only between organs, but within tissue types. Additionally, we put forth the concept that single-cell analyses can be utilized to expose underlying variability in cellular response to drugs, providing a unique examination of drug efficacy, toxicity, and metabolism. We will outline several of these techniques: single-cell RNA-sequencing and mass cytometry to characterize and distinguish different cell types, single-cell proteomics to quantify drug metabolizing enzymes and characterize cellular responses to drug, capillary electrophoresis-ultrasensitive laser-induced fluorescence detection and single-probe single-cell mass spectrometry for detection of drugs, and others. Emerging single-cell technologies such as these can comprehensively characterize heterogeneity in both cell-type specific drug metabolism and response to treatment, enhancing progress toward personalized and precision medicine.

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Significance Statement

Recent technological advances have enabled the analysis of gene expression and protein levels in single cells. These types of analyses are important to investigating mechanisms that cannot be elucidated on a bulk level, primarily due to the variability of cell populations within biological systems. Here, we summarize cell-type specific drug metabolism and how pharmacologists can utilize single-cell approaches to obtain a comprehensive understanding of drug metabolism and cellular heterogeneity in response to drugs.

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Introduction

In terms of morphology, function, and gene expression profiles, cell-to-cell differences are evident between various tissues, and have also been observed among the same cell types (Altschuler and Wu, 2010; Paszek et al., 2010). Paszek *et al*. proposed cellular heterogeneity and coordination of signaling and regulatory systems, such as the cell cycle and apoptosis, allow a tissue as a whole to maintain biological stability (Paszek et al., 2010). In recent years, a variety of comprehensive single-cell transcriptomic and genomic studies have demonstrated that heterogeneity is intrinsic to the nature of individual cells and arises from stochastic influences such as the microenvironment (Peng et al., 2019; Izar et al., 2020). It is no longer a question of whether this heterogeneity exists, but rather how it effects our understanding of cellular function, metabolism, and even response to therapeutics.

Longstanding techniques involving the use of bulk cells or homogenized tissue only allow researchers to measure an average response of all the components of their sample. This type of measurement may not fully reflect physiological differences between subpopulations or individual cells. As a means to address this, recent advances in single-cell technologies have enabled the use of multi-omics approaches to obtain a more comprehensive understanding of disease biology on a single-cell level. For example, analyzing a biopsy lysate prohibits the characterization of the tumor microenvironment at a cellular level; however, single-cell proteomics, epigenetic analyses, and a number of sequencing techniques – whole genome, whole exome, and single-cell RNA – have demonstrated the complexity of the tumor microenvironment (Wilting and Dannenberg, 2012; Gay et al., 2016; Slavov, 2022). This may contribute significantly to the development of resistance to anti-tumor treatment (Wilting and Dannenberg, 2012; Gay et al., 2016; Slavov, 2022). Thus, analyses of tumor biopsies fail to capture the

regional heterogeneity of the tumor itself, potentially missing legions essential for an accurate prognosis (Gay et al., 2016). It can be envisioned that single-cell technologies could be utilized to advance the understanding of intracellular pharmacokinetics and inter-cellular variability in drug response.

Here, we look forward and describe how techniques such as single-cell RNA sequencing (scRNA-seq), a technology that can be enhanced by Drop-seq and mass cytometry, can be used to characterize different cell populations and investigate the expression of drug metabolizing enzymes in individual cells of specific cell populations. Methods that allow the visualization and analysis of cell-to-cell heterogeneity include co-detection by indexing, mass spectrometry-based proteomics and imaging, and whole-cell patch clamp electrophysiology paired with high resolution mass spectrometry. Additionally, we will discuss opportunities for applying technologies such as secondary ion mass spectrometry, single-probe single-cell mass spectrometry, and capillary electrophoresis-ultrasensitive laser-induced fluorescence detection to quantify and locate drugs in individual cells. In this review, we will cover the current knowledge pertaining to organ and cell-type specific drug metabolism and propose how emerging single-cell technologies can be employed to expand the scope of the field of pharmacology and drug metabolism.

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Cell-type Specific Drug Metabolism

Liver

Primary human hepatocytes are a commonly-utilized model for investigating drug metabolism, as this system is often reflective of in vivo hepatic metabolism (Ponsoda et al., 2001; McGinnity et al., 2004). However, hepatocytes only make up approximately 80% of cells in the liver. Other cell types, including hepatic stellate cells, endothelial cells, cholangiocytes, and Kupffer cells, may also influence drug metabolism (MacParland et al., 2018; Aizarani et al., 2019). Importantly, these cells make up the complex framework of the liver and express a degree of intrapopulation heterogeneity themselves, which enables the multifunctionality of the organ (MacParland et al., 2018; Marcu et al., 2018; Payen et al., 2021).

Though a significant proportion of the body's drug metabolism can be attributed to the liver, several other organs — including the kidneys, intestines, lungs, and brain — express metabolizing enzymes such as the cytochromes P450 (P450s), UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), and others (Table 1). Notably, extrahepatic drug metabolizing enzymes can impact drug bioavailability and pharmacokinetics in both an organ and cell-type specific manner (Paine et al., 1996; Zhang et al., 2007; Fonsi, 2014; Shibata and Chiba, 2015). For example, Shibata and Chiba observed significantly higher predicted bioavailability based on hepatocyte metabolism than calculated bioavailability in the plasma of rats, dogs, monkey, and humans (Shibata and Chiba, 2015).

Kidney

Drug metabolism in the kidneys has been extensively studied and has been shown to contribute considerably to the biotransformation of a variety of different drug classes, including

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immunosuppressants such as cyclosporin A, sedatives such as propofol, and antiretrovirals such as zidovudine (antiretroviral) (Dai et al., 2004; Hiraoka et al., 2005; Knights et al., 2016). Using scRNA-seq, ten distinct cell types were classified in adult human kidney. Of these, glomerular parietal epithelial cells and proximal tubule cells accounted for 80% of the cells present (Liao et al., 2020). Primary human proximal tubule cells have been noted to express UGT1A1, UGT1A6, and UGT2B7, as well as CYP1B1, CYP3A4/5, and CYP4A11; however, the only enzymatic activity that was directly observed was that of CYP3A4/5 (Lash et al., 2008). UGTs 1A8/9 and 2B7 have been observed in primary renal epithelial and cortical renal epithelial cells, in addition to proximal tubule cells, but at significantly lower levels than in renal tissue as a whole (Van der Hauwaert et al., 2014). In immortalized cell lines such as HK2 (proximal tubular cell line) and HEK293 (human embryonic kidney cells), UGT transcript expression has not been detected (Van der Hauwaert et al., 2014). Differences between cellular and microsomal/tissue expression data suggest that there could be a range of cell types contributing to renal metabolism. These cells may be overlooked or underappreciated in bulk analyses; however, single-cell technologies have the potential to fill this gap when studying renal metabolism, as well as drug metabolism in other regions of the body.

Intestine

Drug metabolizing enzymes are also expressed throughout the intestines, and have been found to contribute to the overall clearance of drugs such as cyclosporin, midazolam, and docetaxel (Kolars et al., 1991; Thummel et al., 1996; van Herwaarden et al., 2007). RNA sequencing of human intestinal epithelial cells revealed the presence of several phase 1 and phase 2 drug metabolizing enzymes, with notable differences in transcript expression between the duodenum,

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ileum, colon, and rectum. In the ileum, the most abundant P450 transcripts observed were CYP3A4, CYP2C18, CYP3A5, and CYP2J2 (Takayama et al., 2021).

The metabolic activity of enterocytes, the major subtype of intestinal epithelial cells, from the duodenum exhibit nonspecific UGT and SULT activity as well as CYP3A4, CYP2C9, CYP2C19, and CYP2J2 activity (Ho et al., 2017). Of the four intestinal regions examined, these enzymes were more highly expressed in the ileum and duodenum than in the colon or rectum. This region-specific expression also included UGTs and SULTs, where UGT8, UGT1A1, UGT2B7, UGT2A3, and SULT2A1, SULT1B1, SULT1A2, and SULT1A1 were the top four enzymes of each class identified in the ileum (Takayama et al., 2021). An orthogonal study employing immunoquantification in tissue from the ileum and duodenum identified many of the same SULTs, but at slightly different proportions, confirming that whole tissue analyses do not necessarily reflect the protein abundance in specific cell populations (Riches et al., 2009).

Intestinal cellular models such as Caco-2 cells have shown low transcript expression of CYP3A4, which can be artificially induced, but limited endogenous expression of drug metabolizing enzymes make it a poor model for exploring intestinal drug metabolism (Sergent et al., 2009; Li, 2020). Intestinal metabolism has also been explored using precision-cut intestinal slices as a more comprehensive approach than classic in vitro models such as cultured cells or microsomes (de Graaf et al., 2010). In precision-cut intestinal slices taken from both the ileum and the colon, CYP3A and SULT1 metabolite formation was higher in the ileum, while CYP2A and UGT1A metabolite formation was higher in the colon, indicating regional variability (Iswandana et al., 2018). However, this method does not allow for the discernment between different cell types. Understanding cell type-specific metabolism within different intestinal

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regions is especially relevant for regimens that are delivered rectally or drugs that specifically target the intestines.

Lung

Regional differences in metabolizing enzymes have also been noted in the lung, another extrahepatic organ where local metabolism may have significant effects on overall drug biotransformation, particularly for medications administered via inhalation. scRNA-seq has characterized 58 different cell types in the human lung, encompassing epithelial, endothelial, stromal, and immune cell populations (Travaglini et al., 2020).

Parenchymal cells, part of the alveolar epithelial population, have been found to express the following P450 enzymes: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2J2, CYP3A4 and CYP3A5, all at a fraction of the expression levels found in the hepatocytes. However, phase 2 metabolizing enzymes SULT1A1, SULT1A3/4, SULT1C2, SULT2B1/2, and UGT2A1 were expressed more highly in the parenchymal cells. Several other UGTs and SULT1A2 were also observed, but with lower relative expression to hepatocytes (Somers et al., 2007).

Further, primary human bronchial epithelial cells have been noted to express up to 27 different P450 isoforms, including CYP1A1, CYP2C9, CYP2J2, and CYP3A5, which were also observed in alveolar epithelial cells (Courcot et al., 2012). Courcot *et al.* also presented Phase 2 metabolizing enzyme mRNA expression of UGTs 1A1 and 1A6, NAT5, and SULTs 1A3/4, 1B1, 1E1, 2B1, and 4A1 (Courcot et al., 2012). Of particular interest, transcripts of P450s 1A1 and 1B1 have been observed at higher concentrations in the lungs of smokers and ex-smokers as

opposed to non-smokers, indicating that environmental and/or health-related events could impact drug metabolism in the lung (Kim et al., 2004).

Brain

The brain is perhaps the most pharmacologically elusive and heterogenous of the organs discussed here. When considering the presence of drug metabolizing enzymes, there are not only several brain regions, but a multitude of cell types that must be taken into account. In the cortex alone, there have been 180 perceptible areas (Glasser et al., 2016) and 75 cell types identified (Hodge et al., 2019). Between the frontal cortex, striatum, hippocampus, thalamus/hypothalamus, basal ganglia, substantia nigra, amygdala, pons, and cerebellum, the frontal cortex and cerebellum are the most expressive of P450 enzymes, with 11 and 10 P450s identified, respectively, while the thalamus and hypothalamus have only two P450 enzymes expressed: CYP2D6 and CYP3A4 (McFadyen et al., 1998; Toselli et al., 2015; Kuban and Daniel, 2021). Though CYP2B6 mRNA has been observed in five of the different brain regions explored (frontal cortex, striatum, basal ganglia, pons, and cerebellum), a study examining the microsomal metabolism of various CYP2B6 substrates — nicotine, ketamine, and methadone — found no evidence of enzyme activity (Bloom et al., 2019).

Importantly, CYP2E1 plays a role in the metabolism of ethanol in the brain (Carpenter et al., 1996; Upadhya et al., 2000; Zimatkin et al., 2006; Zhong et al., 2012; Toselli et al., 2015) and has also been linked to Parkinson's disease (Shahabi et al., 2008; Kaut et al., 2012). CYP2E1 mRNA is found primarily in neurons, but has also been observed in Purkinje cells of the cerebellum (Upadhya et al., 2000). In a study of the blood-brain barrier (BBB), CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2E1, CYP2J2, CYP3A4, and CYP4A11 mRNA were observed in primary human brain endothelial cells, each at a significantly higher level than

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commercially available immortalized human brain microvascular cerebral endothelial cells and human-derived umbilical vein endothelial cells (Ghosh et al., 2010). Further study of human brain microvessels, however, revealed that CYP1B1 and CYP2U1 were the only two P450s able to be quantified at the protein level (Shawahna et al., 2011).

Regarding phase 2 metabolizing enzymes, UGTs and NATs were not observed in the microvasculature of the BBB, but SULT1A1 was expressed (Shawahna et al., 2011). UGT mRNA expressed in brain tissues includes UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT 1A9, UGT2A3, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 (Court et al., 2012; Jones and Lazarus, 2014). Both UGT 1A6 and UGT2B7 mRNA have been observed specifically in the cerebellum (King et al., 1999). UGT2B7, the enzyme responsible for the biotransformation of morphine (Coffman et al., 1997), had shown activity in some, but not all, human brain microsomes tested in a study by Wahlstrom *et al*. (Wahlstrom et al., 1988). Lastly, the protein of SULTs 1A1 and 1A3 have been identified in neurons and glial cells from the hippocampus, cerebellum, and frontal, occipital, and temporal lobes (Salman et al., 2009). Overall, these data indicate the presence of drug metabolizing enzymes in specific regions of the brain, which is of particular relevance to local metabolism of drugs that cross the blood-brain barrier.

Moving Forward

While the liver is the primary site of metabolism of most drugs, extrahepatic organs such as the kidneys and the intestines have been shown to contribute to the overall clearance of certain drugs. Additionally, extrahepatic metabolism has the potential to impact pharmacology when target tissues exhibit local metabolism. Further, with the complexity of cellular heterogeneity in pathophysiological states such as tumorigenesis and the sensitivity of cell state to the

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microenvironment, investigation of cell-type specific drug metabolism could reveal unique insight into cellular phenotype in these contexts. (Wilting and Dannenberg, 2012; Kirschner et al., 2020; Guan et al., 2021; Xu et al., 2022). We envision the utilization of single-cell technologies in drug metabolism research will aid in more accurate targeting and dosing for a wide variety of treatments.

Current literature primarily uses scRNA-seq to address differences in the expression of drug metabolizing enzyme transcripts between different cell types, but this strategy is most powerful when employed in conjunction with other single-cell technologies. Few studies have sought to uncover intercellular heterogeneity of drug metabolizing enzymes in extrahepatic metabolizing organs, and the recent development of a variety of single-cell analysis tools will be indispensable in remedying this gap in knowledge.

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Current and Emerging Single-Cell Methods

While some progress has been made using scRNA-seq to study drug metabolizing enzyme transcript expression, there is still little known about drug metabolism and action in a single cell. The following methods can be employed to ascertain cell-type heterogeneity in metabolic activity and cellular response to aid in pharmacological research (Table 2).

RNA Transcripts

Transcriptomics at the single-cell level has been utilized as a means to determine therapeutic targets and to identify genes related to drug tolerance. Drop-seq is a technique used to analyze mRNA of individual cells and has been applied to the investigation of genes associated with drug-tolerance (Macosko et al., 2015; Aissa et al., 2021). Using this method, Aissa *et al.* employed PC9 cells to define subpopulations of drug-tolerant cells after treatment with erlotinib, a tyrosine kinase inhibitor, and characterized previously unidentified drug-tolerant markers. In determining drug-tolerant markers in subpopulations, Aissa et al. identified drugs that could downregulate such tolerance markers, sensitizing drug-tolerant cells to erlotinib treatment, a feat not possible using classic bulk cell RNA sequencing technologies. Further, their data determined that higher transcript expression of tolerance markers correlated with worse patient outcomes (Aissa et al., 2021). Interestingly, drug-tolerance was shown to be continual such that cells can transition between tolerance states, which is an important finding relevant to improving therapeutics. Overall, transcriptomics can be used to characterize subpopulation tolerances that can better inform personalized treatments, as well as identify new targets for drug discovery.

In recent years, bioinformaticians have generated various scRNA-seq atlases and databases, as the curation of datasets facilitates the continual analysis of published data. Several scRNA-seq

databases have been established, such as the EMBL-EBI Single Cell Expression Atlas which as of March 2022 includes 304 studies, encompassing 20 species and over 8.5 million cells (Moreno et al., 2022). Other initiatives such as Gene Expression Omnibus (Barrett et al., 2009) and CellDepot (Lin et al., 2022) provide raw sequencing and visually processed data from single-cell sequencing data, respectively. In utilizing these databases, pharmacologists can uncover the transcript expression of drug metabolizing enzymes in specific cell populations and identify potential therapeutic targets.

Proteins

While measurements of transcript abundance are valuable and have the potential to be integrated with other -omics data sets, single-cell protein abundance and localization data can further improve our understanding of complex biological systems and lend insight into drug disposition. Of note, flow cytometry can be used to characterize heterogeneity in cell surface markers and intracellular proteins in many different biological systems. However, this technique is limited in multiplexing capabilities and does not allow for characterization of protein localization within cells and intact tissues (Tracey et al., 2021).

Mass cytometry (also referred to as CyTOF) is a complementary method to flow cytometry that can produce comprehensive data sets. Like flow cytometry, CyTOF uses antibodies to cell surface markers and intracellular proteins to enable detection. However, while flow cytometry multiplexing is limited by spectral overlap of fluorophores, CyTOF uses antibodies conjugated to heavy metals and inductively coupled mass spectrometry to measure the abundance of these metals in each cell. This technique allows for routine 40-plex experiments and theoretically enables 100-plex studies.

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Similar to scRNA-seq and flow cytometry, CyTOF has been used to study the immune tumor microenvironment (Norton et al., 2019; Zhang et al., 2019; Zhu et al., 2021). Zhu *et al*. were able to use such data to link AKT activity to chemotherapy resistance in breast cancer cells and showed that AKT inhibition increased cell death (Zhu et al., 2021). CyTOF provides a unique profile of a tumor and has been used to develop a computational framework to identify the optimal combination therapy for a patient (Anchang et al., 2018). Additionally, Zhang and colleagues incorporated CyTOF data into a multi-omics analysis including RNA-seq and metabolomics data to study the heterogeneity of hepatocellular carcinoma (Zhang et al., 2019).

The same antibodies that enable high multiplexing in CyTOF can also be used to build upon classic immunohistochemistry in imaging mass cytometry and multiplexed ion beam imaging (MIBI) experiments. Both methods involve labeling a tissue section with metal-conjugated antibodies and detecting these metals by mass spectrometry. To transfer the metals from the tissue to the mass spectrometer, imaging mass cytometry and MIBI use laser ablation and a beam of primary ions, respectively. Both techniques can achieve spatial resolution at or below 1 μm, rendering them capable of imaging at the single-cell level. Employing MIBI technology, Keren *et al.* characterized breast cancer tumor immune microenvironments using 36 markers simultaneously at 260 nm spatial resolution (Keren et al., 2019).

An emerging multiplexed protein imaging technique is co-detection by indexing (CODEX). CODEX utilizes oligonucleotide-conjugated antibodies and involves iterative in situ polymerization to selectively incorporate dNTP-tethered fluorophores on specific target barcodes. Following imaging, fluorophores are removed and additional rounds of in situ polymerization are completed, theoretically allowing for limitless protein detection. Goltsev and

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colleagues used CODEX to characterize the architecture of normal and diseased mouse spleens using 28 antibodies on the same tissue section (Goltsev et al., 2018).

Typically, these single-cell methods are used to compare the presence of surface markers and occasionally intracellular proteins. However, these methods can be used to inform knowledge of drug metabolism and pharmacokinetics by applying them to the study of drug transporters and intracellular drug metabolizing enzymes.

MS-based proteomics removes the need for cell surface markers to characterize proteins of a single cell. Combined with other biochemical methods, mass spectrometry can deliver data on regulatory correlation, signaling pathways, metabolic processes, and posttranslational modifications, all of which are factors that can drive cellular pathobiology and drug response (Olsen and Mann, 2013; Carvalho and Matthiesen, 2016; Marx, 2019; Ctortecka and Mechtler, 2021; Meissner et al., 2022).

The standard bottom-up proteomics workflow includes cell lysis, protein extraction and solubilization, reduction of disulfide bonds and alkylation of cysteines, digestion of proteins to peptides, sample desalting, clean-up, chromatographic separation, MS acquisition, and analysis (Aebersold and Mann, 2003; Bantscheff et al., 2007; Gundry et al., 2010). Currently, single cells are isolated by fluorescence-activated cell sorting (FACS) or microscopy-based sorting techniques into multi-well plates where lysis, digestion, and multiplex labeling, if employed, are performed. To date, multiple methods for single-cell proteomics processing have been described, including Minimal ProteOmic Sample Preparation (mPOP) (Budnik et al., 2018), Nanodroplet Processing in One pot for Trace Samples (nanoPOTS) (Zhu et al., 2018), and Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS) (Vallone et al.; Rinke et al., 2014; Hartlmayr et al., 2021).

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The nanoPOTS platform (Zhu et al., 2018) consists of microfabricated nanowell array chips and a nanoliter robotic pipetting system (Figure 1A). The nanoPOTS platform demonstrated enhanced efficiency and sample recovery by downscaling processing volumes to <200 μL to minimize surface losses (Zhu et al., 2018). Recent trials of the nanoPOTS platform have identified and quantified over 1,000 proteins from single mammalian cells (Kelly et al., 2019).

Modern proteomics often relies on the use of chemical "barcodes" to facilitate sample multiplexing, allowing up to an 18-fold increase in sample throughput. Specifically, isobaric tandem mass tags (TMT) have an identical total mass while heavy isotopes are distributed differently across the tag (Budnik et al., 2018; Myers et al., 2018). Thus, the fragmentation of precursor ions generates TMT reporter ions with isotopic loading unique to each TMT tag, and quantification of TMT-labeled peptides relies on reporter ions whose levels reflect both peptide abundances and noise contributions. Multiplexed peptides have an additive signal when combined, which enhances low level samples such as single cells.

Single Cell ProtEomics by Mass Spectrometry (SCoPE2) further exploits the additive nature of multiplexed peptides through the use of a single labeled channel that contains a significantly higher peptide concentration (Figure 1B). This "carrier" or "boost" channel provides sufficient signal to trigger fragmentation enabling peptide sequencing, while the isobaric tags for each single cell are sufficient for obtaining relative peptide quantification. Recent work by Petelski *et al*. described the preparation of single mammalian macrophages, where all stages of preparation were carried out in a total volume of 1 μL (Petelski et al., 2021). Minimal sample loss and efficient sample preparation enabled over 1,000 proteins to be identified per cell.

Our group recently optimized the multiplexed single-cell proteomics methods from SCoPE2 and analyzed human-derived single-cell samples using trapped ion mobility time-of-flight mass

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spectrometry (Orsburn et al., 2022, preprint). When employing a peptide carrier channel to boost protein sequence coverage, we obtained over 40,000 tandem mass spectra and identified over 1,000 proteins in 30-minute LC gradients, providing unprecedented sequence coverage of each identified protein. We described the first observations of multiple classes of protein post translational modifications (PTMs) using liquid chromatography-mass spectrometry (LC-MS) (TIMSTOF Flex) in single human cells. PTMs play an essential role in various cellular processes that modulate the folding, stability, interaction, and functional activity of proteins (Su et al., 2017). Understanding the impact of PTMs on proteins, especially near active sites and the interfaces of protein-protein interaction, is critical for ongoing drug development research and can be leveraged to improve the efficacy of future drug design (Meng et al., 2021). Precisely targeting PTMs or protein isoforms can advance personalized treatment and precision medicine (Meng et al., 2021).

While these methods rely on traditional LC-MS, other groups have explored alternative techniques to further intensify peptide signal. Choi *et al*. described a custom-built capillary electrophoresis (CE) ionization platform using high-resolution mass spectrometry (HRMS) (Choi et al., 2021) (Figure 1C).

This platform combines patch-clamp electrophysiology with subcellular CE-ESI-HRMS to quantify proteins in targeted neurons in brain tissues. Analysis of 1 picogram of protein digest quantified 157 proteins among the somas of three different dopaminergic neurons from a mouse (Choi et al., 2021). Additionally, Webber *et al*. optimized a label-free single-cell proteomics method which mitigates the slow MS2 sampling rate (Webber et al., 2022). A two-column LC system provides two parallel subsystems to multiplex sample loading, desalting, analysis, and column regeneration, which doubles the throughput as an average 621, 774, 952, and 1622

protein groups were identified with total analysis times of 7, 10, 15, and 30 minutes, respectively (Webber et al., 2022).

Small Molecule Measurements

An advancement in single-cell analysis that directly impacts pharmacology is the quantification of intracellular drug concentrations. This approach provides data regarding drug delivery, influx, and efflux at the intended target cell (i.e., CD4+ T-cells in the treatment and prevention of HIV) and can offer a real-time readout of pharmacokinetic parameters. Drug concentrations in single cells can be quantified under ambient conditions using single-probe mass spectrometry. In this technique, a probe containing dual capillaries allows for the delivery of sampling solvent and internal standard to a cell and the withdrawal of cellular extracts by capillary action. The extracts flow through a nano-electrospray ionization emitter and ions are detected by mass spectrometry (Pan et al., 2019). Recent developments have coupled the single-probe system with an integrated cell manipulation platform, allowing for this technique to be used on cells in suspension, thereby increasing the applicability. Using this method, intracellular drug levels determined in single bladder cancer cells from patients treated with gemcitabine revealed significantly different intracellular concentrations of the drug between two patients, even after multiple rounds of treatment (Bensen et al., 2021), unveiling a need for precision medicine.

Single-probe single-cell mass spectrometry can also be utilized to understand the metabolic changes in cells upon treatment with drugs. These data can provide a key understanding of the drugs' mechanisms of action and how cells can become resistant to therapeutics. This technique has been used to understand synergism of metformin and irinotecan in an induced-irinotecan-resistant colorectal cancer cell line where it was shown that the combination therapy had the largest synergistic effect on the reduced abundance of ceramide phosphoethanolamine, likely due

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to a reduction in enzymatic activity of fatty acid synthase, an enzyme overexpressed in cancer cells (Chen et al., 2022). Using this technique to identify changes in metabolite levels in single-cells upon treatment with a drug can assist in understanding the pharmacodynamics and eventual development of new combination therapies.

Another technique in development to quantify drugs in single cells is capillary electrophoresis coupled with ultrasensitive laser-induced fluorescence detection (CE-LIF). In this method, the native fluorescence of drug molecules is exploited to quantitate single-cell drug levels. Importantly, this method can be used in tandem with flow cytometry to correlate intracellular drug levels to the abundance of cell-surface transporters, such as p-glycoprotein (Deng et al., 2011). Studies using this technique identified cell-to-cell variability in drug uptake and how dosing schedules and p-glycoprotein can impact drug uptake heterogeneity, which are factors that can have clinical implications (Deng et al., 2014).

Imaging

Finally, in order to decipher the molecular diversity of single cells, powerful imaging techniques such as mass spectrometry imaging (MSI) can be used to map the localizations of a range of biomolecules including small metabolites, lipids, peptides, proteins, drugs, and drug metabolites. One of the major advantages of MSI over other imaging techniques is that it can be utilized to visualize the spatial distribution of hundreds of biomolecules simultaneously without labeling (Buchberger et al., 2018). The main ionization techniques used for MSI are matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), laser ablation electrospray ionization (LAESI), and secondary ion mass spectrometry (SIMS) (Buchberger et al., 2018). Of these ionization techniques, MALDI MSI is the most frequently employed for simultaneous visualization of the spatial distribution of drugs, their metabolites, and endogenous

lipid molecules at tissue level (Prideaux et al., 2010; Seneviratne et al., 2018; Tang et al., 2019; Seneviratne et al., 2020). Due to recent improvements in MALDI MS sources, this approach can also be utilized for single cell imaging. Several research groups have demonstrated the use of MALDI MSI to image analytes at 5 μm spatial resolution (Zavalin et al., 2013; Korte et al., 2015). However, the observed ion yields were low, and the investigators had to partially overlay individual pixels to remedy this problem (Korte et al., 2015). Technological and methodological advancements in MALDI MSI have enabled high spatial resolution; however, sensitivity is a major challenge. To overcome this, laser desorption/ionization (LDI) is employed, as it avoids spectral interference due to the presence of a chemical matrix (Stopka et al., 2016). Currently, SIMS is the predominant modality for single cell MSI, and it uses a primary ion beam instead of a laser for sample ablation and ionization. Because of this, it can achieve greater spatial resolution and sensitivity than either MALDI or LDI. Interestingly, Malherbe *et al.* demonstrated that a radio frequency plasma oxygen primary ion source coupled to NanoSIMS can image endogenous cations (e.g., calcium) at a 37 nm spatial resolution (Malherbe et al., 2016). While SIMS is typically coupled to time-of-flight mass spectrometers, this ionization method can also be coupled to an orbitrap mass spectrometer to achieve higher mass resolution and improve identification of biological molecules (Passarelli et al., 2017).

Single-cell-level imaging of endogenous metabolites can improve our understanding of the natural heterogeneity of tissues and cell types as well as enhance research in disease biology and drug discovery. These same tools can be used to study patterns of drug absorption, distribution, and metabolism. In addition to lipids and neurotransmitters, Passarelli *et al.* were able to detect and image the distribution of amiadarone, an antiarrhythmic drug, using OrbiSIMS (Passarelli et al., 2017). SIMS can achieve spatial resolution closer to 200 nm when imaging heavy metals and

has been leveraged to image the distribution of platinum-containing drugs (Lee et al., 2017; Proetto et al., 2018).

Recently, there has been a trend toward the combined use of multiple imaging modalities (Veličković et al., 2018; Tuck et al., 2022). For example, imaging mass cytometry can obtain snapshots of tissue and cell microenvironments, and when coupled with MSI, may facilitate the correlation of drugs, drug metabolites, and endogenous molecules with cellular markers. Further, coupling microscopy and mass spectrometry can help unravel biological processes.

Van Acker *et al.* demonstrated a direct comparison between fluorescence confocal microscopy and laser ablation-inductively coupled plasma-MSI techniques through the use of hybrid tracers targeting CXCR4 and EGFR (Van Acker et al., 2019). Additionally, Schoeberl *et al.* developed a high-throughput laser ablation-inductively coupled plasma MS workflow for single cell analysis following cytospin preparation of cells (Schoeberl et al., 2021). Using this technique, the authors investigated cisplatin, an anticancer drug, uptake in macrophage subtypes (Schoeberl et al., 2021). The above innovative multimodal imaging strategies can be employed broadly to high-resolution imaging of drugs/metabolites and single cell analysis in precision medicine.

In addition to these original investigations, the results of single cell studies are often available for meta-analysis through public data repositories. Today, nearly all mass spectrometry journals require that the original data and processed results are made directly accessible through ProteomeXchange Consortium of global public repositories (Vizcaino et al., 2014). Recent efforts by the European Bioinformatics Institute repository PRIDE have enabled the public deposition of MALDI imaging data when converted to a universal XML format (Rompp et al., 2015).

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With the ongoing evolution of bioinformatics tools and computational techniques, the exploration of publicly deposited single cell data will likely lead to further discoveries that those made in the original studies.

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Conclusion

One of the greatest obstacles in pharmacology and drug development is understanding the heterogeneity in response to drug treatment (Calabresi et al., 1979). This may arise from a number of genetic, epigenetic, and environmental factors and can be reflected by differences in morphology, physiology, and pathology (Evers et al., 2019). Intra-organ cell type heterogeneity warrants the study of distinct cell populations and cell-type specific drug metabolism. The development of single-cell technologies will be invaluable in assessing the scope of cell-to-cell variability within a population. These advancements will provide improved analysis of an individual cell's pharmacokinetic and pharmacodynamic responses, including expression or abundance variations in membrane drug transporters, drug metabolizing enzymes, and cellular proteomic profiling changes due to drug-induced toxicity. Further, the combination of single-cell analyses and single-cell omics can provide a wealth of knowledge and insight into biological and pharmacological processes. We expect the elucidation of cellular heterogeneity in drug metabolism to be transformative to biomedical research and will improve our depth of understanding of biological systems.

References

Aebersold R and Mann M (2003) Mass spectrometry-based proteomics. *Nature* **422:**198-207.

Aissa AF, Islam A, Ariss MM, Go CC, Rader AE, Conrardy RD, Gajda AM, Rubio-Perez C, Valyi-Nagy K, Pasquinelli M, Feldman LE, Green SJ, Lopez-Bigas N, Frolov MV, and Benevolenskaya EV (2021) Single-cell transcriptional changes associated with drug tolerance and response to combination therapies in cancer. *Nat Commun* **12:**1628.

Aizarani N, Saviano A, Sagar, Mailly L, Durand S, Herman JS, Pessaux P, Baumert TF, and Grun D (2019) A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature* **572:**199-204.

Altschuler SJ and Wu LF (2010) Cellular heterogeneity: do differences make a difference? *Cell* **141:**559-563.

Anchang B, Davis KL, Fienberg HG, Williamson BD, Bendall SC, Karacosta LG, Tibshirani R, Nolan GP, and Plevritis SK (2018) DRUG-NEM: Optimizing drug combinations using single-cell perturbation response to account for intratumoral heterogeneity. *Proc Natl Acad Sci U S A* **115:**E4294-e4303.

Bantscheff M, Schirle M, Sweetman G, Rick J, and Kuster B (2007) Quantitative mass spectrometry in proteomics: a critical review. *Analytical and bioanalytical chemistry* **389:**1017-1031.

Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Muertter RN, and Edgar R (2009) NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res* **37:**D885-890.

Bensen RC, Standke SJ, Colby DH, Kothapalli NR, Le-McClain AT, Patten MA, Tripathi A, Heinlen JE, Yang Z, and Burgett AWG (2021) Single Cell Mass Spectrometry Quantification of Anticancer Drugs: Proof of Concept in Cancer Patients. *ACS Pharmacol Transl Sci* **4:**96-100.

Bloom AJ, Wang PF, and Kharasch ED (2019) Nicotine oxidation by genetic variants of CYP2B6 and in human brain microsomes. *Pharmacol Res Perspect* **7:**e00468.

Buchberger AR, DeLaney K, Johnson J, and Li L (2018) Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights. *Anal Chem* **90:**240-265.

Budnik B, Levy E, Harmange G, and Slavov N (2018) SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. *Genome Biol* **19:**161.

Calabresi P, Dexter DL, and Heppner GH (1979) Clinical and pharmacological implications of cancer cell differentiation and heterogeneity. *Biochemical Pharmacology* **28:**1933-1941.

Carpenter SP, Lasker JM, and Raucy JL (1996) Expression, induction, and catalytic activity of the ethanol-inducible cytochrome P450 (CYP2E1) in human fetal liver and hepatocytes. *Mol Pharmacol* **49:**260-268.

Carvalho AS and Matthiesen R (2016) Global MS-based proteomics drug profiling, in: *Proteostasis*, pp 469-479, Springer.

Chen X, Sun M, and Yang Z (2022) Single cell mass spectrometry analysis of drug-resistant cancer cells: Metabolomics studies of synergetic effect of combinational treatment. *Anal Chim Acta* **1201:**339621.

Choi SB, Polter AM, and Nemes P (2021) Patch-Clamp Proteomics of Single Neuronal Somas in Tissue Using Electrophysiology and Subcellular Capillary Electrophoresis Mass Spectrometry. *bioRxiv*.

Coffman BL, Rios GR, King CD, and Tephly TR (1997) Human UGT2B7 catalyzes morphine glucuronidation. *Drug Metab Dispos* **25:**1-4.

Courcot E, Leclerc J, Lafitte JJ, Mensier E, Jaillard S, Gosset P, Shirali P, Pottier N, Broly F, and Lo-Guidice JM (2012) Xenobiotic metabolism and disposition in human lung cell models: comparison with in vivo expression profiles. *Drug Metab Dispos* **40:**1953-1965.

Court MH, Zhang X, Ding X, Yee KK, Hesse LM, and Finel M (2012) Quantitative distribution of mRNAs encoding the 19 human UDP-glucuronosyltransferase enzymes in 26 adult and 3 fetal tissues. *Xenobiotica* **42:**266-277.

Ctortecka C and Mechtler K (2021) The rise of single-cell proteomics. *Analytical Science Advances* **2:**84-94.

Dai Y, Iwanaga K, Lin YS, Hebert MF, Davis CL, Huang W, Kharasch ED, and Thummel KE (2004) In vitro metabolism of cyclosporine A by human kidney CYP3A5. *Biochem Pharmacol* **68:**1889-1902.

de Graaf IA, Olinga P, de Jager MH, Merema MT, de Kanter R, van de Kerkhof EG, and Groothuis GM (2010) Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nat Protoc* **5:**1540-1551.

Deng B, Wang Z, Song J, Xiao Y, Chen D, and Huang J (2011) Analysis of doxorubicin uptake in single human leukemia K562 cells using capillary electrophoresis coupled with laser-induced fluorescence detection. *Anal Bioanal Chem* **401:**2143-2152.

Deng B, Wang ZM, Zhou ZH, Liu YM, Yang XL, Song J, and Xiao YX (2014) Quantitative study of cellular heterogeneity in doxorubicin uptake and its pharmacological effect on cancer cells. *Biomed Chromatogr* **28:**1393-1401.

Evers TM, Hochane M, Tans SJ, Heeren RM, Semrau S, Nemes P, and Mashaghi A (2019) Deciphering metabolic heterogeneity by single-cell analysis, ACS Publications.

Fonsi M (2014) Extrahepatic metabolism may complicate the IVIVC in rats. *Drug Metab Lett* **8:**51-66.

Gay L, Baker A-M, and Graham TA (2016) Tumour cell heterogeneity. *F1000Research* **5**.

Ghosh C, Gonzalez-Martinez J, Hossain M, Cucullo L, Fazio V, Janigro D, and Marchi N (2010) Pattern of P450 expression at the human blood-brain barrier: roles of epileptic condition and laminar flow. *Epilepsia* **51:**1408-1417.

Glasser MF, Coalson TS, Robinson EC, Hacker CD, Harwell J, Yacoub E, Ugurbil K, Andersson J, Beckmann CF, Jenkinson M, Smith SM, and Van Essen DC (2016) A multi-modal parcellation of human cerebral cortex. *Nature* **536:**171-178.

Goltsev Y, Samusik N, Kennedy-Darling J, Bhate S, Hale M, Vazquez G, Black S, and Nolan GP (2018) Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging. *Cell* **174:**968-981.e915.

Guan J, Lin Y, Wang Y, Gao J, and Ji G (2021) An analytical method for the identification of cell type-specific disease gene modules. *J Transl Med* **19:**20.

Gundry RL, White MY, Murray CI, Kane LA, Fu Q, Stanley BA, and Van Eyk JE (2010) Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow. *Current protocols in molecular biology* **90:**10.25. 11-10.25. 23.

Hartlmayr D, Ctortecka C, Seth A, Mendjan S, Tourniaire G, and Mechtler K (2021) An automated workflow for label-free and multiplexed single cell proteomics sample preparation at unprecedented sensitivity. *bioRxiv*.

Hiraoka H, Yamamoto K, Miyoshi S, Morita T, Nakamura K, Kadoi Y, Kunimoto F, and Horiuchi R (2005) Kidneys contribute to the extrahepatic clearance of propofol in humans, but not lungs and brain. *Br J Clin Pharmacol* **60:**176-182.

Ho MD, Ring N, Amaral K, Doshi U, and Li AP (2017) Human Enterocytes as an In Vitro Model for the Evaluation of Intestinal Drug Metabolism: Characterization of Drug-Metabolizing Enzyme Activities of Cryopreserved Human Enterocytes from Twenty-Four Donors. *Drug Metab Dispos* **45:**686-691.

Hodge RD, Bakken TE, Miller JA, Smith KA, Barkan ER, Graybuck LT, Close JL, Long B, Johansen N, Penn O, Yao Z, Eggermont J, Hollt T, Levi BP, Shehata SI, Aevermann B, Beller A, Bertagnolli D, Brouner K, Casper T, Cobbs C, Dalley R, Dee N, Ding SL, Ellenbogen RG, Fong O, Garren E, Goldy J, Gwinn RP, Hirschstein D, Keene CD, Keshk M, Ko AL, Lathia K, Mahfouz A, Maltzer Z, McGraw M, Nguyen TN, Nyhus J, Ojemann JG, Oldre A, Parry S, Reynolds S, Rimorin C, Shapovalova NV, Somasundaram S, Szafer A, Thomsen ER, Tieu M, Quon G, Scheuermann RH, Yuste R, Sunkin SM, Lelieveldt B, Feng D, Ng L, Bernard A, Hawrylycz M, Phillips JW, Tasic B, Zeng H, Jones AR, Koch C, and Lein ES (2019) Conserved cell types with divergent features in human versus mouse cortex. *Nature* **573:**61-68.

Iswandana R, Irianti MI, Oosterhuis D, Hofker HS, Merema MT, de Jager MH, Mutsaers HAM, and Olinga P (2018) Regional Differences in Human Intestinal Drug Metabolism. *Drug Metab Dispos* **46:**1879-1885.

Izar B, Tirosh I, Stover EH, Wakiro I, Cuoco MS, Alter I, Rodman C, Leeson R, Su M-J, and Shah P (2020) A single-cell landscape of high-grade serous ovarian cancer. *Nature medicine* **26:**1271-1279.

Jones NR and Lazarus P (2014) UGT2B gene expression analysis in multiple tobacco carcinogen-targeted tissues. *Drug Metab Dispos* **42:**529-536.

Kaut O, Schmitt I, and Wullner U (2012) Genome-scale methylation analysis of Parkinson's disease patients' brains reveals DNA hypomethylation and increased mRNA expression of cytochrome P450 2E1. *Neurogenetics* **13:**87-91.

Kelly R, Zhu Y, Liang Y, Cong Y, Piehowski P, Dou M, Zhao R, Qian W-J, Burnum-Johnson K, and Ansong C (2019) Single Cell Proteome Mapping of Tissue Heterogeneity Using Microfluidic Nanodroplet Sample Processing and Ultrasensitive LC-MS. *Journal of Biomolecular Techniques: JBT* **30:**S61.

Keren L, Bosse M, Thompson S, Risom T, Vijayaragavan K, McCaffrey E, Marquez D, Angoshtari R, Greenwald NF, Fienberg H, Wang J, Kambham N, Kirkwood D, Nolan G, Montine TJ, Galli SJ, West R, Bendall SC, and Angelo M (2019) MIBI-TOF: A multiplexed imaging platform relates cellular phenotypes and tissue structure. *Sci Adv* **5:**eaax5851.

Kim JH, Sherman ME, Curriero FC, Guengerich FP, Strickland PT, and Sutter TR (2004) Expression of cytochromes P450 1A1 and 1B1 in human lung from smokers, non-smokers, and ex-smokers. *Toxicol Appl Pharmacol* **199:**210-219.

King CD, Rios GR, Assouline JA, and Tephly TR (1999) Expression of UDP-glucuronosyltransferases (UGTs) 2B7 and 1A6 in the human brain and identification of 5-hydroxytryptamine as a substrate. *Arch Biochem Biophys* **365:**156-162.

Kirschner K, Rattanavirotkul N, Quince MF, and Chandra T (2020) Functional heterogeneity in senescence. *Biochem Soc Trans* **48:**765-773.

Knights KM, Spencer SM, Fallon JK, Chau N, Smith PC, and Miners JO (2016) Scaling factors for the in vitro-in vivo extrapolation (IV-IVE) of renal drug and xenobiotic glucuronidation clearance. *Br J Clin Pharmacol* **81:**1153-1164.

Kolars JC, Awni WM, Merion RM, and Watkins PB (1991) First-pass metabolism of cyclosporin by the gut. *Lancet* **338:**1488-1490.

Korte AR, Yandeau-Nelson MD, Nikolau BJ, and Lee YJ (2015) Subcellular-level resolution MALDI-MS imaging of maize leaf metabolites by MALDI-linear ion trap-Orbitrap mass spectrometer. *Anal Bioanal Chem* **407:**2301-2309.

Kuban W and Daniel WA (2021) Cytochrome P450 expression and regulation in the brain. *Drug Metab Rev* **53:**1-29.

Lash LH, Putt DA, and Cai H (2008) Drug metabolism enzyme expression and activity in primary cultures of human proximal tubular cells. *Toxicology* **244:**56-65.

Lee RFS, Theiner S, Meibom A, Koellensperger G, Keppler BK, and Dyson PJ (2017) Application of imaging mass spectrometry approaches to facilitate metal-based anticancer drug research. *Metallomics* **9:**365-381.

Li AP (2020) In Vitro Human Cell-Based Experimental Models for the Evaluation of Enteric Metabolism and Drug Interaction Potential of Drugs and Natural Products. *Drug Metab Dispos* **48:**980-992.

Liao J, Yu Z, Chen Y, Bao M, Zou C, Zhang H, Liu D, Li T, Zhang Q, Li J, Cheng J, and Mo Z (2020) Single-cell RNA sequencing of human kidney. *Sci Data* **7:**4.

Lin D, Chen Y, Negi S, Cheng D, Ouyang Z, Sexton D, Li K, and Zhang B (2022) CellDepot: A Unified Repository for scRNA-seq Data and Visual Exploration. *J Mol Biol* **434:**167425.

Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, and McCarroll SA (2015) Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* **161:**1202-1214.

MacParland SA, Liu JC, Ma XZ, Innes BT, Bartczak AM, Gage BK, Manuel J, Khuu N, Echeverri J, Linares I, Gupta R, Cheng ML, Liu LY, Camat D, Chung SW, Seliga RK, Shao Z, Lee E, Ogawa S, Ogawa M, Wilson MD, Fish JE, Selzner M, Ghanekar A, Grant D, Greig P, Sapisochin G, Selzner N, Winegarden N, Adeyi O, Keller G, Bader GD, and McGilvray ID (2018) Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Commun* **9:**4383.

Malherbe J, Penen F, Isaure MP, Frank J, Hause G, Dobritzsch D, Gontier E, Horréard F, Hillion F, and Schaumlöffel D (2016) A New Radio Frequency Plasma Oxygen Primary Ion Source on Nano Secondary Ion Mass Spectrometry for Improved Lateral Resolution and Detection of Electropositive Elements at Single Cell Level. *Anal Chem* **88:**7130-7136.

Marcu R, Choi YJ, Xue J, Fortin CL, Wang Y, Nagao RJ, Xu J, MacDonald JW, Bammler TK, Murry CE, Muczynski K, Stevens KR, Himmelfarb J, Schwartz SM, and Zheng Y (2018) Human Organ-Specific Endothelial Cell Heterogeneity. *iScience* **4:**20-35.

Marx V (2019) A dream of single-cell proteomics. *Nature Methods* **16:**809-812.

McFadyen MCE, Melvin WT, and Murray GI (1998) Regional distribution of individual forms of cytochrome P450 mRNA in normal adult human brain. *Biochem Pharmacol* **55:**825-830.

McGinnity DF, Soars MG, Urbanowicz RA, and Riley RJ (2004) Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. *Drug Metab Dispos* **32:**1247-1253.

Meissner F, Geddes-McAlister J, Mann M, and Bantscheff M (2022) The emerging role of mass spectrometry-based proteomics in drug discovery. *Nature Reviews Drug Discovery***:**1-18.

Meng F, Liang Z, Zhao K, and Luo C (2021) Drug design targeting active posttranslational modification protein isoforms. *Medicinal Research Reviews* **41:**1701-1750.

Moreno P, Fexova S, George N, Manning JR, Miao Z, Mohammed S, Munoz-Pomer A, Fullgrabe A, Bi Y, Bush N, Iqbal H, Kumbham U, Solovyev A, Zhao L, Prakash A, Garcia-Seisdedos D, Kundu DJ, Wang S, Walzer M, Clarke L, Osumi-Sutherland D, Tello-Ruiz MK, Kumari S, Ware D, Eliasova J, Arends MJ, Nawijn MC, Meyer K, Burdett T, Marioni J, Teichmann S, Vizcaino JA, Brazma A, and Papatheodorou I (2022) Expression Atlas update: gene and protein expression in multiple species. *Nucleic Acids Res* **50:**D129-D140.

Myers SA, Klaeger S, Satpathy S, Viner R, Choi J, Rogers J, Clauser K, Udeshi ND, and Carr SA (2018) Evaluation of advanced precursor determination for tandem mass tag (TMT)-based quantitative proteomics across instrument platforms. *Journal of proteome research* **18:**542-547.

Norton SE, Ward-Hartstonge KA, McCall JL, Leman JKH, Taylor ES, Munro F, Black MA, Fazekas de St Groth B, McGuire HM, and Kemp RA (2019) High-Dimensional Mass Cytometric Analysis Reveals an Increase in Effector Regulatory T Cells as a Distinguishing Feature of Colorectal Tumors. *J Immunol* **202:**1871-1884.

Olsen JV and Mann M (2013) Status of large-scale analysis of post-translational modifications by mass spectrometry. *Molecular & cellular proteomics* **12:**3444-3452.

Paine MF, Shen DD, Kunze KL, Perkins JD, Marsh CL, McVicar JP, Barr DM, Gillies BS, and Thummel KE (1996) First-pass metabolism of midazolam by the human intestine. *Clin Pharmacol Ther* **60:**14-24.

Pan N, Standke SJ, Kothapalli NR, Sun M, Bensen RC, Burgett AWG, and Yang Z (2019) Quantification of Drug Molecules in Live Single Cells Using the Single-Probe Mass Spectrometry Technique. *Anal Chem* **91:**9018-9024.

Passarelli MK, Pirkl A, Moellers R, Grinfeld D, Kollmer F, Havelund R, Newman CF, Marshall PS, Arlinghaus H, Alexander MR, West A, Horning S, Niehuis E, Makarov A, Dollery CT, and Gilmore IS (2017) The 3D OrbiSIMS-label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. *Nat Methods* **14:**1175-1183.

Paszek P, Ryan S, Ashall L, Sillitoe K, Harper CV, Spiller DG, Rand DA, and White MR (2010) Population robustness arising from cellular heterogeneity. *Proc Natl Acad Sci U S A* **107:**11644-11649.

Payen VL, Lavergne A, Alevra Sarika N, Colonval M, Karim L, Deckers M, Najimi M, Coppieters W, Charloteaux B, Sokal EM, and El Taghdouini A (2021) Single-cell RNA sequencing of human liver reveals hepatic stellate cell heterogeneity. *JHEP Rep* **3:**100278.

Peng J, Sun B-F, Chen C-Y, Zhou J-Y, Chen Y-S, Chen H, Liu L, Huang D, Jiang J, and Cui G-S (2019) Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell research* **29:**725-738.

Petelski AA, Emmott E, Leduc A, Huffman RG, Specht H, Perlman DH, and Slavov N (2021) Multiplexed single-cell proteomics using SCoPE2. *Nature protocols* **16:**5398-5425.

Ponsoda X, Pareja E, Gomez-Lechon MJ, Fabra R, Carrasco E, Trullenque R, and Castell JV (2001) Drug biotransformation by human hepatocytes. In vitro/in vivo metabolism by cells from the same donor. *J Hepatol* **34:**19-25.

Prideaux B, Staab D, and Stoeckli M (2010) Applications of MALDI-MSI to pharmaceutical research. *Methods Mol Biol* **656:**405-413.

Proetto MT, Callmann CE, Cliff J, Szymanski CJ, Hu D, Howell SB, Evans JE, Orr G, and Gianneschi NC (2018) Tumor Retention of Enzyme-Responsive Pt(II) Drug-Loaded Nanoparticles Imaged by Nanoscale Secondary Ion Mass Spectrometry and Fluorescence Microscopy. *ACS Cent Sci* **4:**1477-1484.

Riches Z, Stanley EL, Bloomer JC, and Coughtrie MW (2009) Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie". *Drug Metab Dispos* **37:**2255-2261.

Rinke C, Lee J, Nath N, Goudeau D, Thompson B, Poulton N, Dmitrieff E, Malmstrom R, Stepanauskas R, and Woyke T (2014) Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics. *Nature protocols* **9:**1038-1048.

Rompp A, Both JP, Brunelle A, Heeren RM, Laprevote O, Prideaux B, Seyer A, Spengler B, Stoeckli M, and Smith DF (2015) Mass spectrometry imaging of biological tissue: an approach for multicenter studies. *Anal Bioanal Chem* **407:**2329-2335.

Salman ED, Kadlubar SA, and Falany CN (2009) Expression and localization of cytosolic sulfotransferase (SULT) 1A1 and SULT1A3 in normal human brain. *Drug Metab Dispos* **37:**706-709.

Schoeberl A, Gutmann M, Theiner S, Schaier M, Schweikert A, Berger W, and Koellensperger G (2021) Cisplatin Uptake in Macrophage Subtypes at the Single-Cell Level by LA-ICP-TOFMS Imaging. *Anal Chem* **93:**16456-16465.

Seneviratne HK, Hamlin AN, Heck CJS, and Bumpus NN (2020) Spatial Distribution Profiles of Emtricitabine, Tenofovir, Efavirenz, and Rilpivirine in Murine Tissues Following. *ACS Pharmacol Transl Sci* **3:**655-665.

Seneviratne HK, Hendrix CW, Fuchs EJ, and Bumpus NN (2018) MALDI Mass Spectrometry Imaging Reveals Heterogeneous Distribution of Tenofovir and Tenofovir Diphosphate in Colorectal Tissue of Subjects Receiving a Tenofovir-Containing Enema. *J Pharmacol Exp Ther* **367:**40-48.

Sergent T, Dupont I, Van der Heiden E, Scippo ML, Pussemier L, Larondelle Y, and Schneider YJ (2009) CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. *Toxicol Lett* **191:**216-222.

Shahabi HN, Andersson DR, and Nissbrandt H (2008) Cytochrome P450 2E1 in the substantia nigra: relevance for dopaminergic neurotransmission and free radical production. *Synapse* **62:**379-388.

Shawahna R, Uchida Y, Decleves X, Ohtsuki S, Yousif S, Dauchy S, Jacob A, Chassoux F, Daumas-Duport C, Couraud PO, Terasaki T, and Scherrmann JM (2011) Transcriptomic and quantitative proteomic analysis of transporters and drug metabolizing enzymes in freshly isolated human brain microvessels. *Mol Pharm* **8:**1332-1341.

Shibata Y and Chiba M (2015) The role of extrahepatic metabolism in the pharmacokinetics of the targeted covalent inhibitors afatinib, ibrutinib, and neratinib. *Drug Metab Dispos* **43:**375-384.

Slavov N (2022) Scaling Up Single-Cell Proteomics. *Molecular & Cellular Proteomics* **21**.

Somers GI, Lindsay N, Lowdon BM, Jones AE, Freathy C, Ho S, Woodrooffe AJ, Bayliss MK, and Manchee GR (2007) A comparison of the expression and metabolizing activities of phase I and II enzymes in freshly isolated human lung parenchymal cells and cryopreserved human hepatocytes. *Drug Metab Dispos* **35:**1797-1805.

Stopka SA, Rong C, Korte AR, Yadavilli S, Nazarian J, Razunguzwa TT, Morris NJ, and Vertes A (2016) Molecular Imaging of Biological Samples on Nanophotonic Laser Desorption Ionization Platforms. *Angew Chem Int Ed Engl* **55:**4482-4486.

Su M-G, Weng JT-Y, Hsu JB-K, Huang K-Y, Chi Y-H, and Lee T-Y (2017) Investigation and identification of functional post-translational modification sites associated with drug binding and protein-protein interactions. *BMC systems biology* **11:**69-80.

Takayama K, Ito K, Matsui A, Yamashita T, Kawakami K, Hirayama D, Kishimoto W, Nakase H, and Mizuguchi H (2021) In Vivo Gene Expression Profile of Human Intestinal Epithelial Cells: From the Viewpoint of Drug Metabolism and Pharmacokinetics. *Drug Metab Dispos* **49:**221-232.

Tang W, Chen J, Zhou J, Ge J, Zhang Y, Li P, and Li B (2019) Quantitative MALDI Imaging of Spatial Distributions and Dynamic Changes of Tetrandrine in Multiple Organs of Rats. *Theranostics* **9:**932-944.

Thummel KE, O'Shea D, Paine MF, Shen DD, Kunze KL, Perkins JD, and Wilkinson GR (1996) Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin Pharmacol Ther* **59:**491-502.

Toselli F, Booth Depaz IM, Worrall S, Etheridge N, Dodd PR, Wilce PA, and Gillam EM (2015) Expression of CYP2E1 and CYP2U1 proteins in amygdala and prefrontal cortex: influence of alcoholism and smoking. *Alcohol Clin Exp Res* **39:**790-797.

Tracey LJ, An Y, and Justice MJ (2021) CyTOF: An Emerging Technology for Single-Cell Proteomics in the Mouse. *Curr Protoc* **1:**e118.

Travaglini KJ, Nabhan AN, Penland L, Sinha R, Gillich A, Sit RV, Chang S, Conley SD, Mori Y, Seita J, Berry GJ, Shrager JB, Metzger RJ, Kuo CS, Neff N, Weissman IL, Quake SR, and Krasnow MA (2020) A molecular cell atlas of the human lung from single-cell RNA sequencing. *Nature* **587:**619-625.

Tuck M, Grélard F, Blanc L, and Desbenoit N (2022) MALDI-MSI Towards Multimodal Imaging: Challenges and Perspectives. *Front Chem* **10:**904688.

Upadhya SC, Tirumalai PS, Boyd MR, Mori T, and Ravindranath V (2000) Cytochrome P4502E (CYP2E) in brain: constitutive expression, induction by ethanol and localization by fluorescence in situ hybridization. *Arch Biochem Biophys* **373:**23-34.

Vallone VF, Ludwik K, and Stachelscheid H cellenONE® as automated single cell seeding platform for hiPSC subcloning.

Van Acker T, Buckle T, Van Malderen SJM, van Willigen DM, van Unen V, van Leeuwen FWB, and Vanhaecke F (2019) High-resolution imaging and single-cell analysis via laser ablation-inductively coupled plasma-mass spectrometry for the determination of membranous receptor expression levels in breast cancer cell lines using receptor-specific hybrid tracers. *Anal Chim Acta* **1074:**43-53.

Van der Hauwaert C, Savary G, Buob D, Leroy X, Aubert S, Flamand V, Hennino MF, Perrais M, Lo-Guidice JM, Broly F, Cauffiez C, and Glowacki F (2014) Expression profiles of genes involved in xenobiotic metabolism and disposition in human renal tissues and renal cell models. *Toxicol Appl Pharmacol* **279:**409-418.

van Herwaarden AE, Wagenaar E, van der Kruijssen CM, van Waterschoot RA, Smit JW, Song JY, van der Valk MA, van Tellingen O, van der Hoorn JW, Rosing H, Beijnen JH, and Schinkel AH (2007) Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. *J Clin Invest* **117:**3583-3592.

Veličković D, Chu RK, Carrell AA, Thomas M, Paša-Tolić L, Weston DJ, and Anderton CR (2018) Multimodal MSI in Conjunction with Broad Coverage Spatially Resolved MS. *Anal Chem* **90:**702-707.

Vizcaino JA, Deutsch EW, Wang R, Csordas A, Reisinger F, Rios D, Dianes JA, Sun Z, Farrah T, Bandeira N, Binz PA, Xenarios I, Eisenacher M, Mayer G, Gatto L, Campos A, Chalkley RJ, Kraus HJ, Albar JP, Martinez-Bartolome S, Apweiler R, Omenn GS, Martens L, Jones AR, and Hermjakob H (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat Biotechnol* **32:**223-226.

Wahlstrom A, Winblad B, Bixo M, and Rane A (1988) Human brain metabolism of morphine and naloxone. *Pain* **35:**121-127.

Webber KG, Truong T, Johnston SM, Zapata SE, Liang Y, Davis JM, Buttars AD, Smith FB, Jones HE, and Mahoney AC (2022) Label-Free Profiling of up to 200 Single-Cell Proteomes per Day Using a Dual-Column Nanoflow Liquid Chromatography Platform. *Analytical Chemistry*.

Wilting RH and Dannenberg J-H (2012) Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. *Drug Resistance Updates* **15:**21-38.

Xu P, Wang M, Song WM, Wang Q, Yuan GC, Sudmant PH, Zare H, Tu Z, Orr ME, and Zhang B (2022) The landscape of human tissue and cell type specific expression and co-regulation of senescence genes. *Mol Neurodegener* **17:**5.

Zavalin A, Yang J, and Caprioli R (2013) Laser beam filtration for high spatial resolution MALDI imaging mass spectrometry. *J Am Soc Mass Spectrom* **24:**1153-1156.

Zhang Q, Lou Y, Yang J, Wang J, Feng J, Zhao Y, Wang L, Huang X, Fu Q, Ye M, Zhang X, Chen Y, Ma C, Ge H, Wang J, Wu J, Wei T, Chen Q, Wu J, Yu C, Xiao Y, Feng X, Guo G, Liang T, and Bai X (2019) Integrated multiomic analysis reveals comprehensive tumour heterogeneity and novel immunophenotypic classification in hepatocellular carcinomas. *Gut* **68:**2019-2031.

Zhang QY, Kaminsky LS, Dunbar D, Zhang J, and Ding X (2007) Role of small intestinal cytochromes p450 in the bioavailability of oral nifedipine. *Drug Metab Dispos* **35:**1617-1623.

Zhong Y, Dong G, Luo H, Cao J, Wang C, Wu J, Feng YQ, and Yue J (2012) Induction of brain CYP2E1 by chronic ethanol treatment and related oxidative stress in hippocampus, cerebellum, and brainstem. *Toxicology* **302:**275-284.

Zhu K, Liu X, Liu C, Xu Y, Fu Y, Dong W, Yan Y, Wang W, and Qian C (2021) AKT inhibitor AZD5363 suppresses stemness and promotes anti-cancer activity of 3,3'-diindolylmethane in human breast cancer cells. *Toxicol Appl Pharmacol* **429:**115700.

Zhu Y, Piehowski PD, Zhao R, Chen J, Shen Y, Moore RJ, Shukla AK, Petyuk VA, Campbell-Thompson M, and Mathews CE (2018) Nanodroplet processing platform for deep and quantitative proteome profiling of 10–100 mammalian cells. *Nature communications* **9:**1-10.

Zimatkin SM, Pronko SP, Vasiliou V, Gonzalez FJ, and Deitrich RA (2006) Enzymatic mechanisms of ethanol oxidation in the brain. *Alcohol Clin Exp Res* **30:**1500-1505.

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Wrote or contributed to the writing of the manuscript: Wheeler, Eberhard, Mosher, Yuan, Wilkins, Seneviratne, Orsburn, and Bumpus

Footnote

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Figure Legends

Figure 1. Three sample preparation strategies for single-cell proteomic analysis. (A) The nanoPOTS platform uses a nanoliter robotic pipetting to dispense cells and reagents onto microfabricated nanoarray chips (Zhu et al., 2018). (B) The ScoPE2 method uses TMT isobaric tags to label peptides of single cells and a 200 cell channel to boost protein sequence coverage (Petelski et al., 2021). (C) Whole-cell patch-clamp electrophysiology involves sample injection into a patch pipette, bottom-up proteomics on collected protein, and identification and quantification using capillary electrophoresis (CE) electrospray ionization (ESA) platform followed by high-resolution mass spectrometry (HRMS) (Choi et al., 2021).

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Tables

Table 1. Summary of extrahepatic drug metabolizing enzymes.

Tissue	P450s	UGTs	NATs/SULTs	References
Kidney				Lash et al., 2008
Proximal Tubule Cells	1B1, 3A4, 3A5,	1A1, 1A6, 1A8, 1A9, 2B7		Van der Hauwaert et al.,
		1A8, 1A9		2014
Renal Epithelial Cells				
Intestines				Iswandana et al., 2018
Epithelial Cells				Ho et al., 2017
Colon	2C18, 2J2, 3A5	1A1, 1A8, 2A3, 2B7	SULTs 1A1, 1A3, 1A4, 1B1	Riches et al., 2009
				Takayama et al., 2021
Duodenum	2C9, 2C19, 2J2, 3A4, 3A5	1A1, 1A8, 2A3, 2B7	SULTs 1A1, 1A3, 1A4, 1B1	
Ileum	2C18, 2C19, 2J2, 3A4, 3A5	1A1,1A4, 1A5, 1A8, 1A9, 1A10, 2A3, 2B7, 2B15, 8	SULTs 1A1, 1A2, 1A3, 1A4, 1B1,1C2, 1C3, 1E1, 2A1	
Rectum	2C18, 2J2, 3A5	1A1, 1A8, 2A3, 2B7	SULTs 1A1, 1A3, 1A4, 1B1	
Lung				Courcot et al., 2012
Bronchial Epithelial Cells	1B1, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A5, 3A7	1A1, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B11, 2B15, 2B17, 2B28, 3A2, 8	NAT1, SULTs 1A1, 1A2, 1A3, 1A4, 1B1, 1C2, 1C4, 1E1, 2B1, 4A1	Somers et al., 2007
Parenchymal (alveolar epithelial) Cells	1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2J2, 3A4, 3A5	1A1, 1A4, 1A6, 2A1, 2B4, 2B7, 2B11	SULTs 1A1, 1A3, 1A4, 1C2, 2A1, 2B1, 2B2	
Brain				Court et al., 2012
Amygdala	2E1, 2U1			Ghosh et al., 2010
Basal Ganglia	1A1, 1A2, 1B1, 2B6, 2C9, 2C19, 2E1, 3A4, 3A5			Jones and Lazarus, 2014
Cerebellum	1A1, 1A2, 1B1, 2B6, 2C9, 2C19, 2D6, 2E1, 2U1, 3A	1A6, 2B7	SULTs 1A1, 1A3	King et al., 1999
				Kuban and Daniel, 2021
Cortex	1A1, 1A2, 1B1, 2A6, 2B6, 2D6, 2E1, 2U1, 2C9, 2C19, 3A4, 3A5		SULTs 1A1, 1A3, 4A1	McFayden et al., 1998
				Salman, 2009
Endothelial Cells	1A1, 1B1, 2A6, 2B6, 2C9, 2E1, 2J2, 3A4, 4A11			Shawahna et al., 2011
Hippocampus	1A1, 1A2, 1B1, 2D6, 2E1, 2C9, 2C19, 2E1, 3A		SULTs 1A1, 1A3	Toselli et al., 2015
Medulla	1B1, 2E1			
Microvasculature	1B1, 2U1			

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Pons	1A1, 1A2, 2B6, 2D6, 2E1, 3A			
Striatum	1A1, 1A2, 1B1, 2B6, 2D6, 2E1			
Substantia Nigra	1A1, 1A2, 1B1, 2D6, 2E1			
Microvasculature	1B1, 2U1			
Thalamus & Hypothalamus	2D6, 3A4			
Unspecified Region		1A1, 1A3, 1A4, 1A6, 1A9, 2A3, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, 2B28		

Table 2. Summary of single-cell methods.

Method	Description	Application	Advantages	Challenges
Single-cell RNA sequencing (scRNA-seq)	Individual cells are isolated and RNA is extracted, reverse transcribed, and barcoded. Following sequencing, read counts of the genes of interest are quantified, and expression profiles are compared.	Characterization of transcriptome heterogeneity within different cell types and tissues (MacParland et al., 2018) Define subpopulations of drug-tolerant tumor cells (Aissa et al., 2021)	High throughput Many data processing programs available	Difficult to measure low expression genes High noise and variability
Mass cytometry (CyTOF)	Metal-conjugated antibodies are hybridized to cell surface markers or intracellular proteins and mass spectrometry is used to measure the abundance of labeled targets in each cell.	Determine diversity and functional similarity of cell types (Norton et al., 2019) Measure cell-to-cell variability in changes in protein markers in response to drug treatment (Anchang et al., 2018)	Greater multiplexing ability than flow cytometry Can be quantitative	Low sensitivity Requires specialized antibodies Indirect detection of proteins
Imaging mass cytometry (IMC) and Multiplexed ion beam imaging (MIBI)	Tissue slices are labeled with metal-conjugated antibodies. Primary ion and laser ablation enables detection of labeled markers present in small regions of tissue by mass spectrometry.	Identification of tumor and immune cell microenvironments (Keren et al., 2019)	Spatial data for cell subpopulations Greater multiplexing than immunohistochemistry (IHC)	Low sensitivity Requires specialized antibodies Indirect detection of proteins
Co-detection by indexing (CODEX)	Tissue slices are labeled with oligonucleotide-conjugated antibodies. Cyclic addition and removal of complementary fluorescently labeled DNA probes enable detection of targeted proteins.	Identifying cell types and cellular microenvironment in tissues (Goltsev et al., 2018)	Greater multiplexing than IHC Spatial data for cell subpopulations Compatible with a variety of hardware	Requires specialized antibodies Indirect detection of proteins Requires validation for each antibody
Nanodroplet Processing in One pot for Trace Samples (nanoPOTS)	NanoPOTS uses nanoliter robotic pipetting and flow cytometry for cell partitioning and peptide generation on microfabricated nanowell array chips. Mass spectrometry is used to measure protein abundance.	Able to identify and quantify over 1,000 proteins from single mammalian cells from tissue (Kelly et al., 2019)	Improved sample recovery Direct detection of proteins	Requires specialized nanowell array chip hardware Can only detect high abundance proteins

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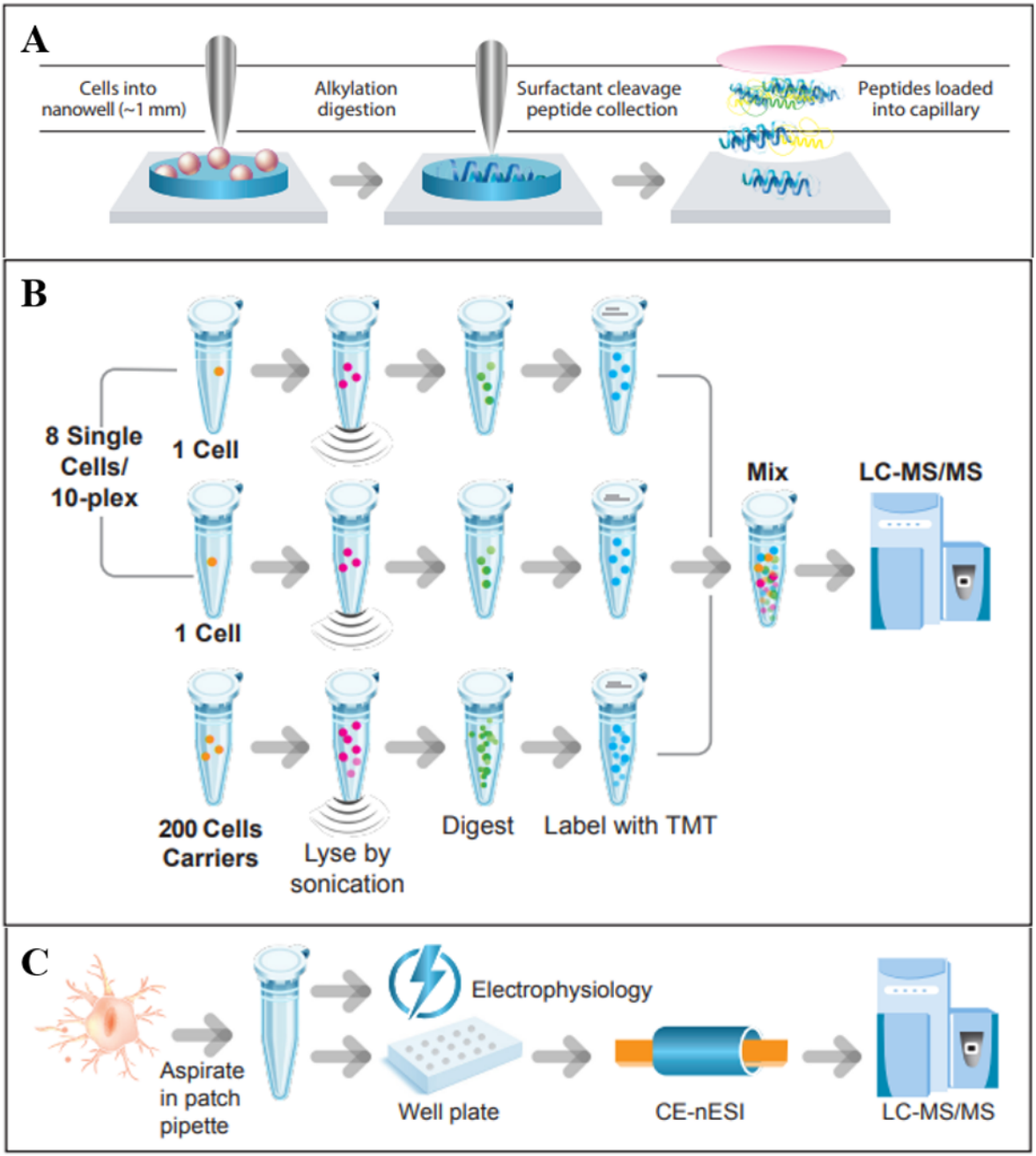
Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS)	A method for the quantification of proteins in single cells by LC-MS/MS. Labeling peptides with isobaric tags and pooling single-cell samples with a multi-cell sample improves sensitivity.	Able to identify over 1,000 proteins per cell from single macrophage sample preparations (Petelski et al., 2021)	Can detect posttranslational modifications Isobaric tags and sample combination enable boosting sensitivity	Limited data processing tools Can only detect high abundance proteins
Subcellular patch-clamp and capillary electrophoresis mass spectrometry	Subcellular sample is collected into a patch pipette, proteins are digested, peptides are separated by capillary electrophoresis and detected using mass spectrometry	Quantification and analysis of proteomic heterogeneity in tissue samples (Choi et al., 2022)	Can work with very small volume samples (subcellular)	Requires specialized hardware Low number of proteins able to be detected
Single-probe single-cell mass spectrometry	A dual-capillary containing probe delivers solvent to a cell and withdraws the extract by capillary action which is subsequently flowed through a nano-electrospray emitter for mass spectrometry detection	Quantification of intracellular drug concentrations in single cells (Pan et al., 2019)	Can obtain subcellular data	Low throughput
Capillary electrophoresis-laser-induced fluorescence (CE-LIF)	The contents of single cells are subjected to electrophoretic separation and the native fluorescence of biomolecules in response to laser irradiation is detected	Quantification of intracellular concentrations of xenobiotics in single cells (Deng et al., 2022)	Sensitive Limited sample loss	Limited to detection of fluorescent compounds Low throughput
Matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI)	A tissue section is coated with a matrix and a laser is used to ablate ions from small regions for detection by mass spectrometry	Imaging of endogenous metabolites as well as parent drugs and drug metabolites within tissues (Seneviratne et al., 2020)	Label-free, direct detection of molecules Can simultaneously map hundreds of molecules	Limited spatial resolution Limited sensitivity Limited quantitative ability
Secondary ion mass spectrometry (SIMS)	In SIMS, tissue sections are struck by a beam of primary ions, generating secondary ions from the sample that are detected using mass spectrometry.	Imaging of the distribution of endogenous metabolites and xenobiotics within tissues (Passarelli et al., 2017)	No matrix required Greater spatial resolution and sensitivity than MALDI	Limited data analysis programs available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Cells into nanowell (~1 mm)
Alkylation digestion
Surfactant cleavage peptide collection
Peptides loaded into capillary
B
8 Single Cells/ 10-plex
1 Cell
1 Cell
200 Cells Carriers
Lyse by sonication
Digest
Label with TMT
Mix
LC-MS/MS
C
Aspirate in patch pipette
Electrophysiology
Well plate
CE-nESI
LC-MS/MS

Figure 1