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Unbiased spatial proteomics with single-cell resolution in tissues

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SUMMARY

Mass spectrometry (MS)-based proteomics has become a powerful technology to quantify the entire complement of proteins in cells or tissues. Here, we review challenges and recent advances in the LC-MS-based analysis of minute protein amounts, down to the level of single cells. Application of this technology revealed that single-cell transcriptomes are dominated by stochastic noise due to the very low number of transcripts per cell, whereas the single-cell proteome appears to be complete. The spatial organization of cells in tissues can be studied by emerging technologies, including multiplexed imaging and spatial transcriptomics, which can now be combined with ultra-sensitive proteomics. Combined with high-content imaging, artificial intelligence and single-cell laser microdissection, MS-based proteomics provides an unbiased molecular readout close to the functional level. Potential applications range from basic biological questions to precision medicine.

INTRODUCTION

The complexity of an organism arises through the intricate functional and spatial adaptation of its cell types and cell states. Humans, for instance, have hundreds of different cell types, and each of these can further change their state over time and depending on where they are in the body (Regev et al., 2017). For centuries, increasingly advanced microscopy technologies have shed light on this complexity and heterogeneity. More recently, in a complementary approach, cells have been molecularly studied in a system-wide, "untargeted" manner using genomics, proteomics, or metabolomics. Tremendous advances in genomic technologies, such as RNA sequencing (RNA-seq), now allow the characterization of the transcriptome of thousands of single cells, revealing important biological insights into cellular heterogeneity (Tabula Muris Consortium et al., 2018).

As proteins in their different forms and modified states are generally the functional units in a cell, it would be very attractive to directly study them at the single-cell level rather than by using transcripts as a proxy. In a targeted manner, this can readily be done by antibodies directed against a number of proteins of interest, for instance, by multiplexed imaging or by FACS sorting. In a somewhat related mass spectrometry (MS)-based technology called CyTOF, heavy metals are coupled to these antibodies and the metal isotope patterns are then recorded by specialized mass spectrometers (Bodenmiller, 2016; Bodenmiller et al., 2012).

Since the introduction of electrospray in 1989 for the analysis of proteins (Fenn et al., 1989), the speed and sensitivity of MS-based technology has continuously increased, which now readily allows the in-depth characterization of the proteome. Importantly, for our purposes, there has been a dramatic boost in sensitivity in just the last few years. In our own laboratory, for instance, the amount of sample needed to identify thousands of proteins in routine 1-h liquid chromatography-mass spectrometry (LC-MS) measurements has decreased more than 100-fold to the nanogram level (Beck et al., 2015; Meier et al., 2020).

Such advances have initiated a quest for measuring single-cell proteomes at a depth similar to that of single-cell RNA-seq (scRNA-seq) and ideally also in a robust and scalable manner. However, proteomics has neither an equivalent to oligonucleotide amplification nor to barcoding that enable multiplexing and high throughput in scRNA-seq. Illustrating the challenge, a single cell contains only about 150 pg of protein material, and its proteome consists of far more than 12,000 different proteins, whose abundance is furthermore distributed over many orders of magnitude (Bekker-Jensen et al., 2017; Muntel et al., 2019; Volpe and Eremenko-Volpe, 1970).

In the first part of this review, we describe how this challenge is being addressed by miniaturizing the entire sample preparation workflow and by dramatically improving the sensitivity of the LC-MS systems themselves. This has now enabled the direct comparison of single-cell proteomes and transcriptomes, which turn out to be quite different from each other in unexpected ways. Initially the above technologies generally require cells to be in suspension or to be disaggregated from tissues before analysis, thereby losing the all-important spatial context of each single cell (Figure 1A).

In the second part of the review, we describe how this important information can be integrated by multiplexed imaging,

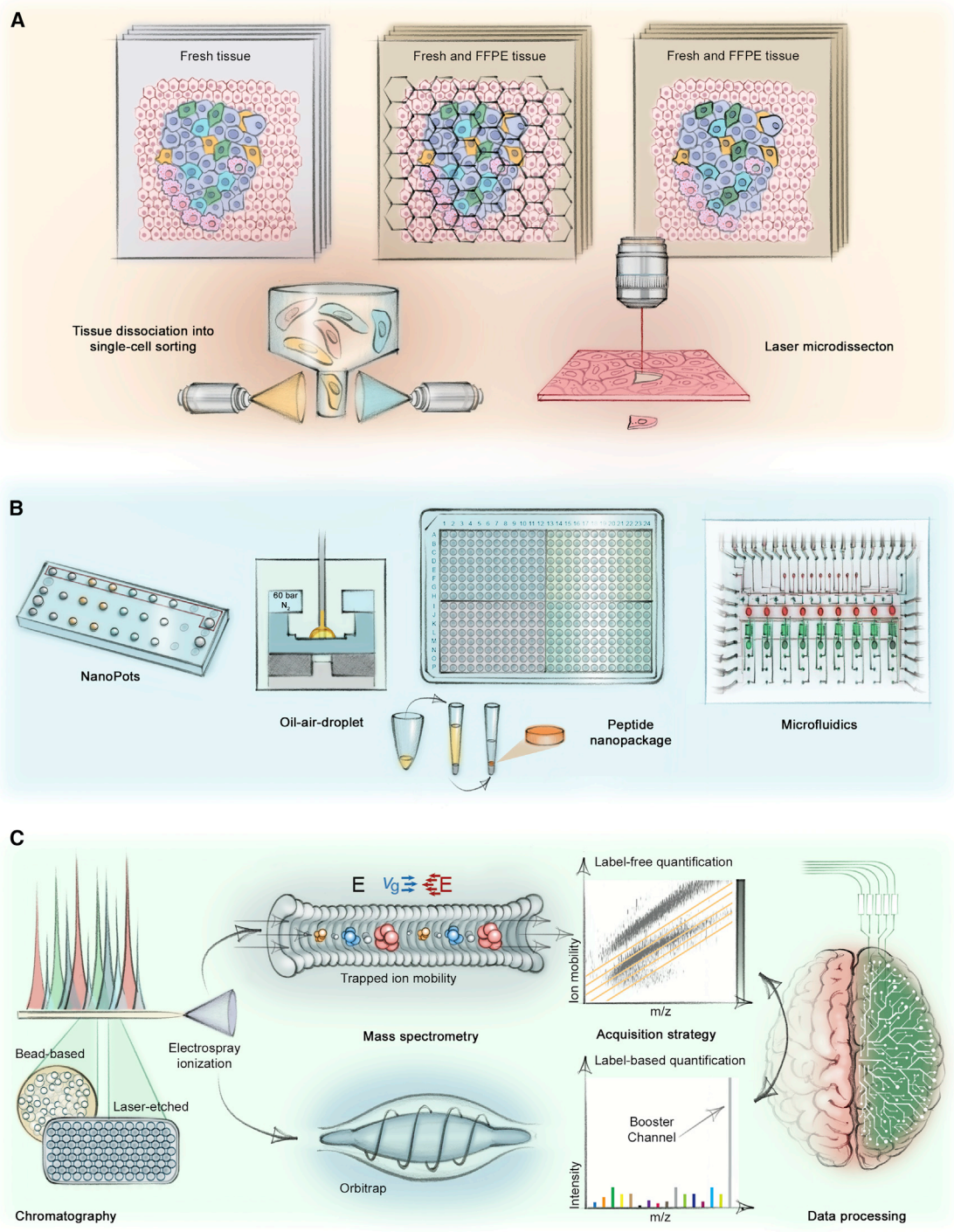

Figure 1. Workflows for single-cell proteomics

(A) Different approaches for isolation of single cells from tissue. Tissues can be treated with enzymes to release single cells followed by single-cell sorting into individual wells. This allows cell-type assignment after downstream analysis but loses the spatial context. Instead, tissues can be sectioned and isolated as octagons by laser microdissection, which results in a merged proteome from neighboring cells and the extracellular matrix. The isolation of single-cell types by laser microdissection after assignment of their spatial position results in a clean isolation with defined spatial information.

(B) Handling of the sample after isolation of the single cells aims at miniaturization to sub μL-scale and lossless preparation. Several options have emerged, which are known as nanodroplet processing in one pot for trace samples, oil-air droplet processing, nanopackage processing, and microfluidics devices.

(legend continued on next page)

spatial transcriptomics, or spatial proteomics. We also introduce a concept that we have termed "deep visual proteomics (DVP)," which combines the advantages of multiplexed imaging with a deep proteomic read out of single-cell types or states (Mund et al., 2022; Spatial characterization of single tumor cells by proteomics, 2022). By combining digital pathology with a molecular readout, these approaches are set to make a transforming impact toward precision medicine.

Ultra-sensitive mass spectrometry for single-cell analysis

There are many different sample preparation techniques for processing low cell numbers down to the single-cell level. Their overall goal is the collection of these cells without loss, miniaturization of sample lysis, protein extraction, and digestion into peptides that are amenable to MS, all aiming at minimizing sample loss and maximizing digestion kinetics (Figure 1B) (Brunner et al., 2022; Gebreyesus et al., 2022; Li et al., 2018; Williams et al., 2020). A major lesson learned in ultra-high sensitivity proteomics is that the analysis of real samples—as opposed to diluted down standards—exposes even subtle imperfections in the workflow, which would otherwise be hidden in bulk analysis. Furthermore, what works in a highly optimized experimental setting for a proof of concept may not be amenable to a high-throughput routine.

As a first step of sample preparation, isolated cells need to be digested to peptides. A pioneering implementation of single-cell sample preparation is called "nanodroplet for processing in one pot for trace amounts (nanoPOTS)" (Zhu et al., 2018a). In nanoPOTS, all sample processing is performed in a 200-nL volume spotted robotically onto a glass slide in a humidity-controlled chamber to prevent evaporation. Similarly, in the "oil-air droplet (OAD)" method, the sample is placed in a nanoscale droplet on the OAD chip, where it is covered with oil, again to prevent evaporation (Li et al., 2018). Proteomics researchers have also adapted microfluidic approaches that are widespread in scRNA-seq. These promise parallel processing in enclosed systems and minimal sample volumes (Gebreyesus et al., 2022). Along these lines, the cellenONE instrument combines single-cell manipulation by acoustic dispensing with a dedicated downstream collection in a manner compatible with further proteomics processing (Ctortecka et al., 2022a).

A limitation of many of these approaches is that they use very dedicated and specialized devices that are not necessarily easily available to use by the community. Furthermore, the absence of a subsequent cleanup step requires enzymatic digestion in electrospray compatible buffers rather than those with digestion enhancing modifiers. In contrast, single cells can be processed efficiently in standard low-bind 384-well plates. Here, the reaction chamber can be used to collect cells in a standardized manner by FACS or following laser capture microdissection (LCM), and samples can be processed in microliter or submicroliter volumes in standard PCR cyclers (Brunner et al., 2022; Specht et al., 2021).

After sample preparation, the digested peptides need to be transferred onto the chromatographic column without loss. A capillary containing chromatographic material can passively extract the peptides after which it can be placed in line with the analytical column (Li et al., 2018; Williams et al., 2020). For a more standardizable and nearly lossless approach, we have found that the digestion mixtures from 384-well plates can be transferred to commercial StageTips that are routinely used for sample cleanup before chromatography (Bache et al., 2018; Binai et al., 2015; Rappsilber et al., 2007). A key advantage is that peptides are concentrated in a "nanopackage" of only 20 nL, which is then eluted onto the analytical column, leaving contaminants on the tip. This approach also allows efficient digestion at low acetonitrile concentrations, which improves digestion kinetics (Brunner et al., 2022).

It has long been appreciated that electrospray sensitivity increases with decreasing chromatographic flowrate, which in turn favors very narrow columns. Whereas standard MS-based proteomics is performed with 75-μm inner diameter columns and flow rates of several hundred nanoliters per minute, dedicated ultra-sensitive setups have used columns that are only 20 μm wide and flowrates in the low nL/min range. Although they often achieve much higher sensitivity, peptide separation power usually suffers, and it is difficult to manufacture them in a reproducible and streamlined manner. The recently introduced μPAC columns may be an attractive alternative, as they make use of lithographically etched micro pillars in regular arrays instead of high pressure packed column material (Malsche et al., 2012). Initial reports already indicate excellent sensitivity (Stadlmann et al., 2019), and this format could readily be adopted to very low flow rates by producing columns with small cross sections.

After eluting from the column, the peptides are transferred to the gas phase by electrospray, which involves small, highly charged droplets evaporating under ambient pressure, thereby ionizing the contained analyte molecules (Figure 1C). Typically, only a small percentage of the total ion population eluting at each time point finds its way into the gas phase and ultimately into the entrance orifice of the MS. Fully enclosed environments such as the captive spray partially address this challenge by increasing desolvation efficiency, as does the addition of chemicals such as DMSO to the solvents of the liquid chromatography system at low percentages (Beck et al., 2015; Hahne et al., 2013). However, the largest contribution to higher sensitivity has been an increase in the diameter of the orifice that admits the ions to the MS, directly boosting the signal available for analysis. Ultimately, it may be possible to electrospray directly into the vacuum, which would remove transfer losses entirely (Page et al., 2008). Once inside the MS instrument, ions are very efficiently guided until they are actually mass analyzed, fragmented, and

(C) Single-cell-derived peptide analytes are separated by high-performance chromatography followed by electrospraying into the mass spectrometer. In the "true single-cell" approach, single cells are injected one by one (upper part of the panel), whereas in the multiplexed "scope" approach a booster channel comprising hundreds of cells increase the overall signal. Novel computational approaches comprising advanced neural networks allow high confidence identification and quantification of single-cell-derived peptide signals.

quantified, for which there are a variety of innovative strategies and scan modes.

One of the first approaches for single-cell proteomics was ''single-cell proteomics by mass spectrometry (SCoPE-MS),'' which multiplexes a number of cells after labeling them with the widely used tandem mass tag (TMT) reagents (Budnik et al., 2018; Thompson et al., 2003). The crucial feature of SCoPE-MS is the introduction of a ''booster channel'' initially consisting of the equivalent of hundreds of cells, which is separately labeled and mixed into the samples. For most aspects of the MS analysis, this reduces the required sensitivity 10- to 100-fold as the vast majority of the signal comes from the booster channel. This method was pioneered on the Orbitrap analyzer, which is capable of resolving multiplexed samples with very small reporter mass differences. Peptide fragmentation is relatively simple, leading to the identification of several hundred proteins per single cell and up to thousands altogether. However, quantification of these single-cell proteomes is complicated by isotope crosstalk between their channels and the dominant booster channel. Furthermore, there is an extensive history of well-documented issues with TMT quantification in general (Bradshaw et al., 2005; Brenes et al., 2019; Cheung et al., 2021). As the sensitivity of MS workflows has increased dramatically, most of these problems could now be alleviated by reducing the booster channel to no more than 25 cells or leaving it out altogether (Cheung et al., 2021). Furthermore, precursor-bound instead of low molecular weight reporter ions would further improve quantification accuracy (Pappireddi et al., 2019).

As an alternative to isotopically multiplexed methods, label-free ''true single-cell'' methods analyze one cell at a time in the mass spectrometer, and therefore the identification and quantification of proteins are based only on the signals of peptides derived from that single cell (Brunner et al., 2022; Dou et al., 2019; Liang et al., 2021). This requires the use of very efficient scan modes. Data-dependent acquisition (DDA), in which peptides are quantified based on their MS1 intensity, has been used in many proof of concept single-cell studies (Brunner et al., 2022; Cong et al., 2020; Li et al., 2018; Liang et al., 2021). Depending on the cell type, more than 1,000 proteins could be identified per single cell. However, DDA converts only a small fraction of the incoming peptide ions into fragments and does not always select the same ones for fragmentation, resulting in relatively low data completeness over many single cells. Data-independent acquisition (DIA) inherently has high data completeness (Gillet et al., 2012; Röst et al., 2015) making this scan mode attractive for single-cell proteomics. Normally, DIA converts just a few percent of the peptide ions into fragments on which identification and quantification are based. Making use of the correlation of ion mobility and mass to charge (m/z) in the ''trapped ion mobility'' time of flight instrument (timsTOF), we have described a scan mode called diaPASEF that converts much of the peptide ion current to quantifiable fragments (Meier et al., 2020). This led to the quantification of up to 2,000 proteins per single cell, with high data completeness (Brunner et al., 2022). Improved software analysis of single-cell spectra by machine learning (ML) and in particular deep learning (DL) has also provided a major boost and is currently a very active field of research (Bruderer et al., 2015; Demichev et al., 2020; Mann et al., 2021). Artificial intelligence (AI) now assists in all stages of the analytical pipeline of MS-based proteomics, predicting the expected measurement points for each identified peptide from the sequence alone. Furthermore, once a clinical study is completed, AI is the method of choice to extract biomarker candidates from the data (Mann et al., 2021).

Many of the methods developed for ''true single-cell'' proteomics would improve isotopically multiplexed approaches as well. Although DIA acquisition is normally incompatible with TMT-based quantification because fragment ions from many precursors are mixed together, ''multiplexed DIA'' may be possible by employing precursor encoding or precursor-coupled reporter ions with sophisticated algorithms to deconvolute the data (Ctortecka et al., 2022b; Derks et al., 2021).

Single-cell transcriptomics versus proteomics

The canonical gene expression cascade starts with the transcription of the genomic blueprint into mRNAs, which are then translated into proteins, the main workhorses and regulators of cellular life. The roughly 20,000 protein-coding genes in the human genome give rise to a number of different active RNA molecules, and in turn to hundreds of thousands of ''proteoforms'' including those subjected to post-translational modifications (PTMs) that dynamically regulate protein function (Aebersold et al., 2018; Melani et al., 2022). This vast complexity of the proteome goes along with a very large dynamic range, with some structural proteins expressed million-fold more than low abundance, regulatory ones (Figure 2A).

Although the ''central dogma of biology'' (that DNA makes mRNA and mRNA makes proteins) appears straightforward in principle, in reality there are numerous control points of immense complexity. Of particular interest here is the translation between mRNAs and their cognate proteins, which is highly regulated and non-linear. This is reflected in the relatively low correlation between the transcriptome and proteome observed in numerous experiments (Pearson correlation coefficients typically in the range of 0.3–0.6) (Buccitelli and Selbach, 2020). This is the case for steady-state levels and is even more pronounced for the correlation of changes in gene expression upon perturbation, which can even be anticorrelated at particular time points, for example, during tumor progression (Zhang et al., 2014).

In single-cell proteomics, the journey starts with obtaining individual cells. In this aspect researchers could build on the extensive experience of single-cell transcriptome analysis. Oligonucleotide sequencing technologies have advanced tremendously and now enable routine analysis and comprehensive coverage of the genome and transcriptome. scRNA-seq has become routine and high throughput, using barcodes to tag all the individual RNA molecules in a cell and quantifying most of them. By far, the majority of these studies employ either suspension cells or fresh tissue cells that have been brought into solution by tissue disaggregation and subsequent cell sorting into single wells. For instance, droplet-based single-cell methods can sort up to 15,000 events per second and have very recently even been coupled to multiparameter image-enabled cell sorting (Schraivogel et al., 2022).

Despite this, even the latest single-cell RNA-seq technologies still report high dropout rates for each individual cell

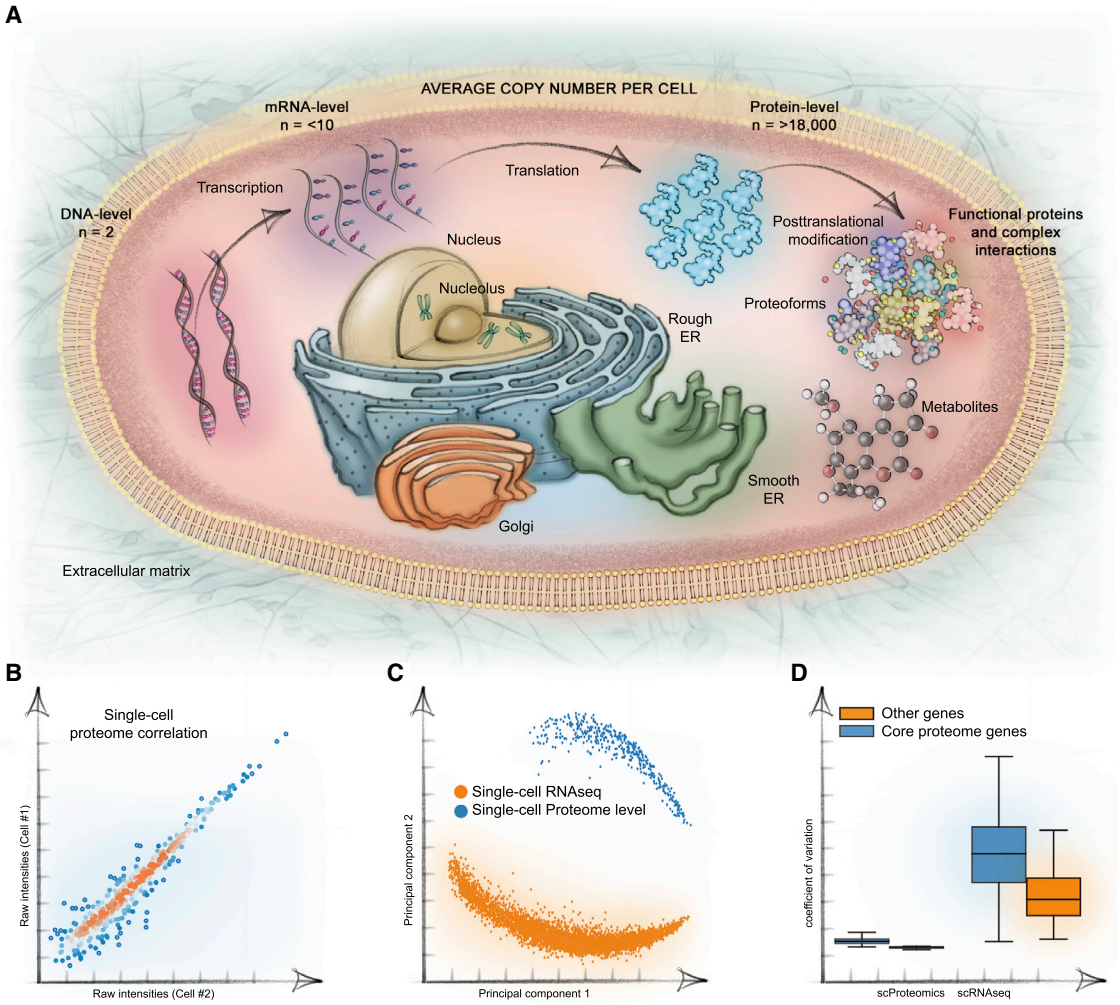

Figure 2. The cellular flow of information and the comparison of transcriptome to the proteome

(A) The molecular flow of information from genome to transcriptome, proteome, and the metabolome. Average copy numbers for each level are indicated.

(B) Single-cell correlation analysis of the proteome highlights a high quantitative protein correlation.

(C) Transcriptome expression levels in single cells are very different from single-cell proteomes in a principal component analysis (PCA).

(D) Distribution of coefficients of variation of the transcriptome and proteome of single cells of the same cell type (blue). A core proteome defined by highest data completeness and least quantitative change at the proteome level is contrasted to matched transcriptome genes (orange). (Box and whiskers: the middle represents the median, the top and the bottom of the box represent the upper and lower quartile values of the data, and the whiskers represent the 1.5× IQR). For further details, see main text.

(Svensson, 2020; Svensson et al., 2017). This is presumably due to the very low number of transcripts per active gene in a given cell. Even in dividing cells the average number of transcripts of expressed genes is less than ten, while for the majority of genes in nondividing tissue cells, copy numbers are less than one (Eberwine et al., 2014). This introduces a high stochastic fluctuation in the number of transcripts per gene and per cell (termed Poisson or shot noise) and raises interesting questions about which mRNA copy numbers are needed to affect biological functions and how cells regulate gene expression in a robust manner, for instance, by transcriptional bursts (Larsson et al., 2019).

At the bulk proteome level, we and others have observed that there is generally at least a 100-fold difference in copy numbers of proteins compared with corresponding mRNAs, well above the level of Poisson noise (Azimifar et al., 2014; Schwanhäusser et al., 2011). This is because each single cell needs a full complement of proteins and functional proteoforms to perform its myriad functions, whereas most transcripts may only be required in particular situations.

In a recent study, we have experimentally explored these questions with the single-cell proteomics technology described above. We drug-perturbed a cancer cell line to profile different cell-cycle stages at the single-cell level and compared our results with equivalent scRNA-seq data (Brunner et al., 2022). Single-cell proteomes correlated highly with each other and proteins significantly changing throughout the cell cycle were known or likely novel actors in this process (Figure 2B). Interestingly, cells clustered quite differently by their transcriptomes and

proteomes, supporting the notion of different modes of regulation (Figure 2C). Strikingly, the coefficients of variation of proteins compared with cognate mRNAs were much lower (Figure 2D). Rather than reflecting differences in technology, we attribute this phenomenon to the above-mentioned stochastic nature of the expression of transcripts compared with the proteins. We further defined a ''core proteome'' as the proteins with the highest data completeness and the least changes throughout the cell cycle. The variability of the core proteome was even much lower than that of their transcripts (median coefficient of variation 0.2 in proteomics versus 1.3 for the corresponding transcripts in dropseq; Figure 2D). Note that MS sensitivity is currently still limited at the single-cell level (up to 2,000 different quantified proteins in this experiment). In general, the throughput and comprehensiveness of deep-sequencing-based technologies is much higher than proteomics, and they increasingly incorporate the spatial aspect as well. Therefore, we believe that it will be advantageous in many situations to combine the two approaches. This can validate transcriptomics or genomic results at the protein level. For instance, these data also allow cataloging mutations in a tumor, which in turn enables proteogenomic approaches, where the impact of mutations on cell function can be accessed directly.

Spatial tissue proteomics with single-cell resolution

The requirement of tissue dissociation described above loses the spatial context important for cell-to-cell interactions between normal and diseased tissue (Goltsev et al., 2018). However, that context is crucial to fully understand cellular functions, their relationships to each other, and their contribution to heterogeneous tissues. Spatial transcriptomics—the 2020 method of the year (Editorial, 2021)—addresses this challenge either by highly multiplexed fluorescence *in situ* hybridization (FISH) or sequencing-based methods (Larsson et al., 2021). In the latter case, areas of tissues with dimensions of 10–100 μm are generally designated to efficiently capture mRNA, and single-cell contribution is inferred by deconvolution with respect to single-cell libraries.

In contrast to proteins, RNA is prone to degradation and easily cross-linked with other biomolecules in formalin-fixed and paraffin-embedded (FFPE) samples (Hoffman et al., 2015), although these challenges can be overcome to some degree (Villacampa et al., 2021). More generally, cell types are not directly visible in spatial transcriptomics but have to be inferred from the data. As mentioned above, RNA expression does not directly predict protein expression (Buccitelli and Selbach, 2020; Liu et al., 2016; Wang et al., 2017; Zhang et al., 2014). Ideally the above-mentioned methods would be complemented by direct measurement of the proteome, which would more accurately reflect the specific cell functions and state.

It is possible to analyze spatially resolved protein distribution or entire proteomes at the tissue, cell, and even subcellular level with immunohistochemistry (IHC), immunofluorescence (IF), MS, and cytometry (Lundberg and Borner, 2019; Uhlén et al., 2015). These methods all have trade-offs between spatial information and depth of coverage, molecular or cellular throughput, and data acquisition time (Hickey et al., 2021; Lewis et al., 2021) and can globally be categorized by whether they use antibodies (or other specific binders) or not. The thousands of tissue IHC images from the Protein Atlas Project provide unprecedented insights into the spatial (sub)cellular organization and composition of tissues at the protein level (Thul et al., 2017; Uhlén et al., 2015). Multiplexed antibody-based imaging methods differ by the principle of antibody tagging (such as metal tag, fluorophore, DNA oligonucleotide barcode, or enzyme) and detection modality (such as MS, spectroscopy, fluorescence, or chromogen approaches) (Hickey et al., 2021). For example, imaging mass cytometry (IMC) now allows detection of 40 antigens and nucleic acid sequences in FFPE with single-cell resolution in their 3D context (Kuett et al., 2022). Multiplexed antibody-based imaging generally defines cell types by the expression of a handful of proteins, but new methods can detect up to a hundred targets in the same tissue section (Table 1). For a detailed discussion of these technologies see (Bodenmiller, 2016; Hickey et al., 2021).

As an alternative to antibody-based approaches, tissues can be scanned by light or particle beams, ionizing some of the surface biomolecules and making them accessible to time of flight (TOF) MS. In matrix-assisted laser desorption/ionization (MALDI), a pulsed laser beam ionizes predominantly small biomolecules and peptides with near single-cell resolution (10–50 μm) (Spraggins et al., 2019). As a first step, surface proteins are digested *in situ* to produce a peptide representation of the proteome. This technology has recently been applied to various states of disease, including cancer, to discover diagnostic, predictive and survival markers (Ahmed et al., 2020). While the laser spot could probably be focused to enable single-cell resolution for MALDI, the digestion of proteins into peptides in slides can lead to analyte delocalization. Furthermore, MALDI is generally less quantitative and has less dynamic range than electrospray, which has led to its displacement in proteomics workflows. However, MALDI has roles in applications such as investigating drug distribution in tissues and tumors, and it can be combined with stable-isotope-labeled nutrient infusion (iso-imaging) to reveal the spatial organization of metabolic activity in tissues (Wang et al., 2022). Furthermore, MALDI is a promising technology for the analysis of single-cell metabolomes, where the aim is to characterize as much of the universe of small molecules (metabolites, lipids, etc.) as possible. For example, a method called SpaceM integrates light microscopy with MALDI-imaging providing a metabolic profile *in situ* for each cell. SpaceM detected more than 100 metabolites and lipids per hour from more than 1,000 co-cultured human epithelial cells and mouse fibroblasts, demonstrating high sensitivity and speed (Rappez et al., 2021). We expect that technological advances, both in sample preparation and in MS equipment, will enable a more extensive and high spatial resolution analysis of metabolic activity over the next few years.

Secondary ion mass spectrometry (SIMS) is a mature technology used for investigating the surfaces of semi-conductors, for instance. NanoSIMS has high spatial resolution in the tens of nanometers. NanoSIMS has been used for visualizing subcellular structures but is limited to very small molecules (Nuñez et al., 2017).

For LC-MS-based proteomics, bulk tissue is most easily analyzed and it is now possible to identify and quantify more than 10,000 proteins in single LC runs and even more after initial fractionation (Bekker-Jensen et al., 2017; Meier et al., 2018; Muntel et al., 2019). However, any tissue homogenization

Table 1. Criteria for the selection and implementation of (spatial) proteomics technologies

(Spatial) proteomics in tissue	Approach	Method	Area	Cellular resolution	Current proteome depth	Tissue preparation	Spatial dimension	Special features
Multiplexed antibody based	fluorophore labeled	CyCIF	whole slide	subcellular	>60	FFPE/FF	2D, 3D	conventional microscopes
		ChipCytometry	mm^2	subcellular	>60	FFPE/FF	2D	specialized instrument, special vendor-supplied kits, or consumables
		Cell DIVE	whole slides	subcellular	>60	FFPE/FF	2D	specialized instrument, special vendor-supplied kits, or consumables
	DNA labeled	CODEX	whole slides	subcellular	>60	FFPE/FF	2D, 3D	specialized instrument, special vendor-supplied kits, or consumables
		GeoMx DSP/ CosMx SMI	whole slides	subcellular	>100/100	FFPE/FF	2D, 3D	specialized instrument, special vendor-supplied kits, or consumables
		immuno-SABER	mm^2	subcellular	>10	FFPE/FF	2D, 3D	conventional microscopes
		Spatial-CITE-seq	mm^2	cellular	Up to 300	FFPE/FF	2D	next-generation sequencer, microfluidic device
	metal labeled	MIBI-TOF	mm^2	subcellular	>40	FFPE/FF	2D, 3D	specialized instrument, special vendor-supplied kits, or consumables
		IMC	mm^2	subcellular	>40	FFPE/FF	2D, 3D	specialized instrument, special vendor-supplied kits, or consumables
Untargeted	label free	DVP	whole slides	subcellular	>5,000	FFPE/FF	2D, 3D	conventional microscopes, specialized software and ultra-high sensitivity MS
		nanoPOTS	whole slides	multi-cellular	>2,000	FFPE/FF	2D, 3D	conventional microscopes, specialized equipment, consumables, ultra-high sensitivity MS
		MALDI	whole slides	multi-cellular	100s	FFPE/FF	2D	specialized equipment
		SCP	–	cellular	up to 2,000 per cell	FF	–	ultra-high sensitivity MS
	labeled	SCP	–	cellular	up to 1,000 per cell	FF	–	ultra-high sensitivity MS

Comparison of technologies representative of different approaches, classified by antibody-based and untargeted as well as the nature of the label. Methods cover spatial dimensions from whole slide to square micrometer or absent spatial information. Proteome depths range from a handful of proteins to a substantial part of the entire cellular proteome. Most but not all spatial proteomics techniques are amenable to FFPE material as well as fresh frozen material (FF). Several approaches can naturally be extended to three dimensions. Finally, special features such as specialized equipment or special vendor-supplied kits are indicated. For a detailed description of these technologies see Hickey et al. (2021). Cyclic immunofluorescence (CyCIF), ChipCytometry (Canopy Biosciences), Cell DIVE (Leica), co-detection by indexing (CODEX, Akoya Biosciences), GeoMx DSP (nanoString), immunostaining with signal amplification by exchange reaction (immuno-SABER), multiplexed ion beam imaging by time of flight (MIBI-TOF), imaging mass cytometry (IMC), deep visual proteomics (DVP), nanodroplet processing in one pot for trace samples (nanoPOTS), matrix-assisted laser desorption/ionization (MALDI), single-cell proteomics (SCP) (Brunner et al., 2022; Budnik et al., 2018; Hickey et al., 2021; Jarosch et al., 2021; Mund et al., 2022). Spatial-CITE-seq utilizes spatial barcoding and high-throughput sequencing for protein mapping with cellular resolution (Liu et al., 2022).

process will unavoidably result in an averaging effect by blurring the spatial and cell-type information.

In LCM, tissue areas of interest are excised using direct microscopic visualization to link positional cellular information to genetic, transcriptomic, or proteomic information (Figure 1A). As an example from transcriptomics, a combination of LCM and Smart-seq2 (LCM-seq) has proven successful for transcriptome analyses of single-cell neurons (Nichterwitz et al., 2016).

FFPE tissue collections are the gold standard in pathology tissue storage and analysis, with hundreds of millions of tissue blocks stored in biobanks (Figure 1A). Therefore, the ability to analyze FFPE material makes any spatial omics technology

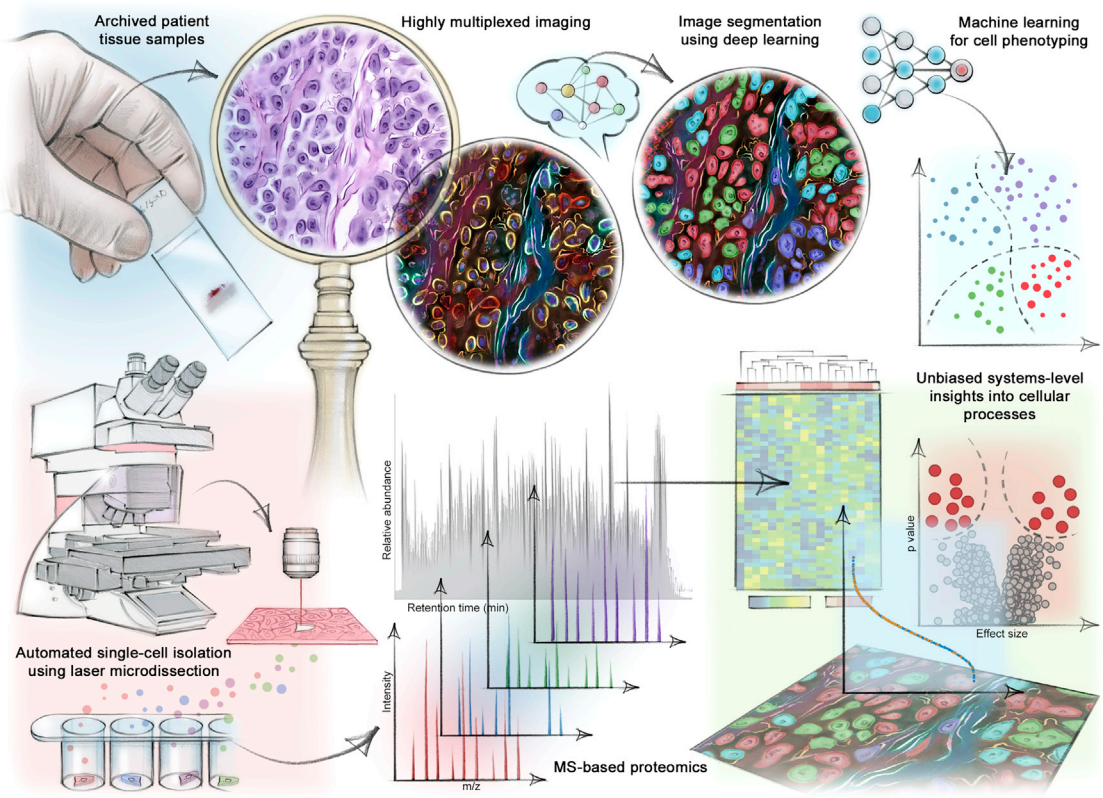

Figure 3. Combining antibody-based bioimaging with the unbiased characterization of proteomes for system-level cellular phenotyping
Deep visual proteomics (DVP) connects high-parametric imaging of biobank tissues with machine-learning-based cell segmentation and classification of cellular phenotypes. The cells or subcellular structures of interest are excised using automated laser microdissection and subjected to ultra-sensitive mass-spectrometry-based proteomic profiling. Next, bioinformatics data analysis discovers protein signatures that provide molecular insights into proteome variation at the single-cell level (Mund et al., 2022).

much more applicable to clinical questions. Initial concerns that proteins might be substantially chemically modified or that they might be inaccessible to extraction and digestion, hampering proteomic analysis by MS, have proven unfounded. On the contrary, FFPE has proven to be an ideal long-term storage medium for the proteome with minimal influence on measured protein abundance as compared with fresh frozen tissue (Coscia et al., 2020; Craven et al., 2013; Shao et al., 2019; Tayri-Wilk et al., 2020; Zhu et al., 2019). Importantly, PTMs are also perfectly preserved over decades in these blocks, awaiting measurement (Friedrich et al., 2021; Ostasiewicz et al., 2010).

Efficient protocols have enabled the streamlined analysis of macro or laser microdissected FFPE tissues (Coscia et al., 2020; Griesser et al., 2020; Zhu et al., 2018b, 2019), leading to insights into tumor heterogeneity (Mardamshina et al., 2021; Xu et al., 2018) and discovery of a novel biomarker of long-term survival following chemotherapy in ovarian cancer (Coscia et al., 2018). Notably, even decades old FFPE material can be analyzed, and functional biomarkers have also been found in the proteins of the extracellular matrix (Eckert et al., 2019). The above-mentioned nanoPOTS method enabled the analysis of grid elements of 100-μm resolution to a depth of 2,000 proteins from fresh tissue (Piehowski et al., 2020). Even though molecular information of the tissue in its spatial context is retained, the grid analysis is not single-cell resolved and therefore results in merged data of cell types and states, or cells and the extracellular matrix.

To gain deeper insights into tissue biology, it would be desirable to directly connect the visual dimension with the molecular phenotype by combining antibody-based bioimaging with the unbiased characterization of proteomes; thereby integrating single-cell and spatially resolved molecular data. To this end, we have developed a new concept, which we have called DVP (Figure 3). DVP combines AI-driven image-based segmentation and classification for the analysis of cellular phenotypes with ultra-high sensitivity MS-based proteomics (Mund et al., 2022). High-content imaging with subcellular resolution provides the required number of cells to identify statistically and analytically robust cellular phenotypes for precise cell-type and state isolation within a spatial region (Palla et al., 2022). Thus, DVP ties together visual information that defines cellular identity and heterogeneity with cellular neighborhoods and the underlying proteomic signatures in an unbiased and system-wide way.

The inherent trade-off between spatial information, throughput, and depth of coverage dictates the methodological choice that needs to be made by the investigator to most efficiently answer

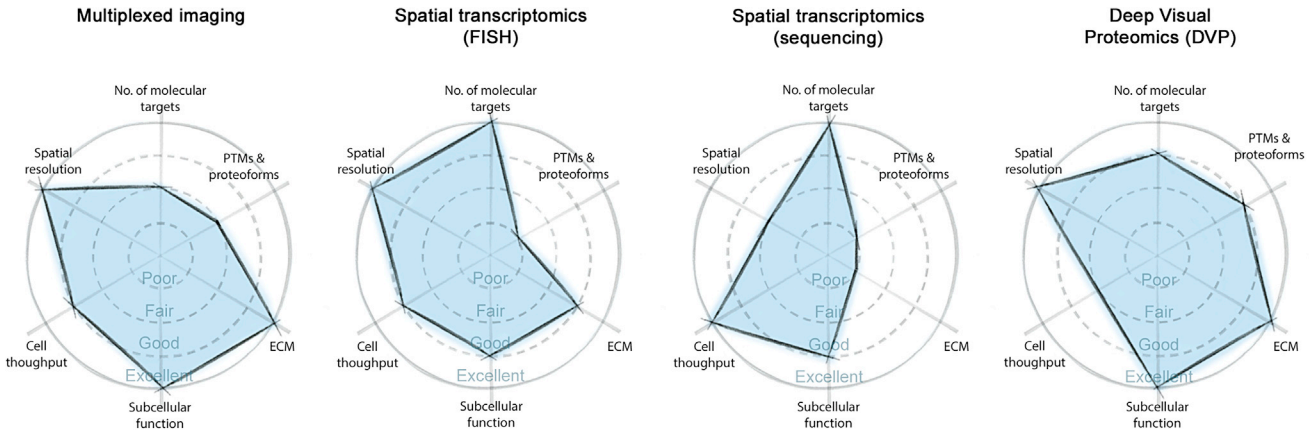

Figure 4. Spider plots ranking each technology for six parameters

Adapted from Lewis et al. (2021). Trade-offs between spatial resolution and subcellular function, molecular or cellular throughput, and type of data acquisition. Highly multiplexed FISH and sequencing-based spatial transcriptomic techniques have great potential for multiomics, combining cellular and molecular throughput with spatial resolution and ease of use. In contrast, multiplexed imaging and deep visual proteomics (DVP) directly address proteins and their post-translational modifications (PTMs) that dictate cellular function or activity. These PTMs include glycosylation, phosphorylation, acetylation, and many more, potentially creating tens of thousands of additional proteoforms. Proteomic methods allow studying extracellular matrix proteins (ECMs), which form complex macromolecule networks that fill the extracellular space in tissues. DVP combines strengths of imaging (high spatial and subcellular resolution) and of the MS-based proteomics worlds (excellent molecular specificity and substantial coverage of the proteome).

the experimental question. Consequently, the final data requirements, samples and formats available, and existing infrastructure should guide the choice of multiplex imaging methods (Table 1). Similarly, the first and crucial step of creating a multiplexed imaging panel is to determine the scientific questions to be solved.

For MS-based proteomics, single-cell analysis is rapidly becoming feasible with the approaches described above, and we expect to see increases in proteome coverage to several thousand proteins as well as throughput of hundreds of cells per day in the next years. It will be interesting to compare the single-cell transcriptome and proteome to pinpoint differences, for instance, in dynamic processes involving protein degradation. In contrast to disaggregated cells, proteomics on cells directly from tissue is more challenging. However, it is crucial not to mix cell types in the analysis as this would entail all the disadvantages of bulk analysis. Here, DVP comes to the rescue as it defines the cell types and cell states upfront from imaging data. This avoids the pooling effect while allowing the analysis of a statistically meaningful number of cells in one go, which also addresses the throughput challenge and provides excellent depth of proteome coverage.

Clinical applications of tissue proteomics with single-cell resolution

In clinical or translational applications, the above-described spatial proteomics approaches are a natural fit to the general area of pathology. For many decades, pathologists have looked through microscopes at stained slides of surgical specimens for diagnostic purposes, to provide prognostic information to clinicians and to explore new approaches of treatment. Today, the rich histological and molecular information embedded in tissue sections can increasingly be extracted through digital pathology (van der Laak et al., 2021). Manual inspection of a narrow field of a microscope is replaced by broader, more diverse, and more precise digital whole-slide imaging (WSI), opening completely new opportunities. A central element in digital pathology is the automated analysis of images using ML methods (van der Laak et al., 2021). By mimicking human capabilities, this promises better and faster data extraction. These technologies require training the algorithms on human annotated features, a fairly complex task. DL can help overcome this problem and aims for end-to-end trainable systems, including feature extraction. In digital pathology, these algorithms allow the discovery of meaningful features, while achieving better robustness regarding intensity or morphology-dependent signal heterogeneity. Pioneering work achieved dermatologist level performance in detecting melanoma from photographs of skin lesions (Esteva et al., 2017).

In the context of protein-based multiplexed image analysis, one wants to retrieve complex information such as single-cell interactions, neighborhood analysis, expression of biomarkers, or immune cell infiltration within the tumor. In oncology, the clinical goals are to define the interplay between individual tumor cells, immune cells, blood vessels, fibroblasts, and the extracellular matrix. Investigating this tumor microenvironment, including the type, density, localization, and organization of immune cells—defined as the immune contexture—could help predict treatment response and clinical outcomes on an individual basis. Thus, digital pathology aims to detect tumors and classify them into subtypes, which involves image segmentation (Chen et al., 2017; Graham et al., 2019; Greenwald et al., 2022; Hollandi et al., 2020), cell detection, and counting (Sirinukunwattana et al., 2016), tumor grading (Nagpal et al., 2019), and others. Human-level performance of tissue segmentation with complex cell morphologies for feature extraction can now be achieved (Greenwald et al., 2022; Mund et al., 2022). As a next step, computational approaches can calculate spatial features and relationships between tissue cells and how frequent certain immune cells are in the tissue microenvironment or parenchyma. Those spatial features allow us to find digital biomarkers that

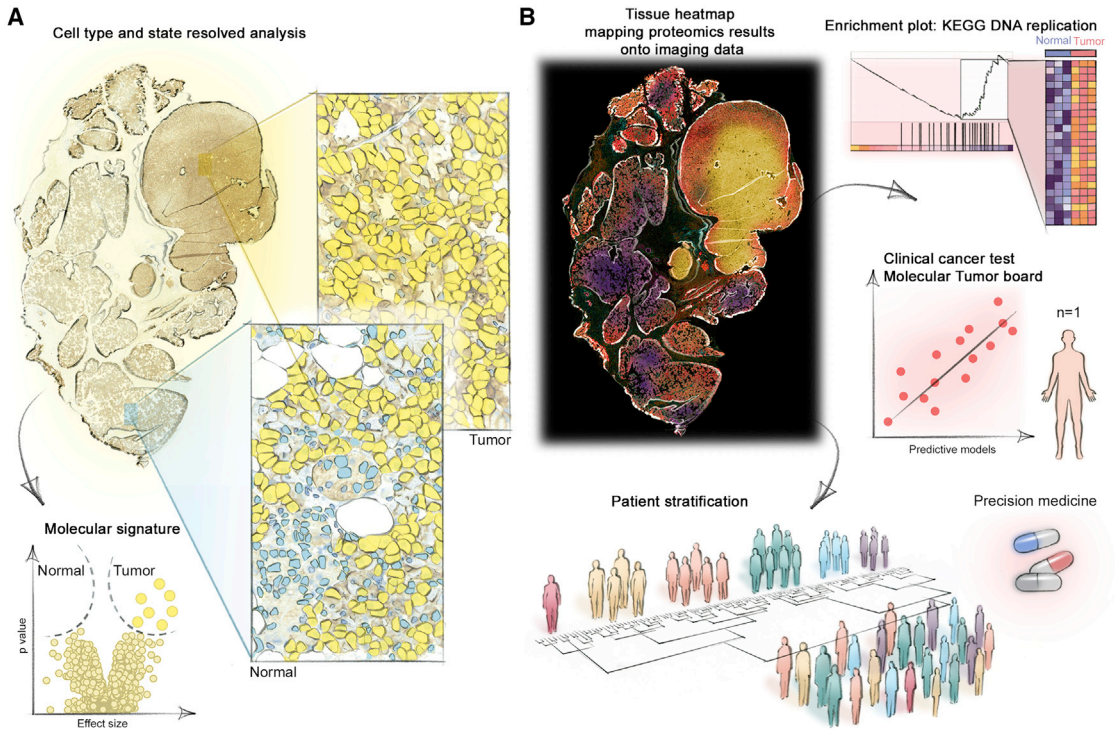

Figure 5. Clinical applications of spatial proteomics for patient phenotyping

(A) High-resolution tissue maps allow machine-learning-based accurate cell segmentation and classification. Spatial proteomics analysis reveals disease-specific molecular signatures in their native tissue context, directly from normal or tumor FFPE tissue slices.

(B) Combining unbiased proteomics with high-content imaging generates a phenotype map including the tissue microenvironment. Out of the detailed and quantitative proteomic map of the tissue, matrices, profiles, enrichment plots, and neighborhood analysis can be generated to define phenotypic relationships and mine the spatial correlations in the data to provide diagnostic decision support.

can potentially predict response to therapy, be prognostic markers themselves, or even predict genomic mutations in patients (van der Laak et al., 2021).

As an illustrative example, a protein-based spatial phenotyping approach using multiplexed immunofluorescence (mIF) was recently evaluated in a meta-analysis to determine whether this approach could improve the diagnostic performance over PD-L1 (programmed cell death ligand 1) IHC, tumor mutational burden (TMB), and gene expression profiling (GEP). From 45 reports, the authors concluded that multimodality biomarker and mIF strategies indeed outperformed PD-L1 IHC, TMB, or GEP alone in terms of specificity and sensitivity (Lu et al., 2019).

Cancer is a genetic and multifactorial disorder associated with the dysregulation of proteins and environmental factors. Hence, acquiring spatial multi-omics profiles may enable the reconstruction of key processes of tumorigenesis in a holistic manner. For example, a new technology called spatial-CITE-seq (spatial co-indexing of transcriptomes and epitopes) combines next-generation sequencing with antibody-based tissue barcoding to map ~300 proteins and the whole transcriptome (Liu et al., 2022). Another example is spatial-CUT&Tag that combines microfluidic deterministic barcoding, next-generation sequencing, and imaging to spatially resolve single-cell epigenome profiling in frozen tissue (Deng et al., 2022). As mentioned above, DVP combines high-content and high-resolution imaging with unbiased, in-depth proteomics to compare and contrast pure cell types and states directly from their native tissue context (Figure 5A). In the context of precision oncology, this allows tracking and functionally describing a single tissue slide. In turn, this enables the visualization of dysregulated key pathways of cancer progression across a two-dimensional tissue section (Figure 5B) (Mund et al., 2022). Future developments will extend this to the analysis of consecutive sections, allowing the reconstruction of enriched key pathways in 3D.

Discussion and outlook

Biology and pathology always occur in a spatial context. As we have described here, omics technologies are now addressing the spatial dimension with a large, diverse, and rapidly growing toolbox. We believe that spatial, high-resolution, and molecular analysis of cells in tissues will be one of the major frontiers of biology in the next years.

As a result of the immense complexity of cellular organization in tissues, no single technology can answer all questions. While genomics is already very mature, we have outlined how MS-based proteomics is reaching an inflection point in its ability to characterize single cells or single-cell types or states in tissue. This unexpected development is the result of a number of breakthroughs in different aspects of the LC-MS-based workflow as

described above. There are many further technological advances for increasing overall sensitivity, including even brighter ion sources, improved chromatography, and smarter data analysis and modeling tools waiting just around the corner. Similar to the rapid recent advances in the scRNA-seq community, this will further expand the myriad potential applications of LC-MS-based single-cell and spatial proteomics.

As proteins are the closest proxy for cellular function, this will add an important dimension to single-cell biology. Although technologically less mature, proteomics has the advantage that proteins are readily extracted from tissue material, such as FFPE slices, which are the common currency of pathology. With further advances in MS-based proteomics workflows, it should have a prime role in pathology, which itself is already undergoing a transformation to digital pathology, in part powered by rapid advances in DL. Although the digital classification of pathology slides is very powerful in itself, we argue that it will truly come to life when coupled to unbiased in-depth molecular characterization. Beyond spatial transcriptomics, this can be done by LCM followed by proteomics analysis. This previously involved the painstaking definition and excision of a large number of cells but can now be achieved by a combination of state-of-the-art imaging, AI, automated single-cell isolation, and ultra-sensitive proteomics using the DVP technology. An obvious next step is to extend these technologies into the third spatial dimension, by stacking and connecting the results of many tissue slices, creating a true 3D multiomics map (Bhatia et al., 2021; Kiemen et al., 2020).

Beyond genomics and MS-based shotgun proteomics, more modalities with a spatial dimension could be addressed. This even extends to the determination of the structures of protein complexes *in situ* and their intracellular localization (Klykov et al., 2022).

All these technologies generate very large datasets on their own that are challenging to analyze and interpret. This is compounded when several omics technologies are brought together, as it is increasingly the case. Therefore, the challenges and opportunities in algorithm development, data analysis, and joint modeling of datasets will be just as great as those in obtaining the data in the first place. Fortunately, this field is under rapid development and seems likely to rise to these challenges (Palla et al., 2022).

DVP is a generic pipeline that can be applied to investigate any type of healthy or diseased tissue sample. In the former case, it could contribute to cell-type-specific cell atlases or by describing developmental trajectories. We envision a strong partnership with pathologists and clinicians to create a future in which cancer biopsies will routinely be imaged with next-generation microscopy technologies, enabling the AI-driven recognition and classification of the affected cells, followed by in-depth characterization of their genome and proteome. In the future, spatial genomics, transcriptomics, and epigenomics could be added (Deng et al., 2022) and perhaps even lipidomics or metabolomics (Tsugawa et al., 2020).

DVP should find one of its major real-world applications when cancer patients first present but will be even more important in deciding on optimal treatment responses after relapse. Besides this prime area of application, spatial proteomics could just as well be used to investigate a wide range of pathologies in organs such as the brain, the liver, the skin and many more.

These methods need to work in a robust and affordable way on a daily basis in the clinic, which will in turn require much technology development and standardization. However, with the required throughput, robustness, and affordability in place, many patients could benefit from unbiased spatial omics and proteomics with single-cell resolution, a worthy goal for our community to strive for.

Box 1. Tissue-based spatial approaches fall into four categories that can be compared along six different criteria (Figure 4)

A key strength of **multiplexed imaging** is its ability to classify a very large number of cells in their spatial context. Even subcellular and extracellular structures are readily accessible. Molecular depth, in contrast, is limited to a relatively small number of proteins, for which specific antibodies have to be available. Bias may also be introduced via the design of the antibody panel.

FISH-based spatial transcriptomics methods have excellent resolution and record positional information for individual mRNA species in a cell and image hundreds to thousands of individual RNA molecules *in situ* through sequential rounds of hybridization (Chen et al., 2015; Lewis et al., 2021).

Sequencing-based spatial transcriptomics also has high throughput and depth at the cost of accurate single-cell spatial resolution. Additionally, as it does not consider cell boundaries, the results need to be deconvoluted (Ståhl et al., 2016). Current developments are set to increase resolution to the sub-um range, as mentioned above.

Spatial transcriptomics detects a proxy for molecular function and consequently, the location of the transcripts is not as functionally informative as that of proteins. In contrast, a key advantage of **spatial MS-based proteomics** is that it is unbiased in the sense that one does not need to decide on the molecular targets to be investigated beforehand. Furthermore, FFPE-embedded clinical samples are readily amenable because proteins from a wide range of sources are preserved and easily analyzable. Currently, throughput is comparatively low and single-cell analysis is still very challenging. However, the DVP method effectively uses high-content imaging for identifying the cell population at its front end and greatly reduces the demands on the MS analysis at the back end. Importantly, the cells that are analyzed together should already be of the same type and state.

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DECLARATION OF INTERESTS

M.M. is an indirect investor in Evosep.

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