nature reviews cancer

https://doi.org/10.1038/s41568-023-00657-4

Review article

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Multiplex protein imaging in tumour biology

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Abstract

Tissue imaging has become much more colourful in the past decade. Advances in both experimental and analytical methods now make it possible to image protein markers in tissue samples in high multiplex. The ability to routinely image 40–50 markers simultaneously, at single-cell or subcellular resolution, has opened up new vistas in the study of tumour biology. Cellular phenotypes, interaction, communication and spatial organization have become amenable to molecular-level analysis, and application to patient cohorts has identified clinically relevant cellular and tissue features in several cancer types. Here, we review the use of multiplex protein imaging methods to study tumour biology, discuss ongoing attempts to combine these approaches with other forms of spatial omics, and highlight challenges in the field.

Sections

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Introduction

The persistence, spread and therapeutic response of tumours depend not only on the cell-autonomous properties of malignant cells but also on their interaction with many other cell types. Immune cells, stromal cells, endothelial cells of blood and lymph vessels, other cell types like adipocytes, and possibly even microbial cells can all have context-dependent tumour-promoting or tumour-suppressive functions[1,2]. It is likely that many of the hallmarks of cancer, the seminal heuristic of Hanahan and Weinberg[1–3], are directly or indirectly affected by the interplay of tumour cells with their microenvironment. If we are to fully understand how cancer develops, spreads, and kills, and thus how to best treat it, we must understand the multidirectional interactions of tumour cells with these other cell types and the signalling networks involved.

Tumour cells are neither uniform nor static, even within a single patient or tumour, but are dynamic and evolving entities[4–7]. This cellular heterogeneity and plasticity contribute to the ability of a tumour to evade the immune system, to spread beyond its primary site and to develop resistance to therapy[8–12]. At each step of disease progression, tumour cells can be seen as learning systems that process information by continuous, reciprocal communication with each other and with other cell types of the microenvironment via a combination of direct contact and soluble factors. This information exchange, together with gradients of nutrients, oxygen and metabolites, shapes cellular communities and environments that enable tumour-promoting functions like immune cell dysfunction or tumour cell migration. The task at hand can therefore be re-stated to embrace this complexity: we must understand which heterogeneous tumour cell phenotypes exist and how these dynamic populations communicate with each other and with equally complex non-tumour cell types in order to understand disease aetiology, progression and response to therapy.

Accordingly, omics studies in cancer biology have moved in recent years from analysis of bulk tumour tissues, which comprise many cell types and subtypes, to single-cell analyses of tumour cells and tumour-associated cells; for instance, we now have single-cell gene expression atlases of tumour, immune and stromal cells from multiple types of cancer[9,13–25]. However, a tumour, like any tissue, is an entity in space. The spatial relationship of cell types with one other as well as with other structures such as blood vessels is an inextricable part of tumour biology and, as such, influences cellular properties, functions and disease outcomes[26]. Tissue architecture based on microscopic and macroscopic patterns in haematoxylin and eosin (H&E) stains is an essential pathological indicator of malignancy. Despite their many important contributions, dissociated single-cell molecular profiling techniques are blind to key spatial information. Molecular studies that retain the spatial organization of the tumour and surrounding tissue are therefore important if we are to understand how intercellular communication shapes tumour growth and treatment response.

Here, we review the development and state-of-the-art of multiplex, protein-based, single-cell resolution imaging methods with a focus on their application to spatial tumour biology; other recent reviews have also discussed this topic[27–29]. Although our focus is on methods that profile proteins, we discuss their integration with techniques that profile other classes of molecules, in particular transcriptomes, as an upcoming area. We are primarily concerned with approaches that can be applied to human tissue, and methods that require genetic modification are outside of the scope of this Review. However, the methods we describe can be equally well applied to model organism tissue or to ex vivo models such as organoids. The study of spatial architecture and information exchange in the tumour microenvironment (TME) with spatial, single-cell-resolved methods will benefit the understanding of many hallmark traits of tumours and will reveal how intercellular communication in tumours can be therapeutically modulated.

Measurable tumour features

A tumour can be conceptualized as an ecosystem of heterogenous cell types that dynamically exchange information to influence one another. In this section, we will discuss the features of tumours that can be tackled with multiplex imaging, ranging from the phenotypes of single cells to larger-scale properties of the TME (Fig. 1a), and will provide an overview of the types of analyses that have so far been performed.

Cell phenotypes, states and functions in a tumour

To understand a tumour ecosystem, an important first step is to identify the cell phenotypes that are present. Single-cell-resolved multiplex imaging (Fig. 1b) is well suited for this purpose since it distinguishes dozens of markers (typically 10–50) rather than the 3 or 4 markers in standard fluorescence imaging. Such large marker panels are necessary to adequately characterize even a handful of cellular phenotypes, since one typically wishes to simultaneously define cell types (for example, $CD3^+$ T cells, $CD68^+$ macrophages, $CD31^+$ endothelial cells and tumour cells), subtypes (for example, $CD4^+$ or $CD8^+$ T cells or forkhead box protein P3 $(FOXP3)^+$ regulatory T (T_{reg}) cells), and functional states (for example, dysfunctional versus active $CD8^+$ T cells, proliferating, hypoxic or apoptotic cells, or cells that show evidence of signalling pathway activation).

Marker panels in multiplex imaging studies are typically designed to identify known cellular subtypes and states of interest within the tumour, immune or stromal compartments. Given the tremendous interest in how the immune system may restrict or enable tumour growth, many studies have focused on immune cells. Immune landscapes have been imaged in breast cancer[30,31], lung cancer[32], brain cancer[33], head and neck cutaneous squamous cell carcinoma (HNSCC)[34], melanoma[35,36], and colorectal cancer[37] using various multiplex techniques. Panel design for immune cells typically draws heavily on prior knowledge but may also use data-driven approaches to further refine cell types. For instance, single-cell RNA sequencing (scRNA-seq) data was used to define a panel that could distinguish macrophage subtypes by multiplex immunofluorescence in breast and colorectal cancer[38], and profiling of metabolic proteins (transporters, enzymes, regulators and transcription factors) was used in multiplexed ion beam imaging (MIBI) to refine T cell subtypes based on proposed functional states[39].

In clinically well-characterized cohorts, cell phenotypes and states can be compared between samples that vary according to some property of interest; for instance, between primary tumour and metastases, between samples with different genomic profiles, or according to pathogen infection or immune infiltration status (Fig. 2). Cell phenotypes and states (or other imaged features) can also be statistically associated with clinical features like tumour grade, clinical subtype, response to therapy and patient outcome (Fig. 2). For instance, patient survival has been positively associated with B cell frequencies in lung adenocarcinoma[32], with endothelial cells in glioblastoma[33], and with the combination of lysosome-associated membrane glycoprotein $(LAMP)^+$ dendritic cells and $CD66B^+$ neutrophils in non-small-cell lung cancer[40]. Myeloid inflammation of tumours, in contrast, has been associated with poor overall survival of patients with HNSCC and pancreatic ductal

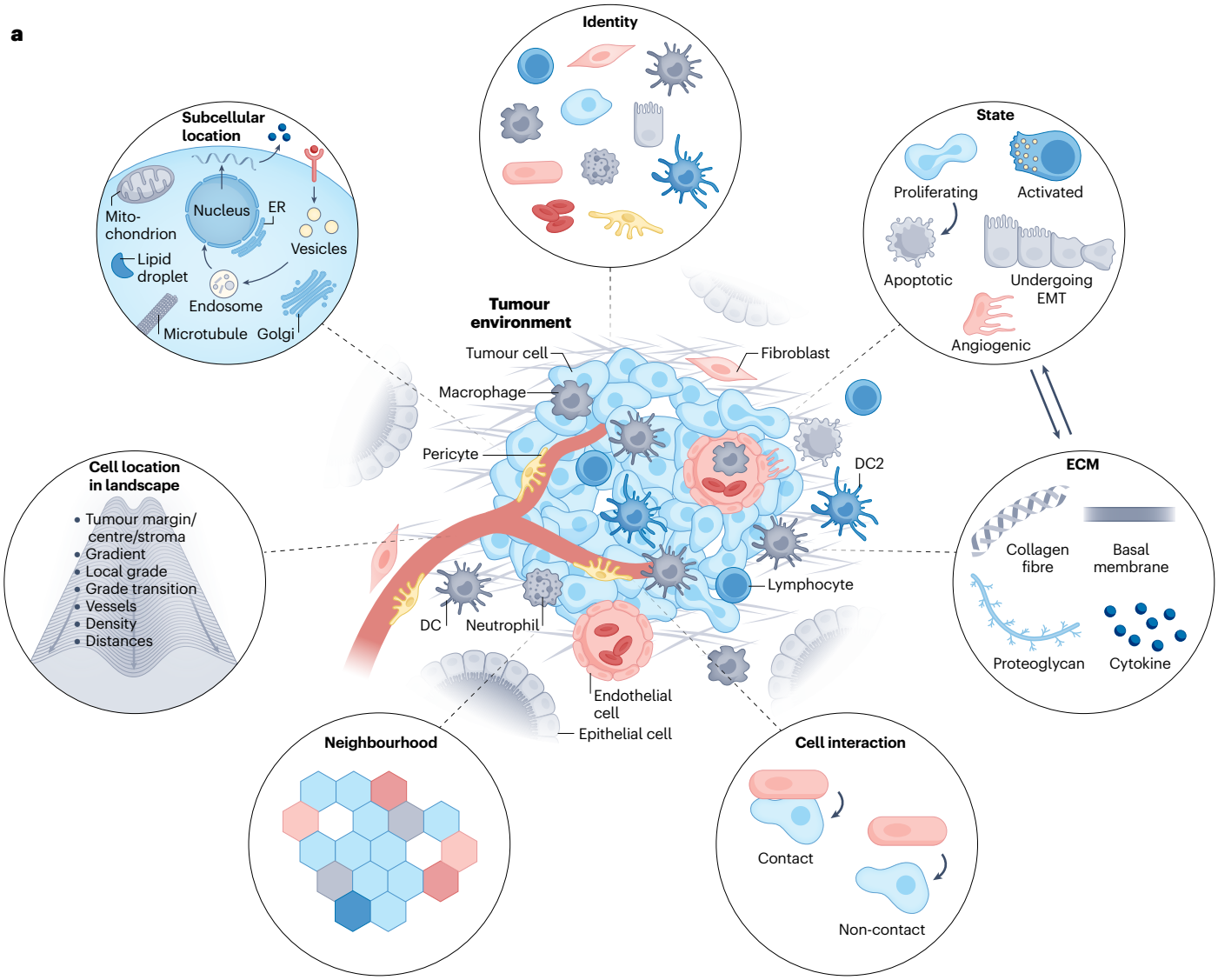

Fig. 1 | Multiplex protein imaging can interrogate a tumour and its microenvironment across scales. a, The schematic illustrates tumour features that can be studied using multiplex protein imaging techniques. Being able to target many markers enables the analysis of subcellular location, cell phenotypes (identity), active signalling or other processes within cells (state), interactions between pairs of cells (cell interaction) or with the extracellular matrix (ECM), local spatial enrichments of cell types (neighbourhood), or larger-scale spatial organization (landscape). Marker panels are typically designed to target the specific features of interest or combinations of these. **b**, Exemplary images to illustrate the nature of imaging mass cytometry data from tissue. DC, dendritic cell; DC2, type 2 dendritic cell; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum.

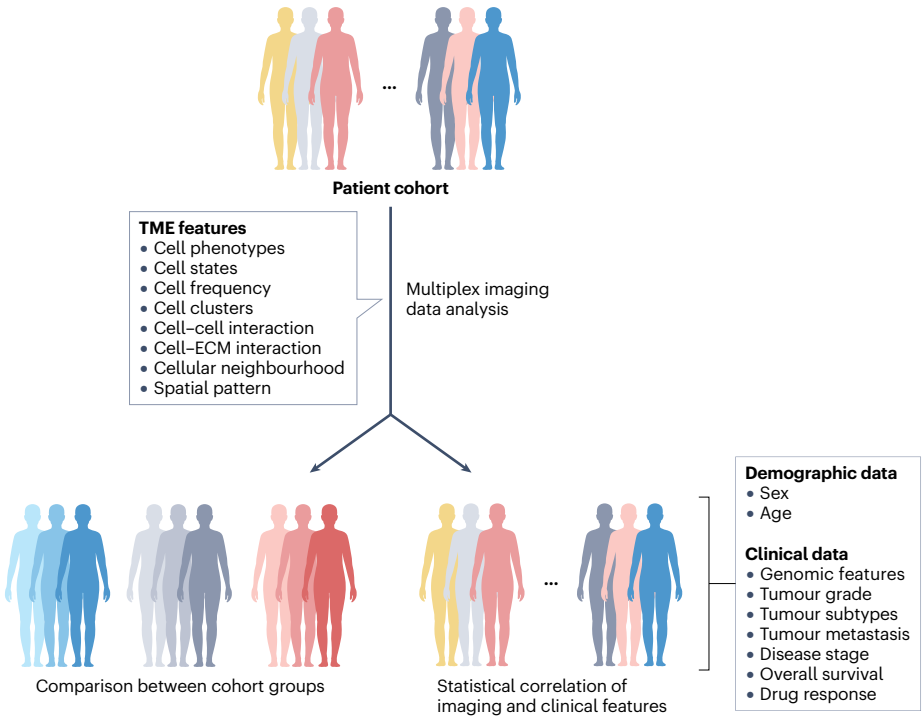

Fig. 2 | Multiplex protein imaging can be applied to clinical cohorts. In studies on well-characterized clinical cohorts, features of the tumour microenvironment (TME) quantified by multiplex protein image analysis (such as cell phenotypes, states, interactions or spatial patterns) can be statistically associated with demographic features like sex or age, with clinical features like tumour subtypes, response to therapy, or overall survival, or can be compared between cohort groups that differ in any defined demographic or clinical feature of interest. Such associations then identify stratifying or prognostic features that inform an understanding of clinical outcome. They could also form the basis of hypotheses on the spatial organization of cancer hallmark processes and their potential clinical relevance. ECM, extracellular matrix.

adenocarcinoma (PDAC)[41] as previously seen in many types of cancer[42], and myeloid cell types are also enriched in recurrent HNSCC tumours[43].

The power of multiplex imaging is even more evident when such association analyses with cell phenotype are performed at high granularity. In lung adenocarcinoma, imaging mass cytometry (IMC) found that proliferating ($Ki67^+$) endothelial cells and hypoxic (hypoxia-inducible factor 1α ($HIF1α)^+$) neutrophils but not all endothelial cells or neutrophils were associated with poor patient survival, emphasizing the need for many markers so that the relevant subsets of cells can be studied[32]. Similarly, a digital spatial profiling (DSP) study with a 44-plex antibody panel (though not strictly an imaging method and not conducted at single-cell resolution) reported that programmed cell death protein 1 ligand 1 (PDL1) expression on macrophages but not on tumour cells themselves was predictive of improved overall patient survival upon anti-programmed cell death protein 1 (PD1) therapy; furthermore, the study identified many potential predictive markers of immunotherapy response[44].

Multiplex immune surveys can reveal interesting patterns even beyond associations with clinical features. Keren et al.[30] used MIBI to propose a structured immune response in triple-negative breast cancer based on the observation that the immune composition varied with the level of immune infiltration: tumours with more immune cells systematically had higher proportions of T cells and lower proportions of macrophages. In our own work, by adapting RNA probes to IMC for the detection of secreted markers[45], we saw that hot (that is, immune infiltrated) metastatic melanoma contained many chemokine-secreting T cells and was associated with antigen presentation on tumour cells yet cold tumours were strongly depleted of these[36].

The phenotypes of tumour cells themselves can also be profiled by multiplex imaging[46–50]. Despite tumour cell heterogeneity being a well-documented phenomenon[4,11,12], functional understanding of tumour cell phenotypes lags behind that of immune cells, which rests on decades of immunological work. One way around this is to draw on prior knowledge, such as a recent cyclic immunofluorescence (CycIF) study that used well-defined markers of the cell cycle to define the proliferation states of tumour cells in several cancer types; notably, the multivariate proliferation index defined using these markers identified proliferating cells that are missed by the more typically used marker Ki67 and that correlated with prognostic clinical features (tumour type, grade and p53 status) in breast cancer[47].

Alternatively, multiplex studies of tumour cell heterogeneity attempt to define cell phenotypic clusters in an unsupervised way and then link these clusters to orthogonal biological or clinical information. In our own 38-plex IMC work on breast cancer, we have used such a strategy to show that the composition of tumour cell phenotypes, which we called single-cell pathology groups, provides a more refined prediction of patient prognosis than standard clinical categories[48]. In parallel IMC work on the genomically characterized Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort, we found that both tumour and stromal cell phenotypes differ between breast tumours of different genomic subtypes and that the nature of the genomic lesion (that is, mutation versus copy number variation) can shape the tumour ecosystem[49]. In a separate IMC study of breast cancer, we found that tumour cell phenotypes diverge substantially between matched primary tumours and lymph node metastases. We identified poor-prognosis tumour cell phenotypes that are prone to disseminating and that frequently deviate from the clinical subtype, suggesting that these are an unused source of prognostic information for breast cancer[50].

In summary, tumour, immune, stromal or any other cell phenotypes can be studied to understand cellular heterogeneity in human tumours and how particular phenotypes may be associated with clinical or molecular features of interest.

The interactions between cell types in a tumour

Cells in a tumour are not isolated entities but sense their environment by constant physical and chemical contact with surrounding cells and matrix. In turn, cells integrate this information with their internal state and send signals to their environment. The ability to detect such interactions and infer such communication between diverse cellular phenotypes in situ and then relate them to essentially any aspect of cancer biology is an exciting aspect of multiplex protein imaging. Such analyses can enable the inference of molecular pathways, such as signalling pathways, that may be active within the tumour, help understand how the neighbours of a cell affect its state, and discern subtle organizational features of a tumour that may have functional consequences. In this section, we focus on the assessment of pairwise interactions between specific cell phenotypes.

The main approach being used to quantify cell interactions in tumours assesses the likelihood of interaction or avoidance between cell pairs, using permutations to control for whether this would occur at random[51]. Notably, a distinction should be made between analyses that report on a statistical association, in which simply whether the presence of cell types is correlated or anti-correlated in images is assessed (that is, without consideration of direct proximity), and those that report on a potential physical interaction, in which directly neighbouring cells are quantified[49]. Both analyses are useful but they report on different scales of organization: a correlation analysis assesses enrichment at the scale of the field of view (for example, hundreds of micrometres in a typical IMC study) whereas an interaction analysis examines proximity over one or two cell diameters.

Multiplex protein imaging studies on many cancer types (for example, breast, lung adenocarcinoma and glioblastoma) have noted that tumour cells, for the most part, are near other tumour cells, in other words, that they show homotypic interactions[32,33,48]. Such homotypic effects have also been shown for tumour proliferation states, with proliferating cells tending to form spatial domains in a CycIF study[47], and for individual tumour cell phenotypes as we have shown in IMC[48]. However, such relationships can be variable across tumour types. Basal-like breast tumours, for instance, showed more homotypic interactions for both tumour and stromal cells than other breast tumour subtypes in an IMC study, indicating a more extreme separation between these compartments in this relatively aggressive breast cancer subtype[49].

Despite being generally less frequent, heterotypic cell–cell interactions are just as relevant, if not more so, in terms of their functional effects. Indeed, interactions between tumour cells and $CD8^+$granzyme B^+ T cells or between tumour cells and B cells, together with the proliferative fractions of $CD8^+$ T cell factor 1 $(TCF1)^+$ T cells and major histocompatibility complex (MHC)-high tumour cells, were the best pre-treatment predictors of response to immune-checkpoint therapy in an IMC study of triple-negative breast cancer[52]. In a CycIF study of colorectal cancer (a cancer type known to be responsive to immune-checkpoint therapy), $PD1^+$ T cells were found to interact with $PDL1^+$ myeloid cells rather than with $PDL1^+$ tumour cells in a small cohort[53]. In another multiplex fluorescence study, in this case, in patients with follicular lymphoma, cell types with predictive value for early relapse were identified and seen to be near tumour cells[54]. As a last example, an IMC study of pairwise cell interactions in glioblastoma identified interactions between endothelial cells and several other cell types, including astrocytes, as expected, but also tumour cells and monocyte-derived macrophages, both of which showed lower levels of a proliferation marker (Ki67-to-cleaved caspase 3 (CC3) ratio) when they were near endothelial cells compared to when they were further away[33], suggesting a different microenvironment in the perivascular region.

Overall, by imaging many cell types within tumours at single-cell resolution, which cell types are more likely to be near each other, or indeed are often direct neighbours, and which cell types tend to avoid each other can be determined, providing insight into the functional effects that cells have on one another and how this may link to clinical and biological features of the disease.

Spatial patterns in a tumour

Beyond interactions between cell pairs, tumours harbour spatial patterns that are likely to be important for the manifestation of disease and that both affect intercellular communication and depend on it. These structures range from smaller local accumulations of cells or molecules to large structures like blood or lymphatic vessels, the tumour–stromal interface, or other tissue elements, such as tertiary lymphoid structures (TLS), that may reflect function. Indeed, tissue architecture encompasses well-known prognostic features such as tumour grade, which reflects cellular abnormality, or histological subtype, which has been defined for many cancer types. A part of the promise of multiplex imaging methods is that they will help to molecularly elucidate such spatial features in tumours and other tissue. We discuss here a few spatial analysis approaches that have been applied to multiplex tumour imaging; for a more complete overview of current spatial analysis approaches, we refer the reader to recent reviews[55–57].

Spatial analysis approaches for multiplex tumour imaging data (Fig. 3) may be broadly classified based on the number of cell types that are simultaneously profiled. Most simply, the spatial distribution of a single cell type within the whole tumour or with respect to some spatial landmark (for example, an intratumoural vessel or a tumour–stromal boundary) is determined; this has been referred to as a first-order analysis (Fig. 3a). For instance, we observed with RNA-IMC that cells expressing specific chemokines, in particular those encoded by the CXC-motif chemokine ligand 10 (*CXCL10*), *CXCL9* and *CXCL13* genes, are not randomly distributed but form patches in metastatic melanoma[36]. In a MIBI study on triple-negative breast cancer, Keren et al.[30] quantified the distribution of several cell types and markers relative to the tumour–immune border, finding this region depleted for B cells across their cohort and observing, in some patients, a gradient in the ratio of histone H3 methylation-to-acetylation levels in tumour cells along the perpendicular axis to the border. Given the known functional associations of these histone marks, this allowed speculation that tumour cells at or near the border are transcriptionally more active than those deeper within the tumour.

In a second-order analysis (Fig. 3b), the goal is to determine how pairs of cell types are distributed spatially with respect to each other and/or to the overall tissue structure. For instance, in their MIBI study on triple-negative breast cancer, Keren et al.[30] grouped tumours into cold (few immune cells), compartmentalized (immune cells present but spatially segregated from tumour) or mixed (immune cells present and spatially mixed with tumour) based on the ratio of tumour cell–immune cell interactions to immune cell–immune cell interactions, and showed that compartmentalized tumours had better prognosis than mixed ones. Interestingly, the spatial organization was also associated with cell type-specific patterns of immunoregulatory molecule expression. Compartmentalized tumours showed PD1 expression on $CD8^+$ T cells as well as PDL1 and indoleamine 2,3-dioxygenase (IDO) expression mostly on immune cells, whilst mixed tumours showed PD1 expression on $CD4^+$ T cells and PDL1 and IDO expression mostly on tumour cells[30].

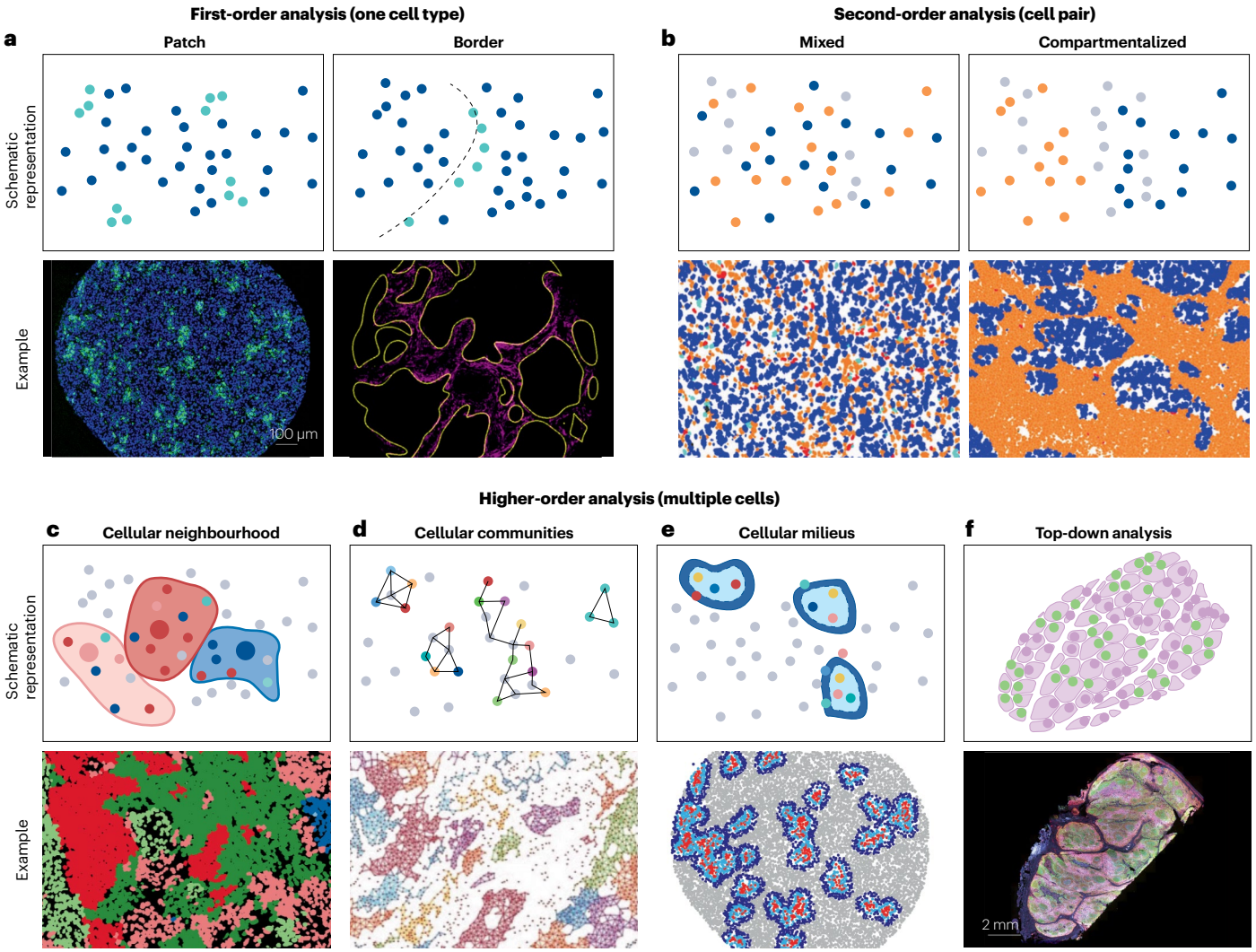

Fig. 3 | An overview of spatial analysis approaches for multiplex protein imaging data. Schematically depicted (top row in each case) are first-order analyses that determine the spatial distribution of a single cell type (light blue) either across a tumour (left) or with respect to a spatial structure of interest (for example, the tumour–stromal boundary, right) (part **a**); second-order analyses that determine the spatial distribution of two defined cell types (blue and orange) (part **b**); and higher-order analyses that define spatial patterns of two or more cell types (parts **c**–**f**). Depicted are higher-order approaches that define cellular neighbourhoods, local cell type enrichment patterns (red, blue or pink) identified in an unsupervised manner around a target cell of interest (part **c**); cellular communities, areas of dense cell–cell interaction defined with a graph-based approach (part **d**); cellular milieus, cell types in the area surrounding a spatial patch of a target cell type (part **e**); and a top-down analysis, in which markers and cell phenotypes that correspond to orthogonally defined spatial patterns (for example, histology) are defined (part **f**). Example images of each class of analysis are shown in the bottom row. Part **a**, bottom row, left panel and part **e**, bottom panel reprinted with permission from ref. 36, AAAS. Part **b**, bottom row, left and right panels reprinted with permission from ref. 30, Elsevier. Part **d**, bottom panel reprinted from ref. 47, Springer Nature Limited.

A similar spatial analysis was used in a MIBI study on colorectal cancer to show that cells tend to spatially cluster with other metabolically similar cells, in some cases even across cell lineages (that is, tumour, immune or stromal), providing evidence for microenvironmentally defined metabolic niches[39].

Higher-order analyses define local enrichment patterns of larger numbers of cell types, and we describe here the main approaches that have been used. Different terms have been used for the resulting local enrichment patterns (for example, neighbourhood, community and milieu). Since spatial analysis methods are still evolving, clear definitions of such terms according to the underlying analyses and the cellular relationships they describe are important.

In a co-detection by indexing (CODEX) study of colorectal cancer, Schürch et al.[58] defined local areas enriched in particular cell types, which they called cellular neighbourhoods (Fig. 3c). Here, the spatial scale of interest, that is, the area or number of cellular neighbours that constitute the neighbourhood of a target cell, must be decided, although we note that a more recently developed approach allows analysis at different scales[59]. In their initial study, Schürch et al.[58] chose 10 cellular neighbours as the relevant scale and determined

the fractions of each of 28 cell types within 10 cells of each target cell type. Cells were then clustered based on the cell type fractions in their neighbourhood, yielding 9 cellular neighbourhoods that could be annotated as, for example, T cell enriched, macrophage enriched, bulk tumour or immune-infiltrated stroma. When comparing tumours that did and did not have TLS at the invasive front, they interestingly observed that inclusion of information on cellular neighbourhoods (other than the TLS itself) improved the ability of a classifier to discriminate between these groups, indicating differences in spatial organization between them[58].

The same authors put cellular neighbourhoods to excellent use in a separate CODEX study of cutaneous T cell lymphoma[60]. In a comparison of responders and non-responders to anti-PD1 immunotherapy, they showed that, for the 21 (mainly immune) cell phenotypes that could be defined in a 51-plex study, cell type frequency showed no difference between the cohort groups but the spatial organization did: responders were enriched for a neighbourhood in which $CD4^+$ T cells are near tumour cells, whereas in non-responders, $CD4^+$ T cells are closer to T_{reg} cells. Using a spatial score to capture these distances and deconvolution of bulk RNA-seq data from sequential sections, a model emerged where responders are primed for $CD4^+$ T cell activation due to their spatial organization whereas non-responders have a relatively suppressed TME, thus possibly explaining the difference in their response to anti-PD1 therapy[60]. Yet again, a consideration of spatial features of the tumour ecosystem revealed a difference between two groups with very different clinical consequences that could not be seen with cellular phenotypes alone. CODEX was also used to define the cellular neighbourhoods of different macrophage subtypes in breast and colorectal cancer[38].

In an IMC study of lung adenocarcinoma, B cell-enriched cellular neighbourhoods were positively associated with overall patient survival, with the presence of T_{reg} cells within the cellular neighbourhood negating this advantage but $CD4^+$ T cells further increasing it[32]. Furthermore, a deep learning model trained on spatial image data from a piece of tissue the size of a biopsy could predict progression in early-stage lung cancer whereas information on only cell phenotypes could not[32]. A study of glioblastoma, again with IMC, identified a macrophage-enriched cellular neighbourhood associated with relatively better patient survival; based on this, the authors narrowed in on a specific macrophage phenotype that may be beneficial in this disease[33]. Recent work has also attempted to analyse the spatial disposition of cellular neighbourhoods with respect to each other to define higher-order motifs in human lymphoid tissue[61].

An alternative approach for spatial analysis, which we have applied in our own work, is to represent the tissue as a graph, with cells as nodes and direct interactions as edges, and then use graph-based methods to detect communities, or areas of dense interaction, within the tissue[48] (Fig. 3d). In this type of analysis, the distance at which cells are defined as direct neighbours must be specified. In our IMC study of breast cancer, communities defined based on tumour cells alone or on multiple cell types were associated with patient survival[48]. For example, a community of densely interacting tumour, stromal, endothelial and T cells, indicating vascularization together with immune cell involvement, was a negative prognostic factor for survival even though it was found within relatively good-prognosis hormone receptor $(HR)^+$ breast tumours[48]. Recently, Danenberg et al.[31] showed how such communities, again defined with a graph-based approach in IMC data, could be further grouped based on cell connectivity to define ten TME spatial subtypes within a breast cancer cohort. TME spatial subtypes were associated with patient prognosis as well as with genomic driver mutations and with clinical and molecular subtypes, further strengthening the case for spatial information in the study of tumour biology and disease outcome. In particular, a TME that represented a dysfunctional niche was associated with mutations in the *BRCA1* and *CASP8* (encoding caspase 8) genes and with poor prognosis in patients with oestrogen receptor $(ER)^+$ tumours but was also enriched in ER^- tumours[31].

In IMC of metastatic melanoma, we used a simple analysis of spatial patterns to infer signalling within tumours. We defined milieus as local environments around patches of chemokine transcript-expressing cells (up to 30 µm away) and then identified which cell types were present in these milieus and thus which intercellular signalling events might occur[36] (Fig. 3e). Different immune and stromal cell types showed different patterns, for instance, $CD8^+$ T cells were enriched most strongly in *CXCL9* milieus, stromal cells in *CXCL12* and CC-chemokine ligand 2 (*CCL2*) milieus, and B cells in *CXCL13* milieus. Furthermore, markers of T cell dysfunction and antigen experience were highly expressed in *CXCL10* and *CXCL9* milieus, which could indicate local areas of antitumour activity[36].

The spatial analysis methods we have discussed so far start by identifying cell phenotypes and then asking whether and how one or more of these phenotypes are spatially organized. However, an analysis may also take a top-down strategy, by starting with spatial structures identified in a histological or classical fluorescence stain, and then using multiplex imaging to characterize the underlying cells and molecules in more detail (Fig. 3f). In our own study of type 1 diabetes, we used low-plex immunofluorescence imaging to identify and select pancreatic islets within larger sections, followed by 35-plex IMC and analysis of beta cell loss in the disease[62]. More recently, Lin et al.[37] conducted a molecular characterization of large-scale, pathologist-selected tissue patterns in colorectal cancer, using 20–30 plex marker panels for immunofluorescence imaging. They found mixed histology between mucinous, glandular, solid and normal tissue regions within some tumours, at length scales ranging from a few cell diameters to the whole imaged section. No histological pattern corresponded to just one of the measured markers but rather these patterns were represented by multiple markers.

By starting with histological data, Risom et al.[63] analysed the early stages of cancer progression, examining the transition from ductal carcinoma in situ (DCIS) to invasive breast cancer. Here, H&E staining was used to first identify the DCIS and stromal areas; these were then analysed with 37-plex MIBI and with RNA-seq on microdissected regions. A combinatorial analysis considering cell phenotype composition, cell type proximities and morphometric features of the myoepithelium and the stroma found that, besides the defining feature of myoepithelial loss, later-stage invasive cancer strongly differed from early-stage DCIS in the stromal compartment, with increased stromal cell proliferation and frequency and fibrillar collagen deposition. By contrast, DCIS had a more complex profile, the most defining features being proliferating myoepithelial cells and the presence of stromal mast cells and $CD4^+$ T cells. A comparison of patients diagnosed with DCIS who did or did not progress showed that loss of myoepithelial integrity, somewhat paradoxically, appears to be protective in DCIS, possibly because this is associated with greater immune and stromal cell activation[63]. Such a conclusion would have been difficult to draw with a study that ignored spatial aspects of the disease or did not include the many markers needed to examine multiple compartments.

Cohort design to identify clinical associations

Together, multiplex imaging methods have demonstrated the potential to identify informative molecular, cellular, multicellular and spatial features in human tumour tissue. For association with clinical data, cohort design is a crucial element and, although this will vary with the particular study, a few general principles apply. Clinical data that are as well-annotated and complete as possible are important as are cohort groups that are balanced for age, sex, genetic ancestry and tumour subtype (where relevant) when a comparison is to be made between groups. For cohorts of patients with cancer, treatment history is often not under experimenter control but a similar history or, at minimum, complete annotation of the history will be essential for data interpretation. Cohort size will ideally be driven by an estimate of the expected effect size but this is also often unknown. Defined biological questions, possibly developed in a pilot study, are of tremendous help in assembling the final cohort; for specific clinical questions, especially regarding treatment response, samples from a controlled clinical trial are likely to be the most fruitful path.

Multiplex protein imaging methods

We provide here a brief overview of the principles, key features and most important practical considerations of the multiplex protein imaging methods that have been applied to the study of tumours. For a more detailed description of these methods, we refer the reader to recent reviews[27,64,65]. Multiplex single-cell-resolved protein imaging or profiling methods can be grouped into four classes based on the readout and probes used (Fig. 4). This may be via mass spectrometry, fluorescence or

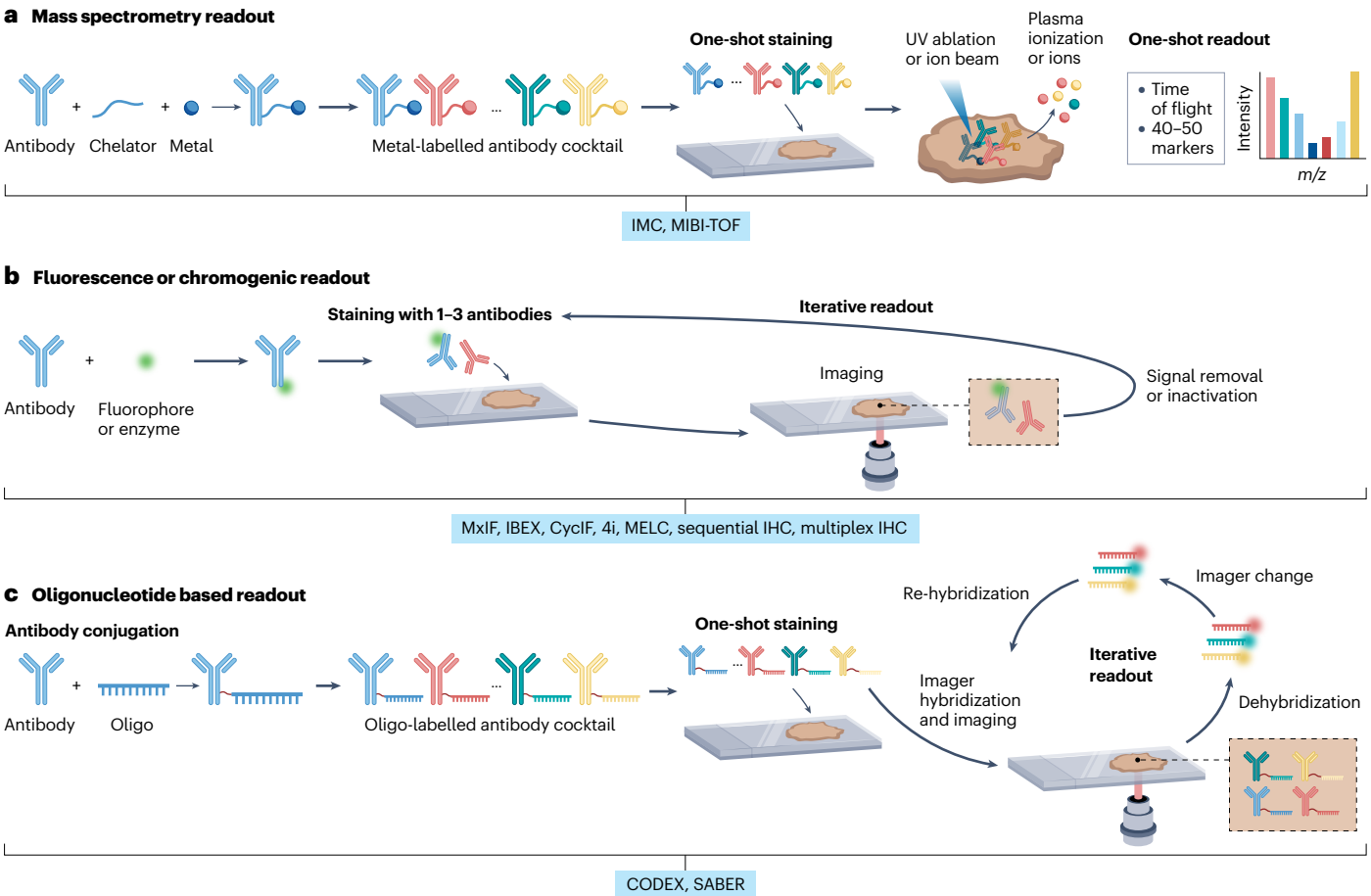

Fig. 4 | The principles of single-cell multiplex protein imaging methods. The methods are grouped into three classes based on the readout that is used. Each panel indicates whether the method is iterative or one-shot. The specific methods that belong to each class are listed below each panel. **a**, Mass cytometry-based methods rely on mass spectrometry to detect metal isotopes coupled to antibodies via a chelating polymer. The metal-labelled antibody cocktail is used to stain a sample of interest; for imaging, ionization of metal isotopes is achieved either with an ion beam or a laser coupled to an ionizing plasma source. **b**, Fluorescence or chromogenic methods rely on conventional imaging of fluorophores or of deposited chromogens. Typically, 1–3 antibodies are used at a time to stain the sample, followed by imaging of a fluorophore (for immunofluorescence) or chromogen (for immunohistochemistry (IHC)). Once imaging is complete, the signal is quenched by fluorophore inactivation, chromogen removal or antibody stripping and the cycle is iterated to reach the desired plex. **c**, Fluorescent oligonucleotide-based methods depend on antibodies conjugated to specific oligonucleotides (oligos), followed by detection of these oligos with complementary DNA imager probes. An oligo-tagged antibody cocktail is used to stain a sample; fluorescent imager probes are then allowed to hybridize, imaged and stripped off, and the process is iterated to reach the desired plex. 4i, iterative indirect immunofluorescence imaging; CODEX, co-detection by indexing; CycIF, cyclic immunofluorescence; IBEX, iterative bleaching extends multiplexity; IMC, imaging mass cytometry; MELC, multi-epitope-ligand cartography; MIBI-TOF, multiplexed ion beam imaging-time of flight; MxIF, multiplexed immunofluorescence; *m/z*, mass-to-charge ratio; SABER, signal amplification by exchange reaction.

chromogenic imaging, oligonucleotide-based fluorescence imaging, or sequencing, all of which have distinct advantages and disadvantages with respect to resolution, plex, throughput and signal-to-noise ratio (Table 1). The different methods vary also in terms of the level of tissue autofluorescence they must contend with, the possibility for signal amplification, and the ability to linearly quantify targets rather than simply give a binary positive or negative signal, all of which affect performance. We also illustrate a typical multiplex imaging analytical workflow (Fig. 5) and provide an overview of the functions of currently used open-source computational tools (Supplementary Table 1). A more detailed description of multiplex image analysis workflows may be found in a recent protocol from our laboratory[66] and associated online documentation of IMC data analysis as well as in several recent reviews[55–57,67–69].

Mass spectrometry readout

MIBI and IMC rely on mass spectrometry to detect metal isotopes coupled to antibodies, with ionization achieved either with an ion beam (MIBI)[70,71] or a laser coupled to an ionizing plasma source (IMC)[72] (Fig. 4a). These techniques allow simultaneous staining with antibodies against 40–50 markers, are also compatible with RNA and DNA detection[36,45,73] and, since they are based on the detection of exogenously added non-biological metal isotopes, avoid the background signal that plagues fluorescence and chromogenic techniques. IMC has been extended to three dimensions by imaging of serial slices, allowing 3D reconstruction of breast tumour samples at single-cell resolution[74]. It has been combined with signal amplification by exchange reaction (SABER) based on cyclic hybridization of metal-labelled oligonucleotides, achieving up to 68-fold amplification while maintaining the signal-to-noise ratio, thus enabling the imaging of low abundance molecules that are difficult or impossible to see with standard approaches[75]. SABER-IMC is compatible with the standard non-amplified IMC workflow, making it possible to tune the imaging of each marker (that is, with or without amplification as well as the level of amplification), depending on its starting signal.

MIBI has also been applied to 3D serial-plane imaging of formalin-fixed paraffin-embedded (FFPE) tissue[71] and of cultured cells at super resolution (30 nm lateral, 5 nm axial) allowing subcellular imaging of the distribution of the platinum drug cisplatin[76]. Both MIBI and IMC enable the detection of high-mass endogenous elements such as iodine and exogenously introduced metals (for example, cisplatin chemotherapy, or gadolinium in magnetic resonance imaging (MRI)). Both approaches were commercialized, with IMC instruments still commercially available as the Hyperion instrument from Standard Biotools, both are applicable to frozen and FFPE tissue samples, and both have been applied in several studies on clinical cohorts[30–33,36,48–50,63]. A key challenge is that the relatively low scanning speed of these approaches means that they are typically used to image smaller areas compared to immunofluorescence or immunohistochemistry (IHC).

Fluorescence readout

Despite the challenges in using a fluorescence readout in a multiplex fashion, fluorescence microscopy is a relatively user-friendly approach and has been adapted for multiplex use. Multiplex fluorescence methods fall broadly into two classes. Multispectral imaging takes a computational spectral demixing approach to separate overlapping fluorophore emission spectra and has achieved up to 20-plex imaging[53,77]; the PhenoImager HT (formerly known as Vectra Polaris) and more recently the Orion are commercially available multispectral instruments. A related one-shot, though lower-plex, approach is Histo-cytometry, which uses conventional high-resolution confocal microscopy with careful spillover correction and voxel gating to identify cells of interest expressing the appropriate marker combinations[78]. Alternatively, fluorescence imaging of 40–50 targets can be achieved using iterative methods, in which each staining and imaging round is followed by antibody stripping or fluorophore inactivation, after which the next round of staining and imaging can take place (Fig. 4b). Fluorophore inactivation has been achieved by chemical quenching in multiplexed immunofluorescence microscopy (MxIF)[46], by bleaching in iterative bleaching extends multiplexity (IBEX)[79] and in multi-epitope-ligand cartography (MELC)[80], or by a combination of the two in tissue-based CyCIF (t-CyCIF)[37,81]; several iterative fluorescence-based approaches have been commercialized (Table 1). Alternative strategies use the stripping of antibodies themselves[82], under mild elution conditions in the case of 4i (iterative indirect immunofluorescence imaging)[83]. Cyclic fluorescence methods can conveniently use commercially available antibodies and have recently begun to be applied to patient cohorts[37,84,85], but they are challenging because of the long times required to reach high-plex and because fluorescent signal removal can alter or destroy epitopes and tissue architecture.

Chromogenic readout

Classical IHC, a well-established clinical pathology technique, has also been used in an iterative mode to achieve multiplex protein imaging (Fig. 4b). Each antibody is detected by horseradish peroxidase (HRP)-based chromogen deposition and imaging followed by de-staining with an organic solvent. In multiplexed immunohistochemical consecutive staining on single slide (MICSSS), effective staining and de-staining required blocking of the previous epitope to counter incomplete de-staining[40]. Multiplexed IHC has been applied to numerous patient cohorts[17,40,41,86,87]. However, even more so than iterative fluorescence imaging, these approaches are time intensive and image registration poses challenges.

Fluorescence or sequencing readout based on oligonucleotide-tagged antibodies

Multiplex imaging via sequential detection has also been achieved with oligonucleotide-tagged antibodies (Fig. 4c) in CODEX, which originally employed fluorescence labelling via primer extension and fluorophore removal by chemical reduction[88], and was then upgraded to more rapid hybridization and stripping of complementary imager probes[58,89]. The approach requires antibody conjugation with DNA barcodes as well as validation of conjugated antibodies (see section on Technical challenges below), has been commercialized by Akoya Biosciences, and has been applied to patient cohorts[58,60]. Since CODEX lacks amplification, in contrast to secondary antibodies or HRP, but rather includes a single fluorophore per imager, sensitivity can be a challenge. The problem has been circumvented by the development of Immuno-SABER (immunostaining with SABER), which can amplify the fluorescent signal up to 188-fold while retaining multiplexity[90]. Oligonucleotide-tagged antibodies are also amenable to a sequencing readout[91–95], as described below.

The rise of spatial multi-omics

In an ideal scenario, it would be possible to directly image cell phenotype, function, state and intercellular communication simultaneously within a tissue, which would most probably require merging the measurement of many types of molecules (that is, transcripts, proteins,

Table 1 | Features of multiplex protein imaging technologies

Technology	Principle	Typical resolution[a] (μm)	Target types	Reported number of protein targets	Background[b]	3D shown?	Protein signal amplification shown?	Tissue types	Commercialization	Key ref.
IMC	Mass spectrometry	1	Protein, nucleic acid	40+	N	Y	y	FF, FFPE	Standard BioTools	72
MIBI	Mass spectrometry	0.26	Protein, nucleic acid	40+	N	Y	Y	FF, FFPE	–	70
DSP	DNA sequencing	50	Protein, nucleic acid	70+	N	N	N	FF, FFPE	NanoString	92
CODEX	Iterative DNA hybridization	0.25	Protein	56+	Y	N	N	FF, FFPE	Akoya Biosciences	58
Immuno-SABER	Iterative DNA hybridization	0.32	Protein	10+	Y	Y	Y	FF, FFPE	–	90
CycIF	Iterative fluorescence inactivation	~0.2	Protein	60	Y	Y	N	FF, FFPE	–	136
IBEX	Iterative fluorescence inactivation	~0.2	Protein	65+	Y	N	N	FFPE	–	79
MxIF	Iterative fluorescence inactivation	~0.2	Protein, nucleic acid	61	Y	N	N	FFPE	–	46
MELC	Iterative fluorescence inactivation	~0.2	Protein	90+	Y	Y	N	FF, FFPE	–	80
4i	Iterative antibody removal	0.165	Protein	40+	Y	N	N	NA[c]	–	83
MICSSS	Iterative antibody removal	~0.4–0.7	Protein	10	Y	N	Y	FFPE	–	40
mIHC	Iterative antibody removal	~0.2	Protein	29	Y	N	Y	FFPE	–	43
MACSima	Iterative fluorescence removal	0.17	Protein	60	Y	N	N	FF, FFPE	Miltenyi	162
InSituPlex	DNA hybridization	~0.2	Protein	12	Y	N	N	FFPE	Ultivue	163
Vectra	Iterative antibody removal	0.25	Protein	6	Y	N	Y	FFPE	Akoya	164
MultiOmyx	Iterative fluorescence inactivation	~0.2	Protein, nucleic acid	9	Y	N	N	FFPE	Leica	165
Orion	Computational spectral demixing	~0.2	Protein	18	Y	N	N	FF, FFPE	Rarecyte	53
COMET	Iterative antibody removal	~0.2	Protein	40	Y	N	N	FFPE	Lunaphore	166
CellScape	Iterative fluorescence inactivation	0.182	Protein	18	Y	N	N	FF, FFPE	Canopy	167

Values are current as of November 2023 but are likely to change over time. 4i, iterative indirect immunofluorescence imaging; CODEX, co-detection by indexing; CycIF, cyclic immunofluorescence; DSP, digital spatial profiling; FF, fresh frozen; FFPE, formalin-fixed paraffin-embedded; IBEX, iterative bleaching extends multiplexity; IMC, imaging mass cytometry; Immuno-SABER, immunostaining with signal amplification by exchange reaction; MELC, multi-epitope-ligand cartography; MIBI, multiplexed ion beam imaging; MICSSS, multiplexed immunohistochemical consecutive staining on single slide; mIHC, multiplexed immunohistochemistry; MxIF, multiplex immunofluorescence; N, no; NA, non-applicable; Y, yes. [a]Note that resolution for all methods depends on the strength of the imaged signal and on the instrument used. [b]Background refers to autofluorescence in most cases. [c]4i has only been reported on cell samples.

glycans, metabolites and lipids) that provide complementary information about biological systems. Multi-omics analyses are accordingly now at the forefront of in situ studies of tumour ecosystems, with most studies so far focusing on merging transcript-level data with protein-level data[96] (Fig. 6). Single-cell transcriptomes can, in principle, provide a more comprehensive and nuanced view of the cell types in a tumour since they measure thousands of features rather than the dozens accessed by multiplex protein imaging. In practice, however, the relative sparsity of spatially resolved single-cell transcript data means that this potential has not yet been achieved.

Multi-omics may be achieved experimentally (that is, via co-detection of two or more classes of molecules) or through data integration. There are so far only a few demonstrations of experimental multi-omics data, where different readouts are obtained on the very same sample and more than one type of molecule is measured for the same single cells. Typically, this requires that all types of molecules being detected are read out in the same way. For instance, we have achieved simultaneous protein and RNA imaging by rendering RNA detectable by metal isotope-labelled probes, such that RNA-IMC can then be used for both proteins and mRNA on the same sample (Fig. 6a).

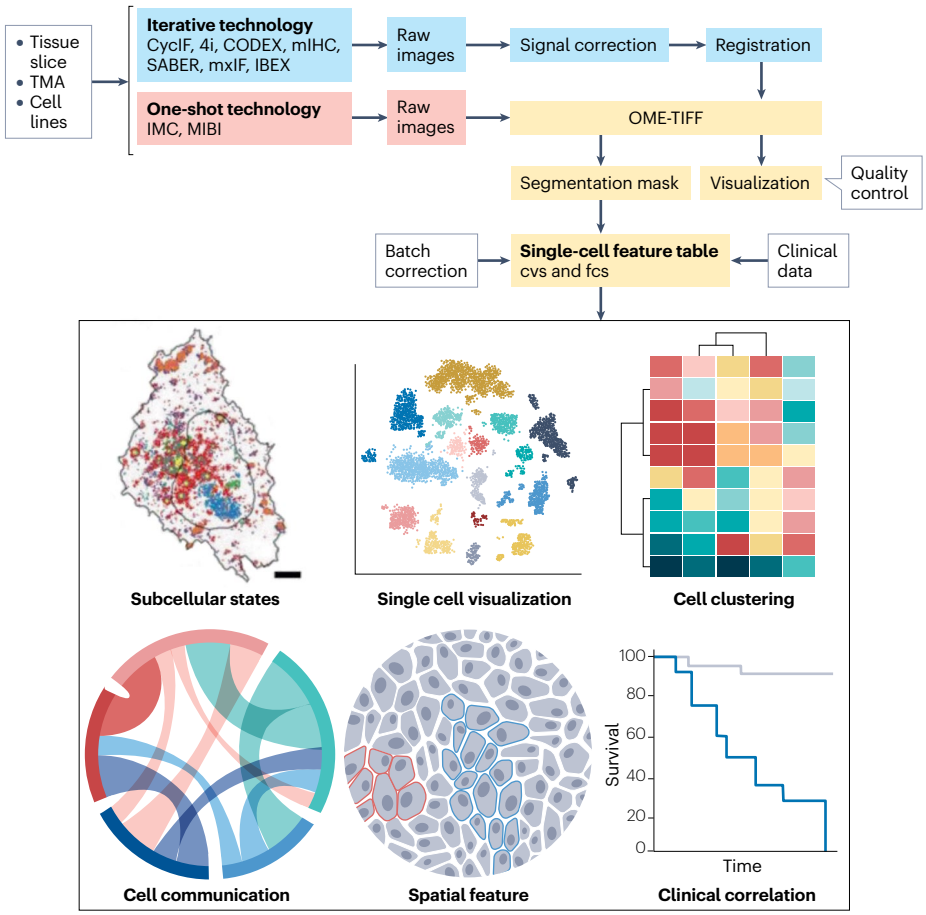

Fig. 5 | Canonical pipeline for multiplex protein image processing. The wealth of information generated by multiplex protein imaging cannot be fully exploited by general image analysis tools; for example, Fiji[168], Qupath[169] and Napari. The schematic briefly illustrates a typical multiplex image analysis workflow; details have been covered in recent reviews[55–57,67–69] and in online documentation of IMC data analysis from our laboratory[66]. Open-source computational tools and their uses are summarized in Supplementary Table 1. After image acquisition, raw data files (typically CZI, TIFF, OME-TIFF or MCD formats) are first visualized using standard tools for quality inspection. Images from iterative methods (typically fluorescence based) are then denoised using tools like BaSiC[170] and spatially registered across all imaging cycles. Images from one-shot methods are denoised by imaging mass cytometry (IMC)[171] or 'hot pixel-filtered' to remove artefactual high-intensity spots and converted to common imaging formats (for example, OME-TIFF or TIFF)[66]. This is followed by cell segmentation using random forest-based pixel classification with Ilastik[172] or deep learning-based methods such as Mesmer[141] and CellPose[140]. Image features, including signal intensities of all imaged markers and cell morphology descriptors (for example, cell area and axis lengths), are then computed per cell or per region. Finally, the resulting spatially resolved single-cell feature table is used for visualization by dimensionality reduction and various quantitative analyses, such as cell phenotyping, differential analyses of marker expression, cell interaction analysis, and various higher-order spatial analyses[66]. There are increasing numbers of graphical user interface (GUI) software or script-based pipelines dedicated to multiplex imaging data analysis, but the need in the field is for open-source, universal, end-to-end frameworks that are compatible with multi-platform file formats, supportive of big data storage and handling, and that can allow for the implementation of existing packages or software as well as ongoing method development, for example, Multiple Choice MICROscopy (MCMICRO)[173] or SpatialData[174]. 4i, iterative indirect immunofluorescence imaging; CODEX, co-detection by indexing; CycIF, cyclic immunofluorescence; IBEX, iterative bleaching extends multiplexity; MIBI, multiplexed ion beam imaging; mIHC, multiplex immunohistochemistry; MxIF, multiplexed immunofluorescence microscopy; SABER, signal amplification by exchange reaction; TMA, tumour microarray. The image for subcellular states is reprinted with permission from ref. 82, AAAS.

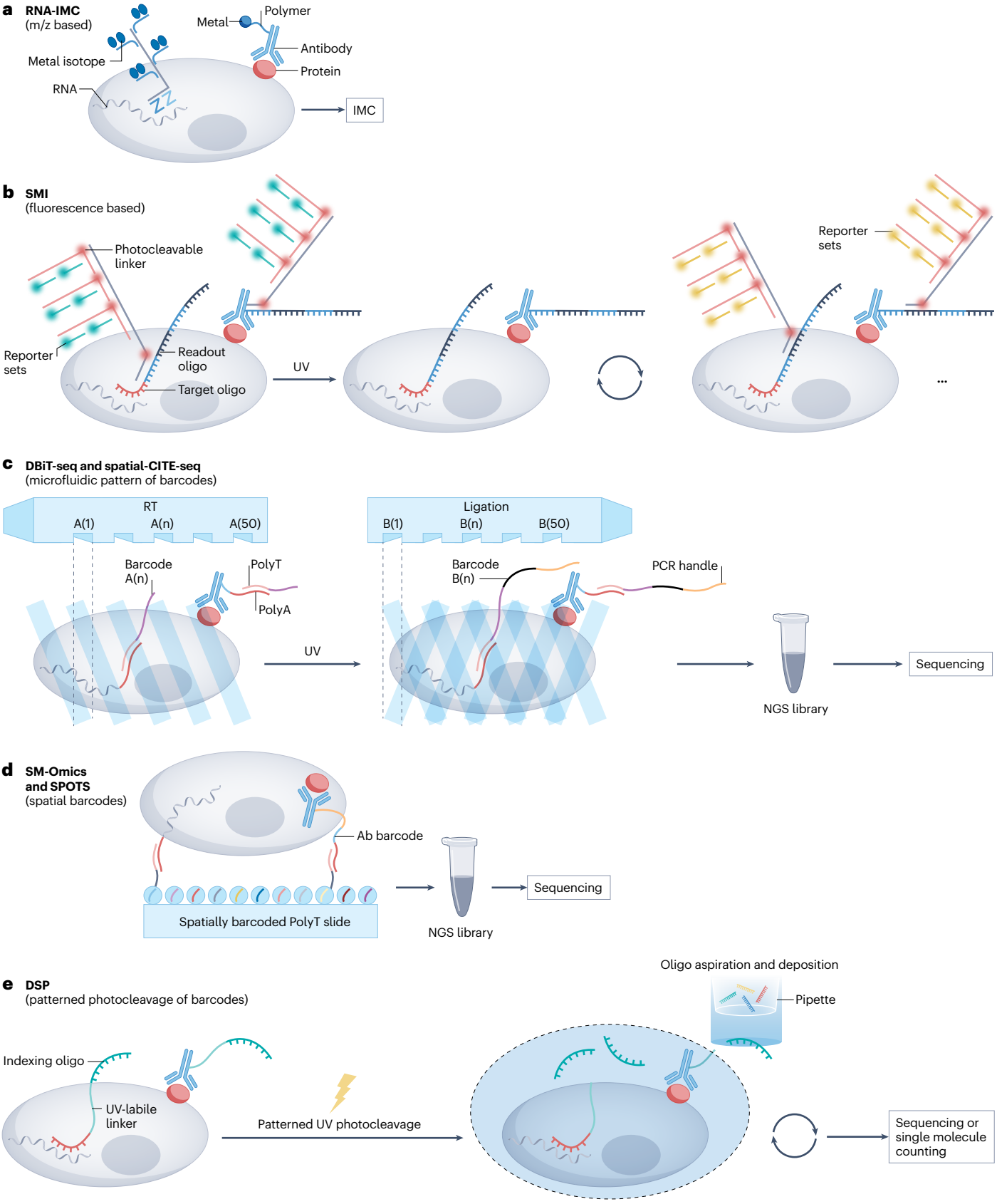
a RNA-IMC
(m/z based)
Metal
Polymer
Antibody
Protein
Metal isotope
RNA
IMC
b SMI
(fluorescence based)
Photocleavable linker
Reporter sets
Readout oligo
Target oligo
UV
Reporter sets
...
c DBiT-seq and spatial-CITE-seq
(microfluidic pattern of barcodes)
RT
A(1)
A(n)
A(50)
Barcode A(n)
PolyT
PolyA
UV
Ligation
B(1)
B(n)
B(50)
Barcode B(n)
PCR handle
NGS library
Sequencing
d SM-Omics and SPOTS
(spatial barcodes)
Ab barcode
Spatially barcoded PolyT slide
NGS library
Sequencing
e DSP
(patterned photocleavage of barcodes)
Oligo aspiration and deposition
Pipette
Indexing oligo
UV-labile linker
Patterned UV photocleavage
Sequencing or single molecule counting

Fig. 6 | The principles of spatial multi-omics techniques. The schematic shows techniques that have been used for multiplex imaging or profiling of both proteins and RNA. From top to bottom, the panels depict: **a**, Imaging of RNA, by rendering this molecule detectable with metal isotopes (RNA imaging mass cytometry (RNA-IMC). **b**, Imaging of RNA or protein with fluorescent and barcoded reporter oligonucleotides (oligos), either directly binding a barcoded oligonucleotide to RNA or via oligo-tagged antibodies (for protein). This spatial molecular imaging (SMI) technique is iterative and has not been demonstrated simultaneously for RNA and protein detection. **c**–**e**, In these panels, we represent sequencing-based profiling to measure RNA (directly) or protein (via oligo-tagged antibodies). Illustrated are different ways of achieving spatial barcoding. In deterministic barcoding in tissue-sequencing (DBiT-seq) and spatial cellular indexing of transcriptomes and epitopes by sequencing (spatial-CITE-seq), this is achieved by spatially controlled microfluidic delivery of probes that are ligated together to generate a unique barcode per position (**c**). In spatial multi-omics (SM-Omics) and spatial protein and transcriptome sequencing (SPOTS), spatial barcoding is achieved via immobilization of barcoded oligos on slides. Barcoded oligo spots are shown schematically (**d**). In digital spatial profiling (DSP), light is used to photocleave light-labile oligos in the desired pattern (**e**). Ab, antibody; *m*/*z*, mass-to-charge ratio; NGS, next-generation sequencing; RT, reverse transcription; UV, ultraviolet.

We applied this dual RNA–protein imaging, of up to 12 transcripts and 21 proteins (plus another 39 proteins on sequential sections), to FFPE samples of metastatic melanoma[36]. This allowed spatial profiling of cell phenotypes that express various chemokines that, being secreted molecules, are difficult to study and to associate with single cells based solely on protein imaging.

In the protein and nucleic acid in situ imaging (PANINI) approach for simultaneous detection of proteins and up to two different RNA or DNA molecules, Jiang et al.[73] alternatively used tyramide-based amplification to deposit haptens, rendering nucleic acids sensitively detectable by antibodies and thus compatible with antibody-based protein detection methods. PANINI was applied to 33-plex protein imaging and 2-plex nucleic acid imaging, detecting single viral integration events to reveal the interplay between human immunodeficiency virus (HIV) infection, viral transcription and cellular immune neighbourhoods in simian lymph nodes[73]. Alternatively, spatial molecular imaging (SMI), commercialized as the Nanostring CosMX platform, measures RNA (directly) or protein (via oligonucleotide-tagged antibodies) by sequential hybridization of fluorescently barcoded oligonucleotide probes[97] (Fig. 6b). SMI has been reported to detect around 1,000 transcripts or 100 proteins in FFPE tissue, but simultaneous imaging of both species has not yet been shown; rather, protein and RNA are imaged on sequential sections that do not typically allow confident cell–cell matching. Notably, SMI has been reported to detect, on average, 200–300 transcripts per cell in the context of FFPE tissue[97]; given this sparsity, targeted approaches are still a sensitive and cost-effective means for spatial single-cell profiling.

Another approach for simultaneous protein and transcript profiling has been to render both types of molecules detectable by sequencing, with different strategies used to spatially map the sequencing readout. In deterministic barcoding in tissue-sequencing (DBiT-seq) and spatial cellular indexing of transcriptomes and epitopes by sequencing (spatial-CITE-seq), spatial barcoding is achieved by controlled microfluidic delivery of the probes used to generate sequencing libraries, with sequencing used to detect both transcripts and antibody-linked DNA oligonucleotide tags[91,94] (Fig. 6c). In DBiT-seq, whole transcriptomes and 22 proteins were profiled in mouse embryos at pixel sizes down to 10 µm (ref. 91). This was then extended, as spatial-CITE-seq, to map about 200 surface proteins together with whole transcriptomes at pixel sizes of 25 µm in mouse and human tissue[94]. Although these methods are approaching the dimensions of single cells, they still fall short of true single-cell resolution within tissue; combination with scRNA-seq data, H&E or immunofluorescence data can be used to help annotate single cells[91,94]. Other potentially higher-resolution spatial barcoding approaches such as Light-seq, which uses light to crosslink barcodes to cDNAs in cells of interest[98], or Zip-seq, which prints barcodes directly on cells[99], have so far only been used to read out transcriptomes from the barcoded cells but could possibly be extended to protein measurements in the future.

The spatial multi-omics (SM-Omics) approach, also based on a sequencing readout, achieves spatial barcoding via immobilization of barcoded oligonucleotides on glass slides, that is, a spatial transcriptomics workflow[93] (Fig. 6d). SM-Omics was applied in proof-of-principle work to measure six DNA-tagged antibodies and whole transcriptomes simultaneously in mouse spleen. DNA-tagged antibodies have also been combined with spatial transcriptomics in spatial protein and transcriptome sequencing (SPOTS) for simultaneous profiling of whole transcriptomes and 32 proteins, achieving differential localization of macrophage subtypes in mouse breast tumours[95], and more recently in Stereo-CITE-seq with the goal of improving spatial resolution[100]. These approaches have so far been used on fresh frozen tissue and performance in FFPE tissue remains to be assessed.

The DSP method[92,101,102], which uses patterned light to achieve spatial barcoding, has been commercialized by Nanostring as the GeoMX platform. In DSP, photocleavable oligonucleotides are linked either to antibodies for protein detection or to nucleic acid probes for transcript detection, photocleaved off in the desired pattern, collected, and then sequenced or counted to identify the target molecules in the selected region (Fig. 6e). The approach has typically been used so far to profile up to 70–80 proteins[92,102–106], in some cases with up to 1,400–2,100 transcripts in sequential sections of FFPE tissue[92,102]. Recently, optimization of sample preparation has achieved co-detection of 100+ proteins and whole transcriptomes in FFPE samples[107]. For abundant proteins and high-quality antibodies, DSP can achieve single-cell sensitivity[92] but, in studies of tumour tissue, it has so far been applied to much larger regions of interest (that is, several hundreds of micrometres in length or diameter), thus profiling many hundreds of cells.

Data integration between transcript-level and protein-level data has also been achieved computationally, without co-detection in the same sample. The prerequisite for any such data integration is that shared molecular features exist and that these molecular features show a relationship to other non-shared molecular features within a given dataset. The STvEA and MARIO approaches achieve data integration by using reference data from CITE-seq, an existing integrated method[108]. In these approaches, single-cell proteomic data from a multiplex imaging dataset are integrated with data from a parallel CITE-seq experiment using an overlapping antibody panel and the same tissue type. This then allows the single-cell transcriptomes from CITE-seq to be mapped onto the imaged cell types, thus enriching and improving cell type annotation in multiplex imaging data[109,110]. The two methods use different mathematical principles for data integration, with MARIO being designed for single-cell proteomic data with fewer overlapping features between

Glossary

Cell segmentation
An image processing step that delineates the boundaries of individual cells.

Chromogen
A compound that can be converted into a coloured product that can be detected by light microscopy.

Co-detection by indexing
(CODEX). A highly multiplex protein imaging technique that uses iterative hybridization and stripping of fluorophore-tagged DNA oligonucleotide probes to image samples stained with DNA barcode-tagged antibodies.

Cyclic immunofluorescence
(CycIF). A highly multiplex imaging technique using iterative staining with fluorophore-tagged antibodies coupled with chemical inactivation of fluorophores between staining cycles to build a multiplex image of a labelled sample.

Digital spatial profiling
(DSP). A highly multiplex profiling technique for mRNA or protein that uses patterned light to release UV-photocleavable oligonucleotide tags attached to antibodies or to RNA probes in a defined spatial region, followed by sequencing or single molecule counting as a readout.

Dimensionality reduction
A data-processing approach whereby high-dimensional data are projected into a low number of dimensions represented by a smaller subset of variables with essentially the same information content as the full measured set.

Expansion microscopy
An approach that uses polymer-based physical expansion of a sample to improve the resolution of fluorescence microscopy beyond the diffraction limit of light.

Haptens
Small molecules that are not intrinsically antigenic but become so in combination with a macromolecule such as a protein.

Image registration
Data processing steps that bring two or more different images into a single coordinate system such that the images can be aligned.

Imaging mass cytometry
(IMC). A highly multiplex protein or RNA imaging technique that couples mass cytometry by time of flight with high-resolution laser ablation to image samples labelled with metal isotope reporter-tagged antibodies.

Imputation
An approach for handling missing data, typically by replacement with substitute values.

Multiplexed ion beam imaging
(MIBI). A highly multiplex protein imaging technique that uses secondary ion mass spectrometry to image samples labelled with metal isotope reporter-tagged antibodies.

Raman microscopy
Spatially resolved chemical analysis of a sample based on the detection of vibrational modes by scattered light.

Signal amplification by exchange reaction
(SABER). A signal amplification approach based on hybridization of imager DNA strands to concatemerized DNA barcodes assembled on antibodies used to label a sample; compatible with fluorescence and mass cytometric multiplex imaging.

Spillover correction
Data processing steps that compensate for fluorescent or metal signals from one channel that are detected artefactually in a different channel.

Synchrotron
A machine that accelerates charged particles (electrons) to almost the speed of light and thereby generates very intense light, mostly in the X-ray region.

Tertiary lymphoid structures
(TLS). Structured multicellular aggregates of immune cells found outside of lymph nodes, in peripheral tissue, and that reflect inflammatory signalling in the tissue.

Tyramide-based amplification
A signal amplification approach in which horseradish peroxidase (typically coupled to an antibody) catalyses the conversion of labelled tyramide to a reactive molecule that covalently labels nearby proteins at high density.

Voxel gating
Data processing steps to identify a cell of interest based on selecting voxels that are positive for expected markers and negative for incorrect or irrelevant markers.

datasets than transcriptomics data; it can be used for data integration across experiments and species as long as the datasets have sufficient marker and biological overlap[110]. The principles behind MARIO were further extended to develop MaxFuse, which can integrate datasets with even fewer overlapping features[111]. MaxFuse does not require the reference CITE-seq dataset for transcriptome and proteome integration and has been applied to integrate multiplex CODEX data with single-cell RNA-seq and single nucleus transposase-accessible chromatin with sequencing (snATAC-seq) data with good performance[111].

Several studies also simply use two or more orthogonal techniques, for instance, multiplex protein imaging and transcriptomics, on the system of interest but do not attempt true data integration. For example, multiplex protein imaging has been used to visualize transcriptomics-defined cell types or TMEs in breast cancer[23,112], small-cell lung cancer[113], ovarian cancer[114] and squamous cell carcinoma[115]. Alternatively, it has provided a detailed view of cell type distribution within tumour areas mapped with lower-resolution spatial transcriptomics[116].

Also, the levels of glycans, lipids or metabolites can reflect functional events or phenotypes that can only be indirectly inferred from protein or transcript measurements. However, measurement of lipids and metabolites in combination with spatial protein analysis is currently not possible because tissue preparation protocols are incompatible or relevant binders do not exist. The method of choice to measure these classes of molecules in a spatially resolved manner is mass spectrometry imaging (MSI), a set of label-free approaches that can detect a range of molecules based on their mass-to-charge ratio[117–119]. Recent technical developments have improved the performance of MSI methods to single-cell or near-single-cell resolution (5–10-µm pixel size) in tissues[120–122], but true single-cell studies are still largely restricted to isolated cells in vitro[123,124]. In the tissue context, MSI has been combined with H&E staining to define metabolite or lipid profiles in histologically different areas of patient tumours in several types of cancer[125–127] or to measure changes in glycan profiles during breast cancer metastasis[128]. Combined with immunostaining, MSI has been used to determine lipid distributions in the pancreas[129], to profile human epidermal growth factor receptor 2 ($HER2)^{+}$ versus $HER2^{-}$ cells in human gastric tumours[130], to image the lipid environment of different macrophage subtypes in mouse atherosclerotic plaque[77], and to study metabolic changes during tissue repair in a mouse kidney injury model[131]. While many challenges

remain, including achieving true single-cell resolution and robust chemical identification of large numbers of molecular species within tissue, multimodal approaches that incorporate MSI could provide an exciting alternative to the more typical protein-centric view of tissue function and dysfunction and bring us closer to a functional understanding of tumours and their microenvironment.

Technical challenges

Like all methods, multiplex protein imaging methods have inherent assumptions and limitations that must be considered if the results they generate are to be meaningful. Here, we address the main technical challenges that apply generally across many of these approaches.

The challenge of undersampling

An ideal multiplex tumour imaging method would be fast enough and cheap enough to rapidly image many whole tumours, in three dimensions, at single-cell resolution without compromising multidimensionality. However, no existing method fits this bill. Even ignoring the issue of three-dimensionality, since there is as yet no scalable multiplex 3D approach, decisions must be made about how much of a patient tumour to sample in 2D to measure representative cell and tissue features, how many tumours to sample, and how the sampling should be spatially distributed.

Typical imaged areas for MIBI and IMC are small (in the millimetre squared range) mainly due to speed limitations, and fluorescence microscopy methods, although faster, face similar problems. Large cohort analyses of whole tumour sections are thus prohibitive and many studies make use of tumour microarrays (TMAs), in which punches of tumours are sectioned and arrayed on glass slides that can be processed and imaged together and in high throughput. TMAs are routinely used in pathology research and have yielded many biomedical findings; their use makes multiplex imaging applicable to large patient cohorts[30,32,48,58,92]. However, recent work using whole-slide imaging with CycIF as the ground truth concluded that, for spatially correlated samples (that is, samples where cellular and supracellular features are not homogenous) from a colorectal cancer cohort, single TMA cores could not capture representative cell abundances or interactions[37]. The extent to which this is generally true will strongly depend on the abundance, spatial distribution and length scales of the feature in question as well as on the tumour type. The area of a tumour that should be imaged therefore depends on the properties of the tissue, and the question to be answered (for example, cell phenotype detection versus characterizing spatial structures).

The most rigorous determination of the appropriate sampling strategy would be based on a matched whole-slide reference sample. However, even if such a sample is not available or measurable, there are approaches that can be taken to increase the likelihood of obtaining representative results. Several groups, including our own, are developing statistical frameworks to help define the best sampling strategies based on known or pilot-experiment determined properties of tissues[132,133]. Given the results of such models, close collaboration with pathologists enables the preparation of TMAs focused on regions of interest to avoid undersampling associated with a given number and size of cores. The number of punches per tumour can also be increased, which further allows direct assessment of intratumour variability of the feature of interest[48]. Furthermore, whole-tissue sections can be imaged first with a low-plex immunofluorescence panel to determine regions of interest; in a second step, informative regions are imaged in high multiplex[62]. Otherwise, if sufficient imaging resources are available and for a question that can be answered using few patient samples, highly multiplexed whole-tissue imaging can be performed. Finally, key findings from multiplex imaging studies should ideally be tested with orthogonal approaches, whether that be whole-slide imaging using a large field-of-view imaging method or transcriptomics data when the spatial element is not relevant[50].

The need for marker choice

Powerful though multiplex protein imaging methods are, in their present iteration, they typically still detect only several dozens of markers. The imaged markers must therefore be judiciously chosen since what can be learned depends directly on this choice. Marker panel design typically strikes a balance between answering specific questions and enabling discovery. For instance, while it is easy to design a marker panel to determine the abundance and spatial distribution of defined immune cell types in a given cohort, it would be advisable to also use valuable patient samples for more exploratory work. When the type of cancer or the cell type being studied is not well characterized — for instance, in our recent work on cancer-associated fibroblasts — it may be expedient to use gene expression patterns as measured by single-cell transcriptomics to help guide antibody panel design[22]. Although transcript levels are far from perfectly predictive of the corresponding protein-level expression[134,135], transcriptomes measure thousands of features rather than dozens, which is a substantial advantage for an initial exploration of cell phenotypes and states where the degree and nature of heterogeneity are not known a priori.

The need for antibody validation

As a technology that depends directly on the sensitive and specific binding of antibodies to targets, all current protein-based multiplex imaging methods require antibody validation[136,137]. Commercially available antibodies cannot be assumed to target the advertised antigens and must be tested in the specific preparation (for example, FFPE tissue) and technology of interest. Furthermore, the specific modification needed to enable any given technique (for example, conjugation of a metal, fluorophore or oligonucleotide) can affect antibody performance. The assembly of validated antibody panels and their optimization for a tissue of interest is therefore time-consuming and expensive yet absolutely necessary for multiplex imaging methods to yield reliable information. The higher the plex, the greater the challenge, as evidenced by approaches that use hundreds of oligonucleotide-labelled antibodies to increase plex but where antibody validation data on FFPE specimens is typically only shown for a few reagents[92,94,97]. In our laboratory, antibodies are validated before and after metal conjugation by testing for expected expression patterns in well-characterized positive and negative control samples prepared with relevant methods. We also compare to expression patterns reported in other resources, such as the Human Protein Atlas, test certain markers (for example, for epidermal growth factor (EGF) signalling) in stimulated and/or inhibited cell lines and, where possible, compare antibodies directed against different epitopes or different markers of the same cell type. Once antibodies have been validated, their use must be optimized, for example, by titrating their concentration for an optimal signal-to-noise ratio. The reader is referred to recent papers for a more detailed discussion of antibody validation[64] and a resource of 200+ validated antibodies targeting markers in several human organs[138].

The challenge of cell segmentation

Analysis of imaging data at the single-cell level requires an initial segmentation step where the spatial boundary of each cell is identified.

The resulting cellular masks then serve as the foundation for most other quantitative analyses, such that errors in cell segmentation can affect the quality of the results. Tumour tissue is particularly challenging to segment. Tumours often have densely packed cells, some with irregular shapes (for example, fibroblasts), and z-dimension overlap will not be apparent in the x–y plane, although recently described pixel-based methods may help with these aspects and enable the study of extracellular features in less dense tissue[139]. Furthermore, many multiplex imaging methods operate at a resolution that only just resolves single cells within tissues, and signal bleed-through can also occur. Segmentation must therefore be conducted with care. State-of-the-art cell segmentation now uses machine learning models (CellPose[140] and Mesmer implemented in DeepCell[141]) that perform as accurately as a human compared with manually segmented ground truth data. If needed, more than one segmentation approach may be tested and segmentation masks varied by a few pixels to check the robustness of the results. Additionally, there is no substitute for human visualization of images, both to assess unlikely marker combinations that may be due to errors as well as to visually check key results.

Research challenges

The high-dimensional data that results from multiplex protein imaging is complex, difficult to visualize in its entirety and typically provides unprecedented views of tumour tissue. Deriving biological and clinical insight from this novel data type is thus an ongoing research challenge, and we discuss here a few of the interpretative difficulties in the field. Some of these issues are not exclusive to multiplex protein imaging but apply more generally to single-cell analysis or work on human samples.

Defining meaningful cell phenotypes

Multiplex imaging looks at single cells in biological samples with many more dimensions than earlier protein-level work (that is, 30–50 protein markers compared to 1–3). The combinations in which these markers can potentially be found are consequently very many indeed, and quickly leading to the problem of deciding which combinations are likely to represent stable phenotypes or sub-phenotypes, dynamic biological states, or stochastic groups that arise due to biological or technical fluctuations that are not inherently meaningful. This is less of a challenge in fields with deep biological knowledge, such as immunology, but defining heterogeneous tumour cell phenotypes in multiplex imaging, where biological understanding is more rudimentary, can easily fracture cells into myriad small clusters of unknown biological importance. There is no single solution to this problem. The definition of phenotypes can be entirely avoided by focusing simply on marker patterns but this loses the critical link to cellular-level function. A pragmatic path can usually be found through some combination of assessment of cluster stability across samples and cohorts, attention to the statistical power engendered by different cluster sizes, incorporation of prior knowledge by separating classical cell phenotype markers (for example, cytokeratins) from markers of cell state (for example, protein phosphorylation), and association of phenotypes with orthogonal clinical or biological information. Additionally, ex vivo or model organism experiments may be needed to understand which phenotypes are stable and meaningful.

Inferring function

When multiplex imaging is applied to carefully designed cohorts with good clinical annotation, a functional role can be suggested for specific features at the level of human outcome, which is arguably the most relevant. Nevertheless, since these methods are applied to fixed samples that are also not amenable to perturbation, the resulting data are temporal snapshots that do not allow functional interrogation. Apart from some well-established markers of cell state or function (for example, Ki67 for cell division, carbonic anhydrase 9 (CAIX) to infer a hypoxic state, cleaved poly(ADP-ribose) polymerase (PARP) to indicate apoptosis or phospho-ribosomal protein S6 to indicate signalling), the most obvious path towards functional understanding is to work in parallel in patient-derived xenografts in mice or in ex vivo models such as organoids. A less direct but also fruitful approach is the design of panels to include the detection of markers known to have functional effects. In our IMC study of metastatic melanoma, for instance, we identified cell types that secrete chemokines, which suggested signalling events that could affect spatial tissue organization[36], although such findings should be treated as hypotheses that need orthogonal testing.

Identifying clinically relevant spatial features

Based on both first principles and the quotidian work of tumour pathologists, it is clear that multiscale tumour architecture is important for tumour biology and therefore for patient outcome and treatment. Recent multiplexed tissue imaging work, as we have discussed in this Review, has detected associations of spatial patterns with clinical features. However, it is frequently observed that non-spatial information, such as cellular phenotype, is at least partially redundant to spatial features in predicting clinical outcome (for example, dysfunctional T cells are often redundant to T cells residing within a tumour). There are many reasons why uncovering the molecular spatial correlates of disease and treatment response is very challenging. Current multiplexed imaging studies only look at single-cell phenotypes but ignore overall cellular shape as well as intracellular shapes and patterns[142]; a possible solution to this may lie in recent developments rendering expansion microscopy compatible with MIBI and IMC, thus improving x–y resolution nearly fourfold[143]. Furthermore, multiplex imaging techniques are typically limited either in the area they can image, thus not sampling large tissue structures, or in the number of patients they can sample, thus reducing their power to detect generalizable patterns. Inherent tumour heterogeneity and the absence of ideal patient cohorts further complicate the picture. A meaningful solution most probably does not lie along the path of ever-more complex but biologically uninterpretable spatial analysis approaches but rather in careful cohort design (as in the recent identification of predictors of immunotherapy response in triple-negative breast cancer[52]), in refinement and reassessment of existing methods and, as recently argued in a study combining multiplex immunofluorescence with classical histology[53], in more actively combining the rich historical knowledge of tumour pathology with multiplex molecular data.

Future perspectives

A decade or two into its existence, the field of multiplex protein imaging is maturing. Numerous experimental and analytical methods are available, some convergence is evident on the basic data processing pipelines, the main technical challenges have been recognized and are being addressed and, as we have summarized in this Review, these approaches are beginning to prove their worth for the study of tumour biology in model systems and clinical samples. However, there remains a great deal of work still to be done.

There will continue to be technical developments on many fronts. New physical principles for detecting proteins in a multiplex manner

are emerging, with advantages or complementarities vis-a-vis existing ones. We have shown that X-ray fluorescence imaging can be used to read out lanthanide metal-conjugated antibodies in high multiplex, with the advantage of being non-destructive and potentially applicable to large 3D tumour volumes; the disadvantage is that the approach currently requires a synchrotron and is less sensitive than IMC[144]. In separate work, the design of photocleavable peptide mass tags linked to antibodies has rendered MSI applicable to direct and targeted visualization of proteins[145,146]. Although this MSI-IHC approach has so far only been shown at up to 12-plex, it could be extended in the future to hundreds of readouts and would thus overcome the current upper limit of approximately 60 metal isotopes available for mass spectrometry and approximately 100-plex for fluorescent methods. Another approach with inherent multiplexity is Raman microscopy, and recent technical developments have shown multiplex Raman imaging of cells and tissues with good sensitivity[147] and in 3D on thick (1 mm) tissue sections[148]. However, as for all methods that require immunostaining, 3D imaging is limited by the efficiencies of antibody penetration into thick samples.

On the computational front, approaches for imputation have been described for single-cell protein imaging data, which can allow more markers to be profiled than are actually measured, either by training deep learning models to learn relationships for imputation or by using approaches that compress marker signals into combinations of a smaller number of reporters[149,150]. However, even very good performance in these approaches is likely to introduce additional uncertainty into already complex biological datasets, such that their value will depend on the tolerance of the study to error. Separately, several recently described machine learning approaches harness prior knowledge about marker expression and spatial patterns to achieve automatic cell type annotation in multiplex imaging data and thus reduce the labour involved in this step as well as dependence on manual clustering[151–153].

Moving away from targeted analyses entirely, the Mann group[154] has harnessed high-sensitivity mass spectrometric instruments to achieve untargeted and spatially mapped proteome profiling of single laser-captured cells from tumour samples. Although such high-resolution untargeted spatial profiling will likely remain the domain of specialized laboratories for some time yet, combining rapid targeted approaches, such as MSI-IHC, to identify cells or areas of interest with untargeted proteomic profiling of these cells or their environs, as recently shown[146], could be a route towards deep insight into specific biological questions.

We expect methodological developments for multi-omics measurements that include multiplex protein imaging as one of their modes (see also the section on Spatial multi-omics). Spatial transcriptomics and MSI, for measurement of RNA and metabolites or lipids, respectively, do not yet perform at single-cell resolution when applied to tissue, thus limiting many types of analyses. However, once this resolution is achieved and compatible tissue processing protocols are developed, cellular-level insights from combined measurements of transcriptomes, proteomes, metabolomes and lipidomes will increase. Expansion microscopy that is compatible with multiple types of molecules is an interesting development in this direction[155].

Computationally, efforts are being made in the development of true data integration methods between measurement modes such as the StabMap approach, which is expressly intended for multimodal data and can integrate different types of datasets at the single-cell level without requiring overlapping features[156]. StabMap requires that a network can be built from the multimodal data, and then uses the topology of this network for integration; initial work has already shown its application to spatial single-cell data[156]. Alternatively, bridge integration methods use a reference multimodal dataset as a 'dictionary' to then bring data from different analysis modes into a shared space[157]; the approach has been used to map multiple (non-spatial) data types, including single-cell transposase-accessible chromatin with sequencing (scATAC-seq), single-cell histone modification and DNA methylation profiles, and cytometry by time of flight (CyTOF) onto a scRNA-seq reference, and could, in principle, be extended to spatial data as well.

Most multiplex protein imaging studies of tumours have so far been on FPPE patient samples and such work will doubtless continue. Multiplex tissue imaging is destined, in our view, to become an essential analytical tool for clinical studies at any stage and ultimately a diagnostic method in the clinics. However, these studies only reveal correlations between tumour tissue and patient features, but the biological processes and mechanisms that underlie these correlations cannot be analysed. Application of multiplexed tissue imaging techniques to cancer models that are amenable to chemical or genetic perturbation and that can be imaged live, specifically mouse or other animal models, tumour organoids, and ex vivo tumour cultures, will overcome this limitation. These models are more tractable for probing function and dynamic cellular processes than fixed patient samples, will be valuable for unbiased perturbation screens, and will offer the possibility to test hypotheses formulated in multiplex imaging studies of patient tumours in situ.

Finally, analysis of many hallmark traits of tumour biology should benefit from spatial, single-cell-resolved methods such that the impact of these methods has the potential to go well beyond the studies of tumour–immune interactions and tumour cell phenotypic plasticity discussed here. Most evidently, spatial processes, like invasion and extravasation during metastasis or the induction of angiogenesis, must be studied using methods with spatial capabilities; the study of such processes in particular will benefit from developments in 3D approaches. Furthermore, intratumoural microbes are now recognized as an integral component of the TME across human cancer types and play important roles in cancer progression, immunosurveillance and treatment[158,159]; multiplex imaging of RNA and protein targets of both bacteria and tumour cells are poised to probe this exciting new area of tumour biology. For instance, recent work used spatial transcriptomics to map the location of bacterial genera within oral and colorectal tumours, combined with 77-plex DSP to show that tumour areas with bacterial presence have higher expression of immunosuppressive markers, lower proliferation and higher vascularization[160]. More generally, studies of tumour–microorganism and tumour–immune cell interactions, particularly when combined with functional studies, will reveal how intercellular communication can be therapeutically modulated.

However, even cancer hallmarks that are less obviously spatially organized, namely replicative immortality, cell death avoidance, metabolic reprogramming, sustained proliferative signalling, growth suppression avoidance and genomic instability, may be influenced by cellular interactions and by spatial features of tumours and may therefore benefit from multiplexed protein and multimodal imaging. Antibodies or RNA probes against relevant players of a hallmark process of interest could be used in multiplex fashion to ask relatively simple questions about its spatial distribution within the tumour or to relate the process to other cellular or spatial features. Antibody-based imaging of oxidative damage[161], for instance, could be used to infer the distribution of an inducer of genome instability within tumours. Intercellular signalling using probes against ligands, receptors and secreted molecules could be used to examine proliferative signalling.

Even whole signalling loops could be mapped if markers for extracellular and intracellular events are included simultaneously. In the absence of robust methods for single-cell and deep phenotypic profiling of tumour tissue, it has simply not been possible to probe such questions so far, especially in human samples. Single-cell-resolved and multiplex protein and multimodal imaging will change this. We envision increasingly colourful and exciting times ahead.

Published online: 05 February 2024

References

1. Hanahan, D. Hallmarks of cancer: new dimensions. *Cancer Discov.* **12**, 31–46 (2022).
2. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 (2011).
   The second iteration of this seminal review of the hallmarks of cancer, synthesizing evidence for immune evasion, inflammation and the TME as important features of the disease.
3. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
4. Tabassum, D. P. & Polyak, K. Tumorigenesis: it takes a village. *Nat. Rev. Cancer* **15**, 473–483 (2015).
5. Chen, B. et al. Differential pre-malignant programs and microenvironment chart distinct paths to malignancy in human colorectal polyps. *Cell* **184**, 6262–6280.e26 (2021).
6. Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306–313 (2012).
7. Heppner, G. H. Tumor heterogeneity. *Cancer Res.* **44**, 2259–2265 (1984).
8. Bi, K. et al. Tumor and immune reprogramming during immunotherapy in advanced renal cell carcinoma. *Cancer Cell* **39**, 649–661.e5 (2021).
9. Bhaduri, A. et al. Outer radial glia-like cancer stem cells contribute to heterogeneity of glioblastoma. *Cell Stem Cell* **26**, 48–63.e6 (2020).
10. Wang, L. et al. A single-cell atlas of glioblastoma evolution under therapy reveals cell-intrinsic and cell-extrinsic therapeutic targets. *Nat. Cancer* **3**, 1534–1552 (2022).
11. Vegliante, R., Pastushenko, I. & Blanpain, C. Deciphering functional tumor states at single-cell resolution. *EMBO J.* **41**, e109221 (2022).
12. Lawson, D. A., Kessenbrock, K., Davis, R. T., Pervolarakis, N. & Werb, Z. Tumour heterogeneity and metastasis at single-cell resolution. *Nat. Cell Biol.* **20**, 1349–1360 (2018).
13. Izar, B. et al. A single-cell landscape of high-grade serous ovarian cancer. *Nat. Med.* **26**, 1271–1279 (2020).
14. Massalha, H. et al. A single cell atlas of the human liver tumor microenvironment. *Mol. Syst. Biol.* **16**, e9682 (2020).
15. Zhou, Y. et al. Single-cell RNA landscape of intratumoral heterogeneity and immunosuppressive microenvironment in advanced osteosarcoma. *Nat. Commun.* **11**, 6322 (2020).
16. Cheng, S. et al. A pan-cancer single-cell transcriptional atlas of tumor infiltrating myeloid cells. *Cell* **184**, 792–809.e23 (2021).
17. Chen, Y. et al. Predicting response to immunotherapy in gastric cancer via multi-dimensional analyses of the tumour immune microenvironment. *Nat. Commun.* **13**, 4851 (2022).
18. Nieto, P. et al. A single-cell tumor immune atlas for precision oncology. *Genome Res.* **31**, 1913–1926 (2021).
19. Bolis, M. et al. Dynamic prostate cancer transcriptome analysis delineates the trajectory to disease progression. *Nat. Commun.* **12**, 7033 (2021).
20. Zheng, L. et al. Pan-cancer single-cell landscape of tumor-infiltrating T cells. *Science* **374**, abe6474 (2021).
21. Kumar, V. et al. Single-cell atlas of lineage states, tumor microenvironment, and subtype-specific expression programs in gastric cancer. *Cancer Discov.* **12**, 670–691 (2022).
22. Cords, L. et al. Cancer-associated fibroblast classification in single-cell and spatial proteomics data. *Nat. Commun.* **14**, 4294 (2023).
23. Tietscher, S. et al. A comprehensive single-cell map of T cell exhaustion-associated immune environments in human breast cancer. *Nat. Commun.* **14**, 98 (2023).
24. Chijimatsu, R. et al. Establishment of a reference single-cell RNA sequencing dataset for human pancreatic adenocarcinoma. *iScience* **25**, 104659 (2022).
25. Chu, Y. et al. Pan-cancer T cell atlas links a cellular stress response state to immunotherapy resistance. *Nat. Med.* **29**, 1550–1562 (2023).
26. Rozenblatt-Rosen, O. et al. The human tumor atlas network: charting tumor transitions across space and time at single-cell resolution. *Cell* **181**, 236–249 (2020).
27. Lewis, S. M. et al. Spatial omics and multiplexed imaging to explore cancer biology. *Nat. Methods* **18**, 997–1012 (2021).
28. Elhanani, O., Ben-Uri, R. & Keren, L. Spatial profiling technologies illuminate the tumor microenvironment. *Cancer Cell* **41**, 404–420 (2023).
29. de Vries, N. L., Mahfouz, A., Koning, F. & de Miranda, N. Unraveling the complexity of the cancer microenvironment with multidimensional genomic and cytometric technologies. *Front. Oncol.* **10**, 1254 (2020).
30. Keren, L. et al. A structured tumor-immune microenvironment in triple negative breast cancer revealed by multiplexed ion beam imaging. *Cell* **174**, 1373–1387.e19 (2018).
    A MIBI study of triple-negative breast cancer that defines immune cell patterns, immunoregulatory protein expression and spatial features of tumour tissue.
31. Danenberg, E. et al. Breast tumor microenvironment structures are associated with genomic features and clinical outcome. *Nat. Genet.* **54**, 660–669 (2022).
    An IMC study of the TME in breast cancer that identifies spatial patterns associated with specific driver mutations and with patient survival.
32. Sorin, M. et al. Single-cell spatial landscapes of the lung tumour immune microenvironment. *Nature* **614**, 548–554 (2023).
33. Karimi, E. et al. Single-cell spatial immune landscapes of primary and metastatic brain tumours. *Nature* **614**, 555–563 (2023).
34. Ferguson, A. L. et al. High-dimensional and spatial analysis reveals immune landscape-dependent progression in cutaneous squamous cell carcinoma. *Clin. Cancer Res.* **28**, 4677–4688 (2022).
35. Xiao, X. et al. Multiplexed imaging mass cytometry reveals distinct tumor-immune microenvironments linked to immunotherapy responses in melanoma. *Commun. Med.* **2**, 131 (2022).
36. Hoch, T. et al. Multiplexed imaging mass cytometry of the chemokine milieus in melanoma characterizes features of the response to immunotherapy. *Sci. Immunol.* **7**, eabk1692 (2022).
    A RNA-IMC study of metastatic melanoma that identifies local patches of chemokine-secreting cells together with the phenotypes of the cells in the surrounding milieus.
37. Lin, J. R. et al. Multiplexed 3D atlas of state transitions and immune interaction in colorectal cancer. *Cell* **186**, 363–381.e19 (2023).
    A CycIF study of colorectal cancer that identifies molecular features underlying large-scale histological patterns and highlights the limitations of imaging small fields of view such as those in tumour microarrays.
38. Matusiak, M. et al. A spatial map of human macrophage niches reveals context-dependent macrophage functions in colon and breast cancer. *Res. Sq.* https://doi.org/10.21203/rs.3.rs-2393443/v1 (2023).
39. Hartmann, F. J. et al. Single-cell metabolic profiling of human cytotoxic T cells. *Nat. Biotechnol.* **39**, 186–197 (2021).
    A MIBI and mass cytometry study that examines metabolic programmes of different cell types in colorectal cancer and maps their spatial organization.
40. Remark, R. et al. In-depth tissue profiling using multiplexed immunohistochemical consecutive staining on single slide. *Sci. Immunol.* **1**, aaf6925 (2016).
41. Tsujikawa, T. et al. Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis. *Cell Rep.* **19**, 203–217 (2017).
42. DeNardo, D. G. & Ruffell, B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat. Rev. Immunol.* **19**, 369–382 (2019).
43. Banik, G. et al. High-dimensional multiplexed immunohistochemical characterization of immune contexture in human cancers. *Methods Enzymol.* **635**, 1–20 (2020).
44. Toki, M. I. et al. High-plex predictive marker discovery for melanoma immunotherapy-treated patients using digital spatial profiling. *Clin. Cancer Res.* **25**, 5503–5512 (2019).
45. Schulz, D. et al. Simultaneous multiplexed imaging of mRNA and proteins with subcellular resolution in breast cancer tissue samples by mass cytometry. *Cell Syst.* **6**, 25–36.e5 (2018).
46. Gerdes, M. J. et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc. Natl Acad. Sci. USA* **110**, 11982–11987 (2013).
47. Gaglia, G. et al. Temporal and spatial topography of cell proliferation in cancer. *Nat. Cell Biol.* **24**, 316–326 (2022).
48. Jackson, H. W. et al. The single-cell pathology landscape of breast cancer. *Nature* **578**, 615–620 (2020).
    An IMC study of breast cancer showing that multiplex imaging-defined single-cell pathology groups correlate with patient survival beyond the information provided by clinical subtypes.
49. Ali, H. R. et al. Imaging mass cytometry and multiplatform genomics define the phenogenomic landscape of breast cancer. *Nat. Cancer* **1**, 163–175 (2020).
50. Fischer, J. R. et al. Multiplex imaging of breast cancer lymph node metastases identifies prognostic single-cell populations independent of clinical classifiers. *Cell Rep. Med.* **4**, 100977 (2023).
    An IMC study showing cell phenotypic divergence between paired primary breast tumours and lymph node metastases, and identifying prognostic phenotypes in metastatic tumours.
51. Schapiro, D. et al. histoCAT: analysis of cell phenotypes and interactions in multiplex image cytometry data. *Nat. Methods* **14**, 873–876 (2017).
52. Wang, X. Q. et al. Spatial predictors of immunotherapy response in triple-negative breast cancer. *Nature* **621**, 868–876 (2023).
    An IMC study conducted on a cohort of patients with breast cancer within a clinical trial and identifying cell phenotypic and spatial predictors of immunotherapy response.
53. Lin, J. R. et al. High-plex immunofluorescence imaging and traditional histology of the same tissue section for discovering image-based biomarkers. *Nat. Cancer* **4**, 1036–1052 (2023).
54. Radtke, A. J. et al. A multi-scale, multi-omic atlas of human normal and follicular lymphoma lymph nodes. Preprint at *bioRxiv* https://doi.org/10.1101/2022.06.03.494716 (2022).
55. Milosevic, V. Different approaches to imaging mass cytometry data analysis. *Bioinform Adv.* **3**, vbad046 (2023).
56. Palla, G., Fischer, D. S., Regev, A. & Theis, F. J. Spatial components of molecular tissue biology. *Nat. Biotechnol.* **40**, 308–318 (2022).

57. Zhang, M. et al. Spatial molecular profiling: platforms, applications and analysis tools.*Brief. Bioinform.* **22**, bbaa145 (2021).
58. Schürch, C. M. et al. Coordinated cellular neighborhoods orchestrate antitumoral immunity at the colorectal cancer invasive front. *Cell* **182**, 1341–1359.e19 (2020).
A CODEX study on colorectal cancer illustrating the definition of cellular neighbourhoods and showing that spatial organization differs in samples with and without TLSs.
59. Chen, Z., Soifer, I., Hilton, H., Keren, L. & Jojic, V. Modeling multiplexed images with spatial-LDA reveals novel tissue microenvironments. *J. Comput. Biol.* **27**, 1204–1218 (2020).
60. Phillips, D. et al. Immune cell topography predicts response to PD-1 blockade in cutaneous T cell lymphoma. *Nat. Commun.* **12**, 6726 (2021).
A CODEX study on cutaneous T cell lymphoma showing that spatial organization but not cell phenotypes is associated with patient response to immunotherapy.
61. Bhate, S. S., Barlow, G. L., Schürch, C. M. & Nolan, G. P. Tissue schematics map the specialization of immune tissue motifs and their appropriation by tumors. *Cell Syst.* **13**, 109–130.e6 (2022).
62. Damond, N. et al. A map of human type 1 diabetes progression by imaging mass cytometry. *Cell Metab.* **29**, 755–768.e55 (2019).
63. Risom, T. et al. Transition to invasive breast cancer is associated with progressive changes in the structure and composition of tumor stroma. *Cell* **185**, 299–310.e18 (2022).
A MIBI study of early-stage breast cancer that used histological staining to define areas of interest for multiplex imaging and identifies features prognostic for disease progression.
64. Hickey, J. W. et al. Spatial mapping of protein composition and tissue organization: a primer for multiplexed antibody-based imaging. *Nat. Methods* **19**, 284–295 (2022).
A detailed practical guide to many aspects of multiplex protein imaging.
65. Moffitt, J. R., Lundberg, E. & Heyn, H. The emerging landscape of spatial profiling technologies. *Nat. Rev. Genet.* **23**, 741–759 (2022).
66. Windhager, J. et al. An end-to-end workflow for multiplexed image processing and analysis. *Nat. Protoc.* **18**, 3565–3613 (2023).
67. Liu, C. C. et al. Multiplexed ion beam imaging: insights into pathobiology. *Annu. Rev. Pathol.* **17**, 403–423 (2022).
68. Kuswanto, W., Nolan, G. & Lu, G. Highly multiplexed spatial profiling with CODEX: bioinformatic analysis and application in human disease. *Semin. Immunopathol.* **45**, 145–157 (2023).
69. Baharlou, H., Canete, N. P., Cunningham, A. L., Harman, A. N. & Patrick, E. Mass cytometry imaging for the study of human diseases-applications and data analysis strategies. *Front. Immunol.* **10**, 2657 (2019).
70. Angelo, M. et al. Multiplexed ion beam imaging of human breast tumors. *Nat. Med.* **20**, 436–442 (2014).
The first methodological report of MIBI applied to human breast tumours.
71. Keren, L. et al. MIBI-TOF: a multiplexed imaging platform relates cellular phenotypes and tissue structure. *Sci. Adv.* **5**, eaax5851 (2019).
72. Giesen, C. et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat. Methods* **11**, 417–422 (2014).
The first methodological report of IMC applied to human breast tumours.
73. Jiang, S. et al. Combined protein and nucleic acid imaging reveals virus-dependent B cell and macrophage immunosuppression of tissue microenvironments. *Immunity* **55**, 1118–1134.e8 (2022).
74. Kuett, L. et al. Three-dimensional imaging mass cytometry for highly multiplexed molecular and cellular mapping of tissues and the tumor microenvironment. *Nat. Cancer* **3**, 122–133 (2022).
75. Hosogane, T., Casanova, R. & Bodenmiller, B. DNA-barcoded signal amplification for imaging mass cytometry enables sensitive and highly multiplexed tissue imaging. *Nat. Methods* **20**, 1304–1309 (2023).
76. Rovira-Clavé, X. et al. Subcellular localization of biomolecules and drug distribution by high-definition ion beam imaging. *Nat. Commun.* **12**, 4628 (2021).
77. Goossens, P. et al. Integrating multiplex immunofluorescent and mass spectrometry imaging to map myeloid heterogeneity in its metabolic and cellular context. *Cell Metab.* **34**, 1214–1225.e6 (2022).
78. Gerner, M. Y., Kastenmuller, W., Ifrim, I., Kabat, J. & Germain, R. N. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity* **37**, 364–376 (2012).
79. Radtke, A. J. et al. IBEX: a versatile multiplex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues. *Proc. Natl Acad. Sci. USA* **117**, 33455–33465 (2020).
A methodological paper describing the rapid, flexible and highly-multiplex IBEX method for cyclic immunofluorescence imaging.
80. Schubert, W. et al. Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat. Biotechnol.* **24**, 1270–1278 (2006).
81. Lin, J. R. et al. Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *eLife* **7**, e31657 (2018).
An early methodological report of highly multiplex CycIF applied to tissue.
82. Wählby, C., Erlandsson, F., Bengtsson, E. & Zetterberg, A. Sequential immunofluorescence staining and image analysis for detection of large numbers of antigens in individual cell nuclei. *Cytometry* **47**, 32–41 (2002).
The first multiplex imaging paper, showing that immunofluorescence signals can be sequentially removed without destroying antigenicity in fixed and paraffin-embedded tissue.
83. Gut, G., Herrmann, M. D. & Pelkmans, L. Multiplexed protein maps link subcellular organization to cellular states. *Science* **361**, eaar7042 (2018).
84. Gaglia, G. et al. Lymphocyte networks are dynamic cellular communities in the immunoregulatory landscape of lung adenocarcinoma. *Cancer Cell* **41**, 871–886.e10 (2023).
85. Nirmal, A. J. et al. The spatial landscape of progression and immunoediting in primary melanoma at single-cell resolution. *Cancer Discov.* **12**, 1518–1541 (2022).
86. Li, K. et al. Multi-omic analyses of changes in the tumor microenvironment of pancreatic adenocarcinoma following neoadjuvant treatment with anti-PD-1 therapy. *Cancer Cell* **40**, 1374–1391.e7 (2022).
87. Chen, M. et al. Spatiotemporal analysis of B cell- and antibody secreting cell-subsets in human melanoma reveals metastasis-, tumor stage-, and age-associated dynamics. *Front. Cell Dev. Biol.* **9**, 677944 (2021).
88. Goltsev, Y. et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell* **174**, 968–981.e15 (2018).
89. Black, S. et al. CODEX multiplexed tissue imaging with DNA-conjugated antibodies. *Nat. Protoc.* **16**, 3802–3835 (2021).
A methodological paper describing the CODEX workflow.
90. Saka, S. K. et al. Immuno-SABER enables highly multiplexed and amplified protein imaging in tissues. *Nat. Biotechnol.* **37**, 1080–1090 (2019).
91. Liu, Y. et al. High-spatial-resolution multi-omics sequencing via deterministic barcoding in tissue. *Cell* **183**, 1665–1681.e18 (2020).
92. Merritt, C. R. et al. Multiplex digital spatial profiling of proteins and RNA in fixed tissue. *Nat. Biotechnol.* **38**, 586–599 (2020).
93. Vickovic, S. et al. SM-Omics is an automated platform for high-throughput spatial multi-omics. *Nat. Commun.* **13**, 795 (2022).
94. Liu, Y. et al. High-plex protein and whole transcriptome co-mapping at cellular resolution with spatial CITE-seq. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-023-01676-0 (2023).
95. Ben-Chetrit, N. et al. Integration of whole transcriptome spatial profiling with protein markers. *Nat. Biotechnol.* **41**, 788–793 (2023).
96. Heumos, L. et al. Best practices for single-cell analysis across modalities. *Nat. Rev. Genet.* **24**, 550–572 (2023).
97. He, S. et al. High-plex imaging of RNA and proteins at subcellular resolution in fixed tissue by spatial molecular imaging. *Nat. Biotechnol.* **40**, 1794–1806 (2022).
98. Kishi, J. Y. et al. Light-Seq: light-directed in situ barcoding of biomolecules in fixed cells and tissues for spatially indexed sequencing. *Nat. Methods* **19**, 1393–1402 (2022).
99. Hu, K. H. et al. ZipSeq: barcoding for real-time mapping of single cell transcriptomes. *Nat. Methods* **17**, 833–843 (2020).
100. Sha, L. et al. Integrated spatial transcriptomic and proteomic analysis of fresh frozen tissue based on stereo-seq. Preprint at: *bioRxiv* https://doi.org/10.1101/2023.04.28.538364 (2023).
101. McNamara, K. L. et al. Spatial proteomic characterization of HER2-positive breast tumors through neoadjuvant therapy predicts response. *Nat. Cancer* **2**, 400–413 (2021).
102. Brady, L. et al. Inter- and intra-tumor heterogeneity of metastatic prostate cancer determined by digital spatial gene expression profiling. *Nat. Commun.* **12**, 1426 (2021).
103. Martinez-Morilla, S. et al. Digital spatial profiling of melanoma shows CD95 expression in immune cells is associated with resistance to immunotherapy. *Oncoimmunology* **12**, 2260618 (2023).
104. Gavrielatou, N. et al. Digital spatial profiling links beta-2-microglobulin expression with immune checkpoint blockade outcomes in head and neck squamous cell carcinoma. *Cancer Res. Commun.* **3**, 558–563 (2023).
105. Schoenfeld, D. A. et al. Immune dysfunction revealed by digital spatial profiling of immuno-oncology markers in progressive stages of renal cell carcinoma and in brain metastases. *J. Immunother. Cancer* **11**, e007240 (2023).
106. Carter, J. M. et al. Distinct spatial immune microlandscapes are independently associated with outcomes in triple-negative breast cancer. *Nat. Commun.* **14**, 2215 (2023).
107. Bonnett, S. A. et al. Ultra high-plex spatial proteogenomic investigation of giant cell glioblastoma multiforme immune infiltrates reveals distinct protein and RNA expression profiles. *Cancer Res. Commun.* **3**, 763–779 (2023).
108. Stoeckius, M. et al. Simultaneous epitope and transcriptome measurement in single cells. *Nat. Methods* **14**, 865–868 (2017).
109. Govek, K. W. et al. Single-cell transcriptomic analysis of mIHC images via antigen mapping. *Sci. Adv.* **7**, eabc5464 (2021).
110. Zhu, B. et al. Robust single-cell matching and multimodal analysis using shared and distinct features. *Nat. Methods* **20**, 304–315 (2023).
111. Chen, S. et al. Integration of spatial and single-cell data across modalities with weakly linked features. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-023-01935-0 (2023).
112. Strand, S. H. et al. Molecular classification and biomarkers of clinical outcome in breast ductal carcinoma in situ: analysis of TBCRC 038 and RAHBT cohorts. *Cancer Cell* **40**, 1521–1536.e7 (2022).
113. Chan, J. M. et al. Signatures of plasticity, metastasis, and immunosuppression in an atlas of human small cell lung cancer. *Cancer Cell* **39**, 1479–1496.e18 (2021).
114. Vázquez-García, I. et al. Ovarian cancer mutational processes drive site-specific immune evasion. *Nature* **612**, 778–786 (2022).
115. Ji, A. L. et al. Multimodal analysis of composition and spatial architecture in human squamous cell carcinoma. *Cell* **182**, 497–514.e22 (2020).
116. Ravi, V. M. et al. Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma. *Cancer Cell* **40**, 639–655.e13 (2022).

117. Müller, W. H., De Pauw, E., Far, J., Malherbe, C. & Eppe, G. Imaging lipids in biological samples with surface-assisted laser desorption/ionization mass spectrometry: a concise review of the last decade. *Prog. Lipid Res.* **83**, 101114 (2021).
118. Balluff, B., Hanselmann, M. & Heeren, R. M. Mass spectrometry imaging for the investigation of intratumor heterogeneity. *Adv. Cancer Res.* **134**, 201–230 (2017).
119. Ma, X. & Fernández, F. M. Advances in mass spectrometry imaging for spatial cancer metabolomics. *Mass Spectrom. Rev.* **2022**, e21804 (2022).
120. Spraggins, J. M. et al. High-performance molecular imaging with MALDI trapped ion-mobility time-of-flight (timsTOF) mass spectrometry. *Anal. Chem.* **91**, 14552–14560 (2019).
121. Niehaus, M., Soltwisch, J., Belov, M. E. & Dreisewerd, K. Transmission-mode MALDI-2 mass spectrometry imaging of cells and tissues at subcellular resolution. *Nat. Methods* **16**, 925–931 (2019).
122. Zavalin, A., Yang, J., Hayden, K., Vestal, M. & Caprioli, R. M. Tissue protein imaging at 1 μm laser spot diameter for high spatial resolution and high imaging speed using transmission geometry MALDI TOF MS. *Anal. Bioanal. Chem.* **407**, 2337–2342 (2015).
123. Neumann, E. K., Comi, T. J., Rubakhin, S. S. & Sweedler, J. V. Lipid heterogeneity between astrocytes and neurons revealed by single-cell MALDI-MS combined with immunocytochemical classification. *Angew. Chem. Int. Ed. Engl.* **58**, 5910–5914 (2019).
124. Cuypers, E. et al. 'On the Spot' digital pathology of breast cancer based on single-cell mass spectrometry imaging. *Anal. Chem.* **94**, 6180–6190 (2022).
125. Bien, T. et al. MALDI-2 mass spectrometry and immunohistochemistry imaging of Gb3Cer, Gb4Cer, and further glycosphingolipids in human colorectal cancer tissue. *Anal. Chem.* **92**, 7096–7105 (2020).
126. O'Neill, K. C., Liapis, E., Harris, B. T., Perlin, D. S. & Carter, C. L. Mass spectrometry imaging discriminates glioblastoma tumor cell subpopulations and different microvascular formations based on their lipid profiles. *Sci. Rep.* **12**, 17069 (2022).
127. Andersen, M. K. et al. Spatial differentiation of metabolism in prostate cancer tissue by MALDI-TOF MSI. *Cancer Metab.* **9**, 9 (2021).
128. Ščupáková, K. et al. Clinical importance of high-mannose, fucosylated, and complex N-glycans in breast cancer metastasis. *JCI Insight* **6**, e146945 (2021).
129. Prentice, B. M. et al. Imaging mass spectrometry enables molecular profiling of mouse and human pancreatic tissue. *Diabetologia* **62**, 1036–1047 (2019).
130. Prade, V. M. et al. De novo discovery of metabolic heterogeneity with immunophenotype-guided imaging mass spectrometry. *Mol. Metab.* **36**, 100953 (2020).
131. Wang, G. et al. Analyzing cell-type-specific dynamics of metabolism in kidney repair. *Nat. Metab.* **4**, 1109–1118 (2022).
132. Baker, E. A. G., Schapiro, D., Dumitrascu, B., Vickovic, S. & Regev, A. In silico tissue generation and power analysis for spatial omics. *Nat. Methods* **20**, 424–431 (2023).
133. Bost, P., Schulz, D., Engler, S., Wasserfall, C. & Bodenmiller, B. Optimizing multiplexed imaging experimental design through tissue spatial segregation estimation. *Nat. Methods* **20**, 418–423 (2023).
134. Edfors, F. et al. Gene-specific correlation of RNA and protein levels in human cells and tissues. *Mol. Syst. Biol.* **12**, 883 (2016).
135. Liu, Y., Beyer, A. & Aebersold, R. On the dependency of cellular protein levels on mRNA abundance. *Cell* **165**, 535–550 (2016).
A paper discussing the imperfect relationship between transcript and protein levels and arguing that protein-level measurements are needed to understand biological systems in many contexts.
136. Du, Z. et al. Qualifying antibodies for image-based immune profiling and multiplexed tissue imaging. *Nat. Protoc.* **14**, 2900–2930 (2019).
A methodological paper that describes a pipeline for antibody validation.
137. Uhlen, M. et al. A proposal for validation of antibodies. *Nat. Methods* **13**, 823–827 (2016).
138. Quardokus, E. M. et al. Organ mapping antibody panels: a community resource for standardized multiplexed tissue imaging. *Nat. Methods* **20**, 1174–1178 (2023).
139. Liu, C. C. et al. Robust phenotyping of highly multiplexed tissue imaging data using pixel-level clustering. *Nat. Commun.* **14**, 4618 (2023).
140. Stringer, C., Wang, T., Michaelos, M. & Pachitariu, M. Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* **18**, 100–106 (2021).
141. Greenwald, N. F. et al. Whole-cell segmentation of tissue images with human-level performance using large-scale data annotation and deep learning. *Nat. Biotechnol.* **40**, 555–565 (2022).
142. Spitzer, H., Berry, S., Donoghoe, M., Pelkmans, L. & Theis, F. J. Learning consistent subcellular landmarks to quantify changes in multiplexed protein maps. *Nat. Methods* https://doi.org/10.1038/s41592-023-01894-z (2023).
143. Bai, Y. et al. Expanded vacuum-stable gels for multiplexed high-resolution spatial histopathology. *Nat. Commun.* **14**, 4013 (2023).
144. Strotton, M. et al. Multielement Z-tag imaging by X-ray fluorescence microscopy for next-generation multiplex imaging. *Nat. Methods* **20**, 1310–1322 (2023).
145. Yagnik, G., Liu, Z., Rothschild, K. J. & Lim, M. J. Highly multiplexed immunohistochemical MALDI-MS imaging of biomarkers in tissues. *J. Am. Soc. Mass Spectrom.* **32**, 977–988 (2021).
146. Claes, B. S. R. et al. MALDI-IHC-guided in-depth spatial proteomics: targeted and untargeted MSI combined. *Anal. Chem.* **95**, 2329–2338 (2023).
147. Wei, L. et al. Super-multiplex vibrational imaging. *Nature* **544**, 465–470 (2017).
148. Shi, L. et al. Highly-multiplexed volumetric mapping with Raman dye imaging and tissue clearing. *Nat. Biotechnol.* **40**, 364–373 (2022).
149. Wu, E. et al. 7-UP: generating in silico CODEX from a small set of immunofluorescence markers. *PNAS Nexus* **2**, pgad171 (2023).
150. Ben-Uri, R. et al. Escalating high-dimensional imaging using combinatorial channel multiplexing and deep learning. Preprint at: *bioRxiv* https://doi.org/10.1101/2023.09.09.556962 (2023).
151. Zhang, W. et al. Identification of cell types in multiplexed in situ images by combining protein expression and spatial information using CELESTA. *Nat. Methods* **19**, 759–769 (2022).
152. Brbić, M. et al. Annotation of spatially resolved single-cell data with STELLAR. *Nat. Methods* **19**, 1411–1418 (2022).
153. Amitay, Y. et al. CellSighter: a neural network to classify cells in highly multiplexed images. *Nat. Commun.* **14**, 4302 (2023).
154. Mund, A. et al. Deep visual proteomics defines single-cell identity and heterogeneity. *Nat. Biotechnol.* **40**, 1231–1240 (2022).
155. Cui, Y. et al. Expansion microscopy using a single anchor molecule for high-yield multiplexed imaging of proteins and RNAs. *PLoS One* **18**, e0291506 (2023).
156. Ghazanfar, S., Guibentif, C. & Marioni, J. C. Stabilized mosaic single-cell data integration using unshared features. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-023-01766-z (2023).
157. Hao, Y. et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-023-01767-y (2023).
158. Sepich-Poore, G. D. et al. The microbiome and human cancer. *Science* **371**, eabc4552 (2021).
159. Cullin, N., Azevedo Antunes, C., Straussman, R., Stein-Thoeringer, C. K. & Elinav, E. Microbiome and cancer. *Cancer Cell* **39**, 1317–1341 (2021).
160. Galeano Niño, J. L. et al. Effect of the intratumoral microbiota on spatial and cellular heterogeneity in cancer. *Nature* **611**, 810–817 (2022).
161. Ozeki, M. et al. Susceptibility of actin to modification by 4-hydroxy-2-nonenal. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **827**, 119–126 (2005).
162. Guilliams, M. et al. Spatial proteogenomics reveals distinct and evolutionarily conserved hepatic macrophage niches. *Cell* **185**, 379–396.e38 (2022).
163. Montanari, N. R. et al. Multi-parametric analysis of human livers reveals variation in intrahepatic inflammation across phases of chronic hepatitis B infection. *J. Hepatol.* **77**, 332–343 (2022).
164. Hoyt, C. C. Multiplex immunofluorescence and multispectral imaging: forming the basis of a clinical test platform for immuno-oncology. *Front. Mol. Biosci.* **8**, 674747 (2021).
165. Hurov, K. et al. BT7480, a novel fully synthetic Bicycle tumor-targeted immune cell agonist™ (Bicycle TICA™) induces tumor localized CD137 agonism. *J. Immunother. Cancer* **9**, e002883 (2021).
166. Rivest, F. et al. Fully automated sequential immunofluorescence (seqIF) for hyperplex spatial proteomics. *Sci. Rep.* **13**, 16994 (2023).
167. Jarosch, S. et al. Multiplexed imaging and automated signal quantification in formalin-fixed paraffin-embedded tissues by ChipCytometry. *Cell Rep. Methods* **1**, 100104 (2021).
168. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
169. Bankhead, P. et al. QuPath: open source software for digital pathology image analysis. *Sci. Rep.* **7**, 16878 (2017).
170. Peng, T. et al. A BaSiC tool for background and shading correction of optical microscopy images. *Nat. Commun.* **8**, 14836 (2017).
171. Lu, P. et al. IMC-Denoise: a content aware denoising pipeline to enhance imaging mass cytometry. *Nat. Commun.* **14**, 1601 (2023).
172. Berg, S. et al. ilastik: interactive machine learning for (bio)image analysis. *Nat. Methods* **16**, 1226–1232 (2019).
173. Schapiro, D. et al. MCMICRO: a scalable, modular image-processing pipeline for multiplexed tissue imaging. *Nat. Methods* **19**, 311–315 (2022).
174. Marconato, L. et al. SpatialData: an open and universal data framework for spatial omics. Preprint at *bioRxiv* https://doi.org/10.1101/2023.05.05.539647 (2023).

Acknowledgements

We thank Nils Eling and Daniel Schulz for critical reading and feedback on the manuscript. We thank all Bodenmiller laboratory members for helpful discussions. B.B. was funded by two SNSF grants (310030_205007, 316030_213512), an NIH grant (UC4 DK108132), the CRUK IMAXT Grand Challenge, and the European Research Council (ERC) under the European Union's Horizon 2020 Program under the ERC grant agreement no. 866074 ("Precision Motifs").

Author contributions

N.d.S. and S.Z. researched data for the article. All authors contributed substantially to discussion of the content. N.d.S. wrote and revised the article. S.Z. prepared and revised the figures and tables with input from the other authors. N.d.S. and B.B. reviewed and edited the manuscript before submission.

Competing interests

B.B. has founded and is a shareholder and member of the board of Navignostics, a precision oncology spin-off from the University of Zurich. N.d.S. and S.Z. declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41568-023-00657-4.

Peer review information *Nature Reviews Cancer* thanks Sizun Jiang who co-reviewed with Hendrik Michel, Christian Schürch and Sean Bendall for their contribution to the peer review of this work.

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