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Applying high-dimensional single-cell technologies to the analysis of cancer immunotherapy

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Abstract

Advances in molecular biology, microfluidics and bioinformatics have empowered the study of thousands or even millions of individual cells from malignant tumours at the single-cell level of resolution. This high-dimensional, multi-faceted characterization of the genomic, transcriptomic, epigenomic and proteomic features of the tumour and/or the associated immune and stromal cells enables the dissection of tumour heterogeneity, the complex interactions between tumour cells and their microenvironment, and the details of the evolutionary trajectory of each tumour. Single-cell transcriptomics, the ability to track individual T cell clones through paired sequencing of the T cell receptor genes and high-dimensional single-cell spatial analysis are all areas of particular relevance to immuno-oncology. Multidimensional biomarker signatures will increasingly be crucial to guiding clinical decision-making in each patient with cancer. High-dimensional single-cell technologies are likely to provide the resolution and richness of data required to generate such clinically relevant signatures in immuno-oncology. In this Perspective, we describe advances made using transformative single-cell analysis technologies, especially in relation to clinical response and resistance to immunotherapy, and discuss the growing utility of single-cell approaches for answering important research questions.

Tumours are a complex mixture of malignant, immune and stromal cells, which often have substantial levels of intratumour and intertumour heterogeneity. The tumour microenvironment (TME) comprises an amalgam of tumour-promoting and anti-tumour signals that are able to modulate tumour growth and influence tumour evolution. Bulk genomic and transcriptomic analyses have provided valuable insights into these processes, but the averaging of signals from large numbers of cells rendered by these methods often

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obscures specific subpopulations and/or cellular states. Indeed, rare and unique cellular subtypes or states that might be instrumental in disease biology, such as potential ‘cancer stem cells’ or immune cells crucial for a therapeutic response, might not be detected in bulk analyses derived by averaging data from millions of cells. For cancer immunotherapy, in particular, single-cell analyses can provide unique insights into the tumour-intrinsic and tumour-extrinsic mechanisms that drive responses, as well as primary or acquired resistance to novel immunotherapeutics[1,2].

The genomic, transcriptomic, epigenomic and/or proteomic examination of individual cells overcomes many of the limitations of bulk analysis, enabling a more refined dissection of the cellular and molecular basis of cancer. The tremendous power of single-cell analysis has been recognized for decades, as shown by the fact that immunohistochemistry, in situ hybridization and flow cytometry have all been indispensable tools in investigating the differences between non-malignant cells and cancer cells, both in the laboratory and in the clinic. These classic methods, however, often only detect a limited number of analytes in any given experiment or test, and this limitation reduces the power to distinguish the diversity of cellular subtypes and states present in the TME.

Breakthroughs in sequencing technologies, cell-isolating microfluidics and analytical bioinformatics have led to a rapid proliferation of single-cell analysis methodologies, and have been reviewed extensively elsewhere[3–6]. These techniques enable the detection of many analytes at single-cell resolution in a large number of cells (initially a few hundred, but now thousands to hundreds of thousands of cells) in an increasingly cost-effective manner. The concomitant blossoming of the availability of bioinformatic tools has enabled researchers to interrogate the massive datasets provided by such methods in order to exploit the covariate power at the heart of single-cell data, visualize the diversity of cellular subtypes and elucidate the interactions between cells. Continual improvements in single-cell methodological workflows and analytics, including the ascertainment of single cells, reduction of high-dimensional data, unsupervised clustering, phylogeny modelling, harmonization of multiple datasets, trajectory inference, RNA velocity, lineage tracing and ligand–receptor interaction mapping, have been crucial to the success of single-cell approaches and have been reviewed in detail elsewhere[7–11]. Single-cell approaches are uniquely poised to help answer many of the questions that persist in immuno-oncology, including: the role of tumour and immune-cell heterogeneity (including heterogeneity in expression of the antigen-presenting machinery); the relationship between T cell clonotype and phenotype; the network of immune-checkpoint interactions present in the TME; and the spatiofunctional relationships between tumour cells and immune cells, as well as between different immune cell types. Herein, we focus on how high-dimensional single-cell analysis has been leveraged to investigate the interplay between tumour cells and their microenvironment, and especially how this relates to response to anti-cancer immunotherapy.

In this Perspective, we first examine how single-cell transcriptomic, proteomic, epigenomic and genomic technologies have transformed our understanding of responsiveness and resistance to anti-cancer immunotherapy, before exploring analytical approaches that integrate multiple modalities. Next, we consider the critical importance of single-cell T cell

receptor (TCR) analysis. We then survey emerging technologies enabling single-cell spatial analysis, with a focus on their applicability to immuno-oncology. Finally, we discuss the future directions of single-cell technologies and their possible prospective roles in advancing the field of immuno-oncology.

Single-cell omics

Transcriptomics

Single-cell RNA sequencing (scRNA-seq) refers to a collection of techniques that enable the untargeted quantification of the transcripts present in individual cells. Svensson et al.[12] have reviewed the remarkable technological advances over the past decade that culminated in the Human Cell Atlas project adopting scRNA-seq as the definitive method for classifying cell types and states[13]. As an untargeted approach to surveying the transcriptome, scRNA-seq can identify novel cell types (such as the pulmonary ionocyte fundamentally responsible for the disease biology of cystic fibrosis[14]), interrogate rare cell populations, and define a spectrum of cellular states and phylogenies (such as the reclassification of human dendritic cells (DCs) and monocytes in the peripheral blood[15]) (FIG. 1). After dissociating a tumour into individual cells, scRNA-seq can be used to analyse the single-cell suspension either without enrichment or after fractionation into defined subsets of cells. In the following subsections, a few illustrative examples of the value of scRNA-seq are discussed in detail. Additional examples highlight the key insights derived from scRNA-seq — especially those that are relevant to the involvement of immune cells in cancer in general and in the response to immunotherapy in particular (TABLE 1).

Tumour cells and immunotherapy response.—The heterogeneous nature of cancer, both between and within individual tumours, is the predominant feature highlighted by scRNA-seq analysis of tumour cells. When clustering malignant cells from multiple patients, authors have noted that transcriptional profiles of individual cells group primarily by patient of origin in those with glioma[16], melanoma[17,18], head and neck squamous cell carcinoma[19] and ovarian cancer[20], among others. This observation is perhaps not surprising and attests to the fact that each tumour has a unique evolutionary trajectory. This patient-to-patient heterogeneity can complicate the use of scRNA-seq analysis of single tumour cells to study response to therapy. Consequently, single-cell analysis of immune and stromal cells has been more fruitful when applied to the identification of cell types and states associated with immunotherapy response that are shared among patients.

Despite this heterogeneity, identifying malignant cells with specific transcriptional states that are shared across patients is possible. Studies of the transcriptomes of single tumour cells have refined the classification of tumour types and their cellular hierarchies[16,19,21–24], and have also enabled the identification of transcriptome-wide signatures associated with therapeutic response or resistance. The powerful potential of this capability is illustrated by data from an scRNA-seq-based analysis of samples from 31 patients with melanoma receiving immune-checkpoint inhibitors (ICIs), in which the authors defined a transcriptional programme expressed in malignant cells that was associated with T cell exclusion and intrinsic resistance to therapy[18]. This programme resulted in increased

expression of *CDK4* with concomitant reductions in the expression of genes encoding proteins involved in antigen processing and presentation (such as *B2M*, *HLA-A*, *HLA-B* and *HLA-E*), interferon signalling or components of the complement system. Iterative multiplex immunofluorescence using tissue-based cyclic immunofluorescence (t-CyCIF) (BOX 1) confirmed the association of this programme with T cell exclusion in situ. By applying single-cell insights to bulk RNA-seq data, the researchers showed that the resistance programme was expressed prior to therapy in a longitudinal cohort of 26 patients with metastatic melanoma, was associated with inferior survival in 473 patients with melanoma included in The Cancer Genome Atlas and was predictive of poor response to anti-PD-1 in an independent cohort of 112 patients. These observations collectively showcase the potential of single-cell analyses of a small cohort to aid in the deconvolution of bulk data from larger cohorts and to identify cellular subpopulations that are amenable to therapy. Testing of these single-cell insights in a mouse model[18] suggested that CDK4/6 inhibition might be a tractable target to reverse the resistance programme and sensitize melanomas to immune-checkpoint inhibition.

Immune and/or stromal cells and immunotherapy response.—Overall, a comprehensive understanding of immune cell composition and state is crucial to understanding responsiveness and resistance to current immunotherapies, and to the rational design of novel immune-modulating therapies and combinations. Initial efforts in this area were focused on creating detailed immune atlases of the cells that reside within non-malignant tissues and then applying those atlases and methods to the neoplastic setting. For example, Szabo et al.[25] generated a reference map of non-malignant human T cell activation by analysing >50,000 resting and activated T cells from the lungs, lymph nodes, bone marrow and blood. The authors then projected published scRNA-seq profiles of tumour-associated T cells from patients with non-small-cell lung cancer[26], colorectal cancer[27], breast cancer[28] or melanoma[29] onto their map of non-malignant T cell activation states, and thereby demonstrated that tumours harbour activated $CD8^+$ T cells, but not functionally activated $CD4^+$ T cells[25]. Furthermore, intratumour $CD8^+$ T cells expressing exhaustion markers also expressed genes associated with conventional $CD8^+$ T cell effector functions and ongoing proliferation.

Extending single-cell transcriptional mapping to the TME across a range of tumour types has revealed several intriguing insights into the heterogeneity and diversity of immune responses in cancer (TABLE 1). An important finding is that clusters based on untargeted transcriptional assessments of cellular states often do not cleanly segregate into conventional immune cell subsets based on cell-surface protein expression, thus leading to a more nuanced understanding of the roles of immune cells in the TME.

Applying these approaches to the immune and stromal cells present in the TME in the context of immunotherapy has enabled the elucidation of the transcriptional states that underlie response and resistance to immunotherapies, such as ICIs. In a prototypical example, Sade-Feldman et al.[29] analysed 16,291 immune cells from 48 well-annotated tumour biopsy samples from 32 patients with metastatic melanoma treated with ICIs and identified 11 immune cell clusters. Focusing on $CD8^+$ T cells revealed two broadly defined cellular states that correlated with response and resistance. Features of the cellular state

enriched in responders included increased expression of genes associated with memory, activation and cellular survival (including *TCF7*) and reduced expression of genes associated with co-inhibitory effects. By contrast, the cellular state enriched in non-responders included increased expression of genes associated with T cell exhaustion (such as *ENTPD1*, *BATF*, *HAVCR2*, *PDCD1*, *LAG3* and *CTLA4*). Notably, this distinction between responders and non-responders was observed in tumour-infiltrating lymphocytes (TILs) from both pretreatment and post-treatment samples, suggesting that these signatures have predictive potential. Indeed, conventional multiplex immunofluorescence analysis of tissue sections from an independent cohort of 33 patients confirmed the association of *TCF7* expression with a response. Additionally, the finding that $CD8^+$ TILs in non-responders are enriched with cells that co-express CD39 and TIM3 prompted the testing of targeted inhibitors of these proteins in a mouse model of melanoma[29]. Enhanced levels of tumour control were observed when inhibiting CD39 in combination with antibodies targeting TIM3, PD-1, or PD-1 and CTLA-4, thus illustrating the potential of single-cell transcriptomics, when combined with protein-level confirmation, to identify new co-expressed targets for novel immunotherapy combinations.

Importantly, analyses designed to map the immune landscapes of a diverse range of tumour types have identified a nascent, but growing portfolio of transcriptional states associated with responsiveness to immunotherapy across lymphocyte, DC, monocyte, macrophage and fibroblast compartments, many of which are now being studied as putative biomarkers (TABLE 1). Having gained a better understanding of the transcriptional landscapes of tumours and their microenvironmental constituents, investigators can now examine how the tumour and immune system co-evolve in concert over the course of treatment and relapse, at the single-cell level of resolution.

Interaction analysis.—Many studies have explored the roles of either tumour cells or immune and/or stromal cells in isolation, despite complex interactions between and within these cell types having a crucial role in cancer biology. Information on these important cell–cell interactions can be inferred from scRNA-seq data by correlating the expression of ligand–receptor pairs between different cell types[30,31]. For example, assessments of the interactome by Zhang et al.[32] have provided insights into the interplay between tumour-associated DCs and peripheral T cells. In this study involving 16 treatment-naive patients with hepatocellular carcinoma, leukocytes from the tumour, adjacent liver, hepatic lymph nodes, blood and ascites were analysed using scRNA-seq. Lineage analysis revealed a novel class of $LAMP3^+$ DCs capable of migrating from tumours to lymph nodes. These $LAMP3^+$ DCs expressed many putative inhibitory ligand–receptor pairs and were associated with multiple T cell subsets, suggesting a role in promoting the dysfunction of peripheral T cells. Novel technologies are also being developed that enable the exploration of physical cell–cell interactions — including spatially resolved scRNA-seq methods (BOX 1) — in an attempt to better understand the intricately choreographed interactions between tumour cells and those of the TME.

Limitations of scRNA-seq.—scRNA-seq has revolutionized the delineation of cell types and cellular states, although this technique also has several notable drawbacks. First,

scRNA-seq data are intrinsically noisy because eukaryotic transcription does not take place at a consistent basal rate; it occurs in pulses[33,34]. For example, monoallelic expression in single cells is predominantly the result of burst-like stochastic transcription[35,36]. Thus, a failure to detect a transcript from a particular gene in a cell at a single specific point in time is an ambiguous result: it could mean that the gene is inactive in that cell, that the gene is active, but the transcript was not detected owing to burst kinetics and the timing of sampling, or that technical deficiencies meant that expression of the gene was not detected. This challenge in substantiating negative findings is particularly relevant for genes with lower levels of expression. Analytical approaches that consider the entirety of the cellular transcriptome (such as those involving clustering by differentially expressed genes) have helped to overcome this limitation of scRNA-seq. Consequently, the interpretation of results should focus on pathway analysis and gene-set enrichment, rather than the expression or effects of any single gene.

Second, the use of transcriptome data alone to establish correlations between genotype and phenotype remains challenging. For example, scRNA-seq data have been used to infer somatic copy number variants (commonly referred to as sCNVs)[37,38], although this only applies to relatively large deletions or amplifications. The larger number of somatic single-nucleotide variants (sSNVs) present in tumour cells enables the assessment of their genetic architecture at a higher level of resolution than sCNVs; nonetheless, the ability to confidently detect sSNVs within scRNA-seq data remains limited. For example, sSNVs in transcripts with low levels of expression are detected only in a minority of cells owing to the combined challenges of transcriptional bursting and technical dropout. Additionally, because the highest-throughput methods involve end counting (by sequencing just the 3′ or 5′ end of the RNA molecule, rather than the full transcript), sSNVs located away from the end of a transcript are often poorly captured and thus under-represented in the data. Several new approaches, including TARGET-seq[39], genotyping of transcriptomes[40] and the approach described by van Galen et al.[41], have incorporated primers for known mutations into otherwise untargeted scRNA-seq protocols in an attempt to address this limitation, but these approaches require a priori knowledge of the variants to target. As a result, scRNA-seq approaches are a suboptimal method of evaluating the implications of somatic mutations in individual tumour cells for therapeutic response or resistance.

In addition to these inherent limitations of scRNA-seq, specific details relating to sample procurement and processing can also confound results, with important implications for the clinical applicability of these technologies. Indeed, scRNA-seq has relied upon viable single-cell suspensions, which are readily available from peripheral blood and/or lymph nodes, but are often more difficult to obtain from solid tumour tissues. Differences in dissociation methods and cryopreservation conditions can also induce considerable transcriptome-wide changes[42]. Even with blood samples, the amount of time blood draws are kept at room temperature before cryopreservation substantially alters the transcriptional profile of individual cells, including a pronounced downregulation of immune cell type-specific genes, in the absence of a loss of RNA fidelity after storage for >2 hours at room temperature[43]. In an attempt to address these issues, a large group of researchers with an interest in single-cell genomic analysis of tumours has begun developing protocols designed to standardize tissue dissociation and the preparation of single-cell suspensions[44].

Investigators studying tissue development have found that single-nucleus RNA sequencing (snRNA-seq) enables a more streamlined workflow that is applicable to many different tissue types, is compatible with frozen samples and can sometimes provide less-biased cellular coverage compared with scRNA-seq[45–48]. This method is beginning to be applied to tumour samples and should substantially improve the clinical utility of single-cell approaches[44].

Finally, batch effects can occur owing to the processing of samples on different days with even slight technical variations, as often happens when comparing samples obtained over a time course. Multiple methods of correcting such batch effects have been developed[49], although the use of these corrections must be balanced with the risk of obscuring true biological differences.

Proteomics

Mass cytometry.—Untargeted detection of proteins using mass spectrometry lacks sufficient sensitivity to be relevant to single-cell analysis[50]. Therefore, single-cell proteomics studies rely predominantly on selective target detection using antibodies or similar affinity reagents (such as affibodies or aptamers)[51–55]. Flow cytometry has been the longstanding workhorse for investigators seeking to assess protein expression at the single-cell level of resolution, although the number of epitopes that can be detected concurrently is limited by spectral overlap between fluorophores. The advent of mass cytometry[56], which involves the use of metal isotopes to label antibodies, has greatly expanded the number of markers that can be analysed simultaneously, thereby enabling the high-dimensional detection of proteins expressed on single cells. For example, Wagner et al.[57] used a 35-marker immune cell-centric panel and a 38-marker tumour cell-centric panel to generate a comprehensive single-cell atlas of breast cancers.

In general, the analytical precision of single-cell proteomics methods is greater than that of current scRNA-seq methods. Furthermore, the longer half-life of proteins compared with RNA means that protein measurements are less prone to the fluctuations often observed in scRNA-seq data. Antibody-targeted approaches also provide additional detection capabilities, such as the detection of post-translational modifications[58] or the assessment of morphological features via cell morphometry[59]. Similar to flow cytometry, however, the targeted nature of mass cytometry analysis requires the prospective selection of analytes, which can bias the identification of cellular groups compared with the untargeted simultaneous detection of hundreds to thousands of RNA transcripts using scRNA-seq.

Mass cytometry is a particularly useful method of characterizing the specific phenotypes of cells involved in responses to immunotherapy at the single-cell level, as illustrated by the results of two investigations of responses to ICIs in patients with melanoma. In the first study[60], investigators used mass cytometry to demonstrate that a high frequency of circulating classic monocytes before treatment is a strong predictor of a response to anti-PD-1 antibodies, which was confirmed by analysing samples from a validation cohort of 31 patients using targeted flow cytometry. In the second study, Gide et al.[61] analysed melanoma biopsy samples obtained from a subset of patients who received either anti-PD-1 antibodies as monotherapy or in combination with anti-CTLA-4 antibodies. Dimensionality reduction

analysis of a 43-marker mass cytometry panel identified 14 immune cell clusters, including three distinct T cell clusters. Key markers of activation, differentiation and exhaustion were found to co-cluster with a $CD45RO^{+}EOMES^{+}$ T cell population, including markers of recent activation and tissue residency (CD69), tissue-resident memory (CD103) and effector memory (T-BET, HLA-DR and low CCR7). This subpopulation expressed the inhibitory receptors PD-1 and TIGIT, but had only low levels of CD57 expression, suggesting they are not terminally exhausted. Responders to combination therapy had a higher proportion of this $CD45RO^{+}EOMES^{+}$ subpopulation among both the $CD4^{+}$ and $CD8^{+}$ cells compared with non-responders, but no difference was observed among patients receiving monotherapy. When the insights gained from mass cytometry were used to examine a larger cohort of 140 samples from 73 patients with melanoma using bulk RNA-seq analysis, a memory T cell phenotype featuring high levels of *CD4* or *CD8*, *EOMES*, *CD69* and *CD45RO* expression corresponded with responsiveness to both ICI regimens and was predictive of a longer progression-free survival duration in those receiving anti-PD-1 antibodies as monotherapy. These observations underscore the importance of evaluating immune cell phenotypes at the single-cell level.

As noted previously, the ability to detect post-translational modifications is a distinct advantage of antibody-targeted approaches, such as mass cytometry. This capability was utilized in a study analysing chimeric antigen receptor (CAR) T cells using a mass cytometry panel that focused on the detection of phosphoproteins with roles in maintaining T cell function[53]. The single-cell resolution provided by mass cytometry enabled a more sophisticated analysis of network dynamics. Analysis of second-generation CD19–28ζ CAR cells indicated that tonic signalling, resulting in antigen-independent CAR activation, is predominantly associated with the CD3ζ, but not the CD28, endodomain. Tonic signalling can affect the efficacy of therapy by inducing CAR T cell exhaustion. On the basis of these single-cell findings, the authors explored an alternative CAR design that circumvents tonic signalling by using γδT cells expressing a chimeric costimulatory receptor. By separating the CD3ζ domain in the γδTCR from the CD28 domain in the chimeric costimulatory receptor, this modified CAR design enabled efficient cytotoxic responses to CD19-positive leukaemic cells without evidence of activation of tonic signalling. The single-cell analysis of post-translational modifications will continue to be of particular benefit to understanding the signalling pathways associated with effectiveness and/or resistance of adoptive cellular therapies and native immune cells in the context of immunotherapy.

Emerging technologies for single-cell proteome analysis.—Next-generation spectral analysers promise to extend the capabilities of fluorescence-based flow cytometry to a level that rivals that of mass cytometry. An advantage of these systems over mass cytometry is the ability to utilize the vast number of fluorescence-labelled antibodies that are already available and, because they do not destroy cells like mass cytometry does, enable the sorting and isolation of cells for subsequent experiments. As another emerging technology, tagging antibodies with barcode oligonucleotides, instead of fluorophores or heavy metal ions, obviates the constraints placed on the number of simultaneously resolvable targets.

Additional antibody-targeted single-cell strategies include tissue-based cytometry (BOX 1), which adds an improved level of spatial resolution, and microfluidic cytometry, which is

exemplified by chipcytometry[62,63] wherein cells are immobilized on a microfluidic chip instead of being analysed in a flow stream. Chipcytometry enables iterative staining; therefore, scores of markers (up to 110) can be detected and quantified on the same sample of cells. Another innovation based on single-cell barcode chip technology[64,65] is the IsoLight system[66], which enables the detection of secreted proteins from single cells. The system utilizes microfluidic chips to capture information from 200 to 2,000 single live cells and sandwich-ELISA-like assays to measure >30 cytokines, chemokines, growth factors and other secreted ligands over a multi-hour incubation period. This system also has the flexibility to incorporate additional analytes, such as metabolites. The preliminary utility of the IsoLight system has been demonstrated in a phase 1 trial in which patients with advanced-stage melanoma received adoptive cell transfer supported by pegylated IL-2, which resulted in enhanced levels of cytokine polyfunctionality among circulating T cells and natural killer cells[67]. These new platforms of spectral analysers and microfluidic systems are still in the early stages of development and need to be validated in additional clinical settings. Nonetheless, the early successes thus far suggest that they will increasingly have a key role in immuno-oncology.

Genomics

Methods enabling whole-genome and whole-exome sequencing of single cells have been developed (reviewed extensively elsewhere by Gawad et al.[68]), although these methods have not yet made the transition to high-dimensional analysis, largely because the high costs of such sequencing preclude scaling to sizeable numbers of cells per patient. A more efficient approach is to first use deep sequencing of bulk tumour DNA samples to identify sSNVs, then use targeted sequencing of DNA from single cells to detect putative driver mutations or mutations implicated in clonal structure. By combining microfluidics with the ability to merge droplets, the Tapestri system uses a two-step process whereby robust lysis and liberation of DNA occurs in a first droplet followed by merging with a second droplet to perform PCR amplification and cell-specific barcoding of target genomic loci[69]. Although not yet applied in the context of immunotherapy, this system has been used to analyse hundreds of thousands of single cells to track specific mutations through diagnosis, remission and relapse in patients with acute myeloid leukaemia[70–74] — thus demonstrating the promise of this technology.

Epigenomics

Epigenomic analysis methods, such as the assay for transposase-accessible chromatin using sequencing (ATAC-seq, which enables the detection of open segments of chromatin), bisulfite-based DNA methylation sequencing, chromatin immunoprecipitation sequencing (ChIP-seq, which detects DNA–protein interactions) and chromosome conformation capture (such as 3C and Hi-C), have all been adapted for single-cell analysis (reviewed in detail elsewhere[75–77]). Of these methods, single-cell (sc)ATAC-seq is currently the only one with sufficiently high throughput and is thus widely adopted for this purpose. For example, application of this method to pretreatment and post-treatment samples obtained from patients with advanced-stage basal cell carcinoma revealed expanded populations of exhausted $CD8^+$ T cells and $CD4^+$ T follicular helper cells after treatment with anti-PD-1 antibodies[78]. In these cells, the *PDCD1* locus contained a +5 kb *cis*-element with specific

accessibility, suggesting that post-treatment $CD8^+$ T cells and T follicular helper cells have a shared enhancer that drives persistent PD-1 expression. Single-cell epigenomics is a rapidly developing field that, particularly when integrated with single-cell transcriptomics[79,80], is likely to be one of the next major advances in single-cell analysis in oncology. In this regard, single-cell reduced representation bisulfite sequencing[81] for DNA methylation analysis and CUT&Tag[82] for single-cell ChIP-seq, are particularly notable, and these methods should further expand the ability to analyse epigenetic changes at the level of individual cells.

Multimodal analysis

Interest in integrating distinct modalities of single-cell analysis into consolidated technologies is increasing[83,84]. The application of such multimodal approaches to investigating responses to immunotherapy has been limited thus far, although several promising methods with broad applicability are briefly reviewed here.

RNA or DNA plus protein.—Techniques incorporating single-cell detection of proteins into scRNA-seq protocols (such as CITE-seq[85] and REAP-seq[86]) use antibodies conjugated with barcode oligonucleotides to enable the detection of protein-based markers via the cDNA sequencing steps of scRNA-seq, thereby enabling protein detection to proceed without the constraints imposed by spectral overlap or the number of resolvable metal ions. For example, the proximity extension assay[87] that uses barcoded antibodies has enabled the development of commercial 96-plex panels for the detection of proteins present in serum and plasma samples. The ability to add the antibody-based detection of tens to hundreds of proteins to untargeted transcriptome analysis will substantially improve the ability to assess the TME by defining transcriptional clusters within the framework of traditional immunology and diminishing ambiguities caused by transcript dropout. The use of oligonucleotide-tagged antibodies has also been incorporated into the Tapestri workflow for single-cell DNA sequencing, providing a direct method of linking genotype and phenotype[88].

Lineage tracing.—scATAC-seq data contain sequencing reads from the mitochondrial genome. Although these signals were originally treated as an experimental nuisance, this feature enables the detection of mitochondrial mutations that can be used to perform clonal tracing[89,90]. Thus, scATAC-seq enables the simultaneous measurement of cell lineage (via the mitochondrial genome) and cell fate (via the nuclear epigenome). Mitochondrial mutations can also be detected in scRNA-seq data[84], although not as robustly as in scATAC-seq data owing to lower and less consistent coverage of the mitochondrial genome. Such lineage tracing analysis could be applied in the future to better understand which tumour cell clones expand or contract in response to a specific treatment, and thus which clones might be linked to therapeutic resistance.

Single-cell T cell receptor analysis

T cells are the main arm of the adaptive immune system responsible for anti-tumour immunity and are thus the primary focus of most FDA-approved cancer immunotherapies (such as ICIs, adoptive cell therapies, peptide or RNA vaccines, cytokine-based therapies

and oncolytic virotherapies). Owing to this focus, the analysis of individual T cell clonotypes is currently of increasing research interest. The TCR is a heterodimer comprising two chains, for which genetic recombination creates a diverse TCR repertoire. The majority of T cells (95%) carry individual TCR α and β subunits, which together recognize a cognate peptide–MHC antigen[91]. On the basis of this TCR–peptide–MHC interaction, naturally occurring TILs in immunogenic tumours have the potential to eliminate tumours[92]. Determining whether a T cell is capable of targeting a tumour cell requires single-cell paired TCR α and β subunit sequences for TCR reconstruction and specificity testing. Furthermore, in order for immunotherapies to generate an effective antigen-specific anti-tumour immune response, tumour antigens must ultimately be presented as antigen–MHC complexes and recognized by cognate TCRs present on tumour-infiltrating T cells. A growing body of evidence suggests that many tumour-infiltrating T cells are simply ‘bystanders’ that are incapable of recognizing and attacking tumour cells[93]. Therefore, a tumour might appear to be highly infiltrated by immune effector cells, but nonetheless be ‘qualitatively cold’ owing to a lack of tumour-specific T cells capable of anti-tumour activity[94]. Beyond quantifying cellular composition and characterizing transcriptional states, single-cell approaches also enable the identification of paired α and β TCR subunits, which is a crucial step in determining the specificity of infiltrating T cells. Such information might not only help explain resistance to current immunotherapies (such as a lack of tumour specificity), but also facilitate the discovery of high-avidity anti-tumour TCRs that can be utilized in novel engineered TCR-based cellular therapies.

Bulk TCR sequencing cannot assign paired TCR subunits to individual T cells; therefore, this information was historically obtained by isolating TILs using in vitro culture methods, then subcloning cells through a low-throughput, labour-intensive process of creating homogeneous populations of T cells that could then be sequenced to determine individual TCR αβ subunit combinations. In the subsequent years, numerous PCR-based techniques were developed to enable sequencing of individual T cell TCR–complementarity determining region (CDR3) sequences in order to unambiguously assign TCR αβ subunits to individual T cells in a tumour[95–98]. Paired αβ sequences can now be obtained at higher throughput using droplet-based methods. Moreover, the identified TCR sequences can be reconstituted in native T cells or reporter cell lines using viral transfection to deduce antigen specificity[99–101]. Combining scRNA-seq with single-cell TCR sequencing (scTCR-seq) links T cell phenotypes, such as activation, memory and exhaustion, with the specific TCR clonotypes of individual T cells. The ability to unambiguously assign TCR sequences to single T cells has led to the development of microfluidic and flow cytometry-based methods designed to directly identify antigen-specific T cells from TIL, tissue and peripheral blood samples[102–104]. Together, the integration of scTCR-seq, antigen specificity determination and the single-cell analysis approaches described previously have enabled more detailed investigations of several important topics in immuno-oncology, such as the roles of clonal replacement versus clonal expansion and of T cell clonotypic phenotypes in immunotherapy responses.

scTCR-seq of mRNA had a key role in the analysis of samples obtained in a phase 1 trial involving personalized vaccination with neoantigen peptides to treat patients with glioblastoma[105]. In one patient, scTCR-seq enabled the identification of clonotypes shared

between TILs and peripheral blood T cells. Cloning and expression of the shared TCRs demonstrated the presence of TILs within the tumour that were specific for a vaccinating peptide, thus highlighting the power of these techniques to track and evaluate individual T cell clonotypes during an immunotherapy response. Tumour material from other patients in this study was not analysed in this manner owing to difficulties in obtaining TILs as single-cell suspensions from clinical samples, which is a persistent issue in applying scTCR-seq to the management of patients with solid tumours.

Integrated scRNA-seq and scTCR-seq provides a particularly effective method of analysing the phenotypic and functional characteristics of immune cells over the course of treatment with immunotherapy. For example, analysis of pretreatment and post-treatment biopsy samples from patients with advanced-stage basal cell carcinoma ($n = 11$) or squamous cell carcinoma ($n = 4$) receiving treatment with an anti-PD-1 antibody revealed an increase in the number of both activated and chronically activated (exhausted) $CD8^+$ TILs following treatment[106]. Notably, the greatest degree of TCR clonality (implying the lowest level of diversity) was observed in post-treatment exhausted $CD8^+$ TILs, and the overwhelming majority of these clonotypes consisted of expanded novel clones that were not detected prior to anti-PD-1 therapy. Exhausted TIL clones that were present before treatment did not expand upon treatment nor did they transition to non-exhausted phenotypes. Bulk repertoire analysis of pretreatment peripheral blood samples revealed that 11.8% of the post-treatment TILs were novel exhausted T cell clonotypes, even though these clonotypes were not detected in pretreatment TILs. These results suggest that pre-existing tumour-specific TILs have only a limited ability to be reactivated and that ICIs can induce the influx and expansion of novel T cell clones (clonal replacement). Additionally, in an analysis of samples from eight primary uveal melanomas and three metastases[107], scTCR-seq revealed expanded populations of specific T cell clonotypes, and scRNA-seq indicated that these expanded T cell clones were largely exhausted. The expression of genes encoding exhaustion-associated immune checkpoints was strongest for *LAG3*, with minimal expression of *PDCD1* and *CTLA4*, which might contribute to the observed ineffectiveness of anti-PD-1 and anti-CTLA4 antibodies in this setting. The results of single-cell studies like these suggest that responsiveness to ICIs might depend on non-exhausted T cells and T cells recruited from the periphery (clonal replacement) rather than only on the reinvigoration of exhausted T cells in the tumour (clonal expansion).

By complementing single-cell phenotypic profiling of T cells (such as via scRNA-seq) with the identification of their respective TCR clonotypes, these integrated approaches add crucial information on T cell antigen specificity to the analyses of immune cells, thereby enabling finer dissection of the roles of antigen-specific T cells in immunotherapy responses. Furthermore, the non-invasive identification of expanded TCR clones in blood samples collected longitudinally over the course of treatment with immunotherapy might provide insights into the identity and functionality of clinically relevant TCRs in tumours. The development of a cost-effective method of identifying paired TCR αβ subunit clonotypes from peripheral blood[108] will make the longitudinal analysis of blood samples obtained from patients receiving immunotherapy in clinical trials even more powerful.

High-dimensional spatial analysis

Spatial variability in the tumour immune microenvironment is increasingly becoming recognized as contributing to the heterogeneous nature and variable therapeutic responses seen with most cancer types. Time-honoured spatial methods dating back to Coons et al.[109] (selective immunostaining of proteins by immunohistochemistry and immunofluorescence) and Gall and Pardue[110] (in situ hybridization of nucleic acid sequences) have been indispensable for cancer diagnosis, classification, treatment selection and prognostication. Contemporary applications include the quantification of PD-L1 expression using immunohistochemistry and HER2 amplification using in situ hybridization, both of which are required by the FDA as companion diagnostics for certain anti-PD-(L)1 checkpoint inhibitors and HER2-targeted monoclonal antibodies, respectively, in defined clinical indications. Over the past few years, these more traditional methods have been substantially expanded, multiplexed and coupled with advances in bioinformatics to enable the simultaneous visualization of multiple proteins, DNA and/or RNA molecules. Several approaches that combine spatial resolution with next generation sequencing are also being developed (BOX 1). Most published data relating to these new approaches are limited to preliminary studies designed to demonstrate proof-of-concept.

Multiplexing of immunohistochemistry and immunofluorescence has already demonstrated preliminary clinical utility in a few immunotherapy studies. A meta-analysis of data from 8,135 patients across ten different cancer types treated with anti-PD-1 or anti-PD-L1 antibodies demonstrated that multiplexed immunohistochemistry plus immunofluorescence strategies outperform tumour mutational burden, gene expression profiling and PD-L1 immunohistochemistry expression biomarkers in predicting response to immunotherapy[111]. The diagnostic benefits of multiplexed imaging were not dependent on a large number of analytes — improved prediction was observed with as few as two or three markers — but from the incorporation of information on the spatial relationships and protein co-expression at the single-cell level of resolution. Indeed, in an investigation of tumour biopsy samples from patients with metastatic melanoma who received anti-PD-1 antibodies, tumours with both increased spatial interactions between PD-L1$^+$ cells (both tumour and myeloid) and PD-1$^+$ non-tumour cells (mostly immune) as well as those featuring higher proportions of tumour cells co-expressing HLA-DR and IDO-1 were most likely to respond to immunotherapy and were associated with improved survival outcomes[112]. These single-cell level spatial findings reinforce the hypothesis that ample and proximal expression of both PD-1 in T cells and PD-L1 in tumour cells is needed to derive therapeutic benefit from anti-PD-1 or anti-PD-L1 antibodies.

High-dimensional protein detection methods have the potential to increase the multiplex capabilities of spatial analysis to a level of 20–100 markers, thus expanding the number of cell types and cell states that can be characterized (BOX 1). This increased dimensionality enables an unprecedented depth of analysis of the complex spatial interactions between networks of single-cell phenotypes, thereby spatially defining ‘communities’ of cells in the tumour and/or their microenvironment that might be better prognostic biomarkers than quantification of single-cell phenotypes alone[113,114]. Cytometry by time of flight (CyTOF) and scRNA-seq-based analyses implicated intratumoural B cell receptor diversity

and memory B cell phenotypes in responses to ICIs in patients with melanoma, with the addition of spatial methods indicating the importance of B cell localization to tertiary lymphoid structures (TLSs)[115]. By using the GeoMx DSP platform to quantify both the expression and location of 60 proteins in B cells, T cells and tumour cells in melanoma biopsy samples, Cabrita et al.[116] found that B cells interact closely with $CD4^+$ T cells in TLSs and are associated with increases in the numbers of $TCF7^+$-naive and memory T cells. Conversely, TILs present in tumours that lack TLSs were more likely to have molecular signatures associated with immunological dysfunction and/or exhaustion. By integrating these key spatial findings with scRNA-seq data, the authors derived a TLS gene signature that predicted an improved response to ICIs[116].

Although still in their technological infancy, spatially-resolved multiplexed profiling approaches are reshaping the ability to interrogate both the intercellular interactions and the architectural relationships between tumour cells and cells of the TME that govern tumour immunology and responsiveness to immunotherapy in patients.

Future directions

scRNA-seq and its various multimodal incarnations are revolutionary research tools that are advancing both our understanding of human cancer immunology and the mechanisms driving responsiveness and resistance to immunotherapies in patients. Although immunotherapies have transformed the management of many advanced-stage malignancies, the majority of patients either do not respond to these agents or will ultimately acquire resistance[1]. Thousands of clinical trials exploring combination immunotherapy approaches, with other immunotherapy agents, with non-immunotherapy agents or both, have either been completed or are currently underway[117]; nonetheless, the design of rational immunotherapy-containing combinations designed to overcome primary and/or acquired resistance will require the comprehensive characterization of the tumour immune microenvironment of each cancer type, and a detailed understanding of the determinants of effective anti-tumour immunity in the context of therapy. Single-cell technologies enable comprehensive profiling of the TME and are therefore especially well-suited to studying both tumour and immune cell heterogeneity and how such differences in tumour biology contribute to therapeutic resistance[118,119].

In order to effectively leverage single-cell technologies to improve our understanding of mechanisms of immunotherapy response and resistance and to aid the development of novel therapeutics, clinical trials will have to be carefully designed to incorporate single-cell approaches in a feasible way. Many of the current technologies are costly and require the isolation of viable single cells from disaggregated tumour tissues, thus posing substantial financial and logistical challenges that preclude their routine use in clinical care. If clinical studies are carefully designed in collaboration with translational scientists, however, incorporating single-cell analysis is feasible in a subset of patients and could yield informative results that can be applied to larger cohorts. Careful consideration must be given to what types (among others, tumour tissue, non-malignant adjacent tissues and/or peripheral blood) and formats (including fresh, snap-frozen and/or formalin-fixed paraffin-embedded) of samples should be collected, the optimal timing of collection (pretreatment, on-treatment

and/or post-treatment) and how sample material will be rapidly processed for immediate single-cell analysis (or cryopreserved for later analysis). While challenging, the insights gleaned from an in-depth investigation of a relatively small number of patients (5–50) can inform the analysis of a larger sample set using a more cost-effective and logistically more straightforward method (FIG. 2). As indicated in a number of studies described in this Perspective, an emerging approach is to exploit published scRNA-seq data rather than to generate these data de novo. This type of resource has now been formalized in the establishment of the Human Tumor Atlas Network[120].

Mining high-dimensional single-cell data to define low-dimensional panels of genes and/or proteins that show promise as predictive biomarkers provides a clear path towards clinical utility. Another avenue for exploiting data-rich single-cell information is provided by the use of bioinformatic algorithms to map bulk RNA-seq data onto the cell types defined by scRNA-seq and related techniques. The use of these in silico dissection approaches is less subject to the compromises inherent in using low-dimensional panels alone. A good example of this approach is provided by CIBERSORTx[121], a machine learning-based method that takes a signature matrix generated from scRNA-seq data and applies it to bulk tissue RNA-seq profiles in order to estimate the composition and abundance of cell types in each tissue sample. Thus, highly refined cell types defined by whole-transcriptome data can be readily quantified in routine clinical samples that might be of limited quality, fixed and/or difficult to disaggregate into intact single cells — thereby helping to substantially reduce the barriers to implementation in future immuno-oncology studies.

High-dimensional single-cell techniques that could be more readily applied clinically include those that expand the information content of traditional methods, such as immunohistochemistry and flow cytometry. For samples from haematological malignancies and others that can be easily dispersed into single cells, mass cytometry and flow cytometry panels incorporating increased numbers of fluorescent markers (such as 20–50) have already begun to be successfully utilized in clinical trials (NCT03734198, NCT03915379 and NCT04181827). For solid tumours, spatially resolved multiplexed technologies (BOX 1) have the ability to add genotypic and phenotypic dimensions to our understanding of how cells interact in the tumour immune microenvironment, and constitute the next frontier in elucidating the mechanisms underlying resistance to immunotherapies. Devices that automate these technologies and are compatible with existing clinical laboratory workflows, such as the in situ sequencing microfluidic tissue processor[122], indicate potential ways forward to the type of advances that will ease the adoption of these techniques.

Conclusions

Single-cell RNA sequencing and related modalities are increasingly becoming critical research tools for characterizing response and resistance to immunotherapy. Additionally, single-cell TCR analysis is required to determine T cell antigen specificity and provides crucial information on the role of the adaptive immune system in tumour formation, growth and treatment. The next frontier in single-cell analysis is the combination of high-dimensional content with precise tissue localization. The clinical utility of high-dimensional single-cell analysis includes identifying high-dimensional biomarkers of immunotherapy

response, robustly informing the design of low-dimensional biomarkers, enabling refined analysis of bulk sequencing data and understanding the complex cell–cell spatial interactions in the TME that drive responses to immunotherapy. Given the growing matrix of possible immunotherapies coupled with the personalized nature of tumour heterogeneity and therapeutic resistance, the future of cancer treatment is combination therapy[123–125]. As a result, multidimensional biomarker signatures will be crucial to making the optimal management choices for each patient with cancer. High-dimensional single-cell technologies will provide the resolution and richness of data required to generate these signatures in immuno-oncology.

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Competing interests

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Box 1 |

Methods of high-dimensional spatial analysis

Immunohistochemistry and in situ hybridization have long been used to localize one or a few proteins and RNAs, respectively, in tissue sections. Multiplex methods that have emerged over the past few years have dramatically increased the number of targets that can be detected.

Protein detection

- Iterative multiplexed immunofluorescence. The use of multiple rounds of labelling with fluorescent antibodies to detect 50–100 targeted proteins in tissue sections at subcellular resolution. The widespread availability of fluorescent antibodies and microscopy cores, and the use of routine formalin-fixed paraffin-embedded (FFPE) pathological specimens make these approaches relatively inexpensive and straightforward to apply in most immunotherapy studies. Examples include:
  - multiplexed immunofluorescence (MxIF)[134]
  - tissue-based cyclic immunofluorescence (t-CyCIF)[135]
  - multiple iterative labelling by antibody neodeposition (MILAN)[136,137]
  - cyclic multiplexed-immunofluorescence (cmIF)[138]
  - chipcytometry for frozen, non-fixed tissue sections[139]
- Mass cytometry. Use of metal-tagged antibodies to detect 30–100 target proteins in FFPE tissue sections. Single-cell detection is possible, although resolution is typically lower than with fluorescence-based methods. These approaches require specialized core facilities and expertise, but enable simultaneous measurement across all targets. Examples include:
  - multiplexed ion beam imaging (MIBI)[140,141]
  - imaging mass cytometry (IMC)[142–144]
- Oligonucleotide barcoding. Use of oligonucleotide-tagged antibodies to detect up to 50 targeted proteins in fresh frozen sections at subcellular levels of resolution. Examples include:
  - co-detection by indexing (CODEX)[145]

RNA Detection

- Iterative multiplexed in situ hybridization. Use of barcoded oligonucleotide probes to greatly expand the capabilities of conventional in situ hybridization in order to detect thousands of transcripts with subcellular resolution in FFPE tissue sections. These approaches require specialized analytics and expertise in order to localize individual mRNA transcripts below the optical diffraction limit. Examples include:

  - multiplexed error-robust fluorescence in situ hybridization (MERFISH)[146]
  - sequential fluorescence in situ hybridization (seqFISH^{+})[147]
- Spatial barcoding with hybrid capture of mRNA. Use of barcoded reverse transcriptase primers attached to slides or beads to enable untargeted whole-transcriptomic sequencing with the resulting resolution determined by spot or bead size. Unlike spatial protein detection methods, these early incarnations of spatial RNA methods are limited by transcript drop-out, overlapping capture of transcripts from neighbouring cells and the need for frozen, non-fixed tissue. Largely amenable to processing by existing single-cell cores with relatively short turnaround times, but do require specialized analytics and expertise. Examples include:
  - spatial transcriptomics with a 55-μm spot size (commercialized as the Visium system by 10× Genomics)[148,149]
  - slide-seq[150] with 10-μm beads
  - high-definition spatial transcriptomics (HDST)[151] with 2-μm beads
- Untargeted in situ sequencing. Use of tailed random hexamer reverse transcriptase primers followed by rolling-circle amplification of transcript sequences and in situ sequencing in frozen, non-fixed tissue sections. Capable of single-cell detection. Examples include:
  - fluorescent in situ sequencing (FISSEQ)[152,153]
- Targeted in situ sequencing. Use of specific probes to initiate rolling-circle amplification of target transcripts followed by in situ sequencing in frozen, non-fixed tissue sections. Capable of single-cell detection. Examples include:
  - spatially-resolved transcript amplicon readout mapping (STARmap)[154]
  - in situ sequencing (ISS)[122]
- Protein and/or RNA detection. This targeted method uses antibodies labelled with oligonucleotide barcodes and barcoded probes for RNA followed by location-specific release of the barcodes. Detection and quantification of the barcodes is conducted using a separate instrument. Although the barcode-releasing mechanism is capable of single-cell detection, current system sensitivity limits practical resolution to regions of interest at the level of 10–100 cells. Examples include:
  - digital spatial profiling (commercialized by Nanostring as GeoMx DSP)[155,156]

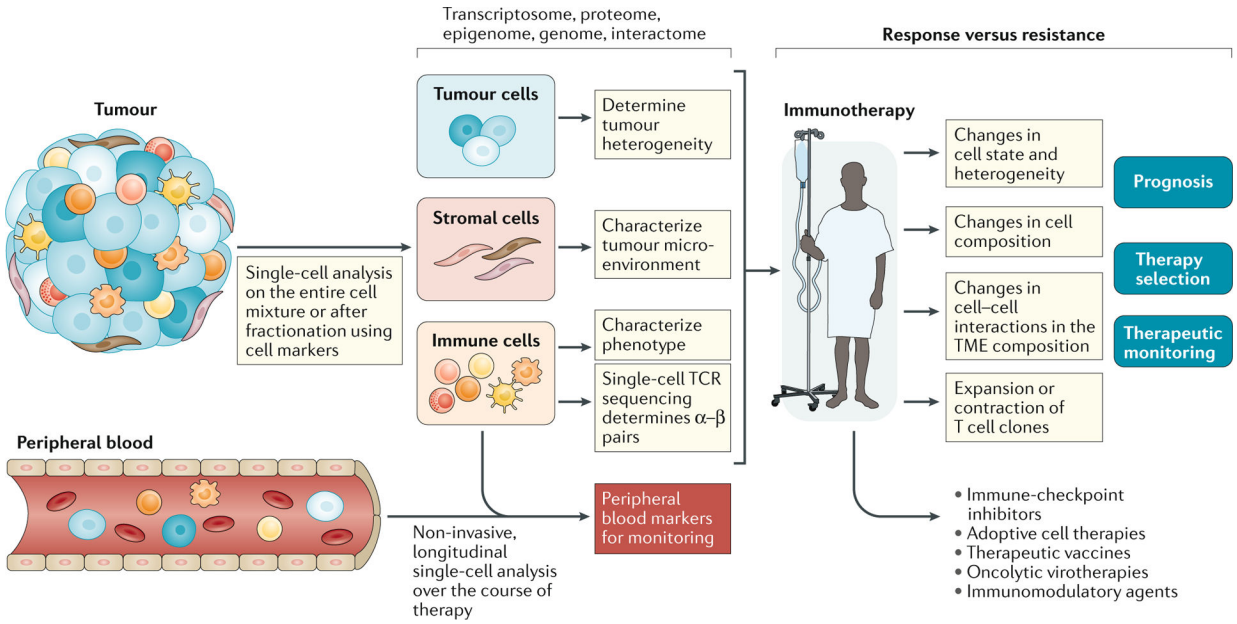

Fig. 1 |. Workflow for single-cell analysis in immuno-oncology.

The tumour microenvironment (TME) is often complex and contains myriad cell types and states that can be disaggregated, with varying degrees of success, into a single-cell suspension. Cells can then be processed without further manipulation, or specific cell subsets can be isolated for downstream analysis. Single-cell analysis — most commonly by single-cell RNA sequencing (scRNA-seq) — enables the assessment of tumour, immune and/or stromal cells to yield high-dimensional information on tumour heterogeneity and an atlas of the immune and/or stromal microenvironment in relation to clinical stage, disease subtype and tumour location. Sampling before and after immunotherapy, especially if patients can be stratified based on their response, facilitates a detailed dissection of the mechanisms underlying response and resistance. The ability to track immune cell populations, specifically T cells through paired T cell receptor (TCR) α and β subunit sequencing, enables the detailed characterization of T cell clones involved in mediating response. These findings can be applied to a wider selection of patients, with the goals of improving prognostication and optimal treatment selection in the face of an ever-expanding range of therapeutic options, and enabling therapeutic monitoring through the identification of cellular populations or markers that can be monitored using high-throughput conventional technologies. Findings from within the TME might also be linked to changes in the peripheral circulation, which are more amenable to regular monitoring.

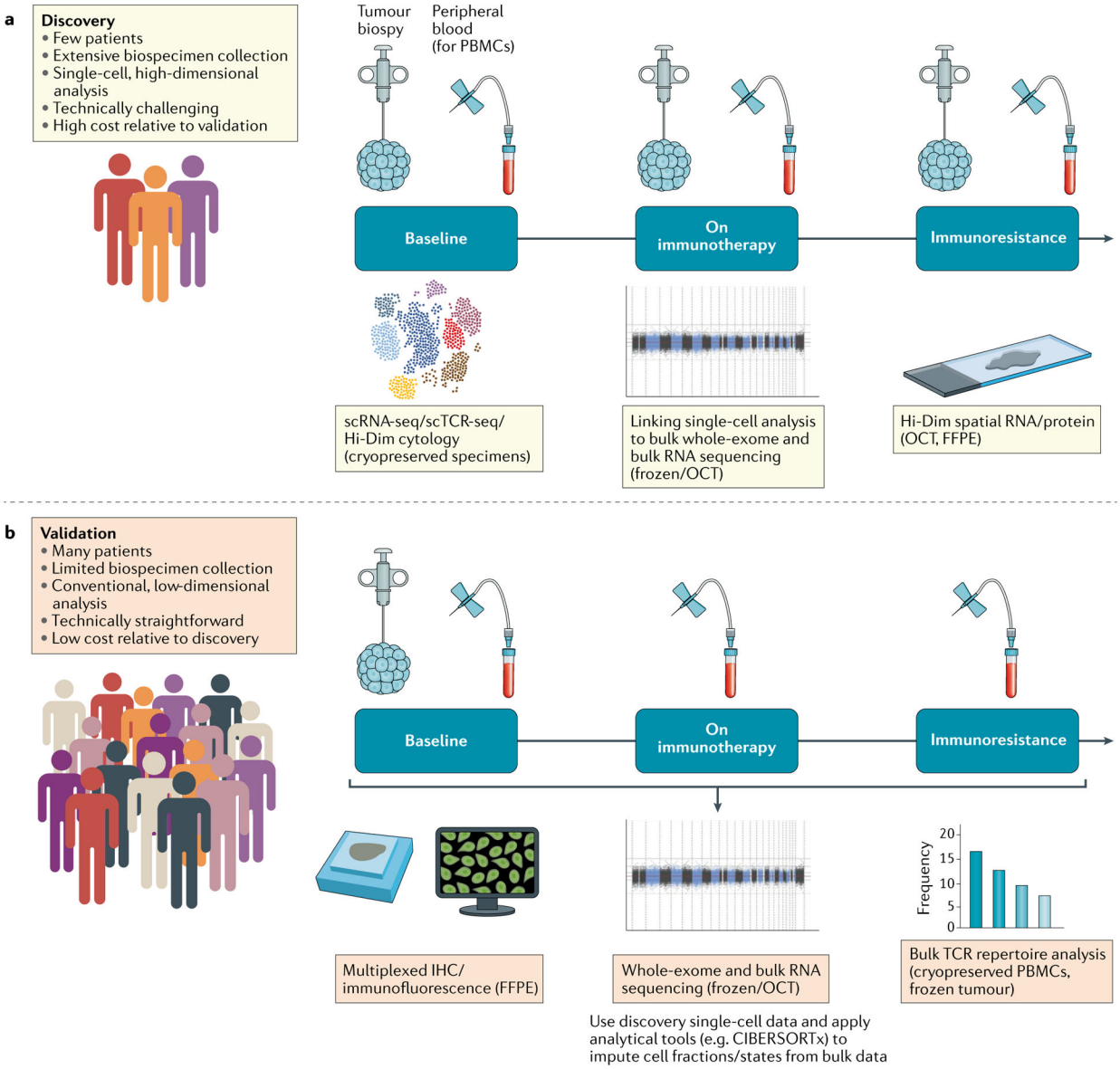

Fig. 2 |. General biospecimen framework for incorporating single-cell analyses in clinical trials involving immunotherapies.

a | Discovery efforts for a limited subset of patients. In-depth characterization of pre-treatment, on-treatment and post-treatment (resistance) tumour biopsies and peripheral immune cells using single-cell and bulk approaches enables the discovery of biological determinants of therapeutic response and resistance. These analyses are technically challenging and expensive, and so would only be performed in a limited number of patients. **b** | Validation using conventional technologies for a larger set of patients. For a larger set of clinical trial patients, more conventional sample collection (such as frozen or formalin-fixed paraffin-embedded (FFPE) tumour tissue at baseline, peripheral blood cells before and during treatment) is feasible. These samples can be used to validate proposed biological determinants of immunotherapy response and resistance (from the 'discovery' efforts depicted in part **a**) using standardized assays. PBMCs, peripheral blood mononuclear cells;

Hi-Dim, high-dimensional; IHC, immunohistochemistry; OCT, optimal cutting temperature compound; scRNA-seq, single-cell RNA sequencing; TCR, T cell receptor.

Table 1 |

Selected immuno-oncology insights from scRNA-seq-based analyses

Cancer type	Key findings	Single-cell technologies used	Cell types characterized	Ref.
Oligodendroglioma and astrocytoma	Microglia/macrophages are the predominant non-malignant cells in the TME with increased infiltration of these cells in *IDH*-mutant astrocytomas compared with *IDH*-mutant oligodendrogliomas	Smart-seq2 (plate-based)	Tumour cells, microglia and TAMs	22
Ovarian ascites	TCGA-based mesenchymal and immunoreactive subtypes of ovarian carcinoma might reflect the relative abundances of CAFs and macrophages, respectively, rather than differences in malignant cells	Smart-seq2, 10× Genomics (droplet-based)	Tumour cells, CAFs and immune cells	20
	Elements of the JAK/STAT signalling pathway are highly expressed in tumour cells and CAFs			
Breast cancer	The presence of gene-expression signatures of three myofibroblastic CAF subpopulations at diagnosis is associated with resistance to anti-PD-1 antibodies	10× Genomics	CAFs	126
Pancreatic cancer	Used a published scRNA-seq dataset to characterize CAFs expressing *LRRC15,* then correlated the LRRC15$^+$ CAF signature with a poor response to anti-PD-L1 antibodies	10× Genomics	CAFs	127
Breast cancer	Identified 38 distinct T cell, 27 myeloid cell, 9 B cell and 9 NK cell clusters	inDrop (droplet-based), 10× Genomics	CD45$^+$ immune cells	28
	Immune cells in the TME have an increased 'phenotypic volume' compared with immune cells in non-malignant breast tissues, indicating increased levels of phenotypic heterogeneity			
	Trajectory analysis revealed a continuum of T cell states along axes of activation, hypoxia and terminal differentiation			
NSCLC	Based on CD8$^+$ T cell phenotypes, higher ratios of progenitor exhausted cells to terminally exhausted cells were associated with a better prognosis	Smart-seq2	T cells	128
HCC	Increased expression of *LAYN* associated with exhausted tumour-infiltrating CD8$^+$ T cells and a poor prognosis	Smart-seq2, 10× Genomics	T cells	129
Colon cancer	Defined trajectory from *CD14*$^+$ monocytes through *FCN1*$^+$ monocyte-like cells to two distinct TAM subpopulations: complement-enriched *C1QC*$^+$ and pro-angiogenic *SPP1*$^+$	Smart-seq2, 10× Genomics	TAMs and dendritic cells	130
	High *SPP1*$^+$/low *C1QC*$^+$ TAM signature associated with a poor prognosis			
B-ALL	Characterized remodelling of the myeloid compartment at diagnosis including an increase in non-classic CD16$^-$ monocyte subpopulations associated with unfavourable survival	10× Genomics	Myeloid cells (all immune cells included in sequencing)	131
Melanoma, HNSCC, NSCLC	Identified TAMs as the predominant cell type responsible for producing the CXCR3 ligands CXCL9, CXCL10 and CXCL11	Published data (Smart-seq2, 10× Genomics, inDrop)	TAMs	132
	Implicated macrophages with elevated levels of CXCL10 and CXCL11 at baseline with response to immune-checkpoint inhibitors in patients with melanoma using published scRNA-seq datasets			

The capabilities of single-cell RNA sequencing (scRNA-seq) technologies that use end counting (such as 10× Genomics) versus whole-transcript sequencing (such as Smart-seq2) have been reviewed in detail elsewhere[3,12,133]. B-ALL, B cell acute lymphoblastic leukaemia; CAFs, cancer-associated fibroblasts; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; NK, natural killer; NSCLC, non-small-cell lung cancer; TAMs, tumour-associated macrophages; TCGA, The Cancer Genome Atlas; TME, tumour microenvironment.