

REVIEW

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Single-cell multi-omics in cancer immunotherapy: from tumor heterogeneity to personalized precision treatment

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Abstract

Cancer immunotherapy has revolutionized clinical oncology; however, the inherent complexity and heterogeneity of cancer present substantial challenges to achieving broad therapeutic efficacy. Tumor heterogeneity manifests not only among different patients but also within individual tumors, further complicating personalized treatment approaches. Single-cell sequencing technologies encompassing genomics, transcriptomics, epigenomics, proteomics, and spatial omics have significantly enhanced our ability to dissect tumor heterogeneity at single-cell resolution with multi-layered depth. These approaches have illuminated tumor biology, immune escape mechanisms, treatment resistance, and patient-specific immune response mechanisms, thereby substantially advancing precision oncology strategies. This review systematically examines recent advances in single-cell multi-omics technologies across various cancer research areas, emphasizing their transformative impacts on understanding tumor heterogeneity, immunotherapy, minimal residual disease monitoring, and neoantigen discovery. Additionally, we discuss current technical and analytical limitations and unresolved questions associated with single-cell technologies. We anticipate single-cell multi-omics technologies will become central to precision oncology, facilitating truly personalized therapeutic interventions.

Keywords Single-cell sequencing, Immunotherapy, Tumor heterogeneity, Personalized therapy

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Introduction

Cancer remains one of the most pressing global health challenges, characterized by its profound molecular, genetic, and phenotypic heterogeneity. This heterogeneity is not only observed across different patients but also exists among multiple tumors within the same individual and even within distinct cellular components of the tumor microenvironment (TME). Such complexity underlies key obstacles in cancer treatment, including therapeutic resistance, metastatic progression, and inter-patient variability in clinical outcomes, ultimately posing a major bottleneck to optimizing personalized therapy outcomes [1]. Conventional bulk-tissue sequencing approaches, due to signal averaging across heterogeneous cell populations, often fail to resolve clinically relevant rare cellular subsets, thereby limiting the advancement of personalized cancer therapies.

The advent of single-cell sequencing technologies has revolutionized our ability to dissect tumor complexity with unprecedented resolution, offering novel insights into cancer biology. By enabling multi-dimensional single-cell omics analyses—including genomics, transcriptomics, epigenomics, proteomics, and spatial transcriptomics [2–5]—researchers can now construct high-resolution cellular atlases of tumors [6, 7] delineate tumor evolutionary trajectories [8, 9] and unravel the intricate regulatory networks within the TME [10, 11]. These advances help bridge the gap between molecular alterations and their functional consequences in the tumor ecosystem. In parallel, the rapid development of single-cell technologies has profoundly impacted cancer immunotherapy, which aims to harness the patient's own immune system against tumors, has advanced rapidly, yet therapeutic efficacy remains inconsistent due to inter-patient response variability and the emergence of resistant cell populations [12]. Single-cell approaches offer critical insights into these issues by identifying immune cell subsets and states associated with immune evasion and therapy resistance [13, 14] thus opening new avenues for investigation. Furthermore, integrative analysis of multimodal single-cell data has accelerated the discovery of predictive biomarkers and enhanced our mechanistic understanding of treatment responses, thereby paving the way for personalized immunotherapeutic strategies [15, 16].

Nevertheless, several technical and analytical challenges remain before these cutting-edge technologies can be fully translated into clinical practice. These include the high cost of sequencing, methodological limitations in cell isolation and molecular profiling, and the computational complexity involved in integrating and interpreting multi-omics datasets. Technological innovation and interdisciplinary collaboration will be critical to

addressing these challenges and unlocking the full potential of single-cell sequencing in clinical oncology.

In this review, we systematically examine recent advances in single-cell multi-omics technologies across various cancer research areas, and comprehensively discuss the transformative role of single-cell multi-omics technologies in linking tumor heterogeneity to personalized immunotherapy, with a particular emphasis on recent breakthroughs in neoantigen discovery and minimal residual disease (MRD) monitoring. Additionally, we summarize current existing technical and analytical hurdles, and highlight unresolved questions. Looking ahead, we envision that single-cell multi-omics will serve as a cornerstone of precision oncology, ultimately realizing the goal of truly individualized cancer treatment.

Overview of single-cell sequencing

The functional characteristics of diverse cell types in the human body arise from a complex system shaped by multidimensional genotype–phenotype regulatory networks. Throughout this process, dynamic interactions across various layers of “omics” — including the genome, epigenome, transcriptome, and proteome — play a pivotal role. Large-scale sequencing technologies based on bulk population analyses have enabled systematic characterization across these omic layers, allowing researchers to obtain comprehensive, population-level molecular profiles spanning genomics, epigenomics, transcriptomics, and proteomics. However, bulk sequencing approaches inherently capture averaged signals that mask cellular heterogeneity and obscure the molecular features of rare or distinct cell populations. In contrast, single-cell technologies have revolutionized the ability to resolve the cellular composition of complex tissues, such as the TME, and to characterize previously inaccessible cell subsets, including cancer stem cells and immunologically relevant rare populations.

To enable single-cell molecular profiling, an essential first step involves the efficient and accurate isolation of individual cells from tumor tissues. Over the past decade, several advanced single-cell isolation strategies have been developed to meet the technical demands of high-resolution analysis (Fig. 1), including micromanipulation, laser capture microdissection (LCM), fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), and microfluidic technologies [3, 17]. Specifically, micromanipulation involves the manual selection of single cells under a microscope, ensuring that each sample contains a solitary cell; however, this method is labor-intensive, low-throughput, and carries a risk of mechanical damage to cells. Similar to micromanipulation, LCM isolates target cells manually under microscopic guidance but employs laser beams to excise specific cells or regions directly from fixed tissue

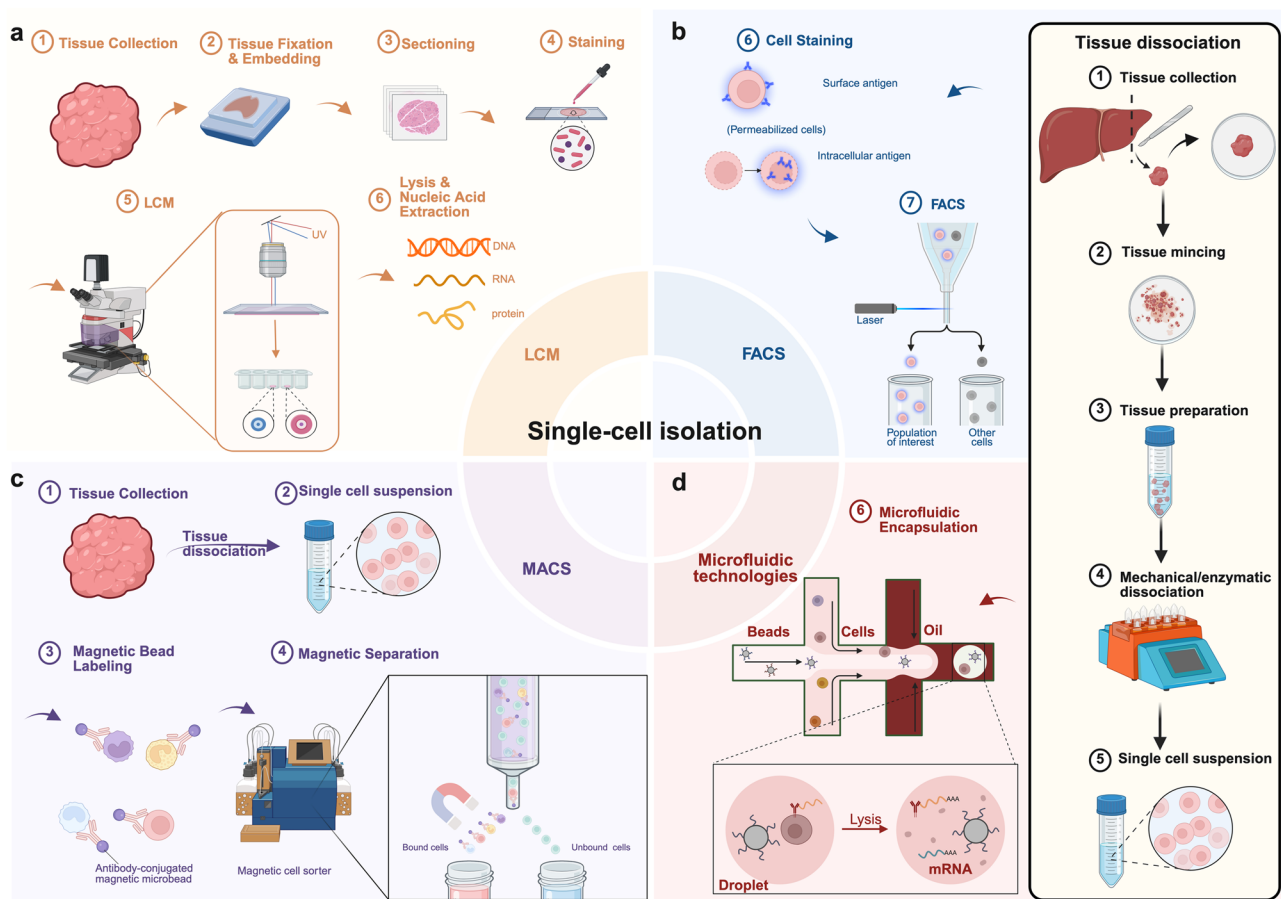


Fig. 1 Representative approaches for single-cell isolation from tissue samples. Several techniques are used to isolate single cells for downstream omics analyses, each suited to different sample types and experimental needs. **a** Laser capture microdissection (LCM): tissue samples are fixed, embedded, sectioned, and stained. Selected cells are visualized and precisely excised using a laser, followed by nucleic acid or protein extraction. **b** Fluorescence-activated cell sorting (FACS): tissues are first enzymatically and mechanically dissociated into single-cell suspensions. Cells are then stained for surface or intracellular markers and sorted by fluorescence intensity to isolate specific populations. **c** Magnetic-activated cell sorting (MACS): dissociated cells are labeled with antibody-conjugated magnetic beads, and target cells are separated using a magnetic field. **d** Microfluidic technologies: single cells are suspended in solution and encapsulated into nanoliter droplets along with barcoded beads and lysis reagents. This high-throughput system enables parallel capture and molecular barcoding of individual cells for downstream analysis

sections. By precisely tuning laser parameters and integrating microscopic control, LCM allows for the targeted acquisition of cells from complex tissues while preserving spatial context, making it particularly suitable for studies of tumor heterogeneity or neural circuitry that require spatial omics data [18]. Nonetheless, LCM remains time-consuming and limited in throughput. FACS represents a substantial advance in cell throughput. In this technique, target cells are specifically labeled using fluorescent dyes or fluorescent proteins (such as GFP) conjugated to antibodies. The cell suspension is hydrodynamically focused into a single-cell stream, which passes sequentially through a laser interrogation zone to trigger multidimensional signal acquisition. When cells matching pre-set parameters are detected, the system generates charged droplets via high-frequency vibration, and an external electric field deflects these droplets to sort target

cells into designated collection devices. This approach enables the efficient and precise isolation of desired subpopulations from heterogeneous cell mixtures. However, FACS requires a large number of starting cells, relies on monoclonal antibodies targeting specific surface markers, and demands experienced operators — all of which may limit its applicability. Compared with FACS, MACS offers a simpler and more cost-effective alternative. This method employs magnetic beads conjugated with various affinity ligands (e.g., antibodies, enzymes, lectins) to capture surface proteins on target cells. Upon application of an external magnetic field, the labeled cells are retained and separated from the unlabeled population. In recent years, the advent of microfluidic technologies has further advanced single-cell isolation. By precisely controlling fluid dynamics within microscale channels, microfluidic platforms leverage principles such as

laminar flow, capillary effects, and microvolume manipulation to achieve highly efficient cell separation [19]. This technology offers significant advantages in terms of high throughput, low technical noise, and minimal cellular stress; however, it is often associated with higher operational costs.

Following cell isolation, a suite of sequencing technologies has been developed to interrogate distinct molecular layers at single-cell resolution. In transcriptomics, single-cell RNA sequencing (scRNA-seq) enables the unbiased characterization of gene expression programs. Due to the low RNA content of individual cells, optimized workflows incorporating efficient mRNA reverse transcription, cDNA amplification, and the use of unique molecular identifiers (UMIs) and cell-specific barcodes have been implemented to minimize technical noise and enable high-throughput analysis [20]. These technical optimizations have enabled the detection of rare cell types, characterization of intermediate cell states, and reconstruction of developmental trajectories across diverse biological contexts [21, 22]. Moreover, UMI-based barcoding strategies have been adapted to other omics modalities, including genome, transcriptome, immunome, and proteome sequencing [23, 24]. Techniques such as Drop-seq and the commercially available 10x Genomics platform have further expanded the scalability and precision of scRNA-seq [25, 26] supported by the development of sophisticated analytical methods for lineage tracing, RNA velocity analysis, and cell–cell communication inference [27–29]. Recent platforms such as 10x Genomics Chromium X and BD Rhapsody HT-Xpress enable profiling of over one million cells per run with improved sensitivity and multimodal compatibility [30]. These advancements, coupled with improved chemistry and barcode resolution, are reshaping single-cell transcriptomic studies and facilitating large-scale clinical applications.

In parallel, single-cell DNA sequencing (scDNA-seq) provides complementary information by directly profiling the genomic landscape of individual cells. Compared to transcriptomic approaches, scDNA-seq provides broader genomic coverage, enabling researchers to directly read the genome and identify mutations at the single-cell level, such as copy number variations and single nucleotide variants. Although some genomic alterations can be inferred indirectly from transcriptome data, scDNA-seq remains the gold standard for accurate mutation detection [8]. Various methods have been developed based on different DNA isolation and amplification techniques, including G&T-seq, SIDR-seq, DNTR-seq, and DR-seq [31]. Currently, multiple displacement amplification has supplanted PCR as the primary method for whole-genome amplification due to its superior genomic coverage and lower error rate [32, 33].

Beyond genomics and transcriptomics, single-cell epigenomic technologies offer crucial insights into the gene regulatory landscape governing cellular identity and plasticity. These approaches enable high-resolution mapping of chromatin accessibility, DNA methylation, histone modifications, and nucleosome positioning — all fundamental determinants of cellular identity and phenotype. Among these, chromatin accessibility serves as a direct indicator of the physical availability of DNA regulatory elements. Single-cell assay for transposase-accessible chromatin sequencing (scATAC-seq) has become a cornerstone technique in this field, leveraging Tn5 transposase-mediated insertion to selectively label accessible chromatin regions, thereby enabling the generation of high-resolution chromatin accessibility maps at single-cell resolution [34]. DNA methylation, characterized by the addition of a methyl group to cytosine residues within CpG dinucleotides, is a key epigenetic modification associated with transcriptional repression. Bisulfite sequencing remains the gold standard for single-cell methylome profiling, operating through chemical conversion of unmethylated cytosines to uracils [35]. However, the harsh chemical treatment inherent to this approach poses a risk of DNA degradation. Recently, enzyme-based conversion strategies have emerged as gentler alternatives, broadening the applicability and resolution of single-cell DNA methylation analyses. Beyond DNA methylation, post-translational modifications of histones exert profound influence over chromatin structure and gene expression. Advances in single-cell chromatin profiling — from the pioneering single-cell ChIP-seq to next-generation platforms such as scCUT&Tag [36, 37] — enable the high-resolution mapping of histone modifications by antibody-guided capture of specific epigenetic marks, offering new avenues to dissect the epigenetic basis of cell fate decisions. At a higher-order level of chromatin organization, nucleosomes — the fundamental units of chromatin comprising DNA wrapped around histone octamers — play a pivotal role in regulating gene accessibility and transcriptional output [38]. Single-cell micrococcal nuclease sequencing (scMNase-seq) represents a powerful approach to resolve nucleosome positioning patterns, coupling enzymatic digestion of linker DNA with high-throughput sequencing to reveal nucleosome architecture at single-cell resolution [39]. Crucially, the integration of multi-omics strategies is redefining the scope of single-cell epigenomic research. By jointly profiling chromatin three-dimensional conformation (such as via scHi-C), histone modification landscapes, and transcriptomic outputs, researchers are now poised to reconstruct the intricate regulatory networks that govern cellular identity and plasticity [40]. Collectively, these technological advances are transforming single-cell

epigenomics from descriptive cataloguing to mechanistic dissection of gene regulation.

Complementing these genomic and epigenomic approaches, single-cell proteomics technologies provide direct quantification of the functional proteome, which more closely reflects cellular phenotype. Proteins represent the main functional machinery of cells and possess a longer half-life than mRNA. Hence, the single-cell proteomics methods are more precise compared to scRNA-seq methods [41]. Single-cell proteomics technologies can be broadly classified into targeted and non-targeted protein detection strategies, depending on whether the target proteins are pre-determined [42]. Targeted protein analysis approaches predominantly rely on antibodies or other affinity reagents to selectively detect proteins of interest [12]. A notable example is mass cytometry, which employs antibodies conjugated to rare earth metal isotopes — elements that are minimally present in biological systems — to enable the simultaneous quantification of over 40 cellular parameters at single-cell resolution. This strategy markedly enhances the ability to characterize complex cellular systems and dynamic processes. Specifically, cellular markers and other expressed proteins are labeled with isotope-tagged antibodies, allowing for the concurrent identification of cell types and high-dimensional profiling of protein expression within individual cells [5]. By contrast, untargeted proteomic approaches do not require prior immunological knowledge, making them particularly suited for discovery-driven research. Moreover, because they are independent of antigen–antibody interactions, untargeted strategies circumvent key limitations of targeted methods, such as epitope accessibility and antibody cross-reactivity [43]. Mass spectrometry is the core technology underpinning untargeted proteomics [44–46]. Among the most widely adopted platforms, Orbitrap-based mass spectrometry offers superior resolution and sensitivity, enabling the detection of thousands of proteins at single-cell resolution [47]. This technological advance has been leveraged in a range of oncological applications, including the identification of early diagnostic biomarkers in lung cancer, the elucidation of mechanisms underlying chemotherapy resistance, and the metabolic profiling of circulating tumor cells derived from gastrointestinal malignancies [48–50]. Recent developments in single-cell proteomics have led to significant improvements in protein coverage and quantification precision. Techniques such as PiSPA now routinely identify over 3,000 proteins per cell, surpassing previous depth limitations [51]. The introduction of timsTOF Ultra 2, a next-generation trapped ion mobility mass spectrometer, further enhances sensitivity and detection accuracy, enabling more comprehensive profiling of post-translational modifications and signaling cascades at single-cell resolution [52].

Despite the rapid evolution of single-cell proteomics, genomics, and epigenomics technologies, the interrogation of individual omic layers alone remains insufficient to fully elucidate the complex molecular architecture of single cells. To address this limitation, integrative multi-modal sequencing approaches (Table 1) — which enable the simultaneous profiling of genomic, transcriptomic, and epigenomic features at single-cell resolution — have emerged as powerful tools for dissecting cellular complexity [31]. In parallel, spatially resolved multi-omics technologies (Table 2) provide additional contextual information by preserving the spatial organization of molecular profiles within tissues.

Notably, in the field of immunology, single-cell T cell and B cell receptor sequencing (scTCR/BCR-seq) has demonstrated unique utility. This technology not only facilitates comprehensive mapping of immune receptor repertoire diversity [127, 128] but also enables the precise tracking of clonal expansion and differentiation dynamics, thereby providing critical insights for vaccine development, immunotherapy optimization, and immune monitoring [129]. Such integrative, functional characterization of single-cell molecular profiles is accelerating the translation of single-cell technologies into clinical applications, advancing the realization of precision medicine.

Together, these technological advances are expanding the frontiers of single-cell biology, enabling unprecedented resolution of cellular heterogeneity and regulatory complexity, and driving significant progress in fields such as oncology, developmental biology, and immunotherapy.

Single-cell sequencing: illuminating tumor complexity

Tumor progression is a complex, multistep process wherein normal cells gradually transform into malignant tumors through a series of genetic alterations and the subsequent accumulation of these mutations in somatic cells. Throughout differentiation, cells undergo recurrent cycles of proliferation and division, resulting in substantial biological and genetic diversity within tumors, which underlies the intricate phenomenon of tumor heterogeneity [130]. Notably, the TME plays a critical role in tumor initiation and contributes significantly to intra-tumoral heterogeneity. Specifically, the TME comprises diverse cell populations, and tumorigenesis requires overcoming multiple proliferative barriers imposed by this microenvironment, converting the surrounding stromal compartment into a supportive niche for tumor growth. Persistent reciprocal interactions between tumors and their microenvironment profoundly impact therapeutic responses and patient survival outcomes [131–133]. Single-cell sequencing technologies spanning various omics approaches have emerged as powerful tools for dissecting the heterogeneity of tumors and their

Table 1 Single-cell multimodal approaches

Omics Category	Year	Method	Reference
Genome + Transcriptome	2015	G&T-seq	[53]
Genome + Transcriptome	2015	SCTG	[54]
Transcriptome + CRISPR screening	2016	Perturb-seq	[55]
Transcriptome + CRISPR screening	2016	CRISP-seq	[56]
Epigenome + Transcriptome	2016	scMT-seq	[57]
Genome + Epigenome + Transcriptome	2016	scTrio-seq	[58]
Transcriptome + Proteome	2017	REAP-seq	[59]
Transcriptome + Proteome	2017	CITE-seq	[60]
Transcriptome + CRISPR screening	2017	CROP-seq	[61]
Genome + Transcriptome	2016	DR-seq	[62]
Epigenome + Transcriptome	2019	scCAT-seq	[63]
Genome + Transcriptome	2018	SIDR-seq	[64]
Epigenome + Transcriptome	2018	sci-CAR	[65]
Genome + Transcriptome	2019	TARGET-seq	[66]
Epigenome + Transcriptome	2019	SNARE-seq	[67]
Epigenome + CRISPR screening	2019	Perturb-ATAC	[68]
Epigenome + Transcriptome	2019	scDam&T-seq	[69]
Transcriptome + Proteome + CRISPR screening	2019	ECCITE-seq	[70]
Epigenome + Transcriptome	2019	Paired-seq	[71]
Transcriptome + Proteome	2019	RAID	[72]
Genome + Transcriptome	2019	sci-L3-RNA/DNA	[73]
Genome + Transcriptome	2020	DNTR-seq	[74]
Epigenome + Transcriptome	2020	SHARE-seq	[75]
Epigenome + Transcriptome	2020	ASTAR-seq	[76]
Epigenome + Transcriptome	2021	SNARE-seq2	[77]
Epigenome + Transcriptome	2021	scChaRM-seq	[78]
Epigenome + Proteome	2021	ICICLE-seq	[79]
Epigenome + Proteome	2021	ASAP-seq	[80]
Epigenome + Transcriptome	2021	Smart-RRBS	[81]
Transcriptome + Proteome	2021	inCITE-seq	[82]
Transcriptome + Proteome	2021	SPARC	[83]
Epigenome + CRISPR screening	2021	CRISPR-sciATAC	[84]
Epigenome + CRISPR screening	2021	Spear-ATAC	[85]
Epigenome + Transcriptome	2021	scNOMeRe-seq	[86]
Epigenome + Transcriptome	2021	Paired-Tag	[87]
Transcriptome + Epigenome + Proteome	2021	TEA-seq	[79]
Transcriptome + Epigenome + Proteome	2021	DOGMA-seq	[88]
Epigenome + Proteome	2022	PHAGE-ATAC	[89]
Genome + Epigenome + Transcriptome	2022	snmCAT-seq	[90]
Epigenome + Transcriptome	2022	scPCOR-seq	[91]
Epigenome + Proteome	2022	scCUT&Tag-pro	[92]
Transcriptome + Epigenome + Proteome	2022	NEAT-seq	[93]
Genome + Transcriptome	2023	scONE-seq	[94]
Transcriptome + Proteome	2023	CaRPool-seq	[95]
Transcriptome + Epigenome + Proteome	2023	CellOracle	[96]
Transcriptome + Metabolome	2024	scMeT-seq	[97]
Transcriptome + Proteome	2024	InTraSeq	[98]
Epigenome + Transcriptome	2024	uCoTargetX	[99]
Transcriptome + Epigenome/TCR/Proteome	2025	UDA-seq	[100]

Table 2 Single-cell spatial approaches

Omic Category	Year	Method	Resolution	Reference
Spatial Transcriptomics	2014	seqFISH	Subcellular	[101]
Spatial Transcriptomics	2015	MERFISH	Subcellular	[102]
Spatial Transcriptomics	2016	corrFISH	Single-cell	[103]
Spatial Transcriptomics	2017	Geo-seq	Single-cell	[104]
Spatial Transcriptomics	2018	Visium	55 µm	[105]
Spatial Transcriptomics	2021	ExSeq	Single-cell	[106]
Spatial Transcriptomics	2019	Slide-seq	10 µm	[107]
Spatial Transcriptomics	2019	HDST	2 µm	[108]
Spatial Transcriptomics + Proteomics	2020	DBIT-seq	10–50 µm	[109]
Spatial Transcriptomics + Proteomics	2020	GeoMx DSP	Single-cell	[110]
Spatial Epigenome + Proteomics	2020	OligoFISSEQ	Single-cell	[111]
Spatial Transcriptomics	2021	Slide-seqV2	10 µm	[112]
Spatial Epigenome	2022	Spatial ATAC-seq	10–50 µm	[113]
Spatial Epigenome	2022	Spatial CUT&Tag	10–50 µm	[114]
Spatial Transcriptomics	2022	Stereo-seq	Single-cell	[115]
Spatial Transcriptomics + Proteomics	2022	SM-Omics	100 µm	[116]
Spatial Genomics + Transcriptome	2022	Slide-DNA-seq	25 µm	[117]
Spatial Transcriptomics + TCR	2022	Slide-TCR-seq	10 µm	[118]
Spatial Epigenome + Transcriptome	2023	spatial CUT&Tag-RNA-seq	10–50 µm	[119]
Spatial Epigenome + Transcriptome	2023	spatial-ATAC&RNA-seq	10–50 µm	[119]
Spatial Transcriptomics + Proteomics	2022	Spatial-CITE-seq	10–50 µm	[120]
Spatial Transcriptomics + Proteomics	2023	SPOTS	55 µm	[121]
Spatial Epigenome + Transcriptome	2023	MISAR-seq	Single-cell	[122]
Spatial Transcriptomics(3D)	2024	Open-ST	Subcellular	[123]
Spatial Transcriptomics	2024	Visium HD	2 µm	[124]
Spatial Proteomics	2024	SCPro	Single-cell	[125]
Spatial Transcriptomics + CRISPR screening	2025	Perturb-FISH	Single-cell	[126]

microenvironments (Fig. 2). These techniques offer substantial promise in elucidating the complex mechanisms driving tumor development, metastasis, and therapeutic resistance, thus opening new avenues for personalized therapeutic strategies [134–136]. In the following section, we highlight pivotal studies leveraging single-cell sequencing to dissect tumor heterogeneity and the TME, underscoring how these insights are accelerating the development of personalized cancer therapies.

Genomics

Single-cell genomic sequencing technology, characterized by its unparalleled resolution at the individual cell level, is reshaping our understanding of the complex ecosystem of the TME. By interrogating genomic landscapes at single-cell resolution, researchers can now systematically identify rare genetic variants, trace evolutionary trajectories of malignant clones, and elucidate mechanisms underlying therapeutic resistance and metastasis.

A major advantage of single-cell genomic sequencing over conventional bulk sequencing approaches lies in its enhanced sensitivity for detecting low-frequency genetic variants (<1% vs. 5–10% variant allele frequency, VAF) [137, 138]. This improved sensitivity has profound clinical implications. For example, in acute myeloid leukemia, single-cell sequencing revealed subpopulations harboring FLT3-ITD mutations previously undetectable by bulk methods. Importantly, these FLT3-ITD-positive cells exhibit stronger proliferative ability and chemotherapy resistance, thus providing critical prognostic and therapeutic insights [139–141]. Similarly, in breast cancer research, single-cell sequencing identified hundreds of subclones and novel mutations that appear at low frequencies (<10%) in tumor bulk. These rare mutations enable tumors to withstand selective pressures in the tumor microenvironment, including from the immune system, hypoxia, and chemotherapy, highlighting their role in tumor adaptability and progression [142].

Single-cell genomic technologies have also provided novel insights into tumor evolution, particularly through comprehensive analyses of CTCs and solid tumors [143]. A comprehensive copy number variation (CNA) analysis of CTCs from 23 patients confirmed that the evolution of CNAs is not random but rather a form of convergent evolution. This suggests that CNA dynamics may be governed by selection-driven, non-random evolutionary pressures, indicating that only a small fraction of highly malignant tumor cells may enter the circulation from primary tumors and subsequently form metastatic lesions [144]. Corroborating these findings, single-cell genomic analyses of breast cancer CTCs indicated high concordance of ERBB2 status (77%) with primary tumors, alongside the acquisition of additional genomic instability. This increased instability reflects ongoing

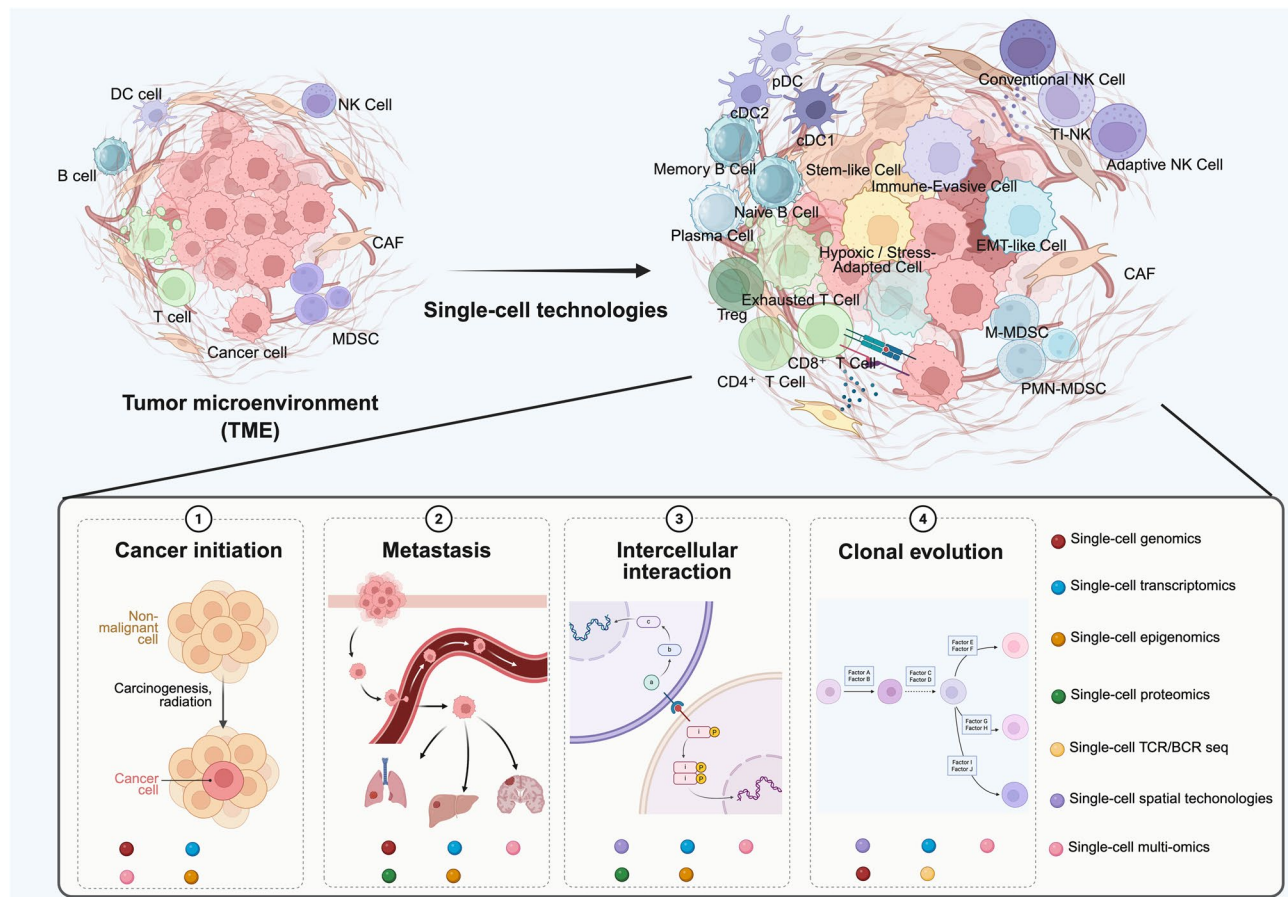


Fig. 2 Single-cell technologies for dissecting tumor heterogeneity across the tumor microenvironment. Single-cell technologies enable high-resolution characterization of the complex cellular composition and dynamic states within the tumor microenvironment (TME), including malignant cells, immune subsets (T cells, B cells, NK cells, dendritic cells, MDSCs), and stromal components (e.g., CAFs). A range of single-cell modalities (genomics, transcriptomics, epigenomics, proteomics, TCR/BCR sequencing, spatial technologies, and multi-omics integration) have been applied to investigate key processes underlying tumor progression, including cancer initiation — identifying early molecular alterations during malignant transformation, metastasis — tracking cell states and routes of dissemination, intercellular interactions — mapping ligand–receptor networks and signaling cross-talk between cells, and clonal evolution — reconstructing lineage trajectories and mutational dynamics

evolutionary adaptation during metastasis [145]. In solid tumor research, single-cell whole-genome-sequencing of hepatocellular carcinoma (HCC) revealed the spatio-temporal stability of early CNAs: consistency of CNAs between primary and metastatic lesions reached 89%, and multi-nodular tumors displayed significantly higher subclonal diversity, suggesting an inherent link between multiclonal origin and invasive phenotypes [146].

Single-cell genomics is also increasingly used to dissect the mechanistic role of the TME in shaping clonal evolution. A colorectal cancer evolution model showed that early monoclonal origin adenomas initiate subclonal differentiation through mutations in the GPCR/PI3K-Akt pathway, while tumor-associated fibroblasts (CAFs) were found to secrete IL-6, driving further subclonal selection via STAT3 activation, directly linking microenvironmental factors to intratumoural diversification [147]. In the context of evolutionary dynamics under treatment

pressure, a neoadjuvant chemotherapy study in triple-negative breast cancer revealed that pre-existing resistant subclones selectively expand through activation of the Wnt/ β -catenin pathway and metabolic reprogramming. Concurrently, these resistant populations promoted CD8⁺ T cell exhaustion, generating an immunosuppressive microenvironment that facilitates tumor persistence and progression [148]. These findings have driven clinical strategies towards “evolutionary interception”—using single-cell dynamic monitoring to identify early resistance signals and block adaptive evolutionary pathways.

In conclusion, single-cell genomic sequencing has transcended simple genomic variation detection to become a core tool for deciphering the multidimensional interaction networks within the tumor microenvironment. By integrating multimodal data such as spatial omics and epigenomics [149, 150] this technology is driving the paradigm shift in cancer research from static classification to

dynamic ecosystem analysis, offering a new spatiotemporal dimension for precision medicine.

Transcriptomics

scRNA-seq has significantly advanced our understanding of tumor biology by enabling high-resolution exploration of transcriptional dynamics within individual tumor cells. By capturing molecular changes at single-cell resolution, scRNA-seq not only reveals the genetic underpinnings of tumor initiation and progression but also provides crucial insights into metastasis mechanisms, intratumoural heterogeneity, immune landscapes, and complex intercellular communication within the TME.

Somatic mutations drive tumor initiation and progression, and scRNA-seq facilitates the precise mapping of transcriptional changes associated with malignant transformation across different tumor stages [151, 152]. For example, in colorectal cancer, APC mutations are often the initiating events in most colon polyps and colorectal cancers. Researchers performed single-cell sequencing on colon polyps from patients with familial adenomatous polyposis carrying APC germline mutations and normal colon tissues from healthy individuals. They found that during the malignant transformation of intestinal epithelial cells, the expression of stem cell marker genes such as LGR5, SMOC2, and RGMB was significantly upregulated. Additionally, the study identified a type of “super stem cell” characterized by high ASCL2 expression, which maintains stemness and drives cancer cell proliferation and invasion [153]. Similar approaches have been applied to studies of gastric cancer [154] head and neck squamous cell carcinoma [155] and lung adenocarcinoma [156] demonstrating that single-cell transcriptomics can be used for early cancer detection and the discovery of new therapeutic targets based on tumor cell heterogeneity.

Metastasis remains the leading cause of cancer-related mortality [157] and scRNA-seq has emerged as a powerful approach for investigating the mechanisms underlying metastatic dissemination. For instance, acral melanoma, a subtype with a high incidence of lymph node metastasis, exhibits significant inter- and intratumor heterogeneity, as revealed by single-cell technology. Wei et al. identified MYC⁺ melanoma cells as closely associated with lymph node metastasis. In these cells, the transcription factor MITF drives fatty acid oxidation metabolic reprogramming, and experiments confirmed that fatty acid oxidation inhibitors effectively suppress MITF-mediated lymph node metastasis [158]. Such studies highlight the potential of single-cell transcriptomics in identifying metastasis-prone cell populations, uncovering metastasis mechanisms, and developing targeted therapies for tumors.

Moreover, single-cell sequencing has further revealed the complexity of intratumoral heterogeneity, enabling the distinction of tumor cell subpopulations (particularly malignant cell groups) and elucidating their roles in disease progression. A single-cell study of tumor cells from liver cancer patients found that 13% of tumor cells (malignant cells) exhibited high heterogeneity. Tumors with greater cellular heterogeneity showed elevated levels of vascular endothelial growth factor (VEGF) expression, promoting angiogenesis and remodeling the TME, thereby enhancing tumor survival [10]. This study was the first to use single-cell sequencing to reveal how tumor heterogeneity remodels the TME, confirming its close association with immune cell dysfunction and abnormal angiogenesis. Similarly, Wang et al. used single-cell transcriptomics to identify tumor subtypes with distinct lineage features in metastatic gastric adenocarcinoma, categorizing cancer cells into two major subtypes: “gastric-dominant” and “gastrointestinal mixed,” and revealing significant prognostic differences between them [159].

Beyond the heterogeneity of tumor cells themselves, immune cells in the TME also exhibit complexity. scRNA-seq provides an advanced tool for dissecting tumor immune heterogeneity, uncovering changes in immune cell composition during disease progression and treatment response. For example, Alcazar et al. discovered that during the progression of ductal carcinoma in situ to invasive breast cancer, the number of CD8⁺ T cells, the proportion of regulatory T cells, and the clonal diversity of TCRs underwent significant changes, reflecting a gradual increase in immunosuppression. This was evidenced by elevated PD-L1 and CTLA4 expression and reduced TCR clonotype diversity [160]. In relapsed hepatocellular carcinoma, single-cell analysis identified a distinct population of innate-like CD8⁺ T cells characterized by diminished cytotoxic potential and clonal expansion, providing mechanistic insight into the impaired antitumor immunity and poor prognosis associated with this disease [161]. Similarly, Liu et al. employed scRNA-seq to investigate liver metastases of colorectal cancer and revealed enrichment of DC3-like dendritic cells and SPP1⁺ macrophages, implicating these populations in facilitating metastatic progression [11]. Notably, single-cell sequencing excels in identifying rare immune cell populations, analyzing their specific contributions to tumor immune responses, and laying the foundation for precision therapy in cancer patients. For instance, Cui et al. discovered a rare subset of SELENOP-expressing macrophages with anti-tumor functions in lung cancer through scRNA-seq, suggesting this population as a potential new target for immunotherapy and underscoring the clinical significance of single-cell technology in precision oncology [162]. In gastric cancer with

peritoneal metastasis, scRNA-seq of patients treated with the anti-PD-1 antibody sintilimab uncovered a distinctive immunosuppressive ecosystem dominated by a stroma–myeloid niche, composed of SPP1⁺ tumor-associated macrophages and Thrombospondin 2⁺ matrix cancer-associated fibroblasts. Disrupting the crosstalk between these stromal and immune compartments markedly improved the therapeutic efficacy of immune checkpoint blockade [163]. Furthermore, in HCC, scRNA-seq revealed that CD103⁺ reactive cytotoxic T lymphocytes, traditionally associated with anti-tumor immunity, can paradoxically promote tumor progression and resistance to immunotherapy by activating the NLRP3 inflammasome in macrophages [164]. Together, these studies illustrate the power of single-cell approaches to dissect the intricate immune landscape of tumors, resolve functionally relevant rare cell populations, and inform the development of precision immunotherapies.

Tumor progression is driven not only by intrinsic cellular properties but also by dynamic intercellular interactions within the TME, mediated through ligand–receptor signaling, paracrine and autocrine mechanisms [133]. ScRNA-seq can dissect intercellular communication in detail to better understand how cell interactions in the TME impact tumor progression. For example, in gliomas, a common malignant brain tumor in adults, single-cell sequencing identified 16 significant autocrine ligand–receptor pairs and revealed that high expression of DLL1 and NOTCH1 was closely linked to tumor cell proliferation. The study also found that the binding of cancer stem cell-derived COL1A1/COL3A1 to macrophage-expressed ITGB1/ITGB3 played a key role in tumor invasion and angiogenesis, correlating with poor patient prognosis [165]. Such detailed characterization of intercellular crosstalk deepens our understanding of how cell–cell interactions within the TME drive malignant progression.

Collectively, scRNA-seq has revolutionized cancer research by providing comprehensive, high-resolution insights into tumor biology—from initiation and progression to metastasis, immune evasion, and therapeutic resistance. Ultimately, scRNA-seq is poised to refine precision medicine approaches, enabling tailored therapeutic strategies grounded in the rich cellular diversity and molecular complexity of tumors.

Proteomics

Single-cell proteomic sequencing has emerged as a powerful approach to unravel the molecular intricacies of the TME, providing high-resolution insights into cellular phenotypes, post-translational modifications (PTMs), and secreted protein networks that shape tumor progression, immune responses, and therapeutic resistance. Unlike transcriptomic approaches, proteomic analyses

offer direct functional readouts at the protein level, capturing dynamic biological processes and enabling the identification of clinically actionable molecular targets.

Single-cell proteomic sequencing has demonstrated superior performance in decoding the tumor microenvironment. For instance, CyTOF-based high-dimensional phenotypic analysis at single-cell resolution on immune cells from surgically resected gliomas and non-tumor control tissues revealed distinct immune landscapes. Primary gliomas were predominantly infiltrated by resident CNS microglia, whereas brain metastases harbored a substantial number of macrophages derived from circulating monocytes. This finding suggests that brain metastases induce a higher degree of peripheral immune cell infiltration, whereas the immune microenvironment of gliomas is primarily composed of resident cells. Further analysis of T-cell activation and functional states demonstrated a significant accumulation of regulatory T cells and exhausted T cells with high PD-1 expression in metastatic tumors, unveiling the immune characteristics of the TME driven by glioma type [166]. Such study illustrates how single-cell proteomics effectively delineates the immune landscapes of tumors, providing critical insights into disease-specific immune cell infiltration and functional states.

Notably, compared to transcriptomic sequencing, single-cell proteomic sequencing provides a more precise depiction of the landscape of PTMs within the tumor microenvironment. Common PTMs, including phosphorylation, acetylation, and ubiquitination, modulate protein activity and function, and tumor behavior [167]. Protein phosphorylation is closely associated with signal transduction, and a study utilizing single-cell phosphoproteomic analysis in glioblastoma revealed dynamic changes in protein phosphorylation sites before and after treatment, as well as under drug resistance conditions. The study found that phosphorylation of the mTOR and Akt signaling pathways was significantly downregulated during CC214-2 (an ATP-competitive mTORC1/mTORC2 kinase inhibitor) treatment but reactivated in drug-resistant states, providing potential combinatorial strategies for targeted therapy [168]. Recently, the development of Chip-DIA has significantly enhanced the sensitivity of single-cell phosphoproteomics. In an analysis of non-small cell lung cancer cell samples, Chip-DIA identified multiple resistance-associated signaling pathways, including MAPK and cytoskeletal remodeling. Additionally, it detected self-phosphorylation sites of drug targets such as EGFR and ERBB2, offering novel therapeutic targets and strategies for personalized treatment in patients with drug-resistant cancers [169]. Fagerholt et al. developed specific antibodies and utilized CyTOF to detect K382 acetylation of the p53 protein in acute myeloid leukemia samples. Upon γ -ray stimulation, K382-acetylated

p53 exhibited a significant increase, influencing cell proliferation and apoptosis by regulating the expression of Ki67, a proliferation marker, and p21 (CIP1/WAF1), a cell cycle regulator. This study highlights the potential of single-cell proteomic sequencing in elucidating cancer regulatory mechanisms [170]. More recently, single-cell proteomics has enabled the identification of various E3 ubiquitin ligases [52]. While there are currently no reported studies leveraging single-cell proteomics to investigate ubiquitination in cancer, future research could integrate this technique to directly analyze ubiquitination patterns at the protein level. This approach could provide deeper insights into the role of ubiquitination in protein function and cancer progression.

Furthermore, microfluidics-based single-cell proteomic sequencing enables the capture of secreted proteins, unveiling paracrine signaling networks that orchestrate tumor–stromal crosstalk and immune evasion. Lu et al. successfully employed this platform to detect 42 immune effector proteins secreted by single immune cells. Their study identified distinct macrophage subpopulations transitioning from quiescence to partial and full activation, with variations in cytokine secretion patterns and response intensities. Notably, a macrophage subpopulation with high secretion of macrophage migration inhibitory factor was identified, which enhanced LPS-induced inflammatory responses and served as a key node in the inflammatory regulatory network [171]. Another study utilized a single-cell barcoded chip to analyze the expression of secreted proteins in glioblastoma cells, identifying key proteins that regulate intercellular communication, including interleukin-6, interleukin-8, vascular endothelial growth factor, hepatocyte growth factor, and migration inhibitory factor. The concentration gradients formed by these secreted proteins guided directional cell migration. When these factors were inhibited, tumor cell movement shifted to a random Brownian motion pattern. This study provided a novel mechanistic perspective on the role of secreted proteins in tumor invasion from a biophysical standpoint, offering new insights into tumor cell migration behavior [172].

Collectively, single-cell proteomic sequencing has significantly expanded our understanding of tumor complexity by directly interrogating functional protein networks, PTMs, and immune landscapes within the TME, promising to deliver actionable molecular insights, facilitating precision oncology strategies aimed at intercepting disease progression, overcoming therapeutic resistance, and improving patient outcomes.

Epigenomics

Epigenetic regulation has long been recognized as crucial for development and disease progression. The advent of single-cell epigenetic sequencing technologies has

propelled the development of various methods to analyze different layers of epigenetic regulation, including chromatin accessibility, DNA methylation, and histone modifications, thereby enabling the investigation of diverse epigenetic processes at the single-cell level [173].

Chromatin accessibility refers to the structural state of DNA within chromatin that determines whether proteins such as transcription factors can physically bind specific DNA regions. As a “molecular switch” for gene expression, chromatin accessibility influences cell fate by modulating transcription factor binding [174]. The introduction of single-cell epigenetic techniques is of great significance in uncovering mechanisms underlying tumor initiation, progression, and metastasis. For instance, a multi-cancer single-cell epigenetic landscape analysis revealed that differences in chromatin accessibility between cancerous and normal cells act as key epigenetic drivers of cancer transition. Within these epigenetic pathways, alterations in TP53, hypoxia, and TNF signaling coincide with changes in chromatin accessibility that promote tumorigenesis. In metastatic cancer cells, the accessibility of EMT-related transcription factors (such as SNAI1 and TWIST1) is significantly elevated, facilitating tumor cell migration and invasion [175]. Moreover, single-cell chromatin accessibility technologies can distinguish mature cell populations from stem cell-like populations, which are critical determinants in many cancers. In glioblastoma, scATAC-seq analysis showed that tumor-initiating cells sustain three survival-related states by regulating specific transcription factor networks, thereby revealing epigenetic targets for therapies aimed at eradicating tumor stem cells [176].

DNA methylation involves the addition of a methyl group ($-\text{CH}_3$) to the 5' position of cytosine nucleotides, typically occurring in CpG dinucleotide regions. Single-cell epigenomic approaches have provided pivotal insights into tumor heterogeneity, including intratumoral epigenetic diversity, tumorigenesis, the TME, and metastasis. For instance, scRRBS studies of patient-derived glioblastoma demonstrated that epigenetic diversity among cancer cells—mediated by critical signaling pathways—drives intratumoral heterogeneity [177]. In a prostate cancer metastasis model, scBS-seq analysis revealed early-stage low-methylation regions; reduced methylation of CD1 and IFI16 led to the failure of NKT cell immune surveillance, a feature commonly observed in metastatic cancers. Restoring the expression of these genes reinstated antitumor immunity, indicating that early epigenetic changes may underpin tumorigenesis [178]. Single-cell DNA methylation profiling also reveals how tumor microenvironmental contexts influence epigenetic patterns. For example, in colon cancer, scBS-seq data showed that certain partially methylated domains exhibit substantial epigenetic heterogeneity among cells

within the same tumor, reflecting how the TME shapes intratumoral DNA methylation patterns [179]. Lastly, scWGBS revealed that, compared with single CTCs, CTC clusters display characteristic hypomethylation at binding sites for stemness- (e.g., OCT4, SOX2, NANOG) and proliferation-related transcription factors. This epigenetic state endows CTC clusters with enhanced stemness and proliferative capacity, thereby increasing their propensity for distant metastases [175]. Single-cell epigenetic profiling also shows promise for biomarker development. For example, a single-cell multi-omics study in gastric cancer revealed widespread DNA demethylation in cancer cells. Methylation variations among these cells are mainly lineage-dependent; in particular, high methylation at the TMEM240 and HAGLROS promoters, as well as low methylation at the TRPM2-AS and HRH1 promoters, may serve as diagnostic and therapeutic biomarkers for gastric cancer [180].

Histone modifications—including methylation, acetylation, and phosphorylation—play essential roles in chromatin structure regulation and transcriptional activity. Dysregulation of these modifications, particularly alterations in activating (H3K4me3) or repressive (H3K27me3) marks, commonly occurs in tumors and contributes significantly to disease progression. ScChIP-seq has begun elucidating how these histone modification alterations promote tumor adaptation and drug resistance. Through scChIP-seq analyses of patient-derived xenograft models of breast cancer, researchers identified a notable decrease in H3K27me3 marks on genes such as IGF2BP3 (insulin-like growth factor binding protein 3) in drug-resistant cell populations, suggesting epigenetic activation of these genes as a mechanism underlying therapeutic adaptation [181]. Such findings illuminate critical epigenetic adaptations that facilitate tumor survival under treatment pressures, offering new opportunities for therapeutic intervention.

Single cell TCR/BCR seq

TCRs and BCRs are both composed of two chains. Each TCR consists of a β chain (comprising V, D, and J segments) and an α chain (comprising V and J segments), both of which have unique sequences. BCRs, on the other hand, are composed of a heavy chain and a light chain. These chains are generated through the recombination of V(D)J gene segments during lymphocyte development [182]. Traditional sequencing methods are largely limited to single-chain analysis and thus cannot capture the dimeric nature of adaptive immune receptors, which significantly hampers the ability to assess antigen specificity [183]. For example, the same TCR β sequence can pair with different TCR α chains and vice versa [184]. Single-cell TCR and BCR sequencing technologies have enabled high-throughput recovery of paired receptor chains,

thereby allowing for more accurate clonotype analysis and facilitating mechanistic investigations and real-time monitoring in cancer immunotherapy.

By resolving immune receptor repertoires at single-cell resolution, these approaches have proven instrumental in elucidating the molecular and immunological underpinnings of immune checkpoint blockade (ICB)-induced tumor rejection, and in guiding the rational design of next-generation immunotherapies. In lung cancer, for example, linking TCR clonotypes with transcriptional states has enabled the reconstruction of tumor-reactive T cell trajectories in responders. Post-treatment expansion of precursor exhausted T cells (Texp), defined by high GZMK and low NR4A2 expression, has been observed. Notably, single-cell data revealed that these Texp cells are not reactivated terminally exhausted cells, as previously assumed, but instead arise via local proliferation and recruitment of peripheral T cells — a process termed clonal revival [185]. In a parallel study, Yost and colleagues reported marked clonal replacement of tumor-specific exhausted T cells following PD-1 blockade in basal and squamous cell carcinoma. The emergent CD8⁺CD39⁺ clones, exhibiting transcriptional signatures of chronic activation and exhaustion, were absent from pre-treatment biopsies, supporting a model in which ICB efficacy is driven by the recruitment of novel peripheral clones rather than re-invigoration of pre-existing intratumoral memory cells [186].

Single-cell receptor sequencing also provides a powerful framework for establishing minimally invasive immune monitoring systems by identifying shared clonotypes between tumor-infiltrating lymphocytes (TILs) and circulating T/B cells. For instance, by profiling TCRs in matched tumor and peripheral blood samples, Lucca et al. identified circulating T cell clones that overlapped with TILs and expressed consistent cytotoxic effector genes, such as GZMB and PRF1, suggesting that circulating TILs may serve as a proxy for tumor-resident cytotoxic responses [187]. This approach offers a promising strategy for enhancing the sensitivity and specificity of liquid biopsy platforms by incorporating immune dynamics into tumor surveillance.

In addition, scBCR-seq has emerged as a powerful tool to interrogate the cellular origins, clonal hierarchies and evolutionary trajectories of B cell malignancies, as well as to uncover early immunological changes during tumorigenesis. In multiple myeloma, Dang and colleagues integrated scRNA-seq and scBCR-seq to map disease progression and substantially improved the detection sensitivity of known genomic aberrations in precursor states with low tumor purity. Their data revealed early emergence of dominant BCR clonotypes in clonal plasma cells, pronounced intraclonal heterogeneity, and diverse evolutionary paths — including both linear and

branching patterns. Moreover, the integration of paired transcriptomic and receptor data enabled identification of transcriptional programs associated with clonal evolution, including a terminal subcluster in MGUS01 specifically enriched for ASS1, suggesting a potential transformation node. These insights offer a foundation for early detection, risk stratification and the development of precision therapies in B cell neoplasms [188].

High-dimensional spatial analysis

TME is a complex ecosystem in which previous studies have demonstrated that the spatial localization of immune cells significantly impacts tumor progression and therapeutic outcomes. Traditional single-cell technologies based on tissue dissociation often obscure the intricate cellular interactions and spatial heterogeneity within the TME. In contrast, spatially-resolved single-cell techniques preserve tissue architecture, enabling a more precise understanding of the spatial variability within the tumor immune microenvironment.

In recent years, spatial proteomics and transcriptomics have emerged as powerful tools to characterize the spatial distribution of cell types within tumors. These techniques directly measure cellular co-localization to reveal intercellular interactions [189]. At present, high-dimensional spatial proteomics have been successfully applied to characterize multiple tumor types, including melanoma, breast cancer, and cutaneous squamous cell carcinoma [189–191]. In the context of immunotherapy, Cabrita et al. employed the Nanostring GeoMx platform to analyze clinical samples from metastatic melanoma patients, discovering that the co-presence of tumor-associated CD8⁺ T cells and CD20⁺ B cells promoted the formation of tertiary lymphoid structures (TLS) within tumors. The associated gene signatures could predict clinical outcomes for patients undergoing ICB therapy [192]. Spatial neighborhood analysis of immune cells in adoptive cell therapy contexts further identified increased CD8⁺ TILs and myeloid cell type I interferon signaling in treatment responders [193]. These findings reveal critical immune responses within responder tumors, enhancing patient selection strategies and informing immunotherapy approaches. Compared with spatial proteomics, spatial transcriptomics analysis provides higher molecular resolution, greatly advancing TME research. For instance, combining scRNA-seq with spatial transcriptomics has been utilized in pancreatic cancer and human squamous cell carcinoma studies, uncovering novel interactions between tumor and immune cells [189, 194]. Both studies demonstrate cancer cell aggregation within fibrovascular niches, while immune cells typically display spatial restriction within distinct compartments, highlighting spatial compartmentalization as a potential mechanism of immune exclusion.

Other single-cell spatial technologies have been extensively reviewed elsewhere [31, 195–197]. Notably, recent spatial sequencing studies have also focused on delineating the landscape of TCR clonality within tissues. In 2022, slide-TCR-seq was developed, enabling whole-transcriptome and TCR sequencing of entire tumor sections at a resolution of 10 μ m, revealing diverse T-cell states and spatial clonal heterogeneity in renal cell carcinoma and melanoma samples [118]. The same team further identified TLS in metastatic melanoma and renal cell carcinoma lesions, observing that T cells within tumor regions exhibited greater clonal expansion than those in TLS. Moreover, TLS-resident T cells were predominantly CD4⁺ T cells, while tumor-infiltrating T cells generally exhibited an exhausted CD8⁺ phenotype, indicating distinct immunological roles for TLS within tumors and suggesting that transcriptional profiles of T cells, monocytes, and tumor cells may be influenced by their spatial relationships within the TME [198].

Multimodal analysis and multi-omics

With continuous advancements in technology and analytical methodologies, single-cell approaches have ushered in a new era of multimodal and multi-omics analyses. These innovations have expanded the investigative scope from transcriptional initiation to protein translation, enabling the identification of rare cellular populations that influence tumor growth, metastasis, and therapeutic resistance. Furthermore, they have elucidated the molecular mechanisms governing cellular state transitions and intercellular interaction networks, offering transformative insights into the TME. For instance, by employing scTrio-seq, which enables the simultaneous profiling of the genome, DNA methylome and transcriptome at single-cell resolution, researchers delineated the intricate interplay between genetic and epigenetic regulation in HCC. This study provided the first direct evidence linking DNA copy number alterations (CNAs), DNA methylation, and gene expression within individual HCC cells. Promoter methylation was found to be inversely correlated with the expression of corresponding genes, whereas gene body methylation (excluding promoter regions) showed a positive association with transcriptional activity. Notably, CNAs influenced gene expression primarily through gene dosage effects, without substantially altering DNA methylation patterns at affected loci. To investigate intratumoural heterogeneity, the authors identified two transcriptionally and genetically distinct subpopulations of malignant hepatocytes. One subpopulation (subpopulation I), comprising only a minor fraction of the tumor mass, exhibited an increased burden of functionally significant CNAs, elevated expression of genes associated with aggressive phenotypes, and signatures suggestive of enhanced immune evasion. These

findings underscore the utility of multimodal single-cell sequencing for resolving tumor complexity and for uncovering rare, yet clinically relevant, malignant subclones in HCC [58].

Beyond tumor cell heterogeneity, single-cell multi-omics approaches have proven instrumental in unraveling the immunological heterogeneity within the TME. Diverse cellular populations from both the innate and adaptive immune systems intricately orchestrate a balance between immunosuppressive and immune-activating signals [133]. CD8⁺ T cells, known for their potent cytotoxic functions, are often considered one of the most critical anti-tumor components in the TME. However, intratumoral CD8⁺ T cells exist in distinct functional states, frequently exhibiting exhaustion or dysfunction, making it imperative to decipher their state transitions within the microenvironment. ScRNA-seq and TCR sequencing have unveiled a continuum of activation states in T cells within the breast cancer microenvironment, characterized by a gradient of activation-associated gene expression, including *GZMA*, *GZMK*, *IL-32*, and *CCL5*, suggesting a progressive functional evolution of T cells within tumors. Furthermore, this study established a correlation between TCR clonotype and cellular phenotype, demonstrating that T cells sharing the same TCR clonotype tend to occupy specific phenotypic spectra, indicating that TCR signaling intensity partially drives phenotypic variation through antigen recognition [199]. CD8⁺PD-1⁺ T cells, a population critical to the efficacy of immune checkpoint inhibitors (ICIs), have been further delineated using single-cell RNA sequencing and mass cytometry, revealing transcriptionally distinct subclusters characterized by heterogeneous expression of *CD161*. Notably, the enrichment of CD8⁺PD-1⁺CD161⁺ T cells within tumor nodules has been associated with favorable clinical outcomes. Mechanistically, co-expression of *CD161* and interleukin-7 receptor (*IL-7R*) appears to sustain the proliferative capacity of CD8⁺PD-1⁺ T cells—at least in part—by enhancing the expression of *IL-2*, *TNF-α* and perforin, thereby mitigating terminal exhaustion. Spatial profiling further revealed a higher frequency of cytotoxic CD8⁺PD-1⁺CD161⁺ T cells in adjacent non-tumor tissue, whereas exhausted CD8⁺PD-1⁺CD161⁻ T cells predominated within the tumor microenvironment. These findings highlight the dynamic spatial reprogramming of CD8⁺ T cells and underscore the complexity of immune compartmentalization in shaping antitumor responses [200].

Macrophages, as key constituents of innate immunity, fulfill diverse roles, including phagocytosis, immune activation/suppression, metabolic regulation, growth support, angiogenesis induction, and immune evasion [201]. ScRNA-seq and ATAC-seq analyses have delineated seven distinct subpopulations of tumor-associated

macrophages (TAMs) in pediatric sarcomas, each exhibiting varying degrees of immunosuppressive potential. Among these, TAM subpopulations with high *SPP1* and *C1QC* gene expression demonstrated the strongest immunosuppressive capabilities, driven by transcription factors *ELF2*, *JUND*, and *RUNX1*. These subpopulations exert their immunosuppressive effects through interactions with T and NK cells [202]. Mounting evidence also underscores the critical role of stromal cells in orchestrating tumor progression and mediating therapeutic resistance [203–205]. For instance, scRNA-seq and spatial transcriptomics have identified distinct stromal cell subtypes in high-grade serous ovarian carcinoma, including fibroblasts, myofibroblasts, and cancer-associated mesenchymal stem cells. High-stroma tumors establish intricate paracrine signaling networks with tumor-infiltrating NK and CD8⁺ T cells via *CXCL12*-*CXCR4* receptor interactions, thereby promoting immune exclusion and suppressing anti-tumor immune responses [205].

Collectively, single-cell multi-omics technologies have profoundly deepened our understanding of tumor heterogeneity and immune dynamics, offering novel insights into mechanisms of therapeutic resistance and paving the way for the development of more effective personalized immunotherapy strategies.

Leveraging single-cell sequencing for personalized cancer therapy

As aforementioned, the integration of single-cell multi-omics technologies have redefined tumors not as homogeneous masses but as dynamic, evolving ecosystems shaped by continuous interplay between malignant cells and their microenvironment. By resolving cellular heterogeneity and revealing rare subpopulations with distinct phenotypic and functional attributes, single-cell sequencing offers a transformative lens through which to reinterpret cancer pathogenesis. Importantly, these high-resolution datasets are increasingly being harnessed to inform therapeutic decision-making, predict treatment responses, and identify mechanisms of resistance at the earliest stages. In the following section, we highlight how the translational application of single-cell technologies is shaping the next generation of personalized cancer therapy, including precision immunotherapy design, MRD detection and neoantigen-targeted interventions.

Tailoring immunotherapy based on single-cell data

Personalized cancer management relies substantially on the accurate identification of specific molecular subtypes or therapeutic targets. Although personalized treatments targeting tumor heterogeneity have advanced significantly in cancer research, the demand for enhancing precision medicine to improve patient survival and prevent late-stage recurrence remains unmet. As discussed

earlier, single-cell technologies represent powerful tools to unravel the complexity and heterogeneity of tumors and their microenvironments. Identifying distinct cellular subpopulations and developing novel therapeutic approaches targeting treatment-resistant cell subsets can guide the design of tailored combinatorial or innovative treatment strategies. For example, Sangeeta Goswami and colleagues analyzed samples from 94 patients with five different cancer types undergoing immune checkpoint therapy. Using scRNA-seq, they identified a distinct population of CD73^{high} macrophages in glioblastoma patients treated with anti-PD-1 therapy. This population exhibited elevated expression of immunosuppressive genes and a reduction in T cell infiltration within the tumor, resulting in diminished sensitivity to immunotherapy. Based on these findings, they proposed a combination therapy strategy targeting CD73, alongside dual blockade of PD-1 and CTLA-4, which could hold substantial clinical promise [206]. Another single-cell proteomics study revealed that certain TAMs are enriched in prostate tumors, especially in high-grade cases, indicating that targeting macrophage subpopulations may serve as a viable therapeutic adjunct in aggressive prostate cancers [207].

For patients with ICB resistance, single-cell technologies are invaluable for unraveling resistance mechanisms by tracking changes in gene expression and cell states within the tumor during treatment. This approach is crucial for designing next-generation therapies to overcome resistance. Specifically, in melanoma, single-cell transcriptomic analyses have uncovered a tumor-intrinsic immune resistance program characterized by suppression of antigen presentation and interferon signaling pathways, coupled with activation of CDK4/6 and Myc signaling, collectively driving T-cell exclusion and the establishment of a 'cold' tumor microenvironment. Notably, this resistance phenotype pre-exists prior to therapy and becomes further intensified upon immune checkpoint blockade (ICB) failure, yet intriguingly, it can be pharmacologically reversed by CDK4/6 inhibition, thereby enhancing therapeutic outcomes. These observations highlight tumor cell-state modulation as a promising therapeutic strategy to overcome immune resistance [208]. Additionally, multimodal single-cell CRISPR screens have further delineated classical resistance mechanisms such as impaired antigen presentation and disrupted IFN γ -JAK/STAT signaling, while simultaneously uncovering an independent immune evasion pathway mediated by CD58 loss. This CD58 deficiency markedly impairs T cell and NK cell-mediated cytotoxicity independently of MHC status and is frequently observed in clinically resistant tumors, suggesting CD58 as a critical and distinct route of immune escape. Targeting novel pathways such as CD58 may thus offer new avenues for

therapeutic intervention to counteract tumor immune resistance [209]. Additionally, scRNA-seq of non-small cell lung cancer samples at pre-treatment, treatment response, and acquired resistance stages revealed that commonly used targeted therapy drugs, such as tyrosine kinase inhibitors, induce the expression of APOBEC3A. This induction increases mutation rates, leading to the emergence of drug-resistant persistent cells that can survive even under therapeutic pressure [210]. In a study on Merkel cell carcinoma, Sc-RNA-seq analysis of tumor samples from patients who initially responded well to immunotherapy but later relapsed and developed resistance indicated that immunotherapy resistance primarily stems from the transcriptional suppression of specific HLA genes (HLA-A and HLA-B), which are responsible for presenting viral-specific epitopes. The suppression of HLA gene transcription may represent a widespread mechanism of resistance to immunotherapy (including ICIs). Encouragingly, this suppression may be pharmacologically reversible with demethylating agents, offering potential to restore immune recognition and therapeutic response in resistant tumours [211]. It is noteworthy that resistance to ICB therapies is mediated not only by tumor cells themselves but also by alterations in immune cells. For instance, regulatory T (Treg) cells are typically viewed as suppressors of endogenous and therapy-induced anti-tumor immunity. By integrating scRNA-seq and TCR-seq analyses of Tregs from NSCLC patients who responded or did not respond to anti-PD-1 therapy, Dykema et al. identified a tumor-resident OX40^{hi}GITR^{hi} Treg subpopulation that highly expresses immunosuppressive molecules (e.g., LAG3, CD39, and EBI3), demonstrating robust inhibitory function, thus facilitating immune evasion and resistance to ICB therapy [212]. Tumor-specific CD8⁺ T cells reciprocally augment the expansion and stability of this Treg subset through OX40L signaling, establishing an immunosuppressive feedback loop [212]. Furthermore, in therapy-resistant tumors, Treg differentiation trajectories preferentially persist in a highly activated suppressive state and lack pro-inflammatory TH1-like Treg subsets, further weakening anti-tumor immunity [212]. Collectively, these mechanisms form a complex Treg-mediated network contributing to resistance against ICB treatment. Similarly, scRNA-seq studies in HCC revealed that intratumoral CD56⁺ NK cells significantly express inhibitory receptors such as PD-1 and TIGIT, exhibiting a highly exhausted and dysfunctional phenotype. This impairs the immune activation capacity of ICB therapy and consequently mediates resistance [213].

Based on the analysis of drug resistance mechanisms, single-cell technologies can further identify unique gene expression patterns in cells that are insensitive to immunotherapy. This capability allows for the precise

identification of patients who respond favorably to treatment and prediction of therapeutic efficacy, assisting clinicians in selecting personalized treatment strategies. For example, Sade-Feldman et al. analyzed transcriptomes of 16,291 individual immune cells from 48 tumor samples of melanoma patients treated with checkpoint inhibitors, revealing that the presence of the transcription factor TCF7 in CD8⁺ T cells could predict clinical response to ICB therapy [214]. Additionally, combined scRNA-seq and TCR sequencing have been utilized to analyze the TME of esophageal squamous cell carcinoma before and after immunotherapy, identifying CXCL13⁺ CD8⁺ T cells as being associated with therapeutic response [215]. By recognizing these predictive biomarkers early in treatment, clinicians can promptly adjust therapeutic regimens to enhance efficacy. Recently, machine learning techniques have been integrated with scRNA-seq data to develop predictive models for immunotherapy response. For instance, the PRECISE analysis pipeline employs the XGBoost algorithm and Boruta feature selection to identify predictive genetic features, achieving high accuracy in predicting treatment responses in melanoma, lung cancer, and breast cancer patients. These models aid in identifying specific genes and cell types related to treatment outcomes, thus improving the development of personalized patient care plans [216].

Moreover, while immunotherapies can enhance some patients' natural defenses against cancer, they can also induce severe side effects, and single-cell platforms hold significant potential in managing immunotherapy-related toxicities. For instance, Das et al. utilized scRNA-seq and BCR sequencing to analyze circulating B cells from 39 advanced melanoma patients before and after the first cycle of ICB therapy. They found that combined CTLA-4 and PD-1 checkpoint blockade therapy caused a decline in circulating B cell numbers, and the extent of early B cell reduction post-treatment was directly correlated with the timing and severity of immune-related adverse events. This indicates that proactive strategies targeting B cells might mitigate toxicity [217]. Another study used scRNA-seq and TCR sequencing to analyze cardiac immune infiltration in mice with ICI-associated myocarditis, revealing that myocarditis was closely associated with a T cell population specifically expressing alpha-myosin [218].

Thus, single-cell sequencing is a powerful tool for tailoring immunotherapy, providing detailed insights into the TME, analyzing immune responses, elucidating mechanisms of resistance, predicting treatment outcomes, and monitoring immunotherapy toxicities (Fig. 3a). Collectively, these advancements significantly enhance the precision and efficacy of immunotherapy in cancer treatment.

MRD detection

MRD refers to the presence of a small number of cancer cells remaining in the body after treatment, even when a patient appears to be in clinical or complete remission. These residual cancer cells exist at extremely low levels, making them difficult to detect using conventional imaging or clinical examinations. The presence of MRD indicates that cancer has not been entirely eradicated and that there is a risk of relapse in the future. Therefore, MRD detection plays a critical role in monitoring remission status and predicting the likelihood of recurrence.

Single-cell technologies have significantly enhanced the sensitivity and specificity of MRD detection in cancer. Due to the accessibility of samples, single-cell sequencing was first applied to MRD monitoring in hematologic malignancies. Compared to traditional sequencing methods, single-cell sequencing offers unprecedented sensitivity in MRD detection, capable of identifying a single cancer cell among one million normal cells. Specifically, it can distinguish between pre-leukemic clonal hematopoiesis and dominant malignant clones while tracking clonal evolution from diagnosis to remission and potential relapse [219]. For instance, a study by Zhang et al. utilized scRNA-seq and B-cell receptor sequencing to generate 161,986 single-cell transcriptomes, capturing the dynamic changes in B-cell acute lymphoblastic leukemia across different disease stages (diagnosis, MRD, and relapse). The study found that certain drug-resistant clones expanded during relapse, while others diminished after chemotherapy. Furthermore, it revealed that activation of the hypoxia signaling pathway in MRD cells is a major driver of drug resistance [220].

With ongoing technological advancements, the application of single-cell sequencing in MRD detection has expanded to solid tumors, establishing itself as a crucial monitoring tool in cancer treatment [221, 222] (Fig. 3b). For example, scRNA-seq has been employed to study melanoma MRD following BRAF and MEK inhibitor therapy, shedding light on how resistant cells survive treatment and lead to relapse [221]. Researchers identified four distinct drug-resistant cell states within MRD: pigmented cells, invasive cells, starvation-like cells, and neural crest stem cells (NCSC). Among these, NCSC was found to be closely associated with tumor resistance and recurrence. Gene regulatory network analysis suggested that the RXR signaling pathway is a key driver of the NCSC state, and targeting the NCSC subpopulation with RXR antagonists could provide a novel therapeutic strategy for preventing relapse. This insight paves the way for the development of more effective treatments that target MRD heterogeneity and prevent tumor recurrence. Recently, spatial omics technologies have also been applied to MRD detection [223]. Researchers identified a specific spatial niche where PD-L1⁺ M2-like macrophages

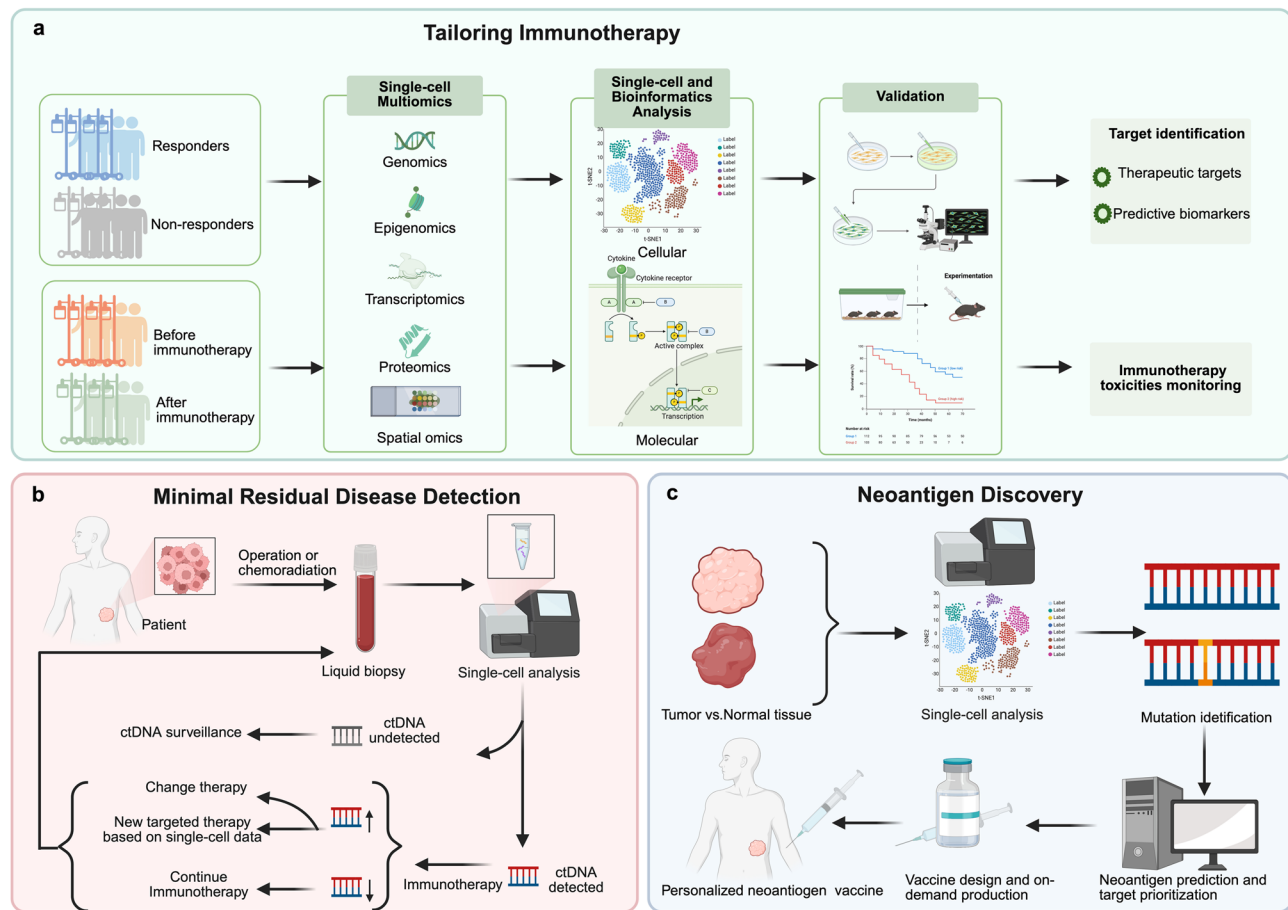


Fig. 3 Applications of high-dimensional single-cell multi-omics analysis in personalized immunotherapy. **a** Samples from responders and non-responders, or collected before and after immunotherapy, are profiled using single-cell multi-omics approaches, including genomics, epigenomics, transcriptomics, proteomics and spatial omics. Integrated single-cell and bioinformatics analyses reveal cellular and molecular features associated with therapy response. Findings are experimentally validated in vitro and in vivo to identify therapeutic targets and predictive biomarkers, and to enable immunotherapy toxicity monitoring. **b** Following tumor resection or chemoradiation, liquid biopsy is performed to obtain blood samples from the patient. These samples undergo single-cell analysis to detect residual tumor-specific molecular signatures and inform downstream treatment strategies. Circulating tumor DNA (ctDNA) analysis is used for ongoing minimal residual disease (MRD) surveillance. If ctDNA is undetectable, patients continue with ctDNA monitoring. In cases where ctDNA is detected, patients may undergo immunotherapy guided by single-cell data. Based on the molecular profile, patients can either continue immunotherapy or receive newly tailored targeted therapies. This dynamic monitoring system allows adaptive treatment modification to improve outcomes. **c** Tumor and matched normal tissues are subjected to single-cell analysis to resolve cellular heterogeneity and detect somatic mutations. Identified mutations are computationally screened to predict candidate neoantigens and prioritize targets. Selected neoantigens inform the design and production of personalized vaccines, which are administered to elicit a tumor-specific immune response

interact with stem-like tumor cells, a phenomenon linked to CD8⁺ T-cell exhaustion and poor survival outcomes. Further investigations revealed that macrophage-derived TGFβ1 mediates the persistence of stem-like tumor cells, suggesting that disrupting this interaction could help prevent HCC recurrence from MRD.

In summary, single-cell multi-omics tools have dramatically improved the sensitivity and specificity of MRD detection, enabling the identification of ultra-low levels of cancer cells while simultaneously assessing genotypic and immunophenotypic characteristics at single-cell resolution. This high sensitivity may inform early therapeutic intervention before clinical relapse becomes

detectable, ultimately advancing more precise and effective cancer treatment strategies.

Neoantigen discovery and immune profiling

An important reason for immune escape in tumor cells is the lack of tumor cell immunogenicity. Genetic mutations and epigenetic alterations in malignant cells lead to abnormal protein expression and the formation of various tumor antigens, which are the primary drivers of immunogenicity. Neoantigens can stimulate anti-tumor immune responses by activating neoantigen-specific T cells; therefore, they hold promise as novel targets for tumor immunotherapy and can be utilized in the development of personalized cancer-specific vaccines [224,

225]. Since not all mutations are immunogenic, distinguishing between immunogenic neoantigens and mere genetic mutations remains challenging. Single-cell sequencing technologies can be employed to detect abnormal mutation sites in tumor cells. By combining these findings with the molecular characteristics of the major histocompatibility complex, mutations with high major histocompatibility complex binding potential can be identified, facilitating the accurate prediction of neoantigens suitable for recombinant use [226, 227] (Fig. 3c). Recent studies have shown that long-read RNA sequencing methods (such as PacBio's Iso-Seq) can capture tumor-specific RNA isoforms and fusion genes, which are often overlooked by conventional short-read sequencing techniques. This approach has led to the identification of neoantigens with high HLA affinity, offering new avenues for cancer vaccine development [228]. Additionally, Ribo-Seq technology isolates ribosome-bound mRNA fragments, revealing actively translated proteins and uncovering neoantigens that traditional DNA sequencing cannot detect, thus enhancing the accuracy of neoantigen prediction [229]. Furthermore, innovations in mass spectrometry have improved the sensitivity of immune peptide analysis in tumor samples, enabling the identification of tumor-specific peptides (including rare neoantigens) that bind to MHC molecules, even in smaller cell populations, thereby expanding the potential of mass spectrometry in clinical applications [230]. Single-cell technologies have also enabled the direct isolation of neoantigen-specific TCR sequences from tumor samples. By stimulating TILs with dendritic cells loaded with neoantigens and utilizing single-cell sequencing, researchers have successfully identified multiple neoantigen-specific TCRs, which can be synthesized and tested for their ability to recognize specific neoantigens [231].

In conclusion, recent advancements in single-cell sequencing for neoantigen discovery have greatly enhanced our understanding of tumor biology and immune responses, paving the way for the development of personalized cancer therapies.

Challenges and future directions in single-cell sequencing

Methodological and technical challenges

The main experimental and technical challenges arise from protocol-induced artifacts, balancing multi-modal sequencing throughput and sensitivity, batch effects, and the limitations of spatial technologies. First, cell separation, a core step in single-cell sequencing, involves trade-offs between cell purity, viability, and throughput. Harsh dissociation conditions can eliminate fragile cells or induce stress-related cell state changes [232–234] leading to the loss of rare and subtle cell populations during pre-processing [199, 235]. Additionally, inherent processes

following cell separation may result in the loss of valuable information. For example, single-cell DNA methylation sequencing requires bisulfite treatment of DNA, and the high temperature and low pH conditions necessary for this step may cause pyrimidine removal, leading to DNA degradation and fragmentation [236]. Incomplete bisulfite treatment can, in turn, lead to an overestimation of methylation levels in the sample [237]. Furthermore, not all single cells can be extracted from fresh samples; many cases involve extracting single cells from frozen or fixed tissues, which can distort the data. For instance, in single-cell chromatin accessibility sequencing, fixatives in tissues can induce protein cross-linking, making the chromatin more compact and less accessible, reducing the ability of Tn5 transposase to interact with and cleave DNA. This results in an underestimation of open chromatin regions in the sample, distorting the apparent genome-wide analysis results [238].

Secondly, although current single-cell multi-omics technologies can simultaneously measure multiple molecular types and provide a comprehensive view of cellular processes [239–244] balancing data quality and throughput remains challenging. Droplet-based microfluidic systems have significantly improved sequencing throughput but still face issues with capture efficiency due to probabilistic collisions, which can result in the loss of rare and small cells [245]. Moreover, due to limited cell coverage, we are unable to capture all desired molecular layers with high sensitivity and selectivity, leading to the loss of critical data. For example, compared to scRNA-seq, epigenomics and proteomics exhibit greater data sparsity and uneven coverage. ScATAC-seq, which relies on transposase-mediated labeling, significantly reduces DNA damage, but due to limited DNA input from single cells and the stochastic nature of Tn5 transposase cutting, the data remain sparse [246]. PCR amplification can introduce systematic biases in certain genomic regions (such as those with high GC content), resulting in uneven coverage [247]. In proteomics, when integrated with other omics, the limitation of antibody panels means that fewer than 200 proteins can typically be detected in a single cell, leading to the potential omission of crucial signaling proteins [248]. Long-read cancer genomics technologies can capture more complete genomic data but are costly and time-consuming [249, 250].

Another technical challenge is batch effects. Existing methods generally assume that cell states across different experiments or data types should be similar. However, differences in batch treatments can introduce technical noise, causing single cells to cluster by technical batch [233]. While computational methods can align data from different batches post-hoc [251] techniques such as nearest neighbor analysis, principal component analysis [252, 253] singular value decomposition [254]

and non-negative matrix factorization may inadvertently “correct” away true biological differences between samples from different batches [255] particularly when dealing with high-dimensional gene expression data that is sparse, noisy, and prone to the “curse of dimensionality.” This can result in the loss of valuable information or the introduction of false data, which is especially problematic when studying subtle cell state changes within the TME.

Finally, although spatial omics methods have advanced rapidly in recent years, high costs and technical complexity limit their accessibility in many laboratories. Even when appropriate experimental conditions are met, 2D tissue section-based spatial omics methods are increasingly inadequate for comprehensive TME analysis because they cannot capture cells above or below the plane of the section, leading to an incomplete understanding of cell interactions and neighborhood structures. 3D spatial omics, which relies on extensive consecutive tissue sectioning, has been applied in oncology [256, 257] but requires considerable effort and processing to reconstruct tissue in three dimensions. Furthermore, stitching slices together and analyzing them separately can introduce alignment errors, causing the loss of continuity between layers [258, 259]. Additionally, the resolution differences between spatial omics technologies on different platforms can vary by orders of magnitude (e.g., 0.2 microns in Stereo-seq vs. 50 microns in Visium) [260] complicating subsequent data integration.

Integration and analysis of multi-omics data

An increasing number of advanced experimental methods enable the simultaneous analysis of multiple biological data types (such as genes, proteins, etc.), providing a comprehensive understanding of cellular function. However, most single-cell data come from different cells, with no direct correspondence between data sets, meaning that single-cell multi-omics approaches still face significant data integration challenges. Currently, tools for analyzing multi-omics data can be classified into those that handle “matched” data and those that handle “unmatched” data. Matched data refers to multiple measurements obtained from the same cell, while unmatched data comes from different experiments and lacks direct correspondence. Integrating unmatched data is more difficult because discrepancies between data sets can lead to information loss. To address this, scientists typically project cell data into a shared space (e.g., Seurat v3 [261], LIGER [255] GLUE [262] etc.) to identify commonalities across data sets. Some methods, such as StabMap [263] Cobolt [264] MultiVI [265] and Seurat v5 [266], map unmatched data to a reference framework for integration by using real multi-omics data as a reference. However, these methods still face challenges with computational efficiency, memory consumption, and

biological interpretability, especially when handling high-dimensional spatial data [267]. Recently, the SIMO method developed by Yang et al. has achieved more efficient multi-modal integration in spatial contexts, offering a new analytical paradigm for TME research [268]. However, a current limitation of this method is the sequential integration error, where biases from earlier steps in single-omics data processing are amplified in subsequent integration stages, and no effective solution has yet been found [268].

Storage and computational challenges associated with multimodal data integration and analysis have become critical bottlenecks restricting further advancements in this field. Recently, constructing similarity matrices for millions of cells has necessitated memory capacities exceeding 7TB [269] and the introduction of spatial omics and high-throughput imaging data has further intensified the demands on data storage and management. Although cloud platforms provide flexible solutions for data storage, the high costs associated with commercial cloud services significantly impede data sharing and broad application [270]. This represents a substantial economic barrier, particularly for resource-limited research institutions and smaller-scale research teams. Furthermore, integrating single-cell multimodal omics data requires robust high-performance computing (HPC) platforms to address data harmonization, batch-effect correction, and machine learning modeling within high-dimensional spaces [271]. However, widespread deployment of HPC is constrained by high economic costs and notable technical barriers. For instance, analyses involving DNA sequencing, methylation, and RNA-seq data on cloud platforms incur storage and transfer expenses ranging from approximately \$40–\$66 per GB per test, making the cumulative costs and resource consumption prohibitively high [272]. Additionally, multimodal integration requires not only large-scale storage and high-speed computing but also relies on complex resource management, parallel scheduling, and workflow optimization. These tasks necessitate comprehensive expertise in systems architecture, DevOps operations, and big data processing [273] which often exceed the capabilities of biology-focused research groups operating independently. Collectively, these factors have led to the concentration of multimodal omics integration capabilities within resource-rich centers, thereby limiting equitable application of this technology across broader research and clinical settings.

Furthermore, the complexity of multi-omics data makes the visualization of multi-modal regulatory networks a significant challenge, particularly due to the absence of cross-modal reasoning logic and authoritative biological annotations. First, the lack of cross-modal reasoning logic can result in incorrect correlation

integration, misleading inferences about tumor disease mechanisms. Regulatory networks typically involve interactions across multiple molecular layers, are large in scale, and are highly complex. Differences in data quality and signal-to-noise ratios between different omics technologies can also lead to spurious correlations during the integration process [274]. As previously mentioned, batch effects can introduce biological variations that may obscure true biological signals, leading to erroneous inferences in regulatory networks. Additionally, existing methods are limited in their ability to effectively capture dynamic cross-modal relationships, hindering more in-depth mechanistic investigations [275]. However, authoritative biological annotations play a crucial role in correcting noise and errors within the analysis, ensuring that the results are both statistically and biologically validated. Despite large-scale efforts to create cell atlases across various tissues [276–279] a consensus on definitive cell type markers and cell states in the field of oncology has yet to be reached.

Translating single-cell sequencing to clinical applications

An ideal clinical single-cell sequencing platform should feature ease of use, high throughput, high resolution, and relatively low cost. However, the technical challenges associated with experimental procedures and data integration significantly limit the translation of single-cell sequencing technology into clinical practice. For fresh tumor tissue, commonly used techniques include ScRNA-seq [3] scATAC-seq [280] ScDNA-seq [8] and integrated omics approaches such as CITE-seq (which simultaneously detects transcriptomes and cell surface proteins) [60] SNARE-seq [67] or SHARE-seq (which simultaneously measures RNA and chromatin accessibility) [281]. These methods require rapid tissue dissociation to ensure data integrity, which presents challenges in terms of sample collection, preservation, and efficient dissociation. While commercially available tissue preservation and dissociation kits can offer some operational flexibility and enhance dissociation efficiency, they cannot facilitate long-term preservation. Additionally, for tumors with poor tissue quality, the cell viability of single-cell suspensions often fails to meet sequencing requirements. Notably, modified single-nucleus sequencing technologies have overcome this issue [282] enabling single-cell sequencing from frozen or fixed tissues. However, compared to scRNA-seq, SnRNA-seq has lower gene counts and exhibits slight differences in gene expression patterns, which could complicate the integration of data across different sequencing methods.

In practice, an ideal single-cell sequencing platform should integrate smoothly into existing clinical workflows, such as those used in clinical pathology. Based on this, more intuitive single-cell spatial omics technologies

are better suited for clinical applications. For example, tumor samples can be sectioned, with one slide used for H&E staining and another for single-cell spatial analysis. To realize this vision, single-cell spatial analysis platforms must support FFPE (Formalin-Fixed Paraffin-Embedded) tissue, which is the standard archival format in clinical pathology. Significant advancements have been made in integrating FFPE samples with spatial analysis technologies [109, 283, 284]. These technologies rely on probe capture or digital profiling strategies to mitigate issues such as RNA degradation and crosslinking in FFPE samples, enabling high-resolution spatial transcriptomics and protein expression analysis. However, pre-designed capture probes may not cover all target transcripts, and digital profiling strategies often fail to provide single-cell resolution, indicating that further optimization is required.

Furthermore, the high cost remains a major barrier to the broader clinical adoption of single-cell and spatial technologies. While the cost of sequencing the human genome has decreased from approximately \$1 million in the early 2000s to less than \$1,000 today, and is expected to approach \$100 soon [285] each platform has different reagent and instrument requirements, making it potentially unfeasible for large-scale clinical implementation. In contrast, spatial technologies depend on advanced imaging equipment, software for processing high-dimensional data, and substantial computational resources, making them more costly than conventional scRNA-seq. For instance, the cost of transcriptomic profiling on a single slide can still run into thousands of dollars, and for spatial proteomics methods using antibodies, the cost per analyte is even higher. Reducing costs is therefore essential to achieve the clinical implementation of single-cell spatial analysis.

Future trends

Based on the challenges described above, future developments in single-cell and spatial multi-omics technologies will push technological boundaries by emphasizing high throughput, enhanced accuracy, multimodality, and reduced costs. These advances will enable researchers to gain a more comprehensive understanding of the TME and facilitate its application in precision cancer therapies and clinical practice. Currently, single-cell sequencing platforms can process over one million cells per experiment [286]. When combined with novel microfluidic chips (e.g., nanopore arrays) and AI-driven cell sorting techniques [265, 287] these technologies are poised to dramatically increase throughput, with future capacities expected to approach tens of millions of cells per day.

In terms of accuracy, omics modalities beyond the transcriptome still have significant room for improvement. For example, current single-cell genomic techniques

cannot reliably detect all somatic mutations in individual cells; missing low-frequency and driver mutations may compromise the detailed characterization of tumor subclonal architectures and the identification of critical events in tumorigenesis and progression [288, 289]. Recent research has made notable advances in single-cell epigenomics—uCoTarget technology, for instance, can simultaneously profile five types of histone modifications in 19,860 single cells [99]. Future developments are expected to expand coverage to include additional modalities such as DNA methylation and chromatin accessibility. Similarly, in proteomics, the latest single-cell workflow, PiSPA, is capable of quantifying up to 3,000 proteins in a single mammalian cell [51]. Next-generation mass spectrometers, such as the timsTOF Ultra 2 system, have improved protein identification by 15–20% and peptide identification by 20–25% in single-cell analyses [290]. It is anticipated that future innovations will further increase protein identifications to over 5,000, while also integrating additional molecular layers. Moreover, current multimodal platforms have already combined transcriptomics with proteomics, epigenomics, and genomics—including spatial dimensions—with future goals aimed at achieving matched, multimodal causal integrative analyses [291].

In single-cell multi-omics analysis, ensuring reproducibility and consistency of analytical outcomes has increasingly emerged as a critical challenge, especially given the ubiquitous issue of batch effects [292]. Although various batch-effect correction methods, such as Harmony, Seurat, and MNN, have significantly improved the integration of single-cell datasets [252, 253, 261] considerable challenges remain. A particularly prominent example is RNA velocity analysis, a crucial tool for inferring cellular dynamics from single-cell transcriptomic data, which critically depends on the accurate measurement and modeling of the proportions of unspliced RNA at the individual gene and cell levels. Current mainstream global batch-correction strategies may inadvertently distort these precise dynamic relationships, thereby compromising the integrity and accuracy of RNA velocity analyses post-correction [293]. Furthermore, inadequate handling of batch-specific and variable proportions of spliced and unspliced RNAs has led to substantial reproducibility issues. Numerous studies have demonstrated significant inconsistencies in RNA velocity results when comparing across different platforms, laboratories, and analytical pipelines [294–296]. These issues highlight the urgent need for developing specialized, robust batch-correction frameworks specifically tailored to dynamic analyses such as RNA velocity. Future advancements in single-cell analytical tools should prioritize robust, standardized methodologies and computational strategies capable of accurately modeling and correcting batch

effects, ultimately enhancing the reproducibility and reliability of biological interpretations across diverse experimental contexts.

Importantly, as the generation of single-cell multi-omics data continues to expand, artificial intelligence (AI) and deep learning will fundamentally transform tool development and data analysis in this field. Deep learning models excel at processing vast datasets, enabling the discovery of patterns that are difficult to capture with traditional analytical approaches, thereby accelerating our understanding of tumor biology and advancing precision medicine. For instance, DeepMAPS constructs heterogeneous graphs that integrate cells and genes to identify joint embeddings in lung tumor single-cell multi-omics data. This approach infers cell type-specific biological networks and has identified biologically meaningful intercellular communication pathways between dendritic cells and tissue-resident memory CD4⁺ T cells [297]. Additionally, CytoMAD automatically corrects for batch effects in image-based cell counting, enhances cell images, and performs comprehensive label-free classification of human lung cancer cell types, effectively distinguishing malignant cells and accurately summarizing their progressive drug responses [298]. Furthermore, PERCEPTION leverages scRNA-seq data to predict the responses of individual cancer cells to specific drugs, thereby assisting clinicians in patient stratification and improving the precision of therapeutic matching for cancer patients [299]. Encouragingly, the continued evolution of AI, such as scGPT, enhances the ability to infer cell states, predict gene regulatory networks, and integrate spatial and temporal dynamics of the tumor microenvironment [300] and is expected to further reduce the costs of single-cell analyses over time, ultimately promoting the integration of single-cell spatial technologies into clinical settings.

Conclusion

Single-cell resolution multi-omics sequencing has revolutionized our understanding of malignancies. By enabling high-resolution characterization of rare tumor subpopulations, immune cell subsets, and cell-to-cell interactions within the tumor microenvironment, this approach has created detailed atlases of tumor heterogeneity and microenvironmental complexity. It has been effectively employed in identifying multiple targets for combinational therapies, monitoring therapeutic responses, and elucidating mechanisms underlying treatment resistance, thus providing novel insights and perspectives for the development of personalized immunotherapy strategies.

However, the clinical translation of single-cell sequencing technology faces significant technical and analytical challenges. These include high experimental costs, limitations inherent in cell isolation methods, potential data

loss and biases introduced during molecular amplification, and computational complexities involved in integrating and analyzing multimodal datasets. To address these issues, continuous improvements in experimental methodologies and computational algorithms are needed to enhance accuracy, throughput, and cost-efficiency.

Ultimately, single-cell sequencing technologies hold tremendous potential for personalized cancer treatment. Integration of multi-dimensional single-cell data will allow precise characterization of patient-specific tumors, real-time monitoring of therapeutic responses, and accurate identification of predictive biomarkers. Achieving these goals necessitates strengthened interdisciplinary collaborations among biology, medicine, bioinformatics, engineering, and artificial intelligence fields, thereby facilitating the transition of single-cell technologies from fundamental research into clinical application.

Abbreviations

AI	Artificial Intelligence
AML	Acute Myeloid Leukemia
APC	Adenomatous Polyposis Coli
ATAC	Assay for Transposase-Accessible Chromatin
BCR	B Cell Receptor
CALGB	Cancer and Leukemia Group B
CNA	Copy Number Alteration
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTC	Circulating Tumor Cell
CTLA	Cytotoxic T-Lymphocyte Antigen
CUT	Cleavage Under Targets
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
FACS	Fluorescence-Activated Cell Sorting
FFPE	Formalin-Fixed Paraffin-Embedded
GC	Gastric Cancer
GFP	Green Fluorescent Protein
GPCR	G Protein-Coupled Receptor
GZMA	Granzyme A
GZMB	Granzyme B
GZMK	Granzyme K
HCC	Hepatocellular Carcinoma
HLA	Human Leukocyte Antigen
HPC	high-performance computing
ICB	Immune Checkpoint Blockade
ICI	Immune Checkpoint Inhibitor
IL	Interleukin
LCM	Laser Capture Microdissection
LPS	Lipopolysaccharide
MACS	Magnetic-Activated Cell Sorting
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
MHC	Major Histocompatibility Complex
MITF	Microphthalmia-Associated Transcription Factor
MRD	Minimal Residual Disease
MS	Mass Spectrometry
NCSC	Neural Crest Stem Cell
NK	Natural Killer (cell)
NKT	Natural Killer T (cell)
RGMB	Repulsive Guidance Molecule B
RNA	Ribonucleic Acid
RRBS	Reduced Representation Bisulfite Sequencing
SELENOP	Selenoprotein P
SHARE	Simultaneous High-throughput ATAC and RNA Expression
SIDR	Simultaneous DNA and RNA sequencing
SIMO	Single-cell Integration for Multi-Omics

SLAT	SWAP-70-Like Adapter of T cells
SM	Small Molecule
SNARE	Soluble NSF Attachment Protein Receptor
TAM	Tumor-Associated Macrophage
TCR	T Cell Receptor
TEA	Transcriptional Enhancer Activator
TEL	Telomerase
TLS	Tertiary Lymphoid Structure
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
Treg	regulatory T
UDA	Unsupervised Domain Adaptation
UMI	Unique Molecular Identifier
VAF	Variant Allele Frequency
VEGF	Vascular Endothelial Growth Factor

Acknowledgements

We thank Biorender(<https://www.biorender.com/>) for the assistance for the illustration.

Authors' contributions

G.D., J.L. designed the study; J.L., Y.D., Z.G., and D.Z., searched for literature; J.L. wrote the manuscript; Y.D., Z.L., X.C. and D.Z. helped edit and revise the manuscript. G.D., and F.Z., provided funding support. All authors have reviewed and approved the article and agree with publication in this journal.

Funding

This work was supported by the National Natural Science Foundation of China (Grant Nos. 82272849 to GD), Huxiang Youth Talent Program (Grant Nos. 2023RC3072 to GD, 2024RC3043 to FZ), Natural Science Fund for Outstanding Youths in Hunan Province (Grant Nos. 2023JJ20093 to GD).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 30 April 2025 / Accepted: 28 July 2025

Published online: 25 August 2025

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