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Review

# Applications of single-cell technologies in drug discovery for tumor treatment

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## SUMMARY

**Single-cell technologies have been known as advanced and powerful tools to study tumor biological systems at the single-cell resolution and are playing increasingly critical roles in multiple stages of drug discovery and development. Specifically, single-cell technologies can promote the discovery of drug targets, help high-throughput screening at single-cell level, and contribute to pharmacokinetic studies of anti-tumor drugs. Emerging single-cell analysis technologies have been developed to further integrating multi-dimensional single-cell molecular features, expanding the scale of single-cell data, profiling phenotypic impact of genes in single cell, and providing full-length coverage single-cell sequencing. In this review, we systematically summarized the applications of single-cell technologies in various sections of drug discovery for tumor treatment, including target identification, high-throughput drug screening, and pharmacokinetic evaluation and highlighted emerging single-cell technologies in providing in-depth understanding of tumor biology. Single-cell-technology-based drug discovery is expected to further optimize therapeutic strategies and improve clinical outcomes of tumor patients.**

## INTRODUCTION

Single-cell technologies refer to a group of cutting-edge technologies that allow researchers to explore biological systems with single-cell resolution and have played mountingly critical roles in the researches of various diseases.[1] As a complement to the averaging of large cell populations, single-cell technologies, including single-cell transcriptomic, proteomic, epigenomic, and genomic technologies, provide a more detailed picture of complex biology and reveal the heterogeneity that exists in tumor tissues.[2,3] Bulk genomic and transcriptomic analyses have significantly improved our understanding of the biological mechanisms within tumors and their microenvironment. However, the averaging of signals from large numbers of cells by these methods may obscure specific cell subpopulations and states that play important roles in tumor progression and therapeutic response, which might impede the development of anti-tumor drugs.[4] Conversely, single-cell technologies can probe the cellular state, interactions, and regulatory mechanisms of tumor cells and their microenvironment at the single-cell level, providing insights into key issues in development of anti-tumor drugs, including tumor evolution, acquisition of therapeutic resistance, and mechanisms of metastasis.[5,6]

Recently, single-cell technologies have accomplished significant achievements in the diagnosis and treatment of various tumors, including breast tumor, lung tumor, and glioma.[7] The main types and platforms of single-cell technologies have been summarized in Table 1. Single-cell technologies have been used to declare tumor heterogeneity and molecular subtypes; explore underlying mechanisms of tumor metastasis, recurrence, and drug resistance; identify rare subpopulations, such as tumor stem cells and circulating tumor cells; and analyze the composition of tumor microenvironment.[8] Single-cell analysis techniques provide new methods to examine intercellular variation and biological mechanisms in tumor cells, which contributes to identifying potential therapeutic targets for drug candidates and combination therapeutic targets to overcome drug resistance in tumor treatment.

Moreover, single-cell technologies also offer advanced insights into tumor biology and provide information about drug perturbation and pharmacokinetic effects in cells with different genetic backgrounds, which will help researchers understand the different responses and ADME (absorption, distribution, metabolism, excretion) mechanisms of drugs at cellular level.

Especially, more detailed maps of cell subtypes and physical locations in tumor tissues have been developed, as microfluidics, electrophysiological measurements, high-resolution imaging, deep sequencing, and mass spectrometry platforms become increasingly sophisticated.[4] In preclinical studies, single-cell technologies can be used to screen perturbations in cellular function and phenotype, evaluate drug toxicology and specific pharmacodynamic markers, and promote the optimization of drug candidates for tumor treatment. In clinical studies, single-cell technologies are applicable in monitoring the response to therapy in tumor patients and further exploring the mechanisms of drug resistance. In particular, the emerging VASA-seq technique enables high-throughput capture of non-coding RNAs, completing

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**Table 1. Summary of main types and platforms for single-cell technologies**

| Technology types | Characteristics | Platforms | Year | Reference |
| --- | --- | --- | --- | --- |
| Single-cell genome | Detecting single-cell SNV, CNV and other genomic sequence or structure variations | DOP-PCR | 2011 | Navin et al.[9] |
| | | MALBAC | 2012 | Zong et al.[10] |
| | | LIANTI | 2017 | Chen et al.[11] |
| | | MDA | 2002 | Dean et al.[12] |
| | | META-CS | 2021 | Xing et al.[13] |
| | | Refresh-seq | 2024 | Wang et al.[14] |
| Single-cell transcriptome | Detecting mRNA expression in single cells | STRT-seq | 2011 | Islam et al.[15] |
| | | Smart-seq | 2012 | Ramskold et al.[16] |
| | | CEL-seq | 2012 | Hashimshony et al.[17] |
| | | InDrop | 2015 | Klein et al.[18] |
| | | Drop-seq | 2015 | Macosko et al.[19] |
| | | MARS-seq | 2019 | Keren-Shaul et al.[20] |
| | | Seq-Well | 2017 | Gierahn et al.[21] |
| | | Microwell-seq | 2018 | Han et al.[22] |
| | | SPLit-seq | 2018 | Rosenberg et al.[23] |
| | | Quartz-seq | 2013 | Sasagawa et al.[24] |
| | | RamDa-seq | 2018 | Hayashi et al.[25] |
| | | SCAN-seq2 | 2023 | Liao et al.[26] |
| | | Seq-Well $S^3$ | 2020 | Hughes et al.[27] |
| | | SMART-seq3 | 2020 | Hagemann-Jensen et al.[28] |
| | | Smart-seq3xpress | 2022 | Hagemann-Jensen et al.[29] |
| | | VASA-seq | 2022 | Salmen et al.[30] |
| Single-cell epigenome | Detecting the epigenomic status of cells, such as DNA methylation, histone modification, and chromatin states | scRRBS | 2016 | Clark et al.[31] |
| | | WGBS | 2015 | Farlik et al.[32] |
| | | CGI-seq | 2017 | Han et al.[33] |
| | | ATAC-seq | 2015 | Litzenburger et al.[34] |
| | | DNase-seq | 2010 | Song and Crawford[35] |
| | | ChIP-seq | 2019 | Park et al.[36] |
| | | Drop-ChIP | 2015 | Rotem et al.[37] |
| | | scBS-seq | 2020 | Kashima et al.[38] |
| | | scAba-seq | 2016 | Mooijman et al.[39] |
| | | CUT&Tag | 2019 | Kaya-Okur et al.[40] |
| | | Single-cell Hi-C | 2013 | Nagano et al.[41] |
| Single-cell proteome | Detecting the protein quantification in single cells | SCoPE-MS | 2018 | Budnik et al.[42] |
| | | SCoPE2 | 2021 | Specht et al.[43] |
| | | sc-CyTOF | 2022 | Iyer et al.[44] |

transcriptome bioinformation at the single-cell level.[30] The NEAT-seq technique facilitates simultaneous measurement of nuclear protein abundance, chromatin accessibility, and the transcriptome in single cells, allowing for the interrogation of regulatory mechanisms that transcend central dogma.[45] In addition, although there are no mainstream non-targeted assays for single-cell proteomics, the emerging SCoPE-MS holds the promise of bridging the technology gap.[46] Furthermore, single-cell sequencing and single-cell multi-omics analyses that preserve spatial information will provide a comprehensive approach to parse the biological mechanisms of tumor development, whereas

advanced integration methods for single-cell data and single-cell sequencing technologies for formalin-fixed and paraffin-embedded (FFPE) samples will further expand the scale of single-cell data. Single-cell clustered regularly interspaced short palindromic repeats (scCRISPRs) and single-cell third-generation sequencing (TGS) technologies will further elucidate gene function and the cellular genomic and transcriptomic landscape.

Collectively, single-cell technologies are revolutionizing the field of drug discovery and development for tumor treatment. Applying single-cell technologies markedly contributed to drug discovery process, including target identification, high-throughput screening, and pharmacokinetic evaluation. In this review, we systematically summarized the applications of single-cell technologies across various processes in drug discovery and development for tumor treatment (Table 2), introduced advanced single-cell technologies, and outlined their cutting-edge questions in drug discovery and development. The application of single-cell technologies has great potential in reducing the time cost and improving the success rate and development efficiency for anti-tumor drugs, which will ultimately provide tumor patients with optimized treatment strategies to improve clinical prognosis.

## SINGLE-CELL TECHNOLOGIES IN THE DISCOVERY OF THERAPEUTIC TARGETS

Target identification is the first and most important process in the development of new drugs, especially in the era of precision medicine. The application of single-cell technologies can provide in-depth insights in analyzing the clonal evolutionary subpopulation characteristics of tumor cells, the metastatic mechanism of tumor circulating cells, and the immune escape mechanism and interaction network in tumor microenvironment, which can provide the theoretical foundation for the identification of anti-tumor drug targets (Figure 1).

### Identification of anti-tumor targets in clonal evolution using single-cell technologies

Tumor development was pioneeringly described as a clonal evolution process by Nowell in 1976, which has been extensively supported in recent era.[88] Malignant cell populations have the property of undergoing further genetic diversification during progression, leading to a shift in clonal structure, genotype, and phenotype over time, which has been known as clonal evolution of tumor. The heterogeneity derived from clonal evolution has been intimately related to tumor progression and the acquisition of drug resistance.[89] Recent advances in single-cell technologies have offered unprecedented opportunities for identifying clonal subpopulations, interpreting mutational patterns, and predicting evolutionary tendencies.[90,91] Utilizing the latest single-cell DNA sequencing (scDNA-seq) technologies and data analysis methods helps researchers identify single-nucleotide variants (SNVs) and copy-number aberrations (CNAs) in tumor cells, on the basis of which cell lineage trees can be inferred.[92] Algorithms including inferCNV and CopyKAT are also available for extrapolating CNAs and cell development trajectories from single-cell RNA sequencing (scRNA-seq) results.[90,92,93] Targeting clonal events to block the evolution of intra-tumor heterogeneity or disrupt the balance of interaction network would provide new therapeutic strategies for tumor treatment.[94] Next, we will discuss the application and cutting-edge issues of single-cell technologies in identifying drug-resistant subpopulations of tumor cells and cancer-stem-cell-related therapeutic targets in detail.

#### *Drug-resistant subpopulations*

Although current anti-tumor therapies may eradicate the majority of tumor cell populations, they also obviously facilitate clonal evolution in tumor development through exerting selection pressure or inducing secondary mutations beyond the pharmaceutical target, which ultimately results in the emergence of therapeutic resistance in tumor patients.[95] Existing studies have demonstrated that exogenous treatment could induce a rapid adaptation and alteration of functional mechanisms in tumor cells, such as stress-induced mutagenesis, the downregulation of DNA repair genes, the expression of error-prone polymerase, and enhanced plasticity.[96] Single-cell technologies quantitatively analyze oncogenic signaling pathways and characterize drug-resistant subpopulations by identifying major cellular components and defining individual genomic and molecular status, which provides potential targets for synthetic lethal therapies in tumor treatment.[97]

Combining lineage tracing and scRNA-seq, Eyler et al. discovered that the copy-number amplification of insulin receptor substrate-1 and substrate-2 (IRS1 and IRS2) in a small subset of dasatinib-resistant clones activated the insulin and AKT signaling programs and permitted a growth predominance, indicating the promising effective therapy to overcome glioblastoma drug resistance.[47] Besides, Zhao et al. identified a drug-resistant subpopulation with unique dominant metabolic pattern by scRNA-seq in liver organoid, where accelerated glucose metabolism initiated hypoxia-induced HIF-1 signaling, the upregulation of NEAT1 in CD44-high cells, and the overactivation of Jak-STAT signaling, which could provide new targets for overcoming drug resistance in hepatobiliary tumor.[48] In the study of drug resistance mechanisms in refractory multiple myeloma (MM) patients, peptidylprolyl isomerase A (PPIA) was identified to be a signature and potential interfering target of drug resistance to DARA-KRD treatment by longitudinal scRNA-seq, and the inhibition of PPIA could induce the restoration of sensitivity to proteasome inhibitors in MM tumor cells.[49]

Moreover, epigenetic plasticity can provide homogeneous tumor populations with the selective advantage required to survive under the pressure of drug therapy, thereby promoting drug resistance in tumor cells.[98] Sharma et al. used scRNA-seq to profile the transcriptional dynamics of distinct stages in cell evolution under the selective pressure of cisplatin in oral squamous cell carcinomas (OSCCs). It was revealed that H3K27 acetylation induced the upregulation of SOX9 expression and the evolution of tumor cells toward drug resistance, and targeting JQ1, a validated inhibitor of histone acetyltransferase BRD4, could revert the sensitivity of cisplatin-resistant cells.[50] Another study integrating scRNA-seq and scATAC-seq demonstrated enhanced transcriptional activation of primitive cells to other lineages besides myeloid in resistant and relapsed pediatric acute myeloid leukemia, which might offer a promising combination therapy strategy for drug-resistant patients.[51]

**Table 2. Application of single-cell technologies in drug discovery for tumor treatment**

| Process | Sample source | Tumor types | Detecting technologies | Application and findings | Drugs | Reference |
|---|---|---|---|---|---|---|
| Target identification | Neurosphere cultures | Glioblastoma | scRNA-seq | Discovered the copy-number amplification of IRS1 and IRS2 in dasatinib-resistant clones | – | Eyler et al.[47] |
| Target identification | Organoid | Hepatobiliary tumor | scRNA-seq | Identified a drug-resistant subpopulation with unique dominant metabolic pattern and NEAT1 as a potential therapeutic target | – | Zhao et al.[48] |
| Target identification | Clinical tumor sample | Multiple myeloma | scRNA-seq | Identified PPIA as a potential novel target of drug-resistance to Dara-KRd treatment | Ciclosporin (targeting PPIA) | Cohen et al.[49] |
| Target identification | Primary cell | Oral squamous cell carcinomas | scRNA-seq | Revealed that H3K27 acetylation induced the drug resistance, and BRD4 was a therapeutic target | JQ1 (targeting BRD4) | Sharma et al.[50] |
| Target identification | Clinical tumor sample | Pediatric acute myeloid leukemia | scRNA-seq and scATAC-seq | Demonstrated enhanced transcriptional activation of primitive cells to other lineages besides myeloid in resistant and relapsed samples and revealed MEF2C as a potential therapeutic target | – | Lambo et al.[51] |
| Target identification | Mouse model | Lung tumor | scRNA-seq | Discovered high expression of TIGIT in stem cells and identified TIGIT as a potential therapeutic target | – | Marjanovic et al.[52] |
| Target identification | Primary cell | Gastric adenocarcinoma | scRNA-seq | Revealed that SOX9 was associated with the maintenance of stemness in CSCs | EC359 (targeting LIF/LIFR) | Fan et al.[53] |
| Target identification | Clinical tumor sample | Glioblastoma | scRNA-seq | Targeting Wnt could eliminate refractory cells and block CTC-mediated recolonization | XAV939 (targeting Wnt) | Liu et al.[54] |
| Target identification | Mouse model | Pancreatic ductal adenocarcinoma | scRNA-seq | Revealed that CTCs displayed upregulated survivin expression | YM155 (targeting survivin) | Dimitrov-Markov et al.[55] |
| Target identification | Clinical tumor sample | Hepatocellular carcinoma | scRNA-seq and multi-regional sampling | CTCs modulated CCL5 through p38-MAX signaling axis to enable immune escape | Antihuman CCL5-neutralizing antibody (targeting CCL5) | Sun et al.[56] |
| Target identification | Clinical tumor sample | Colorectal tumor | Single-cell metabolic fingerprints | Developed a molecular typing system to predict metastasis potential based on the metabolic fingerprints of single CTCs | – | Zhang et al.[57] |
| Target identification | Clinical tumor sample | Lung tumor | CTC-race | Enabled concurrent biophysical and biochemical characterization of migrating CTCs | – | Liu et al.[58] |

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**Table 2. *Continued***

| Process | Sample source | Tumor types | Detecting technologies | Application and findings | Drugs | Reference |
|---|---|---|---|---|---|---|
| Target identification | Clinical tumor sample | Breast tumor | scRNA-seq | Plakoglobin induced the generation of CTC clusters and the development of lung metastasis and was a potential therapeutic target | – | Aceto et al.[59] |
| Target identification | Clinical tumor sample and mouse model | Breast tumor | scRNA-seq | Found the association between neutrophils and CTCs derived the metastatic potential and identified Vcam1 as a potential therapeutic target | – | Szczerba et al.[60] |
| Target identification | Mouse model | Breast tumor | scRNA-seq | ICAM1 induced CTCs cluster formation and lung metastasis | Anti-ICAM1 neutralizing antibody (targeting ICAM1) | Taftaf et al.[61] |
| Target identification | Coculture cell line | Lung tumor | scRNA-seq | Developed an algorithm to quantify mitochondrial transfer from T cells to tumor cells based on scRNA-seq data and identified TNF-α pathway as a potential target | – | Zhang et al.[62] |
| Target identification | Clinical tumor sample | Breast tumor | scRNA-seq and imaging mass cytometry | Analyzed molecular characterization of depletion-like T cells and identified IL-15 as a potential therapeutic target | – | Tietscher et al.[63] |
| Target identification | Clinical tumor sample | Prostate tumor | scRNA-seq | Elevated PTGER4 expression levels in T cells correlated with an exhaustion phenotype marked by impaired cytotoxicity | YY001 (targeting PTGER4) | Peng et al.[64] |
| Target identification | Clinical tumor sample | Nasopharyngeal tumor | scRNA-seq | CD70–CD27 interactions enhanced the development of regulatory T cells and suppressed T cell activity | Cusatuzumab (targeting CD70) | Gong et al.[65] |
| Target identification | Mouse model | Lung tumor | scRNA-seq | Discovered upregulation of VEGF and CCR2-signaling-related genes in Treg-cell-deficient samples | RS-504393 (targeting CCR2) | Glasner et al.[66] |
| Target identification | Naive Treg cells from human cord blood | | scRNA-seq | CYP1A1 was an important regulator of Tregs stability, and CYP1A1 knockdown could diminish the immunosuppressive activity of Tregs | Alizarin (targeting CYP1A1) | Yi et al.[67] |
| Target identification | Mouse model | Lung tumor | scRNA-seq | Discovered enhanced CCR8 expression in activated Tregs | Anti-CCR8 mAb (targeting CCR8) | Van Damme et al.[68] |

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**Table 2. *Continued***

| Process | Sample source | Tumor types | Detecting technologies | Application and findings | Drugs | Reference |
|---|---|---|---|---|---|---|
| Target identification | Clinical tumor sample | Glioblastoma | Mass cytometry and scRNA-seq | Identified a subpopulation of CD73-high macrophages with an immunosuppressive phenotype and CD73 as a potential therapeutic target | – | Goswami et al.[69] |
| Target identification | Clinical tumor sample | Clear cell renal cell carcinoma | scRNA-seq and multi-regional sampling | Higher level of the epithelial–mesenchymal transition program was localized in the tumor-normal interface, which was accompanied by the infiltration of macrophage subpopulations with high IL1B expression, representing a potential therapeutic target | – | Li et al.[70] |
| Target identification | Clinical tumor sample | Colorectal tumor | scRNA-seq and spatial transcriptomics | FAP+ CAFs and SPP1+ macrophages were positively correlated and were potential therapeutic targets | – | Qi et al.[71] |
| Target identification | Clinical tumor sample | Esophageal squamous cell carcinoma | scRNA-seq and bulk RNA-seq | Identified the druggable target CCL18 that mediated macrophage chemotaxis | Peptide Pep3 (targeting CCL18) | Sui et al.[72] |
| Target identification | Mouse model | – | scRNA-seq | Recognized the DC-related pathway involved in the response to PD-1 therapy based on scRNA-seq and accordingly developed a bispecific antibody that promoted PD-1+ T cell-DC interactions for improving the efficacy of PD-1 therapy | BiCE (targeting T cell-DC interaction) | Shapir Itai et al.[73] |
| Target identification | Mouse model | Melanoma | scRNA-seq | The specific inactivation of NF-KB or IFN regulatory factor 1 (IRF1) in conventional DCS 1 (CDC1S) led to impaired expression of IFN-$\gamma$-responsive genes, defective recruitment, and activation of anti-tumor $CD8^+$ T cells and were potential therapeutic targets | – | Ghislat et al.[74] |
| Target identification | Mouse model | Pancreatic ductal adenocarcinoma | scRNA-seq and bulk RNA-seq | Identified a population of CAFs with high LRRC15 expression programmed by TGFB, which was associated with adverse responses to anti-PD-L1 therapy and was a potential therapeutic target | – | Dominguez et al.[75] |

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**Table 2.** ***Continued***

| Process | Sample source | Tumor types | Detecting technologies | Application and findings | Drugs | Reference |
|---|---|---|---|---|---|---|
| Target identification | Clinical tumor sample | Gastric tumor | scRNA-seq | Identified a population of CAFs with high expression of POSTN and exhibiting pro-carcinogenic phenotype, which represented potential therapeutic targets | – | Li et al.[76] |
| Target identification | Clinical tumor sample | – | scRNA-seq and spatial transcriptomics | Discovered the matrix CAFs and inflammatory CAFs associated with tumor angiogenesis and the immunosuppressive microenvironment | – | Ma et al.[77] |
| HTS | Cell line | Glioblastoma | mHTS | Identified the migration inhibitory potential of AZD-6244 | AZD-6244 | Shen et al.[78] |
| HTS | Cell line | – | Cgg-sca | Achieved concentration gradient dilution of nanomedicines and real-time response monitoring at the single-cell level | – | Liu et al.[79] |
| HTS | Cell line | – | FALCOscope | Quantified the response of protein kinase A to different concentrations of isoprenaline, cAMP responses of endogenous GPCR to GPCR agonists and antagonists, and effects of GRP68 on cAMP at different PH in living cells | Ogerin | Greenwald et al.[80] |
| HTS | Primary cell | – | FLECSplate | Quantified the effect of different asthma pro-contractile agonists on the contractility of cells | CAL-101 | Pushkarsky et al.[81] |
| HTS | Cell line | – | Sci-plex | Screened three tumor cell lines exposed to 188 compounds and found that proliferation was inhibited with HDACi by limiting the ability of cells to extract acetic acid from chromatin | – | Srivatsan et al.[82] |

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**Table 2. *Continued***

| Process | Sample source | Tumor types | Detecting technologies | Application and findings | Drugs | Reference |
|---|---|---|---|---|---|---|
| HST | HESC | – | Microwell-seq 2.0 | Analyzed the effect of small molecule combinations on the HESC differentiation process | – | Chen et al.[83] |
| HTS | Human embryonic stem cells | | CP-seq | Analyzed the effects of drug combinations on cells after random pairing | – | Xie et al.[84] |
| Pharmacokinetics evaluation | Epithelial cell from organ donor | | scRNA-seq | Found that levels of target genes and metabolizing enzymes were associated with gastrointestinal side effects | – | Burclaff et al.[85] |
| Pharmacokinetics evaluation | Mouse model | – | CATCH | Allowed the observation of drug-target interactions at subcellular resolution *in vivo* | Cisplatin | Pang et al.[86] |
| Pharmacokinetics evaluation | Cell line | – | Confocal microscopy and mass spectrometry | Measured concentrations of amiodarone and its metabolites at the single-cell level | AMIO | Pedro et al.[87] |

CAF, cancer-associated fibroblasts; CSC, cancer stem cell; CTC, circulating tumor cell; DC, dendritic cell; HCC, hepatocellular carcinoma; HDACi, histone deacetylase inhibitor; HESC, human embryonic stem cell; HTS, high-throughput screening; Treg, regulatory T cell.

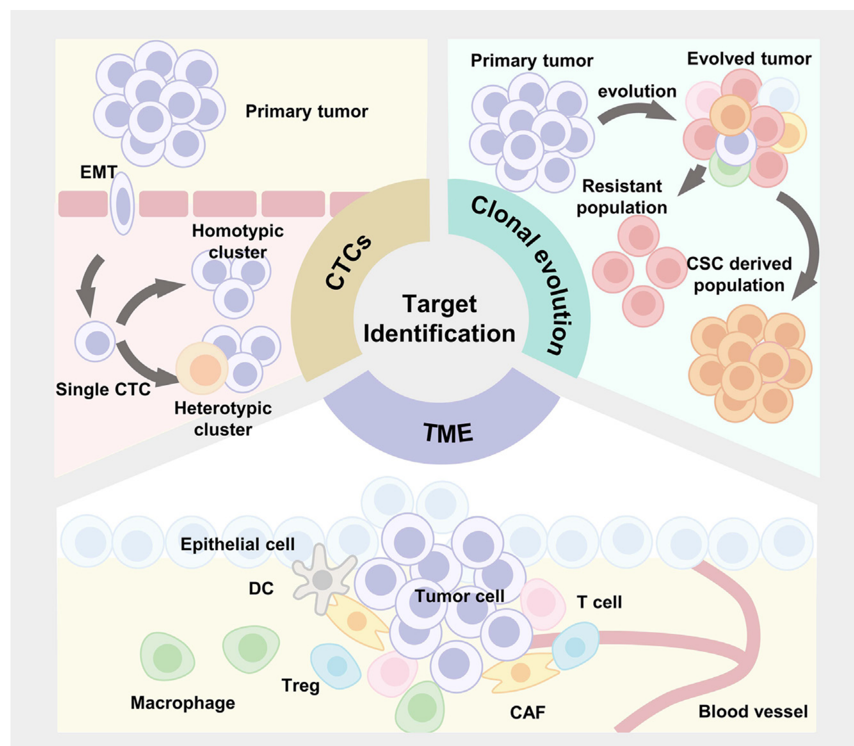


**Figure 1. Single-cell technologies facilitate the identification of drug targets related to clonal evolution, circulating tumor cells, and tumor microenvironment**

Single-cell technologies have revolutionized the field of target identification in anti-tumor drug discovery through providing insights into the molecular mechanisms underlying tumor biology, including clonal evolution, circulating tumor cells, and tumor microenvironment at single-cell level. In clonal evolution, single-cell technologies have been used to identify molecular signatures of drug-resistant subpopulations and stem cell subpopulations. In CTCs, single-cell technologies have been used to characterize molecular features and interactions of single CTC and CTC cluster. In TME, single-cell technologies have been used to explore the regulatory network of T cells, Treg, macrophage, DC, CAF, and tumor cells. CAF, cancer-associated fibroblasts; CSC, cancer stem cell; CTC, circulating tumor cell; DC, dendritic cell; EMT, epithelial-mesenchymal transition; TME, tumor microenvironment; Treg, regulatory T cell.

Taken together, resistance to tumor therapies has been attributed to the presence of variations associated with genetic, epigenetic, transcriptomic, and proteomic modifications.[99] Comparing resistant and non-resistant cell lines, samples from relapsed refractory patients and newly diagnosed patients, and longitudinal time point samples collected before and after treatment using scRNA-seq can facilitate the unraveling of drug-resistant mechanisms and the formulation of targeted combination therapies.[100] Recently, single-cell multi-omics combining proteomics and metabolomics and single-cell imaging technologies have remarkably enriched the detecting dimension in single-cell studies. Considering that scRNA-seq can only provide the transcriptome status of cells at a specific time point, single-cell multi-omics are anticipated to further illuminate the multidimensional molecular details in drug-resistant tumor cells.[101]

### *Cancer stem cells*

In the evolution of tumor, cancer stem cells (CSCs) with extensive reproductive capacity have been considered as the main unit of evolutional selection[102] and closely associated with a variety of biological phenotypes in tumor malignancies, including recurrence, metastasis, heterogeneity, and radiation resistance.[103] In particular, CSCs have generally considered to be multi-drug resistant and may represent one source of drug-resistant subpopulations. Therefore, signaling pathways involved in regulating the maintenance and survival of CSCs have emerged as promising targets for tumor therapy, and the application of single-cell technologies to the characterization of CSCs has provided unprecedented opportunities to identify new targetable molecular pathways and thus design new strategies for the eradication of CSCs.[104]

Nemanja et al. captured a high-plasticity cell subpopulation with stem cell characteristics and significantly high expression of TIGIT, which was found to be an essential driver of tumor progression and heterogeneity in lung tumor, providing a rationale for targeting these highly plastic cells in the clinical treatment of tumor patients.[52] A study on gastric adenocarcinoma (GAC) utilizing scRNA-seq revealed that SOX9, a vital transcription factor for stem cells, was significantly enriched in tumor cells and associated with the maintenance of stemness in CSCs. The study also found that targeting LIF/LIFR, the most abundantly secreted molecule regulated by SOX9, had great potential in eliminating CSCs. This new combination therapy strategy offered a promising approach for the treatment of advanced GAC.[53] Besides, a comprehensive analysis of 34 scRNA-seq datasets by bulk sequencing and clustered regularly interspaced short palindromic repeats

(CRISPR) screening developed a pan-tumor stemness gene signature and identified its correlation with immunotherapy response, which suggested the significance of single-cell technologies in identifying targets to overcome resistance to immunotherapy.[105]

Although single-cell technologies are uniquely qualified in revealing the characteristics of cancer stem cell clusters and recognizing relevant targets for tumor treatment, present studies have focused exclusively on the CSCs of specific time cross-section, while the dynamic evolution of CSCs during tumor progression still requires further investigation. Furthermore, how mutant heterogeneous cells of different clones in tumor entity transform to the CSC phenotype through intrinsic or acquired plasticity, especially the influence of epigenetic plasticity and signals from tumor microenvironment, remains to be further investigated.[104]

Continuously expanding malignancy populations are genetically diverse during clonal evolutionary processes, leading to tumor progression and resistance to treatment.[106] Mapping the heterogeneity and evolution of tumor cells at single-cell resolution contributes to the identification of relevant therapeutic targets for tumor treatment, and technological innovations, including longitudinal sampling, preclinical models, and new computational tools for evaluating phylogeny, provide additional assistance in the clonal deconstruction of tumor evolution.[107] However, relevant studies to date have predominantly relied on scRNA-seq, and the intercellular variation promoting evolutionary selection is also manifested in other dimensions of tumor cells such as epigenetic profiles, spatial distribution, and interactions with the microenvironment. The utilization of multi-omics technologies, including epigenomics, proteomics, metabolomics, and spatial omics at single-cell resolution might promisingly further enhance the understanding of tumor cell evolutionary landscape and the comprehensive identification of targets for early tumor intervention or combinational therapy.[108,109]

## Identification of anti-tumor targets in circulating tumor cells using single-cell technologies

Circulating tumor cells (CTCs) represent one of the essential mechanisms of tumor metastasis, and targeting the regulatory mechanisms associated with CTCs has emerged as a promising anti-tumor strategy. In this section, we systematically discussed the application of single-cell technologies to investigate the molecular characterization and relevant targets of single CTC, homotypic, and heterotypic CTC clusters.

### *Single CTC*

CTCs, which refer to cell populations migrating from primary or metastatic tumors into peripheral blood,[110] have been found to be closely associated with the metastasis tumors.[111] Accumulating evidence indicates that the in-depth understanding of CTCs can be of great help in identifying therapeutic targets and building individualized treatment for tumor metastasis.[112] Conventional CTC detection relies on the identification of classical CTC markers, including E-cadherin, vimentin, and Twist, and has decreased sensitivity because of the heterogeneity in tumor cells.[113] However, the application of single-cell technologies can facilitate further unbiased identification of typical and atypical CTCs populations and provide a more comprehensive insight into the status of CTCs and the interaction with blood microenvironment.[114,115]

Using scRNA-seq, Liu et al. discovered the correlation between Wnt pathway activation and the establishment of CTC stemness in glioblastoma (GBM) drug-resistant circulating subpopulations, indicating that targeting Wnt had the potential to eliminate refractory cells and block CTC-mediated recolonization.[54] Besides, the establishment of xenograft models of CTCs could provide new opportunities for the exploration in the mechanisms of tumor metastasis and drug resistance. In the scRNA-seq analysis of CTCs isolated from a highly metastatic pancreatic ductal adenocarcinoma (PDAC) patient-derived xenograft (PDX) mouse model, researchers revealed that CTCs displayed upregulated expression of survivin, a key regulator of mitosis and apoptosis, and the combination of YM155 (survivin inhibitor) with chemotherapy was effective in preventing tumor metastasis in the PDX model.[55] Furthermore, emerging evidence has indicated that CTCs exhibit spatial heterogeneity during hematogenous metastasis in response to stresses in blood microenvironment.[115] Sun et al. performed multisite sampling from hepatocellular carcinoma (HCC) patients and scRNA-seq to probe the spatiotemporal dynamics of the transcriptome in CTCs during blood transport and found that CTCs modulated the expression of CCL5 through p38-MAX signaling axis to enable the recruitment of regulatory T cells (Tregs) and immune escape, indicating new targets to inhibit the metastasis of HCC tumor.[56] Besides scRNA-seq, novel analysis platform, including single-cell metabolic fingerprints and CTC-Race, have also been developed to further profile the metabolic, biophysical, and biochemical characterization of single CTC.[57,58]

### *Homotypic and heterotypic clusters of CTCs*

During the process of migration in blood, CTCs have been found to interact with other tumor cells, immune cells, and blood components and induce the formation of CTC clusters, including tumor cell-tumor cell homotypic clusters and tumor cell-blood cell heterotypic clusters, promoting the blood dissemination and metastatic potential of CTCs.[116,117] Understanding the characterization and formation mechanisms of CTC cluster will contribute to the development of therapeutic strategies targeting this biological process, which could inhibit the progression of metastatic tumors and improve the survival of tumor patients. In a study using single-cell RNA sequencing, CTC clusters derived from oligoclonal primary tumor cells were found to possess enhanced metastatic potential over individual CTCs. Knockdown of plakoglobin, a cell-linkage component significantly upregulated in CTC clusters, inhibited the generation of CTC clusters and the development of lung metastasis in breast tumor, indicating a potential therapeutic target for breast tumor.[59] Other adhesion molecules, including VCAM1 and ICAM1, were also found to be associated with the formation of CTCs clusters based on single-cell analysis, and blocking these molecules inhibited CTCs clustering.[60,61]

Technologies that allow for in-depth characterization of the single-cell features of CTCs are now providing high-resolution molecular details about the mechanisms of tumor metastasis and treatment resistance. In future research, the generation of CTC cell lines, CTC-derived xenografts, appropriate *in vitro* CTCs expansion for molecular analysis, and drug screening may help further understand the relationship between CTCs and treatment selection.[118] Additionally, the combination of single-cell technologies with artificial intelligence (AI) has been used

to identify and analyze CTCs, and AI-based cell identification technology, "Deepcell," can be used for morphological identification of live cells. Furthermore, MagRC, a new AI technology, can distinguish CTCs from all blood cells and classify heterogeneous CTCs.[119] Therefore, the combination of single-cell technologies and AI can provide a more comprehensive analysis of CTCs that is not affected by interference between operators, making it a promising tool for identifying metastasis-related biomarkers and treatment targets.

## Identification of anti-tumor targets in tumor microenvironment using single-cell technologies

Tumor progression is not only driven by genes within tumor cells but also modulated by the surrounding tumor microenvironment (TME).[120] The main explanation for the failure of many therapeutic approaches directly targeting tumor cells in the clinical practice includes the high heterogeneity and immune escape phenotype of TME. Accordingly, understanding the dynamic functions and interactions of TME will provide critical insights for the development of potent anti-tumor strategies.[121,122] The accelerated development of single-cell technologies provides compelling opportunities to profile the composition, heterogeneity, dynamics, and regulation of TME components, which is essential to further advance existing immunotherapeutic approaches and develop new therapeutic approaches for tumor treatment.[123,124] As immunotherapy has emerged as a promising anti-tumor strategy, the anti-tumor effects of TME immune cells and their regulatory mechanisms require growing concern. Using single-cell technologies to uncover characteristic molecules and functional regulatory pathways of critical immune cell subpopulations, especially T cells, regulatory T cells, macrophages, dendritic cells, and cancer-associated fibroblasts in TME will provide new targets for the development of immunotherapy.

### *T cells*

T cells are critical mediators in both orchestrating the overall immune response and directly killing damaged cells, and the unprecedented success of T cell therapies including Chimeric antigen receptor (CAR)-T cell therapy has demonstrated the tremendous potential of anti-tumor strategies that modulate T cells.[125] Through scRNA-seq analysis, Peng et al. demonstrated that elevated expression levels of PTGER4 in T cells were associated with an exhausted phenotype marked by impaired cytotoxicity. Its antagonist YY001 inhibited the immunosuppressive function of myeloid-derived suppressor cells (MDSCs), promoted the proliferation and anti-tumor function of T cells, and restored the infiltration levels of MDSCs and T cells in TME through chemokines, which can be employed as a combination therapeutic agent to overcome the resistance to PD-1 antibodies in prostate tumor.[64] scRNA-seq and receptor-ligand analysis have also been used to probe the regulatory mechanisms and potential therapeutic targets of T cell in GBM and head and neck cancer. In addition, recent studies have shown that mitochondrial transfer from T cells to tumor cells serves in immune escape. A study developed an algorithm to quantify mitochondrial transfer from T cells to tumor cells based on scRNA-seq data and identified regulatory mechanisms associated with the transfer, which provided the theoretical basis for therapeutic strategies targeting this process.[62] Although scRNA-seq plays an important role in revealing the mechanisms regulating T cell depletion and activity, the processing prior to sequencing has caused the samples to lose the spatial information of TME, which is intimately associated with T cell and tumor cell interactions. Combining scRNA-seq with imaging techniques such as imaging mass cytometry can facilitate the systematic elucidation of regulatory mechanisms and therapeutic targets involved in the anti-tumor effects of T cells.[63]

### *Treg*

Treg represents a unique subpopulation of T cell, which harbors lower proliferative capacity. Recent evidence has demonstrated that Treg subpopulations in tumors exhibited a high degree of heterogeneity, which resulted in their complex biological roles.[124] Profiles at single-cell resolution to clarify their effect in the anti-tumor response network are necessary for the exploration of novel drug targets on Treg cells. Helena et al. utilized scRNA-seq to identify a Tregs subpopulation that transitioned from immunosuppressive state to an effector-like T cell state after stimulation by interleukin-6 (IL-6) pro-inflammatory therapy. The study demonstrated that CYP1A1 was an important regulator of Tregs stability, and CYP1A1 knockdown could diminish the immunosuppressive activity of Tregs, providing a potential new target for tumor treatment.[67] Another study identified an activated Tregs subpopulation with enhanced CCR8 expression in an NSCLC mouse model using scRNA-seq. Further analysis revealed that the depletion of CCR8+ Tregs by natural killer (NK) cells using anti-CCR8 nanobody-Fc fusions significantly inhibited tumor growth, which could serve as a potential treatment strategy for non-small cell lung cancer (NSCLC) patients.[68] Moreover, based on scRNA-seq, CD70-CD27 interactions and vascular endothelial growth factor (VEGF) as well as CCR2 signaling were found to be correlated with the activation of Tregs, respectively, which could be considered as immunotherapeutic targets.[65,66]

### *Macrophages*

Tumor-associated macrophages (TAMs) are derived from circulating monocytes that infiltrate the tumor site and tissue-resident macrophages. Recent evidence suggested that crosstalk and metabolic changes in TAMs and Tregs significantly affected their pro/anti-tumor functions by modulating signaling cascades and epigenetic reprogramming.[126] TAMs are generally associated with tumor-promoting activities such as angiogenesis, immunosuppression, and tissue remodeling, making TAMs a promising target for the development of anti-tumor therapies.[127] In this regard, single-cell technologies have emerged as a powerful tool to explore the heterogeneity and plasticity of TAMs. Applying mass cytometry and scRNA-seq, Goswami et al. identified a subpopulation of CD73-high macrophages with an immunosuppressive phenotype that persisted after anti-PD-1 treatment, which was associated with deficient T cell infiltration and worse prognosis in GBM. These results suggested that targeting CD73 may improve the anti-tumor response to immune checkpoint therapy in GBM, which could serve as an

attractive therapeutic target for tumor treatment.[69] Another study in esophageal squamous cell carcinoma identified the drug target CCL18 that mediated macrophage chemotaxis by integrating scRNA-seq and bulk RNA-seq.[72] Furthermore, single-cell technologies incorporating spatial information can provide further insight into cellular cross-talk in TME and its spatial heterogeneity.[109] In a study conducted by Li et al., multi-regional sampling and scRNA-seq were applied to characterize the spatial heterogeneity of the microenvironment in clear cell renal cell carcinoma (ccRCC). It was observed that higher levels of the epithelial–mesenchymal transition (EMT) program were localized in the tumor-normal interface, which was accompanied by the infiltration of macrophage subpopulations with high IL1B expression. Further *in vivo* results found that IL1B inhibition induced tumor regression in mouse models, indicating a promising therapeutic target for ccRCC.[70] Another study integrating scRNA-seq and spatial transcriptomics found that tumor-specific FAP+ fibroblasts and SPP1+ macrophages were positively correlated with spatial distribution in colorectal cancer, and the interaction promoted the remodeling of extracellular matrix and immune escape in TME, representing a potential target for interference.[71] Future exploration of the spatial and temporal evolution of TAM diversity could further provide theoretical foundation for the identification of TAMs-related targets.

### Dendritic cells

Dendritic cells (DCs) are essential participants in recognizing tumor antigens and initiating the anti-tumor activity of T cells and have emerged as a promising target for tumor immunotherapy.[128] Recent studies have used single-cell technologies to identify unrevealed DCs subpopulations and molecular mechanisms associated with anti-tumor immunity. A deeper understanding of the regulatory mechanisms of DCs in tumor microenvironment can provide opportunities to reverse the immunosuppressive phenotype of tumors with deficient T cell infiltration.[129] Ghislat et al. analyzed the distinct activation states of DCs associated with tumor immunogenicity by scRNA-seq. The major pathways associated with DCs maturation included nuclear factor κB (NF-κB) and interferon (IFN) pathways, and the specific inactivation of NF-κB or IFN regulatory factor 1 (IRF1) in conventional DCs 1 (cDC1s) led to impaired expression of IFN-γ-responsive genes, defective recruitment, and activation of anti-tumor $CD8^+$ T cells. It was also found that the reactivation of NF-κB/IRF1 axis was associated with improved clinical outcomes in melanoma, which may provide a theoretical foundation for the development of new therapeutic targets for tumor treatment.[74] An scRNA-seq study in B cell acute lymphoblastic leukemia (B-ALL) found that TMEM173 was associated with functional activation of NK cells and DCs in TME, which is expected to be a feasible strategy for improving therapeutic efficiency of B-ALL.[130] Yuval et al. recognized the DC-related pathway involved in the response to PD-1 therapy based on scRNA-seq and accordingly developed a bispecific antibody that promoted PD-1+ T-cell-DC interactions and improved the efficacy of PD-1 therapy.[73] Moreover, single-cell technologies hold promises for integrating DC maps across species, which can deepen our understanding of the correspondence between DC subtypes in human and mouse and promote the development of preclinical drug research for tumor treatment.

### Cancer-associated fibroblasts

Besides immune cells, stromal cells are essential components of TME. Cancer-associated fibroblasts (CAFs) are derived from the tumor-driven transformation of a variety of precursor cells, including mesenchymal stem cells and fibroblasts, and can contribute to the regulation of tumor cell plasticity and TME heterogeneity.[109] CAFs interacted with tumor-infiltrating immune cells and modulated their antitumor effects by secreting various cytokines, growth factors, chemokines, exosomes, and other effector molecules. As the promotion of CAFs on the acquisition of therapy resistance, the positive effect of modulating CAFs in combination with immunotherapies has been demonstrated in preclinical tumor models of pancreas and lung tumors.[131] In animal models of pancreatic ductal adenocarcinoma, a population of tumor-associated CAFs with high LRRC15 expression programmed by transforming growth factor β (TGF-β) was identified by the combination of bulk and scRNA-seq. The LRRC15+ CAF signature was found to be associated with adverse responses to anti-PD-L1 therapy in multiple immune-resistant tumors, which could provide a combination therapeutic target for tumor immunotherapy.[75] Li et al. identified a subpopulation of CAFs in gastric tumor characterized by high expression of POSTN using scRNA-seq, which was an important component of the pro-carcinogenic phenotype TME and closely involved in the remodeling of tumor extracellular matrix.[76] Although CAFs have long been studied as an attractive therapeutic target, several therapeutic strategies targeting CAFs or related components have failed to improve clinical outcomes in tumor patients, indicating that CAF heterogeneity and regulatory pathways based on different subsets need to be further explored.[132] By integrating spatial information and scRNA-seq, researchers discovered the matrix CAFs and inflammatory CAFs associated with tumor angiogenesis and the immunosuppressive microenvironment, as targeting the relevant pathways may contribute to improving the immunotherapy response in tumor patients.[77]

Overall, single-cell transcriptome has revolutionized the research methods in analyzing highly complex TME,[133] and the improvements in single-cell technologies, as well as the integration with other high-throughput technologies such as bulk sequencing, *in situ* sequencing, *in situ* molecular imaging, and CRISPR screening have provided a powerful toolkit for understanding various immune cell populations and the interaction between TME and malignant cells.[134,135] However, due to the low sensitivity of most single-cell technologies to sample quality, limitations in cell throughput, and high cost, only a small portion of tumor tissue samples from a few patients can be analyzed to explore relevant anti-tumor targets and improve the efficacy of anti-tumor treatment.[136] The application of single-cell technologies to dissect the TME composition of tumor and develop relevant therapeutic strategies still depends on technological advances. Therefore, new advances in single-cell technologies, including spatial single-cell sequencing, single-cell proteomics, and single-cell epigenomics, will provide clues for understanding the coordinated organization, and interaction between tumor and immune cells in the spatial coordinate system, which could propose new treatment strategies for tumor patients.

## SINGLE-CELL ANALYSIS IN HIGH-THROUGHPUT DRUG SCREENING

High-throughput screening (HTS) is an essential component of the drug development pipeline, which allows rapid and efficient assessment of the effects of numerous drug candidates in tumor treatment.[137] Given the diversity in genetic backgrounds and states of cells within tumors, which reflect varying sensitivities to drug perturbations, it is essential to conduct single-cell phenotypic observations and screenings. Single-cell HTS is crucial for elucidating the differential responses to candidate drugs and for uncovering the underlying mechanisms within distinct subpopulations of tumor cells. Currently, the single-cell technologies integrated into the HTS platform mainly involve imaging-based single-cell technologies and single-cell sequencing technologies.

### HTS with imaging-based single-cell technologies

Imaging-based single-cell technologies represent a group of high-resolution cellular imaging technologies that can be used to observe the structure, morphology, function, and metabolism of a single cell. The advantages of imaging-based single-cell HTS include rapidity, low cost, and availability of temporal and spatial information.[138] Shen et al. developed a platform by combining microchannel technology and single-cell imaging that could quantify the anti-migratory and anti-survival effects of drugs at the single-cell level for HTS of migratory cancer cells.[78] The microfluidic platform developed by Liu et al. could achieve concentration gradient dilution of nanomedicines and real-time response monitoring of tumor cells at the single-cell level, providing an automated platform for HTS of nanomedicines.[79] The FLECSplate technology has achieved high-throughput quantification of cell contractility at the single-cell level.[81] Moreover, FALCOscope was developed as a high-throughput platform integrating automated drug processing, fluorescence imaging, and single-cell analysis for drug screening in living cells.[80]

### HTS with single-cell sequencing technologies

Moreover, combining drug screening with single-cell sequencing helps to reveal deeper pharmacological information, including off-target effects and drug initiation mechanisms, which could in turn interpret the complex physiological responses induced by drugs in biological systems. The technical hurdles in applying single-cell transcriptome sequencing to high-throughput screening mainly include how to index the perturbation information into sequencing results while considering the cost.

In this regard, researchers have developed a single-cell transcriptomics-based high-throughput screening platform that allows for the simultaneous detection of single-cell RNA at low cost by transiently transfecting single-stranded oligonucleotide (SBO)-labeled tumor cells treated with different drugs to explore the explanation for the different response of tumor cells to drugs.[82] Haide et al. reported a high-throughput screening and single-cell sequencing platform that used labeled reverse transcription primers and TN5 transposase to pre-label the transcriptome or genome in tumor cells and captured cells using microtiter plates, followed by a second round of labeling cells using labeled magnetic beads after single-cell sequencing. Then, the transcriptome of individual cells was assembled by combining reads containing the same two barcode combinations and enabling more cost-effective single-cell high-throughput drug screening.[83] Furthermore, combination drug therapy is an effective strategy to reduce tumor resistance and recurrence, and single-cell technologies are advantageous for investigating combinatorial therapeutic regimens for precisely targeting tumor cells and the mechanism of combination drugs.[139,140] In a recent study, Xie et al. employed oligonucleotides to encode drugs, which were then encapsulated along with cells in separate droplets. These droplets were randomly paired on a microwell array chip to complete combinatorial drug treatment and barcode labeling. Subsequent single-cell RNA sequencing enabled simultaneous detection of the single-cell transcriptome and drug barcodes, thereby facilitating the examination of drug efficacy. This high-throughput approach holds significant promise for identifying efficacious drug combinations.[84]

In conclusion, the application of single-cell technologies in high-throughput drug screening has shown great potential to provide a more comprehensive understanding of drug mechanisms and enable the efficient discovery of new drug combinations in tumor treatment. With the development of innovative platforms and decreasing sequencing costs, single-cell transcriptome sequencing is becoming a more cost-effective and accessible option for high-throughput drug screening for tumor therapy. By combining the advantages of both high-throughput screening and single-cell technologies, researchers can accelerate the drug development process and ultimately bring more effective treatments to tumor patients.

## SINGLE-CELL ANALYSIS IN PHARMACOKINETICS EVALUATION

Verification of drug molecule-target interactions and investigation of drug absorption, excretion, distribution, and metabolism patterns in biological systems represent a major challenge in drug development following target identification. Traditional pharmacokinetic studies usually quantify the drug concentration or drug-target interactions in homogenized organs, and combining single-cell technologies in this regard could facilitate the precise identification of pharmacokinetic effects at single-cell resolution.[141] Advanced single-cell sequencing technologies are available to help characterize the physiological mechanisms of drug absorption and excretion organs. In a single-cell sequencing study of comprehensive cellular profiles of the intestinal epithelium for healthy adults, different levels of selected drug target genes and drug metabolizing enzymes between cell subpopulations were found to be associated with gastrointestinal side effects of the drug.[85,142]

Single-cell technologies combining spectroscopic or mass spectrometric imaging have enabled the analysis of drug distribution at the individual living cell or even subcellular level.[87,143,144] However, *in situ* imaging of small molecule drugs *in vivo* at high resolution remained challenging due to the effect of additional labeling on the chemical properties of exogenous small molecule drugs. The CATCH method

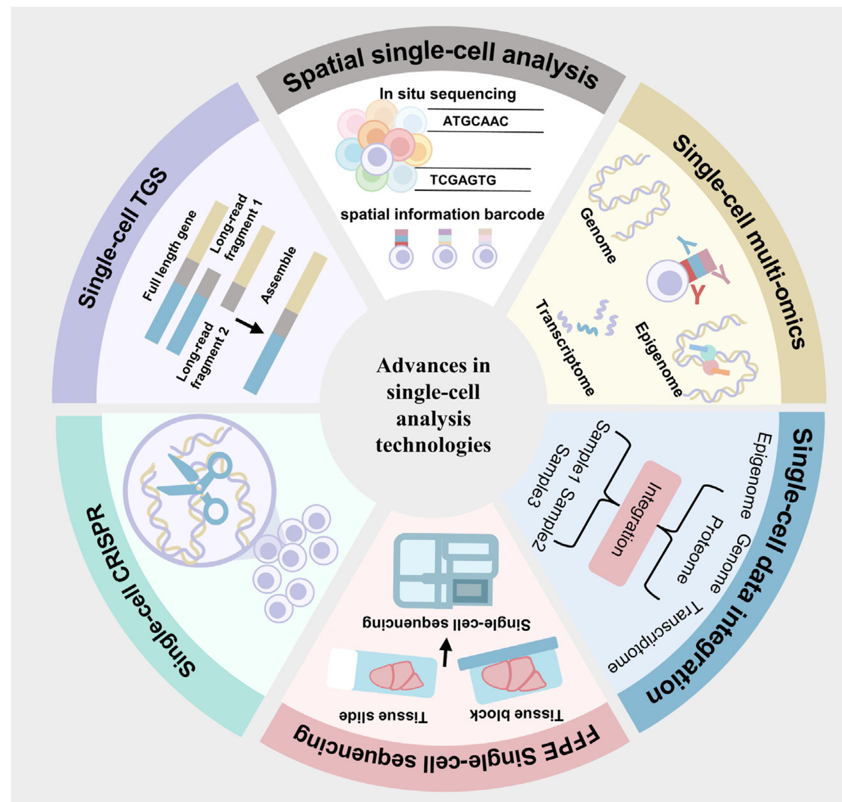


**Figure 2. Advances in single-cell analysis technologies for drug discovery and development**

(1) The emergence of single-cell technologies with spatial information, including *in situ* sequencing and spatial information barcode, have provided researchers with powerful tools to investigate the complex and heterogeneous nature of tumor development. (2) Single-cell multi-omics analysis allows for the simultaneous measurement of multiple types of biological information at the single-cell level, including epigenomics, genomics, transcriptomics, and proteomics. (3) Integration of single-cell data, including sequencing data from different technology platforms of the same samples and multi-model single-cell data from different samples, contributes to the expansion of sample size for single-cell analysis and enhances the availability of multi-dimensional single-cell analysis. (4) Single-cell sequencing technologies applicable to FFPE samples facilitate the utilization of clinical samples and promote the clinical application of single-cell technologies. (5) ScCRISPR provides insights into the effect of genes on single-cell phenotypes. (6) Single-cell TGS allows to obtain more intact genomic and transcriptomic information at single-cell level. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; FFPE, formalin-fixed and paraffin-embedded; TGS, third-generation sequencing.

developed by Pang et al. integrating click chemistry with tissue clearing allowed the observation of drug-target interactions at subcellular resolution *in vivo*, providing a platform for the *in vivo* assessment of small molecule drug pharmacokinetics.[86] A newly developed method for high-precision three-dimensional (3D) visualization of tumor structures based on a micro-optical slice tomography (MOST) system and a fluorescence MOST (fMOST) system can be used to explore the correlation between tumor structure and nanoparticle distribution at high resolution and help to assess the efficacy, distribution of nanoparticle drug delivery system (NDDS).[145]

Collectively, these advanced single-cell technologies hold the promise to further improve the comprehension of mechanisms related to drug absorption, distribution, metabolism, and excretion on cellular level and promote the optimization of drug structures for better therapeutic effects.

## ADVANCES IN SINGLE-CELL ANALYSIS TECHNOLOGIES

Emerging single-cell technologies in drug discovery and development focus on single-cell analysis preserving spatial information, single-cell multi-omics, single-cell data integration, FFPE single-cell sequencing, single-cell CRISPR, and single-cell third-generation sequencing (Figure 2). Table 3 summarized the characteristics and relevant cutting-edge questions of advanced single-cell technologies.

### Single-cell analysis with preservation of spatial information

In contrast to traditional single-cell sequencing methods, preservation of high-resolution spatial data allows the discovery of new cell types, cell interactions, and tissue structures in an unbiased manner, thereby facilitating the understanding of tumor structure and microenvironment and promoting the development of new drugs. Moreover, single-cell sequencing that preserves spatial information relies heavily on

*in situ* sequencing and spatial barcoding. *In situ* sequencing technologies, including Stereo-seq, could enable single-cell even subcellular localization of sequencing reads across the entire slide. Spatial barcoding allows the identification of cell origin by barcoding cells with spatial information prior to sequencing.[199] Meanwhile, some researchers have developed high-resolution tissue separation technologies to enable single-cell sequencing of specific spatial locations.[200,201]

In addition, traditional scRNA-seq and SpatialOmics can be integrated through deconvolution and mapping methods. Deconvolution refers to the identification of discrete cell subpopulations from a mixture of mRNA transcripts at each capture site based on single-cell data, whereas mapping method localizes each cell to a specific ecological niche or tissue region.[202]

Overall, the large-scale application of single-cell technologies integrating spatial information needs to be further investigated, and mapping spatially complete single-cell atlases of tumors is still expected. Further integration of metabolomics, proteomics, and epigenomics information can greatly complement the findings at transcript level, and the integration of three-dimensional and time-series information is expected to enable researchers to probe the biological features of tumor from a new perspective.

### Integrating multi-omics for multidimensional single-cell analysis

Integrating multi-omics single-cell resolution analysis will provide a platform for multi-level and all-encompassing understanding of tumor heterogeneity and dynamic progression. In recent studies, multi-omics sequencing at the single-cell level has become a reality. For example, the sci-L3-RNA/DNA method developed by Yin et al. could simultaneously detect single-cell DNA and RNA.[203] Furthermore, recent studies have developed various methods for simultaneous detection of genomic, transcriptomic, and epigenetic features at single-cell resolution.[204–206] The latest CUT&Tag technology has enabled high-throughput and efficient single-cell chromatin characterization.[207] More importantly, the integration of single-cell protein detection techniques, such as the analysis of single-cell secretory factors[205] and metabolome,[109] can significantly enhance the utilization of single-cell technology for studying cell interactions and drug response. Overall, although innovative technologies can incorporate multidimensional biology into the assay, the specificity and sensitivity of each dimension of detection still needs further improvement, and further technological advances will mainly focus on improving throughput, reducing costs, and simultaneously detecting more dimensions of information.[201,208] The application of multi-omics techniques to the continuous measurement of living cells is also expected based on the versatility and transient nature of cellular molecular features.[209,210]

### Single-cell data integration

Although multi-omics single-cell technologies have facilitated the simultaneous detection of multi-dimensional single-cell resolution information, single-dimensional data still constitute the vast majority of single-cell data. Consequently, new data integration methods are necessary to achieve the amalgamation of multi-dimensional information across different experiments. Furthermore, with the expeditious expansion in size and accessibility of single-cell datasets, new computational methods are required for normalization and joint analysis across samples.

Currently, several algorithms have been designed for integrating scRNA-seq data generated by different techniques to realize the amplification of sample sizes.[206,211] Furthermore, the integration and analysis of distinct single-cell dimensional data can facilitate comprehensive insights into the heterogeneity and regulatory mechanisms of tumor, thereby providing a theoretical basis for identifying drug intervention targets.[212]

The growing scale and accessibility of single-cell data necessitated more computational methods to satisfy the requirements for the integration of single-cell data in different contexts.[213] However, in this area, enhancing the stability and fidelity of integrated data, improving the efficiency of data processing and reducing hardware requirements, and preserving high-dimensional data for downstream biological analysis still remained formidable technical challenges. Furthermore, the accelerated development of artificial intelligence has led to a gradual increase of its impact in the field of single-cell biology computing, which is expected to introduce new solutions for single-cell data integration.

### FFPE samples

The extensive reliance of most high-throughput single-cell technologies on the cellular activity of specimens under examination curtailed their usage to samples other than fresh tissues. Therefore, single-cell technologies that are applicable for non-fresh samples, particularly FFPE samples, hold immense potential in elucidating tumor biological mechanisms and associated drug targets.

The technical obstacles in applying scRNA-seq in FFPE samples mostly comprise chemical cross-linking, extraction of intact cells or nuclei from damaged structures, severely degraded RNA, differences in FFPE sample preparation and storage conditions, and changes in gene expression of cells during fixation. Recently, FFPE single-cell sequencing platforms including Smart-3SEQ, snPATHO-seq, snRandom-seq, and Arc-well have been developed.[169,168] The integration of FFPE single-cell analysis with multiplexed antibody imaging, bulk RNA sequencing, and spatial transcriptome sequencing will provide a more comprehensive understanding for the heterogeneity of tumor in clinical samples.

### Single-cell CRISPR

Gene screening based on clustered regularly interspaced short palindromic repeats (CRISPR) has been proved to be a powerful approach for unbiased functional genomics studies.[214] Positive selection using CRISPR libraries allows detection of surviving cells under specific conditions and further elucidation of drug resistance mechanisms, whereas negative selection allows for the identification of drug targets by detecting

**Table 3. Characteristics and cutting-edge questions of new single-cell technologies in drug discovery**

| Technology types | Platforms | Characteristics | Year | Questions |
|---|---|---|---|---|
| Single-cell analysis with spatial information | Phenocycler[146] | Detecting RNA and protein based on Ab | 2018 | 1. Integrate three-dimensional and time-series information<br>2. Improve feasibility in FFPE samples<br>3. Improve resolution and throughput |
| | DBiT-seq[147] | Detecting RNA and protein based on NGS | 2020 | |
| | Stereo-seq[148] | Detecting RNA based on NGS | 2021 | |
| | ExSeq[149] | Detecting RNA based on NGS | 2021 | |
| | Cell DIVE[150] | Detecting protein based on Ab | 2021 | |
| | Seq-scope[151] | Detecting RNA based on NGS | 2021 | |
| | MOSAICA[152] | Detecting RNA based on mFISH | 2022 | |
| | MICS[153] | Detecting protein based on Ab | 2022 | |
| Single-cell multi-omics | Trio-seq[154] | Combining analyses of genome, epigenome and transcriptome | 2016 | 1. Improve throughput<br>2. Reduce cost<br>3. Incorporate more modalities in a single assay<br>4. Improve the sensitivity and specificity in each modality<br>5. Enable continuous measurement of living cells |
| | CITE-seq[155] | Combining surface proteins with transcriptome | 2017 | |
| | REAP-seq[156] | Combining surface proteins with transcriptome | 2017 | |
| | G&T-seq[157] | Combining genome with transcriptome | 2015 | |
| | DR-seq[158] | Combining genome with transcriptome | 2015 | |
| | scM&T-seq[159] | Combining DNA methylation with transcriptome | 2016 | |
| | scDam&T-seq[160] | Combining protein-DNA contacts with transcriptome | 2019 | |
| | T-ATAC-seq[161] | Combining open chromatin with TCR | 2018 | |
| | SNARE-seq[162] | Combining open chromatin with transcriptome | 2019 | |
| | scCAT-seq[163] | Combining open chromatin with transcriptome | 2019 | |
| FFPE single-cell sequencing | Pick-seq[164] | Detecting single-cell nuclear poly-A RNA | 2021 | 1. Avoid negative impact of the molecular cross-linking<br>2. Remove of excessive tissue debris<br>3. Maximize RNA protection and minimize degradation during FFPE sample preparation |
| | Smart-3SEQ[165] | Detecting single-cell nuclear poly-A RNA | 2019 | |
| | snFFPE-Seq[166] | Detecting single-cell nuclear poly-A RNA | 2022 | |
| | snPATHO-seq[167] | Detecting single-cell nuclear Target mRNA | 2022 | |
| | snRandom-seq[168] | Detecting single-cell nuclear total RNA | 2023 | |
| | Arc-well[169] | Detecting single-cell DNA | 2023 | |

(*Continued on next page*)

**Table 3.** ***Continued***

| Technology types | Platforms | Characteristics | Year | Questions |
|---|---|---|---|---|
| Single-cell CRISPR | Perturb-seq[170] | Combining CRISPRi/CRISPRko screening and detecting single-cell RNA | 2016 | 1. Improve feasibility of *in vivo* screening<br>2. Avoid negative impact of off-target effects<br>3. Develop scCRISPR with metabolomics readout<br>4. Add spatial dimension |
| | CROP-seq[171] | Combining CRISPRko screening and detecting single-cell RNA | 2017 | |
| | crisprQTL mapping[172] | Combining CRISPRi screening and detecting single-cell RNA | 2017 | |
| | SLICE[173] | Combining CRISPRko screening and detecting single-cell RNA | 2018 | |
| | *In vivo* Perturb-seq[174] | Combining CRISPRi screening and detecting single-cell RNA | 2020 | |
| | scCRISPRa screening[175] | Combining CRISPRa screening and detecting single-cell RNA | 2020 | |
| | STING-seq[176] | Combining CRISPRi screening and detecting single-cell RNA + protein | 2021 | |
| | CRISPRa Perturb-seq[177] | Combining CRISPRa screening and detecting single-cell RNA | 2022 | |
| | Perturb-ATAC[178] | Combining CRISPRi/CRISPRko screening and detecting single-cell DNA | 2019 | |
| | Spear-ATAC[179] | Combining CRISPRi/CRISPRko screening and detecting single-cell DNA | 2021 | |
| | CRISPR-sciATAC[180] | Combining CRISPRko screening and detecting single-cell DNA | 2021 | |
| | Pro-Codes[181] | Combining CRISPRko screening and detecting single-cell Protein | 2016 | |
| | Perturb-map[135] | Combining CRISPRko screening and detecting single-cell Protein+RNA+imaging | 2022 | |
| | ECCITE-seq[182] | Combining CRISPRko screening and detecting single-cell Protein+RNA | 2019 | |
| | Perturb-CITE-seq[183] | Combining CRISPRko screening and detecting single-cell Protein+RNA | 2021 | |
| | CaRPool-seq[184] | Combining CRISPR RNA knockdown screening and detecting single-cell Protein+RNA | 2022 | |
| | imaging-based CRISPR screen[185] | Combining CRISPRko screening and detecting single-cell imaging | 2019 | |
| | Optical Pooled Screen[186] | Combining CRISPRa/CRISPRko screening and detecting single-cell Imaging | 2019 | |
| | CRaft-ID[187] | Combining CRISPRko screening and detecting single-cell Imaging | 2020 | |
| | MIC-Drop[188] | Combining CRISPRko screening and detecting single-cell Imaging | 2021 | |
| | Raft-seq[189] | Combining CRISPRko screening and detecting single-cell Imaging | 2022 | |

(*Continued on next page*)

**Table 3. *Continued***

| Technology types | Platforms | Characteristics | Year | Questions |
|---|---|---|---|---|
| Single-cell TGS | Smart-seq2[190] | Single-cell full-length RNA sequencing method that amplifies full-length cDNA from individual cells | 2014 | 1. Improve throughput and accuracy<br>2. Improve sequencing depth<br>3. Reduce cost<br>4. Improve compatibility with commonly used scRNA-seq platforms |
| | ScISOr-seq[191] | UMI and TSO to capture full-length transcripts and identify barcodes for individual cells | 2018 | |
| | RAGE-seq[192] | Incorporates a 3′-adapter to capture the 3′-end of transcripts, which allows for full-length transcript reconstruction | 2019 | |
| | scCOLOR-seq[193] | Enables the correction of barcode and unique molecular identifier oligonucleotide sequences and permits standalone cDNA nanopore sequencing of single cells | 2021 | |
| | LR-Split-seq[194] | Combinatorial barcoding to sequence single cells with long reads and accurately assign them to their cellular origin | 2021 | |
| | scNanoATAC-seq[195] | Investigating the relationship between chromatin accessibility and genome structure combining single-cell ATAC-seq with Nanopore third-generation genome sequencing | 2023 | |
| | scNanoCOOL-seq[196] | Enables joint analysis of CNVs, DNA methylome, chromatin accessibility, and transcriptome in the same individual cell | 2023 | |
| | scNanoHi-C[197] | Explore genome-wide proximal high-order chromatin contacts within individual cells | 2023 | |
| | scGTP-seq[198] | Single-cell parallel genome and transcriptome sequencing | 2023 | |

Ab, antibody; ATAC-seq, assay for transposase-accessible chromatin sequencing; CNVs, copy-number variations; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRko, CRISPR knockout; CRISPRi, CRISPR interference; CRISPRa, CRISPR activation; NGS, next-generation sequencing; TCR, T cell receptor.

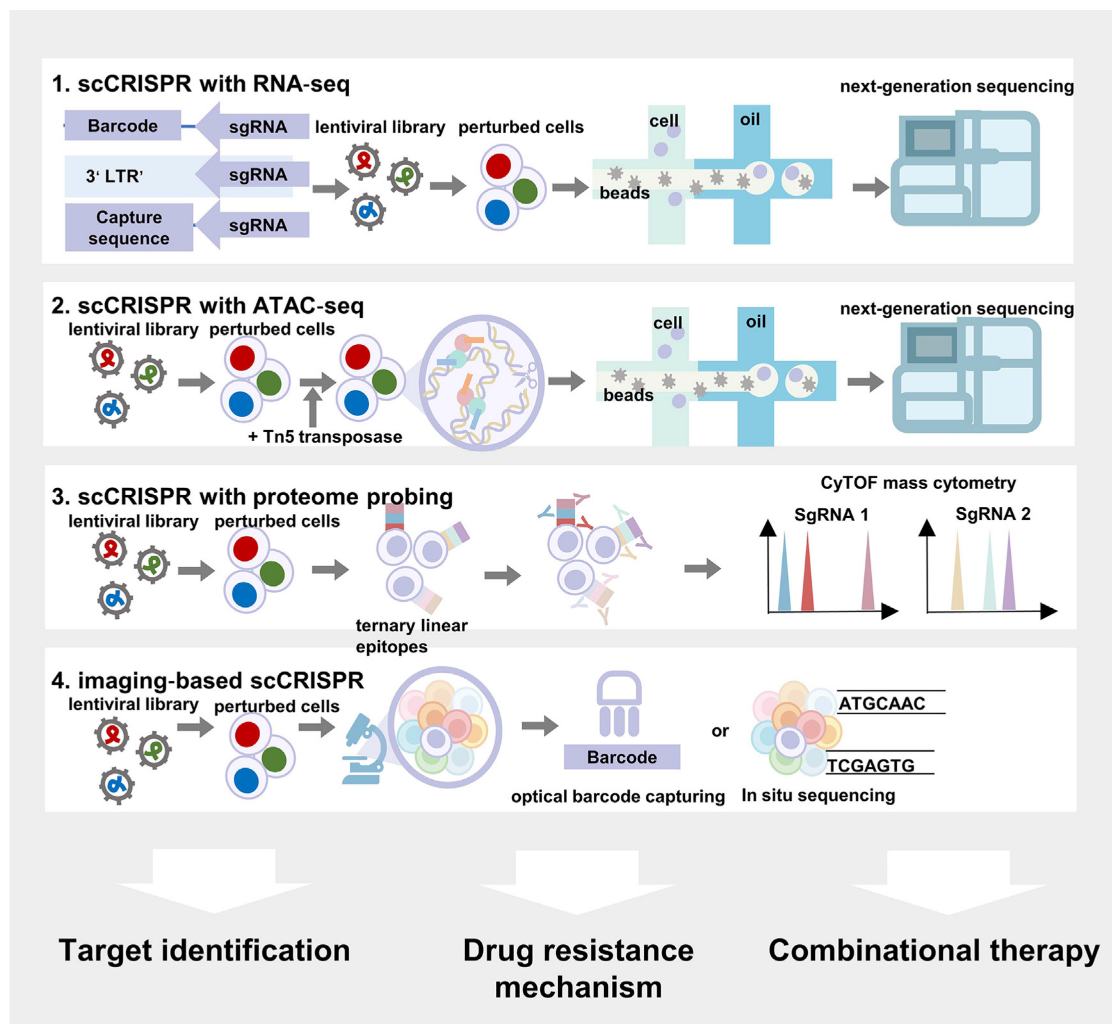


**Figure 3. Applications of scCRISPR in drug discovery for tumor treatment**

Based on the integrated single-cell technologies, scCRISPR can be categorized into scCRISPR with RNA-seq, scCRISPR with ATAC-seq, scCRISPR with proteome probing and imaged-based scCRISPR. (1) ScCRISPR with RNA-seq. Take Perturb-seq for example: tumor cells are transduced with lentiviral library in which lentivirus carries sgRNA with specific barcode, polyadenylated region, or capture sequence. Perturbed tumor cells are eventually used for microfluidic-based scRNA-seq. (2) ScCRISPR with ATAC-seq. Take Spear-ATAC for example: the perturbed tumor cells are used for scATAC-seq after nuclear isolation and transposition. (3) scCRISPR with proteome probing. Take Pro-Codes for example: the perturbed tumor cells are used for mass cytometry after being tagged by linear epitope combinations. (4) Imaging-based scCRISPR. The perturbed tumor cells are used for single-cell imaging after capturing optical barcodes or *in situ* sequencing. scCRISPR may facilitate drug target identification, drug resistance mechanism research, and the discovery of combinational therapy strategies, thereby promoting anti-tumor drug discovery and development. ATAC-seq, assay for transposase-accessible chromatin sequencing; RNA-seq, RNA sequencing; scCRISPR, single-cell clustered regularly interspaced short palindromic repeats.

dead cells and recognizing essential survival genes.[215] In addition, CRISPR screening can be applied to the study of synthetic lethal mechanisms, which are critical for determining the optimal combination of targeted drugs. However, pooled CRISPR screening is limited to low content readout and cannot satisfy the needs of complex mechanism studies.[216]

Different from traditional CRISPR screening, scCRISPR enables multi-omics analysis with single-cell resolution and provides in-depth comprehension of regulatory mechanisms in tumor biology, which is technically accomplished by annotating cells with single guide RNA (sgRNA)-specific barcodes or directly detectable sgRNA.[217] Based on the integrated single-cell technologies, scCRISPR can be categorized into scCRISPR with RNA-seq, scCRISPR with ATAC-seq, scCRISPR with proteomics, and imaged-based scCRISPR (Figure 3). Up to now, scCRISPR has been used to investigate critical complex regulating anti-tumor T cell function, mechanisms of drug resistance in tumor, oncogene interactions, and immune checkpoint regulatory network.[218] The most recent cutting-edge research focuses on exploring the application of *in vivo* scCRISPR in tumor biology research. Through constructing mouse models using tumor or immune cells after transferring CRISPR library and cell barcode, gene functions that affect tumor morphology, histological features, and immune cell recruitment can be further

revealed.[219] Meanwhile, CRISPR technologies that integrate non-invasive single-cell transcriptomics and use single-cell metabolomics readout may open new avenues for research in tumor biology.

### Single-cell third-generation sequencing

ScRNA-seq has revealed gene expression levels in individual cells at an unprecedented resolution, providing valuable insights into cellular state and functional regulation of tumor. However, current scRNA-seq is mainly based on detecting read counts of 3′ or 5′ ends in polyadenylated transcripts, which fails to provide sufficient coverage of mRNA splicing.[220] TGS, also known as long-read sequencing, is a real-time molecular sequencing technology that has overcome this limitation through detecting full-length of cDNA and RNA. Compared to NGS technologies, TGS has the capability to identify complex DNA structural variants, whole transcript selective splicing events, and cell-type-specific mRNA isoform expression.[221] Some of the TGS technologies, such as single-molecule real-time (SMRT) sequencing and nanopore sequencing, have been utilized in scRNA-seq analysis to provide further insights into selective splicing regulation, transcriptome complexity, and isoform diversity of tumor cells at the single-cell level.[222]

In breast cancer, the single-cell TGS technology RAGE-Seq was used to reveal complete antigen-receptor sequences with high accuracy and sensitivity for inferring the clonal evolution of tumor-associated B cells.[192] In another study, simultaneous single-cell genomic and transcriptomic detection was performed on HCC samples based on the scGTP-seq platform to identify tumor-cell-associated structural variants and extrachromosomal DNA.[198] In patients with acute myeloid leukemia, researchers utilized the newly developed single-cell TGS technology, Nanoranger, to improve the resolution of leukemia and immune cell phenotypes.[223] Another recently developed technology has integrated CRISPR and single-cell TGS to perform functional characterization of genes.[224] However, one of the challenges in single-cell TGS analysis is that lower sequencing depth decreases its ability to accurately quantify the expression level of isoforms, which leads to data sparsity and negatively affects the accuracy of results.[221] Although advanced computational tools, such as scNanoGPS, have been developed to optimize the accuracy of single-cell nanopore TGS results and overcome the dependence on short-read sequencing results,[225] it remains necessary for more research to focus on addressing common errors in single-cell TGS.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Collectively, single-cell technologies have greatly expanded our understanding of the complexity and heterogeneity of tumor tissue and provided new insights into the mechanisms of tumor evolution, metastasis, drug resistance, and microenvironment regulation. The application of single-cell technologies has greatly contributed to drug discovery for tumor treatment, thus promoting the development of more effective treatment strategies for tumor patients. During the identification of new therapeutic targets, single-cell technologies have provided further insights into the molecular features of specific clonal subpopulations, CTCs, and regulatory network in TME. Single-cell technologies have also enriched the readout of HTS and facilitated the detection of pharmacokinetics at the single-cell level. However, their application in drug development still has numerous limitations, including the sensitivity to sample quality, the limited cell throughput, and the high cost. Additionally, a unidimensional single-cell analysis is not sufficient to define exact cell subtypes or lineages due to the dynamic nature of gene expression and cell transformation in tumor development. Future research should focus on integrating multi-omics data and spatial information to comprehensively understand tumor biology and develop new treatment strategies.

The application of single-cell technologies in drug development has compelling clinical significance. By identifying the heterogeneity and drug resistance mechanisms of tumor cells, single-cell technologies can promote the development of more effective therapies targeting specific cell populations or pathways. High-throughput drug screening at the single-cell resolution can improve the efficiency of drug development by shortening the development cycle and reducing development costs. Using single-cell technologies for pharmacokinetics research can help optimize lead compounds and increase the success rate of drug development. Additionally, applying single-cell technologies in the regular monitoring of patients in clinical trial, including identifying drug resistance mechanisms, analyzing clone evolution, and screening biomarkers for treatment response, has the potential to further increase the efficiency of drug development and improve the survival of tumor patients.

In conclusion, single-cell technologies are powerful tools that fundamentally change our understanding of tumor biology and have enormous potential in drug development for tumor treatment. The continuous advancement of single-cell technologies and their integration with other high-throughput methods will pave the way to further insights into tumor biology at single-cell level and the development of relevant drugs.

### Limitations of the study

The application of single-cell technologies in drug discovery and development has several limitations, including the sensitivity to sample quality, the limited cell throughput, and the high cost. Moreover, a unidimensional single-cell analysis is not sufficient to define exact cell subtypes or lineages due to the dynamic nature of gene expression and cell transformation in tumor development. Future research is required on integrating multi-omics data and spatial information to comprehensively understand tumor biology and develop new therapeutic strategies.

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## AUTHOR CONTRIBUTIONS

B.L. wrote and edited this manuscript and created figures. S.H. and X.W. reviewed and revised the manuscript. X.W. provided direction and guidance throughout the preparation of the manuscript. All authors read and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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