



Review Article

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Exploring single-cell and multi-omics technologies and their role in unraveling tumor heterogeneity of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Tumor heterogeneity is a major obstacle to effective treatment and is poorly understood using traditional bulk sequencing methods. This review highlights the transformative role of single-cell and multi-omics technologies in determining the cellular and molecular complexities of HCC. We summarize recent advances in single-cell transcriptomics, epigenomics, multi-omics, and spatial transcriptomics platforms, emphasizing their applications in characterizing tumor subclones, cancer-associated fibroblast-immune interactions, circulating tumor cells, and immune-resistant phenotypes. Spatial approaches have revealed the architecture of cancer stem cell niches and tertiary lymphoid structures, providing unprecedented insights into tumor organization and microenvironmental crosstalk. Although still in their early stages, clinical trials have begun to incorporate these technologies, underscoring their translational potential. Single-cell and spatial omics have reshaped HCC research by enabling high-resolution profiling of tumor ecosystems and driving the discovery of biomarkers, therapeutic targets, and strategies for patient stratification. However, high cost, technical expertise, and limited accessibility, particularly in resource-constrained settings, are major barriers to its widespread adoption. Addressing these challenges is critical for translating these powerful approaches into clinical practice and for advancing precision medicine for the treatment of liver cancer. (*J Liver Cancer* 2026;26:104-123)

Keywords: Carcinoma, hepatocellular; Single-cell gene expression analysis; Spatial transcriptomics; Genetic heterogeneity; Neoplasms

INTRODUCTION

Primary liver cancer (PLC) includes a diverse group of malignant tumors such as hepatocellular carcinoma (HCC), cholangiocarcinoma, and combined hepatocellular-intrahepatic cholangiocarcinoma. All of these tumors have distinct histopathological and molecular profiles. Among these, HCC alone, which originates from the malignant transformation of hepatocytes, represents 80-90% of PLC cases.¹ The pathogenesis involves an interaction between environmental and genetic factors. Several factors contribute to the development of HCC, such as hepatitis C virus/hepatitis B virus (HCV/HBV) infection, alcohol abuse, non-alcoholic fatty liver disease, and contact with environmental carcinogens

(e.g., aflatoxin B1). Less common factors include α 1-antitrypsin deficiency, hepatolenticular degeneration or Wilson's disease, erythropoietic protoporphyria, and autoimmune hepatitis.² The precise molecular mechanisms of HCC are complicated and heterogeneous, impeding clinical diagnosis and treatment. It poses a significant health threat to patients because of its high relapse rate and poor response to current therapies. For instance, treatment with tyrosine kinase inhibitors (e.g., lenvatinib and sorafenib) or immune checkpoint inhibitors (e.g., atezolizumab combined with bevacizumab) offers limited clinical benefits.³ The presence of intertumoral and intratumoral heterogeneity is an important factor that contributes to its limited efficacy. This heterogeneity is driven by complex interactions between cancer cells

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and the surrounding tumor microenvironment (TME), comprising stromal, immune, mesenchymal, and endothelial cells, leading to the emergence of various HCC sub-clusters that remodel the tumor ecosystem to favor HCC growth by manipulating neighboring cells (Fig. 1).⁴ Although several studies have shown that liver cancer comprises a variety of cells, the effect of the differential nature of cells on the clinical outcome of HCC remains unclear.^{5,6} Therefore, an in-depth or detailed characterization of the tumor ecosystem involved in HCC initiation, progression, and therapeutic resistance is essential for developing more effective diagnostic and treatment strategies.

Conventional sequencing methods, such as whole-exome sequencing, whole-transcriptome sequencing, and epigenomic profiling, have limited efficacy in understanding the complexity of tumor heterogeneity because they rely on bulk tissue analysis,

which has the limitation of identifying signals from rare cell populations. In contrast, single-cell sequencing is a powerful technique that can overcome this challenge by permitting detailed analysis of the interactions between tumor cells and various cell types within the TME. It also provides valuable information regarding the functional dynamics and composition of the tumor ecosystem. Unimodal approaches to single-cell sequencing study single cells at the genome, transcriptome, epigenome, or proteome level independently. In contrast, single-cell multi-omics integrates multiple unimodal datasets, offering a comprehensive view of the complex relationships between genotypes and phenotypes in patients with HCC. Recent technological advancements in single-cell and spatial omics have provided a more refined and high-resolution understanding of tumor biology, offering new opportunities for precision oncology. For instance, a novel spatial

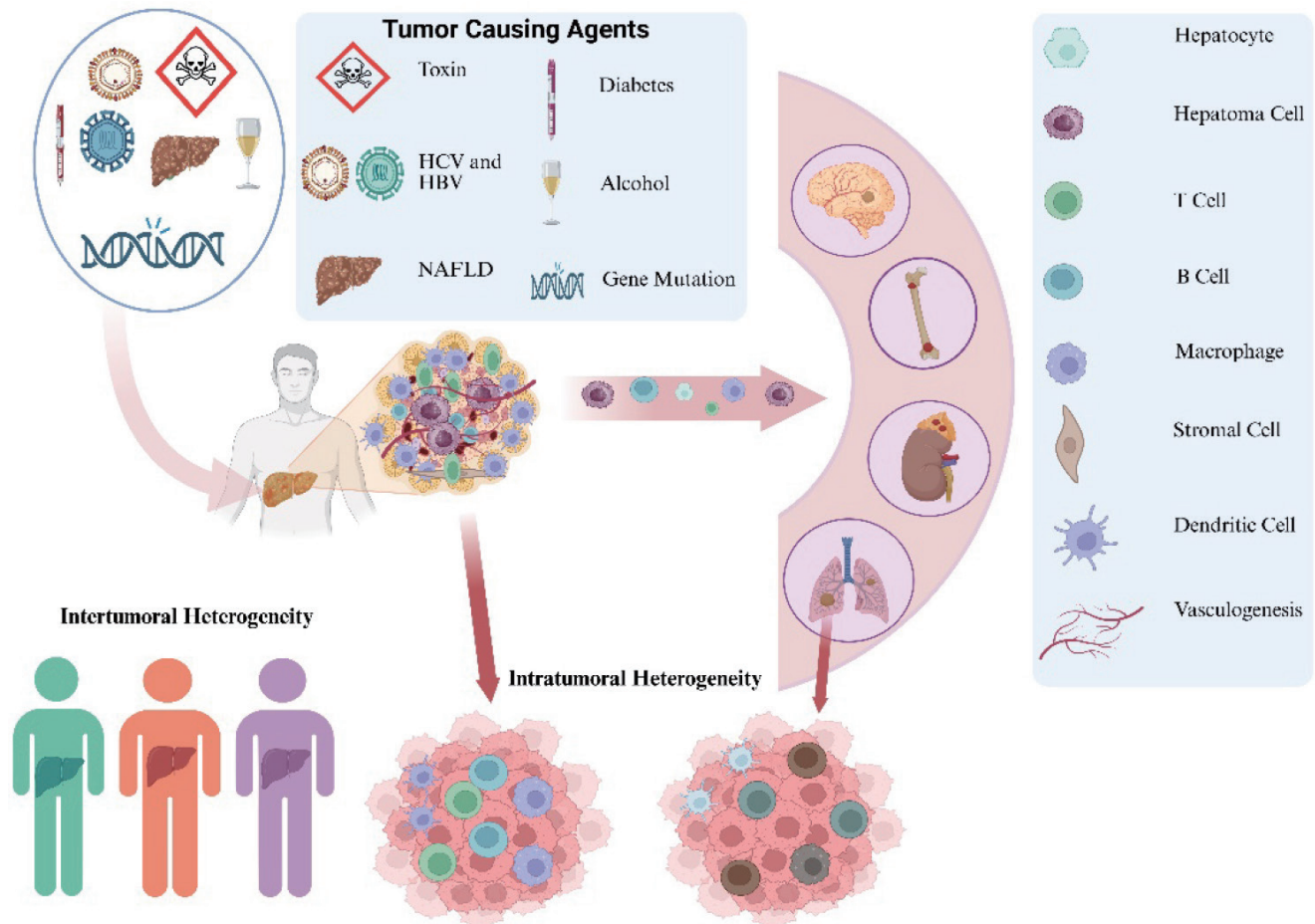


Figure 1. Tumor-causing agents and the ecosystem of primary liver cancer. Multiple tumor-causing agents, including toxins, diabetes, hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, non-alcoholic fatty liver disease (NAFLD), and gene mutations, contribute to hepatic carcinogenesis. These factors induce genetic and cellular alterations in hepatocytes, resulting in intertumoral heterogeneity (variations between patients) and intratumoral heterogeneity (variations within the same tumor). The heterogeneous tumor microenvironment consists of different cell types, including hepatocytes, hepatoma cells, T cells, B cells, macrophages, stromal cells, dendritic cells, and vasculature. The diagram also illustrates possible sites of metastasis (brain, bone, lung, and kidney).

transcriptome platform, Stereo-seq, was used to identify a 500- μm -wide invasive zone centered around the tumor border in patients with HCC.⁷ The same method was used to assess heterogeneity of cancer-associated fibroblasts (CAFs) and identified two subtypes: CAF-fibroblast activation protein (FAP) and CAF-C7. The enrichment of these subtypes strongly correlated with worse outcomes.⁸ These studies suggest that spatial omics, when integrated with single-cell approaches, can help identify the real cause or resistance behavior of a disease. This may accelerate the discovery of effective treatments for this complex disease.

In this review, we provide an overview of the main single-cell sequencing techniques and summarize the impact of these technologies on understanding the heterogeneous behavior of liver cancer cells and the immune cell microenvironment.

SINGLE-CELL OMICS TECHNOLOGIES AND HCC

In contrast to conventional sequencing, single-cell sequencing has dramatically improved accuracy and efficiency. This technique utilizes several methods, including single-cell DNA sequencing (scDNA-seq) for DNA, single-cell RNA sequencing (scRNA-seq) for RNA, cytometry by time of flight (CyTOF) for cell surface proteins, and single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq) for chromatin accessibility. A single cell contains DNA or RNA in picograms. Therefore, whole-genome amplification (WGA) is required to obtain sufficient nucleic acid material. WGA has been previously performed using degenerate oligonucleotide-primed polymerase chain reaction (PCR), interspersed repetitive sequence PCR,

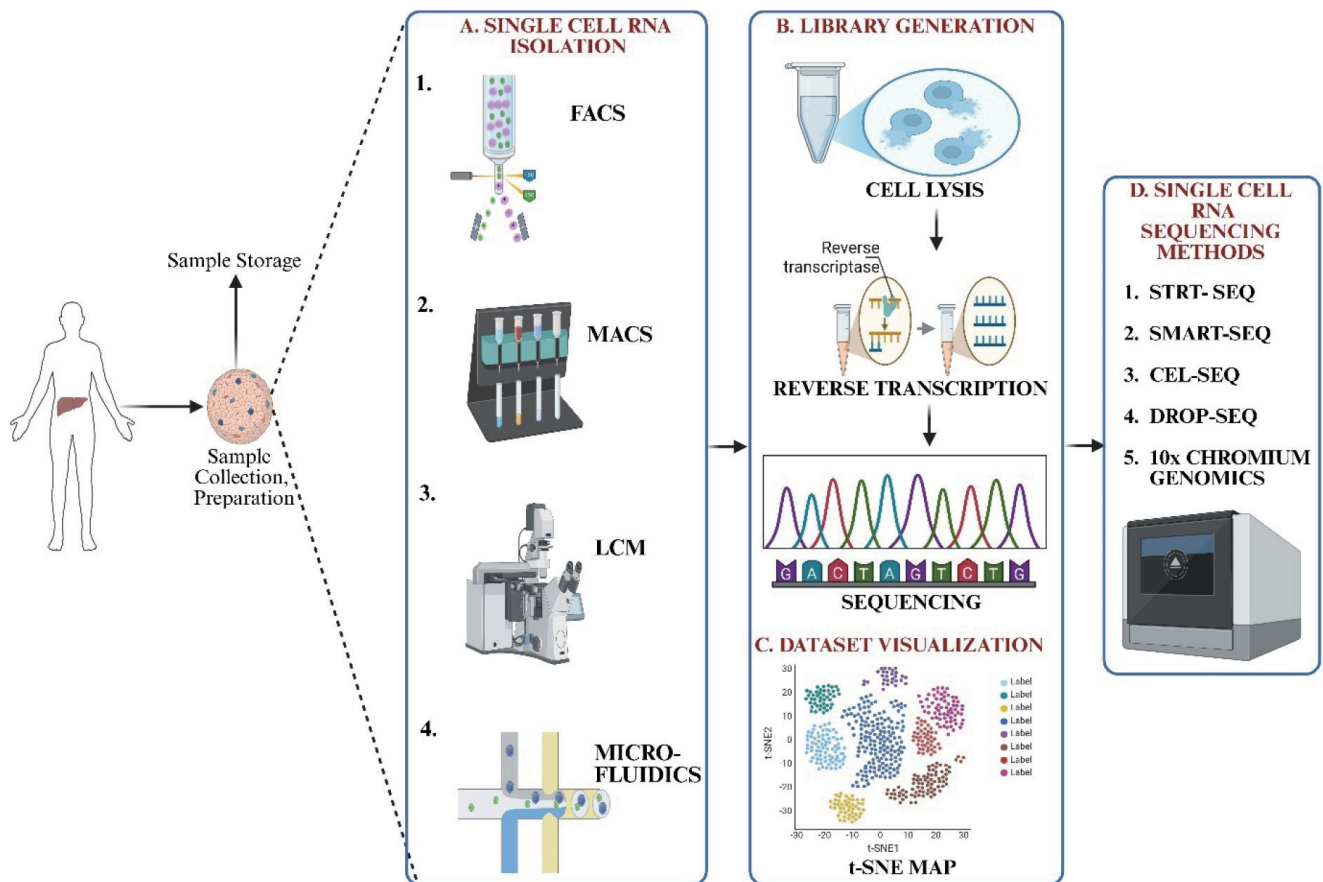


Figure 2. Workflow for single-cell RNA (scRNA) isolation, sequencing, and data analysis. The schematic illustrates the entire procedure of scRNA sequencing, where the sample is collected and stored before it can be isolated using various methods, such as fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), laser capture microdissection (LCM), and microfluidic-based separation. Following isolation, single cells undergo lysis, reverse transcription, and library preparation for sequencing. Various sequencing platforms are used in the sequencing, including single-cell tagged reverse transcription sequencing (STRT-seq), switching mechanism at the 5' end of RNA template sequencing (SMART-seq), cell expression by linear amplification and sequencing (CEL-seq), droplet-based sequencing (Drop-seq), or 10x Chromium Genomics. Dimensionality-reduction algorithms, such as t-distributed stochastic neighbor embedding (t-SNE), are used to visualize the resulting transcriptomic data.

primer extension pre-amplification PCR, and linker-adaptor PCR. These WGA techniques have been replaced in modern scDNA-seq with multiple displacement amplification (MDA), multiple annealing and looping-based amplification cycles (MALBAC), and linear amplification via transposon insertion (LIANTI). In MDA, high-fidelity Phi29 polymerase is used for genome amplification to achieve high coverage of genes and low error rates compared with PCR. In MALBAC, primers have a 27 bp common sequence at the 5'-end and an 8 bp random sequence at the 3'-end, which enables copying of genomic DNA. After the first round of amplification, the amplified product contains complementary sequences at both 5'- and 3'-ends and thus forms a self-loop. This loop prevents further amplification of this strand, thereby preventing amplification bias and increasing genomic coverage.⁹ In LIANTI, transposases (such as Tn5) are used to insert primers or adaptors into genomic DNA at many sites. This process is called tagmentation. Only one primer is used to perform linear amplification. Because no reverse primer is used, each strand is extended once per cycle, thereby preventing exponential doubling. Similarly, the initial step in an scRNA-seq experiment is the isolation of single cells, which can be performed using several methods such as fluorescence-activated cell sorting, chip-based or microdroplet-based microfluidic technologies, or laser microdissection.¹⁰ Following this, scRNA-seq libraries are generated by cell lysis, reverse transcription, second-strand synthesis of complementary DNA (cDNA), and amplification, followed by deep sequencing.

scRNA-seq datasets are usually visualized using a *t*-distributed stochastic neighbor embedding (*t*-SNE) map. In this map, cells are clustered based on their transcriptome similarity, and single or multiple genes can be visualized separately on individual *t*-SNE maps.¹⁰ Several methods are used for scRNA-seq, such as STRT-seq, SMART-seq, CEL-seq, DROP-seq, and 10X Chromium Genomics (Fig. 2). However, these techniques differ in terms of the specific steps and workflows involved in scRNA-seq. These scRNA-seq methods have various limitations; therefore, researchers choose platforms that are best suited to their study objectives. For example, the SMART-seq platform has been used to reveal a high-resolution dynamic immune landscape in HCC. In this study, we demonstrated that LAMP3⁺ dendritic cells (DCs) express diverse immune-relevant ligands. These DCs can migrate from tumors to the hepatic lymph nodes and regulate lymphocytes. The study also characterized tumor-associated macrophage states linked to poor prognosis, providing one of the earliest comprehensive single-cell maps of the HCC immune microenvironment.¹¹ In another study, the SMART-seq2 sequencing method

was used on heterogeneous subclones in HCC, including five HCC and two hepatocyte subclones. The study found that MLX-interacting proteins-like (MLXIPL) were commonly upregulated in HCC and associated with poor survival. These results identified that MLXIPL in HCC is a promising therapeutic target.¹² Similarly, different regions of HCC and intrahepatic cholangiocarcinoma were analyzed using droplet-based 5'-scRNA-seq to determine the spatial distribution of tumor cells and the TME. This study observed that the diversity between HCC and intrahepatic cholangiocarcinoma was higher than that within the tumor, regardless of tumor size and the location of sampling tissues.¹³ While scRNA-seq reveals the transcriptional heterogeneity of tumor and stromal cells in HCC, single-cell epigenomic approaches are critical for understanding the regulatory mechanisms that drive these expression patterns. For epigenomes, several methods are used, including RRBS, WGBS, CGI-seq, ATAC-seq, and CHIP-seq. The mechanisms, advantages, and disadvantages of these scRNA-seq and single-cell epigenomic methods are described in Table 1^{9,14-22} and Table 2,^{9,23-29} respectively.

The most commonly used tools for scRNA-seq analysis include the R package Seurat, Python package Scanpy, SeuratDisk for interoperability between Seurat and Scanpy, scDIOR for rapidly transforming data between R and Python, and CellBridge.³⁰ In addition to these tools, several other tools are also used, including FastQC, CellRanger, Trimmomatic, STAR for preprocessing, doubletFinder, Scrublet for quality control, Seurat, GiniClust for feature selection, *t*-SNE and uniform manifold approximation and projection (UMAP) for dimensionality reduction and visualization, and CellAssign for annotation.³¹ EpiScanpy is a versatile toolkit used for scDNA methylation and scATAC-seq data.³²

MULTI-OMICS AND HCC

Unimodal methods of single-cell sequencing are combined into multi-omics approaches that enable the acquisition of multiple types of information from the same single cell simultaneously. Multi-omics approaches comprise several methods.

CITE-seq

This method is used to study the transcriptome and surface proteins. In this technique, oligo-tagged antibodies with poly(A) tails are used; therefore, in contrast to flow cytometry, there is no upper limit to the number of antibodies that can be used to identify cell surface proteins. In addition, cellular indexing of tran-

Table 1. Comparative summary of scRNA-seq techniques and their applications in single-cell and bulk analysis

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages	Applications
Single-cell tagged reverse transcription (STRT-seq) ²⁰	In this oligo dT primers containing barcode and primer binding sequence is used for the reverse transcription. Consequently, moloney murine leukemia virus reverse transcriptase (MMLV RT) introduces the barcode sequence at the 5'-end of the synthesized cDNA. MMLV RT also adds common sequence at the 5'-end of c-DNA. Sequencing reads are then generated specifically from 5'-end-tagged region. This allows for accurate quantification of gene expression based on transcription start sites	STRTprep pipeline ⁷² This is followed for single cell downstream analysis on Seurat	Early barcoding supports multiplexing Exact location of the 5'-end of transcripts Low RNA input needed ¹⁴	Complex protocol Low sensitivity It only sequences a short region near the 5'-end of each transcript cDNA synthesis starts at the 3'-end of RNA using an oligo dT primer, which can lead to inefficient capture of degraded mRNAs or exclude transcripts without intact poly(A) tails	Especially useful for transcript counting and identifying TSS
Switching mechanism at the 5'-end of RNA template sequencing (SMART-seq2) ²¹	In this MMLV RT adds few nucleotides at 3'-end of cDNA due to the terminal transferase activity of MMLV RT. In this TSO containing LNA is used to enhance the stability and efficiency of reverse transcription. Additionally, betaine is used to reduce secondary structure of transcript. This strategy enables capturing of the 5' cap-proximal region, preserving full-length information	ScPipe ⁷³ nf-core/scrnaseq ⁷⁴	Full-length coverage across transcripts Detects low-abundance transcripts Require as little as 50 pg RNA Use of LNA-modified TSO, optimized oligo dT, and betaine improves reverse transcription efficiency	Barcoding is not done and therefore, high throughput multiplexing is not favoured Expensive Labor intensive Lack of strand specificity Unable to detect nonpolyadenylated (poly(A)) RNA	Suitable for isoforms, splicing, low expression genes
Cell expression level RNA sequencing (CEL-seq) ¹⁷	In this method primers containing oligo dT, barcode, an Illumina 5' sequencing adaptor, and a T7 promoter are used for RT. Briefly, RNA is isolated after cell lysis, and then converted to cDNA with CEL-seq-primer. Once barcoded, the cDNAs from multiple cells are combined into one tube. After completion of second-strand cDNA synthesis, several samples are combined and subjected to IVT reactions	zUMIs ⁷⁵	Minimize amplification bias because the technique uses IVT and not PCR Early barcoding allows pooling of samples and thus reduces batch effects, and cost Highly sensitive and capable of detecting a large number of genes per cell, even at low input RNA levels High reproducibility	Captures just the 3'-ends of mRNA transcripts Multiple enzymatic steps are used and thus it is more technically demanding and time-consuming Usually done in plate formats and thus lower throughput	Suitable for balance throughput and sensitivity

Table 1. Continued

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages	Applications
Indexed droplet sequencing (InDrop) ^{9,19}	Briefly, in this method, individual cells are encapsulated into nanoliter-sized droplets containing lysis buffer, RT mix, and primers composed of poly(dT) sequences, UMIs, cell barcodes, sequencing adaptors, T7 RNA polymerase promoters, and photocleavable spacers. Following photocleavage, primers are released, enabling cDNA synthesis and the incorporation of cell-specific barcodes during reverse transcription. After generating the second cDNA strand, IVT is conducted to amplify the material. The droplets are subsequently disrupted, and the amplified RNA is fragmented via zinc-ion-mediated cleavage. These RNA fragments are then reverse-transcribed to produce a cDNA library suitable for next-generation sequencing. Finally, the cDNA libraries are sequenced on Illumina sequencing platforms	ZUMIs ⁷⁵	High throughput, low cost The method can index over 15,000 cells per hour; demonstrates minimal technical variability, and is highly adaptable for integration with other sequencing-based platforms Scalable and automated	Detects fewer transcripts per cell Captures mainly the 3'-end of mRNAs Requires microfluidic devices and specialized expertise Doublet's formation Barcodes collision or uneven capture	
Geographical position sequencing (GEO-seq) ^{15,18}	It combines LCM with SMART-seq2-based RNA-seq. In this cell are precisely isolated from tissues on the basis of their location. Following that RNA is extracted and reverse transcribed. Full length cDNA is amplified like SMART-seq2	Standard scRNA-seq pipelines ^{76,77}	Avoids biases introduced by enzymatic dissociation or cell sorting, preserving native cell states High-efficiency, high-resolution strategy for spatial transcriptome analysis Wide application potentials such as prospective cell fates, biological functions and gene regulatory networks	Requires high-quality tissue sections Laser capture microdissection is time-consuming and labour-intensive Requires specialized equipment (LCM system) and skilled operators, increasing cost and technical barriers	Study spatial heterogeneity
Multiple annealing and tailing-based quantitative scRNA-seq (MATQ) ^{18,22}	In this method instead of oligo dT, multiple random primers are used that anneal across RNA. Poly(C) tail is added at the 3'-end of first strand of cDNA by terminal deoxynucleotidyl transferase. Similar to SMART-seq, reverse transcriptase adds an Illumina Truseq adaptor to the 5'-end. Because of the random priming and optimized reverse transcription, MATQ-seq captures full-length transcripts, including non-coding and non-polyadenylated RNA	Standard full-length scRNA-seq pipelines ^{76,77}	High sensitivity Captures both poly(A) and non-poly(A) RNAs	Random priming can lead to non-specific amplification Complex library preparation No built-in barcoding Lower throughput	Capture non-coding and mitochondrial RNA

Table 1. Continued

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages	Applications
10x Chromium Genomics ^{9,16,18}	In this method, microfluidic chip is used to combine single cell, barcoded beads having millions of oligonucleotides, enzymes and reagents for RT. RT is initiated inside each droplet. Following that barcodes and UMIs are incorporated. Following this, the GEMs are disrupted to release the barcoded cDNAs, which are then pooled for subsequent amplification and library construction	Cell Ranger ^{78,79} Seurat ^{78,80}	High throughput Automated and reproducible Low technical noise Time saving Supports immune profiling, chromatin accessibility, spatial transcriptomics	Captures only a fraction (to 10-20%) of total mRNA in a cell High cost Requires high-quality single-cell suspensions Doublets or multiplets Only captures the 3'- or 5'-end of transcripts	Analyse thousands of cells

scRNA-seq, single-cell RNA sequencing; dT, deoxythymidine; cDNA, complementary DNA; STRTprep, Single-cell tagged reverse transcription-preparation; TSS, transcription start sites; TSO, template switching oligonucleotide; LNA, locked nucleic acid; IVT, *in vitro* transcription; PCR, polymerase chain reaction; RT, reverse transcription; UMIs, unique molecular identifiers; LCM, laser capture microdissection; GEMs, gel bead-in-emulsions.

scriptomes and epitopes by sequencing (CITE-seq) can be adapted for RNA interference, CRISPR, and other gene-editing techniques. Bioinformatics tools such as the CITE-seq Python package, Cell Ranger,³³ Seurat, total VI, CiteFuse, and LinQ-View provide frameworks for CITE-seq data analysis, including dimensionality reduction, cell clustering, cell type identification, and differential expression of RNA and cell surface proteins.³⁴ The data can also be analyzed using SeqGeq or FlowJo software (Ashland, OR, USA).³⁵ However, CITE-seq has several limitations, such as low throughput, poor resolution, no detection of intracellular proteins, loss of information about the location of cells, availability of antibodies, and optimization of the assay.³⁴

This method was recently used to identify six liver cancer stem cell markers (*CD44*, *EPCAM*, *CD133*, *CD24*, *CD90*, and *CD54*) in five HCC cell lines. These markers characterize the epithelial or mesenchymal state of these cells. The study also identified a robust gene panel representing the hypoxia signature in specific high-yield glycolysis clusters and found a correlation with HCC prognosis.³⁶

Techniques enabling the parallel analysis of genomic DNA and polyadenylated RNA within individual cells

DR-seq

In this preamplification, genomic DNA (gDNA) and RNA are amplified simultaneously to minimize nucleic acid loss. Following this, the reaction is divided into two tubes to complete the gDNA and RNA sequencing libraries separately.³⁷ However, the analysis and interpretation of such data are complicated by the presence of contaminating RNA sequences within the gDNA sequencing dataset.

scONE-seq

In this technique, gDNA and RNA are differentially barcoded; therefore, sequencing of RNA and DNA is possible in a single reaction. It is particularly useful for frozen samples because DNA and RNA are not physically separated.³⁸ However, re-sequencing of genomic material at greater depths is not possible, and the method lacks flexibility for selecting subsets of gDNA or RNA for sequencing.

G&T-seq and DNTR-seq

In these methods, RNA and gDNA are physically separated; therefore, these methods are flexible and scalable.³⁹ However, gDNA sequencing in genome and transcriptome sequencing (G&T-seq) necessitates WGA, making it expensive and time-

Table 2. Comparative summary of epigenomic sequencing techniques in single-cell and bulk liver cancer studies

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages
Reduced representation bisulfite sequencing (RRBS) ^{9,26}	It utilizes MspI restriction enzyme-which cuts DNA at all CCGG sites, regardless of their DNA methylation status. After digestion, selected fragments are size-selected (typically 40-220 bp), enriching for CpG-rich regions such as promoters and CpG islands. Bisulphite convert unmethylated DNA to uracil (read as T in sequencing). In sequencing presence of T confirms unmethylation while presence of C confirms methylated site. It enables the measurement of DNA methylation levels at 5-10% of all CpG sites in the mammalian genome ²⁶	RnBeads ^{81,82}	Relatively low cost Standardized and well-validated	Limited to regions near MspI sites, hence low coverage Not suitable for global methylation patterns
Whole genome bisulfite sequencing (WGBS) ^{83,84}	It involves fragmentation of DNA and did not use MspI. Following this entire genome is then sequenced after bisulphite treatment ⁸⁵	Bismark ^{81,86} MethPipe ⁸⁷ MethylDackel ⁸⁸ MethylKit ⁸⁹ DSS ⁹⁰ RnBeads ^{81,82}	Covers nearly all CpG sites Can detect non-CpG methylation (e.g., CHH, CHG)	Low library complexity More input DNA required than reduced methods
CpG island sequencing (CGI-seq) ^{93,91}	In this after fragmentation of DNA, methylated or unmethylated DNA is enriched either using MBD proteins or using specific restriction enzymes respectively. Following that enriched DNA is sequenced using standard next-generation sequencing platforms	MEDIPS ^{81,92} MethylKit ⁸⁹	High efficiency, simplified procedure Targeted enrichment	Inconsistent and/or low coverage Biased toward CpG islands
Assay for transposase-accessible chromatin using sequencing (ATAC-seq) ^{93,93}	In this Tn5 transposase cut and tags accessible DNA. Tn5 inserts adapters preferentially to nucleosome free regions. Following that tagged fragments are PCR amplified and sequenced	nf-core/atacseq ⁹⁴ ENCODE-ATAC ⁹⁵	High sensitivity for detecting open chromatin Useful for mapping transcription factor footprints	Low recovery of DNA fragments Tn5 sequence bias Low input can lead to noisy data
DNase-seq ^{9,28}	DNase I cuts DNA at accessible site. The cleaved fragments are sequenced after that. Sequences that bound to regulatory proteins are protected from DNase I digestion. Deep sequencing provides identify accurate location of regulatory proteins in the genome	ENCODE DNase-seq ⁹⁶	Simplicity Can detect open chromatin ⁴ No prior knowledge of the sequence or binding protein is required	Large amount of material needed, high error rate DNase I is sequence-specific and hypersensitive sites might not account for the entire genome ⁶ DNA loss through the multiple purification steps limits sensitivity Technically challenging, including optimization of DNase digestion
chromatin immunoprecipitation followed by sequencing (ChIP-seq) ^{27,83}	Cells are treated with formaldehyde to crosslink DNA and proteins. A specific antibody is used to pull down DNA-protein complex. After that cross-linking is reversed and DNA is sequenced	ENCODE ChIP-seq ⁹⁶ nf-core/chipseq pipeline ⁷⁴ DROMPAplus ²⁷	High resolution, high coverage Reveals regulatory landscapes Works with various cell types and conditions	Highly dependent on the quality of antibody Requires high-quality, specific antibodies Crosslinking and immunoprecipitation can be inefficient or biased High input requirement High background noise

Table 2. Continued

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages
Droplet chromatin immunoprecipitation followed by sequencing (Drop-ChIP) ²⁴	Individual nuclei of cells, barcoded beads, and reagents are co-encapsulated in droplets using a microfluidic device. ChIP is performed using antibodies targeting specific histone modifications. DNA fragments are captured on barcoded beads. Following that emulsions are broken, and DNA is purified, amplified, and sequenced	Standard tools for single-cell epigenomic data are used ^{77,98}	High throughput High specificity Provides greater resolution of chromatin state heterogeneity in complex tissues	Low coverage Technically challenging, requires microfluidic devices Limited to histone marks
Single-nucleus methylcytosine sequencing (snmC-seq) ^{23,83}	Unlike standard WGBS, snmC-seq uses post-bisulfite adapter tagging to prevent DNA degradation and loss during bisulfite treatment. Following that DNA is sequenced to read methylation patterns	Bismark ⁸⁶	Ultra-low-input DNA requirement	Suffers from DNA degradation PCR bias can be introduced during amplification
single-cell genome and epigenome transfer sequencing (scGET-seq) ⁹⁹	The method utilizes a recombinant transposase (TnH), created by fusing Tn5 with the chromodomain of HP1- α , which imparts binding affinity for H3K9me3-enriched heterochromatin. The combined use of Tn5 and TnH allows for comprehensive analysis of accessible and compacted chromatin states and their dynamic alterations	Snakemake ^{100,101}	High resolution	Technically complex protocol Requires advanced bioinformatics tools to integrate ATAC-seq and histone signal properly Dependent on quality of antibody
Single nucleus barcoding assay for transposase-accessible chromatin sequencing (SNUBar-ATAC) ²⁹	A single oligonucleotide adaptor containing unique barcodes is used during the tagmentation step, where a transposase enzyme inserts DNA fragments into open chromatin regions. Libraries are prepared for sequencing, and the barcodes are used to identify the origin of each fragment	SNUBar-ATAC pipeline ²⁹	Robust chromatin accessibility profiling even from frozen or difficult tissues, and it is well-suited for tissues where nuclei isolation is more feasible than whole-cell isolation Multiplexing capability Scalable	Requires nuclei isolation optimization Each nucleus provides limited signal, requiring deep sequencing
Single-cell chromatin integration labelling sequencing (scChIL-seq) ^{83,102}	Tn5 transposase is fused to protein A (pA-Tn5) or introduced via secondary antibodies, so it binds to the target histone mark. Transposition occurs in situ at the antibody-bound regions, integrating sequencing adapters directly at the chromatin site. The resulting DNA fragments are then amplified and sequenced	nf-core/chipseq pipeline ⁷⁴	No need for nuclei isolation or bulk chromatin fragmentation Suitable for clinical or frozen samples	Antibody-dependent Require specialized bioinformatics pipelines
Single-cell cleavage under targets and tagmentation (scCUT&Tag) ¹⁰³	In this method cells/nuclei are permeabilized. Primary antibodies bind to the target protein/epigenetic mark (e.g., H3K27me3, H3K4me1). A secondary antibody is used to tether a fusion protein of Protein A/G and Tn5 transposase (pA-Tn5) to the chromatin at the mark. Upon activation, Tn5 integrates sequencing adapters into nearby DNA. In scCUT&Tag, this is done on a single-cell platform. DNA fragments are barcoded per cell and sequenced	ChromHMMand ¹⁰⁴ Segway ¹⁰⁴	Low input requirement High signal-to-noise ratio (less background than ChIP) Captures histone modifications, TF binding, and chromatin accessibility Compatible with frozen or fixed tissues	Dependent on antibody quality

Table 2. Continued

Methods	Mechanisms	Analysis tool	Advantages	Disadvantages
Single-cell combinatorial indexing for transposase-accessible chromatin profiling (sciTIP-seq) ^{12,583}	The transposase inserts sequencing adapters near the bound protein sites. Nuclei are indexed multiple times. In the first round of indexing fixed nuclei are randomly distributed across a 96- or 384-well plate. Each well contains a unique barcode. Barcodes are introduced by tagmentation or ligation. All nuclei are pooled together and then redistributed randomly into a new plate for the second round of indexing, introducing a second barcode. Each nucleus receives a unique combination of barcodes enabling single-nucleus resolution without physically isolating every cell	SnapATAC ¹⁰⁵	High-throughput Cost-efficient Scalable to tens of thousands of nuclei	Dependent on antibody quality Barcoding collisions can occur if not enough barcode diversity

MBD, methyl-CpG binding domain; PCR, polymerase chain reaction; DNase-seq, DNase I hypersensitive sites sequencing; WGBS, whole genome bisulfite sequencing; TnH, Tn5 transposase hyperactive; HP1-α, heterochromatin protein 1-alpha; TF, transcription factor.

consuming.

Gtag&T-seq

In this method, WGA is not performed to sequence the gDNA and RNA of single cells, thus enhancing throughput while minimizing coverage bias, amplification noise, and cost.⁴⁰

In the field of liver cancer, no widely recognized published study has applied DR-seq, scONE-seq, G&T-seq, DNTR-seq, or Gtag&T-seq. There could be several reasons for this, including technical complexity, preference for alternative methods such as single-cell triple omics sequencing (cTrio-seq), single-nucleus chromatin accessibility and mRNA expression sequencing (SNARE-seq), 10X Multiome, or spatial transcriptomics, or a focus on transcriptome profiling using scRNA-seq alone.

SUPeR-seq

This method is used to identify and characterize super-enhancers (SEs) in various cancers, focusing on both full-length and small RNA sequencing. SEs contain approximately 10-fold higher density of transcription factors, making them superior to typical enhancers.⁴¹ SE-associated genes and long non-coding RNAs (lncRNAs) play an important role in the progression and development of HCC. For example, SE-targeted genes (*SPHK1*, *SPIDR*, *AJUBA*, *QK1*, and *SIRT7*), microRNAs (*miR-9*), and lncRNAs (*HCCL5*, *LINC1089*, *lncRNA-DAW*, and *HSAL-3*) play crucial roles in HCC progression.⁴¹ Recently, a study has shown that *E2F1* combined with *LINC01004*, a novel SE-associated lncRNA, is upregulated in liver cancer tissues and is associated with cell proliferation and metastasis with poor patient prognosis.⁴² Therefore, single-cell universal poly(A)-independent RNA sequencing (SUPeR-seq) could be instrumental in uncovering the role of SE-associated target genes and lncRNAs in HCC; however, to date, its use in HCC has not been reported.

scTrio-seq

This single-cell triple omics sequencing technique can be used to analyze genomic copy number variations (CNVs), DNA methylomes, and transcriptomes simultaneously in a single cell. The unique and specific arrangement of DNA methylation abnormalities has been correlated with the diagnosis and prognosis of HCC.² This technique was applied to 25 single HCC cancer cells, and two subpopulations were identified within these cells based on CNVs, DNA methylome, or transcriptome of individual cells.⁴³ Subpopulations expressing high levels of cell invasion

biomarkers tend to escape immune surveillance. Thus, filtering and capturing different subpopulations of tumor cells is an important topic for future cancer research.

SNARE-seq

This method simultaneously measures chromatin accessibility and gene expression.⁴⁴ Briefly, in this technique, accessible sites are captured by Tn5 transposase in permeabilized nuclei to permit DNA barcode tagging together with the mRNA molecules from the same cells.⁴⁵ Similarly, ultra-high-throughput joint analysis of the transcriptome and accessible chromatin can be performed using paired-seq to study millions of individual cells simultaneously. This method simultaneously tags open chromatin fragments generated by Tn5 transposases and cDNA molecules generated from reverse transcription of RNA in millions of cells. This technique was applied to Hep-G2 cells, in which reads from the DNA library were enriched around transcription start sites, whereas reads from the RNA library were enriched upstream of transcription termination sites.⁴⁶

scRNA-seq + scTCR-seq

This technique combines gene expression and T-cell receptor (TCR) sequences from the same immune cells³ and has been applied to HCC. For example, tumors from patients with HCC treated with atezolizumab plus bevacizumab that presented with durable responses were enriched in PD-L1⁺ CXCL10⁺ macrophages and, based on cell-cell interaction analysis, expressed high levels of CXCL9/10/11 and were predicted to attract peripheral CXCR3⁺ CD8⁺ effector memory T cells (CD8 TEM) into the tumor. These data suggest that CD8 TEM cells preferentially differentiate into clonally expanded PD-1⁺ CD45RA⁺ effector-memory CD8⁺ T cells (CD8 TEMRA) with pronounced cytotoxicity. However, contradictory results were obtained in non-responders.³³ Another study analyzed the mutational profiles and evolutionary trajectories of paired primary and recurrent HCC samples and identified differential gene expression in *de novo* versus true recurrence. The abundance of KLRB1⁺ CD8⁺ T cells and growth differentiation factor (GDF) 15 in DCs was high in truly recurrent HCCs with low cytotoxicity. High GDF4 levels in DCs dampen antigen presentation and inhibit antitumor immunity in recurrent lesions. Consistent with these findings, a phase 2 trial of neoadjuvant anti-PD-1 immunotherapy showed better responses in patients with *de novo* recurrent HCC.⁴⁷

SINGLE-CELL OMICS AND MULTI-OMICS IN UNRAVELING HETEROGENEITY IN HCC

Tumor heterogeneity incorporates various subpopulations of cells, either within a tumor (intratumoral heterogeneity) or between different tumors or nodes in the same patient (intertumoral heterogeneity). The biological behavior of these cells differs owing to their different genetic and morphological characteristics, including size, shape, and structure. Heterogeneity also exists before and after tumor treatment, which is known as spatiotemporal heterogeneity. For example, berberine treatment alters the tumor immune landscape in mice by decreasing the proportion of TCR-biPD-1hiCD69⁺ CD27⁺ effector CD8⁺ T lymphocytes and increasing the proportion of Ly6ChiTCRb⁺ CD69⁺ CD27⁺ CD62L⁺ central memory CD8⁺ T lymphocytes.⁴⁸ Heterogeneity is commonly observed in HCC and is thought to result from differences in patient genetics and TME. The diverse pathways involved in HCC contribute to its clinical complexity and are crucial factors in challenges, such as treatment resistance, tumor dormancy, and relapse. Several factors, such as genomic mutations, epigenomic changes, and the TME, contribute to the clonal evolution of cancer, resulting in heterogeneity at the genomic, molecular, and functional levels.

The TME, particularly immune cells and CAFs, plays a key role in shaping HCC heterogeneity and treatment resistance. CAFs promote tumorigenesis by secreting cytokines, chemokines, and growth factors. Notably, the liver is in direct contact with bacterial products or other inflammatory stimuli and has the highest number of immune cells, such as Kupffer cells. Hence, it prevents excessive immune responses to maintain tissue health. Dysregulation of the immunological network due to prolonged inflammation can lead to HCC. Therefore, the interactions between hepatic immune cells and malignant hepatocytes should be studied to improve understanding of HCC and develop new treatment regimens. scRNA-seq studies have revealed CAF subtypes, such as POSTN⁺,^{49,50} CD36⁺,⁵¹ and vascular endothelial growth factor A (VEGFA)⁺,⁵² that promote immunosuppression, recruitment of myeloid-derived suppressor cells, and angiogenesis, respectively. These findings underscore the importance of mapping the immune TME landscape for the development of targeted therapies. Recent scRNA-seq of CAF populations in HCC further revealed the immunosuppressive role of immunoglobulin A (IgA) signaling in the TME. The PD-L1 level in IgA-treated CAFs was increased, and the amount of fibroblast activation protein was elevated, which suppressed the cytotoxicity of CD8⁺ T cells through PD-1-induced interactions. This study

suggests that intrahepatic IgA induces polarization of HCC-CAFs into more malignant matrix phenotypes and attenuates the function of cytotoxic T cells. These findings highlight their potential roles in tumor progression and immune suppression.⁵³

Single-cell sequencing technologies have become essential for uncovering cellular and molecular landscapes.⁵² Alvarez et al.⁵⁴ used single-cell analysis in human liver single nucleus. They identified a proliferating cell type carrying *TP53* and *RB1* somatic mutations that are enriched in HCC tumors and associated with decreased overall survival. The six datasets of scRNA-seq identified five clusters and found that the aggressive HCC subtype is composed of a combination of cluster 1 characterized by high expression of *p53* and *myc* with cluster 3, 4, or 5. Cluster 1 elucidated T cell depletion as an immune resistance mechanism. This study deepens our understanding of the molecular mechanisms and TME in aggressive HCC.⁵⁵ To move toward clinical application, an 11-gene prognostic signature was derived from the dif-

ferentially expressed genes, distinguishing tumor from adjacent non-tumor cells using an scRNA-seq approach. This signature stratifies patients with HCC into two risk groups based on markedly different molecular profiles. The study also identified 14 candidate compounds that may improve outcomes in aggressive HCC in the high-risk group.⁵⁶ These studies collectively highlight the power of single-cell sequencing technologies in identifying distinct tumor subpopulations, prognostic gene signatures, and potential therapeutic targets.

However, scRNA-seq technology still has limitations due to the loss of spatial and morphological information after tissue dissociation, which makes it difficult to determine the spatial architecture of tumors. Methods such as multiplexed error-robust fluorescence in situ hybridization and sequential fluorescence in situ hybridization can capture spatial information; however, they are limited to detecting only a small number of predefined target genes simultaneously.⁵⁷ The newly developed spatial transcrip-

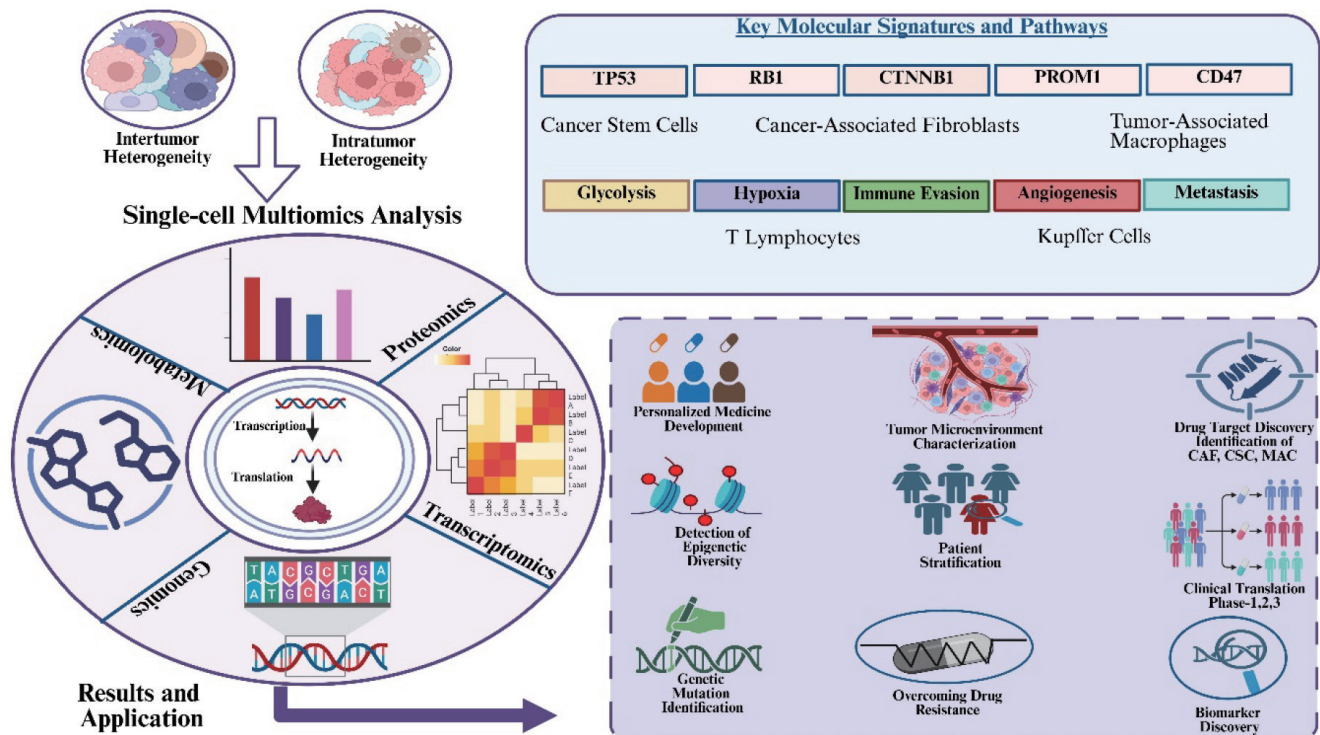


Figure 3. Please check copyright of figure. Importance of single-cell multi-omics in understanding tumor heterogeneity. This figure depicts the role of single-cell multi-omics in cancer diagnosis and analysis of intertumoral and intratumoral heterogeneity. Multi-omics methods can provide a complete picture of tumor biology by combining genomics, transcriptomics, proteomics, and metabolomics. These analyses reveal the main molecular signatures and pathways, including *TP53*, *RB1*, *CTNNB1*, *PROM1*, and *CD47*, as well as changes in various cell types, such as cancer stem cells, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), T lymphocytes, and Kupffer cells. Dysregulated pathways such as glycolysis, hypoxia response, immune evasion, angiogenesis, and metastasis are mentioned as the drivers of tumor progression. Single-cell multi-omics insights aid in the development of personalized medicine, drug targets, biomarker discovery, patient stratification, and clinical translation through multi-phase therapeutic trials. *TP53*, tumor protein p53; *RB1*, retinoblastoma gene; *CTNNB1*, β -catenin gene; *PROM1*, prominin-1 (CD133); *CD47*, cluster of differentiation 47; CSC, cancer stem cells; MAC, macrophages.

tomics technology can overcome these limitations. For example, the spatial transcriptome architecture of seven PLCs characterized the tertiary lymphoid structure composition, stromal and immune cell distribution, cancer stem cell niche diversity, and tumor cluster interactions. In brief, the study revealed that reciprocal ligand-receptor interactions occurring along the 100- μ m cluster boundary are essential for preserving the structural organization within the tumor. PROM1⁺ CD47⁺ cancer stem cell niches contribute to the remodeling of the TME and tumor metastasis.⁵⁷ These findings provide a deeper understanding of the complex ecosystem of liver cancer and can therefore improve individualized cancer prevention and drug discovery.

scRNA-seq and spatial transcriptomics are inadequate for epigenetics, proteomics, and metabolomics. Thus, multi-omics with bulk tissue-resolution profiling has the potential to address such deficiencies. For example, using a combination of scRNA-seq, spatial transcriptomics, and bulk multi-omics, Zhou et al.⁵⁸ revealed that intrahepatic cholangiocarcinoma was the primary source of CAFs, whereas HCC exhibited disrupted metabolism and greater individual heterogeneity of T cells. Similarly, in another study, single-cell transcriptomic, proteomic, and chromatin accessibility data were combined to identify the correlation between heterogeneity and metastatic potential in five HCC cell lines. They identified a rare hypoxic subtype with a high capacity for glycolysis and increased metastatic potential. Additionally, they identified a robust 14-gene panel representing the hypoxia signature that could serve as a prognostic index.³⁶ In a recent study on HCC, single-cell spatial transcriptomics and bulk multi-omics analyses were used to investigate the heterogeneous behavior of HCC and its ecosystems. They identified MI types of tumor-associated macrophages that perturb metabolism, antigen presentation, and immune-killing abilities. In addition, they identified that cirrhotic and HCC lesions in an individual patient shared a common origin and then evolved in parallel; each lesion adopted distinct immune reprogramming strategies to adapt to its local microenvironment.⁴ This dataset revealed the dynamic heterogeneity of HCC across spatiotemporal dimensions. Patients with β -catenin (encoded by *CTNNB1*)-mutated HCC demonstrate heterogeneous responses to first-line immune checkpoint inhibitors. Precision medicine-based treatments are not available for this class. Analysis of bulk and spatial transcriptomic data showed that the mutated β -catenin gene signature overlapped with the Hoshida S3, Boyault G5/G6, and Chiang CTNNB1 subclasses in The Cancer Genome Atlas and HCC spatial datasets. Therefore, this signature may aid in patient stratification to guide precision medicine therapeutics for the *CTN-*

NB1-mutated HCC subclass.⁵⁹ These studies highlight the importance of multi-omics technologies in addressing the spatial, functional, and evolutionary complexities of HCC (Fig. 3).

Given the pressing need for novel liver cancer therapies, scRNA-seq not only uncovers potential drug targets but also enables finer tumor subclassification to better tailor individual treatments. An initial scRNA-seq-based classification system has been proposed. However, it awaits prospective validation before adoption in routine clinical practice. To date, only a handful of single-cell sequencing studies (Table 3) and clinical trials on HCC have investigated therapies based on single-cell analyses, highlighting the gap between their discovery and application (Table 4). Combining scRNA-seq with spatial transcriptomics will deepen our insight into cellular and molecular crosstalk within the TME, driving the discovery of new targets in hepatobiliary cancers. Moreover, these high-resolution approaches promise to improve tumor classification and patient stratification, thereby improving the precision of future clinical trials.

Application of scRNA-seq in biomarker discovery

Using an scRNA-seq approach, a study found that HCC expressing the predictive markers *FKBP10*, *ATP1A2*, *NT5DC2*, *UGT3A2*, *PYCR1*, *CKB*, *GPX7*, *DNMT3B*, *GSTP1*, and *OXCT1* activate a metabolic transcriptional program that influences epigenetic regulation.⁶⁰ In another study, an scRNA-seq approach was used to calculate a metabolic score predicting metastatic potential in HCC based on the combined tumor expression of *DNMT3B* and *PFKFB4*. Emerging evidence suggests that circular RNAs (circRNAs) and lncRNAs contribute substantially to the molecular etiology of HCC.⁶¹ circRNAs and lncRNAs are hypothesized to play important roles in the etiology of HCC. Kaplan-Meier plot analysis showed that of the 55 differentially expressed mRNAs in the circRNA/lncRNA-miRNA-mRNA network, the following genes were most strongly associated with the prognosis of patients with HCC: *CPEB3*, *EFNB3*, *FATA4*, *GH* receptor, *GSTZ1*, *KLF8*, *MFAP4*, *PAIP2B*, *PHACTR3*, *PITPNM3*, *RPS6KA6*, *RSPO3*, *SLITRK6*, *SMOC1*, *STEAP4*, *SYT1*, *TMEM132E*, *TSPAN11*, and *ZFPM2*.⁶²

APPLICATION OF scRNA-SEQ IN LIQUID BIOPSY AND NONINVASIVE MONITORING

Liquid biopsy is a minimally invasive method for detecting tumor-derived materials in blood. It enables the acquisition of genetic, epigenetic, transcriptomic, and proteomic data. This meth-

Table 3. Overview of single-cell sequencing studies in unravelling heterogeneity in liver cancer in the past year

Single-cell technology	Major findings
Tumor immune microenvironment	
scRNA-seq	NQO1 ⁺ macrophages may have an immunosuppressive effect on HCC ¹⁰⁶
scRNA-seq	Four distinct TAMs were identified. Specifically, TAM-c4 was enriched in the advanced-stage patients or those receiving ICT and found to be related to a short survival time and low abundance of CD8 ⁺ T cells in primary liver cancers ¹⁰⁷
scRNA-seq, spatial transcriptomics and transcriptome profiling	A novel FMO2 ⁺ CAF subset serves as a critical regulator of microenvironmental immune properties and a predictive biomarker of the immunotherapy response in patients with HCC. CCL19 in combination with anti-PD-1 therapy may constitute a novel therapeutic strategy for HCC ¹⁰⁸
Spatial transcriptomics and scRNA-seq	High protein kinase, DNA-activated, catalytic subunit expression is associated with shorter survival times and an abnormal tumor microenvironment, highlighting its impact on immune cell infiltration and HCC prognosis ¹⁰⁹
Spatial transcriptomics and scRNA-seq	LAMA4 ⁺ CD90 ⁺ extracellular matrix CAFs provide immunosuppressive microenvironment for liver cancer through induction of CD8 ⁺ T cell senescence ¹¹⁰
scRNA-seq	Glycan-HCCs were associated with multifaceted immune distortion, including exhaustion of T cells and enriched SPP1 ⁺ macrophages and leads to worse survival ¹¹¹
Single-cell, bulk, and spatial transcriptome profiling with multiplexed immunofluorescence	The crosstalk between DAB2 ⁺ TAMs and FAP ⁺ CAFs seem to be a key determinant in shaping the tumor immune barrier. Therapeutic strategies that disrupt this interaction could potentiate immunotherapeutic responses and improve patient prognosis ¹¹²
scRNA-seq, whole-exome sequencing, whole-transcriptome sequencing, and NGS-based HBV integration analysis	In this study, patients were categorized into two groups on the basis of effector CD8 ⁺ T-cell exhaustion markers high and low exhaustion groups. The high-exhaustion group exhibited higher <i>PDCD1</i> expression, higher <i>TP53</i> mutation rates, clonal expansion of CD4 ⁺ regulatory T cells and follicular helper T cells, and more pronounced HBV integrations with elevated intrahepatic covalently cccDNA and pgRNA levels. These findings provide insight into the intricate relationship between high exhaustion, proliferation subtype, increased HBV integrations, and enhanced HBV-induced oncogenic potential in virus-related HCC ¹¹³
Tumor cell heterogeneity & spatial features	
scRNA-seq	The analysis demonstrated regional heterogeneity in cellular composition and malignant potential across the tumor, with the T1 region displaying the greatest degree of malignancy, characterized by the upregulation of HMGB2 and TOP2A ¹¹⁴
Spatial transcriptomics and scRNA-seq	Identified three subtypes in tumor cells, including ARG1 ⁺ metabolism subtype (metab-subtype), TOP2A ⁺ proliferation phenotype, and S100A6 ⁺ pro-metastatic subtype (EMT-subtype) ¹¹⁵
scCPA-Tag	Identified a tumour cell subtype (C2) with more aggressive features. This subtype was characterized by high chromatin accessibility and a lower abundance of <i>H3K27me3</i> on tumour-promoting genes ¹¹⁶
Spatial transcriptomics sequencing	The study identified one LPC cluster, three LPC subpopulations, and four distinct cellular modules, indicating the heterogeneity within LPC and the diversity between LPCs and epithelial cells ¹¹⁷
Single-cell immune repertoire sequencing, mass cytometry, and multiplex immunofluorescence	The study describes the TME landscape and identified six prognosis-related cell subclusters in this landscape ¹¹⁸
Tumor endothelial cells	
scRNA-seq	The analysis uncovered significant heterogeneity among TECs in HCC, delineating two subgroups (TEC1 and TEC2) with distinct functional roles and signaling profiles ¹¹⁹
scRNA-seq	This analysis delineated the intratumoral cellular heterogeneity underlying MVI, identifying MARCKSL1 ⁺ MVI-positive malignant cells as key contributors to MVI progression via activation of the PTN signaling pathway ¹²⁰

od is especially important in HCC because biopsies are rarely performed to diagnose HCC owing to the widespread reliance on imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) scans. Tumor-derived materials for liquid biopsy include cell-free DNA, circulating tumor

cells (CTCs), and extracellular vesicles. The presence of CTCs is regarded as a valuable biomarker for the diagnosis and prognosis of HCC.

scRNA-seq is a promising approach for analyzing gene expression in individual CTCs. However, these cells are present in ex-

Table 3. Continued

Single-cell technology	Major findings
Relapse, prognostic risk, and cellular senescence	
scRNA-seq and bulk RNA-seq datasets	Related genes from senescence-related pathways were used to identify senescence-related molecular subtypes in HCC with <i>G6PD</i> as a key gene, potentially serving as a senescence-related target in liver cancer ¹²¹
scRNA-seq	Analysis of primary versus early-relapsed HCC demonstrated elevated infiltration of CD8 ⁺ T cells and malignant cells in relapsed tumors, alongside a marked decline in CD4 ⁺ T cell populations ¹²²
scRNA-seq	Identified a distinct senescent-associated subset of CD34 ⁺ CLDN5 ⁺ endothelial cells, which mainly enriched in tumor tissue. These cells increased cholangiocellular phenotype in HCC via IGF2-IGF2R signaling ¹²³
Bulk RNA sequencing, proteomic analysis, scRNA-seq, spatial transcriptomics sequencing, and genome sequencing	Reveals a novel subtype of HCC with biological and clinical relevance. Patients with tumor purity-TME high-risk subtypes show pronounced hypoxia and activation of Wnt/ β -catenin, Notch, and TGF- β pathways. Notably, a novel XPO1 ⁺ epithelial subtype shares these high-risk signatures and aggressive behavior ¹²⁴

scRNA-seq, single-cell RNA sequencing; NQO1, NAD(P)H quinone dehydrogenase 1; HCC, hepatocellular carcinoma; TAM, tumor-associated macrophage; ICT, immune checkpoint therapy; FMO2, flavin-containing monooxygenase 2; CAF, cancer-associated fibroblast; CCL19, C-C motif chemokine ligand 19; PD-1, programmed death 1; LAMA4, laminin subunit alpha 4; SPP1, secreted phosphoprotein 1; DAB2, disabled homolog 2; FAP, fibroblast activation protein; NGS, next generation sequencing; HBV, hepatitis B virus; cccDNA, closed circular DNA; pgRNA, pregenomic RNA; HMGB2, high mobility group box 2; TOP2A, topoisomerase (DNA) II alpha; scCPA, single-cell clustering-based pathway analysis; EMT, epithelial-to-mesenchymal transition; LPC, liver progenitor cell; TEC, tumor endothelial cell; MVI, microvascular invasion; PTN, pleiotrophin; IGF2, insulin-like growth factor 2; IGF2R, insulin-like growth factor 2 receptor; TGF- β , transforming growth factor-beta; XPO1, exportin 1.

Table 4. Ongoing and completed clinical trials utilizing single-cell approaches in liver cancer

Clinical trial identifier	Country	Year	Title	Status
NCT05677724	China	2022	Single-cell RNA Sequencing Resolves the Regulatory Role of HBV on the Hepatocellular Carcinoma Immune Microenvironment	Unknown status
NCT05171439	China	2022	Surufatinib in Advanced Hepatocellular Carcinoma Based on Single-cell Sequencing of Tumor Samples	Unknown status
NCT05540925	China	2022	Vascular Invasion Signatures in cfDNA Support Re-staging of Liver Cancer	Completed*

HBV, hepatitis B virus; cfDNA, cell-free DNA.

*No results posted.

tremely low numbers (approximately 1-10 per million white blood cells [WBCs]). Therefore, their enrichment is critical before scRNA-seq and is typically achieved using various immunoaffinity-based or physical property-based methods. The Cell-Search® System (Menarini Silicon Biosystems Inc, Huntington Valley, PA, USA), an epithelial cell adhesion molecule (EpCAM)-based immunoaffinity platform, is currently the only United States Food and Drug Administration (FDA)-approved method for CTC detection. It employs antibodies targeting EpCAMs to isolate and enrich CTCs in metastatic breast, colorectal, and prostate cancers. This system captures CTCs using ferromagnetic beads coated with anti-EpCAM antibodies and isolates them via magnetically activated cell sorting. However, CTCs commonly exhibit epithelial-to-mesenchymal transition (EMT), and in the context of HCC, they arise from hepatocytes rather than from classical epithelial cell lineages. Therefore, the use of anti-EpCAM antibodies alone may not be sufficient for effective enrichment of HCC CTCs. To overcome this, newer strategies have incorporated multiple antibodies to improve the capture ef-

ficiency. Advanced microfluidic platforms, such as CTC-Chip™ (Johnson & Johnson, New Brunswick, NJ, USA) and NanoVelcro Chip™ (University of California, Los Angeles, Los Angeles, CA, USA), have been developed to increase the frequency and strength of interactions between CTCs and functionalized chip surfaces, resulting in markedly improved capture efficiency. Alternatively, physical property-based approaches utilize techniques, such as microfiltration, density gradient centrifugation, dielectrophoresis, and microfluidic systems, to isolate CTCs. All of these methods are antigen-independent; therefore, they can address the issue of EpCAM loss during the EMT of CTCs. Additionally, CTCs do not need to be separated from the coated antibodies, which makes these methods cost-effective. The Parsortix® PC1 System (CellBxHealth, Guildford, UK) represents a microfluidic technology recently cleared by the FDA that isolates CTCs from patients with metastatic breast cancer by exploiting differences in cell size and deformability. However, even with these technologies, more than 1,000 WBCs per CTC often remain in the background. Therefore, immunofluorescence staining is com-

monly used to distinguish CTCs from the background WBCs. Alternative strategies have applied PCR-based assays to characterize CTC expression signatures through the analysis of a restricted set of genes, as shown in recent studies on HCC.⁶³ However, comprehensive whole-genome transcriptomic profiling of CTCs from patients with HCC remains scarce.

Advances in single-cell technology offer a feasible path for obtaining such data, providing a foundation for the development of more precise biomarkers for HCC. However, only a few studies have employed scRNA-seq to detect and characterize CTCs in HCC. D'Avola et al.⁶⁴ reported a sequential approach combining imaging flow cytometry with high-density scRNA-seq to detect and characterize CTCs in patients with HCC. Genome-wide expression profiling of CTCs using this approach demonstrated

that the presence of HCC upregulated lncRNA (*HULC*), *SPINK1*, *IGF2*, *SPP1*, and *BRIC5* in the CTCs of the hepatic lineage. Moreover, the absence of circulating hepatic lineage cells in the blood of healthy individuals and patients with non-malignant chronic liver diseases further supports the malignant origin of CTCs. In addition to the aforementioned genes, multiple other genes exhibited differential expression between the two candidate CTCs detected in patient 1, underscoring the transcriptomic heterogeneity within the CTC compartment. This study suggests that high-density expression profiling using scRNA-seq helps trace cancer heterogeneity in CTCs.⁶⁴ Building on this concept, researchers performed single-cell transcriptomic analysis to identify cell type-specific RNA markers in plasma samples from patients with HCC. In this study, scRNA-seq identified six major

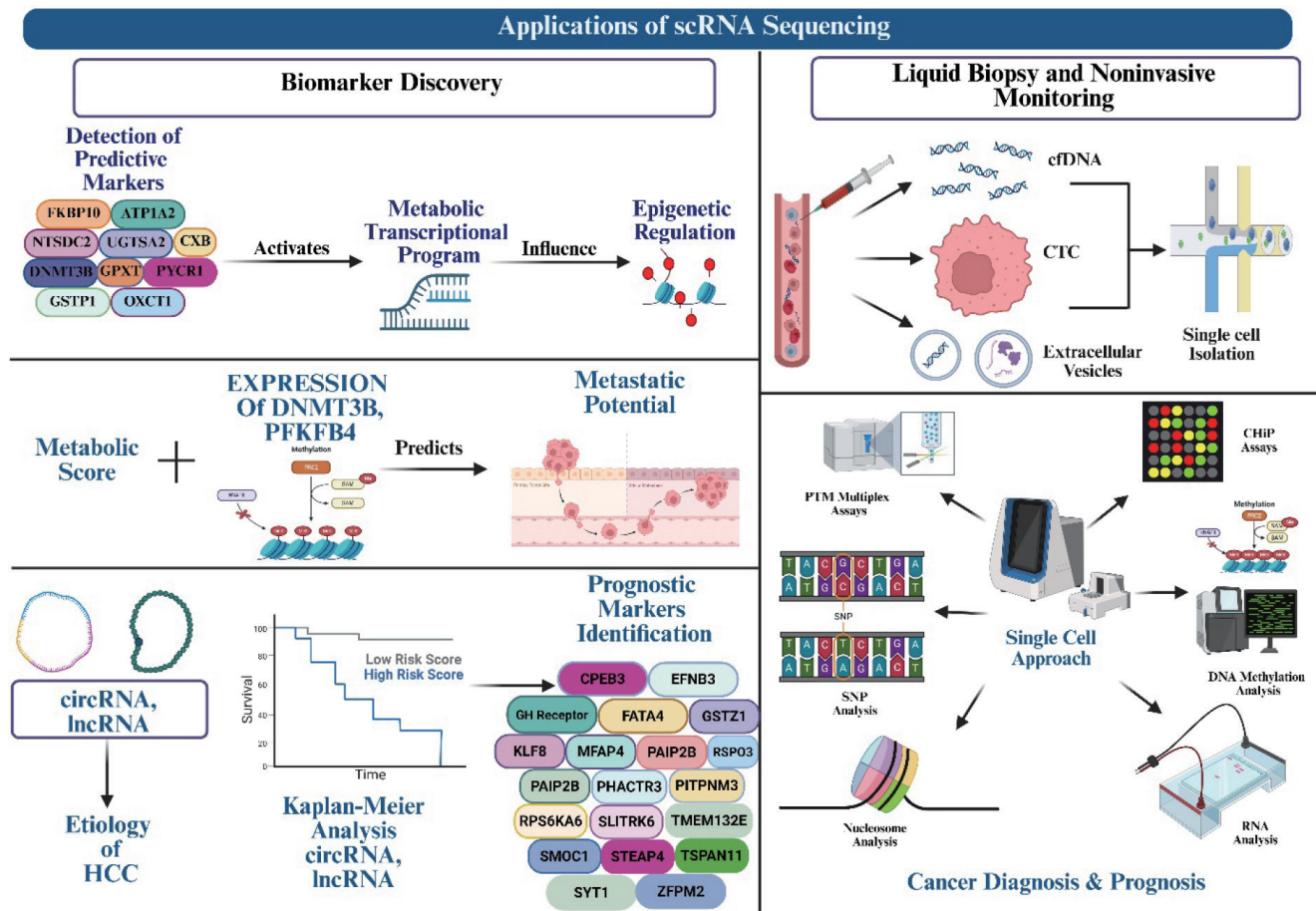


Figure 4. Applications of single-cell sequencing. This figure demonstrates the uses of single-cell RNA sequencing (scRNA-seq) in cancer. The schematic shows the biomarker discovery through predictive markers that trigger metabolic transcriptional programs and affect epigenetic regulation. It also describes the ability to predict metastatic potential and identify prognostic markers with the help of the Kaplan-Meier analysis of circular RNA (circRNA) and long non-coding RNA (lncRNA) profiles. The noninvasive tumor monitoring methods based on liquid biopsy are circulating tumor cells (CTCs), cell-free DNA (cfDNA), and extracellular vesicles, along with single-cell isolation. Multiplex protein analysis, single-nucleotide polymorphism (SNP) genotyping, chromatin immunoprecipitation (CHIP) assays, DNA methylation, RNA, and nucleosome profiling are examples of integrative single-cell assays that contribute to the comprehensive diagnosis and prognosis of cancer.

cell clusters in HCC tissues compared with paired non-HCC tissues. These profiles were then used to derive cell-type-specific gene signature (CELSIG) scores from plasma RNA. Notably, the hepatocyte-like CELSIG score was significantly elevated in the preoperative plasma of patients with HCC compared with that of patients without HCC. These findings highlight the use of combining scRNA-seq with plasma RNA analysis to develop noninvasive and dynamic biomarkers for cancer detection and monitoring.⁶⁵ Collectively, these studies underscore the significance of scRNA-seq as an emerging approach for biomarker identification in HCC using liquid biopsy. Various applications of scRNA-seq in cancer are shown in Fig. 4.

CHALLENGES AND PERSPECTIVES

Although scRNA-seq has transformed our understanding of organ complexity, it faces several challenges: tissue dissociation can alter gene expression and must be finely tuned, the approach remains costly and requires extensive bioinformatics expertise, and spatial transcriptomics, while promising, still requires validation in liver studies. Emerging single-cell multiomics methods will further enrich our insights by integrating protein, RNA, and DNA analyses; however, they must become more affordable.

Finally, the development of reliable histological or imaging biomarkers could help predict treatment responses and patient outcomes without the need for full single-cell profiling. Nevertheless, integrating single-cell and multi-omics analyses into the real-world diagnostics of HCC is challenging. Most clinical specimens are limited to small biopsies or formalin-fixed paraffin-embedded (FFPE) tissues, where RNA degradation and rapid tissue handling pipelines hinder high-quality single-cell isolation and sequencing.^{66,67} Furthermore, these samples may not adequately capture the spatial and cellular heterogeneity of HCC, leading to sampling bias. New methods, including single-nucleus RNA sequencing^{68,69} and FFPE-compatible spatial transcriptomics,^{70,71} address these shortcomings; however, they are costly and technically challenging for routine use in clinical practice.

CONCLUSION

scRNA-seq has transformed our ability to dissect liver biology by resolving the diversity of cell types and states and mapping intercellular communication in health and disease. Selecting an appropriate scRNA-seq protocol requires balancing experimental goals, desired gene coverage, and budget constraints. Advances in computational analysis, spatial transcriptomics, and hybrid work-

flows that combine scRNA-seq with spatial data now enable us to study gene expression within the intact, architecturally complex liver. This approach has yielded key insights into the hepatic zonation, mechanisms of regeneration, and cellular ecosystems that drive chronic liver disease and cancer. Because liver pathology involves intricate networks of multiple cell populations, scRNA-seq offers an unprecedented window into these microenvironments. The current challenge lies in converting these molecular and cellular discoveries into targeted therapies to address the pressing clinical needs of hepatology.

Conflicts of Interests

The authors have no conflicts of interests to declare.

Ethics Statements

This review article is fully based on articles which have already been published and did not involve additional patient participants. Therefore, IRB approval is not necessary.

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