Liu *et al. Journal of Hematology & Oncology* (2024) 17:72
https://doi.org/10.1186/s13045-024-01596-9

Journal of Hematology & Oncology

REVIEW

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Spatial multi-omics: deciphering technological landscape of integration of multi-omics and its applications

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Abstract

The emergence of spatial multi-omics has helped address the limitations of single-cell sequencing, which often leads to the loss of spatial context among cell populations. Integrated analysis of the genome, transcriptome, proteome, metabolome, and epigenome has enhanced our understanding of cell biology and the molecular basis of human diseases. Moreover, this approach offers profound insights into the interactions between intracellular and intercellular molecular mechanisms involved in the development, physiology, and pathogenesis of human diseases. In this comprehensive review, we examine current advancements in multi-omics technologies, focusing on their evolution and refinement over the past decade, including improvements in throughput and resolution, modality integration, and accuracy. We also discuss the pivotal contributions of spatial multi-omics in revealing spatial heterogeneity, constructing detailed spatial atlases, deciphering spatial crosstalk in tumor immunology, and advancing translational research and cancer therapy through precise spatial mapping.

Keywords Spatial multi-omics, Heterogeneity, Spatial-specific atlas, Lineage tracking, Crosstalk, New therapy, Reproduction

Introduction

Single-cell sequencing has been instrumental in providing detailed insights into gene expression at the individual cell level for decades. This technique has revealed the complexity of cellular diversity, exacerbated by processes such as cell proliferation, differentiation, and death, particularly in relation to the local and distant environment of the cell [1]. Single-cell sequencing can detect cellular heterogeneity, enabling detailed analysis of individual cell behavior, mechanisms, and relationships. The high resolution of these methods has allowed for the extensive exploration and characterization of cell diversity on a large scale. However, despite these advantages, single-cell sequencing often fails to retain critical spatial information about cell populations, resulting in the loss of crucial spatial context [1, 2].

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To overcome this limitation, spatial multi-omics has emerged as a transformative technology, enabling the precise localization of cells within tissues and the quantitative measurement of gene expression in situ. This advancement marks an important technological breakthrough in life sciences and biomedicine, with wide-ranging applications in neuroscience, developmental biology, and cancer research [3]. Furthermore, spatial multi-omics allows researchers to investigate the development of multicellular organisms from single totipotent cells, as well as their function, aging, and disease progression. High-throughput multi-omics technologies, such as genomics, epigenomics, transcriptomics, proteomics, and metabolomics, have also facilitated the mapping of diverse molecular layers, significantly broadening the scope of biological analysis and our understanding of complex biological systems. In the current review, we trace the developmental timeline of spatial multi-omics technologies, highlighting their evolution and substantial contributions to modern science. Furthermore, we discuss the current state of these technologies, their integration into research, and their significant applicative value in enhancing our understanding of biological complexity.

Technologies for spatial omics

Spatial mono-omics, such as spatial transcriptomics, was recognized as the "Technology of the Year 2020" by Nature Methods magazine [4] (Fig. 1). Although single-cell sequencing technology has provided valuable insights into cellular heterogeneity, it lacks spatial context. Spatial multi-omics overcomes this limitation by enabling the precise localization and molecular characterization of individual cells within their tissue environments [5]. The innovation in spatial multi-omics builds upon foundational spatial mono-omics methods. In this section, we introduce key spatial mono-omics techniques (Table 1) and discuss their pivotal role in advancing the field of spatial multi-omics.

Spatial transcriptomics

Spatial transcriptomics has significantly enhanced our understanding of cellular organization and intra-tissue interactions based on the systematic measurement of gene expression levels across tissue space. Recent advancements in spatial transcriptomics sequencing have focused on increasing the number of detectable genes or proteins, enhancing sensitivity and resolution, simplifying operation, and expanding the size of the analyzed area. Spatial transcriptomics has been used in various fields, including cancer research [6], developmental biology [7], and disease studies [8]. Fundamentally, spatial transcriptomics technology has the ability to reveal the precise spatial localization of RNA molecules within tissues. In this section, we provide a comprehensive overview of mainstream spatial transcriptomics research strategies and summarize the strengths and limitations of these approaches.

(1) *Image-based in situ transcriptomics*. Image-based spatial transcriptomics primarily includes fluorescence

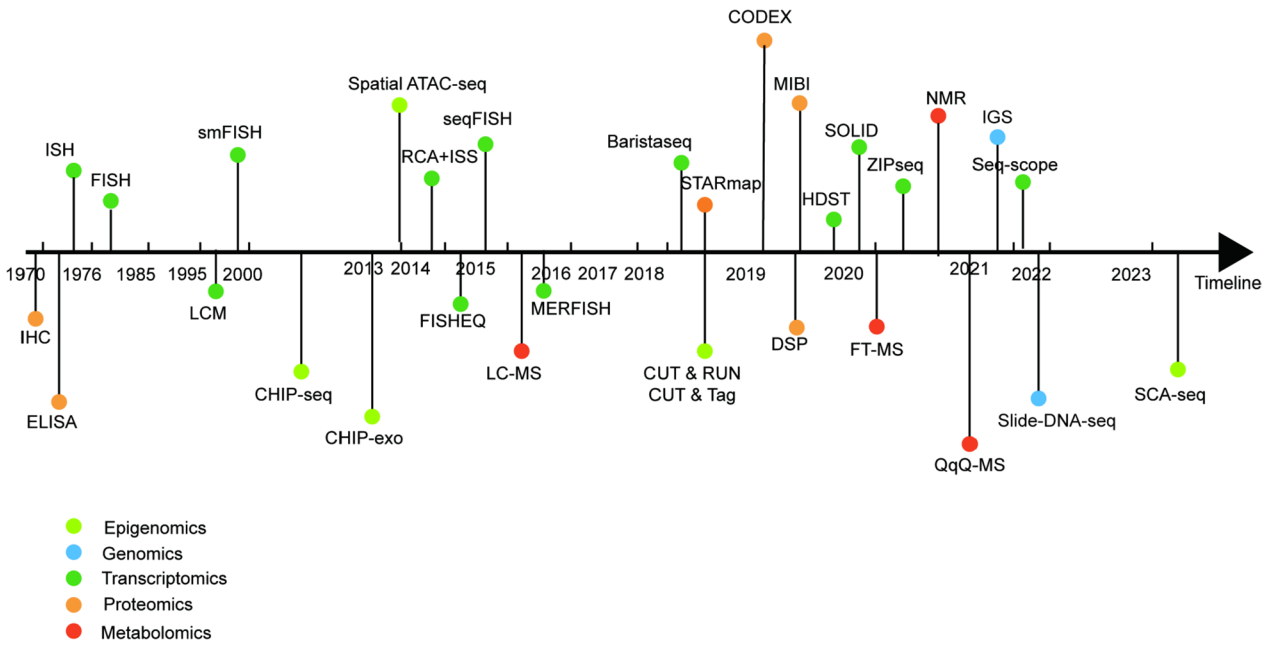

Fig. 1 Timeline of spatial multi-omics. Transcriptomics, genomics, proteomics, metabolomics, and epigenomics are included. In addition to the frequently used techniques, some emerging methods are mentioned

Table 1 Sub-technologies of spatial transcriptomics, spatial genomics, spatial proteomics, spatial epigenomics, spatial metabolomics

Methods	Analyte principles	Features	Limitations	References
Spatial transcriptomics				
smFISH	In situ hybridization of reverse-complementary oligo probes conjugated with fluorophores	Precise localization and quantification of intracellular RNA	Low probe specificity; Low hybridization efficiency; High background fluorescence	[11]
MERFISH	Multiplexed error-robust fluorescence in situ hybridization	Short time, low error rate	Strong hybrid fluorescence background	[151]
seqFISH	Sequential rounds of fluorescent hybridization and imaging	High coding efficiency; high hybridization efficiency	High cost and extended duration due to numerous probes	[13]
FISSEQ	Sequencing of cross-linked complementary DNA (cDNA) amplicons	Unbiased whole transcriptome; less noisy signals	low capture efficiency; low sensitivity	[20]
STARmap	Transcript amplicon readout mapping	High efficiency; high accuracy; high reproducibility	Incomplete coverage of expressed genes	[22]
BaristaSeq	Based on gap padlock probe	High efficiency; high accuracy;	Inapplicability to experimental tissues	[25]
454	Enzyme cascade chemiluminescence reaction	High throughput; low cost; fast; intuitive	Short readout fragments	[35]
Illumina/Solexa	Single molecule array sequencing	High accuracy; high throughput; high sensitivity; low costs	Short readout fragments; Extensive computational splicing work	[36]
SOLiD	Sequencing based on fluorescently labeled base recognition	High accuracy; high throughput; high output	Run slowly	[36]
PacBio	Real-time reading of single molecule based on nanopore	Long readout; high accuracy; no bias based on GC content	Random errors in the judgment of bases	[42]
ONT	Identification of base sequences based on electrical signals	Long readout; low cost; high accuracy	Not suitable for reference-free transcriptome sequencing alone	[42]
Spatial genomics				
IGS	Spatial mapping of paired-end sequences of the whole genome	Simultaneous sequencing and imaging of genomes in complete biological samples	Inability to achieve genome-wide coverage sampling	[47]
Slide-DNA-seq	In situ fragmentation of genomic DNA organized with the addition of barcode adapters containing spatial information for subsequent next-generation sequencing	High resolution; high sensitivity	Low efficiency	[48]
Spatial proteomics				
Mass spectrometry-based methods	Distinction of compounds by relative molecular weight difference	Cheap; self- service operation by trained users possible	Low resolution	[54]
Imaging-based methods	Visualization by affinity-based reagents or expression constructs encoding fluorescent fusion proteins	High sensitivity	Low throughput	[60]
Spatial epigenomics				
CUT&Tag	Insertion of adaptors directly during chromatin cutting based on Tn5 transposable enzyme	Simple operation; short experiment period; high signal-to-noise ratio; good repeatability	More suitable for cell lines	[65]

Table 1 (continued)

Methods	Analyte principles	Features	Limitations	References
Spatial metabolomics				
FT-MS	Mass analyzer determining the m/z of ions based on cyclotron frequency in a fixed magnetic field	High resolution; high sensitivity	Difficult operation; expensive	[72]
NMR	Analysis of metabolites using the resonance of atoms and nuclei in an applied magnetic field	High precision, less restrictions on the sample, no damage to the sample	Low sensitivity; limited dynamic range	[73]
LC/MS	Separation of different metabolites based on varying migration rates	High sensitivity, no derivatization, suitable for polar compounds	Low separation rate; time cost; bias	[77]

Analyte principles, features, and limitations of sub-technologies of spatial multi-omics

in situ hybridization (FISH) and in situ sequencing (ISS). Recent advancements feature highly multiplexed single-molecule FISH (smFISH), which uses reverse complementary oligo probes conjugated with fluorophores [9] for precise mRNA quantification and localization at the single-cell level [10]. The specificity of fluorescent probes to their RNA targets is critical for reliable smFISH results [11]. While smFISH can detect many transcripts due to high hybridization efficiency, signal overlap complicates barcode deconvolution. To address this issue, single-molecule imaging and multiplexed error-robust FISH (MERFISH) (Fig. 2A) have been developed, allowing the identification of thousands of RNA species in single cells by reducing optical crowding, albeit at the cost of increased imaging rounds and time [12]. Sequential FISH (seqFISH) [13, 14] (Fig. 2B), an in situ three-dimensional (3D) multiplexed imaging method, also addresses optical crowding by decreasing the number of transcripts per image, requiring additional imaging rounds. Despite these advancements, smFISH is limited by the spectral overlap of fluorophores, restricting its multiplexing capabilities and its effectiveness in analyzing cell heterogeneity in complex tissues [15]. For example, Long et al. utilized seqFISH to analyze the hippocampus, identifying distinct transcriptional states by quantifying and clustering 249 genes in 16,958 cells [14], thereby demonstrating the effectiveness of this method for detailed transcriptional profiling in complex tissues.

Both ISH and ISS provide similar transcriptomic information, with the primary difference being that ISS-based methods directly read nucleotide sequences within tissues to identify a larger number of RNA-targeting probes, while ISH-based methods image the sequences of barcoded FISH probes [9]. As a targeted spatial transcriptomics technology, ISS facilitates highly multiplexed in situ gene expression profiling through padlock probes, rolling circle amplification (RCA), and sequencing-by-ligation [16, 17] chemistry combined with next-generation sequencing chemistry [18]. In ISS, reverse transcribed cDNA is hybridized with padlock probes containing gene-specific barcode sequences, which are ligated at the specific hybridization site and amplified by rolling circle amplification (RCA) with a circularized padlock primer probe [9]. Chatarina et al. developed a method that combines padlock probes with in situ target-primed rolling-circle amplification to detect and genotype individual transcripts, offering deeper insights into mRNA expression heterogeneity within single-cell populations [19]. Sequential imaging using sequencing-by-ligation allows for the identification of repeatedly amplified barcode sequences in situ, while fluorescent in situ sequencing (FISSEQ) (Fig. 2C) employs an oligonucleotide ligation and detection substrate (SOLiD) for genome and transcriptome sequencing of DNA amplicons [9, 17]. FISSEQ experiences fewer issues with optical crowding compared to ISH-based methods because it is less efficient at converting transcripts into cDNA in situ. However, methods that use padlock probes hybridized with target RNA species require enzyme ligations and have lower detection rates compared to multiplexed FISH methods [12]. Next-generation FISSEQ [20] was developed to complement spatially structured sequencing libraries and includes an imaging method capable of resolving amplicons, which is essential for conducting ISS of cellular RNA for gene expression profiling [17]. RNA is reverse transcribed in fixed cells with tagged random hexamers to generate cDNA amplicons within the cell, which can be repeatedly hybridized with minimal changes in signal-to-noise ratios or position [21]. RNA sequencing libraries can be visualized in different cell types, tissue sections, and whole-mount embryos, enabling 3D visualization spanning multiple resolution scales [17]. Spatially resolved transcript amplicon readout mapping (STARmap) [22, 23] (Fig. 2D) employs dynamic annealing and ligation (SEDAL) to reduce sequencing errors. This technology integrates hydrogel tissue chemistry, targeted signal amplification, and ISS [22], enabling high multiplexing and analysis of thicker tissue slices, although it may detect fewer transcripts in such slices [24]. BaristaSeq, an optimized padlock probe-based technique compatible with Illumina sequencing, significantly enhances amplification efficiency and sequencing accuracy, achieving at least 97% accuracy and a five-fold increase in amplification efficiency [25].

Both ISS and ISH-based methods require image processing to generate gene expression matrices. These images are segmented to create cell-level matrices, which can be done manually for small areas or systematically using computational approaches [3, 26]. RNA hybridization-based spatial transcriptomics provides exceptional detection sensitivity [27]; however, the misassignment of mRNAs during cell segmentation is a significant source of error. To address this, the JSTA computational framework utilizes prior knowledge of cell type-specific gene expression to perform joint cell segmentation and cell type annotation, increasing the accuracy of RNA assignment by over 45% [28]. Spot-based spatial cell-type analysis by multidimensional mRNA density estimation (SSAM) is a robust cell segmentation-free computational framework that identifies cell types and tissue domains in both 2D and 3D [29].

(2) *Oligonucleotide-based spatial barcoding followed by the next-generation sequencing (NGS)* [20]. NGS represents a significant improvement over previous sequencing technologies, offering cost-effective, rapid sequencing with higher throughput, thereby greatly extending our genomic knowledge [30] and addressing

(See figure on next page.)

Fig. 2 Technologies of spatial techniques. **A** The MERFISH technology, a binary barcode scheme that employs different fluorescent probes to sequentially detect each bit. **B** The seqFISH technology. Complete RNA in cells/tissues was imaged by multiple rounds of hybridization. Each round obtains a coded message, corresponding to a bit in the digital code, and then decodes it to correspond to each RNA. **C** FISSEQ incorporates amplification after reverse transcription of cellular RNA into cDNA. **D** STARmap is based on DNA tandem sequencing technology, using complementary pairing principle of DNA and fluorescent dye labeled nucleotide probe for sequence determination. **E** LCM-seq utilizes a laser beam to microdissect tissue regions under a microscope. **F** IGS combines in situ sequencing with high-throughput paired-end DNA sequencing. **G** Slide-DNA-seq is used to fragment genomic DNA in situ by tissue, and barcode connector with spatial information is added for subsequent second-generation sequencing. **H** CUT and Tag guides Protein A/G-Tn5 transposase to cut the target chromatin region through protein-specific antibodies such as transcription factors. At the same time, sequencing joints are added to both ends of the sequence to form a library for high-throughput sequencing by PCR amplification

the time and resource-intensive challenges faced by the Human Genome Project [31]. NGS technologies introduce three main improvements over first-generation sequencing. First, they rely on the preparation of NGS libraries in a cell-free system, eliminating the need for bacterial cloning of DNA fragments [32]. Second, numerous sequencing reactions are produced in parallel, enhancing efficiency [33]. Third, sequencing outputs are detected directly, with base interrogation performed cyclically and in parallel [34]. Several prominent NGS platforms have emerged, including 454 (pyrosequencing) [35], Illumina/Solexa, and Sequencing by Oligo Ligation Detection (SOLiD) [36]. The 454 approach involves the clonal amplification of DNA fragments on beads within emulsion droplets, which are then loaded into wells for sequencing using the pyrosequencing protocol [37]. This approach enables the sequencing of long reads, making it suitable for various applications, although its inherent problem in detecting homopolymers and nucleotide stretches can impact data quality as sequence volume increases [38]. Illumina/Solexa employs an array-based DNA sequencing-by-synthesis technology with reversible terminator chemistry [39]. Primers, DNA polymerase, and four differently labeled reversible terminator nucleotides are used, with each nucleotide identified by color, followed by terminator and fluorophore removal, and the cycle repeating [34]. This platform currently offers the highest throughput and lowest per-base cost, making it the leading NGS platform. In contrast, the SOLiD platform prepares sequencing libraries by emulsion polymerase chain reaction (PCR) and sequences through successive cycles of ligation [39], exhibiting the lowest error rate among the three platforms. However, NGS methods have several drawbacks, notably short reads that fail to cover full-length transcripts in eukaryotic genomes and challenges in detecting larger structural variations. Additionally, the reliance on PCR amplification can lead to difficulties in regions with extreme GC content [40]. The advent of single-molecule, third-generation sequencing technologies, such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), has resolved these issues. PacBio and ONT offer read lengths exceeding 15 kb and 30 kb, respectively, surpassing the length necessary to capture most RNA molecules in eukaryotes. Furthermore, ONT long-read sequencing does not require PCR amplification, thereby reducing potential bias [41, 42].

(3) *Laser capture microdissection (LCM).* A key challenge in transcriptomics is precise segmentation of tissues and accurate assignment of individual cells to specific locations, often resulting in the loss of spatial information [12]. LCM (Fig. 2E), a powerful, microscope-guided cutting system that uses ultraviolet (UV) light as a contact- and contamination-free knife [43], enables accurate isolation of specific tissues or cells of interest from complex tissue structures. The combination of smart-3SEQ and LCM overcomes various experimental design challenges posed by conventional single-cell RNA-sequencing (scRNA-seq). For instance, formalin-fixed, paraffin-embedded archival clinical tissues, which are unsuitable for conventional RNA-seq due to their inability to be physically dissociated, and fresh or frozen non-archival tissue samples that lack sufficient material for clinical studies can be effectively analyzed using the LCM smart-3SEQ technique [11].

Spatial genomics

The proper functioning of tissues relies on the precise spatial organization of cell types, which is influenced by both intrinsic genetic factors and the external cellular environment. In cancer, tumor cells exhibit multiple DNA mutations and large chromosomal rearrangements, resulting in intratumor genetic heterogeneity [44]. Additionally, cells within the tumor microenvironment (TME) interact with each other, forming spatial neighborhoods with distinct biochemical and biomechanical properties. Quantifying these genetic aberrations and environmental cues within tumors is critical for understanding cancer progression and improving treatment [45]. In situ genome sequencing (IGS) (Fig. 2F) and slide-DNA-seq (Fig. 2G) are two exciting methods that promise to fuel the spatial genomics revolution [46]. IGS expands non-targeted genomic samples in a natural spatial environment, creating an in situ sequencing library in a fixed sample using in vitro

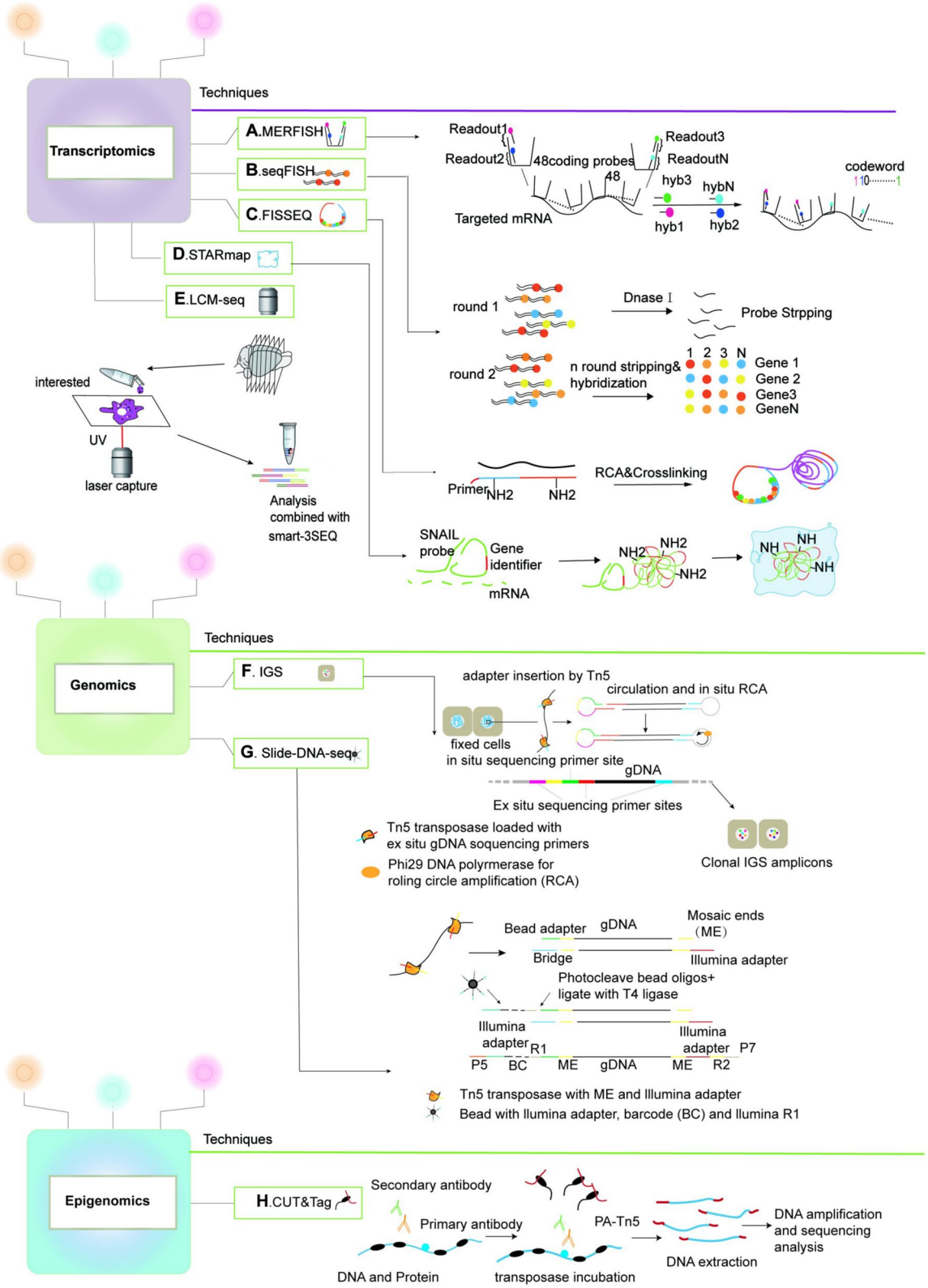

Fig. 2 (See legend on previous page.)

Table 2 Technologies for spatial multi-omics

Method	Technology	Resolution	References
Integrating transcriptomics and genomics			
Slide-DNA-seq	Microarray-based sequencing	25 μm	[45]
DNA-MERFISH	Probe hybridization -based sequencing	Subcellular	[152]
DNAseqFISH +	Probe hybridization -based sequencing	Subcellular	[93]
Integrating epigenomics and transcriptomics			
Spatial ATAC–RNA-seq	Microchannel-based microfluidics sequencing	20–25 μm	[88]
Spatial CUT&Tag–RNA-seq	Microchannel-based microfluidics sequencing	20–25 μm	[88]
MISAR-seq	Microchannel-based microfluidics sequencing	50 μm	[153]
Integrating proteomics and transcriptomics			
SM-Omics	Microarray-based sequencing	100 μm	[96]
Slide-TCR-seq	Microarray-based sequencing	10 μm	[154]
Spatial CITE-seq	Microchannel-based microfluidics sequencing	20 μm	[155]
SPOTS	Microarray-based sequencing	55 μm	[156]
DBiT-seq	Microchannel-based microfluidics sequencing	10–25 μm	[91]
seqFISH	Probe hybridization -based sequencing	Subcellular	[157]
MERFISH	Probe hybridization -based sequencing	Subcellular	[158]
Integrating spatial transcriptomics and metabolomics			
SMA	Mass spectrometry imaging	10–55 μm	[88, 103]
Integrating of spatial transcriptomics, genomics and proteomics			
MIP-seq	Multi-omics in situ pairwise sequencing	Subcellular	[103]

Technologies for spatial multi-omics; DNA-MERFISH, multiplexed error-Robust fluorescence in situ hybridization; DNAseqFISH +, Sequential fluorescence in situ hybridization plus; Spatial ATAC–RNA-seq, Spatially resolved assay of transposase-accessible chromatin with sequencing and RNA sequencing; Spatial CUT&Tag–RNA-seq, spatial assay of cleavage under targets and tagmentation and RNA using sequencing; MISAR-seq, Microfluidic indexing-based spatial assay for ATAC and RNA-sequencing; Spatial CITE-seq, Spatial cellular indexing of transcriptomes and epitopes by sequencing; SPOTS, Spatial protein and transcriptome sequencing; DBiT-seq, deterministic barcoding in tissue for spatial omics sequencing; seqFISH, sequential fluorescence in situ hybridization; SMA, spatial multimodal analysis; MIP-seq, Multi-omics in situ pairwise sequencing;

transposon technology to fragment DNA. Hairpin DNA splices are then connected to DNA fragments to form circular DNA, which is amplified via rolling circle replication mediated by Phi29 DNA polymerase. Sequencing is performed at both in situ and ectopic sites on the circular DNA fragments [47]. IGS spatially locates paired-end sequences of the whole genome in an endogenous environment, combining sequencing and imaging to construct a genome map [47]. This technique specializes in high-resolution imaging of chromosome structure, allowing detailed analysis of tissue sections. Slide-DNA-seq enables spatially resolved sequencing of DNA from intact tissues. The process begins with generating a spatial index array composed of 3 mm beads, each containing unique DNA barcodes corresponding to specific spatial locations. This array is then read through chemical sequencing [48]. Next, a single 10 μm thick fresh-frozen tissue slice is transferred onto the sequencing bead array. Spatial barcoding is performed through photolysis, and the proximal genome fragments are attached and amplified via PCR to create a DNA sequencing library [49]. Following library construction, high-throughput paired-end sequencing is carried out, associating each genome fragment with its spatial location on the bead array using DNA barcoding [45]. Slide-DNA-seq enables detection of clonal heterogeneity, characterization of copy number variations in each clone, and analysis of their spatial distribution within tissue. This technique is particularly useful for large-scale mapping of tumor evolution, providing essential spatial context to the study of clonal heterogeneity [45, 50]. Current methods for characterizing chromatin states or DNA within tissues on a large spatial scale are still in their infancy. The integration of spatial multi-omics technologies aims to achieve spatially resolved whole-exome or whole-genome sequencing. Ultimately, integrating various spatially resolved omics technologies will mark the beginning of the era of molecular anatomy, offering unprecedented insights into tissue organization and function [46].

Spatial proteomics

Proteomics involves the large-scale study of proteins, encompassing their expression levels, post-translational modifications, and protein–protein interactions, thereby providing a comprehensive understanding of processes such as disease occurrence and cell metabolism at the

protein level. Proteins, whether in their native or modified forms, are functional units within the body, making the direct study of proteomics more valuable than relying on transcripts. Targeted localization of proteins within eukaryotic cells can redirect existing proteins to various transport pathways, including nuclear, mitochondrial, ciliary, peroxisomal, endomembrane, and vesicular transport [51], enabling rapid changes in local protein functions. Conversely, protein mislocalization is frequently associated with cellular dysfunction and diseases such as neurodegeneration, cancer [52], cystic fibrosis [53], and metabolic disorders. Therefore, researching protein localization at the subcellular level and capturing subcellular dynamics are crucial for a complete understanding of cell biology. Two primary approaches are used to acquire large-scale spatial proteomic data, including mass spectrometry (MS) and imaging-based methods.

(1) *Mass spectrometry-based methods*: These approaches offer accurate, proteome-wide identification and quantification of proteins. In subcellular proteomics, specific subcellular compartments are often isolated through biochemical fractionation or proximity labeling before MS analysis [54]. Key processes involve the enrichment and quantification of proteins through biochemical fractionation across different stages using MS [55]. Organelles are separated based on properties such as size, density, membrane solubility, or charge, with differential and density centrifugation being common strategies. These methods typically achieve high sensitivity and proteome coverage, although contamination from non-target proteins can occur. Ensuring adequate enrichment of the target organelle is crucial for accurate analysis. Once purified, the distribution profiles of proteins specific to different organelles can reveal the subcellular localization or complex binding of uncharacterized proteins [54]. MS analysis, combined with multivariate statistics and machine learning (ML), is widely used to handle the complex data generated in spatial proteomics [56]. These techniques compare the abundance distribution of proteins with known organelle markers to infer protein locations and trafficking pathways [57]. They can identify trends in organelle protein distribution, even in the presence of structural alterations. Proteins, which can have different morphologies and modified states, function as essential units within cells. The relationship between mRNA and corresponding protein expression is highly regulated and non-linear, making RNA expression an unreliable predictor of protein levels. Unlike the more random expression of transcripts, proteins exhibit a much lower coefficient of variation than their homologous mRNA counterparts. Therefore, directly studying proteins at the single-cell level is far more informative than using transcripts as proxies [58]. Deep visual proteomics (DVP) combines artificial intelligence-driven image analysis of cellular phenotypes with automated single-cell or single-nucleus laser microdissection and ultra-high-sensitivity MS. This technique associates protein abundance with complex cellular or subcellular phenotypes while preserving spatial context [59]. To achieve this, an ultra-sensitive liquid chromatography-mass spectrometry (LC/MS) workflow has been developed, enhancing sensitivity by up to two orders of magnitude to enable true single-cell state proteomic analysis. The data generated by DVP provide molecular insights into proteomic variation at the phenotypic level while retaining complete spatial information.

(2) *Imaging-based methods*: These approaches allow for the visualization of proteins in situ without requiring cell lysis or the physical separation of compartments or organelles. Unlike MS methods, which are faster and suitable for large-scale quantitative analysis, imaging-based approaches visualize the interactions between proteins and affinity reagents. Modern microscopes can simultaneously analyze up to 50 proteins, but each protein of interest requires specific and validated antibodies, limiting high-throughput detection. To minimize the loss of soluble proteins during cellular permeability, it is best to use non-specific crosslinking in proteome-wide studies. It is increasingly evident that protein expression varies even among genetically identical cells. Imaging-based methods can capture this variation by targeting the spatial distribution of proteins at single-cell resolution [60]. However, the number of published global spatial proteomic studies remains small due to the high cost and time-consuming production of affinity reagents for entire proteomes.

MS and imaging methods each have unique advantages and disadvantages and can complement each other. MS offers high sensitivity, high resolution, and powerful quantitative analysis but involves complex and costly sample preparation. In contrast, imaging methods provide high spatial resolution and dynamic observation capabilities, enabling visualization of protein distribution but have limited quantitative abilities and cover only a small number of proteins. Combining these two techniques allows for comprehensive global protein analysis, enabling the observation of the spatial distribution and dynamic changes of key proteins for a more complete understanding of protein spatial organization.

Spatial epigenomics

Spatial epigenomics examines modifications to the DNA sequence and chromatin structure that regulate gene activity without altering the genetic code itself [61]. Nucleosomes, the fundamental units of chromosomes, are organized into higher-order chromatin structures. Epigenetic modifications, such as histone acetylation,

methylation, phosphorylation, ubiquitination, and DNA methylation, play crucial roles in regulating chromatin structure and DNA accessibility [62, 63]. These modifications impact key cellular processes, including gene transcription, DNA replication, recombination, and repair [61]. Unlike other omics fields, epigenomics relies heavily on bioinformatics to uncover the mechanisms by which the epigenome operates at the molecular level. Developing powerful, repeatable, and process-based techniques is essential for generating data that can be integrated into existing omics databases. The ultimate goal is to create a comprehensive picture of the epigenome by combining information on DNA methylation, chromatin dynamics, accessibility, and gene expression [64]. Epigenomic MERFISH combined with the recently developed Cleavage Under Targets and Tagmentation (CUT&Tag) approach (Fig. 2H) enables the mapping of more than 100 epigenomic loci in tissues [65]. These maps can be used to study patterns of active and silent promoters and potential enhancers, providing deeper insights into the spatial organization and regulation of the epigenome [65].

Spatial metabolomics

Metabolites play a crucial role in various cellular activities, such as cell signaling, energy transfer, and intercellular communication [66]. Metabolomics is an emerging discipline that involves the qualitative and quantitative analysis of all low-molecular-weight metabolites within an organism or cell during specific physiological states [67]. Analyzing metabolites presents challenges due to their dynamic nature and susceptibility to environmental influences during cellular processes [68]. Spatial metabolomics involves the initial detection and quantification of metabolites present in biological material [69]. Depending on experimental objectives, researchers can employ either targeted approaches, focusing on quantifying specific analytes, or untargeted approaches, focusing on biomarker discovery and global metabolite profiling [70].

(1) *Targeted metabolomics* [71]: This approach analyzes specific subsets of compounds to address particular biochemical questions or hypotheses. The two primary methods include Fourier transfer mass spectrometry (FT-MS) [72] and nuclear magnetic resonance (NMR) [73], both of which offer significant advantages in data acquisition due to their specificity and quantitative reproducibility. FT-MS generates mass data for infused samples, allowing for the identification and matching of metabolites with entries in metabolomics databases. The major drawback of this method is that it does not establish a one-to-one correspondence relationship between entities, which means that a single data point can potentially match with multiple metabolites. NMR produces signals based on the chemical environment of protons present in each metabolite, enabling tentative identification [74]. The development of triple quadrupole (QqQ) MS provides a robust and sensitive method for high-throughput measurement of a substantial number of biologically significant metabolites. This technique is particularly effective for quantifying low-concentration metabolites that are difficult to detect using NMR [75].

(2) *Untargeted metabolomics*: Untargeted metabolomics aims to globally analyze biological compounds, permitting the simultaneous detection of as many metabolites as possible and the exploration of cellular biochemical pathways. LC/MS is the most commonly used platform for untargeted metabolomics [76, 77], producing numerous signals during the detection of biological samples. The structural diversity of metabolites is vast, and the acquired data often include both known and unknown metabolites. When searching metabolomics databases for the mass–charge ratio of each detected feature, only a small percentage match the database entries, making the identification of unknown metabolites challenging [78]. The number of detected unknown metabolites is often overestimated due to several factors. A high concentration of ^{13}C can cause a mass shift, leading to the detection of multiple features for a single metabolite through naturally occurring isotopes. Additionally, a single metabolite can be ionized into various adducts, including isomers, increasing the demand for selective analytical techniques. Furthermore, metabolites can fragment or form non-covalent interactions with other metabolites upon entering the mass spectrometer. These factors collectively increase the complexity and diversity of detected metabolites [79].

LC/MS data analysis addresses the complexity of metabolite detection through two main approaches: (1) grouping metabolites with similar features and (2) annotating the type of ion species. These steps facilitate the identification of excimer ions, which are essential for further metabolite identification, such as determining elemental composition or conducting tandem MS based on accurate mass and isotope patterns. CAMERA (an integrated strategy for compound spectral extraction and annotation of LC/MS datasets) can effectively identify most features corresponding to isotopes, adducts, and fragments [80]. Isotopic labeling methods can also be used to identify and analyze isotope ratio outliers [81]. Despite these advancements, many metabolites remain uncharacterized. Variations in metabolites within the cellular environment are closely linked to health and disease development. Metabolomics enhances disease analysis at the genomic and protein levels by providing semi-quantitative and quantitative measurements of metabolite levels, which serve as chemical mediators defining specific phenotypes [70]. The rapid expansion of omics technologies has provided holistic molecular information,

enabling the comprehensive study of biological systems. Small molecules and metabolites are essential for numerous cellular functions [82], offering unique insights into the phenotypic characteristics associated with genome sequences [83].

Integration of spatial multi-omics

While single-cell multi-omics yields valuable insights into gene regulation across various omics layers [84, 85], it lacks the spatial information necessary for understanding cellular functions within tissues. Recently, spatial transcriptomics, proteomics, genomics, epigenomics and metabolomics have emerged, with extensive application in various fields [86, 87]. These techniques typically capture only one layer of omics information, and computational methods for integrating data from different omics layers cannot fully overcome the lack of mechanistic links between them. Spatial multi-omics enables the simultaneous analysis of multiple data modalities, such as transcriptomics, proteomics, genomics, epigenomics, and metabolomics, with the same tissue section (Table 2).

Integration of spatial transcriptomics and (epi)genomics

Spatial ATAC&RNA-seq and spatial CUT&Tag RNA-seq have revolutionized genome-wide co-mapping of the epigenome and transcriptome by simultaneously profiling chromatin accessibility and mRNA expression, or histone modifications and mRNA expression, respectively. These technologies integrate the chemistry of spatial ATAC-seq or CUT&Tag with spatial transcriptomics on the same tissue section at the cellular level via deterministic co-barcoding [88], combining microfluidic deterministic barcoding in tissue (DBiT) strategies for spatial ATAC-seq [89] and CUT&Tag [90] with DBiT-seq poly(A) transcript profiling [91]. Spatial-ATAC-seq enables high-spatial-resolution genome-wide mapping of chromatin accessibility in tissue at the cellular level by applying a spatial barcoding scheme to DNA oligomers inserted into accessible genomic loci by Tn5 transposition [89]. This technology advances our understanding of cell identity, cell state, and cell fate decisions related to epigenetic bases in development and disease. Spatial-CUT&Tag analyzes spatial histone modification profiling at the pixel level on frozen tissue sections without requiring dissociation. This method addresses spatially distinct and cell type-specific chromatin modifications during mouse embryonic organogenesis and postnatal brain development, adding a new dimension to spatial biology by mapping epigenetic regulation related to development and disease [90]. DBiT-seq creates a 2D grid of spatially barcoded tissue pixels, each defined by a unique combination of barcodes A and B [88]. After reverse crosslinking, barcoded complementary DNA and genomic DNA fragments are released, and NGS constructs separate libraries for gDNA and cDNA. Sequencing reads are then combined with microscopy images of the tissue section based on spatial barcodes, allowing multi-omics sequence information to be spatially mapped [88]. These techniques have been applied to co-map embryonic and juvenile mouse brains, as well as the adult human brain. Spatially resolved, genome-wide co-sequencing of the epigenome and transcriptome at the cellular level provides an informative tool for a wide range of biological and biomedical research. Transcriptomics focuses on gene expression from the perspective of mRNA, presenting a global perspective on molecular dynamic changes induced by environmental factors or pathogenic agents [92]. Benefiting from mature in situ RNA hybridization strategies, targeted capture of DNA sequences or chromosomal loci facilitates spatial genomics detection. DNA-seq FISH + can be applied for studying the spatial structure of the genome based on multi-round probe hybridization imaging. Takei et al. [93] reported the imaging of 3660 chromosomal sites in a single mouse embryonic stem cell (ES) using DNA-seq FISH + and the imaging of 17 chromatin markers and subnuclear structures by sequential immunofluorescence and expression profiles of 70 RNAs. Genomic regions and chromosomes associated with nuclear bodies and chromatin marks in different cells were revealed by genomic regions. Some of these regions appear to be related to cell types, whereas others (mostly spot-related regions) are more conserved among different cell types [46].

Integration of spatial proteomics and transcriptomics

Single-cell multi-omics has been highly successful in capturing diverse biological processes at the level of individual cells and nuclei but lacks spatial information [94]. Gene expression is regulated at multiple levels, from transcription to protein degradation, with RNA and protein levels conveying distinct information about gene function and cell state. These processes occur in various contexts, such as tumors and single-cell suspensions [95]. Recent progress in spatial in situ profiling has enabled the simultaneous profiling of location and expression. Spatial transcriptomics provides a global spatial tissue profile and has been applied to the study of diverse diseases. Spatial proteomics acquires large-scale spatial proteomic data through MS- and imaging-based experimental approaches. However, few platforms have successfully integrated spatial proteomics and transcriptomics data. Vickovic et al. [96] developed Spatial Multi-Omics (SM-Omics), an end-to-end framework that leverages a liquid handling platform for high-throughput transcriptome and antibody-based spatial tissue profiling. Using DNA-barcoded antibodies, this automated system enables the

simultaneous profiling of the epitopes and transcriptomes within single cells, offering detailed molecular characterization of tissues in situ by quantifying both spatial transcriptomics and multiplex protein detection [96]. Compared to Visium by 10X Genomics, SM-Omics provides an automated workflow that extends combined spatial transcriptomics and antibody-based protein measurements into a scalable all-sequencing-based technology.

NanoString GeoMx Digital Spatial Profiler (DSP) facilitates high-plex profiling at both the protein and RNA level, permitting spatial and temporal assessment of tumors in frozen or formalin-fixed, paraffin-embedded limited tissue samples [97]. This platform quantifies protein or RNA abundance by counting unique indexing oligos assigned to each target of interest, using oligonucleotides to study a higher number of biomarkers. Additionally, DSP is a non-destructive technique, allowing the same slides to be used for subsequent studies after the assay is completed [97].

Spatial co-indexing of transcriptomes and epitopes (Spatial-CITE-seq) offers high-plex protein and whole-transcriptome co-mapping. This approach involves the staining of a tissue slide with a cocktail of approximately 200–300 antibody-derived tags (ADTs), followed by deterministic in-tissue barcoding of both DNA tags and mRNAs. Each tag contains a unique spatial address code AiBj (i = 1 − 50, j = 1 − 50), co-indexing all protein epitopes and the transcriptome. Barcoded cDNAs are subsequently retrieved, refined, and amplified via PCR to create two NGS libraries for paired-end sequencing of ADTs and mRNAs. This process enables computational reconstruction of spatial protein or gene-expression maps [98].

Integration of spatial transcriptomics and metabolomics

Gene expression and metabolite distribution in tissues are influenced by a variety of factors, including cell type, microenvironment, signaling pathways, and gene regulation. To elucidate the interplay among these factors, it is essential to employ methods that can simultaneously measure molecular evidence of different patterns in tissues while preserving spatial distribution information. Researchers have developed a spatial multimodal analysis (SMA) protocol that combines spatially resolved transcriptomics and mass spectrometry imaging (MSI) in a single tissue slice, while maintaining the specificity and sensitivity of both analytical methods [88]. This integrated approach reveals associations and heterogeneities between transcriptomes and metabolomes across different tissue regions. Combining spatial transcriptomic and metabolomic data, Vicari et al. identified a reduced proportion of midbrain dopaminergic neurons (MBDOP2) in the lesioned substantia nigra pars compacta and ventral tegmental area, and specified the localization of multiple neurotransmitters and metabolites, including taurine, 3-methoxytyramine, 3,4-dihydroxy-phenylacetaldehyde (DOPAL), 3,4-dihydroxy-phenylacetic acid, norepinephrine, serotonin, histidine, tocopherol, and gamma-aminobutyric acid [88]. Oral submucous fibrosis (OSF) is a well-established precancerous lesion, but the molecular mechanisms underlying its malignant transformation into oral squamous cell carcinoma (OSCC) remain unclear [99]. Yuan et al. integrated spatial transcriptomics and metabolomics to obtain spatial location information on cancer cells, fibroblasts, and immune cells, as well as the transcriptomic and metabolomic landscapes of OSF-derived OSCC tissues. Moreover, they revealed the malignant progression from in situ carcinoma (ISC) to partial epithelial-mesenchymal transformation (pEMT), and identified significant metabolic reprogramming, including abnormal polyamine metabolism, which may play a key role in promoting tumorigenesis and immune escape [100]. Zheng et al. [101] combined spatial transcriptomic and metabolic analyses to reveal metabolic heterogeneity and complex transcriptome regulation in injured human brain tissue, facilitating the design of reagents for functional analysis of specific genes. The simultaneous application of these advanced technologies reveals the spatial composition of functional maps within tissues, heterogeneous distribution of cell populations, and differential gene expression in different locations. This comprehensive spatial expression mapping of genes holds significant research value and potential for advancing our understanding of complex biological systems.

Integration of spatial transcriptomics, genomics, and proteomics

The integration of spatial multi-omics aims to expand our understanding of mechanistic relationships across different omics layers and uncover molecular roles essential for cellular function by jointly profiling the transcriptome, genome, epigenome, proteome, and metabolome. Spatially resolved joint analysis of multi-omics can facilitate the identification of novel cell subtypes and measurement of intracellular and intercellular molecular interactions [102]. Therefore, the need for advanced spatial multi-omics methods has become increasingly important. Multi-omics in situ pairwise sequencing (MiP-seq) is a high-throughput targeted in situ sequencing technique that simultaneously detects multiplexed DNA, RNA, proteins, and biomolecules at subcellular resolution, providing comprehensive data for studying cellular functions and disease mechanisms [103]. The in situ detection of proteins and biomolecules is achieved using padlocking probes that target antibody-conjugated nucleic acids,

while the detection of DNA and RNA is accomplished through direct padlock probes targeting nucleic acids [103]. Compared to current in situ sequencing methods, MiP-seq utilizes a pairwise-sequencing strategy and dual barcoded padlock probes, markedly increasing decoding capacity and requiring fewer sequencing rounds (10^N vs. 4^N). Consequently, MiP-seq can reduce sequencing time by approximately 50%, lower sequencing and imaging costs, and minimize laser damage, thereby improving signal decoding accuracy, a key issue in in situ sequencing [104]. MiP-seq has been applied to mouse brain tissue, enabling the in situ detection of Rbfox3 and Nr4a1 gene loci, which are located on different chromosomes and spatially localized within the nucleus. MiP-seq has also been used to study PK-15 cells co-infected with porcine circovirus 2 (PCV2) and classical swine fever virus (CSFV), simultaneously detecting mRNA from eight cytokine or chemokine genes and two virus-specific proteins (CSFV E2 protein and PCV2 Cap protein) by binding antibodies to nucleic acids [103]. Thus, MiP-seq demonstrates versatility and high sensitivity in multi-omics in situ analysis, detecting specific DNA sequences, RNA transcripts, and proteins at single-cell resolution, and is a powerful tool for studying cell function, disease mechanisms, and cell–cell interactions in complex biological systems.

Applications of spatial multi-omics

Deciphering spatial-specific atlas production of molecular and cellular profiles

A comprehensive spatial-specific atlas of molecular and cellular profiles in both healthy and diseased states is essential for developing new therapeutic targets and disease interventions (Fig. 3A). Spatial transcriptomics combined with single-cell sequencing has been widely used to decipher molecular profiles. Fang et al. constructed a spatial atlas of the human middle and superior temporal gyrus using MERFISH, revealing differences in the cellular composition of these cortical regions between humans and mice [105]. Single-nucleus RNA-seq (snRNA-seq), single-nucleus assay for transposase-accessible chromatin with sequencing (snATAC-seq) [106], and spatial transcriptomics have been applied to generate a spatially resolved multi-omics single-cell atlas of the entire human maternal–fetal interface, including the myometrium, enabling resolution of the full trajectory of trophoblast differentiation [107]. Kuppe et al. used snRNA-seq and spatial transcriptomics to create an integrative high-resolution map of cardiac remodeling, enhancing the spatial resolution of cell-type composition and providing spatially resolved insights into the cardiac transcriptome and epigenome with identification of distinct cellular zones of injury, repair, and remodeling [106]. Advanced spatial epigenome-transcriptome co-sequencing has revealed how epigenetic mechanisms control transcriptional phenotypes and cell dynamics at both spatial and genome-wide levels, providing new insights into spatial epigenetic initiation, differentiation, and gene regulation within tissue structures. Spatial ATAC-RNA-seq and spatial CUT&Tag-RNA-seq were first introduced in analyzing mouse embryos, successfully distinguishing each organ with epigenetic and transcriptome data [88]. In some mouse brain tissue regions, the epigenetic signature of certain genes persisted with development, but the gene expression was different. In addition, the results of the joint analysis also found that epigenetic regulation and gene expression in different regions of the brain of young mice have unexpected correlations, and that different epigenetic features can cooperate with each other to regulate gene expression. The integration of spatial multi-omics not only opens a new field of spatial omics but also provides novel research avenues for biological and biomedical research.

Spatial multi-omics decodes spatial-based heterogeneity in human diseases

The complex interactions among tumor cells, surrounding tissues, infiltrating innate immune cells, and adaptive immune cells create a unique environment characterized by inter-related, coexisting, and competitive dynamics [108]. The characteristics of this tumor immune microenvironment vary significantly due to both intrinsic (e.g., tumor type) and extrinsic factors (e.g., environment). Tumor heterogeneity plays a crucial role in enabling tumor cells to adapt to changes in the microenvironment, thereby promoting tumor resistance and progression (Fig. 3B).

Tumor heterogeneity includes both intratumor and intertumor heterogeneity [109]. Metastatic prostate cancer exhibits a wide spectrum of diverse phenotypes, but the extent of these heterogeneities has not yet been established [110]. Brady et al. integrated spatial transcriptomics and proteomics to analyze multiple discrete areas of metastases, discovering heterogeneity among tumors at different metastatic sites and within the same site. They also identified significant intra-patient heterogeneity in regions with varying androgen receptor (AR) and neuroendocrine activity. Most metastases lacked significant inflammatory infiltrates and PD1, PD-L1, and CTLA4 expression, while the B7-H3/CD276 immune checkpoint protein was highly expressed, particularly in metastatic prostate cancers with high AR activity [111]. These findings correlate with the clinical observation that metastatic prostate cancers often fail to respond to immune checkpoint blockade therapies such as anti-CTLA4, PD1, and PD-L1 antibodies, suggesting that

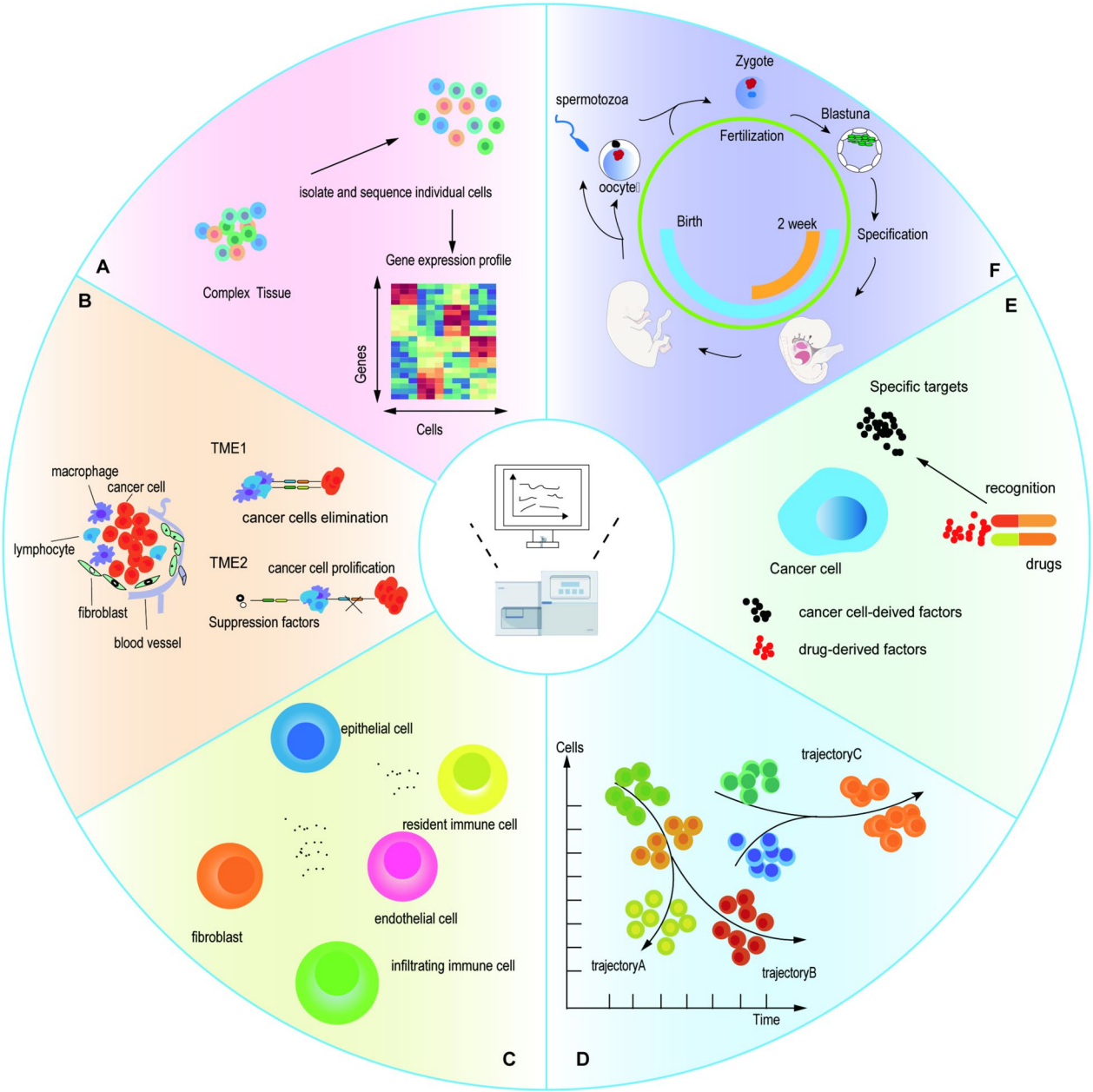

Fig. 3 Applications of spatial-based technologies. Spatial multi-omics technology is employed to investigate various cell biology. This diagram provides an overview of the application of spatial multi-omics. **A** Spatial-based molecular and cellular atlas. **B** Spatial-based heterogeneity in human diseases. **C** Spatial-related crosstalk in tumor immunology. **D** Spatial trajectory and lineage tracking in human diseases. **E** Potential targets for therapeutic applications. **F** Reproduction and development research

B7-H3/CD276 could be a potential therapeutic target. Non-small cell lung cancer (NSCLC) is characterized by substantial heterogeneity among individual tumors and within regions of a single tumor [112]. Intratumor heterogeneity has been shown to contribute to treatment failure and drug resistance through the expansion of pre-existing resistant subclones [113, 114]. Previous studies using multi-region profiling to decode the spatial patterns of heterogeneity were limited by the small number of regions analyzed per tumor [115]. Wu et al. employed multi-region matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), cyclic immunofluorescence (CyCIF), and multi-region single-cell copy number sequencing to conduct spatial multi-omics analysis of tumors from 147 lung adenocarcinoma patients. They developed a novel analysis approach to quantify

intratumor spatial heterogeneity: clustered geographic diversification (GD), where molecularly similar cells cluster together, and random GD, where molecularly similar cells are randomly distributed. Patients with random GD exhibited higher recurrence rates and risk of death, characterized by fewer tumor-interacting endothelial cells, higher infiltrating immune cells, and similar GD patterns observed in both proteomic and genomic data [116], providing insights into spatial heterogeneity and innovative ideas for cancer research. A non-targeted MALDI-MSI analysis [117] followed by spatial segmentation using different algorithms allowed to highlight molecular heterogeneity among glioblastomas. Three sub-regions were identified (A, B and C regions). Duhamel et al. performed a spatially resolved proteomic analysis to decode the biological pathways involved in these three regions: region A is enriched in genes related to neurotransmission and synaptogenesis; proteins overexpressed in region B are associated with immune infiltration; region C identified proteins involved in RNA processing and metabolism. Finally, they identified PPP1R12A and RPS14 are favorable prognostic markers while ALCAM, ANXA11, and AltProt IP_652563 are unfavorable prognostic markers [118]. These results highlight the potential of spatial proteomics and spatial metabolomics to decipher the molecular heterogeneity of glioblastoma and identify markers associated with survival.

Understanding how reprogrammed metabolic networks impact tumor growth is crucial for identifying metabolic vulnerabilities that improve cancer treatment. Sun et al. [119] combined mass spectrometry imaging-based spatial metabolomics and lipid-omics with microarray-based spatial transcriptomics [120] to visualize intratumor metabolic heterogeneity and cell metabolic interactions within the same gastric cancer sample. They imaged tumor-associated metabolic reprogramming at metabolic-transcriptional levels, linking marker metabolites, lipids, and genes within metabolic pathways and colocalizing them in heterogeneous cancer tissues. The integrated data revealed unique transcriptional features and significant immune-metabolic changes at the tumor invasion frontier. Furthermore, glutamine was overutilized in tumor tissue, genes related to lipids, fatty acid synthesis (FA), and fatty acid elongation were enriched in the tumor tissue region, and long chain polyunsaturated fatty acids were significantly up-regulated in borderline lymphoid tissue, even exceeding levels in tumor tissues [121]. These findings enhance our understanding of tumor molecular mechanisms and potential targets for cancer therapy. Spatial multi-omics technology accurately depicts gene expression in different tumor tissue locations, addressing the lack of spatial context in single-cell sequencing. Thus, the advancement of spatial omics provides essential support for exploring tumor immune microenvironment dynamics and identifying corresponding therapeutic targets.

Novel insights from spatial multi-omics analyze spatial-related crosstalk in tumor immunology

Spatial multi-omics has provided new perspectives on the complex interactions within the tumor microenvironment. Tumor tissue comprises various cell types, including epithelial, endothelial, fibroblast, vascular smooth muscle, resident immune, and infiltrating immune cells, all of which interact within a 3D environment to support cancer cell growth [122] (Fig. 3C). By integrating mass spectrometry imaging-based spatial metabolomics and lipidomics with microarray-based spatial transcriptomics, researchers have identified a distinct interface at the junction of tumors and neighboring tissues, termed cluster9, within which peritumoral lymphoid tissue (PLT) and distal lymphoid tissue (DLT) are defined [121]. The PLT exhibits significantly increased uptake and metabolism of glutamine, as well as certain fatty acids, essential for tumor energy metabolism and signaling. Genes associated with fatty acid synthesis, such as FASN, SCD, and ELOVL, as well as ALOX5AP, which promotes arachidonic acid metabolism into leukotriene inflammatory mediators, are also up-regulated in PLT. These results suggest that PLT has a stronger inflammatory response than DLT and inhibits tumor cell proliferation [121]. Identification of this crosstalk between PLT and tumor cells has enhanced our understanding of tumor molecular mechanisms. ScRNA-seq studies on glioblastomas have highlighted the dynamic plasticity across cellular states [123], including mesenchymal-like (MES-like), neural progenitor cell-like (NPC-like), astrocyte-like (AC-like), and oligodendrocytic precursor cell-like (OPC-like) states, which are markers of malignant brain tumors [124]. However, single-cell analysis provides only indirect inferences about cell interactions, often neglecting the role of the local microenvironment in tumorigenesis. Ravi et al. [125] utilized spatial transcriptomics, metabolomics, and proteomics to quantify the relationship between tumor cells and myeloid and lymphoid cells, discovering increased interactions in inflammation-related gene-rich areas and confirming enhanced interactions between tumor cells and virus-free compartments within transcriptionally defined reactive immune regions. Annika et al. [126] combined spatial multi-omics and scRNA-seq data from epithelial and stromal compartments to examine immune cell composition during intestinal damage and regeneration, finding that activated B cells decreased and disrupted the essential crosstalk between stromal and epithelial cells during mucosal healing. Spatiotemporal multi-omics allows for consideration of the microenvironment in cell–cell crosstalk studies, enhancing the accuracy of research findings.

Spatial trajectory and lineage tracking in human diseases

Lineage tracking technology is crucial for studying the developmental trajectory and differentiation process of cells (Fig. 3D). This technology can help determine how individual cells differentiate from a founder cell and how they evolve during development and disease [127]. Traditionally, lineage tracing involves labeling cells with heritable marks and tracking the trajectory of their offspring. The diversity of cell types produced from a founder cell reflects its differentiation potential. To predict the potential and evolutionary trajectory of founder cells, a wide array of markers is needed for accurate cell type classification. However, the limited availability of markers can mask the variability within cell subsets expressing the selected marker genes [128], potentially biasing the interpretation of organ complexity. Spatial transcriptomics not only enables comprehensive transcriptomic analysis of thousands of cells but also offers considerable insights into the spatiotemporal relationships among cells. This approach enhances cell-type identification, deepening our understanding of organizational complexity [129]. By constructing transcriptional atlases of adult tissues and developing embryos, spatial transcriptomics reveals the molecular mechanisms underlying differentiation from stem cells to mature cells. This detailed record elucidates the sequence of events and molecular mechanisms by which cells attain their final identity in embryogenesis or tissue regeneration. It also provides clues to the origins of developmental pathologies and cancer, allowing intervention in pathogenic pathways and replication of cell differentiation processes in vitro [130]. Densely sampling cells at various stages can describe state manifolds, which visualize the continuum of cell state changes in a multidimensional space and the trajectory of cell differentiation. To understand the instantaneous state of the cell, it is necessary to consider its molecular composition, inter-relationships, tissue position, and physical and regulatory interactions with surrounding cells. This comprehensive approach provides deeper insights into the state and function of cells [130]. Given the complexity of cells within different species, lineage tracing has expanded to include additional approaches, such as tracer dyes, cell transplantation, and in vivo genetic recombination. Advances in confocal and light-sheet microscopy have enabled the direct tracking of individual cell division patterns in complex vertebrates. However, these methods are limited to only a few measurements of cell state. Recent spatial transcriptomics approaches overcome spectral limitations by allowing genome-scale measurements in fixed in situ samples. High-throughput sequencing employs DNA sequence barcodes to encode clonal information, which can later be read and integrated with other sequence-based omics data. Zhang et al. applied single-cell and spatial transcriptomics to demonstrate extensive diversification of cells from a few multipotent progenitors to numerous differentiated cell states, including several novel cell populations. Furthermore, they identified lineage-specific clusters radiating from the center of six mesenchymal states and active transcription factor network modules associated with the progression of each lineage. They also observed that chondrocyte lineages increased over time, shifting from progenitor cells to more mature clusters [131]. Bao et al. [132] revealed that microglia and perivascular macrophages exhibit parallel differentiation processes, although the developmental origins of other tissue-resident macrophages require further exploration using single-cell and spatial transcriptomics. Spatial multi-omics have been applied in several fields, such as tumor progression, immune-associated diseases and metabolism-related disorders. Renal fibrosis, a critical pathological feature in chronic kidney disease progression, has significant global health implications. Spatial multi-omics techniques, such as Cut&Tag with DBiT-Seq [133], have been crucial in elucidating the complex epigenetic reprogramming during the transition from acute kidney injury to chronic kidney disease, underscoring the importance of multi-omics in understanding and addressing renal fibrosis pathogenesis [134]. The integration of imaging and sequencing-based omics has led to significant progress in spatial technologies, enabling spatially resolved single-cell detection [135]. These technologies preserve spatial resolution and large fields of view, allowing for detailed analysis of the microenvironment, spatial neighborhoods, and niche networks in kidney injury. Compatibility with formalin-fixed, paraffin-embedded tissue also facilitates the establishment of kidney injury cohorts, filling a critical gap in prognostic research [136].

Investigation of new therapies via spatial multi-omics

Targeting nucleotide metabolism is a well-established metabolic therapy in clinical oncology and practice [137]. However, efforts to target non-nucleotide metabolism in clinical trials have faced challenges due to drug toxicity, inconsistent dietary interventions, lack of biomarkers, and imprecise combination treatments, collectively leading to suboptimal trial outcomes. Additionally, cells within the TME can significantly influence treatment efficacy and undergo substantial changes during tumor progression and treatment response [138]. Therefore, developing biomarker-guided personalized precision metabolic therapies and targeted metabolic reprogramming is critical to improve the sensitivity of cancer therapy. Rational combinations of chemotherapy, radiation therapy, and other targeted therapies should also be considered. Integrating spatial multi-omics could enhance our understanding of

tumor metabolic regulation, offering new therapeutic targets and identifying diagnostic and prognostic markers for various diseases.

Through multi-omics analysis of patients with triple-negative breast cancer (TNBC), researchers discovered that *Clostridiales* and the associated metabolite trimethylamine N-oxide (TMAO) induce pyroptosis in tumor cells by activating the endoplasmic reticulum stress kinase PERK, which amplifies $CD8^+$ T cell-mediated antitumor immunity in vivo. These findings suggest that microbial metabolites, such as TMAO or its precursor choline, could serve as a new therapeutic strategy to enhance the efficacy of TNBC treatment [139], offering insights into the crosstalk between microbiota and metabolite immunology. Metastasis remains the leading cause of death in patients with breast cancer; however, the dynamic changes in dissemination evolution remain poorly understood. High-resolution technologies, such as spatial transcriptomics and metabolomics, have been used to map the metabolic landscape. Combined spatial transcriptomics and scRNA-seq have revealed metabolic changes in tumor cells during their transition from the primary site to the leading edge and metastatic lymph nodes, highlighting the potential of incorporating metabolic therapies in treating breast cancer with lymph node metastasis [140]. *Eclipta prostrata* L. [141] has long been used in traditional medicine for its liver-protective properties. Wedelolactone (WEL) and demethylwedelolactone (DWEL) are the primary coumarins found in *E. prostrata* L. Using a mature thioacetamide (TAA)-induced zebrafish model, Chen et al. integrated spatial metabolomics and transcriptomics and discovered that both WEL and DWEL can improve metabolic disorders induced by nonalcoholic fatty liver disease (NAFLD), primarily through the regulatory effects of WEL on steroid biosynthesis and fatty acid metabolism. Their study successfully mapped the biological distribution and metabolic characteristics of these compounds in zebrafish, revealing the unique mechanisms of WEL and DWEL in improving NAFLD and proposing a multi-omics platform to develop highly effective compounds that improve therapeutic outcomes [142]. Previous studies have highlighted the role of ferroptosis in a variety of neurological diseases [143], although its precise role in multiple sclerosis (MS) remained uncertain. Wu et al. integrated data from snRNA-seq, spatial transcriptomics, and spatial proteomics to define a computational metric of ferroptosis levels and identify the ferroptosis landscape in neuroimmunity and neurodegeneration in MS patients [144]. Results showed that active lesion edges exhibited the highest ferroptosis scores, associated with phagocyte system activation, while remyelination lesions had the lowest scores. Elevated ferroptosis scores were also observed in cortical neurons, linked to multiple neurodegenerative disease-related pathways [144], while significant co-localization was detected between ferroptosis scores, neurodegeneration, and microglia. They also established a diagnostic model for MS based on 24 ferroptosis-related genes in peripheral blood. These findings suggest that ferroptosis may play a dual role in MS, associated with both neuroimmunological and neurodegenerative processes, making it a promising therapeutic target and diagnostic marker for MS. Vedolizumab (VDZ) is known to inhibit lymphocyte trafficking to the intestine and is effective in treating ulcerative colitis (UC). However, its broader effects on other cell subsets are less understood. Using comprehensive spatial transcriptomic and proteomic phenotyping, Mennillo et al. identified mononuclear phagocytes as an important cell type impacted by anti-integrin therapy in UC and revealed changes in the spatial distribution of cell subpopulations in tissues before and after VDZ treatment [145]. Notably, they highlighted the cellular and genetic factors of UC and VDZ therapy, potentially aiding in the development of more precise treatment strategies and the prediction of treatment responses (Fig. 3E).

Multi-omics in reproduction and development research

Mammalian fertilization begins with the fusion of an oocyte and a sperm cell [146], with the reproductive system creating an environment for embryonic development (Fig. 3F). In-depth exploration of the reproductive system requires an understanding of the function of each cell type and their interactions. Spatial multi-omics techniques have been used to examine interactions between adjacent cells and gametes or embryos within the natural tissue environment, preserving the spatial context of the analyzed cells. These technologies have the potential to transform our understanding of mammalian reproduction [147]. Winkler et al. used scRNA-seq and spatial transcriptomics to profile the remodeling of the female reproductive tract during the estrous cycle, decidualization, and aging and discovered that fibroblasts play a central and organ-specific role in female reproductive tract remodeling by coordinating extracellular matrix (ECM) recombination and inflammation. They also revealed the unexpected costs of repeated remodeling required during reproduction and illustrated how estrus, pregnancy, and aging collectively shape the female reproductive tract [148]. Yang et al. conducted scRNA-seq, scATAC-seq, and spatial transcriptomic analyses of fetal samples from gestational week (GW) 13–18, generating a large-scale multi-omics atlas of the developing human fetal cerebellum. They found that PARM1 exhibits inconsistent distribution in human and mouse granulosa cells, and identified gene regulatory networks that control the diversity of

Table 3 Applications of integrated spatial-based technologies

Disease	Integrated spatial-based technologies	Findings	References
Spatial-based molecular and cellular atlas			
Human middle and superior temporal gyrus	Spatial transcriptomics combined single-cell sequencing	Spatial atlas of the human middle and superior temporal gyrus	[105]
Human maternal–fetal interface	Spatial transcriptomics, snRNA-seq and snATAC–seq	Generation of the entire human maternal–fetal interface atlas including the myometrium	[107]
Cardiopathy	Spatial transcriptomics and snRNA-seq	Generation of an integrative high-resolution map of cardiac remodeling	[106]
Mouse embryos	Spatial ATAC–RNA-seq and spatial CUT & Tag-RNA-seq	Unexpected correlations in epigenetic regulation and gene expression across different brain regions of young mice expression	[159]
Spatial-based heterogeneity in human diseases			
Metastatic prostate cancer	Spatial transcriptomics and spatial proteomics	Heterogeneity in tumors at different metastatic sites, as well as in different regions within the same site	[111]
Non-small cell lung cancer	MALDI-TOF (matrix-assisted laser desorption ionization-time of flight), CyCIF (cyclic immunofluorescence) and multi-region single-cell copy number sequencing	A novel approach for quantifying intra-tumor spatial heterogeneity	[116]
Glioblastoma	Non-targeted MALDI-MSI analysis	Molecular heterogeneity among glioblastomas	[118]
Gastric cancer	Mass spectrometry imaging-based spatial metabolomics and lipid-omics with microarray-based spatial transcriptomics	Intratumor metabolic heterogeneity and cell metabolic interactions in same gastric cancer sample	[121]
Spatial-related crosstalk in tumor immunology			
Gastric cancer	Mass spectrometry imaging-based spatial metabolomics and microarray-based spatial transcriptomics	Distinct tumor-neighboring tissue junction interface called cluster9	[121]
Glioblastoma	Spatial transcriptomics, spatial metabolomics, and spatial proteomics	Enhanced interactions between tumor cells and virus-free compartments in transcriptionally defined reactive immune regions	[125]
Intestinal damage and regeneration	Spatial multi-omics and scRNA-seq	Composition of immune cells during intestinal damage and regeneration	[125]
Spatial trajectory and lineage tracking in human diseases			
Human embryonic limb	Spatial transcriptomics and single-cell sequencing	Human embryonic limb cell atlas	[125, 131]
Macrophages	Spatial transcriptomics and single-cell sequencing	Parallel differentiation processes in microglia and perivascular macrophages	[132]
Renal fibrosis	Spatial multi-omics (Cut&Tag with DBiT-Seq)	PDGFRα + /PDGFRβ + mesenchymal cells as the primary cellular source of extracellular matrix	[134]
Potential targets for therapeutic applications			
Tumor metastasis	Spatial transcriptomics and metabolomics	Metabolic changes in tumor cells during transition from primary site to leading edge and metastatic lymph nodes	[160]
Nonalcoholic fatty liver disease	Spatial metabolomics and transcriptomics	WEL and DWEL's improvement of NAFLD-induced metabolic disorders, with effects primarily through WEL's regulation of steroid biosynthesis and fatty acid metabolism	[160]
Multiple sclerosis	Spatial transcriptomics, spatial proteomics and snRNA-seq	Ferroptosis score association with phagocyte system activation at the edges of active lesions	[144]

Table 3 (continued)

Disease	Integrated spatial-based technologies	Findings	References
Ulcerative colitis	spatial transcriptomics and proteomics	Anti-integrin therapy inhibits lymphocyte trafficking in ulcerative colitis	[145]
Reproduction and development research			
Female reproductive tract	Spatial transcriptomics and scRNA-seq	Profile of how the female reproductive tract is remodeled during the estrous cycle, decidualization, and aging	[148]
Human cerebellum	Spatial transcriptomics, scRNA-seq and scATAC-seq	Generation of a large-scale multi-omics atlas of the developing human fetal cerebellum	[149]
Human spinal cord	Spatial transcriptomics, scRNA-seq and hybridization-based in situ sequencing	A comprehensive atlas of developmental cells in the human spinal cord	[150]

Applications of integrated spatial-based technologies: (1) Deciphering spatial-specific atlas production of molecular and cellular profiles, (2) Decoding spatial-based heterogeneity in human diseases; (3) Analyzing spatial-related crosstalk in tumor immunology; (4) Analyzing spatial trajectory and lineage tracking in human diseases; (5) Investigating new therapies via spatial multi-omics; (6) Exploring spatial characteristics in reproduction and development research

Purkinje cells and unipolar brush cells [149]. These key regulatory factors can be harnessed in vitro to generate small brain cells for future clinical applications and enhance our understanding of the link between molecular variation and cell types in neurodevelopmental disorders. Li et al. employed scRNA-seq, spatial transcriptomics, and hybridization-based in situ sequencing to analyze 16 human embryonic and fetal spinal cord samples from post-conceptional weeks 5–12, providing a comprehensive atlas of developmental cells and identifying novel molecular targets and genetic regulation of childhood spinal cancer stem cells [150] (Table 3).

Perspectives

The rapidly evolving field of spatial omics technologies aims to achieve higher resolution, deeper coverage, greater multiplexity, and enhanced versatility in analyzing diverse samples, including formalin-fixed, paraffin-embedded, fresh-frozen, and living tissues. These advancements enable 3D reconstruction of larger tissue regions and comprehensive analysis of spatiotemporal multi-omics, enhancing our understanding of the complex molecular mechanisms underlying cellular interactions within tissues. Effective acquisition, manipulation, analysis, and visualization of spatial omics data are critical components for their successful application. Integrating datasets from different omics modalities is essential to unlock their synergistic potential, although this is challenging due to differing spatial features of the data. Consequently, there is an urgent need for specialized hardware and software to visualize these complex datasets effectively. Key steps include normalizing data matrices, removing low-quality data, improving signal-to-noise ratios, smoothing data to increase sensitivity, and eliminating unwanted technical and biological variations. Developing an independent benchmark of spatial omics integration algorithms should greatly assist researchers in selecting appropriate integration strategies and designing experiments. Without suitable analytical tools, even costly experiments can yield unusable data. To mitigate bias, the scientific community must provide open datasets for comparative analysis of tissues and develop novel methods for accurate detection or capture efficiency. The path to widespread adoption of these technologies remains long. A thorough understanding of the cellular and molecular mechanisms within specific normal or pathogenic microenvironments is crucial for advancing personalized precision medicine. This approach is anticipated to become the primary treatment option in the near future. Expected advancements include increased throughput, reduced costs, integration of more detection modes, and enhanced sensitivity and specificity. Ultimately, multi-omics techniques with spatial single-cell resolution will revolutionize our understanding of cell biology.

Conclusions

The integration of multi-omics with spatial analysis is a rapidly evolving field that holds great promise for a wide range of applications. Spatial multi-omics enables a deeper understanding of complex biological systems, providing novel insights into disease mechanisms, drug target identification, and biomarker discovery. However, integrating multi-omics data presents technical challenges, necessitating advanced computational and statistical methods. Moreover, the interpretation of spatial multi-omics data is further complicated by spatially varying environmental factors and technical noise. Thus, the development of sophisticated computational tools and analytical methods capable of managing large-scale spatial multi-omics datasets is essential for fully leveraging the potential of this approach.

Acknowledgements
Thank you for drawing materials from SMART—Servier Medical ART and SciDraw | Scientific Drawings.

Author contributions
P. W. and X. L. conceived the whole article and completed the original draft preparation. P. W. and C. C. performed major reviews and edited the structure of the article. The primary drawing effort was done by T. P. and C. X. During subsequent revisions of the article, S. L., B. H., T. C., B. L., Y. X., W. D., and L. L. have touched up the language of the article and participated in the revision process of the later article. All authors reviewed the manuscript.

Funding
The National Key R&D Program of China (2021YFC2701201 to P.W.), the Natural Science Foundation of China (82072895 and 82372929 to P.W., 82141106 to M.D., 82203453 to C. C.), and Foundation of Tongji Hospital (24-2KYC13057-08 to C. C.).

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 22 May 2024 Accepted: 9 August 2024
Published online: 24 August 2024

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