Molecular Cancer

REVIEW

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From morphology to single-cell molecules: high-resolution 3D histology in biomedicine

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Abstract

High-resolution three-dimensional (3D) tissue analysis has emerged as a transformative innovation in the life sciences, providing detailed insights into the spatial organization and molecular composition of biological tissues. This review begins by tracing the historical milestones that have shaped the development of high-resolution 3D histology, highlighting key breakthroughs that have facilitated the advancement of current technologies. We then systematically categorize the various families of high-resolution 3D histology techniques, discussing their core principles, capabilities, and inherent limitations. These 3D histology techniques include microscopy imaging, tomographic approaches, single-cell and spatial omics, computational methods and 3D tissue reconstruction (e.g. 3D cultures and spheroids). Additionally, we explore a wide range of applications for single-cell 3D histology, demonstrating how single-cell and spatial technologies are being utilized in the fields such as oncology, cardiology, neuroscience, immunology, developmental biology and regenerative medicine. Despite the remarkable progress made in recent years, the field still faces significant challenges, including high barriers to entry, issues with data robustness, ambiguous best practices for experimental design, and a lack of standardization across methodologies. This review offers a thorough analysis of these challenges and presents recommendations to surmount them, with the overarching goal of nurturing ongoing innovation and broader integration of cellular 3D tissue analysis in both biology research and clinical practice.

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Introduction

High-resolution 3D histology captures and analyzes the three-dimensional architecture of tissues, cells, and molecules using advanced imaging, spatial omics, computational methods, and 3D tissue reconstruction. It provides detailed insights into tissue organization, cellular interactions, and subcellular structures at micrometer to nanometer scales, enhancing our understanding of tissue dynamics in their natural spatial context.

The vast complexity of biology predominantly manifests in three dimensions. Although the spatial organization of proteins and macromolecular machines is well-understood, investigating the structure of simple organisms or individual tissues necessitates not only decoding molecular mechanisms within cells but also grasping how the microenvironment affects their behavior. Recent innovations in multiplexed measurements for reconstructing 3D tissues—through both digital and experimental approaches—are embracing the inherent complexity of biological systems, ushering in a new era of scientific discovery and clinical application.

Currently, tissue biopsy stands as the primary clinical method for tumor detection. Traditionally, imaging techniques such as ultrasound imaging, computed tomography, and magnetic resonance imaging could help doctors observe tissue through physical methods. To better understand the soft tissue structure in sarcoma, 3D computational methods could be used to analyze optical coherence tomography (OCT) images. As a computer-aided high-resolution automatic recognition technology can provide effective indicators for the diagnosis and surgical resection of soft tissue sarcoma [1–3]. Additionally, spatial gradients of physical and chemical properties play a crucial role in the differential regulation of biological processes across all scales, especially during the development of multicellular organisms. However, its requirements for tissue sampling limit the accuracy of the assessment, resulting in significant delays in the completion of surgical excision [4]. Therefore, advanced surgical techniques based on real-time microscopic imaging and detection are needed to reduce the chance of local recurrence, minimize the excision area, and improve the efficiency of surgical treatment.

The advancement of single-cell 3D histology has been driven by two main catalysts: spatial omics and imaging techniques. Spatial omics enhance our understanding of intracellular processes by providing detailed insights into the molecular characteristics of cells within their native 3D context. This approach allows for a more detailed and comprehensive understanding of 3D structural tissues through controlled and optimized microenvironments, thereby reconstructing inherent cell morphology and structure [5, 6]. On the other hand, imaging technologies provide a comprehensive overview of whole tissues and organs. 3D imaging has proven to be one of the most powerful tools for examining cellular networks and interactions between different cell types [7]. Understanding the structure and function of cells within these networks is critical for documenting dynamic changes during tissue development and regeneration [8]. Therefore, single-cell 3D histology is a cutting-edge technique that involves the high-resolution analysis of individual cells within three-dimensional tissue structures. It combines the spatial information of cells in their native tissue context with the molecular and structural details obtained through advanced imaging technologies. This approach allows researchers to study the intricate organization, interactions, and characteristics of individual cells within complex tissue environments, providing a deeper understanding of cellular behavior and function at a level of detail previously unattainable with traditional histological methods.

Traditional single-cell omics are made on disaggregated cells or nuclei lack spatial information, prompting scientists to develop techniques capable of measuring the molecular characteristics of cells in their native 3D context. Spatial omics technologies have emerged, unveiling the 3D structure of cell. Unlike the non-spatial counterparts, single-cell 3D histology methods offer spatial information integrated with imaging, presenting a more detailed and visualized atlas. These methods have facilitated the treatment of more complex diseases, shed new light on regenerative medicine, and helped to create detailed organ atlases. Although advanced techniques exist for studying single cells and tissues in two dimensions, biological processes occur in a spatial context, and the three-dimensional arrangement of cells and their microenvironment profoundly affects their functions. As an example, the organization of cells within a tumor can influence its growth and response to treatment [9–11].

Recent advancements in tissue-clearing techniques have enabled the acquisition of high-resolution images of the fine internal structures of non-sliced biological tissues and organs. These techniques can transform intact opaque tissues into optically transparent and macromolecular permeable states, allowing the collection of three-dimensional data in complex biological systems without destroying the original tissue structure [12, 13]. Three-dimensional volumetric analysis using tissue clearing techniques provides comprehensive information about the volume of epidermal cells in a larger skin space than traditional two-dimensional methods [14].

Despite several limitations such as cost and accessibility, lack of standardization, and challenges in experimental design and data analysis, these powerful technologies have provided substantial insights into various fields,

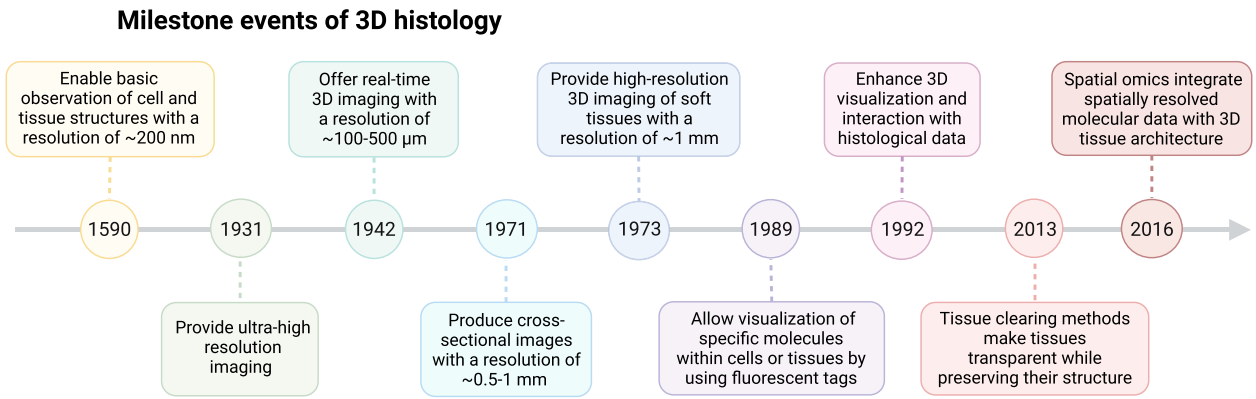

Fig. 1 Milestone events of 3D histology. Key events in the development of 3D histology were retrospectively summarized from 1590 to the present day

including oncology, cardiovascular diseases, neuroscience, immunology, developmental biology, and regenerative medicine. As technology advances and methods become standardized, 3D histology is poised to revolutionize our approach to studying complex biological systems, offering unprecedented insights into the spatial dynamics of life.

Overview of 3D histology

3D tissue reconstruction has become a transformative tool in biomedical research, providing critical insights into tissue organization, cellular interactions, and subcellular structures at micrometer to nanometer scales. This process involves several key stages, including sample preparation, imaging, spatial omics analysis, and advanced computational methods, each essential for preserving tissue integrity and achieving high-resolution 3D reconstructions. By carefully examining these stages, we can appreciate the precision and complexity required to successfully reconstruct tissue structures in three dimensions. Especially over the past two decades, innovations in tissue clearing techniques, imaging methods, virtual reality (VR), augmented reality (AR), and spatial omics have propelled 3D histology forward at an unprecedented pace. Tissue clearing methods, which make biological tissues transparent while preserving their structural integrity, have been instrumental in enhancing imaging clarity. Advances in imaging technologies, such as light-sheet microscopy and two-photon microscopy, have further enabled high-resolution, deep-tissue imaging. Recently, VR and AR have revolutionized how researchers interact with 3D histological data, providing immersive experiences for studying complex tissue structures. Furthermore, spatial omics technologies—covering transcriptomics, epigenomics, and proteomics—allow for the mapping of molecular landscapes within tissues, enabling detailed exploration of cellular organization and function in three dimensions (Fig. 1).

Sample preparation

To begin with, sample preparation is a fundamental process that ensures the preservation of tissue structure for subsequent imaging. Fixation and embedding are the initial steps in this process. Fixatives can crosslink proteins and nucleic acids, thereby maintaining the structural integrity of tissues and preventing degradation [15]. Following fixation, tissues are embedded in supportive media like paraffin or resin. Paraffin embedding is commonly used for light microscopy, offering ease of sectioning and long-term storage [16]. In contrast, resin embedding provides superior support for ultra-thin sections required in electron microscopy, facilitating high-resolution imaging of cellular ultrastructure [17]. In addition, there is a method called optimal cutting temperature (OCT) embedding, which involves a water-soluble polymer mixture. During the frozen sectioning process, OCT embedding agents effectively support and protect tissue structures, preventing damage caused by the formation of ice crystals [18]. Compared to the other two methods, OCT embedding is suitable for tissue samples that require rapid processing, particularly for frozen tissue samples, immunohistochemical staining, and in situ hybridization experiments. This is because it can preserve the antigenicity of proteins and the integrity of RNA molecules [19]. Sectioning is the next crucial step, involving slicing the embedded tissue into thin layers. This is typically done using microtomes for paraffin-embedded tissues or cryostats for frozen tissues. The former produces thin sections suitable for light microscopy, making it a staple in histological studies to examine tissue architecture and cellular organization [20]. The latter is particularly advantageous for immunohistochemistry

and in situ hybridization, as it preserves antigenicity in tissues, making it ideal for detecting specific proteins [21]. Staining, as the last step, enhances the contrast of tissue sections, highlighting specific structures for visualization. Hematoxylin and eosin (H&E) staining is the most common technique, providing a general overview of tissue morphology [15]. Besides, immunohistochemical staining uses antibodies to detect specific proteins, offering insights into protein expression and localization, crucial for understanding disease mechanisms [21].

Given that opaque tissue is unsuitable for obtaining high-resolution images [22], the employment of a clearing agent becomes imperative to render the tissue or organ transparent for the acquisition of precise 3D images. Enhanced depth imaging can be accomplished via tissue-clearing methodologies, facilitating the examination of extensive biological tissue specimens [23]. Stabilization to Handle Insoluble Embedded Lipids for Enhanced Detection (SHIELD) is often the first step, stabilizing proteins and nucleic acids, and preserving the overall tissue architecture. By chemically stabilizing tissue components, SHIELD allows repeated imaging cycles without significant fluorescence loss. This robust protection is vital for maintaining the integrity of the samples during the extensive analysis [24].Next, System-Wide control of Interaction Time and kinetics of Chemicals (SWITCH) is essential for achieving uniform labeling and clearing across large tissue volumes. SWITCH uses electrophoretic and hydrophilic reagents to control the penetration and interaction of clearing agents and fluorescent labels, significantly enhancing the clarity and signal-to-noise ratio of deep tissue imaging [25]. Additionally, the complex structures of biological materials often result in unnecessary light scattering and absorption, leading to opacity [26, 27]. In most tissues, the scattering coefficient is 10 to 1000 times greater than the absorption coefficient [27, 28], which significantly limits the imaging depth and spatial resolution in conventional microscopy [29]. Light scattering in tissues arises from the differences between low refractive index (RI) water-based components, such as interstitial fluid and cytosol, and high RI lipid and protein-based components, such as cell membranes, myelin, and myofibrils. To further refine the clearing process, 3D Nanofluidic Clearing (3DNFC) and Immunolabeling-enabled three-dimensional Imaging of Solvent-Cleared Organs (iDISCO) utilize nanofluidic channels to enhance the penetration of clearing agents, enabling rapid and uniform clearing of large tissue samples. These methods are particularly effective for preparing tissues for high-resolution 3D imaging [30]. On the other hand, iDISCO [31] and its innovative versions such as ultimate three-dimensional imaging of solvent-cleared organs (uDISCO) [32] and three-dimensional imaging of solvent-cleared organs (3DISCO) [33], use organic solvents to achieve tissue transparency and are compatible with immunolabeling, allowing for detailed visualization of specific proteins and cellular structures within cleared tissues [34, 35]. This technique is widely used for whole-organ imaging and offers excellent preservation of fluorescent signals. Although 3DISCO provides the highest degree of optical transparency, this technique alters the lipid structures associated with adipose tissues such as the breast [36]. Another limitation is that organic solvents rapidly quench endogenous fluorescence, requiring quick imaging [37]. To address this issue, a new approach using a clearing agent containing fructose, urea, and glycerol for imaging (FUnGI) [37, 38] was developed, referred to as large-scale single-cell resolution 3D (LSR-3D) imaging. Notably, the combination of fructose and glycerol effectively prevents tissue shrinkage and distortion associated with the use of urea [39]. In contrast to many previously reported clearing agents, FUnGI is a non-toxic, effective clearing agent that preserves organ structure integrity. Another significant advantage is that it allows tissues to be stored at 20°C before microscopic analysis, with no noticeable difference in fluorescence between freshly prepared and frozen samples [37]. Finally, CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ-hybridization-compatible Tissue-hYdrogel) is employed to render tissues transparent while preserving structural and molecular integrity [12, 40]. The CLARITY employs hydrogel embedding to retain biomolecules and structural proteins, followed by lipid removal to achieve transparency. This technique enables multiple cycles of immunostaining and imaging, making it ideal for the comprehensive 3D analysis of complex tissues [41]. To improve tissue transparency, the Clear Unobstructed Brain Imaging Cocktails and Computational Analysis (CUBIC) [42] method employs a range of hydrophilic solutions that efficiently eliminate lipids while safeguarding fluorescent signals and tissue morphology. This technique is particularly beneficial for whole-organ imaging, providing detailed visualization of neural and vascular networks. The ability to maintain structural detail while achieving transparency makes CUBIC an indispensable tool in 3D histology [43, 44]. Water-soluble dyes within the visible spectrum can effectively reduce the RI contrast between water and lipids, thereby enhancing optical transparency in live biological tissues [45, 46]. A recent study discovered that strong absorbent dye molecules could render the skin, muscle, and abdominal regions of living mice transparent, enabling non-invasive, high-resolution imaging of deep tissues [47]. Besides, tissue transparency is influenced by lipid content and average RI values [48–52]. However, due to the extracellular matrix (ECM) richness in tissues

like tendons, skin, and bones, the impact of lipids on the transparency of protein-rich tissues is minimal. Therefore, RI modulation is more beneficial for achieving transparency in these tissues, and materials that exhibit high RI and good tissue penetration are desired [53]. MXDA has been identified as a novel tissue-clearing chemical with a high RI in aqueous solutions [54]. Studies have shown that progressively cultivating biological specimens with increasing concentrations of MXDA can be used for clearing entire organs or organisms [55]. By improving MXDA-based RI solutions, a method was developed to enhance the tissue penetration of RI-matching reagents by combining MXDA with sucrose or iodixanol. MAX (MXDA-based aqueous RI adjustment solution X) [53] enables better tissue penetration without compromising tissue clearing activity, achieving a one-step tissue clearing protocol. It can also be combined with modular processes for de-fatting, decolorization, and decalcification, resulting in higher resolution.

Imaging methods

Experimental imaging methods

Tissues contain a variety of interconnected cells with different functional states and shapes, and this complex organization cannot be captured on a single plane. For example, tumors have been shown to be highly heterogeneous, requiring large-scale spatial analysis to reliably assess their cellular and structural composition [56]. Volumetric imaging allows for the visualization of entire biological samples, revealing the spatial phenotype and dynamic features of complex tissues [57]. A comprehensive understanding of pathophysiological processes requires non-invasive 3D imaging of deep tissues across multiple spatial and temporal scales to link transient subcellular behaviors with long-term physiological events. The advances in 3D histology have revolutionized the landscape of biomedical research by providing unparalleled insight into tissue architecture and cellular arrangement. Through various imaging methods, researchers can capture intricate, high-fidelity tissue images and construct 3D representations, offering valuable insights into the complex mechanisms of diseases and the efficacy of potential treatments.

Optical microscopy is one of the most widely used techniques for 3D histological imaging. Among the various modalities, confocal microscopy stands out as a pioneering tool, which utilizes point illumination and spatial pinholes to eliminate out-of-focus light, resulting in high-resolution images suitable for 3D reconstruction. This technique is widely employed in cell biology to visualize the spatial distribution of proteins within cells [58, 59]. By capturing optical sections of samples at different depths, confocal microscopy has revolutionized the understanding of complex cellular dynamics such as migration and differentiation, providing a clear and detailed 3D representation of tissues [60, 61].

Building upon the success of confocal microscopy, multiphoton microscopy introduced a deeper tissue penetration capability, which is crucial for imaging live tissues. Unlike confocal microscopy using visible light, multiphoton microscopy employs longer wavelengths and multiphoton excitation technology. This unique approach enables enhanced tissue penetration with reduced photodamage, making it particularly useful for observing dynamic biological processes like neuronal activity [62]. Recent advancements in multiphoton imaging, such as spectral imaging, have further improved the technique's ability to collect detailed and quantitative data from biological samples [63].

Light-sheet fluorescence microscopy (LSFM) is another emerging tool, with recent advancements that have improved imaging resolution and speed, significantly expanding the range of targets and endpoints that can be imaged while reducing phototoxicity [57]. This technique is particularly valuable in developmental biology and neuroimaging, where it has enabled researchers to observe tissue development and function in real-time [64, 65]. Moreover, congenital and adaptive immune factors mediate asthma responses within the complex 3D structure of the lungs, characterized by multiple distinct lobes and a branching airway network known as the bronchial tree [66]. Therefore, to understand the pathogenesis of asthma and develop effective treatments, achieving a multi-scale 3D spatial lung immune map is crucial, including information on lung anatomical structures, pathological tissue sites, and the distribution and phenotypes of individual immune cells [67]. LSFM can visualize the macro- and mesostructures of the entire lung tissue in 3D by using optical clearing methods. Through simple incubation in a series of immersion solutions, tissues are rendered transparent and optically accessible by homogenizing the RI of cellular and non-cellular components (such as phospholipids, extracellular matrix, proteins, and DNA) in the tissue [48, 68]. For 3D LSFM, less toxic or non-toxic organic solvents, such as dibenzyl ether (DBE) and ethyl cinnamate (ECi), are typically used to achieve greater transparency in large tissue volumes and whole mouse organs after alcohol-mediated tissue dehydration [69–71]. After optical clearing based on organic solvents, LSFM generates thin light sheets and scans the entire tissue sample, minimizing damage while providing rapid 3D imaging [65, 72, 73].

Although optical microscopy provides significant insight into cellular structures, electron microscopy (EM) offers even higher resolution, capable of revealing ultrastructural details at the nanometer scale. There

are two primary types of electron microscopy used in 3D histology: scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM captures high-resolution images of the surface topography of tissues by scanning a focused electron beam over the specimen. It is particularly effective for examining the morphology of tissue surfaces, such as the epithelial lining of organs [74–76]. SEM has also seen advancements with methods like cryo-SEM and focused ion beam (FIB)-SEM, which allow for the imaging of frozen samples in their native state and serial sectioning for 3D reconstruction [77, 78]. In contrast, TEM provides insights into the internal structure of cells, capturing detailed images of organelles like mitochondria and the endoplasmic reticulum, which are crucial for understanding cellular function and pathology [79]. The capacity of TEM to unveil intricate cellular details renders it an indispensable tool for investigating the fundamental mechanisms of diseases at the cellular level. For tissues and cells that are too thick to image using traditional microscopy such as LSFM, cryo-electron tomography (Cryo-ET) offers a viable alternative. Cryo-ET uses ion beams to thin specific areas of interest within cells, yielding high-resolution 3D images of biological specimens while preserving their native state [80]. This technique has proven to be crucial in structural biology, particularly for studying complex cellular structures and molecular assemblies [81, 82].

In addition to traditional optical and electron microscopy, several emerging techniques have further expanded the capabilities of 3D histology. One such technique is atomic force microscopy (AFM), which measures surface topography using a sharp probe. AFM provides nanoscale resolution and allows researchers to study the mechanical properties of tissues and cells, such as stiffness and elasticity [83]. This technique has proven particularly valuable in tissue engineering, where understanding cellular responses to mechanical forces is critical. Recent developments in AFM, including high-speed imaging and force spectroscopy, have enhanced its application in the detailed study of cell mechanics [76, 84]. Another breakthrough technology is laser capture microdissection (LCM), which enables the precise isolation of specific cells or tissue regions using a laser. This technique is particularly useful for studying heterogeneous tissues and rare cell populations, which can be difficult to analyze using conventional methods [85–88]. Advances in LCM, such as improved precision and automation, have made it an indispensable tool for molecular biology research, allowing for targeted analysis of specific cell types [76, 89–91].

Finally, mass spectrometry imaging (MSI) has revolutionized tissue analysis by providing spatial distribution maps of chemical compounds, such as metabolites, lipids, and proteins, within tissues. MSI enables researchers to visualize tissue composition in 3D, offering valuable insights into disease biomarkers and tissue pathology [92]. Recent improvement in sensitivity and the integration of MSI with other imaging modalities result in a more comprehensive analysis of tissue structures [93, 94].

In conclusion, the development of various imaging technologies has profoundly impacted 3D histology, enabling researchers to explore tissue architecture and cellular organization with unprecedented detail. These imaging technologies play different roles in 3D histology, complementing each other, and through multimodal imaging and integrated analysis, they drive the advancement of 3D histology.

Clinical imaging methods

The evolution of 3D imaging technologies has significantly enhanced diagnostic precision and treatment planning in medical imaging, and when applied within the framework of 3D histology, these technologie further enrich our understanding of tissue organization, cellular interactions, and subcellular structures, providing deeper insights into disease mechanisms. Here we introduce various 3D imaging modalities, their development over time, and their typical applications in clinical practice (Fig. 2).

Initially, CT, which is based on X-ray technology with the transmission of multiple X-ray beams through the body at diverse angles [95], revolutionized medical imaging by providing detailed cross-sectional images of tissues. The introduction of CT in the 1970s marked a significant breakthrough, offering unprecedented clarity in visualizing internal structures [96]. By the 2000s, dual-energy CT emerged, further refining CT imaging by employing two distinct energy levels to distinguish tissues with similar densities. This advancement improved tissue characterization and enabled more accurate detection of subtle abnormalities [96, 97]. Dual-energy CT has shown particular benefits in vascular imaging, oncology, and musculoskeletal assessments [98, 99]. This technique allows for better differentiation of tissue types and enhanced visualization of pathological changes [100, 101]. Unlike conventional CT, a 3D imaging technique that can clearly understand the internal microstructure of a sample without destroying it has gradually been developed. Micro-CT scanning, introduced for small animal imaging, provides ultra-high resolution imaging at the micron scale for pre-clinical studies. This modality allows for detailed visualization of anatomical and pathological features at a microscopic scale, contributing to advancements in research and development [102]. The high resolution of micro-CT has been instrumental in studying disease models and evaluating therapeutic interventions [103]. Moreover, cone beam computed

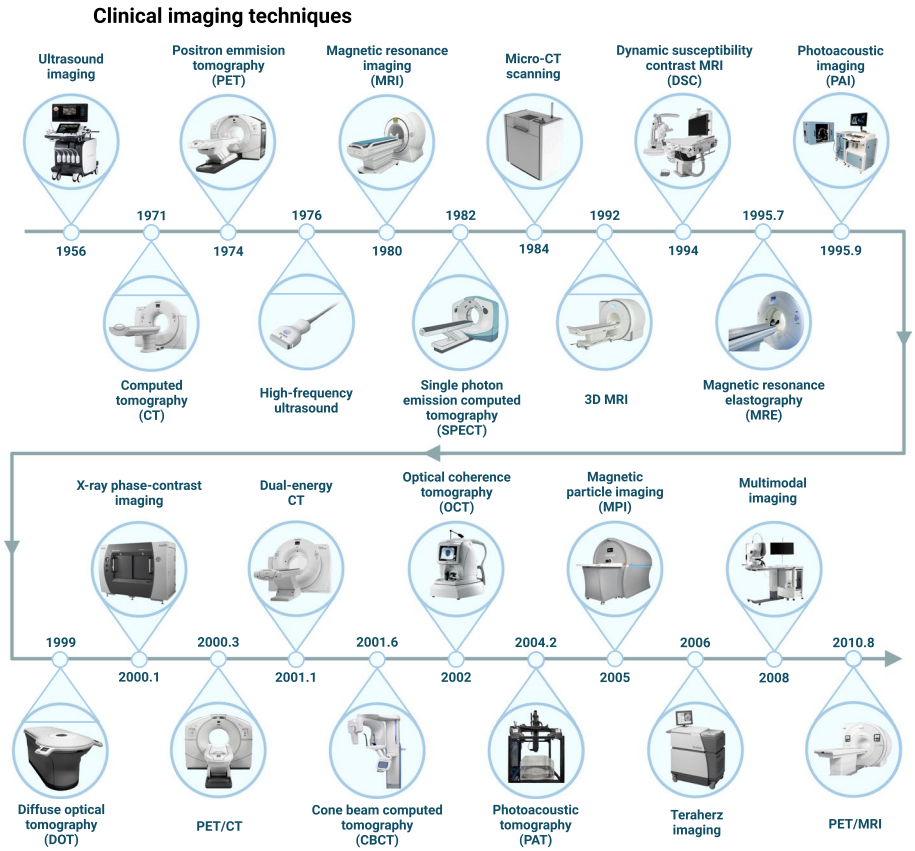

Fig. 2 Clinical imaging techniques. Key events in developing clinical imaging techniques were retrospectively summarized from 1956 to the present

tomography (CBCT) has revolutionized dental and maxillofacial imaging with its ability to deliver detailed 3D images. CBCT's compact design and lower radiation dose compared to conventional CT make it advantageous for applications such as dental implants and orthodontics [104, 105]. The technology has improved patient safety and diagnostic accuracy, offering detailed views of dental and craniofacial structures [106, 107].

On the other hand, magnetic resonance imaging (MRI), using strong magnetic fields and radiofrequency (RF) waves to generate detailed images of soft tissues [108, 109], further advanced the field of medical imaging with its superior ability to visualize soft tissues. MRI has become indispensable for diagnosing neurological, musculoskeletal, and oncological conditions since its development in the 1980s [110]. The advent of 3D MRI allowed for the reconstruction of images in three dimensions, offering a more comprehensive assessment of complex anatomical structures. This modality significantly improved soft-tissue contrast and diagnostic accuracy, facilitating detailed analysis of brain structures and tumors [111, 112]. 3D MRI has been pivotal in both clinical and research settings, enhancing our understanding of various diseases and their progression [111, 113]. A recent development, namely magnetic particle imaging (MPI), uses superparamagnetic nanoparticles to create high-resolution images with excellent spatial and temporal resolution. MPI's ability to provide detailed and dynamic imaging has expanded its applications in preclinical research and clinical diagnostics [114, 115]. This technique offers advantages in terms of imaging speed and resolution compared to traditional modalities [116, 117].

Ultrasound imaging (sonography), known for its real-time capabilities and safety, uses sound waves to create images of internal structures. As an advancement in this technology, high-frequency ultrasound enhances resolution and provides detailed images of superficial tissues and small structures. It has become indispensable in obstetrics, cardiology, and musculoskeletal imaging due to its non-invasive nature and dynamic imaging features [118]. This modality has improved diagnostic accuracy in applications such as dermatology and ophthalmology, offering detailed insights into tissue structures. High-frequency ultrasound's ability to capture real-time images has made it a valuable tool for guiding

interventions and monitoring treatment outcomes [119, 120].

Positron emission tomography (PET) provides functional imaging using radiotracers to visualize metabolic activity, particularly in oncology for detecting cancerous tissues [121, 122]. The integration of PET with MRI in hybrid imaging systems, known as PET/MRI, synergistically merged PET's functional imaging capabilities with MRI's intricate anatomical details. This hybrid approach has provided a comprehensive view of physiological and structural data, enhancing diagnostic precision and treatment planning [123, 124]. PET/MRI remains indispensable for assessing disease progression, treatment response, and guiding personalized therapy [125]. Similarly, PET/CT hybrid imaging has further improved diagnostic accuracy by combining metabolic and anatomical information [126, 127]. Following PET, single photon emission computed tomography (SPECT) provided another method for functional imaging using different radiotracers. SPECT has been particularly useful in cardiology and neurology for evaluating blood flow and metabolic processes [128, 129]. The ability of SPECT to assess dynamic physiological changes complements anatomical imaging modalities, offering a more complete view of disease processes [130].

Terahertz imaging and X-ray phase-contrast imaging represent recent innovations with unique imaging capabilities. Terahertz imaging uses terahertz radiation to detect hidden features, with applications extending into security and material science [131]. X-ray phase-contrast Imaging enhances image contrast by exploiting phase shifts of X-rays, improving the visibility of soft tissues and fine structures [132, 133]. Both modalities offer advancements in imaging technology that extend beyond traditional methods, providing new insights into material and biological samples [134, 135].

In the field of optical imaging, OCT has been instrumental in capturing high-resolution images of tissues using light waves. OCT has found extensive applications in ophthalmology for diagnosing retinal diseases and has expanded into cardiology for imaging coronary arteries [136, 137]. The technique's ability to provide detailed cross-sectional images has been crucial for assessing tissue health and guiding clinical decisions [138]. Moreover, photoacoustic imaging (PAI) combines optical and acoustic technologies to create detailed images of tissue structures and functions. PAI has proven effective in imaging vascular structures and tissue oxygenation, offering insights into tumor physiology and guiding cancer treatment [139–141]. The integration of optical and acoustic signals in PAI allows for high-resolution images with anatomical and functional information, making it a valuable tool in various biomedical applications [142, 143]. In contrast, photoacoustic tomography (PAT) merges the advantages of optical and ultrasound imaging, offering detailed tissue structure and function information [144, 145]. Therefore, multimodal imaging integrates various imaging techniques to leverage their strengths, providing a comprehensive diagnostic approach. Such integration enhances diagnostic capabilities and provides a holistic view of pathological conditions, improving disease diagnosis and treatment planning [146, 147].

With the advancement of biotechnology, more and more imaging techniques have been developed for the visualization of human tissues or organs. For instance, dynamic susceptibility contrast (DSC) imaging has been utilized for visualizing blood vessels and detecting abnormalities such as aneurysms [148, 149]. Magnetic resonance elastography (MRE) assesses tissue stiffness, aiding in diagnosing liver fibrosis and other diseases [150, 151]. Moreover, diffuse optical tomography (DOT) provides functional imaging based on light absorption and scattering, which is useful for brain imaging and cancer detection [152, 153].

In summary, the advancements in these 3D imaging technologies reflect a continuous progression toward greater diagnostic precision and enhanced treatment planning, showcasing the remarkable evolution of medical imaging techniques over time.

Spatial omics

Spatial omics is widely regarded as a new frontier in life sciences. It combines omics technologies (such as transcriptomics, proteomics, epigenomics, and metabolomics) with spatial information, offering a deeper understanding of biological samples by analyzing the spatial distribution of biomolecules within tissues or cells [154]. Traditional omics technologies typically lose spatial information because they require tissue or cell samples to dissociate into homogeneous mixtures [155]. In contrast, spatial omics preserve the spatial structure of the sample, allowing the analysis of biomolecular data while considering their spatial locations. Spatial omics enable the observation of the distribution of genes, proteins, or metabolites in two-dimensional (2D) space and reveal the specific locations of these biomolecules and their interrelationships within the tissue structure [154]. This is of great significance for understanding the complex structure of tissues, cell-to-cell interactions, and the microenvironment of diseases [156–158]. The ability to measure molecular characteristics of cells within their native 3D environment has driven the development of spatial omics (Fig. 3).

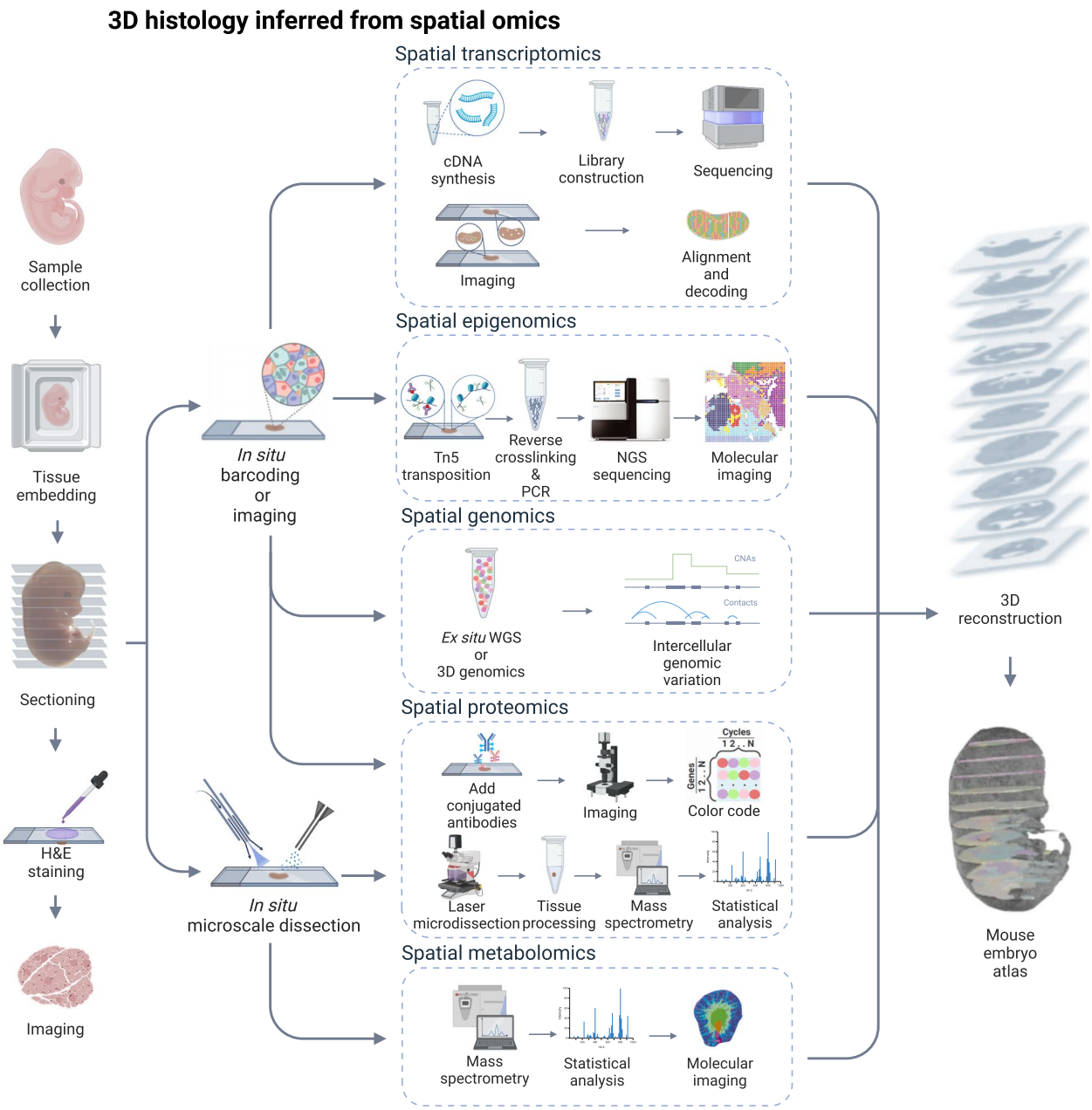

Fig. 3 3D histology inferred from spatial omics. This figure outlines the step-by-step workflow used to investigate the spatial distribution of various cell types within tissues. Firstly, tissue samples were prepared through a series of steps, including collection, embedding, sectioning, H.E. staining, and imaging. Following that, the tissue slices adjacent to the previously imaged sections were subjected to spatial multi-omics analysis, incorporating techniques such as spatial transcriptomics, spatial epigenomics, spatial genomics, spatial proteomics, and spatial metabolomics. Next, the resulting spatial omics data were processed using a range of tools and platforms, facilitating the spatial mapping of cell types and their expression levels. This comprehensive workflow allows for the evaluation of histological features at the 3D level, advancing research related to cellular dynamics and informing relevant therapeutic strategies

Spatial transcriptomics

Spatial transcriptomics involves mapping gene expression at different locations within tissue sections, and connecting gene data to their spatial coordinates within the tissue. This approach unveils the spatial diversity of gene expression and sheds light on the biological roles of distinct tissue regions. [159]. Deciphering the principles and mechanisms through which gene expression orchestrates the intricate cellular

organization in multicellular organisms holds profound implications for research in the life sciences [160].

The earlier development of single-cell RNA sequencing (scRNA-seq) provided comprehensive insights into the transcriptome, revolutionizing the ability to identify cell sub-populations [161]. However, limitations such as relatively low RNA transcript capture efficiency and coverage potentially result in the omission of crucial gene expression in downstream analyses [162]. Additionally, tissue dissociation procedures may influence the transcriptome, triggering transcriptome-wide changes such as ectopic gene expression. These alterations can introduce signal contamination, potentially leading to the misclassification of cell subtypes during analysis [163]. With the advancements in spatial transcriptomics, these challenges have been progressively addressed. Each cell is now assigned a specific and unique spatial tag that includes spatial coordinate information, allowing for the precise localization of identified cell subpopulations within the original tissue section [164]. By leveraging spatial transcriptomics, researchers can acquire transcriptomic data directly from intact tissue sections, thus acquiring spatial distribution information and elucidating patterns of cellular interactions [164].

Traditionally, analyzing multiple mRNAs necessitated cell lysis, which prevented determining the precise localization of mRNAs within cells. Fluorescent in situ RNA sequencing (FISSEQ) addresses this limitation by exposing environment-specific transcripts while maintaining the tissue structure essential for RNA localization [165]. This method is applicable to tissue slices and whole embryos, facilitating extensive parallel detection of genetic elements and supporting the analysis of cell phenotypes, gene regulation, and in situ environments [166]. Another spatial technique based on imaging, multiplexed error-robust fluorescence in situ hybridization (MERFISH), allows the identification and quantification of a large array of RNA species at the single-cell level, ranging from hundreds to thousands. It is achieved through a tailored fluorescent labeling approach that allows for the concurrent detection of multiple RNA molecules [167]. Fluorescence in situ hybridization (FISH) methods such as Vizgen MERSCOPE [168], NanoString CosMx [169], and seqFISH + [170] are capable of capturing hundreds to thousands of transcripts at subcellular resolution [171].

Due to the unbiased spatial composition provided by spatial transcriptomics (ST) technologies, these methods have been instrumental in generating tissue atlases, which serve as invaluable resources for reference mapping [160]. ST-based approaches have successfully established detailed maps of the entire mouse brain [172], as well as specific regions including the visual cortex [173], the primary motor cortex [174], the middle temporal gyrus [175], the preoptic area of the hypothalamus [176], hippocampus [176, 177], and cerebellum [178]. Spatial transcriptomics technologies serve as powerful tools for studying the dynamics of complex structures, tissues, and organ systems, as well as their underlying mechanisms [159]. Such methodologies offer invaluable biological insights by revealing intricate details of tissue architecture, developmental trajectories, and disease pathogenesis. Processes like infection and inflammation can substantially disrupt the cellular organization within tissues [179]. Anomalous spatial arrangements in tissues are often recognized as key indicators of pathological conditions [180]. Beyond normal development and physiology, spatial transcriptomics can identify mechanisms underlying cancers, where the tissue architecture that supports normal physiological functions has been disrupted [181–186]. It has been utilized to investigate the relationship between cancer cells in various states to analyze the tumor microenvironment [185, 187–189]. This approach enables the study of molecular characteristics that span the boundaries between cancerous and normal tissues [190].

While cutting-edge spatial transcriptomics technologies currently face limitations such as relatively low resolution and insufficient sequencing depth, which hinder precise insights into normal and abnormal tissues [162], future advancements are expected to enable the study of larger-scale tissue specimens. This will facilitate the mapping of organ-level tissue topography, allowing for a more comprehensive and continuous interpretation of tissue architecture [159]. It is not feasible to achieve single-cell resolution at the spatial level in certain spatial transcriptomics methods, particularly in widely used spatial barcode-based techniques. Conversely, scRNA-seq fails to capture the spatial distribution of individual cells. By integrating spatial transcriptomics with scRNA-seq data, it is possible to retrospectively query publicly available datasets to uncover new biological insights that may have previously been concealed within the original data [158, 159, 162].

Slide-tag is a spatial transcriptomics technique that combines spatial barcoding with high-throughput sequencing [191]. In this method, spatial barcode probes are pre-fixed on a slide, and when these barcodes bind to mRNA within the tissue, reverse transcription and amplification are carried out. High-throughput sequencing is then performed to capture gene expression information, along with its corresponding spatial location. Slide-tag enables genome-wide gene expression profiling in a single experiment while preserving the spatial context of the tissue, producing spatial heatmaps of gene expression [192]. However, the resolution of Slide-tag is dependent on the distribution density of the barcode probes

on the slide, typically achieving single-cell or subcellular resolution [193]. In contrast, the 10X Genomics Xenium [194] technology leverages in situ fluorescence imaging to capture RNA expression at single-cell or subcellular resolution, significantly enhancing spatial precision. This technology directly maps and quantifies the spatial distribution of millions of cells and billions of RNA molecules, enabling the simultaneous detection of RNA and proteins within the same tissue Section [158]. It is compatible with a variety of tissue types, including fresh frozen (FF), formalin-fixed paraffin-embedded (FFPE), tissue microarrays (TMA), and organoids [158, 195]. At the subcellular level, 10X Genomics Xenium reconstructs the morphological structure of the tissue microenvironment, offering new insights into the relationship between pathological tissue architecture and cellular function[196]. Patho-DBiT, a method for spatial whole transcriptome sequencing in formalin-fixed paraffin-embedded (FFPE) tissues, enables the analysis of diverse RNA species, splicing isoforms, and single-nucleotide RNA variants, which enhances our understanding of tumor clonal architecture and progression in clinical pathology [197].

Spatial epigenomics

The mechanisms of cell interactions involve not only the transcriptome but also upstream factors such as epigenetics, which play a critical role in gene and transcriptional regulation. Chromatin states govern genomic function and are regulated in a cell type-specific manner [198, 199]. Simultaneously, a strong correlation exists between the cellular arrangement within tissues and their functional responsibilities. Spatial epigenomics reveals how these modifications function in different tissue or cellular contexts by examining the spatial distribution of epigenetic modifications such as DNA methylation, histone modifications, and chromatin accessibility. Genome-wide analyses of transcription factors and chromatin modifications offer insights into regulatory processes such as gene transcription, cell differentiation, and cellular responses. Transcription factors control near gene transcription by targeting specific genomic regions and interpreting the DNA code [200]. The patterns of gene expression specific to each cell type are partly driven by the interplay between transcriptional mechanisms and chromatin regulatory elements, a process that can become dysregulated in diseases [201, 202]. Several approaches have been developed to simultaneously measure gene expression and chromatin accessibility at the single-cell level [203–205]. However, our capacity to investigate chromatin accessibility at a significant spatial resolution is currently constrained by the necessity for custom microfluidics or microdissection in existing spatial chromatin analysis methods [206, 207].

Since the development of chromatin immunoprecipitation (ChIP), it has become a widely utilized and powerful methods for chromatin analysis [208, 209]. The ChIP process involves cross-linking and fragmenting chromatin into smaller segments in solution. An antibody is used to selectively capture the target chromatin epitope, after which genomic DNA is extracted for high-throughput sequencing. However, the cross-linking and sonication steps in ChIP-seq and its variants require a substantial number of cells, can potentially mask epitopes, produce high background noise, and often yield a relatively low signal-to-noise ratio [200]. Alongside ChIP, new enzyme-tethering techniques have emerged for use in live cells, including CUT&RUN (Cleavage Under Targets and Release Using Nuclease) [210] and single-molecule method CUT&Tag (Cleavage Under Targets and Tagmentation) [211]. The CUT&RUN protocol uses a fusion of MNase and protein A (PA-MNase) to cleave chromatin at the genomic locations where antibodies bind. Similarly, CUT&Tag targets chromatin associated with specific proteins in situ by using a fusion of Tn5 DNA transposase and protein A (pA-Tn5). CUT&Tag offers distinct advantages over ChIP-seq, such as requiring only about one hundred cells, reduced background noise, a higher signal-to-noise ratio, enhanced reproducibility, and a shorter protocol [212]. This method has been applied to the analysis of transcription factors and chromatin modifications across various species, including humans [213], mice [214], piglets [215], cattle [216, 217], zebrafish [218], fruit flies [219], Toxoplasma [220], and plants [221, 222].

Despite recent breakthroughs in single-cell sequencing that allow for the analysis of single-cell epigenomes [223–225], integrating spatial information of individual cells within their native tissue context remains challenging [204, 226–230]. Spatial-CUT&Tag [231], used for analyzing histone modifications in a spatially resolved manner, combines deterministic tissue barcoding [232, 233] with CUT&Tag chemistry [234, 235] to enable in situ analysis of chromatin states and transcription factors with high spatial resolution. Specifically, Spatial-CUT&Tag employs specific antibodies to mark histone modifications and then utilizes the pA-Tn5 transposase for antibody-guided in situ tagmentation of target-bound DNA in native cells. This approach leverages microfluidic chips to achieve spatial encoding of histone modifications. Once encoding is complete, sequencing is employed to acquire comprehensive gene sequences, followed by the application of computational methodologies to reconstruct the spatial distribution of modifications. Similarly, spatial epigenomics also encompasses spatial ATAC-seq (Assay for Transposase-Accessible Chromatin Sequencing) [207], which maps chromatin accessibility

and reveals regions of open chromatin that are associated with active gene regulation. Spatial-ATAC-seq utilizes the Tn5 transposase to cleave open chromatin regions followed by spatial encoding and next-generation sequencing. And computational methods are then employed to reconstruct the distribution of chromatin accessibility across the genome [236]. Histone modifications and chromatin accessibility represent two distinct aspects of epigenetic regulation. Comprehensive analysis of these diverse epigenetic dimensions enhances our insight into the intricate influence of epigenetic regulation on gene expression and cellular functionality. Significantly, these innovative methodologies afford unprecedented observation of epigenetic processes in tissue development, spanning both spatial and genome-wide scales, thereby enabling the spatial delineation of epigenetic regulatory landscapes intertwined with developmental and pathological contexts.

Spatial genomics

Spatial genomics is used to study how the spatial positioning of genomic sequences or structural variants within the nucleus changes from one cell to another, and between different cell types, at specific locations in multicellular tissues or organs [237]. This can be exemplified by using multi-region sequencing to investigate intratumoral genetic heterogeneity [238]. The genome exhibits spatial organization across different length scales, from individual base pairs to entire chromosomes. This organization is thought to regulate gene expression and control cellular function, and it varies between different cell types within an organism [239]. By integrating genomic technologies with spatial information, it is possible not only to examine gene expression within individual cells but also to explore the organization, spatial arrangement, and interactions of genes within multicellular systems. This approach aids in the study of complex biological systems and the mechanisms underlying disease pathogenesis.

In a recent study, DNA seqFISH + (sequential fluorescence in situ hybridization with DNA encoding) was used to image 3,660 genomic loci in over 2,000 cells from mouse cortical tissue sections [240], along with 76 RNAs and 8 histone marks, and nuclear bodies [241]. This revealed the presence of genomic regions associated with nuclear bodies and chromatin marks in different cells. Currently, in situ genome sequencing (IGS) is an exciting approach poised to drive the spatial genomics revolution [242]. IGS combines in situ sequencing (ISS) with standard next-generation sequencing to decode and map the spatial distribution of hundreds of thousands of paired-end reads from thousands of randomly targeted DNA loci. ISS is capable of mapping the spatial organization of repetitive DNA elements, chromatin domains, and colocalization events between specific genomic regions and nuclear bodies at various stages of mouse embryonic development (zygote, two-cell, and four-cell stages), as well as in human fibroblasts [242]. Furthermore, in early mouse embryos, ISS allows for the spatial resolution of parental genomes, revealing parent-specific features of genome organization [242]. OligoFISSEQ (oligonucleotide fluorescence in situ sequencing) is a multiplex hybridization technique based on Oligopaint probes and ISS. This method utilizes non-fluorescent Oligopaint probes during in situ hybridization, combined with in situ sequencing technology (such as ligation sequencing), to read the barcodes of labeled probes [243]. By hybridizing and ligating single fluorescent nucleotides or dye-labeled dinucleotides to the barcode regions, the barcode data is read sequentially, offering spatial genomic details base by base. Unlike traditional FISH methods, OligoFISSEQ can detect more genomic targets with fewer imaging cycles, thereby increasing detection throughput [237]. By integrating high-throughput sequencing and spatial imaging, OligoFISSEQ allows researchers to detect multiple genomic regions at high resolution, advancing the study of 3D genome organization. Although it is currently limited to studying certain genomic regions, its potential multiplexing capacity indicates that it could be expanded to cover broader genomic areas in the future.

Another promising recently emerged technique is slide-DNA-seq [244]. In slide-DNA-seq, DNA sequences are directly extracted from fixed tissue sections onto a slide coated with polystyrene beads. These beads contain Illumina sequencing adapters and unique spatial barcodes, ensuring the preservation of spatial location information for each DNA sequence. Due to the 10 μm diameter of the beads, slide-DNA-seq is currently limited in achieving subcellular spatial resolution [237]. Applying slide-DNA-seq to tissue sections from mouse metastasis models and primary human cancers enables the identification and spatial localization of tumor clones according to the genomic landscape of DNA copy number [244]. Thus, alongside ISS, slide-DNA-seq holds potential for cancer diagnostics by investigating tumor genetic and phenotypic diversity, thus contributing to personalized cancer treatment [245].

Ductal carcinoma in situ (DCIS) is a common precursor to invasive breast cancer, yet the clonal diversity and genomic evolution that lead to recurrent disease remain poorly understood [246]. Additionally, generating a genomic profile of DCIS, especially at single-cell genomic resolution, is challenging due to the nature of tissue collection, which predominantly involves FFPE blocks [247, 248]. Although a previous study demonstrated the feasibility of performing single-cell DNA sequencing (scDNA-seq) on a small number of FFPE samples, the

approach had limited cell throughput (n = 96), was costly, time-consuming, and unsuitable for high-throughput sequencing of large-scale samples [249]. To address this issue, the first high-throughput single-cell DNA sequencing method for FFPE tissues, known as archive nanowell sequencing (Arc-well), was developed [250]. This method enables the genomic analysis of thousands of single cells from FFPE tissues simultaneously and can also be applied to fresh and frozen tissue samples. Arc-well provides an efficient high-throughput solution, making it feasible to leverage single-cell technologies to study large collections of archived FFPE clinical samples [251]. In addition to cancer research, Arc-well can be used to investigate cellular mutations and copy number variations in both normal and diseased tissues, offering new insights into the understanding of human diseases[252–255].

Although these techniques can currently only probe limited portions of the genome, the rapid advancements in sample preparation methods, imaging and sequencing technologies, and high-performance computing are expected to facilitate spatially resolved whole-exome or whole-genome sequencing in the near future [237].

Spatial proteomics

The primary objective of spatial proteomics is to characterize the abundance and spatial distribution of proteins, along with their post-translational modifications within a two-dimensional space [256]. This is crucial for understanding the functional roles of proteins across different cellular and tissue regions, as well as their involvement in intercellular signaling. The function of a protein is intimately linked to its subcellular localization, as different compartments provide distinct chemical environments (such as pH and redox conditions), potential interaction partners, or substrates [257]. Therefore, the tight regulation of protein subcellular localization is a critical aspect of controlling cellular physiology [258]. Most cellular processes involve changes in protein subcellular localization, such as the nucleocytoplasmic shuttling of transcription factors, the relocalization of mitochondrial proteins during apoptosis, and the endocytic uptake of cargo receptors and signaling receptors at the cell surface [257]. Conversely, the mis-localization of proteins is often associated with cellular dysfunction and disease, including neurodegenerative disorders, cancer, and metabolic abnormalities [259–262]. Thus, understanding the spatial distribution of proteins at the subcellular level and capturing protein subcellular dynamics is essential for a comprehensive understanding of cell biology.

Immunohistochemistry (IHC), immunofluorescence (IF), mass spectrometry (MS), and cytometry [257] can be utilized to analyze the spatially resolved distribution of proteins or even the entire proteome at tissue, cellular, and subcellular levels [263]. These approaches each balance trade-offs between spatial information and depth of coverage, molecular or cellular throughput, and the time required for data acquisition [264]. The vast collection of tissue IHC images from the Protein Atlas Project provides unparalleled insights into the spatial (sub)cellular architecture and composition of tissues at the protein level. Spatial proteomics techniques form the foundation for two fundamental spatial imaging technologies: imaging mass cytometry (IMC) and multiplexed ion beam imaging (MIBI) [154]. IMC integrates immunocytochemistry and immunohistochemistry techniques with high-resolution laser ablation integrated into CyTOF (cytometry by time-of-flight mass spectrometry or mass cytometry) mass cytometry. This approach complements current imaging methods by mapping cell subpopulations and cell–cell interactions, thereby highlighting tumor heterogeneity [265]. Similarly, MIBI uses secondary ion MS to image antibodies labeled with metal isotopes, allowing the analysis of samples with up to one hundred distinct metal isotope-labeled antibodies [266]. Emerging MS techniques for protein identification and quantification not only measure the abundance of proteins and post-translational modifications (PTMs) in individual cells but also the assessment of protein complexes and their subcellular distribution [267].

Nonetheless, these ion MS-based methods face limitations in obtaining sufficiently pure metals. Traditional fluorescence immunohistochemistry, meanwhile, struggles to achieve single-cell analysis due to optical limitations and the difficulty of visualizing more than 7 biomarkers in a single sample [158]. Fortunately, Cell DIVE and CODEX (CO-Detection by indEXing) circumvent this limitation through multiple rounds of staining, directly labeled with fluorescent dyes [268]. Both Cell DIVE and CODEX provide high-dimensional imaging of dozens of proteins within individual cells, facilitating the analysis of cellular spatial organization, cell interactions, and signaling states within tissue. In Cell DIVE, antibodies are directly labeled with a fluorescent dye, followed by multiple rounds of staining, imaging, and fluorescence quenching [268]. In contrast, CODEX's core design involves a unique oligonucleotide "barcode" attached to each antibody, whose complementary sequence binds to fluorescent dyes for subsequent imaging [269–271]. Additionally, emerging single-cell spatial in situ imaging technologies, such as GeoMx DSP (GeoMx digital spatial profiler) spatial omics technology, expand the detection limit, enabling spatial analysis of over 570 protein targets and the entire transcriptome, either individually or simultaneously [157, 272]. These advanced techniques allow researchers to overcome the limitations imposed by the number of fluorescence channels in the visible

spectrum, enabling the concurrent detection and analysis of 50 or more protein markers.

Compared to RNA, proteins are less susceptible to degradation and unprone to cross-linking with other biomolecules in FFPE samples [248]. Additionally, cell types are not directly visible and must be inferred from the spatial transcriptomic data [273]. Furthermore, RNA expression cannot directly predict protein expression [274–277]. Therefore, complementing RNA data with direct proteomic measurements provides a more accurate reflection of specific cellular functions and states. In summary, it would be highly valuable to integrate spatial transcriptomics datasets with high-throughput proteomic in situ imaging as well as the spatial distribution of histone marks within tissues [243, 278, 279].

Spatial metabolomics

Adaptation of cellular metabolism is critical for the tissue immune microenvironment (TIM) to maintain homeostasis or initiate damage responses [280]. Changes in metabolic flux determine chromatin accessibility through the direct modification of histones by metabolites, thereby influencing subsequent downstream transcriptional responses and ultimately dictating cell fate [281, 282]. Spatial metabolomics enables the mapping of metabolite distributions within tissues or cells, allowing for the investigation of metabolic activity variations across different spatial locations and providing in-depth analysis of the metabolic microenvironment in distinct tissue regions.

The current demand for spatial metabolomics approaches has been driven by advancements in MSI technologies [283]. MSI is a powerful method for performing in situ analysis of the molecular composition of biological tissues while preserving spatial information [284]. Initially, studies were constrained by limitations in spatial resolution and molecular classification. However, the rapid advancement of MSI instrumentation now enables the imaging of thousands of molecules, including metabolites, lipids, proteins, and glycans, at (sub)cellular resolution [285, 286]. Desorption electrospray ionization (DESI) and matrix-assisted laser desorption ionization (MALDI) MSI are complementary techniques for identifying hundreds of metabolites with near-single-cell resolution in a spatial context [287]. Metabolic reprogramming is a key regulator of cancer progression [288, 289] and may contribute to treatment resistance [290], underscoring the necessity of evaluating metabolites and metabolic fluxes. MALDI-MSI allows for the spatial visualization of metabolites, particularly lipids, in tissues via laser ionization [291]. The matrix is directly applied to tissue sections, forming a co-crystal with metabolites [292]. Upon laser irradiation, the matrix is ionized (by gaining or losing a proton), transferring charge to the metabolites, leading to their desorption and ionization [292, 293]. The number of metabolites detected depends on matrix application, instrument parameters, and metabolite stability. DESI-MSI employs electrospray ionization to extract metabolites from tissues through a fine spray of charged solvent droplets [294]. A key advantage is the minimal matrix interference, enabling multiple analyses of the same tissue section with different ionization modes and spatial resolutions, or tandem mass spectrometry for metabolite confirmation [295]. High-speed data acquisition at low resolution can be followed by high spatial resolution analysis of selected regions. Due to the lack of matrix, which typically interferes with mass-to-charge ratios (m/z) below 500 Da, DESI-MSI is particularly well-suited for measuring small metabolites [296–304].

To infer biological mechanisms within spatial contexts, it is ideal to integrate spatial metabolomics with other omics layers in the same tissue section. Like other omics technologies, spatial metabolomics generates extensive datasets containing molecular information from a considerable number of pixels [305]. Within the broader field of omics, a significant emphasis lies on integrating data from diverse modalities to create a holistic, multi-modal perspective of cellular states [306, 307]. Ultimately, further efforts are needed to standardize sample preparation, metabolite detection, and identification to achieve broad approval for patient care [287].

Computational methods

The integration of computational methodologies has become a cornerstone in modern biomedical research, revolutionizing the way extensive and complex datasets are processed and interpreted. These advanced tools significantly enhance the accuracy and efficiency of diagnostics, scientific research, and personalized medicine by unlocking deeper insights into biological systems. In particular, computational analysis has facilitated the extraction of meaningful conclusions from vast datasets that were once difficult to handle. Among the most notable advancements is the application of these techniques in 3D histology, where computational methods have drastically improved 3D tissue reconstruction and analysis [308]. By focusing on spatial mapping, gene expression profiling, protein interactions, and segmentation, these computational tools offer unprecedented capabilities, ultimately pushing the boundaries of what can be achieved in tissue analysis (Fig. 4; Tables 1 and 2).

Analysis software

In the rapidly evolving field of biomedical research, computational tools have become indispensable for 3D tissue

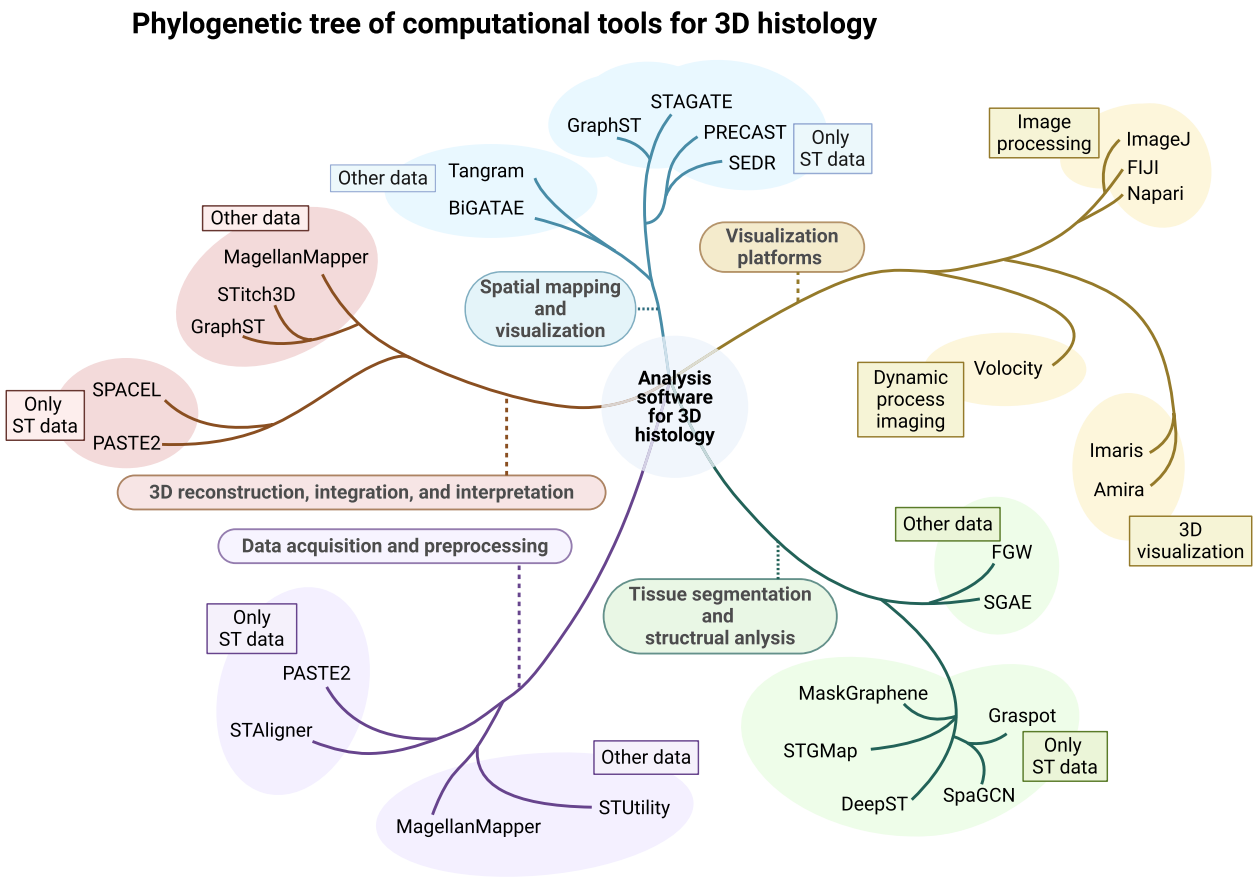

Fig. 4 Phylogenetic tree of computational tools for 3D histology. The categorization of analysis software for 3D histology can be visualized as a tree structure, with a central "root" labeled "Analysis Software for 3D Histology" and "Visualization Platforms". The main branches extending from this root represent the primary areas of expertise for each tool, categorized into four key sections: "Data Acquisition and Preprocessing", "Tissue Segmentation and Structural Analysis", "Spatial Mapping and Visualization", and "3D Reconstruction, Integration, and Interpretation". Each tool or subgroup's input data types are indicated by colored boxes for clarity. In total, the diagram illustrates 19 distinct tools, providing a comprehensive overview. For detailed information, including summaries and repository availability, please refer to Tables 1 and 2

analysis, allowing researchers to study biological structures with unprecedented detail. These tools facilitate the extraction of meaningful biological insights from complex datasets, providing a deeper understanding of tissue organization, function, and pathology. A recommended workflow for 3D tissue analysis includes four main stages: "data acquisition and preprocessing," "tissue segmentation and structural analysis," "spatial mapping and visualization," and "3D reconstruction, integration, and data interpretation." Each stage involves specific computational tools designed to address the unique challenges of interpreting high-dimensional data in a three-dimensional context. Below, we categorize these tools based on their functions, highlighting their contributions to the field of 3D tissue analysis.

Data acquisition and preprocessing

Effective 3D tissue analysis begins with the critical step of acquiring high-quality data. This involves advanced imaging techniques like confocal and light-sheet microscopy or spatial transcriptomics, which generate detailed, multi-dimensional datasets. Preprocessing this raw data is essential, encompassing the correction of imaging artifacts, alignment of tissue slices, and enhancement of image clarity. MagellanMapper offers a user-friendly interface for data acquisition and includes multi-scale modeling and 3D visualization capabilities, allowing researchers to clean, align, and enhance images before further analysis [309]. Additionally, STUtility plays a crucial role in preprocessing spatial transcriptomics data by aligning histological images and synchronizing spatial expression data, which are vital for accurate downstream analyses [310, 311]. For handling large datasets and aligning multiple tissue sections, PASTE2 improves stitching accuracy by aligning adjacent spatial transcriptomics slices, maintaining the coherence of tissue structures during preprocessing [312, 313]. STAligner further enhances preprocessing with deep learning techniques,

Table 1 3D histology tools. This table summarizes various computational tools developed for analyzing 3D histology data derived from spatial omics techniques. Here we provide information on the most powerful field(s), algorithm, input, output, languages, URL, and the first article introduces the tools. These tools facilitate a deeper understanding of tissue architecture and cell distribution, advancing the application of spatial omics in biological and biomedical research

Tool	Feature	Most powerful field(s)	Algorithm	Input	Output	Languages	URL	PMID
SpaGCN	a graph convolutional network to integrate gene expression and histology to identify spatial domains and spatially variable genes	Tissue segmentation and structural analysis	network-based tools	gene expression data; spatial coordinates; histoloigical pixel intensites; graph structure	spatial domains; cell-cell interactions	Python; Jupyter Notebook	https://github.com/jianhuupenn/SpaGCN	34711970
SGAE	learn discriminative spot representation and decipher accurate spatial domains	Tissue segmentation and structural analysis	network-based tools	ST data; graph structure	discriminative spot representation; spatial domain clustering; 3D tissue reconstruction	Jupyter Notebook; Python	https://github.com/STOmics/SGAE/	38373745
DeepST	a deep learning toolbox for spatio-temporal data	Tissue segmentation and structural analysis	network-based tools	ST data; spatial coordinates; batch information	spatial domains; batch-integrated data; finer-scale spatial insights	Python; Batchfile	https://github.com/amirkhango/DeepST	36250636
STAGATE	spatial clustering and denoising expressions of spatial resolved transcriptomics (ST) data	Spatial mapping and visualization	network-based tools	ST data; spatial coordinates; graph structure	latent embeddings; reconstructed expression profiles; spatial domains; extracting 3D spatial domains	Python	https://github.com/QIFEIDKN/STAGATE	35365632
MaskGraphene	better aligning and integrating different ST slices using both self-supervised and contrastive learning	Tissue segmentation and structural analysis	network-based tools	ST data; spatial coordinates; graph structure	joint embeddings; shared and unique cell/domain types; batch-corrected data	Python; Jupyter Notebook	https://github.com/maiziezhoulab/MaskGraphene	Hu, Yunfei, et al. "MaskGraphene: Advancing joint embedding, clustering, and batch correction for spatial transcriptomics using graph-based self-supervised learning." bioRxiv (2024): 2024-02.
Graspot	a graph attention network for spatial transcriptomics data integration with optimal transport	Tissue segmentation and structural analysis	network-based tools	ST data; spatial coordinates; graph structure	unified latent space embeddings; partially aligned spots; spatiotemporal reconstruction	Python	https://github.com/zhan009/Graspot	Gao, Zizhan, Kai Cao, and Lin Wan. "Graspot: A graph attention network for spatial transcriptomics data integration with optimal transport." bioRxiv (2024): 2024-02.

Table 1 (continued)

Tool	Feature	Most powerful field(s)	Algorithm	Input	Output	Languages	URL	PMID
SEDR	learns a low-dimensional latent representation of gene expression embedded with spatial information for spatial transcriptomics analysis	Spatial mapping and visualization	network-based tools	ST data; spatial coordinates; graph structure	low-dimensional latent representations; spatial clustering	Python	https://github.com/JinmiaoChenLab/SEDR	38217035
GraphST	incorporates spatial location information and gene expression profiles to accomplish three key tasks, spatial clustering, spatial transcriptomics data integration, and single-cell RNA-seq transfer onto ST	Spatial mapping and visualization; 3D reconstruction, integration, and data interpretation	network-based tools	ST data; scRNA-seq data; graph structure	spatial domains; batch-corrected data; cell attribute projection	Python	https://github.com/JinmiaoChenLab/GraphST	36859400
BiGATAE	a bipartite graph attention auto-encoder enhancing spatial domain identification from single-slice to multi-slices	Spatial mapping and visualization	network-based tools	ST data; spatial coordinates; adjacency matrix	integrated spatial domains; enhanced clustering performance; extended applicability of single-slice methods	Python; Jupyter Notebook	https://github.com/fdu-wangfeilab/BiGATAE?tab=readme-ov-file	38385877
STitch3D	construction of a 3D whole organism spatial atlas by joint modelling of multiple slices with deep neural networks	3D reconstruction, integration, and data interpretation	network-based tools	ST data; scRNA-seq data; spatial coordinates	3D tissue reconstruction; 3D spatial regions; spatial distribution of cell types	Python; R	https://github.com/YangLabHKUST/STitch3D	Wang, Gefei, et al. "Construction of a 3D whole organism spatial atlas by joint modelling of multiple slices with deep neural networks." Nature Machine Intelligence 5.11 (2023): 1200-1213.
Tangram	aligns the single-cell data in space by fitting gene expression on the shared genes	Spatial mapping and visualization	difference-assembly-based tools	single-cell gene expression data; spatial gene expression data; shared genes	aligned spatial single-cell data; spatially resolved cell types	Jupyter Notebook; Python	https://github.com/broadinstitute/Tangram	34711971
PRECAST	a probabilistic embedding and clustering with alignment for spatial transcriptomics data integration	Spatial mapping and visualization	difference-assembly-based tools	SRT datasets; partially shared clusters	unified spatial embeddings; spatial clustering; integrated spatial analysis	R; C++; C	https://github.com/feiyoung/PRECAST	36653349

Table 1 (continued)

Tool	Feature	Most powerful field(s)	Algorithm	Input	Output	Languages	URL	PMID
MagelLanMapper	a graphical imaging informatics suite for 3D reconstruction and automated analysis of whole specimens and atlases	Data acquisition and preprocessing; 3D reconstruction, integration, and data interpretation	difference-assembly-based tools	3D image data; annotations	3D reconstruction; automated analysis; cross-platform scalability	Python; Shell; R; Jupyter Notebook; Batchfile; Dockerfile NSIS	https://github.com/sanderslab/magellanmapper	32981139
STUtility	facilitates the analysis of spatial transcriptomics experiments, where gene expression is measured in tissue sections while preserving spatial information	Data acquisition and preprocessing	difference-assembly-based tools	10x Genomics visium data	aligned 3D models; regional annotations; data processing and analysis	R	https://github.com/jbergenstrahle/STUtility.git	Bergenstråhle, J., Larsson, L., & Lundeberg, J. (2020). Seamless integration of image and molecular analysis for spatial transcriptomics workflows. BMC genomics, 21, 1-7.
STGMap	a universal framework for spatial transcriptomics data mining with interpretable unsupervised graph representation learning	Tissue segmentation and structural analysis	difference-assembly-based tools	ST data; graph structure	interpretable embeddings; spatial clustering	Python	https://github.com/YuBinLab-QUST/STGMap	Liu, Long, et al. "A Universal Framework for Spatial Transcriptomics Data Mining with Interpretable Unsupervised Graph Representation Learning." Available at SSRN 4791548.
STAligner	alignment and integration of spatially resolved transcriptomics data	Data acquisition and preprocessing	difference-assembly-based tools	ST data; spatial neighbor network	batch-corrected embeddings; aligned spatial domians	Jupyter Notebook; Python	https://github.com/zhoux85/STAligner	38177758
PASTE2	partial alignment and 3D reconstruction of spatial transcriptomics slices when they do not fully overlap in space	Data acquisition and preprocessing; 3D reconstruction, integration, and data interpretation	expression-permutation-based tools	SRT data; spatial coordinates; optional histological images	partially aligned multislice data; 3D reconstruction; model selection	Jupyter Notebook; Python	https://github.com/raphael-group/paste2	37553263
SPACEL	cell type deconvolution	3D reconstruction, integration, and data interpretation	expression-permutation-based tools	ST data; spatial coordinates	spoint; splane; scube	Python	https://github.com/QuKunLab/SPACEL	37990022

Table 1 (continued)

Tool	Feature	Most powerful field(s)	Algorithm	Input	Output	Languages	URL	PMID
FGW	applicable between graphs with different number of nodes and with any type of label/feature on the nodes	Tissue segmentation and structural analysis	expression-permutation-based tools	graphs or structured objects; node features/labels; graph structures	soft node assignments; FGW diatance; barycenter	Python	https://github.com/tvayer/FGW	Vayer, Titouan, et al. "Fused Gromov-Wasserstein distance for structured objects." Algorithms 13.9 (2020): 212.

Table 2 Tools' functional assessment. This table evaluates the functional capabilities of various computational tools designed for 3D histology analysis in spatial omics. The assessment is organized into categories including slice alignment, data integration, aligning partially overlapped datasets, non-rigid coordinate transformation, integrating partially overlapped datasets, batch effect removal, cell-type deconvolution, clustering, spatial domain identification, denoising, and 3D reconstruction. Each tool is assessed based on the original article. This functional assessment provides valuable insights into the strengths and limitations of available tools, guiding researchers in selecting the most suitable options for their specific applications

Tool	Slice Alignment	Data Integration	Aligning Partially Overlapped Datasets	Non-rigid Coordinate Transformation	Batch Effect Removal	Cell-type Deconvolution	Clustering	Spatial Domain Identification	Denoising	3D Reconstruction
SpaGCN							√	√	√	
SGAE							√	√	√	√
DeepST							√	√	√	
STAGATE							√	√	√	
MaskGraphene							√	√	√	
Graspot							√	√	√	
SEDR						√	√	√		
GraphST							√	√	√	
BiGATAE							√	√	√	
STitch3D		√					√	√		√
Tangram		√					√	√		
PRECAST		√					√	√		
MagellanMapper		√					√	√		√
STUtility		√					√	√	√	
STGMap		√					√	√		
STAligner	√		√	√						
PASTE2		√			√		√	√		√
SPACEL		√					√	√		√
FGW							√	√	√	

ensuring complex tissue samples are aligned and ready for subsequent analysis [314].

Tissue segmentation and structural analysis

Once data acquisition and preprocessing are complete, the focus shifts to the segmentation and structural analysis of tissues. This step is essential for distinguishing between different tissue components, identifying cell types, and delineating structural features. Segmentation tools like DeepST [315], which leverages convolutional neural networks, enable precise delineation of intricate tissue structures. To further refine segmentation, STG-Map provides clustering and alignment tools that aid in distinguishing between various regions and cellular components, especially in tissues with complex architectures [316]. Additionally, graph-based approaches such as those utilized by MaskGraphene [317] and SpaGCN [318] are invaluable for analyzing tissue networks, mapping cell interactions, and identifying unique spatial domains. Moreover, Sparse Graph Autoencoder (SGAE) [319] simplifies the analysis of complex regulatory networks within tissues by identifying sparse representations of gene expression data. By focusing on dimensionality reduction and feature extraction for high-dimensional data, particularly in the analysis of gene expression, SGAE provides researchers with a manageable approach to studying gene regulation, facilitating the discovery of new insights into tissue function and disease mechanisms [320]. For heterogeneous datasets, Fused Gromov-Wasserstein (FGW) [321] applies optimal transport theory to integrate multi-modal data, enhancing the resolution and accuracy of 3D models of cellular interactions [322]. By providing detailed and accurate models of tissue structures, Graspot enables researchers to gain a more comprehensive understanding of tissue architecture [323]. These tools collectively enhance our understanding of the tissue microenvironment, offering insights into both healthy and pathological conditions.

Spatial Mapping and Visualization

Spatial mapping focuses on understanding the spatial arrangement of cellular components and visualizing gene expression patterns within tissues in a three-dimensional context. This step is pivotal for elucidating the relationships between spatial positioning and biological function. Tools like GraphST [324] and STAGATE [325] are instrumental in spatial mapping, employing graph-based techniques to integrate spatial and gene expression data. Moreover, Spatially Embedded Deep Representation (SEDR) addresses this challenge by employing deep autoencoders and a masked self-supervised learning mechanism to construct low-dimensional latent representations of gene expression, while simultaneously embedding the corresponding spatial information through a variational graph autoencoder [326, 327]. Bipartite Graph Attention Auto Encoder (BiGATAE), a shell-like tool, can seamlessly integrate with existing single-slice clustering methods, adapting them for multi-slice clustering to enhance ST data by incorporating information from adjacent slices [328, 329].These tools allow for the visualization of gene expression changes across various regions, uncovering patterns that might be overlooked in two-dimensional analyses.

For reconstructing spatial landscapes, Tangram excels in aligning scRNA-seq data with tissue architecture, unveiling critical spatial patterns that enhance our understanding of tissue complexity [330]. In addition, Principal Component Regression and Statistical Analysis Tool (PRECAST) has been developed as a data integration method for multiple spatial transcriptomics datasets that exhibit complex batch effects and/or biological effects between slides [331–333].

3D Reconstruction, integration, and data interpretation

The final phase in 3D tissue analysis encompasses reconstructing a comprehensive 3D model from multiple data slices and interpreting the integrated dataset. This phase transforms separate two-dimensional slices into a cohesive and detailed 3D structure, enabling a holistic visualization of tissue architecture and fostering meaningful biological insights. STitch3D is a key tool in this phase, assembling serial tissue sections into a continuous 3D dataset [334]. It ensures precise alignment and corrects distortions to maintain an accurate spatial representation. PASTE2 contributes to creating seamless 3D structures by aligning multiple tissue slices while preserving spatial and gene expression data integrity [312, 313]. Advanced data integration follows, merging multiple data modalities—such as gene expression and spatial data—for a more profound understanding of biological functions. Tools like GraphST utilize graph-based neural networks to integrate and cluster similar regions, identifying distinct cell types [324].

Visualization of reconstructed 3D tissues is equally critical. MagellanMapper offers robust 3D visualization capabilities, allowing for multi-scale modeling that spans from macroscopic structures to microscopic features [309]. For accurate biological interpretation, SPACEL corrects imaging artifacts, ensuring reconstructed models faithfully represent the original biological tissue [335]. This comprehensive phase, blending 3D reconstruction with advanced data interpretation, offers unprecedented insights into the complexity of tissue architecture and function.

Visualization platforms

ImageJ is a highly versatile, open-source software platform widely used for image processing and analysis in biomedical research [336–339]. FIJI (Fiji Is Just ImageJ) is an enhanced version of ImageJ, bundled with a comprehensive set of plugins that facilitate a broad range of imaging tasks, including image segmentation, 3D reconstruction, and quantitative analysis. One of the significant advantages of ImageJ/FIJI is its customizability, allowing users to write macros and plugins tailored to their specific research needs [340]. It supports various image formats and integrates seamlessly with other software tools, making it a staple in many laboratories worldwide [341].

To view the smaller ones, Imaris provides high-resolution 3D visualization and analysis capabilities, making it an essential tool for studying cellular and subcellular structures [342]. Its advanced algorithms for image segmentation, tracking, and visualization allow researchers to gain deep insights into dynamic biological processes. Imaris is particularly valuable for applications in cell biology and neuroscience, where precise tracking of cell movements and interactions is crucial [343]. The software's interactive visualization tools enable researchers to explore complex datasets intuitively, facilitating the discovery of new biological insights [344].

Napari is an open-source, Python-based visualization platform designed for the analysis of multi-dimensional data, particularly in the field of biological imaging. It provides high-performance visualization for 2D, 3D, and even 4D (time-lapse) datasets, making it an ideal tool for exploring complex biological structures and dynamic processes. One of the key strengths of Napari is its extensibility; users can integrate custom plugins and leverage its support for a wide range of image formats, allowing for tailored analysis workflows. Napari's interactive features and efficient memory management make it particularly valuable for high-throughput analysis in life sciences, particularly in applications like single-cell imaging and multi-channel fluorescence microscopy [345].

As for data, Amira is a sophisticated software platform designed for visualizing and analyzing 3D biomedical data [346–349]. It excels in handling large datasets and offers advanced features for image segmentation, 3D modeling, and quantitative analysis. Amira's user-friendly interface and comprehensive toolset enable detailed structural and functional analyses of biological tissues. This software is particularly effective for complex visualizations and simulations in developmental biology and neuroscience. Amira's ability to integrate various data types, including MRI, CT, and microscopic images, makes it a versatile tool for multidisciplinary research [350].

Focusing on dynamic processes, Volocity offers features such as image reconstruction, object segmentation, and quantitative analysis, making it a powerful tool for 3D imaging and analysis [351, 352]. Its intuitive interface and robust analysis capabilities make it suitable for a wide range of applications, including live cell imaging and developmental biology. Volocity's strength lies in its ability to handle multi-channel and time-lapse data, providing a comprehensive view of dynamic processes. The software's compatibility with various imaging modalities ensures it can be seamlessly integrated into existing workflows, enhancing productivity and data accuracy [353].

3D tissue reconstruction

3D reconstruction technologies are revolutionizing biomedical research by enabling the precise creation of complex tissue structures. This section discusses two main approaches to 3D reconstruction: cellular reconstruction and computational reconstruction, both of which are essential for understanding tissue architecture and function. While cellular/ histological reconstruction focuses on the physical fabrication of tissues and cell models, computational reconstruction uses digital technologies to create and analyze 3D representations of tissue structures.

Cellular recounstruction

Bioprinting and fabrication have emerged as groundbreaking technologies for creating complex tissue structures using 3D printing. These methods allow for the precise deposition of cells, biomaterials, and growth factors to fabricate customized tissue models and potentially functional organs [354]. As a new industrial method, 3D bioprinting could create 3D tissues by depositing material layer by layer [6]. In the biomedical field, 3D printing has been applied to the production of transplant organs and instruments, such as pelvis, mandible, joints, prosthetics, and other support structures. Recent developments in bioprinting technology include improvements in material formulations and printing techniques, enabling the creation of more complex and functional tissue constructs [355, 356]. With the significant growth of 3D bioprinting in medicine and bioengineering, the fabrication of tissues, organs, prosthetic devices, and drug-delivery technologies will become possible [5, 357].

In parallel, 3D cell cultures such as organoid cultures [358] and spheroid cultures have become essential for studying tissue function and drug responses in 3D environments. Organoids are self-organizing structures that mimic the architecture of real organs [359], they can be grown from both embryonic, induced stem cells and also from adult stem cells [360]. With the continuous

development of organoid culture systems and experimental techniques, organoid culture has been applied to various tissues and organs, including intestine (small intestine/colon), stomach, liver, heart, lung, prostate, pancreas, kidney, breast, brain, retina, and inner ear [361–369]. Organoids derived from tumor stem cells are also beginning to show great potential in helping to understand the mechanism of tumor development, screening drug sensitivity, and promoting precision medicine and personalized diagnosis and treatment [370]. Organoids have unique advantages compared to cell line and patient-derived xenotransplantation models, but there is no standardized method to guide the cultivation of organoids, which has led to confusion in organoid research. Therefore, continuous technical improvement and improved repeatability are the keys to successfully solving this problem [371].

In contrast, spheroids are compact cell aggregates used to study cellular interactions and responses. The tight spatial arrangement of cells in the body enables complex interactions between individual cells and between cells and the extracellular matrix. This arrangement provides cells with unique cues to guide specific cell functions and growth. To replicate this spatial arrangement in vitro, cells can be grown in a 3D culture system that makes cell-to-cell contact to form spheres [372, 373]. Compared to monolayer or scaffold-based cultures, the cell spheres contribute to cell growth and have better cell function in terms of potency, angiogenesis, and differentiation. At present, cell spheres have been increasingly used in many fields such as cancer research, drug screening, and tissue assembly [374–376]. Recent developments in these culture methods include improved protocols for generating organoids and spheroids that more accurately replicate in vivo conditions [377].

Computational reconstruction

Simultaneously, 3D reconstruction provides a more comprehensive understanding of tissue architecture by digitally assembling 2D images into detailed 3D models. Computational reconstruction uses advanced algorithms and digital technologies to generate 3D representations of tissue structures from various imaging modalities, such as histology or microscopy images. These digital models allow researchers to study tissue at unprecedented levels of detail and complexity. As previously discussed in Sect. "4) Spatial proteomics", computational methods primarily focus on the algorithms involved in 3D reconstruction. This section, therefore, will complement that discussion by focusing on the application of digital reconstruction through the use of AR (Augmented Reality) and VR (Virtual Reality) technologies, which enhance the visualization and interaction with these 3D tissue models.

VR and AR have revolutionized 3D histology by enhancing the understanding and analysis of complex tissue structures. AR enhances anatomical visualization by superimposing virtual 3D information onto the real world, allowing researchers and medical professionals to interact with tissue structures within their actual environment. This is particularly beneficial for histological studies, where AR can project 3D tissue structures directly onto real-world objects, facilitating a deeper understanding of their spatial relationships [378]. For instance, pathologists can use AR to overlay virtual histological slices onto actual tissue samples, providing a comprehensive view that combines microscopic details with macroscopic context [379]. Similarly, VR offers an immersive environment where users can interact with 3D histological models from multiple angles and scales. This technology allows researchers to zoom in, rotate, and slice through virtual tissue models, providing an in-depth understanding of cellular and tissue morphology that is challenging to achieve with traditional 2D methods [380]. For example, VR can reconstruct histological sections into a 3D model, enabling comprehensive analysis of the spatial distribution of cells and extracellular matrix components within tissues.

The clinical implication and research progress of single-cell 3D histology

3D tissue analysis is reshaping biomedical research and clinical practice by providing unique insights into the intricate structures and functions of tissues. Through the integration of cutting-edge imaging techniques, experimental methodologies, and computational tools, single-cell 3D histology advances our understanding of disease mechanisms. This essay explores the profound impact of single-cell 3D histology across various fields, including oncology, cardiovascular diseases, neuroscience, immunology, developmental biology, regenerative medicine, and other medical specialties. The following sections delve into specific applications and advancements in each area, illustrating how single-cell 3D histology is revolutionizing diagnostics, treatment planning, and therapeutic outcomes (Fig. 5).

Oncology

Single-cell 3D histology has not only revolutionized our understanding of tumor biology but has also transformed the broader landscape of cancer research by enabling researchers to explore the intricate interplay between tumors and their microenvironments in unprecedented detail. This innovative approach is essential for dissecting the complex mechanisms underlying tumor progression,

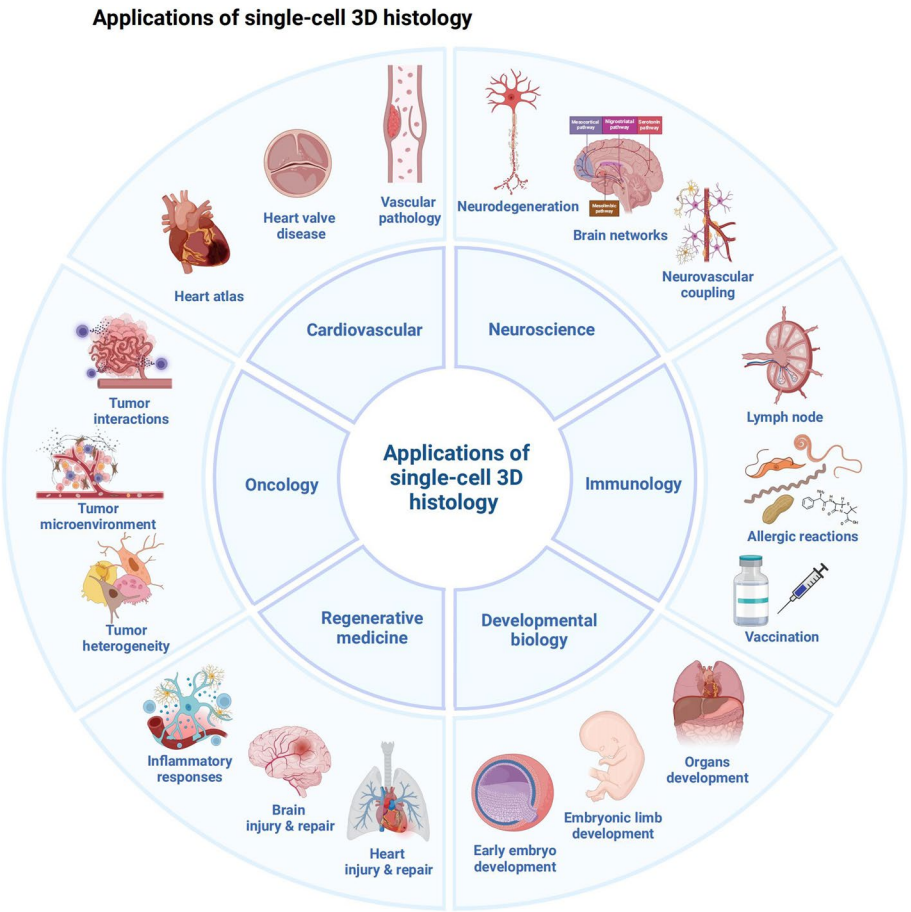

Fig. 5 Applications of single-cell 3D histology in clinical research. This circular figure illustrates the diverse applications of single-cell 3D histology in clinical research, segmented into six key areas: 1) Oncology focuses on tumor interactions, the tumor microenvironment, and tumor heterogeneity. 2) Cardiovascular highlights issues such as coronary artery disease, heart valve disease, and vascular pathology. 3) Neuroscience encompasses neurodegeneration, brain networks, and neurovascular coupling. 4) Immunology explores lymph node function, allergic reactions, and the cytokine storm phenomenon. 5) Developmental Biology examines aspects like placenta development, early embryo development, and the growth of the uterus and ovaries. 6) Regenerative Medicine emphasizes vascularization, the detailed visualization and reconstruction of skin tissue, and the implantation of biomaterials for tissue regeneration. Each segment underscores the potential of single-cell 3D histology to provide critical insights into various medical challenges, ultimately aiding in the development of more effective treatment strategies

immune evasion, and treatment resistance, providing a multifaceted view that traditional methodologies cannot achieve.

Moreover, the integration of spatial omics technologies with single-cell analyses has enhanced our ability to uncover the complex relationships between tumor cells and the immune system [381–391]. Spatiotemporal metabolomic approaches, in conjunction with 3D histology, have significantly advanced our understanding of how metabolic processes evolve within the tumor microenvironment, effectively linking cellular interactions to structural changes over both time and space in cancer biology [381]. For example, Open-ST is a high-resolution, cost-effective, and scalable spatial transcriptomics method capable of capturing subcellular resolution while reconstructing cellular organization in tissues. This technique provides crucial insights into molecular mechanisms across both 2D and 3D contexts, particularly revealing spatially organized cell states and biomarkers at the tumor/lymph node boundary, which is vital for understanding cancer metastasis [382]. Moreover, multiplex digital spatial profiling (mplxDSP) technology is utilized to dissect the tumor microenvironment of pancreatic ductal adenocarcinoma (PDAC) at the single-cell level, unveiling distinct gene expression patterns in cancer-associated fibroblasts (TAFs) and immune cells. These findings inform immune tolerance mechanisms and highlight potential prognostic markers linked to patient survival [383]. Recent advances in

single-cell omics technologies have elucidated the molecular diversity of tumor-associated macrophages (TAMs), proposing a consensus model for TAM classification and emphasizing the urgent need for standardized nomenclature and comprehensive annotation to enhance targeted therapeutic strategies in cancer treatment [384]. Furthermore, single-cell and spatial transcriptomic analyses reveal critical interactions between FAP^+ fibroblasts and $SPP1^+$ macrophages in colorectal cancer, demonstrating their role in creating an immune-excluded microenvironment that limits T-cell infiltration. This suggests that targeting these interactions could enhance the efficacy of immunotherapies, such as anti-PD-L1 treatments [385]. The spatial heterogeneity of the tumor microenvironment in hepatocellular carcinoma (HCC) was explored through profiling 401 samples with 36 biomarkers, identifying vimentin (VIM) high macrophages as immunesuppressive, which in turn enhances regulatory T cell activity and promotes tumor progression. These insights highlight the potential for personalized treatment strategies in HCC by targeting specific spatial features of the tumor microenvironment [386]. Another innovative approach, Perturb-map, integrates clustered regularly interspaced short palindromic repeats (CRISPR) screening with spatial transcriptomics to effectively identify regulators of the tumor microenvironment (TME) in a lung cancer model. This method reveals how the loss of TGFβ-receptors in cancer cells can reshape the TME, enhancing its immunosuppressive properties by promoting a fibro-mucinous state and T cell exclusion [387]. Additionally, the prognostic significance of myeloid immune cells in colorectal cancer has been highlighted through multiplexed immunofluorescence and spatial analysis. 3D in vitro cell culture models designed to mimic the hypoxic TME and its immunosuppressive effects further illustrate the advantages of single-cell 3D histology over traditional 2D models. These advanced systems can replicate TME-immune interactions more accurately and also address challenges in integrating key hallmarks that influence model functionality [389]. Higher densities of mature $CD14^+HLA\text{-}DR^+$ cells correlate with lower cancer-specific mortality, whereas immature $CD14^+HLA\text{-}DR^-$ cells show increased mortality risk, underscoring the importance of spatial distribution and multi-marker evaluations in the tumor microenvironment [392]. Spatial transcriptomics has been utilized to uncover spatial gene expression patterns in HER2-positive breast cancer, revealing tumor-associated cell type interactions and shared gene signatures that underscore immune responses and intra-tumor heterogeneity. This high-resolution mapping aids in understanding tumor biology and guiding treatment strategies [390]. Researchers are also employing multiplexed tissue imaging, 3D reconstruction, spatial statistics, and machine learning to create detailed atlases of cellular state transitions and immune interactions in colorectal cancer. This comprehensive approach reveals spatial gradients and complex molecular dynamics that shape tumor morphology and immune suppression, particularly at the tumor invasive margin. As the field of cancer research continues to evolve, these innovative techniques are paving the way for more effective diagnostic and therapeutic strategies, ultimately enhancing patient outcomes [391].

The TME is crucial for understanding cancer progression and therapy resistance [393–398]. Recent advancements in single-cell spatial transcriptomics, particularly through techniques like spatially constrained optimal transport interaction analysis (SCOTIA), allow researchers to reveal how therapy-associated remodeling of the TME impacts cancer dynamics. For example, changes in ligand-receptor interactions between TAFs and malignant cells have been shown to contribute significantly to chemotherapy resistance in pancreatic cancer, highlighting the intricate interplay within the TME [393]. Moreover, the development of open-source software pipelines enables the quantification of tumor-infiltrating lymphocytes (TILs) and the tumor-stroma ratio (TSR) in colorectal cancer using whole-slide images. This tool effectively classifies cancers into five subgroups, with the CMS4-like subgroup correlated with poorer survival outcomes. Such advancements underscore the potential of machine learning in analyzing the tumor-immune microenvironment, thereby improving prognostic capabilities [394]. Furthermore, recent advancements in bulk and single-cell sequencing technologies have significantly improved our understanding of tumor biology. These innovations allow for spatial mapping techniques that address tumor heterogeneity, microenvironments, and biomarkers, thus highlighting the potential for these technologies to inform therapeutic strategies and future developments in oncology. By integrating single-cell 3D histology, researchers can achieve a more comprehensive understanding of tumor architecture, heterogeneity, and the TME, providing spatially resolved omics profiles that are crucial for advancing treatment strategies [399].

Traditional 2D cell culture models often fail to replicate the complex interactions between tumor cells and their surrounding stroma, leading to a limited understanding of tumor biology [396]. In contrast, 3D models, such as organoids and spheroids, mimic the architecture and cellular heterogeneity of actual tumors, offering a more accurate representation of in vivo conditions. These 3D systems facilitate the study of cancer stem cell niches, which are critical for tumor initiation, maintenance, and resistance to therapy [397]. TAFs play a significant role in modulating the TME and promoting cancer progression.

By leveraging single-cell 3D histology, researchers can better study the interactions between TAFs and tumor cells, revealing their contributions to tumor growth, invasion, and therapy resistance [398]. This comprehensive understanding of TAFs and their interactions is essential for developing more effective treatment modalities and improving patient outcomes in various cancer types.

The progression of cancer, particularly metastatic HCC, can be profoundly understood through integrated multi-omics profiling. This approach uncovers the spatiotemporal evolution of tumors, revealing significant genomic divergence between primary and metastatic lesions, as well as polyclonal dissemination and neoantigen heterogeneity, which contribute to immune evasion. Such studies emphasize the critical roles of somatic copy number alterations and the pro-metastatic microenvironment in facilitating tumor spread [400]. In the realm of breast cancer, whole-genome sequencing, along with highly multiplexed base-specific in situ sequencing and single-cell resolved transcriptomics, allows scientists to create comprehensive maps of genetic subclones. These maps reveal complex growth patterns and unique features of the tumor microenvironment, enhancing our understanding of cancer evolution and the spatial ecology of tumors [401]. Additionally, spatially resolved transcriptomics have been utilized to investigate copy number variations across more than 120,000 regions in both benign and malignant prostate tissues. This research uncovers distinct clonal patterns that shed light on early genomic instability in benign tissues, challenging existing treatment paradigms [402]. A comprehensive spatiotemporal analysis has identified COL1A1 as a key target for disrupting oncostreams in glioblastoma, which are dynamic multicellular structures contributing to tumor heterogeneity and progression [395]. Targeting COL1A1 could potentially reprogram glioma characteristics and alter the tumor microenvironment, thereby enhancing treatment efficacy and improving patient survival [403]. The architectural features of tissues play a crucial role in tumor initiation and progression, influencing the mechanical microenvironment, stromal cell accessibility, and invasion pathways [404]. Moreover, spatial genomics and population genetics models illustrate how tumor structure can dictate evolutionary patterns, such as clonal expansion or branching. These insights highlight the effectiveness of spatial modeling techniques in predicting and possibly controlling cancer progression [405].

The role of single-cell 3D histology in understanding tumor angiogenesis is another critical area of exploration [406, 407]. Angiogenesis, the formation of new blood vessels, is a hallmark of tumor progression and a significant contributor to metastasis. Advanced 3D angiogenesis models, including organ-on-chip and vascular organoid systems, allow researchers to study the mechanisms driving tumor-associated vascular changes in a controlled environment [406]. For example, the use of U-net algorithms for vessel segmentation has enabled detailed analysis of vascular structures, revealing how tumors manipulate blood vessel formation to secure nutrients and promote growth. Understanding these processes at a spatial level not only aids in elucidating tumor biology but also helps identify potential therapeutic targets to disrupt the blood supply to tumors [407].

Another most compelling aspects of single-cell 3D histology is its ability to reveal tumor heterogeneity at a spatial level [244, 390, 399, 408–412], allowing for the examination of how different cell populations coexist within a tumor. Recent advancements in bulk and single-cell sequencing technologies have profoundly transformed our understanding of tumor biology, elucidating how tumor architectures influence cancer mechanisms [408]. These innovations enable spatial mapping techniques that effectively address tumor heterogeneity, microenvironments, and biomarkers, emphasizing their potential to inform therapeutic strategies and future developments in oncology. The insights gained from spatial and temporal intratumor heterogeneity analyses also prove invaluable in conditions like neuroblastoma, where high variability in somatic mutations and copy-number alterations poses challenges to the reliability of single biopsies in treatment decisions [409]. This highlights the necessity for comprehensive multi-regional profiling to effectively inform targeted therapies. In HER2-positive breast cancer, spatial transcriptomics uncovers spatial gene expression patterns, revealing critical tumor-associated cell type interactions and shared gene signatures that underscore immune responses and intra-tumor heterogeneity [390]. Such high-resolution maps are essential for understanding tumor biology and guiding treatment strategies, showcasing the dynamic changes within tumors over time, including the roles of genetic and epigenetic alterations in driving tumor progression and therapy resistance [410]. Researchers have also leveraged spatially resolved paired whole-genome and transcriptome sequencing to demonstrate that intratumor heterogeneity in colorectal cancer primarily arises from transcriptional plasticity rather than genetic ancestry. This finding reveals that most genetic variations do not significantly affect phenotypic traits, emphasizing the common occurrence of spatial intermixing among tumor clones [411]. Techniques like slide-DNA-seq, which capture spatially resolved DNA sequences while preserving tumor architecture, enable the discovery of clonal heterogeneity and interactions within the tumor microenvironment when integrated with spatial transcriptomics [244]. By integrating single-cell analyses with spatial context,

these advanced methods reveal the complex interactions and variations among cancer subclones, paving the way for future research and innovative therapeutic strategies. As these technologies continue to evolve, they promise to deepen our insights into tumor biology and refine our approaches to personalized medicine in oncology [412]. On the other side, three-dimensional imaging mass cytometry (3D IMC) stands out as a groundbreaking technique, allowing for the multiplexed detection of up to 40 molecular constituents within tissues at single-cell resolution [412]. This capability significantly enhances our grasp of cellular and microenvironmental heterogeneity in human breast cancer samples, facilitating the exploration of complex spatial phenomena, such as tumor cell invasion, which traditional two-dimensional imaging cannot capture.

Cardiology

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality worldwide, significantly impacting public health and healthcare systems. By enabling the simultaneous analysis of multiple molecular layers within tissues, spatial multi-omics offers critical insights into the mechanisms underlying CVDs, thus enhancing our ability to develop personalized treatment strategies [413].

Mapping the adult human heart at single-cell resolution has revolutionized our understanding of its intricate biology, revealing cellular heterogeneity across six distinct regions, including specialized atrial and ventricular subsets [414–416]. This approach also elucidates unique immune, vascular, and cardiomyocyte networks, serving as a vital reference for cardiovascular research [414]. Employing single-nucleus RNA sequencing has further enhanced this exploration by characterizing the cellular and transcriptional diversity within the non-failing human heart. This method identifies nine major cell types and 20 subclusters, such as macrophages, endothelial cells, and fibroblasts, highlighting chamber- and sex-specific gene expressions linked to various cardiac traits and diseases [415]. Additionally, the integration of single-cell analyses with spatial transcriptomics allows researchers to map cellular niches within the human heart effectively. This innovative approach reveals pacemaker-specific regulatory networks and immune niches, which are crucial for understanding heart function and pathology. Moreover, these insights pave the way for developing tools for drug target prediction, providing novel perspectives on cardiac electro-anatomy and immunology [416].

Recent advancements in single-cell and single-nucleus RNA sequencing have revolutionized the study of heart diseases by uncovering distinct cell-type-specific transcriptional changes associated with heart failure [417, 418]. These techniques have revealed how cardiomyocytes converge toward disease states, while fibroblasts and myeloid cells exhibit significant diversification, highlighting the complex cellular responses within failing hearts [417]. In another study, single-nucleus RNA sequencing profiled 880,000 nuclei from hearts affected by dilated and arrhythmogenic cardiomyopathy. The data uncovered genotype-specific cellular changes, unique transcriptional states, and intricate intercellular interactions, providing critical insights into the molecular mechanisms driving heart failure. These discoveries have the potential to inform new therapeutic targets aimed at slowing or reversing the progression of heart diseases [418].

A comprehensive spatial multi-omics map of human myocardial infarction further enriches our understanding of the intricate processes involved in injury, repair, and remodeling. By integrating single-cell gene expression, chromatin accessibility, and spatial transcriptomics data, this map reveals cell-type-specific changes and molecular interactions in different zones of the heart. These insights are crucial for identifying the cellular mechanisms underlying tissue repair and regeneration, paving the way for novel therapeutic approaches to support heart recovery post-infarction [419].The integrative analysis combining spatial transcriptomics and single-nucleus RNA sequencing in animal models, such as murine myocardial infarction, has indicated that the mechanosensitive genes, particularly Csrp3, have shown their critical roles in left ventricular remodeling, especially in the border zones during the early stages post-infarction. Understanding how these genes regulate tissue repair and adaptation in response to mechanical stress can guide the development of therapeutic interventions aimed at preventing heart failure following a heart attack [420]. As for human, vascular pathology, including conditions like atherosclerosis, benefits from high-resolution imaging methods such as 3D intravascular ultrasound (IVUS) and optical coherence tomography, which provide detailed views of the vascular lumen and wall. These techniques are essential for evaluating plaque composition, vascular anomalies, and the extent of coronary artery disease (CAD). 3D histology aids in identifying high-risk plaques prone to rupture, thereby improving risk stratification and guiding interventional strategies [421]. Pulmonary hypertension, characterized by elevated pressure in the pulmonary arteries, can be better understood through imaging techniques such as 3D echocardiography and MRI, which provide detailed assessments of right ventricular function and pulmonary artery anatomy, aiding in the diagnosis and management of this condition [422].Moreover, techniques like 3D coronary CT angiography and 3D perfusion MRI enable detailed visualization of coronary

arteries and myocardial blood flow, helping detect stenosis, evaluate myocardial ischemia, and assess the efficacy of revascularization procedures [423].

Furthermore, 3D imaging techniques, such as CT and MRI, have become indispensable tools in the diagnosis and management of cardiovascular diseases. These modalities provide comprehensive views of structures like the aorta and heart valves, aiding clinicians in assessing conditions such as aneurysms, identifying their size, location, and potential risk of rupture [424, 425]. Additionally, MRI and 3D echocardiography are invaluable in evaluating the morphology and function of heart valves, particularly in the context of valvular heart disease. These imaging technologies are crucial for planning and monitoring interventions, including valve repairs and replacements, to ensure precise and successful outcomes [426]. In cases where patients receive prosthetic heart valves, 3D imaging techniques also play a critical role in post-surgical evaluation. Detailed 3D echocardiography and CT scans help detect complications like thrombosis, endocarditis, or structural degeneration, ensuring that any potential issues are identified early, which is vital for maintaining the long-term functionality of the implants [427]. In addition, by enabling precise localization of arrhythmic foci, 3D histology supports the development of highly targeted electrophysiological mapping systems. These systems allow for 3D reconstructions of the heart's electrical activity, which is particularly valuable in treating arrhythmias through catheter ablation. Accurate targeting of abnormal electrical pathways is essential for ensuring successful outcomes in patients undergoing treatment for arrhythmias, improving both treatment precision and patient safety [428].

Neuroscience

Single-cell 3D histology has revolutionized neuroscience by providing comprehensive insights into the intricate structures and functions of the brain [429]. This advanced approach allows researchers to study brain connectivity, and the mechanisms underlying various neurological conditions.

Advancements in 3D histology and spatial multi-omics technologies have revolutionized the study of brain cell types, providing unprecedented insights into neuronal diversity, gene regulation, and cellular organization in the brain [430–439]. HybISS (Hybridization-based in situ sequencing) is one such innovation, enhancing spatial transcriptomics in both human and mouse brain tissues. By utilizing a novel barcoding system, HybISS improves RNA transcript detection, allowing for high-throughput imaging of complex tissues. This technique enables researchers to analyze challenging human samples with greater precision, ultimately shedding light on the complex architecture and function of different brain regions [430]. A significant breakthrough in understanding the brain's cellular complexity comes from the construction of a DNA methylation atlas of the mouse brain. This atlas, created by profiling over 103,982 nuclei, revealed 161 distinct cell clusters, uncovering the epigenetic landscapes that regulate neuronal diversity, spatial organization, and enhancer-gene interactions. Such insights are crucial for understanding how gene expression is modulated across various brain regions and how epigenetic changes influence brain function [431]. Adding to the understanding of brain diversity, a cell type atlas of the bearded dragon brain has illuminated both conserved and divergent neuron types, particularly in the thalamus. This research highlights the role of developmental origins and circuit allocation in driving neuronal identity and evolution, providing a comparative model to deepen our understanding of amniote neuron diversity and the molecular mechanisms that shape it [432]. To complement these findings, a spatially resolved single-cell epigenomic profiling method has been developed to reveal histone modifications at the individual cell level. This innovative approach generates high-resolution spatial maps of active promoters and enhancers in mouse brains. By revealing how the epigenome regulates gene expression spatially, the method identifies important promoter-enhancer interactions critical for the regulation of developmental genes, contributing to our knowledge of how brain function is shaped during development [433]. Single-cell multi-omics technologies have also been employed to identify conserved and divergent cis-regulatory elements within the mouse brain. These elements play a vital role in shaping species-specific traits and help researchers understand the contribution of transposable elements to human-specific regulatory features. These studies are not only deepening our understanding of brain gene regulation but also helping to develop machine learning tools capable of predicting regulatory features, offering a new layer of understanding regarding 3D genome architecture and its implications for neurological diseases [434]. The snATAC-seq, a single-nucleus assay for transposase-accessible chromatin sequencing, has further contributed to creating a comparative atlas of chromatin accessibility across 1.1 million cells in the human brain. By identifying 107 distinct cell types, this research links chromatin accessibility to neuropsychiatric disorders and demonstrates the conservation of key regulatory elements between mouse and human brain cells. This work offers profound implications for understanding the genetic underpinnings of mental health conditions and neurodevelopmental disorders [435]. In a broader effort to map the brain's cellular architecture, a comprehensive cell atlas of the adult mouse brain has

been created using advanced spatial transcriptomics. This atlas, which spans over 10 million cells and includes 5,000 distinct cell clusters, provides critical insights into the molecular and cellular architecture that underlies brain function. The atlas serves as a foundational resource for future research, offering a detailed view of the brain's cellular diversity [436]. Additionally, a single-cell DNA methylome and 3D multi-omic atlas of the adult mouse brain has been established using snmC-seq3 and snm3C-seq, further enhancing our understanding of the brain's molecular landscape [437]. By revealing spatial methylation patterns and their associations with gene expression, this atlas offers invaluable insights into the regulatory networks that shape brain cell diversity. Such detailed molecular maps are crucial for understanding the mechanisms behind neurological diseases and brain development. Further emphasizing the importance of regulatory elements, single-cell multi-omics methods continue to unravel the role of cis-regulatory elements in both conserved and divergent traits between species. The contribution of transposable elements to human-specific regulatory features, along with the development of machine learning approaches, underscores the increasing significance of 3D genome architecture in shaping brain function and its relationship to neurodevelopmental and neurodegenerative diseases [438]. One of the most exciting developments in this field is the MiP-seq technique, a high-throughput method that enables simultaneous detection of DNAs, RNAs, and proteins at subcellular resolution [439]. MiP-seq not only reduces costs and enhances decoding efficiency compared to other methods but also facilitates the identification of gene mutations and RNA modifications. This method's integration with calcium and Raman imaging has created spatial multi-omics atlases of mouse brain tissues, allowing researchers to correlate gene expression with neuronal activity. This multi-dimensional approach is revolutionizing our understanding of how genes influence brain activity at the cellular level, opening new avenues for research into brain function and dysfunction[439, 440].

Advancements in 3D histology and spatial multi-omics techniques are revolutionizing our understanding of various regions of the brain, including the hypothalamus [441], cerebrum [442], cortex [443–445], and hippocampus [446]. These innovations are allowing researchers to map cellular diversity, gene regulation, and spatial organization with unprecedented precision. In the hypothalamus, the novel technique EASI-FISH has made significant strides in mapping thick brain sections, providing a detailed spatial organization of cell types within the lateral hypothalamus [441]. This technique successfully revealed nine distinct subregions, which has greatly enhanced our understanding of hypothalamic architecture and its diverse cell populations. This work is crucial for understanding the functional roles of the hypothalamus in regulating behaviors like hunger, thirst, and sleep, as well as its involvement in neuroendocrine signaling. Moving to the cerebrum, gene regulatory elements across 160 distinct cell types have been mapped in the adult mouse brain, identifying over 491,818 candidate cis-regulatory DNA elements [442]. These elements are linked to specific neuronal and glial functions, offering insights into the transcriptional programs that govern brain cell diversity. Understanding these regulatory elements provides crucial information on how genetic mutations or dysregulations in these pathways might lead to neurological diseases such as Alzheimer's, schizophrenia, or epilepsy. In the cortex, advancements in spatial transcriptomics have been particularly impactful. For instance, Slide-seqV2 enhances the spatial resolution of transcriptomic data, achieving near-cellular resolution with an impressive ~ 50% RNA capture efficiency [443]. This technology allows researchers to identify localized mRNA expression patterns in neurons and provides a clear picture of spatiotemporal development in the mouse neocortex. Further insights come from MERFISH, which helped create a spatially resolved cell atlas of the mouse primary motor cortex [444]. This study profiled approximately 300,000 cells and identified 95 distinct neuronal and non-neuronal clusters, providing an intricate view of spatial organization and connectivity patterns that are critical for motor function. These findings help elucidate how various neuron types contribute to motor control and how disruptions in these networks could lead to motor disorders such as Parkinson's disease or amyotrophic lateral sclerosis (ALS). Further extending our understanding of the cortex, research on the macaque cortex has mapped 264 transcriptome-defined cell types using single-nucleus RNA sequencing and spatial transcriptomics [445]. This work revealed regional preferences and complexities, including the identification of primate-specific cell types enriched in layer 4 of the cortex. These findings provide insights into the evolution of the primate brain and offer a comparative model for studying human brain functions. Advanced single-cell multiomics and 3D genomic profiling techniques also play a critical role in understanding the evolutionary divergence of gene regulatory programs in the mammalian neocortex. By integrating these methods, researchers can explore the interplay between conserved and divergent regulatory features, which is crucial for advancing our understanding of neurological diseases and traits that have evolved in primates [447]. In the hippocampus, a region critical for memory formation and spatial navigation, researchers have utilized in vivo barcoding and single-cell spatial transcriptomics to trace

lineage relationships within the mouse brain [446]. These methods have identified fate-restricted progenitor cells, which give rise to various cell types during development and have revealed the origins of microglia—the brain's resident immune cells. By tracing the development of microglia from a limited number of primitive myeloid precursors, scientists can better understand the role these cells play in both normal brain function and in the response to injury or disease, such as in conditions like multiple sclerosis or neurodegeneration. Additionally, the ability to map the development of distinct cell populations in the hippocampus offers deeper insights into tissue architecture and cell phenotypes that are crucial for understanding brain plasticity and regeneration.

Recent innovations like Polony gels have transformed the way researchers approach the molecular underpinnings of chronic pain. These gels provide a scalable and cost-effective method for fabricating DNA arrays with unique barcodes, enabling detailed mapping of the mouse parabrachial nucleus—a brain region associated with pain processing—through Pixel-seq. By allowing researchers to perform single-cell spatial transcriptomics, Polony gels have facilitated the identification of neuropathic pain-regulated transcriptomes and the communication between cells following nerve injury [448]. Moreover, neural circuit mapping is enhanced by tools like CLARITY and light-sheet microscopy, which provide detailed, transparent views of the brain's neural circuits [449]. These tools enable the visualization of entire neural networks in a three-dimensional space, making it possible to trace the intricate pathways that neurons use to communicate. By enabling precise mapping of brain circuits, these technologies provide the groundwork for developing more effective treatments for a wide range of brain diseases.

One of the notable advancements in the area of central nervous system (CNS) is the use of STARmap PLUS, which has enabled the creation of a spatial atlas of the mouse CNS by mapping over 1.09 million cells and profiling 1,022 genes in 3D [450]. This method led to the identification of 230 distinct molecular cell types and 106 molecular tissue regions, offering unparalleled insights into brain anatomy and aiding the development of gene delivery tools for therapeutic applications. Understanding the dynamic connectivity of brain networks is crucial, as neural communication occurs over different timescales and adapts in response to environmental stimuli and internal states. As for neurodegeneration research, the application of single-cell 3D histology has been groundbreaking in Alzheimer's disease research, particularly through the STARmap PLUS method, which integrates high-resolution spatial transcriptomics with protein detection [451]. This technique has revealed a comprehensive cellular map of Alzheimer's disease progression in mouse models, highlighting a core–shell structure where disease-associated microglia—the brain's immune cells—interact with amyloid-β plaques. The plaques are hallmark features of Alzheimer's disease, and STARmap PLUS has allowed researchers to track how oligodendrocyte precursor cells and hyperphosphorylated tau—another pathological marker of Alzheimer's—accumulate in specific brain regions.

Techniques like diffusion tensor imaging (DTI) and 3D MRI allow researchers to visualize and analyze the intricate network of neural connections within the brain [452]. These techniques are essential for creating detailed maps of neural pathways, which are crucial for understanding how different brain regions interact and coordinate various functions. This is particularly important in the study of neurodevelopmental disorders and neurodegenerative diseases, where disruptions in brain connectivity play a significant role in disease progression. Advanced imaging techniques such as 3D MRI and PET have been crucial in visualizing amyloid plaques [453], tau tangles, and other pathological features in the brain [454], which are central to diseases like Parkinson's disease and ALS [455]. Additionally, by enabling detailed views of spinal cord architecture, 3D histology has also advanced the study of understanding axonal regeneration—the process by which nerve fibers regenerate following injury—and neural network reorganization, both of which are crucial for developing therapies aimed at restoring function after spinal cord injuries [456]. The ability to visualize how axons regenerate and form new networks in real time through live-cell imaging and 3D time-lapse microscopy offers hope for improving recovery outcomes in spinal cord injury patients, as well as for enhancing rehabilitation strategies [457]. For example, an integrative strategy that merges 15-color multispectral imaging with mass spectrometry can be employed to categorize myeloid subsets within murine atherosclerotic plaques [458]. This approach establishes connections between their phenotypes and the cellular and metabolic microenvironments, surpassing the constraints of conventional single-cell and histological techniques. Moreover, chniques like functional MRI (fMRI) and 3D laser speckle imaging provide valuable insights into how neural activity influences cerebral blood flow, offering a window into the intricate balance of energy and blood supply that maintains normal brain function. These imaging methods are especially useful for studying neurovascular disorders, where disrupted blood flow can lead to cognitive impairment and neurodegeneration [459]. By capturing real-time changes in blood flow in response to neural activity, these tools help researchers identify the early

signs of diseases like Alzheimer's and vascular dementia and recognize the dynamic relationship between neural activity and cerebral blood flow [460], aiding in both diagnosis and treatment planning.

Immunology

Single-cell 3D histology has revolutionized the study of the immune system by offering detailed insights into the architecture and dynamics of immune responses, which were previously limited by traditional 2D techniques. From studying lymph nodes to mapping immune cell behaviors, single-cell 3D histology has opened new avenues for examining immune responses, vaccine development, autoimmunity, infections, and transplant immunology.

One of the most promising applications of single-cell 3D histology in immunology lies in its ability to enhance our understanding of immune responses, particularly in vaccination and immune cell behavior [461–463]. For example, spatial CITE-seq allows detailed profiling of immune responses by co-mapping high-plex protein expression with whole transcriptomes at cellular resolution, revealing germinal center reactions in tonsils and early immune responses in the skin following COVID-19 mRNA vaccination [461]. By profiling hundreds of proteins and transcriptomes in human tissues, single-cell 3D histology provides critical data on how immune systems respond at the cellular level, which is invaluable for the development of new vaccines. Beyond vaccination, 3D histology advances cancer research by analyzing immune cell behaviors. Techniques like Amplification by Cyclic Extension (ACE) for mass cytometry allow for signal amplification of over 30 protein epitopes, enabling precise quantification of low-abundance proteins and dynamic signaling in T lymphocytes [462]. This is particularly useful in the TME, where CD8 + cytotoxic T lymphocytes (CTLs) often face challenges such as exhaustion, limiting their efficacy in targeting cancer cells. ACE and similar tools provide key insights into how T cells interact with innate immune cells and how therapies, such as immune checkpoint blockades, can be optimized to improve CTL function [463].

Moreover, single-cell 3D histology has deepened insights into immune regions within tissues, especially lymph nodes, which are critical for initiating immune responses. Tools like Spatial Graph Fourier Transform (SpaGFT) improve gene expression imputation, enabling accurate characterization of immune regions and aiding in the detection of rare immune cells [464]. Confocal and multiphoton microscopy allow researchers to explore the spatial organization of lymph nodes, including the arrangement of T cells, B cells, and dendritic cells, offering a better understanding of immune response initiation and maintenance. Furthermore, single-cell 3D histology enables the real-time tracking of cell migration and interactions, through techniques like intravital microscopy. This provides dynamic views of T and B cell movements and interactions with other immune cells, helping researchers gain unprecedented insights into immune surveillance, activation, and migration. By integrating all these tools and techniques, 3D histology offers transformative potential in improving immunotherapies and vaccine development by providing a deeper understanding of immune cell behavior and interactions within their physiological context [465].

Autoimmune diseases [466, 467], infectious diseases [468, 469], and transplant immunology [463] are all critical areas where single-cell 3D histology has significantly enhanced our understanding of immune responses and tissue interactions. In autoimmune diseases, where the immune system mistakenly attacks the body's own tissues, chronic inflammation is a major concern. This prolonged activation of immune cells and the constant release of inflammatory mediators can result in severe tissue damage [466, 467]. 3D imaging techniques provides a detailed insights into how immune cells interact with tissues during these inflammatory responses, aids in elucidating the mechanisms involved in conditions such as allergic inflammation and hypersensitivity disorders [468]. The infection model, enabled by 3D histology, helps researchers study the speed and pathways of pathogen transmission, as well as the immune system's dynamic response to infection [470, 471]. These insights are invaluable in developing targeted therapies to manage chronic inflammation and autoimmunity more effectively [469]. In the context of transplant immunology, 3D analysis provides a comprehensive view of the immune processes involved in graft acceptance and rejection. This detailed perspective is vital for improving transplant outcomes, as it allows scientists to better understand the immune mechanisms behind graft rejection and tolerance [463].

The maternal–fetal interface is a dynamic environment that involves the coordination of immune tolerance to prevent the maternal immune system from attacking the developing fetus. The complex interactions between maternal immune cells and fetal tissues are crucial for a successful pregnancy[472, 473]. 3D imaging enables detailed visualization of placental architecture and the spatial distribution of immune cells, which is critical for understanding how immune tolerance is maintained throughout pregnancy [472]. Additionally, single-cell 3D histology provides insights into processes like decidualization, which supports pregnancy by transforming the endometrial lining to accommodate fetal development [474]. This technology has proven instrumental in

studying pregnancy complications like preeclampsia and recurrent miscarriages, offering potential pathways to improve maternal and fetal health outcomes [473].

Additionally, techniques like immuno-SABER improving imaging mass cytometry (IMC) and revealing immune cell characteristics, such as T cell co-receptors, that are often undetectable with traditional methods, which contribute to understanding immune markers and their spatial distribution [475]. This advancement aids in identifying new therapeutic targets, particularly in cancer immunotherapies. Real-time visualization of T cells in solid tumors using advanced imaging techniques like confocal and two-photon microscopy further enhances our understanding of CAR T-cell therapies by uncovering factors like T cell exhaustion and multi-killing potential [476]. Spatial and kinetic data derived from these techniques are crucial for improving the effectiveness of CAR T-cell therapies in solid tumors. As for tools, slide-TCR-seq, a 10-μm-resolution method for mapping T cell receptors (TCRs) and whole transcriptomes in intact tissues [477]. It reveals spatially distinct TCR repertoires in human lymphoid germinal centers and heterogeneous immune responses in renal cell carcinoma and melanoma, enhancing understanding of T cell interactions and gene expression in adaptive immunity.

Developmental biology

Single-cell 3D histology is revolutionizing the field of developmental biology, providing unprecedented insights into the intricate processes of organismal formation, growth, and differentiation. By enabling researchers to explore the spatial organization of tissues and cells in three dimensions, this technology allows for a more comprehensive understanding of key biological events such as organogenesis, embryogenesis, and tissue morphogenesis.

Organogenesis is a pivotal phase of development that encompasses the formation and differentiation of organs from embryonic tissues [478]. Recent advancements in single-cell transcriptomics and spatial biology have allowed researchers to explore these intricate processes at unprecedented resolution, shedding light on the cellular diversity, spatial architecture, and regulatory mechanisms involved in early organ formation. One of the most comprehensive resources available is the single-cell transcriptome atlas of 4–6 week human embryos, which reveals 313 distinct cell clusters across 18 developmental systems. This detailed mapping has elucidated the spatial arrangement of cell populations and uncovered human-specific regulatory pathways essential for early organogenesis [478]. The mouse model has been instrumental in furthering our understanding of organogenesis. The development of the Mouse Organogenesis Spatiotemporal Transcriptomic Atlas (MOSTA) has provided high-resolution, dynamic maps of transcriptional activity during mouse embryonic development. Using DNA nanoball-patterned arrays and in situ RNA capture, MOSTA enables detailed investigation of cellular heterogeneity and fate specification across different time points in organ development [479]. Further enriching the field, a three-dimensional spatial transcriptomic atlas of mouse embryogenesis at day 5.5–7.5 and 13.5 offer a new perspective on the molecular mechanisms that regulate organ development [480, 481]. By combining single-cell transcriptomics with spatial data, this atlas illuminates the cellular interactions and molecular pathways that guide cell fate divergence during organogenesis. This integrative approach is particularly valuable for organ engineering and regenerative medicine, as it provides a blueprint for replicating organ development in vitro. The application of 3D histology and spatial transcriptomics in these studies allows for a detailed understanding of how cells communicate and organize to form complex tissues. Additionally, in model organisms, researchers have generated high-resolution 3D spatiotemporal transcriptomic maps of developing Drosophila embryos and larvae by employing Stereo-seq. These maps reveal critical insights into the functional subregions within organs like the midguts and the larval testis, along with the transcription factor networks (regulons) that govern these developmental processes. The comprehensive data sets from these models offer valuable resources for studying complex developmental pathways, serving as references for understanding similar processes in higher organisms [482]. In vertebrates, spatial transcriptomics has illuminated distinct chondrocyte populations during embryonic limb development, highlighting the molecular pathways involved in chondrogenesis. These findings are significant for understanding the formation of cartilage, not only during development but also in the context of diseases like osteoarthritis, where cartilage regeneration is a therapeutic target. The identification of key molecular mechanisms in chondrogenesis opens new avenues for developing therapeutic interventions that aim to regenerate or repair damaged cartilage in degenerative conditions [483]. To further explore cell–cell communication during development, the COMMOT framework—which utilizes collective optimal transport—has proven effective for analyzing spatial transcriptomics data. This analytical tool has demonstrated its robustness across various data sets, providing novel insights into the communication pathways that govern cell differentiation and tissue formation during organogenesis [484].

Organs such as the heart [485, 486] and brain [487–489], undergo highly complex developmental processes that are difficult to capture using traditional 2D

techniques. However, advancements in organoid culture systems—which allow for the growth of 3D, organ-like structures in vitro—have revolutionized the study of organogenesis. By integrating single-cell RNA-sequencing with high-resolution fluorescence in situ hybridization, researchers have uncovered that various cardiac cell types in the developing human heart form distinct cellular communities [485]. This approach sheds light on the complex cell–cell interactions and signaling pathways crucial for ventricular wall morphogenesis, providing valuable insights into heart development and potential strategies for repair. Researchers unveiled a spatiotemporal gene expression and cell atlas of the developing human heart by employing spatial transcriptomics and single-cell RNA sequencing. This pioneering methodology enabled the mapping of cell-type-specific gene expression profiles across three distinct developmental stages, culminating in a meticulously detailed subcellular spatial map. This invaluable resource, now publicly accessible, stands poised to significantly advance the field of human cardiogenesis research [486]. The innovative approach of MAGIC-seq, a cost-effective spatial transcriptome sequencing method, integrates microfluidics and spatial combinatorial indexing to achieve a notable eightfold increase in throughput while approaching near single-cell resolution [488]. This advancement has facilitated the generation of a comprehensive 3D transcriptomic atlas of the developing mouse brain, empowering researchers to conduct dynamic analyses of transcriptional variations across different tissue types. Sequential fluorescence in situ hybridization (seqFISH) was employed to investigate mouse embryos, integrating spatial and single-cell transcriptomic datasets to delineate cell types and elucidate differentiation trajectories [489]. This approach significantly augmented our comprehension of organogenesis, with a specific emphasis on the midbrain-hindbrain boundary and the development of the gut tube. These systems provide an experimental model to visualize organ development in real-time and offer a platform for studying the molecular mechanisms driving tissue patterning and organ differentiation [490].

Early embryonic development represents a crucial phase in an organism's life, where the foundational structures and tissues begin to form. A spatial multiomics atlas of the maternal–fetal interface reveals trophoblast differentiation pathways and potential transcription factors driving EVT invasion, providing insights into placental development and implications for understanding pregnancy disorders like pre-eclampsia [491]. The application of single-cell 3D histology begins with early embryonic development, including processes such as embryo morphogenesis, gastrulation [492], and neurulation. Moreover, utilizing advanced 3D cell culture techniques, such as 3D prolonged in vitro culture (pIVC) system, could help us discover embryo development from several days post-fertilization [493]. These capabilities to visualize and quantify cellular behaviors in three dimensions has significantly advanced our understanding of the fundamental mechanisms driving early embryogenesis.

The Stereo-seq technology has enabled a comprehensive spatial transcriptomic analysis of zebrafish embryogenesis, profiling 91 sections across six critical time points. This study revealed gene expression landscapes and developmental trajectories, correlating microenvironmental cues with cell-fate decisions. By capturing these dynamic processes, Stereo-seq provides a vital reference for studying how embryonic cells differentiate and respond to their environments throughout development [494]. In mouse embryogenesis, researchers utilized multiple single-cell RNA-sequencing datasets to reconstruct cellular trajectories spanning 19 developmental stages, from E3.5 to E13.5. This work led to the creation of the TOME graph, a powerful tool for identifying key transcription factors that regulate development. It also highlighted evolutionary homologs, deepening our understanding of how similar cell types evolve and function across species [495]. Innovative techniques such as IGS have further advanced our understanding of embryogenesis by combining DNA sequencing with spatial imaging. IGS allows researchers to localize thousands of genomic loci within intact samples, revealing parent-specific structural changes during development. This method provides a detailed view of single-cell chromatin domains, linking genomic sequence with spatial organization from base pairs to entire organisms [239]. Additionally, Genome-wide RNA Tomography offers a high-resolution, 3D atlas of gene expression in zebrafish embryos by combining histology, low-input RNA sequencing, and mathematical image reconstruction. This technique enables the identification of spatial gene expression patterns, proving its potential for broader applications in spatially resolved transcriptomics [496]. The DBiT-seq technique, which utilizes deterministic barcoding in tissues, allows for the simultaneous mapping of mRNAs and proteins in fixed samples via next-generation sequencing. By employing microfluidic channels for in situ barcoding, DBiT-seq has facilitated the identification of tissue types and cell distributions in mouse embryos, making it applicable across fields such as cancer biology and clinical pathology [232].

Regenerative medicine

The integration of 3D histology with cutting-edge single-cell genomic technologies has provided remarkable insights into various organ systems, contributing significantly to our understanding of complex biological

processes such as neurogenesis, organ regeneration, and tissue remodeling. This advancement is particularly evident in studies involving the brain, kidney, heart, and immune systems, where researchers have been able to map cell populations, identify transcriptional dynamics, and uncover the regenerative capabilities of various tissues.

In the field of neuroscience, 3D histology combined with single-cell genomic profiling has been instrumental in unveiling the complexity of the nervous system [497, 498]. Studies on the axolotl telencephalon, for example, have provided valuable insights into neurogenesis and brain regeneration. Single-cell genomic methodologies have unveiled critical neuronal subpopulations such as glutamatergic and GABAergic neurons. Furthermore, these techniques have captured the transcriptional dynamics linked to the regenerative capabilities of ependymoglia, specialized glial cells with the remarkable capacity to replenish lost neurons following brain injury [497]. The spatial resolution achieved through techniques like Stereo-seq has further identified injury-induced progenitor cell populations in the axolotl brain, offering clues about how certain neurons can revert to an immature state to facilitate tissue repair. These findings not only deepen our understanding of neurogenesis but also highlight evolutionary parallels between tetrapods, providing important perspectives on the broader mechanisms underlying nervous system regeneration and organization [498].

In parallel, single-cell 3D histology provides valuable insights into the mechanisms of kidney disease progression and potential therapeutic targets, particularly through technologies like the ACE. This innovative technique enhances mass cytometry sensitivity, enabling the simultaneous quantification of over 30 proteins. Such capabilities are crucial for studying molecular reprogramming during epithelial-to-mesenchymal transitions (EMT) and for profiling pathological states in tissues, including polycystic kidneys [462]. Recent comprehensive multi-omics analyses of human kidneys have revealed four distinct microenvironments: glomerular, immune, tubule, and fibrotic. Each of these microenvironments plays a critical role in kidney function and pathology, particularly highlighting the significance of the fibrotic microenvironment in disease classification and prognosis [499]. Further investigations into kidney injury have led to the development of a comprehensive multi-omics atlas detailing 51 distinct cell types and 28 altered cellular states. This atlas, which incorporates spatial transcriptomic profiles and 3D imaging analyses, effectively links cellular neighborhoods to immune responses and outcomes following injury [500]. A particularly significant finding is the identification of distinct subpopulations of pericytes and fibroblasts as the primary sources of myofibroblasts in human kidney fibrosis. Single-cell RNA sequencing has elucidated the cellular origins and differentiation pathways leading to myofibroblast formation, a process integral to the progression of fibrosis. The identification of NKD2 as a potential therapeutic target in this context opens new avenues for intervention, aiming to prevent or reverse fibrotic changes in the kidney [501]. Moreover, the integration of high-spatial-resolution metabolomics methods, such as MALDI-MSI combined with isotope tracing, has further enhanced our understanding of kidney repair dynamics. This approach allows for the analysis of cell-type-specific metabolic changes during recovery from ischemia–reperfusion injury, shedding light on how different kidney regions respond metabolically during the healing process [502].

Further contributions of single-cell 3D histology are seen in cardiac development and repair, where advanced single-cell RNA sequencing and high-resolution imaging techniques have revealed the organization of diverse cardiac cell types into specialized communities [485]. This work has significantly advanced our knowledge of how cell–cell interactions and signaling pathways contribute to critical processes like ventricular wall morphogenesis. By mapping these specialized cellular interactions, researchers are gaining important insights into both normal heart development and potential strategies for repairing damaged cardiac tissue.

Beyond these organ-specific discoveries, the application of single-cell 3D histology in immunology has been pivotal in clarifying the role of immune cells in tissue repair and regeneration. Spatial transcriptomics has been instrumental in uncovering injury-specific gene expression patterns and cellular interactions in models of kidney ischemia–reperfusion injury, revealing the critical roles played by immune cells in facilitating tissue repair [503]. Inflammatory responses, while essential for defending against microbial infections, also play a vital role in facilitating tissue regeneration. However, the exact mechanisms by which different immune cell subtypes contribute to these regenerative processes remain a subject of ongoing investigation [466].

Other applications and progress

3D histology has found significant applications and made substantial progress in orthopedics, dentistry, and dermatology. This advanced imaging approach provides critical insights into complex structures and processes, leading to improved diagnostics, treatment planning, and therapeutic outcomes in these medical specialties.

In orthodontics, 3D imaging allows for the accurate assessment of dental and skeletal relationships,

improving the planning and outcomes of orthodontic treatments [504]. Advancements in spatial biology techniques for analyzing osteoarthritis (OA) microenvironments have been instrumental in elucidating the disease's complexities. By integrating genomics, transcriptomics, and advanced imaging methods, researchers have enhanced their understanding of OA phenotypes [505]. This integrated approach not only sheds light on the disease processes but also paves the way for the development of more precise diagnostic and therapeutic strategies. Moreover, detailed single-cell and spatial maps of the human endometrium reveal signaling pathways that influence epithelial cell fate and enhance our understanding of endometrial cancers and endometriosis to inform future therapeutic strategies [506].

Dermatology has also benefited significantly from advancements in 3D histology. For skin cancer diagnosis and treatment, 3D imaging techniques such as OCT and confocal microscopy provide detailed images of skin layers, enabling early detection and precise excision of cancerous tissues [507]. In conditions like psoriasis and atopic dermatitis, 3D imaging helps in assessing the extent and severity of lesions, facilitating better treatment planning and monitoring [508, 509]. Additionally, 3D histology plays a critical role in wound healing and scar assessment by providing detailed visualization of skin remodeling processes and aiding in the development of effective therapies for burns, cellulitis, and other skin injuries [510].

Challenges and perspectives

The emergence of 3D tissue imaging technologies represents a significant advancement in research capabilities. While these tools offer immense potential, researchers must exercise caution in their application. It is essential to formulate research questions that genuinely stand to benefit from 3D imaging and to design experimental plans based on robust statistical principles. Addressing these challenges is crucial for leveraging the full potential of 3D imaging technologies in scientific inquiry.

Challenges in 3D tissue imaging

Cost and accessibility

The barrier to entry for 3D tissue imaging is high due to the significant costs and accessibility issues associated with the specially required equipment. For instance, imaging methods like PET and CARS require costly facilities, necessitating substantial financial investment from research institutions. Maintaining and operating these sophisticated instruments require extensive training, further escalating the overall cost [511]. This financial burden can limit access to state-of-the-art imaging technologies, particularly for smaller laboratories and institutions with constrained budgets. Moreover, the need for specialized training to operate and troubleshoot these complex imaging systems is also disturbing. The intricacies involved in setting up experiments, optimizing imaging parameters, and interpreting the results demand a high level of expertise. Consequently, the lack of skilled personnel can hinder the adoption and effective use of 3D imaging technologies in various research settings [58].

Significant robustness in technologies

3D tissue imaging technologies temporarily facing significant robustness issues, particularly concerning resolution limitations and artifact reduction. The resolution of 3D images is often constrained by the optical properties of the imaging system and the sample itself. For instance, light scattering and absorption in thicker tissues can degrade image quality, limiting the achievable resolution and depth penetration [29]. Despite advancements in imaging techniques, such as light-sheet microscopy, these limitations persist. Artifact reduction is also a critical challenge. Artifacts, which can arise from various sources including sample preparation and imaging processes, can obscure critical details and lead to misinterpretations. Addressing these artifacts requires meticulous optimization of imaging protocols and advanced image processing techniques, which can be resource-intensive and complex [512].

Lack of best practices for experimental design and data analysis

Sample preparation is a crucial step that significantly impacts the quality of 3D images. Techniques such as tissue clearing, staining and labeling, and fixation and preservation must be optimized to preserve tissue integrity and enhance image quality [39]. However, there is considerable variability in these methods, and standardized protocols are often lacking. Additionally, the large volumes of data generated by 3D imaging require efficient storage, processing, and analysis capabilities. However, the need for high computational resources to handle and analyze large datasets can be a limiting factor for many laboratories [513]. Image stitching and registration are essential for creating coherent 3D datasets from individual images, but these processes can be computationally intensive and prone to errors [514]. In addition to this, segmenting and quantifying complex 3D structures require sophisticated algorithms and software tools, which makes image analysis tools difficult to develop and implement. Automation of these processes is still in its infancy, and manual intervention is often required, increasing the potential for variability and bias [341]. Furthermore, the biological

relevance of the quantified data must be carefully interpreted, necessitating thorough validation with biological controls and comparative studies.

Standardization

Standardization is a critical issue in 3D tissue imaging, affecting the reproducibility and comparability of results across different studies and laboratories. The absence of standardized protocols for sample preparation, data acquisition, processing, and analysis creates inconsistencies in research that hinder the advancement of the field [515]. For instance, variations in tissue-clearing methods can lead to differences in transparency and staining, affecting the quality and interpretability of the resulting images [39]. Ethical and regulatory issues further complicate standardization efforts. Obtaining patient consent and ensuring data privacy are paramount, particularly in clinical research settings. Researchers must navigate complex regulatory landscapes to comply with ethical guidelines, which can vary significantly across regions and institutions [516] Additionally, the lack of standardized ethical guidelines for the use and sharing of 3D imaging data can impede collaborative efforts and data sharing [517, 518].

In conclusion, while 3D tissue imaging holds immense promise for advancing biomedical research, several significant challenges must be addressed to unlock its full potential. To overcome these obstacles, the following methods can be considered:

(a) It is crucial to increase funding and support for acquiring and maintaining 3D imaging systems. Training programs and workshops can help build expertise in 3D imaging technologies, ensuring that researchers are well-equipped to utilize these tools effectively. Collaborative efforts and shared resources among institutions can also alleviate the financial burden and enhance access to cutting-edge technologies.
(b) Technological advancements, such as improved imaging systems [29, 58, 511] and standardized sample preparation methods [39], will enhance the robustness and quality of 3D imaging. Developing imaging systems with higher resolution and better depth penetration, such as adaptive optics and advanced light-sheet microscopy, can help overcome current limitations. Standardizing sample preparation protocols, including tissue clearing and staining, can reduce variability and improve the quality of 3D images.
(c) Establishing clear best practices for experimental design and analysis is crucial for advancing the field. Developing standardized protocols for sample preparation, data acquisition, and processing can enhance reproducibility and comparability of results. Additionally, ensuring ethical and regulatory compliance through standardized guidelines can facilitate the responsible use and sharing of 3D imaging data [517, 518].

Future directions

The future of 3D tissue imaging is brimming with potential as technological advancements continue to enhance our understanding and capabilities in biomedical research. Here, we explore several key areas that are poised to revolutionize the field.

Improved equipment and imaging techniques

The development of portable and miniaturized imaging devices is set to democratize access to advanced imaging technologies. Handheld microscopy systems and compact optical coherence tomography (OCT) devices are making high-resolution imaging more accessible in various settings, including clinical environments and field research [519, 520]. These portable systems enhance the ability to perform real-time analysis and diagnostics, thus broadening the scope of applications in both research and clinical practice.

Advancements in super-resolution imaging techniques are also pushing the boundaries of spatial resolution, allowing us to visualize cellular structures at the nanometer scale. As the first far-field microscopy imaging technique to break the optical diffraction limit, stimulated emission depletion (STED) microscopy achieves a three-dimensional resolution of 30 ~ 50 nm through the use of nonlinear effects [521–523]. Expansion microscopy, for example, involves physically enlarging tissue samples, enabling high-resolution imaging of otherwise obscured details [524]. These techniques are breaking the diffraction limit, revealing intricate cellular details previously unattainable [525, 526].

Integration of multimodal imaging techniques

The integration of multimodal imaging techniques is another promising development. Combining different imaging modalities, such as fluorescence microscopy, electron microscopy, and MRI, provides a comprehensive view of tissue structures [527]. Each modality offers unique strengths: fluorescence microscopy for detailed molecular imaging, electron microscopy for high-resolution ultrastructural data, and MRI for revealing tissue composition and function. This multimodal approach allows researchers to obtain a holistic understanding of complex biological systems [528].

Real-time 3D imaging and functional imaging techniques are also transforming our ability to observe dynamic biological processes. Advances in light-sheet fluorescence microscopy and multi-photon microscopy now allow for live, high-resolution imaging of tissues in action, essential for studying phenomena such as cell migration, tissue development, and therapeutic responses [526, 529, 530]. Functional imaging techniques, such as calcium imaging and functional MRI, complement structural imaging by capturing physiological activities and biochemical changes in real-time, enhancing our understanding of processes like neural activity and vascular dynamics [531, 532].

Artificial intelligence and machine learning

Artificial intelligence (AI) and machine learning are revolutionizing the analysis of 3D tissue imaging data. AI-powered algorithms significantly enhance data processing efficiency and accuracy, enabling the extraction of meaningful insights from complex datasets [533]. Deep learning techniques automate tasks such as image segmentation, feature extraction, and pattern recognition, which accelerates research and improves reproducibility [534, 535]. There is an urgent need for a universal method to convert existing NGS protocols into spatially resolved technologies. Spatial transcriptomics by reoriented projections and sequencing (STRP-seq) achieves this transition by using a tissue sampling strategy based on multi-angle slicing and a probabilistic image reconstruction algorithm, which can reconstruct complex 2D spatial patterns [536]. However, more integrated technologies need to be developed to combine AI with spatial proteomics [537], metabolomics, and other omics fields. By tracking the spatial distribution of gene expression, protein changes, and metabolic activities, researchers can better understand the dynamic changes in tissues during development, disease, and treatment processes.

Innovative contrast agents

Innovative contrast agents are also advancing the capabilities of 3D tissue imaging. Novel agents, such as targeted fluorescent probes and nanoparticle-based markers, improve imaging sensitivity and specificity [538, 539]. These agents allow for precise visualization of molecular targets and cellular processes, providing valuable information for both research and clinical applications [540].

Impact on personalized medicine

The convergence of these advancements is paving the way for significant strides in personalized medicine. Tailored treatments based on precise imaging and analysis of individual patient tissues are becoming more feasible. The ability to visualize and analyze tissues at high resolution and in real-time allows for more accurate diagnosis, treatment planning, and monitoring of therapeutic responses [160, 162].

Further, the integration of single-cell RNA sequencing with CRISPR/Cas screening library allows researchers to perturb gene expression at the single-cell level and examine resulting transcriptional changes. Techniques like Perturb-Seq [541], CRISP-seq [542], and CROP-seq [543] facilitate high-throughput investigations into how genetic perturbations influence gene expression and cellular functions. Moreover, methods such as genome editing of synthetic target arrays for lineage tracing (GESTALT) [544, 545] and lineage tracing by nuclease-activated editing of ubiquitous sequences (LINNAEUS) [546] enable the identification of cellular lineage relationships, cataloging of cell identities in diverse tissues, and simultaneous lineage tracing with transcriptome profiling in thousands of single cells [547]. Although CRISPR/Cas technology has advanced single-cell research significantly, its utilization in spatial genomics necessitates additional refinement. By combining spatial genomics, researchers can use CRISPR/Cas9 to knock out or activate genes in specific tissue regions (such as the cerebral cortex or tumor microenvironment) to study spatial gene expression changes and their effects on tissue development or disease progression [548, 549].

The future of 3D tissue imaging is characterized by remarkable advancements in equipment, imaging techniques, and analysis methods. Portable and miniaturized devices, super-resolution imaging, and real-time functional imaging are expanding our capabilities. The integration of multimodal imaging techniques and the application of AI and innovative contrast agents are further enhancing our understanding of tissue biology. These developments are set to revolutionize personalized medicine, offering deeper insights into complex biological processes and improving diagnostic and therapeutic strategies.

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Authors' contributions

Conceptualization: XZ, JF and FM; Methodology: XX, JS, RZ, KL; Writing and editing: XX, JS, XZ, JF and FM; Supervision: XZ, JF and FM.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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