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Review

Spatial omics techniques and data analysis for cancer immunotherapy applications

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In-depth profiling of cancer cells/tissues is expanding our understanding of the genomic, epigenomic, transcriptomic, and proteomic landscape of cancer. However, the complexity of the cancer microenvironment, particularly its immune regulation, has made it difficult to exploit the potential of cancer immunotherapy. High-throughput spatial omics technologies and analysis pipelines have emerged as powerful tools for tackling this challenge. As a result, a potential revolution in cancer diagnosis, prognosis, and treatment is on the horizon. In this review, we discuss the technological advances in spatial profiling of cancer around and beyond the central dogma to harness the full benefits of immunotherapy. We also discuss the promise and challenges of spatial data analysis and interpretation and provide an outlook for the future.

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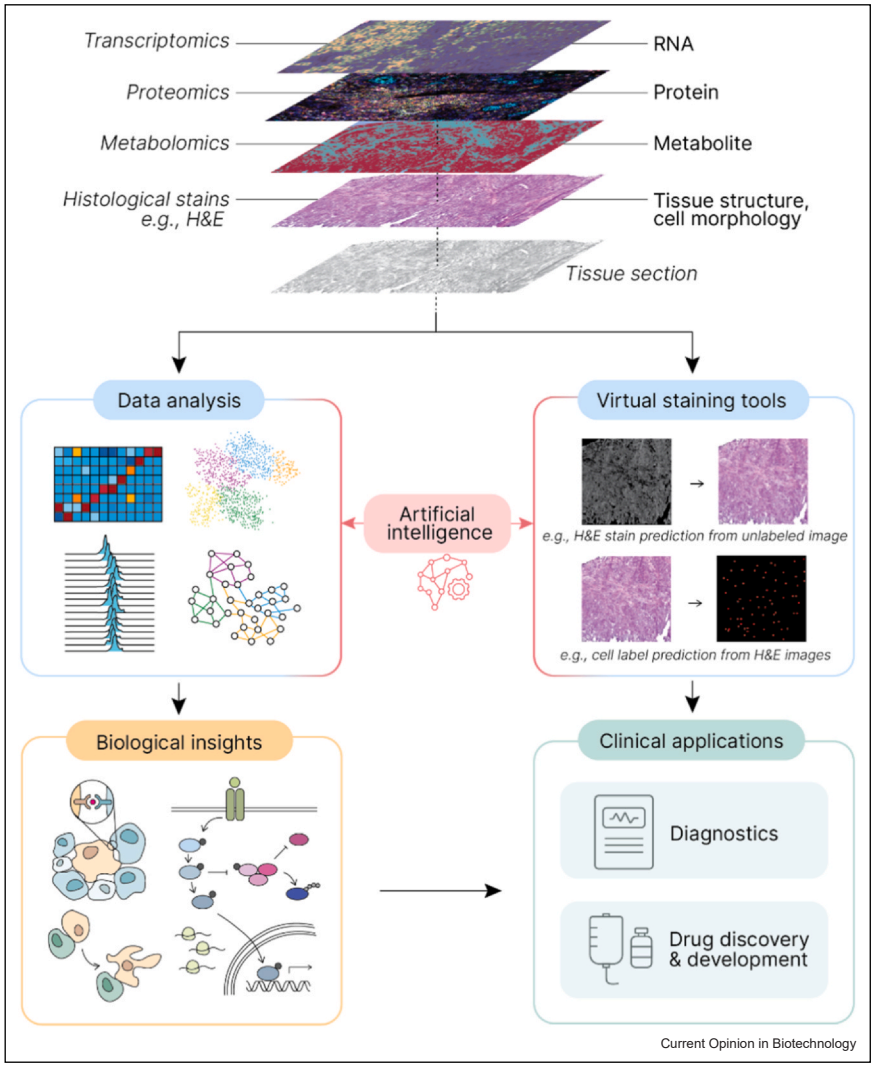
Introduction

Cancer cells exist in a highly intricate tumor microenvironment (TME) that significantly affects their biology and function. Immune cells are critical players in the TME that help determine the fate of cancer cells, either promoting or inhibiting carcinogenesis, progression, metastasis, and recurrence [1,2]. Recent immunotherapy strategies, such as immune checkpoint blockade, adoptive cell transfer, cytokines (e.g. interleukin-2), and cancer vaccines, have shown promising clinical efficacy by promoting the antitumor state of the immune system. However, many patients do not respond to these therapies, and some develop resistance or even immunotoxicity. Consequently, novel approaches are required to decipher the complexity of tumor immunity and thus enhance immunotherapy efficacy [3–5].

Recent breakthroughs in omics techniques have enabled high-throughput molecular profiling of tissues at the single-cell level but require tissue dissociation, leading to the loss of spatial organization. By preserving cell–environment interactions, spatial omics techniques can decipher the heterogeneity of the TME in various cancers [6,7]. One such technique, multiplex immunohistochemistry/immunofluorescence (mIHC/IF), facilitates simultaneous mapping of up to 100 markers in

Figure 1



Spatial multiomics analysis of tissue samples to extract biological insights for clinical applications. This process enables multilevel biological learning from various spatial omics data, including RNA, protein, metabolite, and cellular organization. Distilling high-dimensional spatial data into interpretable information, such as cell phenotypes and functions, requires effective bioinformatics tools, such as dimension reduction, clustering, and network analysis. Additionally, high-resolution molecular readouts from spatial omics techniques can serve as image predictors for clinical prognosis or diagnosis via DL-based virtual staining.

a single tissue sample, acting as a powerful discovery and validation tool in cancer immunology research [8,9]. Similarly, spatial transcriptomics capture both the breadth and depth of biomarker sequence coverage with adequate resolution and spatial information [10]. Other spatial omics techniques, including epigenomics, metabolomics, and proteomics, are emerging to facilitate multilayer exploration of cell identity and state *in situ*. To fully leverage these techniques, uniform analysis pipelines, standardized image processing frameworks, and consistent application are essential. Furthermore, spatial omics development will also be accelerated by artificial intelligence (AI), improved image visualization and storage capacity, and effective validation of genetic and transcription findings at the protein level (Figure 1).

Here, we review the latest advances in omics technology and its data analysis within the field of spatial profiling of molecules around and beyond the central dogma to unravel the complexities of the TME and harness the full potential of cancer immunotherapy. We highlight platforms to showcase the insights these techniques

promise and propose new developments in AI application, data sharing, and protein level validation to accelerate spatial omics advances.

Hyperplex immunohistochemistry/immunofluorescence: beyond multiplex

mIHC/IF allows simultaneous detection of up to 100 protein biomarkers on a single tissue section. While its multiplexing capabilities are limited compared with spatial transcriptomics, mIHC/IF offers a particular advantage in that protein levels provide a better representation of cellular activity than RNA levels. Evidence-based mIHC/IF platforms are now expected to offer diagnostic and prognostic insights in clinical settings, as well as the ability to predict immunotherapy responses across various cancer types. This possibility is further supported by the requirement for only small numbers of tissue sections, automation, and increased affordability [11–13].

As an extension of this technology, hyperplex IHC/IF platforms have now been developed to facilitate even more comprehensive immune characterization through simultaneous mapping of 40 to over 300 protein markers in a single slide (Table 1).

PhenoCycler (previously co-detection by indexing, or CODEX) offers ultra-high-plex imaging for *in situ*, cellular and subcellular analysis and has been used to analyze cutaneous T-cell lymphoma, spleen, tonsil, and lymph node tissues [24,25]. COMET offers an automated sequential cyclic workflow consisting of staining, imaging, and elution of three markers each round. This approach not only decreases steric hindrance by staining only three markers per cycle but also improves signal stability by reducing incubation time [26] (Figure 2I). MACSima or MACSima imaging cyclic staining (MICS) is an automated imaging platform that uses an iterative cyclic process of staining, image acquisition, and signal erasure. The integrated analysis software allows concurrent data acquisition and analysis. Moreover, the absence of tissue degradation after label erasure allows the use of MICS in multiple analyses of a single tissue [20,21]. Multiplexed ion beam imaging (MIBI) detects metal-tagged antibodies by secondary-ion mass spectrometry (MS) and provides a robustness, sensitivity, and reproducibility comparable to that of individual IHC stains [27,28]. However, the high costs and lengthy acquisition periods are potential disadvantages of MIBI.

The combined application of these hyperplex IHC/IF platforms with technologies for spatial transcriptomics profiling at the single-cell level represents a significant advance in deciphering the complexity and regulation of the cancer microenvironment and tumor immunity. These improvements will facilitate the identification of genes and biomolecules that determine anticancer treatment efficacy, further aiding the development of precision-targeted therapies using an immunological strategy.

Single-cell resolved spatial transcriptomics techniques

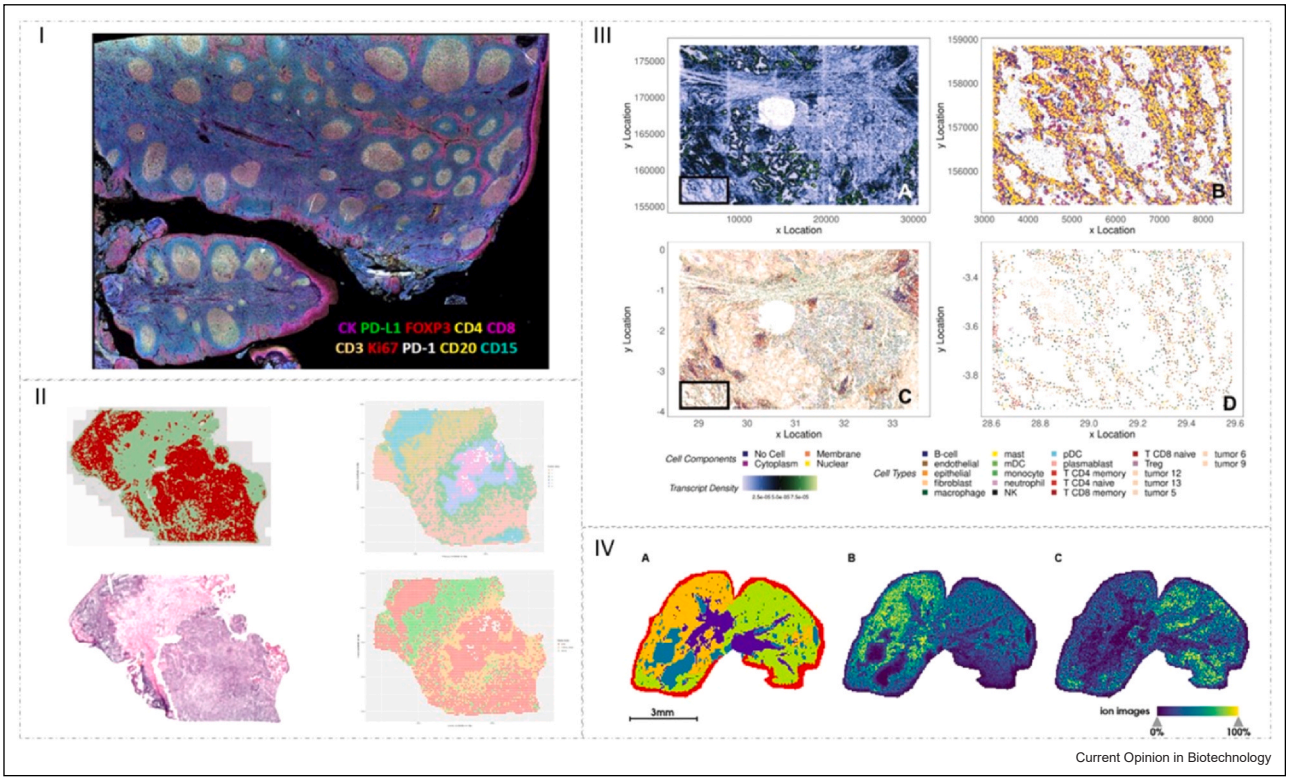
Spatial transcriptomics profiling has progressed from tools that analyze only a few gene targets (e.g. fluorescence-based *in situ* hybridization [FISH]-based technologies) to new developments that allow whole-transcriptome analysis [29]. Next-generation sequencing-based technologies, such

Table 1

Hyperplex IHC/IF platforms and their associated applications.

Hyperplex IHC/IF platform	Profiling technology	Plexing	Example application
PhenoCycler (Akoya Biosciences, USA)	Multiparametric fluorescent labeling with DNA-conjugated antibodies probed with fluorescent reporters [14]	Around 100 proteins (three per cycle) in a single tissue section [15]	Schürch et al. analyzed CRC tissue samples and used 56 protein markers to identify nine conserved cellular neighborhoods, each defined by its unique composition of TME cell types[16]
COMET (Lunaphore, Switzerland)	Fluorophores directly affixed to secondary antibodies [17]	Up to 40 proteins in a single tissue section [18]	Almeida et al. discovered the spatial restriction of myeloid and T regulatory cells in primary lung malignancies and also detected distinct myeloid cell populations within the same TME by using machine learning [19]
MICS (Miltenyi Biotec, Germany)	Antibody-fluorophore conjugates [20]	Analysis of ≥300 proteins in tissues and single cells [21]	Ali et al. identified EPCAM/THY1 as potential targets for AND-gated CAR T-cell therapy based on the co-expression of these antigens in ovarian cancer cells [21]
MIBI (Ionpath, USA)	Metal-tagged antibodies detected by secondary-ion MS [22]	Up to 100 proteins in theory with 40-plex panel developed at subcellular resolution [22,23]	Angelo et al. confirmed the reliability of MIBI versus validated IHC procedures for the detection of estrogen receptor alpha, progesterone receptor, and human epidermal growth factor receptor 2 in breast cancer samples [23]

Figure 2



Representative spatial omics images captured and visualized through the COMET, Stereo-seq, CosMx SMI, or MALDI MSI systems and their associated data analysis pipelines. (I) *Representative 10-plex image (as labeled by color-coded biomarkers) of a tonsil formalin-fixed, paraffin-embedded (FFPE) section, part of a comprehensive 40-plex panel analyzed using the COMET system. (Courtesy of Li Yen CHONG).* (II) *TME interpretation using Stereo-seq data with Seurat analysis. Left: QuPath annotation (top) of the H&E image (bottom) of a nasopharyngeal carcinoma (NPC) sample. The tumor region is colored red in the QuPath image and dark purple in the H&E staining; the stroma is colored green in the QuPath image and light pink in the H&E staining. Right: Seurat clusters show clear localization of six clusters with resolution of BIN100s. BIN1 refers to the unit square space of 500 × 500 nm occupied by an mRNA-capturing spot on the Stereo-seq chip; a BIN100 is about the size of four single cells ([2 × 25 μm] × [2 × 25 μm]). (Courtesy of Yang WU). (III) CosMx SMI images of NSCLC FFPE tissue.* **(a)** *Transcript density (hexbinned transcript counts).* **(b)** *Cellular compartment distribution of the transcripts in the zoomed region of panel A.* **(c)** *Cell type heterogeneity and spatial structure (annotation provided in the processed Giotto object by NanoString Technologies).* **(d)** *Cell type distribution of the zoomed region in panel* **c**. *Images rendered in R using ggplot2. (Courtesy of Chin Wee TAN).* (IV) *MALDI MSI images of mouse lung tissue sections.* **(a)** *Spatial segmentation map of the entire lung section, discriminating specific regions by clustering mass spectra profiles. The distributions of lipid analytes, including* **(b)** *sphingomyelin (SM 34:1;2 O at 741.5313 m/z) and* **(c)** *phosphatidylcholines (PC 16:0_18:5 752.5213 m/z) are visualized after root mean square normalization. (Courtesy of Xue GUO).*

as Visium Spatial Gene Expression (10× Genomics, USA) and GeoMx Digital Spatial Profiler (DSP; NanoString Technologies, USA), facilitate genome-wide spatial transcriptomics characterization of tissues and have been used widely in the field of immunotherapy [30–33]. This progress has facilitated impartial data-driven spatial analysis and biomarker discovery by avoiding marker selection bias. However, these tools are limited as they do not show spatial heterogeneity and cell–cell interactions at a single-cell level. A list of single-cell resolved platforms that have been developed to address this issue is presented in Table 2.

Spatial enhanced resolution omics sequencing (Stereo-seq) is a next-generation sequencing (NGS)–based spatial transcriptomics platform that integrates spatial transcriptome profiling with hematoxylin and eosin (H&E) staining of serial tissue sections. This technology not only offers high plexing capability, subcellular resolution, and a large capture area (see Table 2) but also provides high throughput. It enables the capture of an average of over 133,000 unique molecular identifiers, which equates to over 133,000 mRNA molecules, per 100 × 100 μm square bin [34,35,43]. Unlike traditional single-cell RNA sequencing (scRNA-seq) without spatial information or spatial transcriptomics technologies without cellular resolution, Stereo-seq can (1) construct a spatiotemporal transcriptomics atlas to study developmental dynamics (e.g. embryogenesis and tumorigenesis, developmental diseases) through transcriptome

Table 2

Single-cell spatial transcriptomic profiling platforms.

Platform	Profiling technology	Features
Stereo-seq (BGI, China)	DNA nanoball-patterned arrays combined with tissue RNA capture and next-generation sequencing [34]	Detects over 17 000 genes in one assay, enables analysis of up to approximately 280 000 cells per run with subcellular resolution (220 nm spot size and 500 or 715 nm spot center-to-center distance) within a maximal capture area of 13.2 × 13.2 cm per chip [34,35]
CosMx SMI (NanoString Technologies, USA)	Cyclic ISH chemistry and high-resolution imaging, with multiplexing enabled by 64-bit barcoding [36]	Detects up to 980-plex RNAs and 108-plex proteins simultaneously in tissue samples [36]
Xenium (10× Genomics, USA)	Automated microscopy-based *in situ* platform using padlock probe rolling circle amplification [37]	Supports up to 480-plex gene panels [38]; showcase study of breast cancer tissues uses a 313-gene panel for cell type identification[37]
MERSCOPE (Vizgen, UK)	Automated MERFISH-based platform [39]	High-resolution multiplexing of up to 500 genes at the subcellular level within a 1 × 1 cm capture area [39,40]
Molecular Cartography (Resolve BioSciences, Germany)	smFISH-based platform [41]	Detects up to 100 RNA molecules at a resolution of ~300 nm in x, y, and z coordinates [42]

comparison across different developmental stages [34]; (2) identify subsections within tissues that are characterized by distinct cell subpopulations and states of cellular metabolic reprogramming and damage [44]; and (3) detect ligand–receptor interactions [45], copy number variation [46], and virus infections [47] with subcellular resolution. Data from Stereo-seq are processed using BGI's bioinformatics pipeline and popular R packages such as Seurat [34] (Figure 2II).

The CosMx Spatial Molecular Imager (SMI) overcomes the lack of single-cell resolution obtainable with the GeoMx DSP [31] by utilizing advanced *in situ* hybridization (ISH) chemistry, high-resolution imaging, and a 64-bit multiplexing method to detect up to 980 RNA and 108 protein targets simultaneously at a subcellular resolution [36]. CosMx SMI detected > 96% of the cells in samples from five non–small cell lung cancer (NSCLC) tumors [36]. This high detection efficiency and accuracy were independent of the overall RNA quality of the samples [36], making it suitable for a wide range of sample types. Moreover, its unique chemistry makes CosMx highly scalable, and NanoString Technologies has already announced an improved 6000-plex RNA version [48], with the ultimate aim of reaching whole-transcriptome coverage (Figure 2III).

In 2022, 10× Genomics launched the automated Xenium microscopy-based *in situ* platform for subcellular spatial profiling, mapping hundreds of transcripts (up to 480 plex with stand-alone custom panels or predesigned panels of up to 377 genes with the option to include up to 100 additional genes in a custom add-on panel) at subcellular resolution [38]. Janesick et al. showcased a 313-plex cell type identification panel for human breast cancer [37]. With high-plex and high-throughput capabilities, Xenium generates multimodal readouts from a large imageable area to provide spatial context without damaging tissue integrity. It also integrates gene expression with histological images (e.g. H&E and IF stained) on the same tissue section and supports custom RNA targets as add-ons [49,50]. Similar to CosMx, Xenium was also designed for scalability, with the next version promising to support a 5000-plex gene panel [51].

MERSCOPE highlights the application of multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) for profiling *in situ* gene expression at subcellular resolution. The MERFISH technology incorporates combinatorial barcoding of transcripts and sequential rounds of imaging to decode the barcodes and the associated RNA species, even transcripts at low abundance, allowing comprehensive mapping of up to hundreds to thousands of RNA species [52]. MERSCOPE combines MERFISH with spatial imaging, fluidics, and image processing on an automated instrument to allow high-resolution multiplexing [39]. Using a MERFISH library detecting over 450 genes, Price et al. revealed distinct spatial organizations and multicellular interaction networks within the TMEs of mismatch repair deficient versus mismatch repair proficient human colorectal cancer (CRC) samples [53].

Molecular cartography offers an automated process to analyze up to 100 transcripts based on combinatorial single-molecule fluorescent *in situ* hybridization (smFISH) while preserving sample integrity [41,54]. Similar to the MERFISH technique, this method identifies individual RNA molecules using transcript-specific probes carrying unique barcodes that are decoded by applying a proprietary colorizing and decolorizing chemistry across multiple imaging rounds. The platform has been integrated with single-nucleus RNA sequencing (snRNA-seq) to study the intratumoral heterogeneity of medulloblastoma with extensive nodularity at the single-cell level and reveal the spatial and

development relationships between internodular and nodular compartments [55].

Other spatial techniques

Spatial metabolomics

Metabolomics elucidates intermediates and end products of metabolic reactions crucial for cellular growth, molecular function, and aging [56,57]. Conventional MS techniques, coupled with separation methods such as liquid chromatography or gas chromatography, fail to retain spatial information about metabolites; this spatial information is crucial for comprehending complex metabolic processes within cellular microenvironments [58]. Mass spectrometry imaging (MSI) overcomes this limitation by detecting the spatial distribution of biomolecules in whole tissue sections. In this process, a probe, employing either a laser or ion beam, scans the tissue surface in a raster pattern to desorb and ionize molecules from their natural environment. This approach ensures the retention of precise localization information, avoiding the obscuration that often occurs in conventional MS due to tissue homogenization. Matrix-assisted laser desorption/ionization MS imaging (MALDI MSI) allows simultaneous mapping of hundreds of low molecular weight metabolites while preserving tissue morphology, making it a valuable tool in spatial metabolomics research [59,60] (Figure 2IV).

Over the last decade, MALDI MSI has been integrated into many areas of disease mechanism and drug development. For instance, it has been instrumental in uncovering metabolic rewiring process in diseases, notably by observing the distribution of biomolecules in pathways such as glycolysis and the tricarboxylic acid cycle with high spatial resolution [61]. In oncology, MALDI MSI has been used to investigate the role of dysregulated lipids in metastases through mapping lipid profiles in the TME [62]. Furthermore, recent advances have enabled MALDI MSI to analyze samples with single-cell resolution [63,64] and to probe dynamic changes of cell-type-specific metabolic fluxes *in situ* [65]. Overall, spatial metabolomics techniques enable comprehensive analyses of the metabolic constituents of the TME, leading to an improved understanding of cancer biology.

Mass spectrometry–based, label-free spatial proteomics

Understanding the spatial proteome is essential for elucidating biological and physiological processes within a complex tissue architecture. Methods such as hyperplex IHC/IF generally depend on labels or antibodies, which require a prior knowledge of protein targets and largely limit multiplexing. MS-based spatial proteomics allows a comprehensive, label-free observation of up to hundreds of biomolecules, showcasing the natural protein distribution [66]. Recent advances have introduced multiple complementary methods. In Expansion Proteomics (ProteomEx), tissues are embedded into swellable hydrogels and physically expanded, facilitating the microdissection and subsequent MS-based proteomic analysis of tissue regions with a resolution of hundreds of microns [67,68]. Laser capture microdissection coupled with MS-based proteomics has identified over 2000 proteins from tissue at 100-μm resolution, highlighting its utility in studying microenvironment heterogeneity [69,70]. The application of the aforementioned MALDI MSI in proteomics profiling has revealed protease activity alterations in gastric cancer and neuronal loss in a mouse model of Huntington's disease [71,72]. The in-depth proteome mapping achieved through novel MS workflows demonstrates the exciting potential of spatial proteomics in uncovering disease mechanisms.

Spatial epigenomics

Spatiotemporal regulation of gene expression through epigenetic modifications plays a crucial role in the development and functionality of diverse cell types within the TME. Recent advancements in spatial epigenomics offer valuable insights into the intricate mechanisms governing cell fate and function in normal physiology within complex tissues. One such technique, spatial-CUT&Tag, combines in-tissue cleavage under targets and tagmentation (CUT&Tag) with microfluidic deterministic barcoding and NGS to generate spatially resolved genome-wide profiles of histone modifications in tissues [73]. Another tool, called spatial-ATAC-seq, combines *in situ* Tn5 transposition chemistry, microfluidic deterministic barcoding, and NGS for spatially resolved chromatin accessibility profiling [74]. Built on these two microfluidics and NGS-based techniques, a commercial platform, deterministic barcoding in tissue for spatial omics sequencing (AtlasXomics, USA), has been launched to provide comprehensive spatial epigenomic mapping at cellular resolution [75,76]. Another method, epigenomic MERFISH, combines CUT&Tag and MERFISH to interrogate active and inactive promoter and enhancer histone marks in single cells [77]. However, none of these innovative technologies have yet been employed in the investigation of cancer immunology.

Analysis of spatial data

Data normalization

By associating omics data with physical locations in cells and tissues, spatial omics data formats necessitate the development of data normalization methods that are distinct from those currently used for conventional single-cell data analysis. However, the similarity in structure with scRNA-seq data formats inevitably led early adopters to extend established scRNA-seq data normalization methods to spatial data. For example, the first step of both scRNA-seq and spatial transcriptomics

data analysis involves normalization to account for differences in mRNA capture between cells [40]. Established scRNA-seq normalization methods include *sctransform* in the R package Seurat, which divides each cell in a gene-spot matrix by the total number of cells in the spot [78], and the Python package *stLearn*, which uses a Spatial Morphological Gene Expression normalization approach. These methods adjust data based on the assumption that sequencing depth or library size represents technical variation, but this assumption may not be suitable for spatial data due to its unique characteristics [40,78]. A recent report by Bhuva et al. suggested that better clustering was achieved when library size effects were not explicitly normalized for spatial data sets, particularly for those obtained using the latest subcellular localized technologies [79]. Therefore, normalization for library size is not generally recommended for spatial data unless specific circumstances justify it.

Normalization methods specifically tailored for analyzing spatial data are therefore needed. Currently, normalization of spatial data is often handled by separate pipelines for spatial autocorrelation analysis, spatial trajectory inference, and spatial cell type mapping.

Cell segmentation

Cell segmentation is the first step in many biological image analysis workflows, especially those designed to obtain cell-specific information. Most conventional methods used for fluorescent tissue images are based on an approximation of cell boundaries from the nucleus and are limited by the variability of cell morphology. The high level of accuracy provided by recently developed deep learning (DL)-based models has provided an effective solution to this problem and the pretrained StarDist [80] and Cellpose [81] convolutional neural network–based models have already been widely adopted. The recently released Mesmer model, which was trained on more than one million expert-annotated cells in the 'TissueNet' data set, demonstrated human-level performance for tissue images [82]. These models are publicly available and have been made accessible on the popular open-source image analysis software QuPath and ImageJ.

Cell segmentation solutions are also emerging in commercial image analysis software, such as HALO (Indica Labs, USA), or as options in specialized imaging analysis software, such as SMI for spatial transcriptomics data. Unfortunately, these proprietary algorithms are not publicly available and have not been officially benchmarked against open-source solutions.

The current cell segmentation solutions vary in their performance, speed, cost, and ease of application. The open-source algorithms are not user-friendly, while the commercial ones are locked behind a paywall. The speed and processing power required to run these models can also be prohibitive. Furthermore, the potential for generalized application of these models to the wide variety of tissue and sample types seen in clinical settings remains to be established. Nevertheless, these issues are being addressed actively to achieve accessible and fully automated cell segmentation.

Analytic pipelines

The rapid development of spatial technologies has highlighted the need for computational tools to tackle various challenges unique to spatial analysis; for instance, spatial data normalization (standR [83]), image preprocessing, image registration, cell segmentation (inForm [84], HALO, CellProfiler [85], ilastik [86], Mesmer [82]), unsupervised cell phenotyping (Giotto [87]), cell type deconvolution (DestVI [88], CellDART [89]), identification of spatially variable genes (SpatialDE [90], scGCO [91]), computational resolution enhancement (BayesSpace [92]), cross-sample spatial transcriptomic profiling (PRECAST [93]), and identification of *in situ* ligand–receptor interactions (GCNG [94]).

However, existing analytic methods still have several limitations that currently represent the main bottleneck in spatial biology research. First, the lack of method generalizability as a result of insufficient and unrepresentative training data. As the pathophysiologic processes that drive development (etiology) and progression of disease, particularly cancer, are complex and not fully understood, a model (or parameter settings) trained on a specific tumor type may not be applicable to other tumor types. Second, the absence of tissue-specific databases of gene and protein markers hinders accurate cell type annotation, which is a critical step in cancer spatial research. To address this issue, canonical markers are now being replaced with tumor type–specific markers annotated using high-dimensional single-cell approaches. Finally, nonprogrammers/computational researchers rely mainly on commercial image analysis tools, such as inForm, HALO, and Visiopharm software (Visiopharm, Denmark). The inaccessibility of these commercial algorithms hinders the evaluation of their reproducibility and validation for the crucial image analysis aspect of spatial data pipelines.

Computing power and storage

The large data volume produced by the high-resolution imaging techniques involved in spatial omics presents significant computational and storage challenges [95,96]. In addition, integration of multiple spatial omics data sets poses difficulties due to differences in data types, scales, and resolution. Addressing these challenges requires effective data preprocessing techniques and dimensionality reduction methods, such as principal component analysis or t-distributed stochastic neighbor

embedding, to reduce dimensionality and extract essential information while reducing computational demands [97].

Effective and scalable visualization tools for large-scale and multiscale spatial omics data remain to be developed. Cloud computing platforms such as Amazon Web Services [98] or Google Cloud [99] offer opportunities to provide scalable computational resources on-demand. Furthermore, computing frameworks, such as Apache Spark or Hadoop, could be leveraged to distribute the computational load across multiple machines and accelerate data analysis, while Graphics Processing Unit (GPU) parallel algorithms would speed up image processing, clustering, and other computational tasks. Collaborative efforts are also required to establish platforms for sharing resources such as centralized data repositories, standardized data formats, and computational pipelines and codes [100–102].

Future perspectives

Artificial intelligence

AI has been leveraged in many areas of spatial analysis, such as image preprocessing (color deconvolution, normalization, cell detection, tissue classification), cell phenotyping (unsupervised clustering and deconvolution using large panels of molecular markers), analysis of proximity (direct distance, k-nearest neighbors, ecological indices), and identification of ligand–receptor interactions. Such success has raised researchers' confidence in using AI to predict protein or gene expression information from low-content images, such as H &E images, which are routinely generated for cancer diagnosis due to their low cost and fast turnaround time. An AI-powered approach called virtual staining has been developed for the simultaneous prediction and visualization of the molecular signatures of tumors solely based on H&E slides, generating pseudo-IHC staining [103]. Examples of virtual IHC stains generated from H&E include Ki-67 for neuroendocrine tissue [104], HER2 for breast samples [105], and SOX10 for skin and lymph node samples [106]. Currently, these methods are only able to predict a limited number of IHC stains using a single algorithm [107]. However, as more ground truth data sets become available, advancement in AI methodologies could facilitate the prediction of more complex mIHC/IF information. Beyond protein level prediction, algorithms have been developed to infer spatially resolved transcriptomic data from H&E-stained tumor tissues [108,109] through learning from advanced spatial transcriptomics data. This innovation greatly extends the diagnostic capability of traditional H&E staining, reducing the need for additional tissue staining or processing. As a result, it has the potential to greatly lower equipment requirements and costs, making precision medicine more accessible and affordable.

Image data visualization and repository formation

Spatial omics technologies generate large multidimensional tissue images, revealing important biomolecular expression and localization patterns that may reflect disease biology and etiology. The ability to visualize and compare these images is critical for the validation of new antibodies, staining procedures, and imaging devices and facilitating the development of advanced image processing and AI algorithms. Several existing software tools with graphical user interfaces [110,111] can provide intuitive and interactive visualizations of single or multiple combinations of markers,

Figure 3

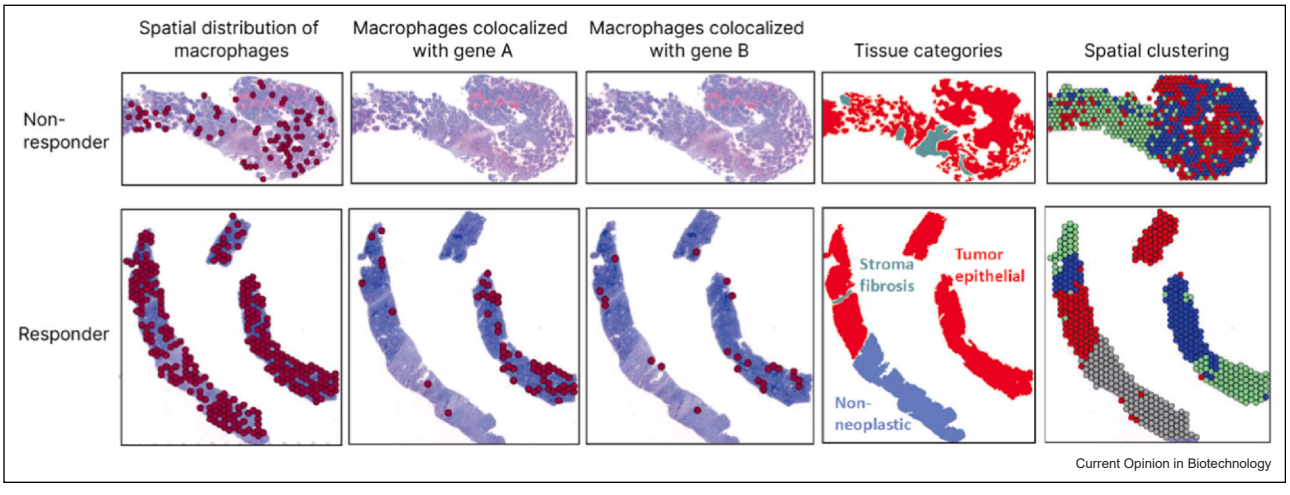


Illustration of the application of visual comparison of cell phenotype distribution patterns between patients who respond and patients who do not respond to treatment (Courtesy of Mai Chan LAU).

Table 3

Current public spatial omics data repositories.

Repository	Data	Features	Reference
NCBI Gene Expression Omnibus	NGS and high-throughput functional genomics data	Browse, search, query, or download curated and annotated gene expression data	[113]
NCI Imaging Data Commons	Radiological, H&E, and multispectral microscopy images and results	Share, visualize, or analyze multimodal tissue images for clinical and basic cancer research	[114]
ImmunoAtlas	Hyperplexed IHC/IF and H&E images and results	Share, visualize, or download large or whole-slide images from immuno-oncology studies generated by common platforms, such as CODEX, COMET, MIBI, and Vectra Polaris	[115]
Image Data Resource	Image data with author-supplied metadata (e.g. information on genes, phenotypes, and experimental perturbations)	Search, visualize, query, or analyze large biological image data sets with annotations, defined regions, etc.	[116]
Minerva	Cyclic immunofluorescence (CyCIF), mIHC/IF, and H&E images	Share, interact with, and interpret large tissue images by creating waypoints and annotations in the form of 'stories'	[117]

image zooming/panning, or displays of phenotypic feature measurements [112]. These technologies also reveal differences in the visual distribution patterns of cell phenotypes between patient groups, such as responders and nonresponders to treatments (Figure 3). Moreover, these visual differences are quantifiable and can be used to help further patient stratification.

Online spatial omics data repositories (Table 3) enable the sharing and visualization of large raw images generated in spatial omics studies, though these capabilities are currently limited. All these tools are essential for the accessibility and reproducibility of spatial omics technologies.

While most current online image management or visualization tools were originally designed for multiplexed tissue images, ongoing efforts are focused on making them compatible with other types of spatial omics data, such as spatial transcriptomics and metabolomics images. This will eventually allow the analysis, visualization, and interpretation of complex TMEs based on spatial multiomics data.

Protein level validation

Spatial transcriptomics is the most widely used spatial omics technique; however, due to inconsistent correlation between mRNA and protein levels [118–120], protein level validation is crucial for integration of transcriptomic findings into our protein-based understanding of cellular function. Despite the potential of mIHC/IF techniques as solutions, several challenges remain. Antibody validation is a time-consuming but essential step in the development of IHC/IF assays [121–124]. In our experience, it takes approximately 70 hours to validate a commercial antibody for IHC, a process that also requires an experienced pathologist to interpret signal accuracy. Setting up staining panels for multiplex and hyperplex IHC/IF takes considerably longer because of the extended imaging times (including autofluorescence capture) and the inclusion of additional controls to exclude antibody crosstalk, on top of antibody validation (approximately 67.5 hours/antibody for sequential immunostaining systems and approximately 80 hours/antibody for combinatorial immunostaining systems). For many laboratories, time and cost constraints in addition to the required technical expertise may limit access to multiplex and hyperplex IHC/IF.

Although the signal intensities and patterns of IF may not be replicated in multiplex IF, the long set-up time could be expedited by using antibodies already validated for IHC. Furthermore, as more mIHC/IF studies are published, access to more validated antibodies and antibody panels will facilitate future assay development. Moreover, technological advances, such as automated sample preparation and image scanning for stripping efficiency, combined with the development of new methods to standardize mIHC/IF experimental workflows, will increase the efficiency of protein level validation.

Conclusion

Remarkable advances in spatial omics and computational methodologies have unveiled many previously underappreciated roles of tumor-immune interplay, thereby enhancing our comprehension of cancer immunology and its implementation in practical applications. Future progress is likely to arise from the integration of spatial multiomics data via innovative techniques, data processing, and the utilization of AI, as well as the protein level validation of spatial transcriptomics. By uncovering novel targets/biomarkers and spatial patterns associated with clinical outcomes, we anticipate that the spatial omics field, spanning multiple molecular strata, will be a major driving force in establishing precision medicine for cancer patients.

CRediT authorship contribution statement

Yue Zhang, Ren Yuan Lee, Chin Wee Tan, Xue Guo, Willa Wen-You Yim, Jeffrey Chun Tatt Lim: Conceptualization, Writing – original draft, review & editing, Visualization. **Yu Ting Felicia Wee, Yang Wu, Malvika Kharbanda, Jia-Ying Joey Lee, Nye Thane Ngo, Wei Qiang Leow:** Writing – original draft, review & editing. **Tony Kiat Hon Lim, Radoslaw Mikolaj Sobota, Mai Chan Lau, Melissa J Davis, Joe Yeong:** Conceptualization, Writing – review & editing, Supervision.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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