Spatial architecture of the immune microenvironment orchestrates tumor immunity and therapeutic response

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Abstract

Tumors are not only aggregates of malignant cells but also well-organized complex ecosystems. The immunological components within tumors, termed the tumor immune microenvironment (TIME), have long been shown to be strongly related to tumor development, recurrence and metastasis. However, conventional studies that underestimate the potential value of the spatial architecture of the TIME are unable to completely elucidate its complexity. As innovative high-flux and high-dimensional technologies emerge, researchers can more feasibly and accurately detect and depict the spatial architecture of the TIME. These findings have improved our understanding of the complexity and role of the TIME in tumor biology. In this review, we first epitomized some representative emerging technologies in the study of the spatial architecture of the TIME and categorized the description methods used to characterize these structures. Then, we determined the functions of the spatial architecture of the TIME in tumor biology and the effects of the gradient of extracellular nonspecific chemicals (ENSCs) on the TIME. We also discussed the potential clinical value of our understanding of the spatial architectures of the TIME, as well as current limitations and future prospects in this novel field. This review will bring spatial architectures of the TIME, an emerging dimension of tumor ecosystem research, to the attention of more researchers and promote its application in tumor research and clinical practice.

Keywords: Tumor immunity, Tumor immune microenvironment, Spatial architecture, Immunotherapy

Background

Over the past few centuries, the concept of tumor has evolved from a simple aggregation of abnormally proliferating cells into a highly organized “organ”. Various components that compose tumors are termed the tumor microenvironment (TME) (1). Although the specific composition of the TME varies between tumor types, most of them share hallmark characteristics, including tumor cells, immune cells, stromal cells, extracellular matrix (ECM), vessels, soluble factors, and physical properties (Table 1) (2–5). Within the TME, all immune components are specifically defined as the tumor immune microenvironment (TIME) because of their unique internal interactions and essential roles in tumor biology, which comprises innate immune cells, adaptive immune cells, extracellular immune factors, and cell surface molecules (4, 6, 7). Studies have focused on the composition of immune cells in the TIME, and established mature theories and clinical applications (4, 8). For example, triple-negative breast cancer (TNBC) with more T cell infiltration generally presents better prognosis than those with less T cell inflamed (9). However, findings that patients with similar compositions of infiltrating immune cells have different prognoses are not well explained (10, 11), suggesting further exploration is needed on the TIME.

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| Components                          | Classification | Molecular characteristics | Function in TME                                                                 | Major Role          |
|------------------------------------|----------------|---------------------------|--------------------------------------------------------------------------------|---------------------|
| T lymphocytes                      |                |                           |                                                                                  |                     |
| CD8⁺ T cell                        | TME & TIME     | CD3⁺CD8⁺                  | Production of IL-2 and IFN-γ                                                   | Anti-tumoral        |
| Th1 cell                           | TME & TIME     | CCR6⁺CXCR3⁺               | Production of IL-4, IL-5 and IL-13                                              | Anti-tumoral        |
| Th2 cell                           | TME & TIME     |                           | Production of IL-17, IL-17f, IL-21, IL-22                                       | Pro-tumoral         |
| Th17 cell                          | TME & TIME     |                           |                                                                                  | Ambiguous           |
| Treg cell                          | TME & TIME     | CD4⁺FOXP3⁺CD25⁺           | Immune suppression                                                             | Anti-tumoral        |
| B lymphocytes                      | TME & TIME     | CD19⁺CD20⁺CD138⁺          | Antibody production, antigen presentation, TLSs formation                      | Anti-tumoral        |
| Innate lymphoid cell (ILCs)        |                |                           |                                                                                  |                     |
| ILC1s                              |                |                           |                                                                                  |                     |
| NK cells                           | TME & TIME     | Expression of T-bet; Eomes⁺CD27⁻ | Cytoxic functions without prior sensitization                                 | Anti-tumoral        |
| Non-NK cells                       | TME & TIME     | Expression of T-bet; Eomes⁻CD27⁺ | Secretion of IFN-γ and TNF-α to induce tumor dependent anti-tumor immune responses | Ambiguous           |
| ILC2s                              | TME & TIME     | Expression of GATA3; secretion of type 2 cytokines | Secrretion of IL-5, IL-13, IL-4 to inhibit anti-tumor immunity | Pro-tumoral         |
| ILC3s                              | TME & TIME     | Expression of RORγt       | Heterogeneous cells secreting various cytokines and chemokines                 | Ambiguous           |
| Tumor-associated macrophages       |                |                           |                                                                                  |                     |
| M1                                 | TME & TIME     | HLA-DR⁺CD68⁺CD11c⁻       | Promoting anti-tumor T₉₁ and T₉₁,17 immune responses                           | Anti-tumoral        |
| M2                                 | TME & TIME     | CD86⁺CD80⁺NGO⁺           | Supporting angogenesis, tumor progression, and metastasis; immune suppression  | Pro-tumoral         |
| MDSCs                              | TME & TIME     | CD11b⁺HLA-DR⁻            | Differentiating into TAMs, immune suppression                                   | Pro-tumoral         |
| Dendritic cells                    | TME & TIME     | Siglec-H⁺CD317⁺           | Binary immune regulatory function shaped by TME                                | Ambiguous           |
| Neutrophils                        | TME & TIME     | CD14⁺HLA-DR⁺CD206⁺CD86⁻  | Immune respond initiation, antigen presenting                                 | Anti-tumoral        |
| N1                                 | TME & TIME     | Supported by TGF-β and G-CSF | Remodeling ECM, promoting angiogenesis and tumor growth                        | Pro-tumoral         |
| N2                                 | TME & TIME     | Supported by IFN-γ and hepatocyte growth factor |                                                                       |                     |
| Components | Classification | Molecular characteristics | Function in TME | Major Role |
|------------|----------------|---------------------------|-----------------|------------|
| Stromal cells | TME | Cellular markers: α-SMA, FAP-α, FSP-1/S100A4, PDGFRβ | Promoting tumor cell proliferation and invasion, angiogenesis, ECM remodeling, bidirectional immune regulation | Pro-tumoral |
| Cancer-associated fibroblasts | TME | Promoting tumor cell proliferation and invasion, angiogenesis, ECM remodeling, bidirectional immune regulation | Pro-tumoral |
| Endothelia cells | TME | Cellular markers: CD31; consisting of blood vessels | Angiogenesis, tumor metastasis | Pro-tumoral |
| Percytles | TME | Cellular markers: Calponin, CD90, DLK, NG2, PDGF-A, SMA | Promote primary tumor growth, negative regulator of metastasis, Pro-tumoral |
| Adipocytes | TME | Cellular markers: CD34 | Secretion of hormones, metabolites, growth factors, enzymes and cytokines | Pro-tumoral |
| Mesenchymal stem cells | TME | Cellular markers: CD105, CD90, CD117, CD133 | Forming the premetastatic niche; promoting tumor initiation and progression | Pro-tumoral |
| Extracellular matrix | TME | Complex noncellular three-dimensional macromolecular network | Physical scaffold for cell, tumor cell dissemination, depot for cytokines and growth factors | Pro-tumoral |
| Collagen, fibronectin, elastin, laminin | TME | Promoting metastasis and angiogenesis | Pro-tumoral |
| Matrix metalloproteases, Cathepsins | TME | Fostering tumor metastasis, physical link between lymph nodes and tumor | Pro-tumoral |
| Immune molecules | TME | ECM remodeling, aiding metastasis, angiogenesis and inflammation | Pro-tumoral |
| Cytokines | TME & TIME | Mainly secreted by immune cells; acting in a paracrine, autocrine or endocrine manner | Promoting leukocyte growth, survival and activation, promoting tumor immunogenicity, tumor cell proliferation, angiogenesis | Ambiguous, mainly anti-tumoral |
| Chemokines | TME & TIME | Regulation of cell movement and leukocyte attractants | Promoting tumor cell migration, invasion and metastasis; promoting immune cell migration, maturation | Ambiguous, mainly anti-tumoral |
| Extracellular nonspecific chemicals | TME | Aerobic cellular respiration | Favor immunosuppressive phenotypes | Ambiguous |
| Components   | Classification | Molecular characteristics                                                                 | Function in TME                                                                 | Major Role            |
|--------------|----------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------|
| Amino acids  | TME            | Components and substrates for various critical processes in cell metabolism and physiology | Supporting physiological processes of both tumor and immune cells, immune regulation | Ambiguous             |
| Glucose      | TME            | Major source of energy for cells                                                           | TME dependent influence of energy metabolism in both immune and tumor cells     | Ambiguous             |
| Lactate      | TME            | Products of glycolysis                                                                     | Inhibiting anti-tumor immunity, decrease the pH within the TME                  | Pro-tumoral           |
| Carbon dioxide | TME              | Products of oxidative phosphorylation                                                     | Causing tumor tissue acidosis                                                  | Ambiguous             |
| Fatty acids  | TME            | Involvement in biological progression and cell structure                                  | Heterogeneous consequences for different cells, leading to immunosuppressive effects | Ambiguous             |
| Metal ions   | TME            | Involvement in biological progression                                                     | Regulating tumor and immune cells                                              | Ambiguous             |

TME, tumor microenvironment; TIME, tumor immune microenvironment; MDSCs, myeloid-derived suppressor cells; TLSs, tertiary lymphoid structures
To obtain diversified information about the TIME, emerging researches focus on not only its compositions and molecular features, but also the spatial organization of components in the TIME, summarized as its spatial architecture. The spatial architecture of the TIME is now described mostly from the following four aspects (Fig. 1 and Table 2): (1) location of immune cells in the tumor compartments (Fig. 1a); (2) distance between cells, evaluated by the distance between cell and its nearest neighborhood (10), or the density of cells around the defined cell (12) (Fig. 1b); (3) spatial distribution of immune regulators (Fig. 1c); (4) spatial patterns formed by the well-organized TIME components characterized morphologically and molecularly (Fig. 1d). Moreover, distance-dependent immune interactions like paracrine, autocrine or cell contact also support that spatial relation matters in the research of TIME. While some previous studies have provided insights into the spatial architecture of the TIME using conventional techniques (13, 14), innovative technologies have portrayed the TIME with higher flux, higher dimensionality and higher resolution ratio. The evolved knowledge about the spatial architecture facilitated revealing its effect on the clinical prognosis and immunotherapy efficacy (10, 15–21).

In this review, we will first summarize the emerging spatial analysis technologies, together with their features and scope of application. Methods used by scientists to describe the complex architectural traits of the TIME are subsequently discussed. Next, we discuss the implication of the TIME architectures in tumor initiation, expansion, invasion and metastasis. We also included gradients of extracellular nonspecific chemicals (ENSCs), a regulatory factor usually ignored in the TIME, to emphasize its importance in TIME function. Then, we reviewed the clinical potential of the spatial architecture of the TIME. Finally, discussions and recommendations for methods to overcome current setbacks and future development in this field are proposed.

Emerging technologies used to characterize the spatial architecture of the TIME

Emerging technologies with a higher flux, higher dimensionality and higher resolution ratio have extensively broadened researchers’ horizon of the spatial architecture of the TIME, which has not been fully characterized previously due to its microcosm and complexity. Here, we reviewed some representative emerging technologies for identifying the spatial architecture of the TIME (Fig. 2 and Table 3) (10, 19, 22–41), which might facilitate further mechanistic research.

Deep learning methods for H&E staining slides

Hematoxylin and eosin (H&E) staining, which is one of the most common techniques adopted by pathologists, has now been rekindled by deep learning method. Conventionally, pathologists observe the spatial architecture of the TIME in formalin-fixed and paraffin-embedded (FFPE) or fresh-frozen samples under a microscope (42–44). However, manual observation of slices is laborious and may result in considerable interobserver discrepancies (45, 46). The introduction of deep learning methods based on whole slide images (WSIs) can automate and standardize the process.

The convolutional neural network (CNN), a universal methodology for processing medical images (47–50), is the most commonly used algorithm for processing WSIs (Fig. 2a). Basically, before adopting CNN, the program must be trained with manually annotated images according to recognition purposes, e.g., marking out certain types of cells or regions. Then, the trained CNN can process coarse images and generate automatically annotated images. Next, a manual quality inspection step often follows to verify the results. Using the collected data, deeper investigations can be performed, such as cell quantification, spatial clustering, intercellular interaction analyses, significance testing with clinical phenotypes, and correlation predictions with sequencing data (22–25).

The integration of deep learning and routine H&E staining techniques can reveal surprising pathological traits that might have been previously ignored by human eyes, with nearly no extra cost and impressive accuracy and efficacy. Moreover, the high-flux deep-learning-based analysis might revive prodigious historical databases of H&E-stained samples, which is promising for large-scale retrospective studies. However, despite the convenience and low cost of H&E staining, it is usually not capable of classifying specific subtypes of immune cells in the TIME.

Probe-based in situ imaging

Discriminating immune cell subtypes by biomarker-probe pairs has been well established, as represented by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) (51, 52). One primary restriction of IHC or FISH is the limited quantity of detectable targets in one section because of the overlap of the emission spectrum of fluorescent reporters. In addition, the techniques arouse the concern of consuming too many precious sections if the detection of a large number of targets is required. To solve these problems, two technology roadmaps were conceived: one by batching the imaging of fluorescent signals and the other by substituting electromagnetic wave with particle flow.
For the first strategy, several methods were proposed. Adopted from Codetection by indexing (CODEX) (28), CODEX-FFPE is a system based on special antibodies conjugated with single-strand DNA, which is suitable for both FFPE samples and tissue microarrays (Fig. 2b). The target protein is detected by DNA-conjugated antibodies, and then, imaging cycle starts. In each imaging cycle, only a few kinds of complementary DNA chains linked with different fluorescent reporters are added to image. Then, these complementary DNA chains are striped to undergo another cycle. And there have been commercial version of CODEX-FFPE provided (10). CorFISH (29), seqFISH + (30) and seqFISH (31) are a series of technologies that exploit two classes of oligonucleotide DNA probes (Fig. 2c). Each primary probe contains 5 domains including one for binding target mRNA and 4 for binding secondary probes. In each cycle of imaging, an exquisitely predesigned mixture of secondary probes labeled

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**A. Location**

- Immune cells in invasion margin
- Immune cells in tumor stroma
- Immune cells in tumor core

**B. Distance**

- Distance to the nearest cell
- Density of immune cells in a certain area around the cell
- Window: a certain number of nearest spatial neighbors around the cell
- Density of the nearest immune cells around the defined cell

**C. Distribution of immune regulator**

- Compartment-based location
- Spatial proximity of paired receptor and ligand
- Cell-specific spatial expression and co-location

**D. Special spatial patterns**

- Tertiary lymphoid structure
- Pre-metastatic niches
- Organ-specific stromal cells
- Immune cells
- Morphological observation
- Cellular resolution in situ imaging

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**Fig. 1** Definition and components of the spatial architecture of the tumor immune microenvironment (TIME). The spatial architecture is described according to the location of immune cells (a), distance between cells (b), distribution of immunoregulators (c), and specific spatial patterns (d).
with different fluorescence reporters is added, and thus, mRNAs will be marked with different colors. Then, these secondary probes are eluted, and a new hybridization cycle starts. The mRNAs will be hybridized with another mixture of secondary probes. After several cycles, nucleotide sequences of mRNA molecules in situ can be deciphered from the unique array of colors in corresponding location.

Mass spectrometry is another strategy for improving IHC and FISH (53, 54). While signal of fluorescent reporters consists of a consecutive range of light spectrum, mass spectrometric signal is discrete peaks, endowing it with higher resolution to discriminate different reporters. Besides, the cell-by-cell scanning manner used in mass-spectrometry-based techniques can also achieve a higher spatial resolution. Multiplexed ion beam imaging (MIBI) (19) and multiplexed ion beam imaging by time of flight (MIBI-TOF) (32, 33) take advantage of antibodies labeled with isotopes (Fig. 2d). And during scanning, these isotopes can be excited by primary oxygen ion beam to form secondary ion beam. Another technique called imaging mass cytometry (IMC) works in an analogous way but uses laser to activate isotopes (Fig. 2d). And notably, IMC does not necessarily need antibody probes to label cells, but also uses endogenous or exogenous tissue-specific chemicals, such as iodine isotopes in the thyroid, to achieve imaging (34, 35).

After the generation of raw images, pathologists can perform further processing and refinement, such as the segregation of cells, classification of immune cells and other analyses, facilitated by bioinformatics tools. Probe-based in situ technology is characterized by its detailed preservation of spatial information and relatively low cost in large-scale applications. Nevertheless, probe-based technologies only detect known targets with existing probes. Thus, they are not suitable for detecting novel biological events, such as undefined molecules.

### Spatial transcriptome and proteome

Omnis techniques are ideal for conducting de novo investigations or revealing the landscape of the TIME due to their probe-free traits, high flux, and large capacity. Currently, the spatial transcriptome and spatial proteome are the two most commonly used spatial omics technologies.

One representative kind of spatial transcriptome is microarray-based spatial transcriptomics (Fig. 2e) (36). On the microarray chip used for the detection of histological sections, there are massive probes containing unique positional barcodes for locating. The barcodes can be sequenced in the subsequent sequencing, thus enabling the mapping of transcriptome information to histological sections. Microarray-based spatial transcriptomics combines transcriptome and histology to extend transcriptome to a 2-D dimension, and has been commercialized and adopted in the researches of several malignancies (55–58). However, the spatial resolution of microarray-based spatial transcriptomics is limited that it only has a minimal resolution power of discriminating 10–200 cells. Thus, Moncada et al. (37) combined microarray-based spatial transcriptomics with single-cell RNA

| Components                  | Definition                                                                 | Detection methods                                                                 | Detecting characteristics                                      |
|-----------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------|
| Location of immune cells    | Identification and quantification of immune cells in different compartments of tumor | H&E staining, digital pathology                                                   | Morphological differences; visual distinction of tumor compartiments (i.e., STILs, iTILs) |
| Distance between immune cells | The shortest distance between cells                                       | Probe-based in situ imaging                                                       | Cellular markers                                               |
| Distribution of immune regulators | Compartment-based distribution                                            | Cellular-resolution imaging and analysis algorithms                               | Expression signature                                           |
| Identification of specific spatial patterns | Robust spatial architecture of immune cells with specific aggregation and distribution patterns (i.e., TLSs, peri-vascular niches) | Probe-based in situ imaging and/or spatial omics                                  | Spatial protein or mRNA expression at cellular or subcellular resolution |
| TILs, tumor infiltrated lymphocytes; TLSs, tertiary lymphoid structures | Location of immune cells Identification and quantification of immune cells in different compartments of tumor | H&E staining, digital pathology                                                   | Morphological differences; visual distinction of tumor compartiments (i.e., STILs, iTILs) |
| Distance between immune cells | The shortest distance between cells                                       | Probe-based in situ imaging                                                       | Cellular markers                                               |
| Distribution of immune regulators | Compartment-based distribution                                            | Cellular-resolution imaging and analysis algorithms                               | Expression signature                                           |
| Identification of specific spatial patterns | Robust spatial architecture of immune cells with specific aggregation and distribution patterns (i.e., TLSs, peri-vascular niches) | Probe-based in situ imaging and/or spatial omics                                  | Spatial protein or mRNA expression at cellular or subcellular resolution |
Fig. 2  Emerging techniques used to identify the spatial architecture of the tumor immune microenvironment. Pink area (a), deep-learning-based HE techniques. Blue area (b–d), probe-based in situ technologies. b, CODEX-FFPE; c, seqFISH+; d, IMC and MIBI/MIBI-TOF. Green area (e–f) spatial omics. e, microarray-based spatial transcriptomics + sc-RNA-seq; f, MALDI MSI. Consult Table 3 for more detailed information. H&E, hematoxylin and eosin; CNN, convolutional neural network; MALDI MSI, matrix-assisted laser desorption/ionization mass spectrometric imaging; UV, ultraviolet; sc-RNAseq, single-cell RNA sequencing; IMC, imaging mass cytometry; MIBI, multiplexed ion beam imaging; MIBI-TOF, multiplexed ion beam imaging by time of flight; CODEX, codetection by indexing; FFPE, formalin-fixed and paraffin-embedded.
### Table 3  Emerging techniques to identify spatial structure of the tumor immune microenvironment (TIME)

| Category                  | Name                                 | Level                      | Sample preparation | Labels                              | Visualization                                    | Comments                                                                 | Refs. |
|---------------------------|--------------------------------------|----------------------------|--------------------|--------------------------------------|--------------------------------------------------|--------------------------------------------------------------------------|-------|
| Non-specific technique    | H&E staining                          | Nonspecific structure      | FFPE               | Hematoxylin and eosin                | Visible light                                    | Low-cost; high flux helped by deep learning; poor discrimination of cell subtypes | [22–25] |
| Probe-based in situ imaging | IHC                                  | Peptide and protein        | FFPE               | Antibody-reporter (usually fluorescent protein) | Fluorescence, insoluble pigment, etc.           | Limited detectable targets simultaneously                                                                 | [26]  |
|                           | FISH                                 | DNA                        | FFPE               | Oligonucleotides-fluorescent reporter | Fluorescence                                    | Limited detectable targets simultaneously                                                                 | [27]  |
|                           | CODEX                                | Peptide and protein        | Fresh-frozen       | Antibody-oligonucleotides            | Fluorescence                                    | Extended detectable targets simultaneously                                                                 | [28]  |
|                           | CODEX-FFPE                           | Peptide and protein        | FFPE               | Antibody-oligonucleotides            | Fluorescence                                    | Extended detectable targets simultaneously                                                                 | [10]  |
| seqFISH, seqFISH +, corFISH | seqFISH, seqFISH +, corFISH           | mRNA (sub-transcriptome)   | Live section       | Oligonucleotides-fluorescent reporter | Fluorescence                                    | >10,000 detectable genes simultaneously; subcellular resolution ratio                                                                 | [29–31] |
|                           | MIBI, MIBI-TOF                        | Peptide and protein        | FFPE, immobilized cell suspension | Antibodies-isotypes                  | Secondary ion beam                               | Extended detectable targets simultaneously                                                                 | [19, 32, 33] |
|                           | IMC                                  | Protein, etc.              | FFPE               | Antibodies-high mass tag             | Ion beam                                        | Subcellular resolution ratio; probes are not necessarily needed                                                                                  | [34, 35] |
| Spatial transcriptome     | Microarray-based spatial transcriptomics | Transcriptome              | Live section, FFPE | Spatial transcriptome                | Pathology + Computational analysis              | Restricted cellular resolution                                                                                                                    | [36]  |
|                           | Microarray-based spatial transcriptomics + sc-RNAseq | Transcriptome              | Live section + single-cell suspension | Spatial transcriptome                | Computational matching                           | Mapping is based on region-cluster matching by multimodal intersection analysis                                                                | [37]  |
|                           | ZipSeq                               | Transcriptome              | Live section       | Antibody/lignoceric-oligonucleotides-zipcode | Computational matching | Exquisite design in advance is required for accuracy                                                                                     | [38]  |
| Spatial proteome          | MALDI MSI                            | Proteome                   | Fresh frozen, FFPE | –                                    | Ion imaging                                      | Label-free; de novo investigation                                                                                                                  | [39, 40] |

H&E, hematoxylin and eosin; FFPE, formalin-fixed and paraffin-embedded; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; DNA, deoxyribonucleic acid; CODEX, co-detection by indexing; MIBI, multiplexed ion beam imaging; MIBI-TOF, multiplexed ion beam imaging by time of flight; IMC, imaging mass cytometry; sc-RNAseq, single-cell RNA sequencing; MALDI MSI, matrix-assisted laser desorption/ionization mass spectrometric imaging.
sequencing (scRNA-seq) to integrate their advantages. With reference maps generated from microarray-based spatial transcriptomics, data from the scRNA-seq of the rest of the tissue can be mapped back.

There are also some other technology roadmaps in spatial transcriptomics, such as ZipSeq. Researchers first hybridized live tissue with cell-marker-specific antibody probes containing unique zipcode oligonucleotides that are initially blocked by photocleavable protecting group. Then, region of interest (ROI) of any shape was radiated with ultraviolet to remove blocking groups, making it possible to combine with complementary oligonucleotides conjugated to fluorescent reporters. Then, processed tissue was imaged to obtain reference map and digested to undergo scRNA-seq, whose outcome will be matched back to the reference map ultimately (38).

Apart from the spatial transcriptome, the spatial proteome represented by matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI MSI) is also capable of depicting in situ omics information (Fig. 2f). As a label-free system, although the spatial proteome also takes advantage of mass spectrometry for imaging, the excitation beam in the spatial proteome is much stronger and is able to directly ionize the components of sections and preserve spatial information simultaneously during scanning (39, 40).

Spatial omics has enabled researchers to better understand the actual biological events occurring in the architecture of the TIME. Spatial omics not only quantifies and locates immune cells but also further reveals their functional status and potential intercellular reactions. However, concern persists about the matching accuracy and precision of spatial omics. In addition, complex protocols and sizable expenses are also setbacks and challenges for large-scale studies or clinical use.

Software for TIME analysis
In-depth data mining is necessary to take full advantage of massive data derived from the techniques reviewed above. Some basic functions, such as cell segregation, classification, quantification and other primary extended tools, are inlaid to the solutions mentioned above. In addition, developers have also proposed general analysis software for deeper analyses. For instance, spatial variance component analysis is a computational algorithm able to analyze cell–cell communication in spatial architecture of the TIME. It deconstructs overall effects that cells receive into four aspects: intrinsic effects, environmental effects, cell–cell interactions, and residual noise (17). In the near feature, an explosion of data is foreseeable as researchers increasingly focus on the spatial architecture of the TIME. More useful tools will be necessary for an in-depth analysis of these precious and complex data.

Aspects to describe the spatial architecture of the TIME
Several strategies are proposed to profile complex TIME information generated by the innovative tools reviewed above or other conventional techniques (Table 2). Different strategies can provide us with different silhouettes to comprehend the complexity of the TIME.

Distribution of immune cells based on tumor compartments
The most widely utilized mode to describe the distribution of immune cells is categorizing them according to the compartments in which they are located in the tumor tissue (Figs. 1a and 3a). The compartment in which immune cells reside potentially reflects their relationships with tumor cells, other immune cells and other various components within the TME (59).

Typically, tumor tissue compartments consist of three parts: the tumor core (TC, also referred to as the tumor nest or tumor cluster), which accommodates the majority of tumor cells in some bordered area; the tumor stoma (TS), where abundant stromal components are situated around TC; and the invasive margin (IM), which represents the transition zone of the TC and TS. These spatial compartments can be observed in a variety of solid tumors such as breast cancer, colorectal cancer, melanoma and oral-tongue cancer (60–64). Thus, the location of immune cells can be described based on the specific compartment. Several papers have previously reported that the distribution of immune cells in these three regions is not significantly different (65, 66), while others found differences in the distribution and functional status of immune cells among these different locations (67, 68). For example, immune cells within TCs are universally considered to have the tightest interaction with tumor cells due to their close juxtaposition to tumor cells (69). The IM is thought to be the front line of the battle between the tumor and immune system, and unsurprisingly, the density and function of effective immune cells at this site are higher than those in either the TC or TS (70, 71). Immune cells in the TS are involved in stromal remodeling and angiogenesis, which may exert profound effects on tumor growth and invasion (72).

Emerging imaging and analyses have further revealed intrinsic differences among specific types of immune cells within these three regions. For instance, TNBC is a highly aggressive and heterogeneous subtype of breast cancer. An analysis of spatial-based microdissection gene expression data in TNBC revealed the differences...
A. Compartment based disturbance

| Exclusion | Aggregation | Tumor | Peritumor |
|-----------|-------------|-------|-----------|
| Treg      | Lymphocyte  | Activated T cell | Active anti-tumor immune response |
| M2 macrophage | Apoptosis tumor cells | Dendritic cell | Immunee cell from peripheral blood |
| Minimum apoptosis tumor cells | Various lymphocytes | Plasma cell secreting antibody | Macrophage engulfing antigen |
| Median apoptosis tumor cells | CD8+ T cell | M1 macrophage | Memory T cell |
| Various apoptosis tumor cells | T cell and tumor cell interaction | M0 macrophage | Memory B cell |
| Various apoptosis tumor cells | Epithelial-mesenchymal transition cell | Tumor cell invaded blood vessel | Blood vessel |
| Diffused infiltration | Epithelial-mesenchymal transition cell | Plasma cell secreting antibody | Lympathic vessels |

Tumor core | Invasive margin | Tumor stroma

Cellular components

- Lymphocyte
- T cell
- CD4+ T cell
- CD8+ T cell
- Treg cell
- B cell
- Tumor cell
- Plasma cell
- Dendritic cell
- Neutrophil
- Nature killer cell
- MDSC

Dying tumor cell
- Macrophage
- Pericyte
- Fibroblast

B. Patterned structure

**Fig. 3** Representative spatial architecture of immune cells in the tumor microenvironment. **a** Primary tumors are divided into the tumor core, tumor stroma, and invasion margin based on tumor compartments. **b** Special immune structures, such as perivascular niches and tertiary lymphoid structures (TLSs), are also involved in the construction of architectures. Moreover, computational technology identified cellular neighborhoods (CNs) as regions with a characteristic local stoichiometry of cellular components. CXCL4, C-X-C chemokine ligand type 4; CCL2, C-C chemokine ligand type 2; CX3CL1, C-X3-C motif ligand 1; TGF-β, transforming growth factor-β; IFN, interferon; IL-2, interleukin 2; ADCC, antibody-dependent cellular cytotoxicity; MDSC, myeloid-derived suppressor cell

in tumor-infiltrating CD8+ T cells with respect to functional markers, interferon signatures, and immune inhibitory molecule cells among these three regions (59). Furthermore, T cells located in different compartments possess a heterogeneous T cell receptor repertoire (73) and exclusion gene expression (74, 75).

Although the specific definitions of the TC, IM and TS are slightly different from one study to another, describing the spatial architecture of TIME according
to compartments has been widely acknowledged for its practicability, clarity and intuitiveness. And notably, there also exists heterogeneity in the spatial distribution of immune cells within these three regions, probably due to different tissue origin of tumor and high mobility of immune cells.

**Distance between immune cells and other cells in the TME**

If we take all immune cells as a universal system (and this is exactly how they function in fact), describing immune cells according to the compartment they located in will actually carve up this kind of totality. Contrarily, measuring the distance between immune cells and other cells can provide a much more meticulous, precise and direct view to comprehend the spatial architecture of immune cells (Figs. 1b and 3a).

The distance between immune cells and tumor cells might directly reflect the lethality of immune cells toward tumors or, in contrast, the editing of immune cells by tumor cells (12, 76–79). The distance between different immune cells potentially reflects the ubiquitous interactions within immune cell populations and helps researchers to better understand all immune cells as a totality (80, 81). To date, the distance between immune cells and stromal cells is not well understood in this field. However, with more emphasis being attached to the stromal components of the TME, such as cancer-associated fibroblasts (CAFs) and tumor-associated adipocytes, a future trend might surge to investigate the effects of spatial relationships on the interactions between immune cells and stromal cells (82–84).

Nevertheless, the application of this parameter for describing the spatial architecture of immune cells also has its own deficiency, primarily because of its inconvenience and intricacy.

**Patterned structures of the TIME**

When analyzing the distribution of immune cells inside tumors, some patterned structures composed of well-organized immune cells show relative intratumor, intertumor, and interpersonal consistency. These structures have been recognized in multiple tumors and were found to be of great clinical value (Figs. 1d and 3b).

Tertiary lymphoid structures (TLSs) are ectopic hyperplasia lymphoid tissues located outside the immune organs (85) that are present in a fraction of specific types of tumors. TLSs are composed of a T cell-rich zone containing mature dendritic cells (DCs), a CD20+ B cell-rich follicle with follicular DCs, plasma cells, and antibodies. Immune cells in TLSs activate antitumor immune response through antibody-dependent cellular cytotoxicity and/or a direct cytotoxic function. Recently, three studies were published back-to-back, further illustrating the localization, spatial composition and function of TLSs, as well as their relationship with the immunotherapy response and survival, highlighting the significance of TLSs as a new area worthy of further exploration (86–88).

Another group of special organization patterns of immune cells is a series of niches that are engaged in multiple biological processes, among which the perivascular niche is best described. The spatially heterogeneous blood flow within tumors constructs perivascular niches that supply oxygen, nutrients and growth factors, as well as remove toxic metabolites (89). According to previous studies, multiple behaviors of tumor cells and immune cells are regulated by regulatory factors in the perivascular niche (90–95). During tumor development, chemokines and cytokines in the TME attract circulating immune cells from the blood. Thus, as the first station of immune infiltration, the density of immune cells in the perivascular area is relatively high (96). Notably, macrophages might play an important role in the TIME around blood vessels due to their bilateral tumor-promoting or tumor-suppressive functions in multiple cancers (96–99). To our knowledge, many gaps remain in our understanding of other perivascular immune cells, which require further documentation.

Other niches, such as the stem cell niche, premetastatic niche and metastatic niche, are also proposed as novel concepts in tumor development, where immune cells seem to play an essential role. Related research is relatively sparse but has been growing rapidly over the past few years (100, 101), which is discussed in detail later in this review.

In addition to the aforementioned structures with certain morphological and pathological characteristics, the development of computational image processing has facilitated the identification of specific structures of the TIME that are undetectable with the human eye, which are termed cellular neighborhoods (CNs) (10). The algorithm-defined CNs helped to reveal the spatial organization of the TIME in different patients, and the results showed that the local aggregation of PD-1+CD4+ T cells was associated with a better prognosis of high-risk patients. However, due to the black box of computational analysis, the recognizable biomarkers, biological characteristics, and functions of CNs in tumor immunity and therapeutic responses are unclear. Therefore, subsequent studies designed to reveal the biological nature and clinical application of CNs should be important.

**Spatial architecture of immune targets**

In clinical application, usually it is more practical to detect certain immune targets than immune cells to obtain spatial information about the TIME. Thus,
research in the spatial distribution of immune targets in the TIME has also attracted great attention (Fig. 1c).

Given the emerging prevalence of immunotherapies that modify antitumor immunity, a variety of immunomodulatory molecules, represented by immune checkpoints, are favorable immune targets (102). For example, antibodies targeting the T cell inhibitory checkpoint proteins programmed cell death 1 (PD-1), its ligand PD-L1, and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) can enhance antitumor immunity. These antibodies have achieved durable clinical efficacy in some patients with various tumor types and have been approved by the U.S. Food and Drug Administration. The spatial localization of these immune targets has been measured in various cancers, including breast cancer (19, 59), non-small cell lung cancer (NSCLC) (103, 104), melanoma (105), colon cancer (106), and craniopharyngioma (107). Given the cell-specific expression of immune targets and well-organized spatial architecture of cells in the TME, the spatial distribution of these immune targets also showed a certain pattern (Fig. 1c). Taking the PD-1/PD-L1 axis as an example, PD-1 is mainly expressed on the surface of CD8+ T cells, while its ligand PD-L1 is expressed on multiple cells, including tumor cells, B lymphocytes, and tumor-associated macrophages (TAMs) (108–110). The results showed that the overall spatial distribution of immune targets is highly correlated with cells, although the levels of these targets vary among the same type of cells in different spatial locations (107).

Moreover, since most immunoregulators depend on ligand-receptor binding, the distance between two targets is crucial for the immune response. Studies have assessed the spatial interaction based on the distance between immune target positive cells, the spatial proximity of targets, or their colocalization in one cell (103, 105, 111). Additionally, taking PD-1/PD-L1 as an example, the PD-1/PD-L1 interaction score, an indicator of the spatial ligand-receptor relationship, was evaluated in patients treated with anti-PD-1. Patients with high interaction scores were more likely to respond, although individual biomarkers were not associated with the response or survival (110). Other tumor-related pathways, such as Notch, interleukin 6 (IL-6)/JAK/STAT, Toll-like receptor, C-X-C chemokine ligand type 12 (CXCL12)/C-X-C chemokine receptor type 4 (CXCR4), Wnt-β-catenin, and transforming growth factor (TGF-β), are also activated by a distance-dependent interaction between immune cells and their environment (112–116). Extracellular paracrine and juxtacrine signaling molecules also act in this manner (117). Furthermore, the relationship between spatial proximity and the functional status of these signal pathways is awaited to be investigated.

**Spatial architecture of the TIME and tumor biology**

The spatial architecture of the TIME is closely related to tumor biology, which coordinates with the development of tumors and simultaneously, exerts effects on tumors. Here, we reviewed the current knowledge of the spatial architecture and function of the TIME in tumor initiation, expansion and metastasis, to discuss the potential mechanism of the evolution of TIME’s spatial architecture and its implications for clinical outcomes (Fig. 4).

**Construction of the TIME at tumor initiation**

The spatial distribution of immune components during tumor initiation has been described by some studies (Fig. 4a). Studies of the somatic mutation burden in morphologically normal precancerous tissues have revealed that “normal” cells accumulate a series of mutations prior to pathologically observable morphological changes (118, 119). Mutations within cells can be detected by the immune surveillance and initiate the immune response to eliminate “nonself” cells in the localized region where precancerous transformation occurs. In most cases, these cells are cleared by immune surveillance system. However, in immunosuppressive microenvironment where immune surveillance is diminished due to chronic inflammation or oncolytic virus, the infiltration of immune cells will decrease, and the cytokine interleukin-6 (IL-6) will activate the JAK/STAT3 signaling pathway in monocytes (120), which ultimately contributes to abnormal cells escaping immune elimination and proliferating into carcinoma in situ (CIS). The landscape of the TIME of CIS has been profiled, and the distribution...
and immune pattern of tumor-infiltrating immune cells are affected by the heterogeneity of tumor cells (121, 122). Therefore, the spatial distribution of immune components is involved in the transition of “normal” cells to tumor cells and CIS.

Knowledge about the spatial architecture of the TIME in the tumor initiation helps to reveal the evolutionary trajectory of premalignancy toward malignancy. However, conventional technologies have difficulties in detecting occult lesions, limiting the conduct of research (123, 124). It is believed that the availability of spatial information about the TIME expands the information dimension of precancer lesions, which can help researchers understand tumor initiation and guide clinical practice.

Spatial architecture of the TIME in tumor expansion and invasion

Developing tumors are primary targets for research in spatial architecture of the TIME because of their appropriate size, enrichment in immune components, and the bidirectional roles of spatial architecture of the TIME within these tissues (Fig. 4b). Tumor expansion is accompanied by physiological processes, such as tumor cell proliferation, angiogenesis and immune cell infiltration. Description of the immune cell infiltration patterns based on spatial information provides additional insights into the tumor invasion. In some samples, immune cells are distributed diffusely, while in others, they tend to aggregate to form CNs, which are considered able to enhance immune cell function. In most cases, tumor-infiltrating immune cells are tumor-killing cells, and thus CN formation can fortify antitumor immunity. Nevertheless, some other CNs may consist of immunosuppressive cells, which reversely attenuate antitumor immunity (10, 19). The formation and impacts of those known structures on antitumor immunity are underexplored, and will probably be the focus of subsequent study.

Invasion, which is necessary for tumors to metastasize, occurs on the border of the tumor and is regulated by the frontier of the TIME. One of the most crucial events in tumor invasion is the epithelial–mesenchymal transition (EMT) (125). Studies of head and neck tumors identified a fraction of cells that were spatially located close to CAFs and immune cells in the leading edge of the tumor, expressing a signature related to a partial EMT program. This study reflected the relationship between the active immune response at the invasion margin and the occurrence of metastasis (126).

Except for EMT and partial EMT cells, previous studies have reported a higher abundance of immune cells and a stronger antitumor immune effect of the IM than the TC at metastatic lesions of gastroesophageal adenocarcinomas (127), hepatocellular carcinoma (128), melanoma (12), and colorectal cancer (129–131). This phenomenon may be driven by the intense struggle in the ecological niche between marginal tumor cells and immune cells, which is similar to that at the invading edge during ecological species invasion. Based on this analogy, the Darwinian dynamics model in ecology has been used to model the spatiotemporal distribution and evolutionary shifts of immune components at the invasion edge of the TIME in silico (132, 133).

Spatial architecture of the TIME and metastases

After invasion, tumor cells traverse the stroma, enter the vascular system and colonize a secondary site, namely metastasis occurs. Current studies about the TIME’s spatial architecture in metastases mainly focus on specific spatial patterns (Fig. 4c).

As mentioned above, spatial patterns indicated the structure formed by well-organized TIME components. Representative spatial patterns in metastasis is the niche induced by exosomes and/or factors from the primary site (134). Before the seeding of circulating tumor cells (CTCs), the primary tumor-derived components facilitate the recruitment of immune cells into the niche, while immune cells subsequently, modify the local microenvironment of niches to form a feedback loop that ultimately promotes the formation of premetastatic niche (100, 135, 136). Specifically, premetastatic niches construct an immunosuppressive microenvironment composed of myeloid-derived suppressor cells (137), regulatory T cells (Tregs) (138), TAMs (139, 140), and tumor-associated neutrophils (141) to abolish local antitumor immunity that is more conducive to CTC colonization. The immunoregulatory components and their functions within niches have been thoroughly studied and recently reviewed (100, 142). Several studies have progressively revealed the spatial distribution of immune and host stromal components within niches (143–145).

After the seeding of CTCs, a premetastatic niche becomes a metastatic niche, which inherits the spatial architecture and functional status of the premetastatic niche, including emerging vessels, vascular leakiness and an immunosuppressive TIME. Moreover, immune components regulate spatiotemporal tumor cell stemness and plasticity (146, 147). Tumor cells also exert a spatially dependent effect on their surrounding cells (148). The formation and development of premetastatic niches and metastatic niches are still complex, and the evolution of these processes should be further explored.

Tumor cells in niches proliferate and ultimately grow into metastasis. Current investigations about the spatial heterogeneity of metastases tend to explore differences among primary sites and metastases in different sites, which expands the conception of spatial heterogeneity

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Tumor cells in niches proliferate and ultimately grow into metastasis. Current investigations about the spatial heterogeneity of metastases tend to explore differences among primary sites and metastases in different sites, which expands the conception of spatial heterogeneity
to a more macro level with greater clinical value. In addition to heterogeneity, studies have identified some consistencies between the spatial arrangement of immune cells in the TIME of metastatic and primary foci (149). For instance, in ovarian cancer, researchers found that subpopulations of T cells in primary tumors or different metastatic sites are relatively consistent, indicating their ability to track disseminated tumor cells through space (56, 106, 150–152). Meanwhile, no difference was observed in the distance between CD8+ T cells and tumor cells in primary and metastatic melanoma foci (12). The heterogeneity and consistency discussed above highlight the complexity of TIME’s spatial architecture, and more researches are required for clear elucidation.

Spatially-based extracellular nonspecific chemical (ENSC) gradients orchestrate immune cell function

In addition to “tangible” structures in the TME, “intangible” extracellular nonspecific chemicals (ENSCs) are also critical components of the TME. In contrast to immune molecules, such as diverse interleukins (ILs), ENSCs refer to extracellular molecules participating in the metabolism of almost all cells and exerting extensive effects on them, including immune cells in the TIME. ENSCs manifest spatial heterogeneity in the form of “gradient”. And though ENSCs are not components of TIME, their unignorable role in the regulation of the spatial architecture of TIME emphasizes their importance in the research of TIME. In this section, we will discuss the current knowledge of the distribution of ENSCs in the TME and briefly review the effects of the ENSC distribution on immune cells.

Basic principle of the distribution of ENSCs

Some basic physical and biological principles can help us to understand the distribution of ENSCs. ENSCs include a variety of chemicals, whose distribution is complex and interdependent. Depicting a general map of all ENSCs in the TIME is infeasible and unnecessary because they vary among different species, tissue origins, patients, lesion locations, stages, and systemic conditions (28, 52). Nevertheless, the distribution of ENSCs is also spatially regulated due to some basic principles it must obey, which can help us grasp the outline of its landscape.

These basic principles are described below, and most of them are self-evident but essential.

1. The appearance and disappearance of ENSCs are in partial hemostasis and depend on two mechanisms, blood circulation and in situ metabolism, which are tightly interlaced.

2. Powered by the concentration gradient, ENSCs passively diffuse from areas of high density toward those of low density.

3. Competition exists between tumor cells and immune cells for most ENSCs, and tumor cells typically have an advantage (153, 154).

4. The distribution of ENSCs observed at a specific time point is a cross section of consecutive biological processes.

Oxygen is pivotal for ENSCs

Oxygen is capable of being the hallmark molecule among all ENSCs. Various types of ENSCs are present in the TME, i.e., oxygen, glucose, carbon dioxide, lactate, amino acids, metal ions, lipids, etc. Considering the high heterogeneity and intricacy of ENSCs in the TME, a judicious approach is to select some representative ENSCs to better understand their distribution and biological effects. As far as we are concerned, oxygen should be critical for several reasons.

First, cellular respiration is the basis of cell metabolism and survival, in which oxygen is irreplaceable in the long term. The transportation and utilization of oxygen among most types of tumors are highly homogeneous, which dramatically facilitates associated studies. Meanwhile, the oxygen gradient serves as an ideal marker for the distance from vessels, and is closely related to other ENSCs by metabolism directly or indirectly. In addition, alterations in oxygenation and its subsequent outcomes are deeply incorporated into the biological transformation of tumors, such as angiogenesis, progression, and necrosis. Taken together, we choose oxygen as a representative ENSC when studying the spatial architecture of the TIME.

The oxygen distribution in normal tissue is regulated by an exquisite mechanism to form balanced dynamic homeostasis, but processes occurring in tumors are aberrant (155–157). The rapid proliferation of tumor cells exceeds the oxygen and nutrient supply and subsequently leads to angiogenesis. Neovascularization, characterized by an abnormal vessel wall structure with unbalanced distribution and immature function, causes an insufficient and unbalanced oxygen supply (158–160). These factors all contribute to the abnormal distribution of oxygen, featuring a consecutive normoxia-hypoxia-anoxia gradient from feeding vessels toward the tumor center, which has been certified in vivo, in vitro and in silico (159, 161–165) (Fig. 5).

The gradient of oxygen in the TME exerts comprehensive effects on the biological performance of diverse immune cells, including infiltration, migration, polarization, function, and metabolism (166). Here, we use
macrophages, one of the most well-studied immune cells in this field, as an example.

Macrophages are innate immune cells residing in all tissues derived from monocytes in blood. M1 macrophages, a subset of macrophages displaying an antitumor phenotype, are more commonly located in normoxic areas close to feeding vessels, while M2 macrophages, the protumor subset, are more dominant in hypoxic TCs (167). Although in vitro experiments have correlated the polarization of macrophages with hypoxia inducible factor (HIF)-1 and HIF-2 (168), in vivo research revealed that hypoxia does not directly affect the activation of macrophages but exerts its effect through hypoxic tumor cells (167, 169, 170). The migration of macrophages from the TS toward the TC is mediated by the interaction between receptors on macrophages and chemotactic molecules released by tumor cells. In the TC, which lacks oxygen, dying tumor cells release damage-associated molecular patterns (DAMPs) and adenosine triphosphate (ATP). Hypoxic tumor cells around dying tumor cells in the TC can also produce various kinds of cytokines regulated by HIF, such as C–C chemokine ligand type 5 (CCL5), CXCL12, vascular endothelial growth factor (VEGF), endothelin (ET)-1, ET-2 and semaphorin-3A (Sema3A). All these molecules bind to receptors primarily regulated by HIF on macrophages, which promotes the migration of macrophages toward the TC (166). Then, polarization toward M2 macrophages occurs with the intervention of lactate, IL-4, transforming TGF-β, oncostatin, eotaxin, and other molecules secreted by hypoxic tumor cells. In summary, the function of macrophages is closely related to the intratumor spatial heterogeneity of oxygenation.

Fig. 5 Oxygen serves as a pivotal extracellular nonspecific chemical (ENSC), and its gradient orchestrates the tumor immune microenvironment (TIME). The aberrant structure of tumor vessels and the abnormal distribution of oxygen in tumors feature a consecutive normoxia-hypoxia-anoxia gradient from feeding vessels toward the tumor center. Chemotactic factors such as CXCL12, CCL5, ET-1, ET-2, VEGF-A and Sema3A released by hypoxic tumor cells, as well as damage-associated molecular patterns (DAMPs) and ATP released by dead/dying tumor cells, attract macrophages to infiltrate into the hypoxic tumor core. Cytokines such as oncostatin, IL-6, IL-10, TGF-β, and HMBG-1 and lactate produced by hypoxic tumor cells further promote the differentiation of macrophages into protumor M2 macrophages, while macrophages remaining next to normoxic feeding vessels display an antitumor phenotype. The oxygen gradient may serve as a marker of the distance from feeding vessels and correlates with other ENSC gradients in the TIME, such as glucose, lactate and hydrion. CXCL12, C-X-C chemokine ligand type 12; CCL5, C–C chemokine ligand type 5; ET, endothelin; VEGF, vascular endothelial growth factor; IL, interleukin; TC, tumor cell; TGF, transforming growth factor; HMBG-1, high mobility group box 1 protein; Sema3A, semaphorin-3A.
The functions of other immune cells are also correlated with the oxygen gradient, such as the migration of neutrophils, natural killer (NK) cells, and Tregs toward hypoxic regions and the stagnancy of CD4+ T cells and CD8+ T cells in normoxic areas. The corresponding retention or alteration of their functions has been elaborated previously (166, 171–173). Nevertheless, the relations of various immune cells with the oxygenation spatial gradient still need further exploration.

The distribution of other ENSCs in the TME and their effect on immune cells
The distributions of other ENSCs (or their uptake) have also been partially revealed in vivo or in vitro to varying degrees, such as glutamine, amino acids, and metal ions (165, 174–177). The distributions of some of these molecules are correlated with the function of immune cells. For instance, the altered gradient of Na+ is correlated with the polarization of macrophages, probably in a tissue-specific manner (176), and increased K+ levels in tumors can suppress effector T cell function and prevent immune cells from maturing (177). However, researchers still do not comprehensively understand the significance of their effects on immune cells in a complex real TME, which must be further established.

Recently, tumor metabolomics has attracted increasing interest from researchers. Using high-flux tools to reveal the metabolism of tissue and cells, a series of metabolites were found to play an important role in the interaction between tumor cells and immune cells (178, 179). With further investigations of their spatial distribution, additional mechanisms and potential therapeutic targets might be proposed.

Spatial architecture of the TIME in clinical application
The clinical application of spatial architecture of the TIME can be roughly divided into two aspects, prognosis prediction and clinical treatment.

Spatial architecture of the TIME in prognosis prediction
Many prognostic implications of compartment-based features have been described. The Immunoscore is a simplified tool based on the immune context that is used to evaluate the abundance of T cell populations in the TC or IM jointly and shows great clinical potential in colorectal cancer (180–183). Apart from the Immunoscore, other researchers also examined the distribution of some specific subtypes of immune cells in different compartments within the TME. The specific distributions of CD8+ T cells and CD163+ macrophages in breast cancer (184), cytotoxic T cells (CTLs) and memory T cells in colorectal cancer (185), CD8+ T cells and Tregs in NSCLC (186), and CTLs in primary melanoma (81) residing in different compartments were found to be significantly related to clinical outcomes, improving our knowledge of a precise diagnosis.

Diverse parameters focusing on the distances of different cell–cell pairs are also well established. In breast cancer, patients with a mixed distribution of tumor and immune cells experienced prolonged survival compared with those whose tumor cells and immune cells were segregated from each other, even if the latter may have more abundant immune cells (19). Quantitative research found that enriched CD8+ cells within distant stroma (farther than one tumor cell diameter away from the TC) rather than adjacent stroma (within one tumor cell diameter) were related to prolonged disease-specific survival in patients with breast carcinoma (187). Mezheyueski et al. (188) calculated the shortest distance from each immune cell to the nearest neighboring cancer cell and discovered that the spatial proximity of Tregs and tumor cells was correlated with a significantly unfavorable survival in patients with NSCLC. Nearcho et al. (79) analyzed the number of CD8+ T cells within 100 μm radii from tumor buds and found that their totality and distance were significantly associated with disease-specific survival. Enfield et al. (189) discovered that the frequencies of CD3+CD8+ T cells around tumor cells were a more powerful marker to predict low recurrence than their density in lung adenocarcinoma. For spatial architecture of the TIME based on immune targets, researches are relatively limited. Lazarus et al. revealed that CTLs were more abundant around PD-L1+ epithelial cells than PD-L1+ epithelial cells, and the engagement between CTLs and epithelial cells was correlated with favorable overall survival (106).

As for patterned structures of the TIME, clinical data showed that the existence of TLSs is significantly related to improved overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) in patients with early stage NSCLC (190). Further studies found that TLSs are associated with increased lymphocyte infiltration into tumors with extended DFS in breast cancer and colorectal cancer (191). However, the role of TLSs in predicting the prognosis is still under debate because another study of breast cancer showed that patients with abundant TLSs exhibited worse DFS and OS than those lacking TLSs (192). The cellular component in TLSs has also aroused the interest of scientists. One study found that TLSs in patients with spontaneous prostate tumor regression contain fewer Tregs and more T-helper 1 (Th1) and CD8+ T cells, which are commonly considered antitumor components (193). The detection of premetastatic
Spatial architecture of the TIME in clinical treatment
To remodeling the spatial architecture of the TIME, diverse therapeutic strategies have also been proposed, including those that promote (116, 198) or prevent (199) immune cells from infiltrating into designated areas of the TIME, modify angiogenesis or oxygenation of the TIME (200–202), induce the formation of TLSs (85, 86) or modulate members of TLSs (87), regulate stromal components in the TIME (203, 204), and transform ENSCs in the TIME (178, 205). Regretfully, there are few researchers focusing on the value of the spatial architecture of the TIME in treatment response prediction.

Conclusions and perspectives
In this article, we provide a systematic summary of technological advances and related researches on the spatial architecture of the TIME. Recently, an explosion of researches witnessed profound advances in high-dimensional techniques that preserve the spatial information, which has revolutionized our understanding about the spatial architecture of the TIME.

Despite the recent rapid development, there are still many unresolved issues in the study of TIME’s spatial architecture. First of all, spatial proximity between cells does not necessarily mean that actual interactions are occurring. Current analyses assume that cells in close proximity have a higher probability of potential interaction, but whether an interaction exists and its extent must be further verified. The in vitro study of cell-to-cell interactions is feasibly facilitated by cell culture techniques, such as coculture and organoids (206). However, these models do not fully reflect the situation in vivo. The mCherry niche labeling strategy devised by Ombrato et al. showed the in vivo intercellular material flow of tumor cells and their neighborhood in metastatic niches through the secretion and uptake of marker proteins. However, due to the disparity in the phagocytic capacity of different neighboring immune cells themselves, the simple secretion-uptake model does not fully reflect interactions within the TIME (148). Therefore, future in-depth studies of in situ spatial interactions and space-dependent functions will depend on the further development of in vivo and in vitro models. Moreover, only limited studies have revealed mechanisms underlying the formation, development and regulation of spatial architecture of the TIME, partially due to the lack of suitable experimental models.

Conventionally, in vitro experiments are widely favored because of their controllability and ease of use. Experimental techniques, such as extracellular matrix culturing cancer cells in biomimetic scaffolds (207), reproduce the spatial distribution of tumor cells in artifically constructed meshwork in vitro to some extent. However, currently, immune cells cannot be cultured in scaffolds due to the difficulty in culturing immune cells and their short survival time in vitro. Organoids and spheroids are popular platforms adopted in the investigation of tumors recently, which can partially preserve the spatial information of original malignancies. Typically, immune cells are not included in organoids or spheroids. Novel strategies such as the air–liquid interface have been proposed to introduce immune cells into these structures, and their clinical potential has been approved (208–210). The technological modification for the culturing and editing of organoids and spheroids might bring us to a new era of the investigation of the TIME. Recent studies about the TIME rely more on transplanted tumors as well as patient-derived xenograft. Although in vivo models can relatively better reflect the real situation, the uncontrollability of animals increases the technical difficulty and confounding of the experiment (211). We hope that in the future, the complex structural networks of the TIME will be constructed in robust experimental models.

In addition, the majority of existing studies on spatial architecture of the TIME are descriptive, with most samples obtained from retrospective cross-sectional studies. We suggest analyzing the spatial architecture of the TIME in terms of temporal dynamics. For example, with superresolution intravital fluorescence microscopy, a prospective study can be carried out to investigate T cell behavior within specimens collected before and after immunotherapy to identify spatial structural changes (212). The description and tracking of the spatiotemporal heterogeneity and evolution of the TIME will provide additional insights into tumor diagnosis and treatment.

In summary, we consider the spatial architecture of the TIME a promising direction for academic research and clinical application in this field (Fig. 6). As an emerging field, the spatial architecture of the TIME has a large
number of unknowns waiting to be explored. With the foreseeable demand for a more comprehensive landscape of the spatial architecture of the TIME, the near future will probably witness newer technologies with better precision, faster scanning, more convenient protocols, lower cost, and public online archives. Improved technologies facilitating sharing and robust softwares or algorithms to query massive databases will also increase accessibility for other researchers in this field.

Abbreviations

CAFs: Cancer-associated fibroblasts; CIS: Carcinoma in situ; CNN: Convolutional neural network; CNs: Cellular neighborhoods; CTCs: Circulating tumor cells; CTLs: Cytotoxic T cells; DFS: Disease-free survival; EMT: Epithelial–mesenchymal transition; ENSCs: Extracellular nonspecific chemicals; FFPE: Formalin-fixed and paraffin-embedded; FISH: Fluorescence in situ hybridization; H&E staining: Hematoxylin and eosin staining; HIF: Hypoxia inducible factor; IDO: Indoleamine2, 3-dioxygenase; IHC: Immunohistochemistry; IM: Invasive margin; NSCLC: Non-small cell lung cancer; OS: Overall survival; PD-1: Programmed cell death 1; scRNA-seq: Single-cell RNA sequencing; TAMs: Tumor-associated macrophages; TC: Tumor core; TIME: Tumor immune microenvironment; TLSs: Tertiary lymphoid structures; Tregs: Regulatory T cells; TS: Tumor stoma.

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Authors’ contributions

Z.-M.S., Y.-Z.J., D.M., and T.F. designed and finalized the study; T.F. and L.-J.D. wrote the paper; S.-Y.W. and Y.X. participated in revising the review. All the authors approved the final version submitted.

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Non-small cell lung cancer; OS: Overall survival; PD-1: Programmed cell death 1; scRNA-seq: Single-cell RNA sequencing; TAMs: Tumor-associated macrophages; TC: Tumor core; TIME: Tumor immune microenvironment; TLSs: Tertiary lymphoid structures; Tregs: Regulatory T cells; TS: Tumor stoma.

Fig. 6 Future development of the tumor immune microenvironment. The development of technologies in high-dimensional in situ imaging, analytical algorithms and in vitro/vivo models will promote the further elucidation of mechanisms underlying the tumor immune microenvironment (TIME) (boxes with cloud marks refer to content that future online databases of TIME might include). Profound advances in the clinical application of TIME rely on deeper insights into the TIME, which will help physicians with determining both a precise diagnosis and therapy...
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