Molecular Cancer

REVIEW

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Spatiotemporal metabolomic approaches to the cancer-immunity panorama: a methodological perspective

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Abstract

Metabolic reprogramming drives the development of an immunosuppressive tumor microenvironment (TME) through various pathways, contributing to cancer progression and reducing the effectiveness of anticancer immunotherapy. However, our understanding of the metabolic landscape within the tumor-immune context has been limited by conventional metabolic measurements, which have not provided comprehensive insights into the spatiotemporal heterogeneity of metabolism within TME. The emergence of single-cell, spatial, and in vivo metabolomic technologies has now enabled detailed and unbiased analysis, revealing unprecedented spatiotemporal heterogeneity that is particularly valuable in the field of cancer immunology. This review summarizes the methodologies of metabolomics and metabolic regulomics that can be applied to the study of cancer-immunity across single-cell, spatial, and in vivo dimensions, and systematically assesses their benefits and limitations.

Keywords Cancer-immunity, Immunometabolism, Metabolomics, Metabolic regulomics, Single cell, Spatiotemporal omics

Background

The immune system interacts with cancer cells at multiple levels [1, 2]. The body's immune responses can eliminate cancer cells, preventing the development, spread, metastasis, and recurrence of cancer [3, 4]. On the other hand, cancer cells can influence the immune system in ways that create an immunosuppressive tumor microenvironment (TME) and disrupt systemic immune balance, leading to cancer progression and resistance to treatments [1]. Current cancer immunotherapies aim to disrupt this interaction, enhancing anti-tumor immune responses and alleviating immunosuppressive TME, offering new hope for successful treatment [5]. While there have been notable successes in treating melanoma and leukemia, many types of tumors, especially solid tumors, still do not respond well to immunotherapies and can cause severe side effects [6, 7]. Therefore, the development of more effective and safer anticancer immunotherapies requires a deeper understanding of the complex interactions between cancer and the immune system.

Recent studies have highlighted metabolic reprogramming as a key factor in cancer-immune interactions at various levels [8–11]. In the TME, a unique metabolic niche is created due to scarce nutrients and the build-up of harmful metabolites [8, 9]. This environment forces both cancerous and non-cancerous cells to compete for restricted nutrients, leading to adaptations in their bioenergetics. Within the TME, cancer cells demonstrate robust metabolic adaptability, which refers to their

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capacity to optimize the utilization and processing of metabolites in response to fluctuating nutrient conditions [12]. Consequently, these cancer cells outcompete anti-tumor immune cells by depriving them of vital resources [13]. Moreover, metabolites produced by cancer cells and their educated counterparts, along with those present in the TME, act as signaling molecules that can disrupt immune cell homeostasis, impair their anti-tumor functions, or even push them towards a pro-tumor phenotype [14–16]. At a systemic level, tumors can induce cachexia by depriving the host of nutrients and can also impact the neuroendocrine system and microbiome, thus reshaping the hematopoietic-immune system [11, 17, 18]. Given the crucial role of metabolism in these processes, investigating the metabolomic landscape of cancer-immunity is an increasingly attractive area of study.

While conventional techniques like extracellular flux analysis (EFA), isotopic tracing, and bulk metabolomics have laid the foundation for metabolic characterization in cancer-immunity, they struggle to fully capture the intricate and spatiotemporal heterogeneity of metabolism [19]. For example, Otto Warburg first discovered that cancer cells preferentially utilize glycolysis over oxidative phosphorylation (OXPHOS) for energy metabolism, even in the presence of oxygen, a phenomenon known as aerobic glycolysis or the Warburg effect [20]. In the decades that followed, this effect was initially thought to be a distinctive characteristic of cancer cells resulting from mitochondrial dysfunction [21]. However, as research into the TME advanced, it became evident that aerobic glycolysis is not uniformly prevalent. Instead, it is heterogenous across cancer cells, and also observed in various tumor-infiltrating (TI) immune cells, such as tumor-associated fibroblasts, TI macrophages, and $CD8^+$ TI lymphocytes (TILs), which exhibit a higher glucose uptake than cancer cells [22–25]. The emergence of single-cell and spatially resolved metabolomics, along with other advanced omics technologies, has revealed previously unknown heterogeneity in the TME, providing researchers with valuable insights into the metabolic principles of cancer-immunity. This review explores how these emerging metabolomic technologies enhance our understanding of cancer-immunity from a methodological perspective, emphasizing their role in characterizing the dynamic and heterogeneous nature of cancer-immunity.

Overview of metabolism in cancer-immunity

To provide a foundational understanding of the metabolic interactions in cancer-immunity, the following offers a minimum overview of cancer immunometabolism, which draws primarily from bulk and in vitro experiments. It is important to acknowledge that a more thorough comprehension of this field can be obtained by referring to additional detailed reviews within the field [8, 9, 26].

Dampened signal 4 blocks the cancer-immunity cycle

$CD8^+$ T cells play a pivotal role in anti-tumor immunity by engaging in the iterative cancer-immunity cycle, which recruits and trains tumor-specific $CD8^+$ T cells [4, 27]. Through multiple cycles, $CD8^+$ T cells continuously recognize antigens and become activated both within (tertiary lymphoid structures, TLSs) and outside the tumor (draining lymph nodes). They adapt to tumor progression and maintain an effective immune response [4]. Activation of $CD8^+$ T cells, essential for anti-tumor immune response, is explained by a recently developed four-signal model [27]. Naive $CD8^+$ T cells require optimal levels of the three classic signals (Signal 1: MHC molecules with antigenic peptides; Signal 2: co-stimulatory molecules; Signal 3: cytokines) and nutrients (Signal 4) to differentiate into high-quality effector and memory $CD8^+$ T cells [27]. Signal 4, dependent on the TME metabolism, is crucial for the bioenergetics of $CD8^+$ T cells, influencing their gene expression and signal transduction as signaling molecules [28].

In many clinical scenarios, the TME often does not provide sufficient glucose and amino acids for the activation of $CD8^+$ T cells [13]. On the other hand, an accumulation of lipids within the TME results in the upregulation of CD36, a critical receptor for lipid uptake, in intratumoral $CD8^+$T cells. This process subsequently induces ferroptosis and dysfunction in these T cells [29, 30]. Furthermore, within the TME and tumor organismal environment (TOE), cancer cells undergo metabolic changes that interact with the host immune system [11]. This communication hinders the anti-tumor immune response, disrupting the effective cancer-immunity cycle and potentially leading to a standstill in cancer-immune interactions or disease progression.

Nutrient competition in the TME

Primarily influenced by vascular system disorders and the robust nutritional demands of cancer cells, the typical TME of solid tumors is a distinct ecological niche characterized by low nutrition, hypoxia, and the accumulation of toxic metabolites (Fig. 1a) [9]. Within this challenging environment, different cell types are forced to compete for nutrients, resulting in changes in the distribution of key metabolites (Fig. 1b) [13].

Both tumor cells and anti-tumor immune cells in the TME rely on adequate glucose intake. Key anti-tumor immune cells, such as conventional T cells (Tconvs) [31, 32], natural killer cells (NKs) [33], M1 macrophages [34],

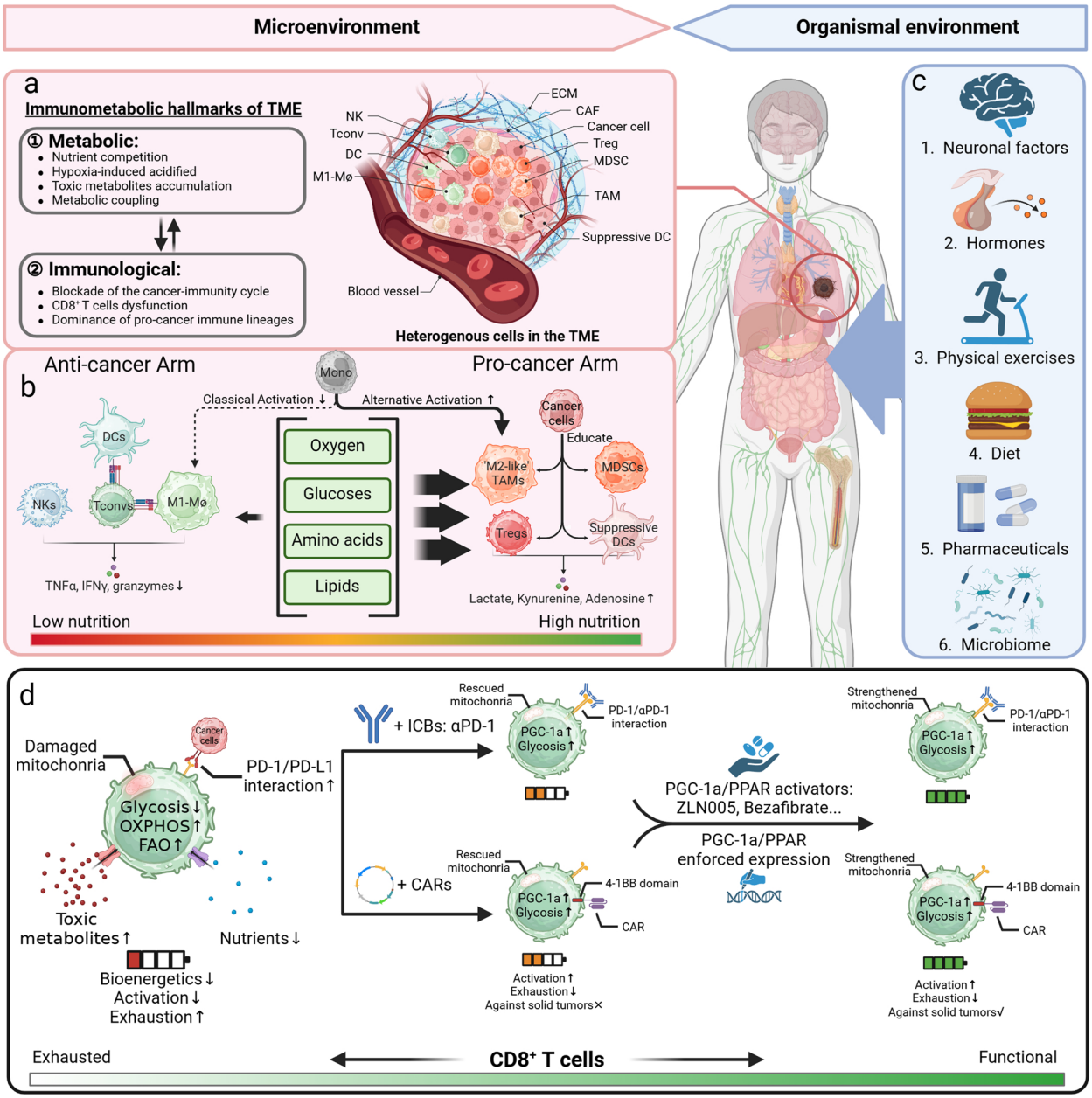

Fig. 1 Overview of the immunometabolic landscape in cancer across multiscales. **a** Schematic depicting the typical immunosuppressive TME of solid tumors: immunometabolic hallmarks (left) and heterogenous immune components (right) within the TME. **b** Nutrient competition between anti-cancer arm (left) and pro-cancer arm (right) in the TME. Core resources, including oxygen, glucoses, amino acids, and lipids, are priorly occupied by pro-cancer immune cells. **c** Factors from TOE regulate immunometabolism of cancer, including neuronal factors, hormones, physical exercises, diets, pharmaceuticals, and microbiomes. **d** Immunometabolic interventions rescue exhausted $CD8^+$ TILs. Prior to these interventions, $CD8^+$ TILs face limitations due to poor nutrition, toxic metabolites build-up, mitochondrial dysfunction, and excessive co-inhibition (left). Treatment with ICBs or 'equipped' with CARs can partially alleviate $CD8^+$ TILs exhaustion and rescue their metabolic phenotypes, but they may not completely eliminate solid tumors (middle). The activation of PGC-1α/PPAR through pharmacological or genetic means can enhance the strength of $CD8^+$ TILs/CAR-T cells, empowering them to effectively combat solid tumors (right). CAF, cancer-associated fibroblast; CAR, chimeric antigen receptor; DC, dendritic cell; ECM, extracellular matrix; FAO, fatty acid oxidation; ICB, immune checkpoint blocker; IFNγ, Interferon-gamma; MΦ, macrophage; MDSC, myeloid-derived suppressor cell; Mono, monocyte; NK, natural killer cell; OXPHOS, oxidative phosphorylation; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PGC1-α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; TAM, tumor-associated macrophage; Tconv, conventional T cell; TIL, tumor-infiltrating lymphocytes; TME, tumor microenvironment; TNFα, tumor necrosis factor-alpha; TOE, tumor organismal environment; Treg, regulatory T cell; αPD-1, anti-programmed death 1

and dendritic cells (DCs) [35], typically undergo aerobic glycolysis during their proliferation or effector phases. However, tumor cells often outcompete anti-tumor immune cells for nutrients, leading to immune dysfunction [9]. Amino acids, particularly glutamine essential for the tricarboxylic acid (TCA) cycle, are another critical resource in the TME [36]. Most cancer cells exhibit 'glutamine addiction', depleting this resource heavily [37]. The effects of amino acid deprivation extend beyond the TCA cycle. For example, glutamine serves as a principal substrate for the synthesis of glutathione (GSH), a crucial endogenous antioxidant. Increased levels of GSH bolster the ability of tumor cells to withstand oxidative stress, thereby imparting resistance to both radiotherapy and chemotherapy [38]. Besides, tumor-educated immune cells, like tumor-associated macrophages (TAMs) [39], myeloid-derived suppressor cells (MDSCs) [40], and suppressive DCs [41], inhibit effector T cell responses by upregulating indoleamine 2,3-dioxygenases (IDOs), which increases local tryptophan consumption.

The TME offers a lipid-rich milieu that supports immune cells exhibiting metabolic flexibility, such as regulatory T cells (Tregs) and TAMs, which preferentially utilize fatty acid oxidation (FAO) for their metabolic needs [9, 42, 43]. Conversely, specific lipids, such as ketone bodies and oleic acid, may serve as optimal energy sources during the effector phase of anti-tumor immune responses [44, 45]. Additionally, abnormal metabolic conditions in the TME, like hypoxia [46] and accumulation of lactate [47], adenosine [48], and kynurenine [49] are associated with impaired anti-tumor immune responses. Collectively, these factors allow tumors to establish a diverse immunosuppressive TME through metabolic reprogramming, hindering the cancer-immunity cycle at different stages.

Immunometabolic regulations in the TOE

Tumors are now understood to be systemic disorders that involve immune and metabolic dysregulation [11]. Various factors from TOE, including the neuro-endocrine network, physical exercise, metabolites derived from diet or drugs, and microbiome, can disrupt the balance of immune and metabolic functions (Fig. 1c) [11, 50].

The neuro-immune-metabolic network, crucial for maintaining internal homeostasis, involves intricate interactions at multiple levels [51]. Various immune cells express receptors for neurotransmitters on their surface, facilitating communication between neural and immune systems. For example, macrophages express olfactory receptors, and T cells express catecholamine receptors [52, 53]. Yang et al. [54] demonstrated that stress-induced neuroendocrine responses can suppress the immune system both locally in the TME and systemically, reducing the effectiveness of immunotherapy. Endocrine factors like hypothalamic-pituitary hormones [55], fibroblast growth factor 21 (FGF21) [56], glucagon-like peptide 1 (GLP-1) [57], and sex hormones [58] also play roles in immune regulation. In addition, physical exercise, which is well-established to be effective for reprogramming systemic immunometabolism, has been demonstrated to increase abundance of the anti-tumor immune cells in the TME [59]. Furthermore, environmental factors, such as specific dietary metabolites, have been linked to alternations of the immune system [60]. Recent research has revealed that dietary trans-fatty acids like elaidic acid [61] and trans-vaccenic acid [62], previously considered harmful to the heart, unexpectedly enhance the function of $CD8^+$ T cells and improve anti-tumor immune responses. This novel mechanism suggests a potential nutritional strategy for advancing future immunotherapies.

Furthermore, the composition of the host microbiota significantly influences cancer immune responses [63]. For example, *C. cateniformis* in the gut can enhance cancer immunotherapy by downregulating the expression of programmed cell death ligand-2 (PD-L2) on DCs and guidance molecule b (RGMb) on T cells, thereby inhibiting their interactions [64]. Recent studies have highlighted the differences between intratumoral and extratumoral microbiota, emphasizing their distinct compositions, distributions, and impacts on tumor development [65]. For example, the intratumoral microbiome in lung cancer tissues promotes M2 macrophage polarizations through the production of butyrate [66].

Immunometabolism affects anti-cancer therapies

In addition to its significant impacts on tumor-host interactions, immunometabolism affects the effectiveness of various anti-tumor therapies. First, recent studies have underscored the relationship between the host's immunometabolic state and the success of immunotherapy, particularly immune checkpoint inhibition (ICI), such as anti-programmed cell death protein 1 (PD-1)/ programmed cell death ligand 1 (PD-L1), as well as adoptive cell transfer (ACT) therapy, including chimeric antigen receptor (CAR)-T and CAR-NK cell therapies [67, 68]. Intense PD-1/PD-L1 interactions in the TME lead to metabolic changes that result in energy deficiencies in TILs by inhibiting their peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α function [69]. Blocking PD-1 can reprogram T cell metabolism and restore aerobic glycolysis, which is beneficial for anti-tumor responses [13, 70]. On the other hand, CAR-T therapy has shown limited effectiveness in solid tumors due to its reduced ability to compete for glucose [71]. Activating the PGC-1α/

peroxisome proliferator-activated receptor (PPAR) complex in $CD8^+$ T or CAR-T cells, either pharmacologically or genetically, to enhance their mitochondrial function has shown promise in maintaining $CD8^+$ T cell effector functions and preventing rapid exhaustion in preclinical studies (Fig. 1d) [72, 73].

Additionally, the interaction between immunotherapy response and systemic factors should not be overlooked. Obesity, a harmful metabolic condition, has been found to impair the function of anti-tumor immune cells, especially $CD8^+$ T cells, in many cancers [74]. However, the 'obesity-immunotherapy paradox' is evident in certain cancers like melanoma, where a higher body mass index (BMI) is associated with better clinical response and overall survival with ICI treatment [75]. This paradoxical finding underscores the complex relationship between immune responses and metabolism at a systemic level.

Immunometabolism also plays a significant role in the effectiveness of conventional anti-tumor therapies. While radiotherapy induces extensive immunogenic death of tumor cells, it often fails to elicit a sustained anti-tumor immune response. This limitation is likely attributable to the elevated level of reactive oxygen species and lactate present in the post-radiotherapy TME [76]. Similarly, chemotherapy can lead to immunometabolic rewiring. For example, neoadjuvant chemotherapy in patients with pancreatic ductal adenocarcinoma results in an accumulation of oleic acid, which markedly diminishes the cytotoxicity of $CD8^+$ TILs [77]. Additionally, antiangiogenic therapy, such as those targeting vascular endothelial growth factor (VEGF) and its receptors (VEGF-receptors, VEGFRs), have been shown to mitigate immunosuppression within the TME by vascular normalizing and restoring oxygen supply [78]. For instance, anti-VEGFR2 treatment has been observed to inhibit the M2-like phenotype in TAMs, particularly in well-oxygenated regions adjacent to normalized blood vessels [79]. This observation suggests a sophisticated interplay between vascular disorders, hypoxia, and immunosuppression within the TME.

In summary, the host's immunometabolism is a key factor influencing the success of contemporary anti-cancer therapies. Therefore, it merits consideration as an independent target for the development of innovative treatments in the future.

High-throughput metabolomic measurements in the single-cell science era: beyond metabolomics

Transitioning from bulk to single-cell: evolving tools for decoding the cancer immunometabolism

Insights into cancer immunometabolism from last section can be summarized by addressing four key questions in sequence: (i) What is the abundance of the metabolites of interest? (ii) Are these metabolites being accumulated or consumed in comparison to their precursors, derivatives, or substances in other metabolic pathways? (iii) What factors lead to changes in metabolites, including the status of enzyme systems that control their synthesis and degradation, as well as the regulatory networks they form? (iv) Considering that cancer-immune interactions involve various cell subsets, how are these metabolites, pathways, and regulatory networks spatially and temporally organized within specific tumor or immune cell subsets?

To address these questions, previous researchers typically employed a hypothesis-driven approach with bulk metabolic measurements (Fig. 2a) [80]. Despite the success of these strategies in specific cell lines and tissues, they inadequately address the intricate spatiotemporal

(See figure on next page.)

Fig. 2 Bulk metabolic measurements vs. single-cell metabolomic measurements. **a** Conventional workflow for studying cancer immunometabolism typically begins with formulating a hypothesis to identify cell populations of interest, through various low-throughput or low-resolution methods, including: (i) bulk steady-state metabolomics using mass spectrometry (MS). (ii) Extracellular flux analysis *via* Seahorse analyzers. (iii) Stable isotope tracing-based fluxomics. (iv) Bulk transcriptomics. These data can be used to construct genome-scale metabolic models (GEMs), thus inferring the state of the metabolic network. **b** A high-throughput single-cell metabolomic workflow involves preprocessing samples using automated systems such as fluorescence activated cell sorting (FACS), microfluidics, and microsampling. The preprocessed samples are then quenched, enriched (or not), and ionized for MS analysis. Various types of MS, such as time-of-flight (TOF), Orbitrap (OT), and Fourier transform ion cyclotron resonance (FTICR), can be utilized for single-cell metabolomics analysis. Subsequently, the abundance levels of metabolites are quantified, allowing for the clustering of cells into distinct subsets based on their metabolomic signatures. **c** The workflow for single-cell metabolic regulomics involves using fresh tissues, FFPEs, and frozen sections for single-cell or single-nucleus RNA-seq workflows such as microwell-based and droplet-based methods. Subsequently, the raw data can be analyzed using standard toolkits like Scanpy and Seurat, with metabolic signatures further examined through metabolic scoring tools like scMetabolism and network modeling techniques like scFBA. Additionally, fresh tissues can be utilized for proteome-level single-cell analysis, where key immune and metabolic markers are identified using labeled antibodies and then analyzed using spectral FCM or CyTOF. CE, capillary electrophoresis; CyTOF, cytometry by time-of-flight; FACS, fluorescence activated cell sorting; FCM, flow cytometry; FFPE, formalin-fixed paraffin-embedded section; FTICR, Fourier transform ion cyclotron resonance; GEM, genome-scale metabolic model; MS, mass spectrometry; OT, Orbitrap; RNA-seq, RNA sequencing; scFBA, single-cell Flux Balance Analysis; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing; ssGSEA, single-sample gene set enrichment analysis; TOF, time-of-flight

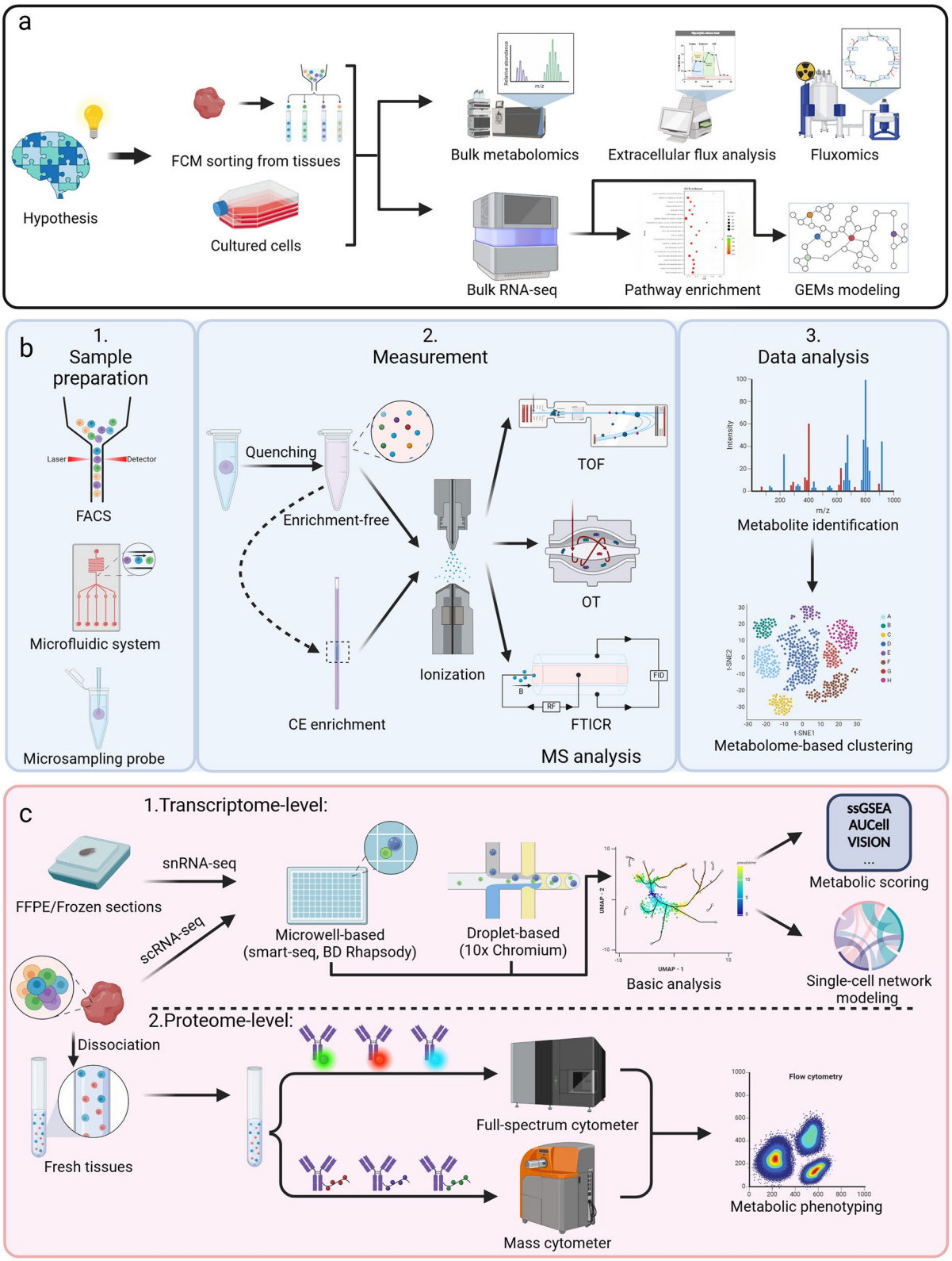

Fig. 2 (See legend on previous page.)

heterogeneity present in cancer immunometabolism. First, conventional metabolic measurements, such as bulk metabolomics and EFA, require 10^6 to 10^7 cells per sample, along with 4 to 5 biological replicates per condition. Given the varied abundance of different immune cells within the TME, isolating and analyzing low-abundance intratumoral immune cells, such as DCs and γδ T cells, necessitates a larger volume of tumor tissue, which is often unattainable when working with human clinical samples, such as those obtained from fine-needle aspirations. Second, although metabolic data can be successfully collected, these measurements typically reflect a convolution of metabolic profiles from predefined immune populations. We remain unable to delineate the distribution of metabolites and metabolic pathways within individual immune cells, thereby limiting our understanding of metabolic heterogeneity in the TME. Additionally, conventional workflows are frequently hypothesis-driven, which restricts comprehensive analysis of multiple components within the TME and immune response due to the inherent trade-off between throughput and resolution [81].

However, single-cell metabolomics and metabolic regulomics have overcome these limitations, offering unbiased panoramic profiling and transitioning from hypothesis-driven to data-driven approaches [19]. In the following sections, we will introduce how these technologies are transforming cancer immunometabolism research.

Single-cell metabolomics

Single-cell metabolomics is rapidly advancing, aiming to quantify the metabolome directly at the single-cell and even subcellular levels (Fig. 2b) [82]. This presents notable challenges in cancer-immunity research, including: (i) the limited sample volume obtained from a single cell compared to bulk metabolomics, which cannot be amplified using conventional methods. (ii) The minimum number of cells for analysis should be at least 100 to 1000, aligning with other types of single-cell omics and the goal of gaining significant immunological insights. Due to the volatile nature of the metabolome, these samples must be processed as synchronously as possible. Despite these obstacles, substantial advancements have been achieved in single-cell metabolomics, allowing for its initial and future application in cancer-immunity research.

To address these challenges, an optimal single-cell metabolomics workflow for cancer-immunity research should involve: (i) automated collection and processing of numerous single-cell samples; (ii) enrichment and ionization of samples, coupled with mass spectrometry (MS) analysis; (iii) analyzing and extracting biological insights from extensive MS datasets [83]. These processes may not align seamlessly with traditional liquid chromatography (LC)/ gas chromatography (GC)-MS workflows and necessitate enhancements in sample preparation, ionization methods, and data analysis.

Analyzing a larger number of cells is crucial for distinguishing genuine biological phenomena from background noise, especially in metabolomic studies where sample-to-sample variability is significant. To manage the extensive workload, it is essential to implement automated sample preparation systems [84]. In the field of cancer immunology, single-cell suspensions derived from tumor dissociation, peripheral blood mononuclear cells (PBMCs), or in vitro experiments are commonly used. Therefore, utilizing flow cytometry (FCM) for automated sample preparation provides a cost-effective and accessible solution. Yao et al. [85] developed a single-cell metabolomic analysis platform called CyESI-MS, which integrates FCM and electrospray ionization (ESI)/MS. This platform allows for online extraction of single-cell samples through label-free FCM, followed by desorption and ionization in a gas-assisted electrospray process, and real-time analysis using MS. Shen et al. [86] utilized CyESI-MS to reveal the single-cell metabolomic profile of human hepatocellular carcinoma (HepG2) cells under NK cell-induced killing, uncovering the metabolic diversity of NK cell cytotoxicity at the single-cell level. Despite the technical accessibility of CyESI-MS, it is important to consider that the shear stress experienced by cells during FCM sorting can significantly impact their metabolic profiles, potentially biasing the results [87]. Microfluidic systems, which minimally disrupt cellular homeostasis, have integrated various designs into single-cell metabolomics workflows [88]. These systems offer a streamlined process of cell lysis, extraction, and ionization, showing promise for automated sample preparation [89]. Microfluidic systems are particularly advantageous for manipulating delicate cells within the TME, such as neutrophils, which necessitate gentle isolation techniques to preserve their metabolic activity [90]. Furthermore, the integration of microfluidics with tumor immune organoids facilitates the development of in vitro models that accurately replicate the metabolic characteristics of the human TME and are well-suited for high-throughput screening applications [91]. For example, Ayuso et al. [92] developed a microfluidic system to model the nutrient and pH gradients present in the TME of human breast cancer, enabling the quantification of their impacts on NK cell functions. This device intuitively illustrated how environmental stress induces immunosuppression, and might be expanded to investigate the role of other specific metabolites on tumor-immune interactions.

Microsampling probe-based methodologies enable metabolic profiling at the level of individual cells and even

subcellular structures. In the section of 'Non-destructive continuous characterization on single living cells', we will delve deeper into how such technologies are driving advancements in 'live single-cell' metabolomics and metabolomic regulomics. Furthermore, formalin-fixed paraffin-embedded sections (FFPEs) and fresh-frozen sections are commonly used samples in cancer immunology. Metabolomic analysis on these samples can also achieve single-cell resolution in situ, which we will explore further in the section of 'Spatial metabolomics in the TME', covering the protocols for sample preparation, ionization, and data analysis.

Enriching and ionizing target metabolites in single-cell metabolomics involving suspended cells presents another significant challenge due to the limited volume of metabolites within individual cells. The direct ionization-mass spectrometry (DI-MS) approach, which involves immediate post-ionization analysis without prior sample enrichment, is highly recommended for single-cell metabolomics [83]. Hiyama et al. [93] introduced a technique called 'Direct Lipido-Metabolomics', where suspended cells are aspirated through a capillary, homogenized using in situ ultrasound, and then directly ionized for MS analysis. This method has been initially used to analyze the lipidomic and metabolomic profiles of PBMCs and circulating tumor cells (CTCs) from neuroblastoma patients. DI-MS offers several advantages for studying tumor-immune interplays in an untargeted manner: (i) The absence of pre-separation and compatibility with derivatization allow comprehensive coverage of the metabolome, thus excelling in untargeted capture of low-abundance metabolites from intratumoral immune cells [94]. For example, Direct Lipido-Metabolomics can detect trace streptomycin from individual CTCs, enabling pharmacodynamics monitoring at the single-cell level [93]. This strategy could be extended to detect diverse responses of immune cells following anticancer therapies. (ii) DI-MS accelerates the processing time for single-cell analyses, and facilitates its integration with the automated preparation systems, thereby enhancing the throughout within a single batch [95]. However, the direct introduction of all cellular components into the MS ionization chamber may result in ionization suppression, highlighting the need for sample enrichment methods suitable for single-cell metabolomics [96]. For example, capillary electrophoresis (CE) is effective at enriching target metabolites from small samples, reducing ionization suppression and broadening the range of identifiable metabolites in embryonic single-cell metabolomics [97, 98]. Furthermore, CE can be combined with a microsampling probe for dynamic in vivo metabolomic monitoring [97]. Ion mobility-mass spectrometry (IMS) complements the single-cell metabolomics workflow by providing increased orthogonality for identifying isomers [99]. In summary, despite the trade-off in processing speed, these enrichment strategies facilitate more personalized metabolomic analyses in a targeted manner. Therefore, the additional enrichment step could be particularly advantageous for the targeted investigation of metabolites within specific immune components of interest, following a comprehensive metabolic profiling of the TME *via* the DI-MS approach.

To summarize, single-cell metabolomics, especially for suspended cells, is rapidly advancing but faces several challenges. First, most of the methods discussed do not have user-friendly commercial solutions, and there are no out-of-the-box data analysis tools designed specifically for single-cell metabolomics. Fundamental issues such as data standardization, combining datasets, and integrating multi-omics data still lack universal agreement [82, 83]. In addition, processes of dissociating and sorting inevitably perturb initial metabolic states of target cells, regardless of the gentleness of handling, while also neglecting their spatiotemporal context in vivo. Therefore, the most promising approach for single-cell metabolomic analysis of the TME in solid tumors appears to be the mass spectrometry imaging (MSI)-based methods (Sect. 'Spatial metabolomics in the TME'). The techniques discussed in this section are more appropriate for liquid-based samples, and might provide valuable insights into the immunometabolism of hematological tumors and metastasis. Their potential in non-invasive clinical diagnostics would also be anticipated. Overall, despite many of these techniques being in the prototyping phase, they are expected to make a significant contribution to future cancer-immunity research [100].

Single-cell metabolic regulomics

The limited coverage and identification of metabolites in single-cell metabolomics currently results in significant signal loss, making it less preferred for uncovering the metabolic landscapes of TME/TOE. On the other hand, single-cell metabolic regulomics at the transcriptomic and proteomic levels have become more accessible in recent years. Various analytical strategies have been developed to map metabolic regulatory networks and redefine the metabolic landscape in the context of cancer-immune interactions (Fig. 2c) [19]. This section focuses on the usefulness of these technologies in exploring the metabolic characteristics within cancer-immune interactions.

Single-cell/nucleus transcriptomics

Over the past decade, single-cell RNA sequencing (scRNA-seq) has significantly impacted the fields of oncology and immunology, ushering in the 'single-cell

science era' [101]. The top three commercial platforms for scRNA-seq, including the 10x Genomics Chromium [102], smart-seq [103], and BD Rhapsody system [104], are tailored to different experimental contexts [105, 106]. The 10x Genomics Chromium, using microfluidics, can process over 10,000 cells in a single batch, rendering it particularly effective for global TME analysis [102]. Smart-seq and BD Rhapsody, which are microwell-based, excel in capturing cells with low mRNA content, such as tumor-resident neutrophils [107]. Furthermore, single-nucleus RNA sequencing (snRNA-seq) is adept at analyzing large or irregularly shaped cells, including neurons and skeletal muscle cells, thereby providing a valuable tool for investigating the interactions between the TME and adjacent non-malignant tissues [108]. SnRNA-seq has also expanded the range of analyzable samples to include both FFPE and fresh-frozen tissues, facilitating systematic and retrospective investigations on archival tumor samples [109]. In terms of data analysis, two prominent toolkits, Seurat and Scanpy, provide user-friendly and scalable approaches [110, 111]. Currently, metabolomic strategies utilizing scRNA-seq/snRNA-seq data are mainly classified into two main types:

(i) Methods based on pre-defined metabolic pathways

Workflows in Seurat and Scanpy aid in visualizing gene expression related to metabolic pathways across different cell subpopulations. Databases like KEGG and Reactome, housing pre-defined metabolic pathways, can assist in enrichment analyses for these cell subpopulations, following practices from bulk RNA sequencing (RNA-seq) data [19]. The sparsity inherent in scRNA-seq data often leads to a low signal-to-noise ratio, prompting the use of strategies like imputation and pseudo-bulk merging to enhance data smoothness [112]. These methods are commonly used to improve enrichment methodologies from the bulk RNA-seq era. Scoring methods utilizing pre-defined databases, such as single-sample gene set enrichment analysis (ssGSEA) [113], Seurat AddModuleScore [114], AUCell [115], singscore [116] and Z-score, are frequently employed to assess the metabolic status of different immune cell subpopulations in TME/TOE. For instance, Wu et al. [117] developed a pipeline called scMetabolism for quantifying metabolic activity in scRNA-seq data. They utilized high-quality metabolic gene sets from KEGG, Reactome, and manually curated genes, applying scoring methods like VISION [118], AUCell, and ssGSEA to quantify the metabolic activity of individual immune cells or cell subpopulations. Through the scMetabolism pipeline, they identified that $MRC1^{+}CCL18^{+}$ M2-like macrophages in the liver metastatic microenvironment of colorectal cancer (CRC) exhibit a highly metabolically activated state, which could be specifically affected by neoadjuvant chemotherapy. Expanding upon these scoring methodologies to reveal further metabolic signatures of immune cells within the TME and TOE, or to employ innovative metabolic gene sets to develop personalized workflows, represents a valuable strategy. However, given potential biases arising from inefficient mRNA captures and improper data smoothing, the scRNA-seq-derived metabolic regulomes would be recommended as an exploratory tool, requiring additional phenotypic validations.

(ii) Network-based methods

Metabolic networks, which are highly interconnected and dynamic, cannot be easily understood by studying individual genes or gene sets alone. To infer global metabolic flux networks in TME/TOE using scRNA-seq data, various genome-scale metabolic model (GEM) methods have been adapted for single-cell analysis [119]. For instance, Damiani et al. [120] introduced the single-cell Flux Balance Analysis (scFBA) framework, which inferred intercellular metabolic interactions by integrating xenograft scRNA-seq data from lung adenocarcinoma (LUAD) and breast cancer patients into a bulk FBA model, using gene expression to define flux boundaries. Similarly, Wagner et al. [121] introduced the Compass method, which estimates the distribution coefficients of metabolic reactions within individual cells based on assumed FBA boundaries, rather than directly predicting fluxes. Additionally, the METAFlux framework, based on the Human1 GEM, allows for the analysis of both bulk and single-cell RNA-seq data [122, 123]. Using METAFlux, Huang et al. [122] clustered and merged single cells into communities from various TME scRNA-seq datasets during CAR-NK treatment, enabling a comparison of metabolic competitiveness between different CAR-NK subtypes and cancer cells. Validation studies confirmed that METAFlux predictions closely matched Seahorse data. Independently of GEMs, Alghamdi et al. [124] developed the single-cell Fluxomics Enrichment Analysis (scFEA) workflow, which utilizes graph neural networks to infer fluxomics information from scRNA-seq data without relying on extracellular flux assumptions. Overall, these investigations contribute insights into the metabolic dynamics at the single-cell level within the TME, shedding light on the tumor-immune interplays. Whether such approaches could facilitate the understanding of nutrient competition among diverse immune components and cancer cells would be exciting for future research.

In addition, exploring metabolic traits from public scRNA-seq datasets holds promise in the field of cancer immunology. A wealth of high-quality scRNA-seq data covering various cancer types such as pan-cancer T

cells [125], NK cells [126], myeloid cells [127] and more has been accumulated, facilitating targeted re-mining of metabolism. It is crucial to note the discrepancies that can arise between the transcriptomic profile and the metabolic characteristics. Therefore, regulomic information obtained from scRNA-seq should be considered as an initial step in assessing immunometabolic status, with further validation being essential.

Protein-targeted single-cell metabolic regulomics

FCM, a well-established technique, continues to be a crucial tool in current cancer-immunity research. In contrast to scRNA-seq, FCM enables a direct evaluation of metabolic enzymes abundance and activities at the protein level, offering a more precise representation of the true metabolic status. Additionally, FCM can quickly and cost-effectively analyze millions of cells in a single run, a throughput that far exceeds that of scRNA-seq (which typically analyzes hundreds to tens of thousands of cells). This high-throughput capacity makes FCM particularly well-suited for comprehensive characterization of populations in TME/TOE.

Advancements in full-spectrum techniques and low-leakage fluoresceins have significantly improved the capabilities of spectral FCM [128]. Current full-spectral FCM can simultaneously measure around 20 to 50 different markers that characterize cell lineages, metabolic enzymes, and various metabolites [129–131]. For instance, Ahl et al. [132] developed Met-Flow, which utilizes a panel of fluorescein-labeled antibodies targeting 17 key human immune markers and 10 human metabolic enzymes. In their research, Ahl et al. conducted both Met-Flow and scRNA-seq on human PBMCs, revealing that the Met-Flow profiles of just 10 metabolic signatures were adequate to categorize PBMCs into different metabolic subgroups with a resolution comparable to that achieved by scRNA-seq using approximately 500 genes. Conversely, clustering biologically relevant subgroups based solely on the same 10 genes from scRNA-seq was found to be challenging [132]. This underscores the nuanced ability of spectral FCM in identifying cellular metabolism and immune cell heterogeneity. This method provides an efficient and reliable approach for comprehensive evaluation of key metabolic pathways across diverse immune components. Extending these analyses to explore the metabolic regulomic heterogeneity of additional immune cells in the TME and TOE presents a next challenge.

Mass cytometry, also known as cytometry by time-of-flight (CyTOF), is an innovative immunotyping technique that utilizes heavy metal-labeled antibodies, mass spectrometry, and FCM. Heavy metal-labeled antibodies provide clearer signals, potentially reducing signal overlap seen with traditional fluorescent markers [133]. While the available range of heavy metal-labeled antibodies is more limited compared to fluorescent ones, designing a complex CyTOF panel for immunometabolism studies is relatively straightforward. For instance, Hartmann et al. [134] developed a comprehensive CyTOF panel targeting key metabolic pathways and immune cell types, conducting a detailed metabolic analysis on TI immune cells from CRC patients. Their research revealed that $CD8^+$ TILs displayed increased uptake of neutral amino acids, as indicated by higher expression of transporters such as L-type amino acid transporter 1 (LAT1) and CD98. This metabolic feature was associated with the TILs exhaustion, characterized by elevated levels of PD-1 and CD39 expression. This approach offers an alternative avenue for evaluating metabolic regulomic heterogeneity of immune cells [135]. With the capability to detect over 50 markers, CyTOF facilitates the synchronized assessment of metabolic regulomics across a broader range of immune components within the TME and TOE in a single batch. This capability allows for comparative studies of regulomics between between less abundant subsets, such as DCs and innate lymphoid cells, and more prevalent subsets, such as TAMs. Furthermore, the compatibility of CyTOF with MS provides the opportunity to integrate it with single-cell metabolomics. This multimodal integration might help address the challenge faced by single-cell metabolomics in accurately identifying diverse cell identities within the TME/TOE.

FCM/CyTOF vs. scRNA-seq

FCM and CyTOF, along with scRNA-seq, offer complementary insights into cancer immunometabolism, each with unique advantages:

(i) Number of cells and targets: FCM and CyTOF can analyze millions of cells simultaneously and assess dozens of targets per cell using antibodies panels labeled with fluoresceins or heavy metals, while scRNA-seq can profile the expression of thousands of signatures in a few thousand cells per run. Furthermore, FCM detects a relatively limited number of markers among these methods, and does not reach the proteomic-level analysis, thus should not be classified within the 'omics' category.

(ii) Accessibility– Cost and Speed: FCM and CyTOF require the use of expensive high-end full-spectrum cytometers or mass cytometers. Similarly, scRNA-seq also requires a substantial investment per sample, but this cost is decreasing rapidly, mirroring the trend seen in Moore's Law [136]. In terms of speed, FCM/CyTOF analysis is generally faster than scRNA-seq, although designing high-

dimension panels for FCM/CyTOF can be challenging and laborious.

(iii) Hierarchy of evidences: FCM and CyTOF primarily provide protein-level expression profiles of enzymes, and can also detect some non-protein metabolites directly. This offers a more accurate representation of the true metabolic phenotype compared to scRNA-seq. In some TME components that are susceptible to perturbation (e.g., platelets), proteins demonstrate greater stability compared to RNA. Thus, protein-based approaches could be effective in minimizing the likelihood of false-negative outcomes.

(iv) Sample applicability: FCM and CyTOF are best suited for fresh samples, while scRNA-seq is suitable for fresh, fresh-frozen, and FFPE tissues, making it compatible with clinical sample archives and extensive patient cohorts [109].

In summary, it is crucial for researchers to carefully plan their studies, taking into account the availability of technological resources (Table 1). In the current era of single-cell science, the data-driven approach improves the effectiveness and objectivity of research in cancer immunometabolism [101]. Therefore, we propose the following approach: first, focus on uncovering metabolic insights from existing scRNA-seq datasets, then use advanced FCM/CyTOF panels for thorough validation at the protein level, and finally, conduct targeted analysis to investigate specific metabolites of interest.

Multiscale spatial analysis of cancer immunometabolism: from TME to TOE

Spatial relationships are another critical dimension for understanding the metabolic interactions in cancer-immune interactions. The single-cell techniques mentioned in last section mostly involve analyzing cells that have been dissociated from tissues, leading to the absence of spatial information on cells and metabolites. Historically, researchers have explored spatial relationships using targeted histological methods, which had limitations in terms of throughput and compatibility. Recent advancements in metabolomics and metabolic regulomics are moving towards spatially resolved analyses, as highlighted in recent reviews [137, 138]. This section focuses on the application of these technologies in cancer-immunity research.

Spatial metabolomics in the TME

Spatial metabolomics is increasingly utilized in cancer immunology, particularly in characterizing the TME [139]. Immune cells within the TME are often clustered into TLSs [140], stem-immunity hubs [141], and other microanatomical structures, or they may infiltrate as single cells. A key consideration in capturing these cells is achieving high spatial resolution. However, this situation presents a dilemma: as the size of individual pixels decreases, fewer analytes can be obtained from a single pixel, leading to a lower metabolic coverage. Therefore, the trade-off between spatial resolution and coverage must be critically considered when designing such metabolomic studies. Furthermore, it is important to ensure that common sample types such as FFPE and fresh-frozen tissues are compatible with the sampling and analytical platforms. In recent years, spatial metabolomics has made significant advancements using MSI, with three pioneering technologies and their derived innovations: matrix-assisted laser desorption/ionization-MSI (MALDI-MSI) [142], secondary ion mass spectrometry-MSI (SIMS-MSI) [143], and desorption electrospray ionization-MSI (DESI-MSI) [139]. These methods offer sophisticated solutions for various resolution scales, compatible samples, and targeted metabolites. Similar to single-cell metabolomics, the workflow for MSI-based spatial metabolomics is primarily optimized in sample preparation, ionization, and data analysis (Fig. 3a).

Sample preparation is crucial for high-quality MSI data [144]. Fresh-frozen tissue sections are the preferred choice for spatial metabolomics platforms due to their ability to effectively capture the metabolic snapshot of

(See figure on next page.)

Fig. 3 Spatial measurements of cancer immunometabolism. **a** Spatial metabolomic workflow includes the following steps: (1) Utilizing samples such as FFPEs and fresh-frozen sections for spatial metabolomics. (2) Preprocessed samples can undergo ionization using MALDI, DESI, and SIMS techniques, followed by MSI analysis with TOF, OT, or FTICR. (3) Quantification of metabolite abundance levels and mapping to their respective spatial locations. **b** Spatial metabolic regulomic workflow: (1) Both FFPEs and fresh-frozen sections are suitable for iST and sST workflows. The raw data should be combined with scRNA-seq data and then modeled to extract immunometabolic signatures. (2) Fresh tissues are suitable for proteome-level spatial analysis. Key immune and metabolic markers can be labeled with fluorescein or heavy metal-coupled antibodies and analyzed using multiplexed fluorescence or MS imaging. CODEX, co-detection by indexing; CyCIF, cyclic immunofluorescence; DESI, desorption electrospray ionization; FFPE, formalin-fixed paraffin-embedded section; FTICR, Fourier transform ion cyclotron resonance; iST, imaging-based spatial transcriptomics; MALDI, matrix-assisted laser desorption/ionization; MERFISH, multiplexed error-robust fluorescence in situ hybridization; MIBI-ToF, multiplexed ion beam imaging by time of flight; MS, mass spectrometry; MSI, mass spectrometry imaging; OT, Orbitrap; scRNA-seq, single-cell transcriptome sequencing; SIMS, secondary ion mass spectrometry; sST, sequencing-based spatial transcriptomics; TOF, time-of-flight

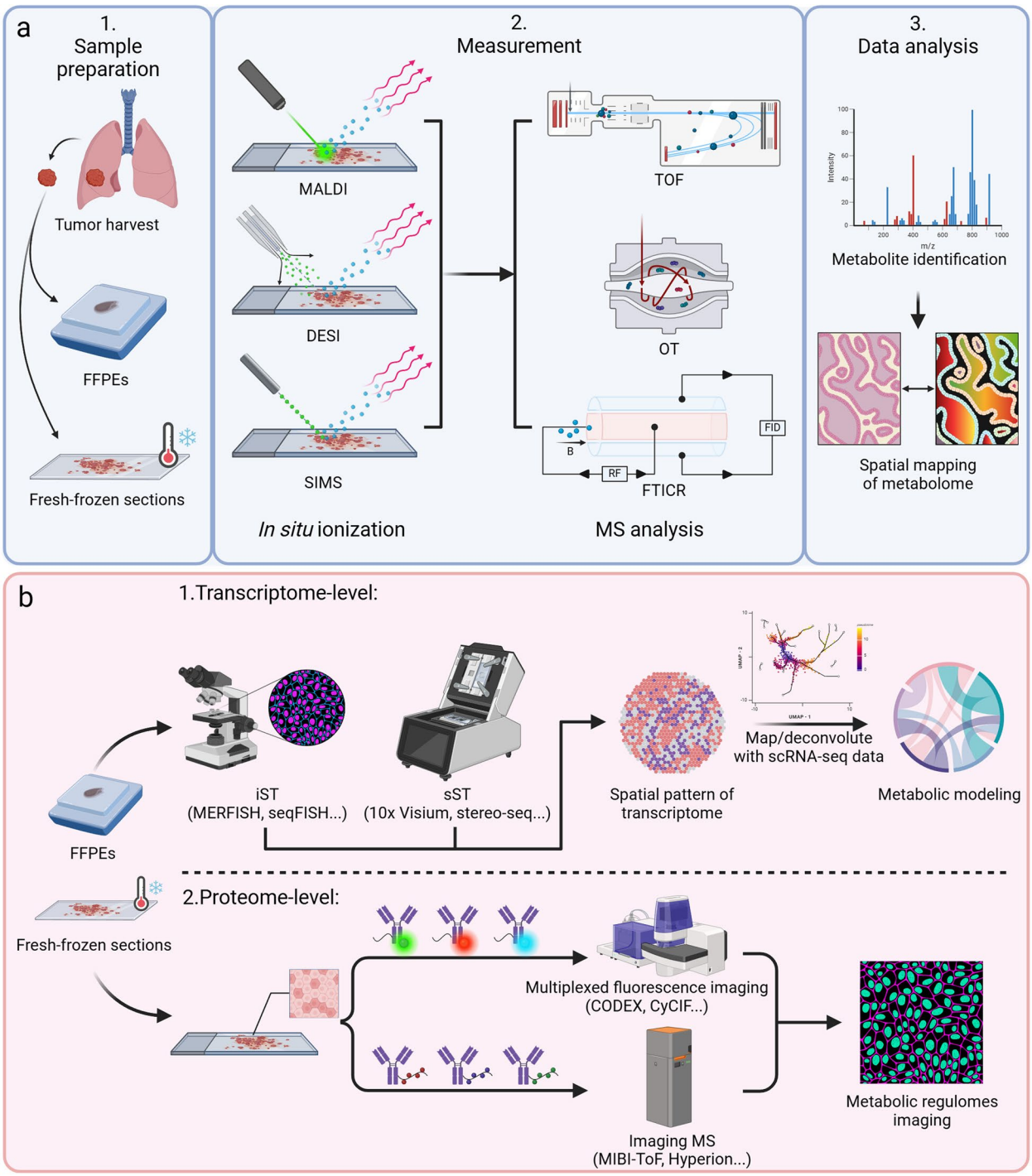

Fig. 3 (See legend on previous page.)

tissues. These sections are now being used in intraoperative MSI procedures, offering rapid diagnostic insights for tumor margins in cases like pancreatic cancer and glioma [145, 146]. On the other hand, FFPE tissues, commonly archived in cancer immunopathology, are not recommended for DESI-MSI and SIMS-MSI-based spatial metabolomics, due to potential alterations in metabolite profiles during the fixation and production process [147]. However, MALDI-MSI, the most popular spatial metabolomics platform, has undergone optimizations for compatibility with FFPE samples, allowing for analysis of proteins, lipids, N-glycans, and small molecule

metabolites [148–150]. These technologies offer a compelling approach to re-use tumor samples within retrospective cohorts for longitudinal metabolic analysis.

When selecting an optimal ionization method, key factors to consider include spatial resolution and the specific analytes of interest. MALDI-MSI utilizes a laser to convert tissue samples, co-crystallized with matrix molecules, into the gas phase under vacuum conditions [151]. These ionized samples are then introduced into either a time-of-flight (TOF) MS or a Fourier transform ion cyclotron resonance (FTICR) MS with ultra-high mass resolution [139, 152]. MALDI-MSI can achieve spatial resolutions ranging from 1.4 to 200 μm and is mainly used for analyzing lipidomes, proteomes/peptidomes, small molecule metabolomes, and N-glycanomes [153–155]. The updated MALDI-2 platform incorporates a secondary laser beam for enhanced ionization efficiency and sensitivity by two to three orders of magnitude [156]. In the field of cancer immunity, MALDI-MSI still necessitates additional tools to attribute the observed metabolomic variations to specific immune components. For example, Rappez et al. [157] have recently introduced SpaceM, an open-source spatial single-cell metabolomics workflow. This approach combines microscopic imaging analysis to correlate metabolite images generated by MALDI-MSI with individual cells, enabling a democratic single-cell-level metabolomic analysis in situ. Such a workflow would provide an innovative spatial avenue for understanding the interplay between metabolism and the TME. In sum, MALDI-based methods stand as predominant approaches for spatial metabolomic analysis of the TME. However, high performance of MALDI depends on diverse matrices, leading to two noteworthy issues: (i) the matrix effect, which can introduce errors in metabolites quantitation; and (ii) the varying efficiencies of ionization assistance among different metabolites, possibly resulting in the biased output [139]. Accordingly, it is essential to meticulously determine the targets of interest before conducting MALDI metabolic analysis.

Second, DESI-MSI utilizes a fast-moving charged droplet to aid in the desorption and ionization of the target sample, similar to ESI in single-cell metabolomics [158]. This approach is carried out in an unconfined setting at atmospheric pressure, removing the need for matrix assistance [159]. DESI-MSI has the potential to provide a thorough profile of metabolites, making it particularly suitable for small molecules [160]. The enhanced Air Flow-Assisted DESI-MSI (AFADESI-MSI) has emerged as a prominent platform for non-targeted spatial metabolomics [161]. Building upon DESI, AFADESI-MSI uses a high-velocity air stream to transport charged ions over longer distances, improving their enrichment and ionization. This progress enables the simultaneous detection of numerous metabolites and significantly widens the field of view (FOV) [162, 163]. However, the spatial resolution achievable with DESI-MSI typically falls between 50 and 200 μm, while AFADESI reaches approximately 100 μm [164, 165]. Neither technique has the capability to resolve at the single-cell level. In contrast, SIMS-MSI utilizes a focused ion beam to desorb and ionize the sample, then directs the resulting secondary ion beam into MS [166], achieving spatial resolution at the subcellular level, typically between 50 and 100 nm [164]. However, its ionization efficiency is relatively low, resulting in reduced metabolite coverage compared to MALDI-MSI and DESI-MSI [167]. Consequently, SIMS-MSI is better suited for characterizing small areas within regions predefined by broad FOV techniques, such as MALDI or DESI. For instance, Tian et al. [168] conducted tissue-level metabolomics of mouse livers using DESI-MSI, then they performed in situ SIMS analysis on certain region of interest to measure the metabolic profiles of individual liver-resident immune cells. Weighing up the pros and cons of these methods, the combined approach opens the opportunity to investigate the nutrient partitioning among individual intratumoral immune cells.

Other emerging ionization strategies such as Matrix-Assisted Laser Desorption Electrospray Ionization (MALDESI) [169], Laser Ablation Electrospray Ionization (LAESI) [170], and Laser Ablation Inductively Coupled Plasma (LA-ICP) [171], each have their own specific applications. These mainstream MSI workflows can also be combined with IMS to improve the differentiation of isomeric metabolites [172]. Another emerging trend in spatial metabolomics is the transtion towards spatial fluxomics. Technologies, such as iso-imaging [173] and ^{13}C-SpaceM [174], which are compatible with the MALDI system, are showing promise as potential strategies for studying the dynamic changes in metabolites within the TME.

As the spatial resolution and coverage of mainstream spatial metabolomics platforms continues to increase, the scale of spatial metabolomics datasets expands explosively, presenting new challenges for analysis. Several established toolkits such as SCiLS Lab [175], Cardinal [176], MSIReader [177], and METASPACE [178], can conduct basic analyses on MSI-generated data. These leading MSI toolkits allow for visualizing spatial patterns of targeted metabolites in the TME, offering unique data inputs for clinical prognostic models [179]. However, significant computational difficulties persist in this field: (i) Absolute quantification remains problematic due to inconsistent resolution/ionization efficiencies across pixels. This issue is likely related to inadequate control over matrix effects and ionization suppression, yet cannot be adequately compensated for by conventional

normalizations (e.g., total ion count) [180]. (ii) Metabolites annotation with MSI-generated data is hindered by the lack of chromatographic separation and incompatibility with tandem MS [181]. While METASPACE offers a foundational option, there is an urgent need for more user-friendly compound libraries and tools specifically designed for MSI.

Finally, the lack of cell identity identification in most current spatial metabolomics workflows poses a challenge in directly exploring the metabolomic landscape in individual intratumoral cells [182]. Consequently, a combination with other spatial omics approaches is now recommended as the preferred strategy. For instance, Hu et al. [183] developed a framework called scSpaMet for single-cell level spatial metabolomics analysis, which combines untargeted spatial metabolomics with targeted multi-plex protein imaging. Through this framework, they were able to identify and map multimodal spatial single-cell metabolic profiles in the microenvironments of human lung cancer, tonsils, and endometrial tissues, revealing a neighborhood-dependent metabolite competition pattern. These integrations of lineage markers detection and metabolomic imaging, remain the most reliable approaches for discerning the metabolic signals of individual immune cells that infiltrating into tumors.

Overall, spatial metabolomics is rapidly advancing in resolution and compatibility. When compared to other spatial omics methods, spatial metabolomics stands out for its quick processing and cost-effectiveness, positioning it as a promising next-generation tool for large-scale cohorts in cancer-immunity phenotyping.

Spatial metabolic regulomics in the TME

Spatial transcriptomics

Spatially resolved transcriptomics technologies have become the cornerstone of high-throughput approaches in spatial biology [184]. These technologies are generally classified into two categories: imaging-based spatial transcriptomics (iST) and sequencing-based spatial transcriptomics (sST) [184]. iST utilizes microscopic imaging to directly detect and quantify target RNA transcripts within tissue sections, primarily through techniques like multiplexed fluorescence in situ hybridization (M-FISH) and in situ sequencing (ISS), enabling precise localization and quantification of transcripts at subcellular resolutions [185]. Despite their high precision, iST methods are costly and have limited throughput, making them more suitable for targeted analyses [138]. On the other hand, sST employs next-generation sequencing (NGS) to unbiasedly quantify RNA transcripts that have been spatially isolated from tissue sections, providing comprehensive coverage of the transcriptome [185]. The spatial selection methods in sST can be further divided into three approaches: (i) identifying and acquiring regions of interest (ROI) through physical or optical microdissection; (ii) using spatial barcodes for geographic indexing; and (iii) *de novo* reconstruction of spatial information [138]. Early studies have utilized methods (i) and (iii), which also show promise for future expansion into three-dimensional spatial analysis [186–189]. Among sST techniques, those utilizing spatial barcodes are currently the most accessible. Popular and user-friendly sST workflows like 10x Visium [190], stereo-seq [191], slide-seq [192], and DBiT-seq [193] fall into this category. sST has emerged as a crucial tool in cancer spatial biology, particularly in uncovering metabolic features in the TME [182].

At present, conducting metabolic regulation analysis in samples from TME using most spatial transcriptomics platforms is challenging due to two main limitations. Firstly, the resolution of most sST platforms has not yet reached the single-cell level, making it difficult to directly measure scattered immune cells in the TME. Secondly, while iST offers superior resolution, its limited throughput restricts the ability to deduce the global metabolic landscape within individual immune cells.

To elucidate the details of each pixel in spatial transcriptomics data, researchers often integrate these data with scRNA-seq data using two main strategies. (i) Deconvolution, which estimates cellular subtype proportions or enrichment levels across all pixels by leveraging reference single-cell data, is particularly beneficial for low-resolution sST [194]. High-performance deconvolution methods for spatial transcriptomics data include cell2location [195], SpatialDWLS [196], robust cell type decomposition (RCTD) [197], among others. (ii) Mapping, which assigns annotated cell types to specific pixels in spatial transcriptomics data based on reference single-cell profiles, is commonly used in iST [198]. Noteworthy mapping methods such as Tangram [199], gimVI [200], SpaGE [201], and the low-resolution spatial transcriptomics-compatible CellTrek [202] are frequently recommended [203]. These strategies enable the utilization of robust metabolic inference tools within the scRNA-seq analytical framework (mentioned in Sect. 'Single-cell/nucleus transcriptomics'). For instance, Wu et al. [204] integrated scRNA-seq with stereo-seq to investigate the immunometabolic status in the marginal areas of human primary liver cancer samples. Similarly, Janesick et al. [205] combined scFFPE-seq [206], Visium CytAssist [207], and Xenium [208] from the 10x Genomics multi-omics platform to create a highly detailed atlas of spatial transcriptomics landscape in human breast cancer TME, identifying a rare malignant cell subgroup of ductal carcinoma in situ (DCIS) with metabolic relevance (Fig. 3b).

In summary, spatial transcriptomics has become a valuable tool for studying the metabolic diversity of the

TME. When deciding on a technology to use, both sST and iST offer unique benefits that complement each other in terms of resolution and throughput. Therefore, careful consideration is essential during experimental planning. Recent advancements in sST, such as the 10x Visium HD and stereo-seq, have enhanced resolution down to the single-cell level [191, 209]. Concurrently, improvements in iST, like the CosMx Human 6K panel and seqFISH+, have increased the number of targets to a quasi-genome-wide level, leading to the development of a bona fide 'high-throughput spatial single-cell transcriptomics' approach [210–212]. These innovations may reduce the need for integration with single-cell omics in the near future.

The potential impact of revolutionary technologies on cancer immunometabolism is highly anticipated. First, those measurements will enhance our capacity to identify novel microstructures within the TME. For detection of intratumoral microbes, low-resolution spatial transcriptomics offers only a general abundance of them, masking their specific interactions with single cells or subcellular structures, and still requires one-by-one verifications through targeted technologies [213]. In contrast, high-throughput spatial single-cell transcriptomics will enable a direct mapping of microbe-cell interactomes with high confidence. On the other hand, future data analysis is likely to increasingly utilize network-based methodologies for spatial metabolic modeling of the TME. For example, initial efforts by Wang et al. [214] to establish spatial GEMs in low-resolution sST were limited by resolution constraints, hindering the understanding of the metabolic functions of infiltrating immune cells. However, with the advent of high-throughput spatial single-cell transcriptomics, these foundational models are expected to be extended to the single-cell level. Additionally, as discussed in Sect. 'Single-cell/nucleus transcriptomics', data mining strategies in spatial single-cell transcriptomics (potentially in three-dimensional space in the future) are likely to become the standard pipeline for metabolomics and metabolic regulomics studies. These approaches are poised to be widely adopted by the spatial analysis community.

Spatial multiplexed proteomics

Antibody-based proteomics has shown remarkable success in analyzing the metabolic profile of single-cell suspensions in the TME. This approach is also suitable for spatial analysis of FFPEs or fresh-frozen tissue sections [182]. Due to the inherent instability of metabolites and RNAs in different spatial samples, spatially resolved proteomic quantification offers a valuable method for understanding the key regulatory landscape of metabolism in the TME. Similar to the analysis of single-cell suspensions, the success of spatially resolved metabolic regulomics analysis on sections of the TME depends on the careful selection of an appropriate antibody panel. These metabolic panels typically fall into two main categories:

(i) The use of multiplexed immunofluorescence techniques, such as cyclic immunofluorescence (CyCIF) and co-detection by indexing (CODEX), has been highlighted in recent studies [215, 216]. For example, Ringel et al. [74] developed a 23-plex CyCIF panel that targeted major immune lineage markers and rate-limiting enzymes in key metabolic pathways. This panel was employed to assess the metabolic phenotype of CRC TME in the context of high-fat diet (HFD)-induced obesity. The findings revealed that HFD alters the nutrient distribution pattern in CRC TME, particularly affecting infiltrating $CD8^+$ T cells by limiting their access to free fatty acids (FFAs), resulting in exhaustion and dysfunction.

(ii) The second approach involves using heavy metal-labeled antibodies for imaging mass cytometry (IMC), utilizing techniques like Multiplexed Ion Beam Imaging by Time of Flight (MIBI-ToF) and Hyperion [217, 218]. To analyze the metabolic regulomes in human CRC TME, Hartmann et al. [134] adapted a metabolic panel initially designed for CyTOF for combined use with MIBI-ToF. They found that the tumor-immune border in CRC represents a unique metabolic environment. Immune cells near this border exhibit a 'glutamine addiction' phenotype, marked by high expression of ASC amino-acid transporter 2 (ASCT2) and CD98, regardless of their lineage. On the other hand, $CD39^+PD\text{-}1^+$ $CD8^+$ T cells, situated farther from this border, display a suppressed metabolic state, suggesting that the functionality of TI immune cells undergoes spatial-induced reprogramming.

In summary, the antibody-based metabolic analyses offer a high-resolution spatial approach for studying heterogeneity of the TME, dispensing with the additional cell identification. This approach might be suitable for the large-scale analysis of archived human samples, potentially transforming the study of cancer immunology into clinical suggestions. Furthermore, the emergence of untargeted spatial proteomics techniques based on MSI, like Laser Capture Microdissection coupled with MS (LCM-MS) [219], is poised to provide a more integrated and comprehensive addition to other spatial omics technologies [220].

Spatial metabolism tracing at the organismal level

In line with previous research discussed in the Background, it is crucial to evaluate the metabolic aspects of cancer-immune interactions at the organismal level, particularly concerning the regulation in the TOE [11, 50]. Analyzing this metabolic dimension not only enhances our understanding of cancer-immunity on a global scale but also helps reduce sampling biases. To achieve this, sampling technologies with a broader FOV are necessary to encompass the entire organism [221]. Considering the Modifiable Areal Unit Problem (MAUP), analytical strategies for TOE must diverge from those used in narrow-FOV imaging [222]. These tailored strategies are expected to be more applicable in clinical settings.

Expanding spatial metabolomics and metabolic regulomics to whole-body wide-field imaging has shown promise in preclinical animal models [191, 223–225]. For example, Luo et al. [223] pioneered the application of AFADESI-MSI to map the organism-level distribution of drugs and endogenous metabolites in whole-body sections of rodents with glioma following chemotherapy. This innovative approach visualized pharmacological activity, and would be valuable for investigating multiple pre-metastatic and metastatic niches, as well as other systemic events within the TOE. Additionally, this approach faces significant challenges, particularly in the preparation of whole-body section samples, which remains a daunting task in many research labs [226]. Another hurdle is the proper interpretation of these data to integrate them with existing biological frameworks [221, 227]. Additionally, utilizing in vivo imaging methodologies for analyzing TOE could be a compelling direction to explore. A more detailed discussion of this approach is provided in Sect. 'In vivo tracing at the organismal level'.

In vivo tracing of metabolomic dynamics in cancer-immunity

The dynamic nature of metabolism plays a crucial role in shaping the intricate interplay between cancer and the immune system. Traditionally, our understanding of the metabolomic dynamics of cancer-immunity has been derived from studying ex vivo tissues and cells, using techniques such as EFA and Nuclear Magnetic Resonance (NMR)-based bulk fluxomics [80]. The emergence of single-cell and spatial omics approaches has allowed for the modeling of individual samples through pseudo-time trajectories, providing further insights into these dynamics [228–233]. However, the complex and rhythmic milieu interior makes the dynamics of immunometabolism more susceptible to disruptions, potentially undermining the existing frameworks [234–237]. As a result, there is a growing demand for in vivo metabolomic tracing strategies that can more accurately depict the dynamics of the TME/TOE. Current and future in vivo tracing strategies for understanding cancer-immunity metabolomic dynamics can be broadly categorized into two levels: those tailored to individual living cells and those designed for the organismal level.

Non-destructive continuous characterization on single living cells

The precise microsampling techniques required for the non-destructive, continuous analysis of metabolic states within individual living cells present a significant challenge in maintaining the cell's homeostasis [238]. The patch-clamp technique, widely used in neuroscience research, provides an elegant solution for the continuous sampling and monitoring of single living cells [239]. Besides monitoring ion flux in single living neurons, this technique has been adapted for studying ex vivo immune cells associated with TME, including T cells and macrophages [240, 241]. The integration of patch-clamp and metabolomics enables metabolomic profiling for individual cells and even subcellular structures. For instance, Zhu et al. [242] established a pipeline for single-lysosome metabolomics, termed single-lysosome mass spectrometry (SLMS). This method utilizes a glass micropipette from the patch-clamp platform to conduct real-time metabolomic sampling in manually isolated single lysosomes. These collected samples are subsequently introduced into induced nanoESI and MS for analysis. This approach allowed for the generation of a single-lysosome metabolome atlas of human urinary system cancer cells (T24), revealing cancer-specific metabolite signatures in autolysosome-like subgroups. These techniques, with minimal disruption to cellular physiology, could be further used to decode the metabolomic responses within single cells or organelles during tumor-immune interactions, such as antigen presenting in the TLSs. Additionally, the integration of whole-cell patch-clamp with the snRNA-seq platform has given rise to patch-seq [243], a novel method that enables the study of real-time transcriptional dynamics at the single-cell level. In conclusion, given its robust capabilities of in vivo and multimodal monitoring, the patch-clamp technique holds promise for in vivo characterization of TME metabolomic dynamics.

Microprobes such as tungsten electrodes [244] and borosilicate capillaries [245] have been specifically designed for profiling metabolomic dynamics in single-cell/subcellular metabolomics workflows. These microprobes are engineered as integrated analytical devices that handle sampling, ionization, and MS injection. For instance, Pan et al. [246] developed 'The Single-Probe'

pipeline for in situ metabolomic analysis of individual living cells. This method involves inserting a probe with a sub-10 μm tip diameter into the cell, followed by direct ionization and MSI analysis. Such techniques reduce the volume of cellular content obtained per run, thus minimizing the risk of ionization suppression [83]. Besides, the Fluidic Force Microscopy (FluidFM) platform has made significant strides in commercialization by integrating microprobing, microfluidics, and Atomic Force Microscopy (AFM) [247, 248]. The platform's advanced force feedback surpasses that of traditional microsampling methods, allowing for more precise and less invasive sampling from living cells [248]. Chen et al. [249] established live-seq, a real-time scRNA-seq workflow using FluidFM, which maintains cellular viability and minimizes physiological perturbation across various cell types. Live-seq was initially employed to recode temporal trajectories of polarization in individual macrophages before and after lipopolysaccharide treatment. Whether these microprobing-based approaches could be advantageous for understanding the temporal metabolic dynamics of TAMs' polarization, or even other transitory events during cancer immune responses (e.g., T cell exhaustion), would be eagerly anticipated.

Overall, these strategies enable the non-targeted acquisition of metabolomes or transcriptomes from single living cells, and hold immense promise for future investigations into the temporal dynamics of TME metabolism. (Fig. 4a; Table 1). However, they face technical challenges, such as analyzing samples with minute quantities and monitoring a limited number of cells simultaneously. Further advancements accessible to users are necessary in these techniques.

In vivo tracing at the organismal level

Imaging strategies offer a non-invasive alternative for continuous, dynamic monitoring at the organismal level (Fig. 4b; Table 2) [250]. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) provide detailed anatomical and morphological information of tumors, indirectly characterizing the immunometabolic status of cancer patients [251–253]. Positron Emission Tomography (PET) offers insights into molecular dynamics at the organismal level, focusing on functional characterization of immunity and metabolism [254]. PET, often integrated with CT and MRI, is a standard approach for monitoring cancer metabolism in both preclinical and clinical practices [255, 256]. The clinical PET-CT system utilizes ^{18}F-FDG to discern morphological and glucometabolic features of proliferative tumors throughout the body [257]. Reinfeld et al. [24] used ^{18}F-FDG PET to show that immune cell subsets exhibit greater glucose uptake capacity than cancer cells, enhancing our understanding of nutrient distribution within the TME. However, the limited specificity of the conventional ^{18}F-FDG probe complicates the direct identification of specific cell subsets with high glucometabolic activity in vivo, posing challenges in differentiating between tumor proliferation and inflammation [258–260]. As a result, the direct characterization of cancer immunometabolism using this technique remains elusive.

The next-generation PET imaging system, designed for characterizing cancer immunometabolism, has seen improvements in two key areas:

(i) Optimization of tracers

The current PET system now includes a wider range of tracers for metabolism, such as amino acid transport (^{18}F-FET) [261], nucleic acid metabolism (^{18}F-FLT) [262], lysosomal status (^{18}F-FPYGal) [263], and hypoxia [264]. These tracers have been utilized in both preclinical and clinical settings. For instance, ^{18}F-fluoroethyl-L-tyrosine (^{18}F-FET) is a new amino acid tracer that addresses the limitations of ^{18}F-FDG [265]. It is able to differentiate between radiotherapy-induced inflammatory responses and cancer recurrence in glioblastoma patients.

Furthermore, the expansion of PET probe panel has taken a new direction with the development of antibody-radioisotope conjugates, known as immuno-positron emission tomography (immunoPET) [266]. ImmunoPET is primarily used in two contexts within cancer-immunity research. The first application is to measure the spatiotemporal pharmacokinetics or pharmacodynamics (PK/PD) of immune checkpoint blockers (ICBs) labeled with radioisotopes, commonly utilized in animal models and clinical trials [266, 267]. For example, Bensch et al. [268] utilized immunoPET with ^{89}Zr-atezolizumab to visualize PD-L1 in humans and validated its predictive value for the clinical response to atezolizumab treatment across different cancer types. Similarly, Meyblum et al. [269] employed ^{89}Zr-labeled anti-CTLA-4 for immunoPET-based PK/PD analysis following intratumoral and intravenous administration, demonstrating that a single intratumoral ICB injection can trigger a systemic therapeutic response with reduced unintended exposure. The second use of immunoPET is to monitor specific immune lineages and their functional characteristics by employing targeted antibodies labeled with PET isotopes [270]. The range of immunoPET tracers for T cell subsets, along with their activation and exhaustion markers, is rapidly expanding, with some progressing to various stages of clinical trials [271]. Additionally, while panels targeting NK cells and TAMs are currently in the pre-clinical phase, tracers for other immune lineages are yet to be developed [272–274].

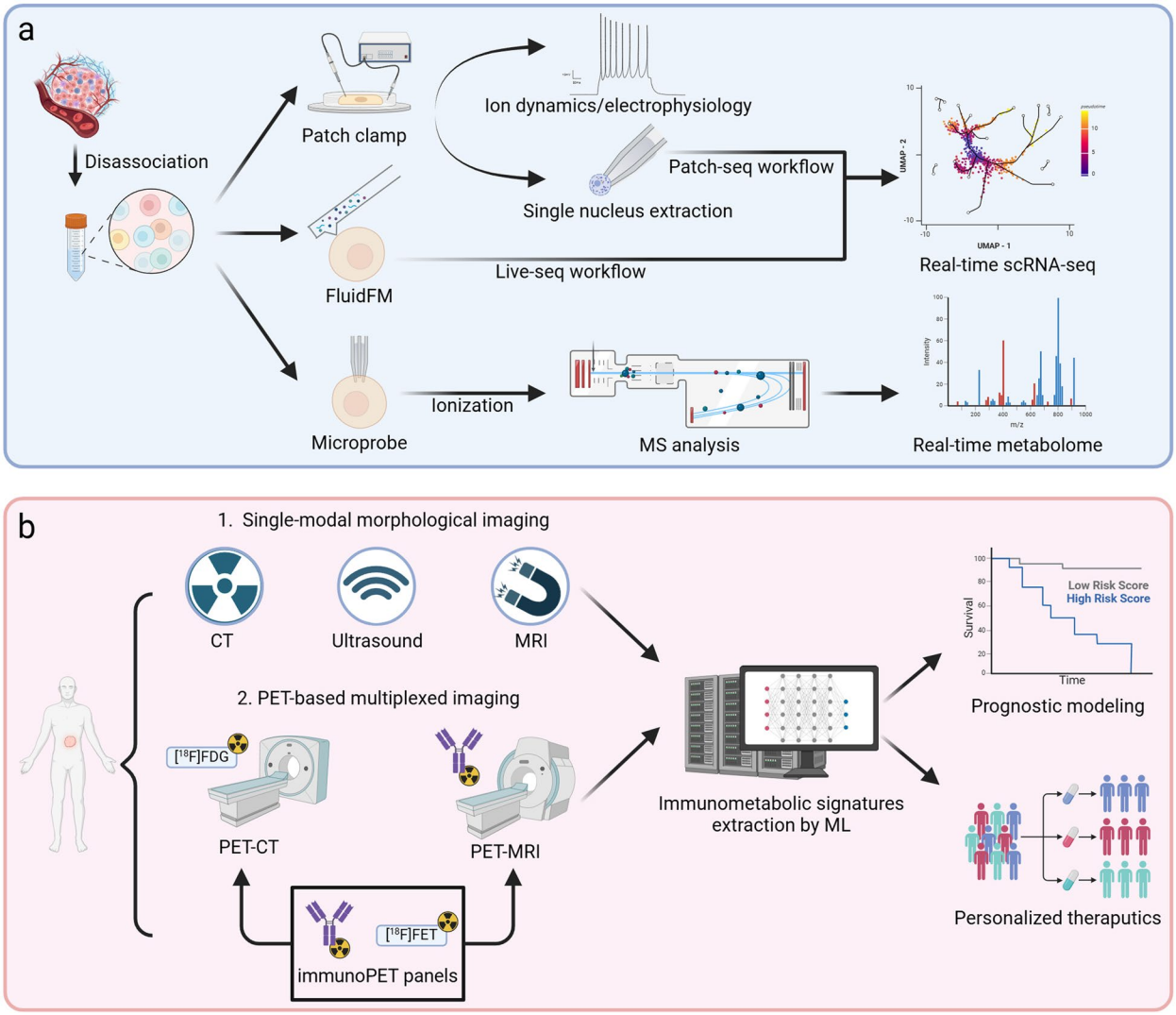

Fig. 4 In vivo measurements of cancer immunometabolism. **a** Single living cells can be continuously and non-destructively sampled by patch clamps, FluidFM, and microprobes. These time-serial samples can then be input into specialized RNA-seq workflows (e.g., patch-seq, live-seq) or metabolomics workflows for real-time analysis. **b** Imaging-based strategies for immunometabolic measurement encompass a variety of approaches. First, single-modal imaging methods such as CT, MRI, and ultrasound offer insights into morphological features, serving as indirect parameters for immunometabolic modeling. Second, PET-based imaging systems like PET-CT and PET-MRI provide multiplexed immunometabolic information through specific immunoPET panels. These data can be directly input for ML modeling, facilitating personalized clinical decision-making. CT, Computed Tomography; FluidFM, Fluidic Force Microscopy; immunoPET, immuno-positron emission tomography; ML, machine learning; MRI, Magnetic Resonance Imaging; MS, mass spectrometry; PET, Positron Emission Tomography; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing

(ii) Deployment of a multiplexed imaging workflow

To achieve metabolomic characterization of cancer-immunity in vivo, a multiplexed PET imaging workflow using both metabolic and immune tracers is essential. Schwenck et al. [254] utilized multiplexed tracers to design a clinical-grade multistep PET-CT/MRI workflow. The workflow begins with the standard ^{18}F-FDG PET-CT to assess patients' overall metabolic and immunological profiles, followed by a targeted PET-MRI using a specific immunoPET panel. Subsequently, machine learning (ML) algorithms are employed to integrate and train these multimodal imaging data, resulting in a multiparametric map of the whole-body immunometabolic dynamics [254, 275]. This innovative approach provides valuable insights for personalized clinical decision-making.

Overall, PET-based multimodal imaging is considered the most effective strategy for in vivo tracing, providing a technical foundation for investigating

Table 1 Targets, available samples, and characteristics of representative metabolomic and regulomic methods at single-cell level for analyzing cancer immunity

Method	Targets	Available samples	Characteristics	
			Pros	Cons
CyESI-MS	♦ Metabolites	♦ Fresh single-cell suspensions	♦ Compatible with FCM ♦ Real-time measurement for single cells	♦ Potential disturbance to metabolism due to the shear stress ♦ No standardized solutions
SLMS	♦ Metabolites ♦ Ion dynamics	♦ Single lysosomes	♦ Real-time measurement for single lysosomes ♦ Multimodal measurement	♦ Limited number of analyzable samples per run ♦ No standardized solutions
PESI-MS	♦ Metabolites	♦ Single living cells	♦ Subcellular detection available ♦ Real-time measurement for live cells	♦ Limited number of analyzable cells per run ♦ No standardized solutions
scRNA-seq/snRNA-seq	♦ RNA	♦ Fresh single-cell suspensions ♦ Single-nucleus suspensions from fresh/frozen tissues, or FFPEs	♦ User-friendly commercial solutions ♦ Unbiased analysis ♦ Diversified toolkits for data analysis ♦ Suitable for FFPEs	♦ Indirect metabolic characterization at mRNA level ♦ Costly and time-consuming
Patch-seq	♦ RNA ♦ Ion dynamics	♦ Single living cells ♦ In vivo	♦ Unbiased analysis ♦ Compatible with snRNA-seq workflow ♦ Multimodal measurement	♦ Indirect metabolic characterization at mRNA level ♦ Costly, time-consuming, and labor-intensive
Live-seq	♦ RNA	♦ Single living cells	♦ Unbiased analysis ♦ Compatible with scRNA-seq workflow ♦ Real-time and continuous measurement for live cells ♦ Minimize disturbance to live cells	♦ Indirect metabolic characterization at mRNA level ♦ Costly, time-consuming, and labor-intensive ♦ FluidFM is not available in most labs
SCENITH	♦ Protein synthesis levels	♦ Fresh single-cell suspensions	♦ Compatible with FCM ♦ Simple method for complex metabolic profiling ♦ Affordable and time-saving	♦ Indirect functional analysis by puromycin ♦ Targeted and biased analysis
Met-Flow	♦ Proteins	♦ Fresh single-cell suspensions	♦ Compatible with FCM ♦ Direct quantitation of metabolic regulomes at protein level ♦ Time-saving	♦ Targeted and biased analysis ♦ Potential fluorescence spillover ♦ Costly in antibodies and high-parameter flow cytometers
scMEP	♦ Proteins ♦ Metabolites	♦ Fresh single-cell suspensions	♦ Direct quantitation of metabolic regulomes at protein level ♦ User-friendly for designing high-dimension panel ♦ Without signal overlap ♦ Time-saving	♦ Targeted and biased analysis ♦ Costly in antibodies and mass cytometers

FCM Flow cytometry, *FFPE* Formalin-fixed paraffin-embedded section, *MS* Mass spectrometry, *PESI-MS* Probe electrospray ionization-mass spectrometry, *SCENITH* Single-cell energetic metabolism by profiling translation inhibition, *scMEP* single-cell metabolic regulome profiling, *scRNA-seq* single-cell RNA sequencing, *SLMS* Single-lysosome mass spectrometry, *snRNA-seq* single-nucleus RNA sequencing

human cancer immunometabolism in large populations. We recommend prioritizing the use of these in vivo methods for the efficient discovery and identification of immunometabolic phenotypes. However, it is important to complement these strategies with ex vivo multimodal measurements for additional validation [276–278].

Informatic approaches to analyze and integrate spatiotemporal metabolomics: current development and challenges

With the emergence of innovative technologies uncovering the spatiotemporal metabolomic heterogeneity within tumor-immune interplays, how to appropriately using informatic approaches for interpreting these diverse outputs has become increasingly critical. A

Table 2 Representative spatially resolved methods of metabolomics and metabolic regulomics for analyzing cancer immunity

Method	Targets	Available samples	Characteristics	
			Pros	Cons
MALDI-MSI	♦ Metabolites ♦ Proteins	♦ FFPEs ♦ Fresh-frozen sections	♦ User-friendly commercial solutions for multiple metabolites ♦ Ionizable at atmospheric pressure ♦ Compatible with FFPEs	♦ Matrix effect
DESI-MSI	♦ Metabolites ♦ Proteins	♦ Fresh-frozen sections	♦ Suitable for small molecules ♦ Ionizable at atmospheric pressure	♦ Low spatial resolution: 50–200 μm
SIMS-MSI	♦ Metabolites	♦ Fresh-frozen sections	♦ Highest spatial resolution: 50–100 nm (suitable for subcellular imaging)	♦ Ultrahigh vacuum needed for ionization ♦ Low ionization efficiency
MALDI-2	♦ Metabolites ♦ Proteins	♦ FFPEs ♦ Fresh-frozen sections	♦ High spatial resolution: 0.6–5 μm (suitable for single-cell imaging) ♦ Higher ionization efficiency than MALDI ♦ Compatible with FFPEs	♦ Matrix effect
AFADESI-MSI	♦ Metabolites	♦ Fresh-frozen sections	♦ Current best solution for small molecules ♦ Higher ionization efficiency than DESI ♦ Ionizable at atmospheric pressure	♦ Low spatial resolution: 100 μm
SpaceM	♦ Metabolites	♦ Fresh-frozen sections	♦ Single-cell level imaging ♦ Compatible with MALDI workflow ♦ Simple design and relatively low requirements	♦ No off-the-shelf solutions ♦ Matrix effect
Iso-imaging	♦ Metabolic flux	♦ Fresh-frozen sections	♦ Direct measurement of spatial fluxomics ♦ Compatible with MALDI workflow	♦ Cannot analysis at single-cell level ♦ No off-the-shelf solutions
^{13}C-SpaceM	♦ Metabolic flux	♦ Fresh-frozen sections	♦ Direct measurement of spatial fluxomics at single-cell level ♦ Compatible with MALDI workflow	♦ No off-the-shelf solutions
iST	♦ RNA	♦ FFPEs ♦ Fresh-frozen sections	♦ User-friendly commercial solutions ♦ Subcellular imaging ♦ Diversified toolkits for data analysis ♦ Compatible with FFPEs	♦ Indirect metabolic characterization at mRNA level ♦ Targeted and biased analysis ♦ Costly and time-consuming
sST	♦ RNA	♦ FFPEs ♦ Fresh-frozen sections	♦ User-friendly commercial solutions ♦ Unbiased analysis ♦ Diversified toolkits for data analysis ♦ Compatible with FFPEs	♦ Indirect metabolic characterization at mRNA level ♦ Relatively low spatial resolution ♦ Costly and time-consuming
Multiplexed immunofluorescence-based spatial proteomics (e.g., CyCIF, CODEX)	♦ Proteins	♦ FFPEs ♦ Fresh-frozen sections	♦ Subcellular imaging ♦ Direct quantitation of metabolic regulomes at protein level ♦ Compatible with FFPEs	♦ Targeted and biased analysis ♦ Costly
MSI-based spatial proteomics (e.g., MIBI-ToF)	♦ Proteins	♦ FFPEs ♦ Fresh-frozen sections	♦ Subcellular imaging ♦ Direct quantitation of metabolic regulomes at protein level ♦ Compatible with FFPEs	♦ Targeted and biased analysis ♦ Limited commercial antibodies ♦ Costly

Table 2 (continued)

Method	Targets	Available samples	Characteristics	
			Pros	Cons
CT	♦ Morphological and functional information	♦ Organisms	♦ Non-invasive, real-time and continuous measurement at organismal level ♦ Suitable for clinical and cohort monitoring ♦ Affordable and time-saving	♦ Indirect prediction for immunometabolism ♦ Risk of radiation exposures ♦ Single modal imaging
MRI	♦ Morphological and functional information	♦ Organisms	♦ Non-invasive, real-time and continuous measurement at organismal level ♦ Suitable for clinical and cohort monitoring ♦ Affordable and time-saving ♦ No radiation risk	♦ Indirect prediction for immunometabolism ♦ Single modal imaging
PET-based multiplexed imaging	♦ Morphological and functional information ♦ Spatial and quantitative assessment of specific molecules	♦ Organisms	♦ Direct measurement of immunometabolic dynamics ♦ Non-invasive, real-time and continuous measurement at organismal level ♦ Multimodal imaging workflow ♦ Suitable for clinical and cohort monitoring	♦ Targeted, biased and low-throughout analysis ♦ Limited commercial antibodies ♦ Risk of radiation exposures ♦ Costly

AFADESI Air flow-assisted desorption electrospray ionization, *CODEX* Co-detection by indexing, *CT* Computed Tomography, *CyCIF* Cyclic immunofluorescence, *DESI* Desorption electrospray ionization, *FFPE* Formalin-fixed paraffin-embedded section, *iST* imaging-based spatial transcriptomics, *MALDI* Matrix-assisted laser desorption/ionization; *MIBI-ToF* Multiplexed ion beam imaging by time of flight, *MRI* Magnetic Resonance Imaging, *MSI* Mass spectrometry imaging, *PET* Positron Emission Tomography, *SIMS* Secondary ion mass spectrometry, *sST* sequencing-based spatial transcriptomics

series of algorithms have been developed for analyzing bulk metabolomics and metabolic regulomics. However, they are not suitable for data generated by novel technologies, which tend to be high-dimensional and frequently enriched with additional spatial or temporal information. Although developments have addressed on this issue, numerous challenges persist in advancing our understanding in this field.

(i) Interpreting the high-dimensionality

A critical distinction of spatiotemporal metabolomics and metabolic regulomics, compared to bulk omics, lies in the high-dimensional nature of the data they generate, which enables the analysis of metabolic, transcriptional, and protein profiles for individual cells or pixels. However, these high-dimensional data are often sparse with inevitable noises, and would request excessive computational costs. Thus, it is essential to reduce the dimensionality for further exploration and visualization [112]. In the context of single-cell and spatial transcriptome analyses, tools like Seurat enable efficient dimensionality reduction of data, principally utilizing unsupervised ML algorithms, including principal components analysis (PCA) [279], t-distributed stochastic neighbor embedding (tSNE) [280], and uniform manifold approximation and projection (UMAP) [281]. Following this, clustering is applied to discern subgroups or subregions for further investigation, employing methods like Louvain community detection [282]. Spatiotemporal proteomic analysis follows this workflow. For instance, in Hartmann et al.'s study of the human CRC TME, FlowSOM was utilized for the visualization and clustering of data generated by CyTOF and MIBI-ToF [134, 283]. This method, based on self-organizing maps, is suitable for rapid processing of samples with a large number of cells/pixels [283]. Additionally, tools such as viSNE [284], PhenoGraph [285], and SPADE [286] have been successfully applied to FCM/CyTOF/IMC. This framework is also compatible with single-cell metabolomics; notably, Seurat has been directly adapted as the downstream analysis pipeline of SLMS, facilitating its data denoising, dimensionality detection, and identification of lysosomal subpopulations with distinct metabolic heterogeneity [242].

Overall, the consistency of analytical approaches underscores the profound influence of single-cell and spatial transcriptomics pipelines on the high-dimensional statistics community, paving the way for future endeavors in multi-omics integrations [287]. it is also important to note that, due to the dynamic nature of metabolic processes, sample-to-sample variability in metabolomics

data often exceeds that observed in transcriptomics. Future researchers should evaluate the robustness of those RNA-seq-based analytical methods within the context of metabolomics. We also eagerly await the development of dedicated tools for analyzing high-dimensional features in single-cell and spatial metabolomics. Such tools would be instrumental in identifying metabolic subpopulations that play a pivotal role in tumor-immune interactions, thereby advancing our understanding of these intricate biological processes.

(ii) Integrations with multi-omics and datasets

Integrating metabolomics with other omics would facilitate a comprehensive and multi-dimensional understanding of the immunometabolic landscape. Canonical lineage markers at the protein level have been regarded as the 'golden standard' for immune cell identification. Given the weakness of metabolomics in independently resolving cell identities, the combinations of spatial metabolomics and proteomics, particularly in the context of caner immunology, is gaining increasing attention [182]. For example, the scSpaMet framework (detailed in Sect. 'Spatial metabolomics in the TME') performs image registration to align SIMS and IMC data, subsequently using Cellpose, a deep learning tool, for the precise segmentation of single-cell nuclei [183, 288]. This comprehensive approach facilitates a detailed characterization of both cellular identities and their metabolic compositions. While spatial transcriptomics, notably the sST, often falls short of achieving single-cell resolution for cell identification, it offers significantly higher throughput compared to targeted proteomics. The integration of spatial metabolomics with transcriptomics could provide detailed biological insights through correlation analysis within metabolically active subregions. For instance, Sun et al. [289] employed 10x Visium, AFADESI-MSI, and MALDI-MSI to conduct a comprehensive multi-omics study of the TME in human gastric cancer. By establishing metabolome-transcriptome correlation networks across different sampling sites, they identified metabolic reprogramming signatures at the tumor interface regions enriched with immune cells. These approaches could be used to uncover spatial dynamics of immunometabolic reprogramming in additional cancer types. Looking ahead, we anticipate that the integration of metabolomics with other omics, such as V(D)J sequencing and epigenomics, will enhance our understanding of cancer immunometabolism.

Integrating multiple datasets is another critical consideration, essential for the systematic analysis of cancer immunometabolism with public datasets. The field of transcriptomics has seen the development of several sophisticated tools for datasets integration [203, 290]. For example, Harmony, a popular integration method for scRNA-seq, effectively reduces batch effects by detecting and adjusting similarities between samples in low-dimensional space [291]. Similar tools were also developed for spatiotemporal proteomics. CytofIn, for instance, employs healthy control samples as generalized anchors for batch normalization, enabling to integrate CyTOF data from different sources [292]. Additional integration tools, such as Spectre [293], CyCombine [294], and scMerge2 [295], have each demonstrated substantial efficacy in reducing batch effects of FCM/CyTOF/IMC datasets. These tools could be further used to conduct systematic re-analysis on data with metabolic panels.

Furthermore, it is important to recognize that the higher between-sample variation necessitates effective management of batch effects in metabolomics analysis. To date, no specific measures have been reported for addressing these challenges in single-cell metabolomics. Current strategies in spatial metabolomics analysis primarily focus on standardizing experimental designs and eliminating outliers to minimize intra-dataset variability; however, they do not resolve the issues of inter-dataset integration [296]. The MSI communities, such as METASPACE and MALDISTAR, have made significant contributions to the standardization and quality enhancement in this field. The development of integration tools for spatiotemporal metabolomics is highly anticipated, as it could facilitate the comprehensive mapping of metabolomic atlases across various cancer types, thereby enhancing our understanding of cancer immunometabolism.

Conclusions

This review presents core concepts in cancer immunometabolism, and conducts a systematic review of spatiotemporal immunometabolomic technologies. It emphasizes their key features and discusses their significance in cancer-immunity research and clinical applications. These technologies offer unprecedented insights into the spatiotemporal heterogeneity of metabolism within immune cells in the TME, surpassing traditional metabolic measurements. Nevertheless, the availability of these technologies is variable, with many still in the developmental stage and not yet available for commercial use. We highlight several key challenges that they continue to confront:

First, the current spatiotemporal metabolomics struggle to establish a dominant role in the study of cancer-immunity. As illustrated in Fig. 5a, application of metabolomics (red dashed curve) has lagged behind transcriptomics (blue curve) and proteomics (orange curve) in this field over the past two decades. This disparity persists despite the recent burgeoning of single-cell

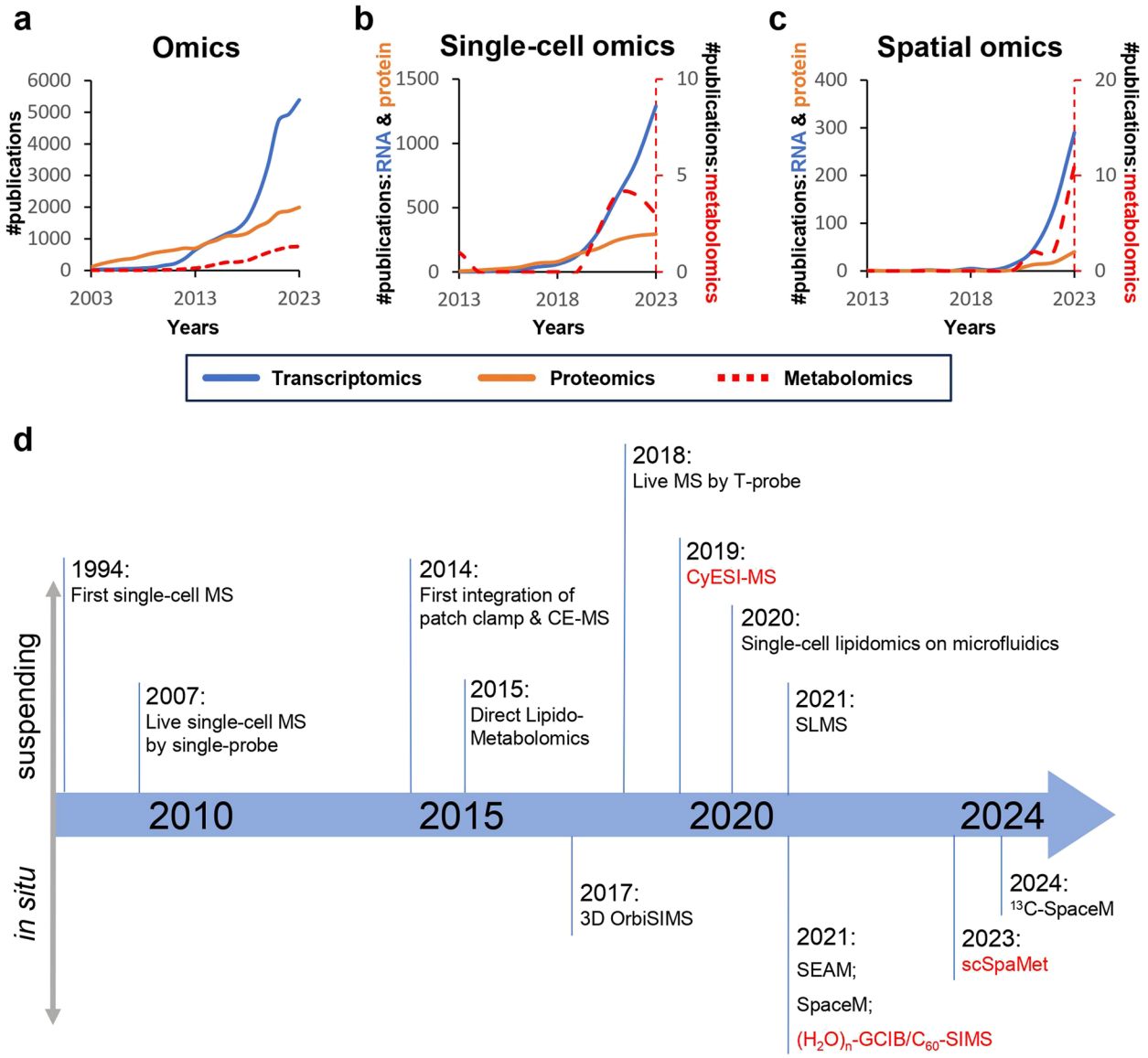

Fig. 5 Publication statistics of spatiotemporal metabolomic measurements in studying cancer-immunity. **a**, **b**, **c** Number of publications focused on caner-immunity (keywords: (((cancer immun*) OR tumor immun*) OR "tumor microenvironment")) and different measurements, including omics (**a**), single-cell omics (**b**), and spatial omics (**c**), reported by PubMed. Blue curves show the total number of publications by adding the following keywords to search: transcriptom* (**a**), ((("single-cell transcriptom*") OR "scRNA-seq") OR "single-cell RNA-seq") (**b**), and (("spatial transcriptom*") OR "spatial RNA-seq") (**c**). Orange curves show the total number of publications by adding the following keywords to search: proteom* (**a**), ((("single-cell proteom*") OR "mass cytometry") OR "CyTOF") (**b**), and (("spatial proteom*") (**c**). Red dashed curves show the total number of publications by adding the following keywords to search: metabolom* (**a**), ((("single-cell metabolom*") OR "single-cell mass spectrom") OR "single-cell MS") (**b**), and (("spatial metabolom*") (**c**). **d** Timeline of the milestone single-cell-level metabolomic techniques. Techniques marked in red have been used to study cancer-immunity. CE, capillary electrophoresis; GCIB, gas cluster ion beam; MS, mass spectrometry; SEAM, spatial single nuclear metabolomics; SIMS, secondary ion mass spectrometry; SLMS, single-lysosome mass spectrometry

and spatial omics (Fig. 5b, c). Although there have been notable advancements in single-cell-level metabolomics to date, such techniques remain underutilized in dissecting tumor-immune interplays (Fig. 5d). A fundamental cause could be the persistently high incidence of false-negative signals in metabolomic data, posing challenges to achieve optimization on par with those of other omics. In single-cell metabolomic analyses, this limitation leads to increased data sparsity compared to other single-cell/spatial omics, complicating the efforts to perform in-depth data analysis. Accordingly, we propose that diminishing signaling loss in the non-targeted metabolomics

is essential for achieving data quality which is comparable with that of other omics. Recently, Guo et al. [297] employed DNA barcodes for chemical labeling of specific amino acids side chains, followed by a quantitative PCR amplification, thus enabling a novel 'amplification sequencing' method for detection of low-abundance proteins. While most metabolites are diverse in structures and cannot be simply amplified, extending the barcoding strategy to the detection of low-abundance metabolites might be a promising avenue for enhancing signals in single-cell metabolomics.

Another significant challenge in understanding metabolic networks is the need to integrate data from multiple sources. How can we effectively analyze the diverse types and scales of data mentioned in this review, as well as any new data that may emerge in the future, to gain a comprehensive understanding of metabolomics [298]? Furthermore, considering the complexity of spatial data analysis, is it possible to develop a suitable model to address the challenges of MAUP and create a unified theory of metabolism that incorporates existing data from the organismal, microenvironmental, and subcellular levels [222]?

The integration and interpretation of multi-sourced data also play a crucial role in shaping research paradigms in cancer immunometabolism [299]. Nonetheless, current data-driven approaches mainly rely on metabolic regulomics, lacking a well-established framework exclusively through multimodal metabolomics [19]. The primary challenge lies in interpreting these complex datasets and the lack of standardized protocols for data processing and sharing, especially in those prototypical methods. With the advancements in generative artificial intelligence (AI) in the field of medicine, could an AI-based approach potentially address these challenges [300–302]?

Moreover, immunometabolism, which is highly responsive to perturbations and varies considerably among individuals, can be considered as the 'immune phenotype'. This makes it a promising biomarker for monitoring responses to immunotherapy. Metabolomics-based spatiotemporal techniques offer rapid and cost-effective strategies for large-scale cohorts in cancer-immunity phenotyping.

In conclusion, the spatiotemporal metabolomic technologies discussed in this review are transforming the field of cancer-immunity research. With advancements in their resolution and accessibility, these technologies are poised to enhance the development of more effective and safer anticancer immunotherapies.

Abbreviations

ACT	Adoptive cell transfer
AFADESI-MSI	Air Flow-Assisted DESI-MSI
AFM	Atomic Force Microscopy
AI	Artificial intelligence
ASCT2	ASC amino-acid transporter 2
BMI	Body mass index
CAFs	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
CE	Capillary electrophoresis
CODEX	Co-detection by indexing
CRC	Colorectal cancer
CT	Computed Tomography
CTCs	Circulating tumor cells
CyCIF	Cyclic immunofluorescence
CyTOF	Cytometry by time-of-flight
DCIS	Ductal carcinoma in situ
DCs	Dendritic cells
DESI-MSI	Desorption electrospray ionization-mass spectrometry imaging
DI-MS	Direct ionization- mass spectrometry
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EFA	Extracellular flux analysis
ESI	Electrospray ionization
FACS	Fluorescence activated cell sorting
FAO	Fatty acid oxidation
FBA	Flux balance analysis
FCM	Flow cytometry
FFAs	Free fatty acids
FFPEs	Formalin-fixed paraffin-embedded sections
FGF21	Fibroblast growth factor 21
FluidFM	Fluidic Force Microscopy
FOV	Field of view
FTICR	Fourier transform ion cyclotron resonance
GC-MS	Gas chromatography-mass spectrometry
GCIB	Gas cluster ion beam
GEMs	Genome-scale metabolic models
GLP-1	Glucagon-like peptide 1
GSH	Glutathione
HFD	High-fat diet
ICBs	Immune checkpoint blockers
ICI	Immune checkpoint inhibition
IDOs	Indoleamine 2,3-dioxygenases
IFNγ	Interferon-gamma
IMC	Imaging mass cytometry
immunoPET	immuno-positron emission tomography
IMS	Ion mobility-mass spectrometry
ISS	In situ sequencing
iST	imaging-based spatial transcriptomics
LAESI	Laser Ablation Electrospray Ionization
LA-ICP	Laser Ablation Inductively Coupled Plasma
LAT1	L-type amino acid transporter 1
LCM-MS	Laser Capture Microdissection coupled with Mass Spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LUAD	Lung adenocarcinoma
MALDESI	Matrix-Assisted Laser Desorption Electrospray Ionization
MALDI-MSI	Matrix-assisted laser desorption/ionization-mass spectrometry imaging
MAUP	Modifiable Areal Unit Problem
MDSCs	Myeloid-derived suppressor cells
MERFISH	Multiplexed error-robust fluorescence in situ hybridization
M-FISH	Multiplexed fluorescence in situ hybridization
MIBI-ToF	Multiplexed ion beam imaging by time of flight
ML	Machine learning
Mono	Monocyte
MRI	Magnetic Resonance Imaging
MS	Mass spectrometry
MSI	Mass spectrometry imaging
MΦ	Macrophage
NGS	Next-generation sequencing
NKs	Natural killer cells
NMR	Nuclear Magnetic Resonance

OCR	Oxygen consumption rate
OT	Orbitrap
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral blood mononuclear cells
PCA	Principal components analysis
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PESI-MS	Probe electrospray ionization-mass spectrometry
PET	Positron Emission Tomography
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PK/PD	Pharmacokinetics or pharmacodynamics
PPAR	Peroxisome proliferator-activated receptor
RCTD	Robust cell type decomposition
RGMb	Guidance molecule b
RNA-seq	RNA sequencing
ROI	Regions of interest
SCENITH	Single-cell energetic metabolism by profiling translation inhibition
SCFAs	Short-chain fatty acids
scFBA	single-cell Flux Balance Analysis
scFEA	single-cell Fluxomics Enrichment Analysis
scMEP	single-cell metabolic regulome profiling
scRNA-seq	single-cell RNA sequencing
SEAM	Spatial single nuclear metabolomics
SIMS-MSI	Secondary ion mass spectrometry-mass spectrometry imaging
SLMS	Single-lysosome mass spectrometry
snRNA-seq	single-nucleus RNA sequencing
ssGSEA	single-sample gene set enrichment analysis
sST	sequencing-based spatial transcriptomics
TAMs	Tumor-associated macrophages
TCA	Tricarboxylic acid
Tconvs	conventional T cells
TI	Tumor-infiltrating
TILs	Tumor-infiltrating lymphocytes
TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
TNFα	Tumor necrosis factor-alpha
TOE	Tumor organismal environment
TOF	Time-of-flight
Tregs	Regulatory T cells
tSNE	t-distributed stochastic neighbor embedding
UMAP	Uniform manifold approximation and projection
VEGF	Vascular endothelial growth factor
VEGFRs	Vascular endothelial growth factor receptors
αPD-1	anti-programmed death 1

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Declarations

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Competing interests
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