Intratumor Heterogeneity Analysis: From Whole Body to Single Cell

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Abstract: Cancer heterogeneity, including both intertumoral and intratumoral heterogeneity (ITH), represents a major challenge in cancer diagnosis, prognosis, and therapeutic response. ITH arises through genetic, epigenetic, and microenvironmental alterations that drive phenotypic diversity and contribute to metastasis, therapy resistance, and disease recurrence. Conventional bulk tumor analyses often fail to detect rare but clinically significant subclonal populations, highlighting the need for higher-resolution analytical approaches.

This review summarizes current methodologies for ITH analysis, including single-cell omics, spatial transcriptomics, molecular imaging, mass cytometry, and integrative radiomics. Spatial techniques preserve tissue architecture and cellular localization, whereas single-cell approaches provide detailed characterization of genomic, transcriptomic, and proteomic variability among individual tumor cells. In addition, multimodal strategies integrating imaging, molecular profiling, and computational analysis offer improved insight into the dynamic interactions between tumor subpopulations and their microenvironment.

We further discuss major technical and analytical limitations associated with current ITH methodologies, including amplification bias, dissociation-induced transcriptional artifacts, loss of spatial information, reproducibility challenges, and difficulties in integrating multi-modal datasets. Emerging artificial intelligence and machine learning approaches may help address some of these limitations through automated image analysis, multimodal data integration, and predictive modeling, although issues related to interpretability, standardization, and external validation remain significant barriers to clinical translation.

Overall, comprehensive characterization of ITH through multi-region and multi-omics approaches may improve precision oncology by enabling more accurate identification of aggressive, treatment-resistant, and metastatic tumor subpopulations. Continued advances in spatially resolved and single-cell technologies, together with robust computational frameworks, are expected to enhance the understanding of tumor evolution and support the development of more adaptive therapeutic strategies.

Keywords: Cancer Heterogeneity, Subpopulation, Intratumor heterogeneity, Single-Cell Analysis, Spatial Methods.

INTRODUCTION

Heterogeneity in cancer refers to differences between different tumors of the same type, known as intertumoral heterogeneity, or differences within a tumor's cell population, known as intra-tumor heterogeneity (ITH). These differences are characterized by variations in cellular morphology or biomarkers associated with the disease [1], ranging from specific protein expression levels to mtDNA copy number.

ITH is not a static phenomenon, as it changes during tumor growth and cancer progression due to factors such as hypoxia [2]. Its tendency to increase as tumors grow, fluctuate during anti-cancer treatments, and its association with poor clinical prognosis highlight its importance [3].

ITH is a result of genetic mutations and/or modifications at the epigenetic level, leading to various phenotypes within the same cell type. In addition to intrinsic variables within the cell population, the tumor microenvironment can also influence ITH [4].

Morphological intratumor heterogeneity can be studied through pathological analyses. For instance, various research groups have endeavored to categorize Gastric Cancer based on morphological features [5]. A recent study on pancreatic cancer delved into intratumor heterogeneity, focusing on structural and functional diversity and categorizing tumors according to their morphological characteristics [6].

Since morphological intratumor heterogeneity does not fully capture the overall heterogeneity governing tumors, researchers have directed their efforts towards associating morphological features with gene expression patterns to establish more meaningful relationships between morphological ITH and clinical outcomes [6, 7]. While these studies aim to integrate gene expression data and correlate it with morphological features, some mouse model studies underscore the strong dependence of ITH on changes

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in genomics, transcriptomics, or epigenomics levels, even in the absence of obvious morphological alterations [8].

For example, gene expression levels for crucial biomarkers such as Ki67 in breast cancer [9] or pancreatic cancer [6] differ among cells with similar morphology. Consequently, omics studies remain an integral component of understanding ITH alongside morphological and pathobiological concepts.

Studying the bulk population alone does not allow for a comprehensive understanding of the phylogenetic tree within the tumor cell population, as informative and rare cell populations are often ignored [10] as shown in Figure **1**. Metastasis, for example, is caused by a rare population of tumor cells with unique properties. These cells originate from the primary tumor [11-13].

A significant drawback in cancer treatment is viewing cancer as a homogeneous disorder and targeting a single pathway or receptor [14]. Relying on a single biopsy to determine the tumor's state underestimates the abilities of the tumor population [15]. Multi-site tumor sampling is proposed to uncover more details about ITH and provide a broader understanding of the tumor's state [16]. Unfortunately, even new therapeutic programs focusing on precision medicine do not fully consider ITH in their approach [14].

ITH can also be understood as spatial heterogeneity, where the degree of heterogeneity in different regions of a primary tumor indicates the presence of different subclones [17]. For example, in primary renal cell carcinoma, metastasis often arises from specific regions of the tumor [15]. Integrating multi-omics analysis that accounts for ITH and spatial heterogeneity within primary tumors can improve diagnosis, targeting, and treatment in cancer [3].

HETEROGENEITY IN CULTURE AND TUMORS

During the growth of a tumor, there is an accumulation of genetic mutations that create different subclones. However, not all of these subclones contribute to the overall tumor burden due to the tumor environment being unsuitable for their growth [18]. Research has shown that a small number of tumor cells, around five, exhibit around 70% of the expression profile of the entire population [19]. Different subpopulations within the tumor mass have varying proportions and play important roles in cancer progression, drug resistance, and pre-metastatic cells. These subpopulations exist as rare subsets in the primary tumor [20]. The different subclones that arise during tumor growth have varying proportions and provide diverse sources for studying intra-tumor heterogeneity [21].

In addition to studying intra-tumor heterogeneity using primary tumor cells (PTCs), other sources such

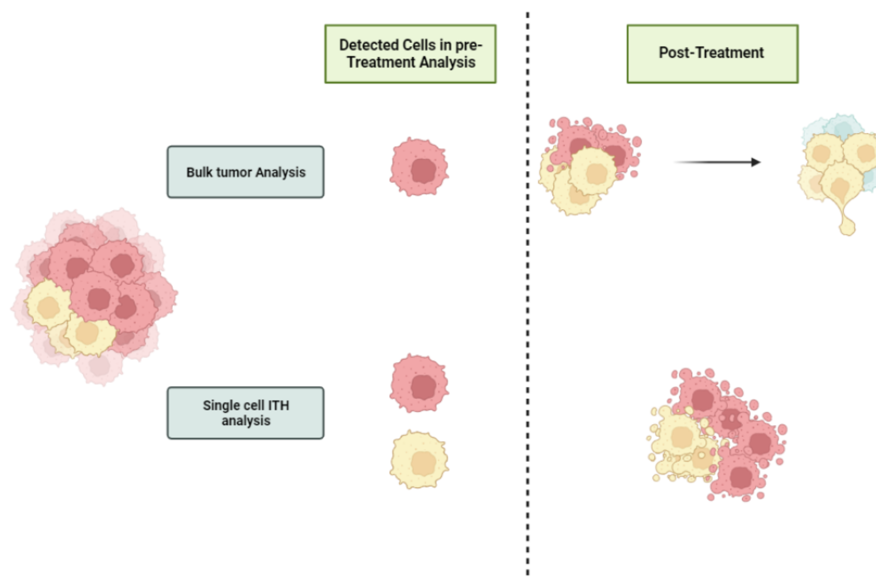

Figure 1: Importance of considering Intra Tumor heterogeneity: Neglecting ITH in bulk tumor analysis overlooks rare subpopulations with distinct pathways, such as drug-resistant pathways, leading to the potential for recurrence or metastasis post inadequate treatment. However, employing appropriate analysis methods capable of identifying these subpopulations enhances the likelihood of selecting a more effective therapeutic plan, thereby improving the overall success of treatment (Created with BioRender.com).

as metastatic tumor cells (MTCs) and circulating tumor cells (CTCs) can be utilized. Circulating tumor cells are considered a reliable and noninvasive window into the landscape of intra-tumor heterogeneity [14]. Although isolating and analyzing circulating tumor cells poses challenges due to their low population, they can play a crucial role in diagnosing and planning treatments before and after therapy.

Cell lines can also be used to study cancer heterogeneity [22]. For example, a bladder carcinoma cell line called UCRU BL 17CL has demonstrated heterogeneity in both histology and functional level due to the existence of around nine sublines. This makes it an appropriate model for studying tumor heterogeneity [23]. Another example is the cell line COLO829, in which single cell analysis revealed subclone heterogeneity even in chromosome 8 copy number within individual cells [24]. Even the culture conditions in cell lines can influence heterogeneity. For instance, using a higher passage number, up to 15, can demonstrate heterogeneity even in non-cancerous cell lines such as the ARPE-19 cell line, which is a spontaneously transformed line of the human RPE (retinal pigment epithelium) [25].

Although cell lines can demonstrate tumor heterogeneity, they may not fully illustrate the truly heterogeneous nature observed in genomic and non-genomic profiles [4, 26].

There are various methods available for analyzing heterogeneity (ITH), which can be categorized as spatial and non-spatial methods according to their impact on space (Figure **2**). Spatial heterogeneity can be assessed using methods that utilize the entire body or tissues. In other hand these methods can divided as unimodal or multimodal methods.

SPATIAL AND NON-SPATIAL METHODS

Spatial ITH Analysis Methods

In the field of clinical and research radiology, traditional imaging methods such as Positron Emission Tomography (PET) are used to determine general tumor features like size. However, in molecular imaging, additional tracers like 18F-fluoro-deoxyglucose (FDG) and radiopharmaceuticals are employed to observe the heterogeneous distribution and uptake, resulting in reflection of ITH [27]. Advanced imaging technologies such as Diffusion Weighted Imaging (DWI), Diffusion Tensor Imaging (DTI), Dynamic Susceptibility Contrast (DSC)-MRI, Dynamic Contrast Enhanced (DCE)-MRI, and MR Spectroscopy (MRS) can enhance the detection of ITH [28].

In addition to conventional molecular imaging, the integration of imaging technology with pathology, genomics, and computational analysis, commonly referred to as radiomics and radiogenomics, provides a more comprehensive framework for investigating spatial intratumor heterogeneity (ITH). Radiomic approaches extract quantitative imaging features related to tumor shape, texture, signal intensity, vascularization, and metabolic activity from modalities such as MRI, CT, and PET. These features can subsequently be correlated with histopathological findings, genomic alterations, and clinical outcomes to characterize biologically distinct tumor regions [27, 29].

Radiogenomic studies have demonstrated that imaging-derived heterogeneity patterns may reflect underlying molecular and genomic subclones. For example, MRI and PET imaging combined with pathological analysis have revealed spatial associations between radiologic features and genomic heterogeneity in high-grade serous ovarian cancer. Similarly, advanced MRI-based radiomic analyses have been utilized to investigate invasive phenotypes and treatment resistance in glioblastoma. Such integrative approaches may improve noninvasive assessment of tumor evolution and therapeutic response [30].

Despite their potential, radiomic methods face substantial challenges regarding reproducibility and standardization. Variability in scanner hardware, acquisition parameters, image reconstruction algorithms, segmentation protocols, and preprocessing workflows can significantly influence extracted radiomic features and reduce cross-study comparability. In addition, batch effects and feature instability may compromise the robustness of predictive models, particularly in multicenter studies. Consequently, standardized imaging protocols and harmonized computational pipelines are necessary to improve the clinical reliability of radiomic biomarkers [31].

A novel whole-body imaging approach utilizing transparent rodent models, known as vDISCO, has further expanded the ability to investigate spatial heterogeneity across the entire organism. Using tissue clearing, multiplex fluorescent labeling, and deep learning-assisted image analysis, this method enables visualization of disseminated tumor cells and micro-metastatic subclones at near single-cell resolution throughout the body. These approaches provide

valuable opportunities for studying metastatic dissemination and tumor evolution within intact biological systems [32].

However, whole-body imaging frameworks such as vDISCO remain technically and computationally demanding. Accurate quantitative analysis requires robust image registration, signal normalization, segmentation algorithms, and high-capacity computational infrastructure. Furthermore, reproducibility may be affected by variability in tissue clearing procedures, fluorescent labeling efficiency, imaging depth, and deep learning model performance across datasets. Although these technologies hold substantial research potential, further validation and standardization are required before broader translational implementation becomes feasible [32].

On a tissue level, spatial methods for uncovering ITH can be performed using conventional Immunohistochemistry (IHC). However, the intensity of labeling in IHC can vary due to non-pathological properties, making it less reliable for assessing heterogeneity. Alternatively, digital slide pathology and image analysis methods provide a more reliable approach for quantifying the expression of biomarkers that reflect ITH [33].

Fluorescent In Situ Hybridization (FISH) techniques, such as single-molecule FISH (smFISH), are now commonly used for detecting spatial ITH. smFISH (Table **1**) allows for the observation of different aspects of RNA, such as copy number and spatial organization, with single-cell resolution. It can be performed on individual cells or within a tissue context [34]. The advantage of smFISH is its ability to detect RNA copy numbers without amplification bias [35]. Despite its benefits, smFISH has a limitation in the number of RNA species that can be detected simultaneously [36]. Recent advancements have increased the number of RNA measurements to 10-30 species simultaneously using methods such as FISSEQ, seqFISH, and MERFISH, which have the capability to measure 140 RNA species simultaneously [37, 38].

Another FISH-based method, known as multiplex interphase FISH (miFISH), enables the detection of DNA copy numbers in interphase cells. As genomic

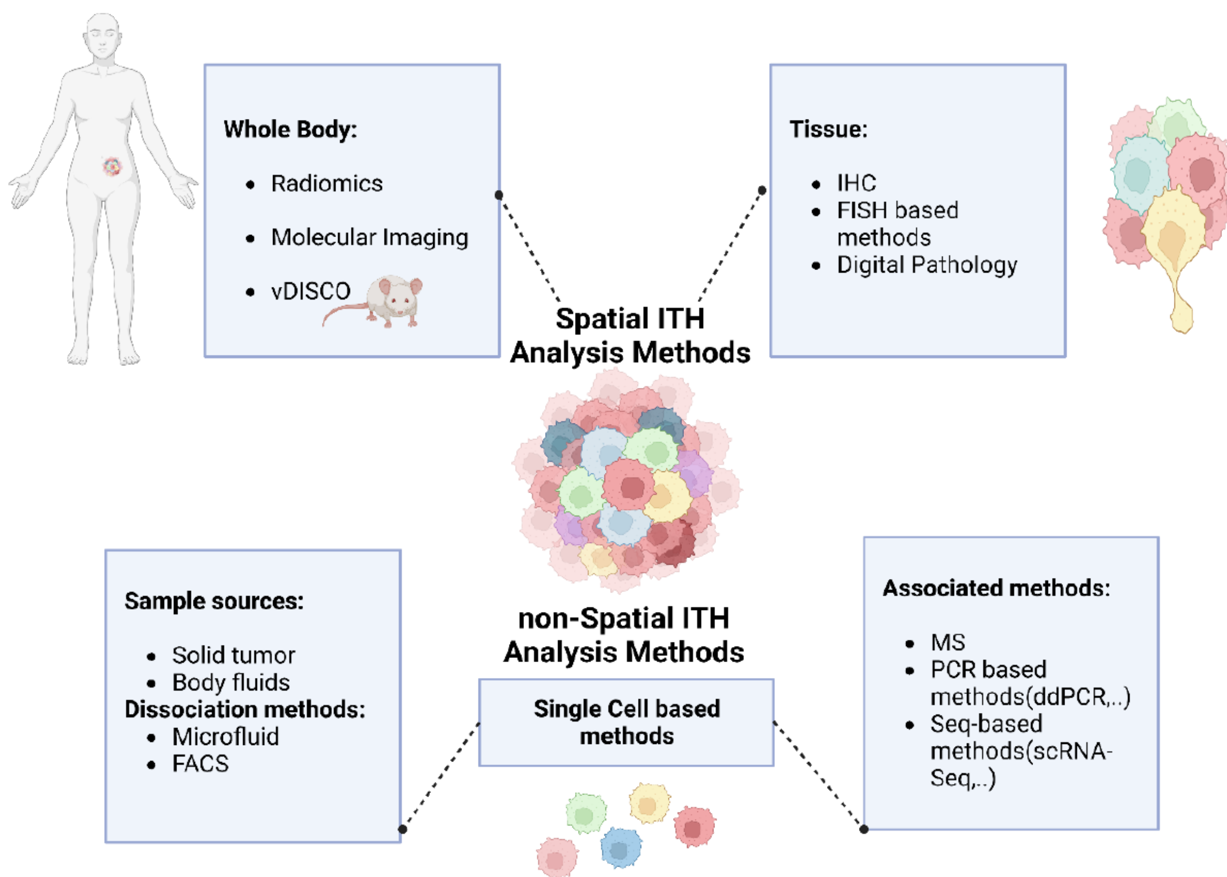

Figure 2: Intratumor Heterogeneity (ITH) Analysis methods: Spatial vs non-Spatial methods (IHC: Immunohistochemistry, FISH: Fluorescent In Situ Hybridization, FACS: Fluorescence-activated cell sorting, MS: Mass spectrometry, ddPCR: droplet digital PCR, scRNA-Seq: single cell RNA sequencing) (Created with BioRender.com).

Table 1: Presents a Comparative Overview of Various In Situ Transcriptomics Techniques

Technique	Full Name	Max RNA Species (Approx.)	Amplification	Sequencing/Imaging Cycles	Strengths	Limitations
smFISH [34]	Single-molecule Fluorescence In Situ Hybridization	~1–5	No	1	High spatial resolution, single molecule	Low multiplexing
MERFISH [40]	Multiplexed Error-Robust FISH	~10,000 (with barcoding)	No	Many	High multiplexing, error correction	Complex barcoding and decoding
seqFISH [38]	Sequential FISH	~10^4	No	Sequential hybridizations	High-throughput, spatial resolution	Time-consuming, optical crowding
FISSEQ [37]	Fluorescent In Situ Sequencing	1,000s (theoretical)	Yes	Sequencing-based	Unbiased, de novo RNA detection	Low sensitivity, difficult read mapping
osmFISH [41]	Optimized Single Molecule FISH	~100	No	Sequential imaging	Improved resolution, whole tissue imaging	Medium throughput
STARmap [42]	Spatially Resolved Transcript Amplicon Readout Mapping	~1,000	Yes	Sequencing-based	3D mapping, high resolution	Moderate multiplexing
ExFISH [43]	Expansion Microscopy FISH	10–100	No	After tissue expansion	Super-resolution spatial data	Complex sample prep
HybISS [44]	Hybridization-based In Situ Sequencing	100s	Yes	Iterative sequencing	Compatible with FFPE*, relatively fast	Lower sensitivity than some FISH methods

Comparative overview of in situ transcriptomics techniques. The table highlights key features of major in situ transcriptomic methods. *FFPE: Formalin-Fixed, Paraffin-Embedded.

instability is associated with ITH, this method can be utilized to determine spatial heterogeneity [39].

Non Spatial ITH Analysis Methods

Non-spatial methods utilize single-cell technology to dissociate individual cells within a tumor to obtain more detailed information at the single-cell level. These methods have been shown to reveal the simultaneous occurrence of over 10,000 somatic mutations in a tumor subpopulation [45]. Although spatial information is not captured in these methods, they have been widely adopted in recent studies on ITH.

Different protocols involve various ways of capturing cells, preparing libraries, and determining throughput. However, the initial step in these methods is to digest the solid tumor [46], removing large debris and blocking cellular activity [47]. In cases where tissue cannot be easily dissociated or preserved, alternative methods such as DroNc-seq or microwell-based single-nucleus RNA-seq are available [48]. It is worth noting that tumor dissociation can affect the RNA transcriptome [49].

After preparing the cell suspension, the next step involves isolating individual cells. Microfluidics and Fluorescence-activated cell sorting (FACS) are commonly used high-throughput methods that provide accurate and automated isolation of unbiased samples [49]. FACS is the most widely used approach in these studies [50]. Prior to FACS, the cellular suspension is stained with suitable stains to attach to target molecules. Additionally, a viability stain is used to determine viable cells for further experimentation [47].

Once sorted by FACS, cells can be cultured [51] to obtain additional genetic or non-genomic material, or they can be deep-frozen for preservation and further experiments [47]. Mass spectrometry (MS) can be employed after FACS to investigate ITH at the proteome level [52]. Label-free MS has been used to discover heterogeneity in tumor metabolome [53]. Mass cytometry, which combines flow cytometry and mass cytometry using a time-of-flight ICP-MS instrument [54], allows the simultaneous study of over 30 markers [55]. Metal isotopes are tagged to antibodies in mass cytometry, enabling subcellular

resolution without compromising antigen specificity [56, 57].

For body fluid samples such as whole blood, plasma, and serum, droplet digital PCR (ddPCR) can be performed directly on the samples without the need for cell sorting using FACS [58].

In terms of analysis, single-cell methods can encompass different "omics" aspects such as genomics, epigenomics, proteomics, etc. Genomics can involve studying the whole genome or a panel of targeted genes [59]. However, there are challenges in single-cell genome studies, such as allelic dropout, which can result in regions of one chromosome not being amplified [20, 60]. To minimize amplification errors, alternative genomics variables such as SNV or CNV can be studied [59].

Single-cell RNA sequencing (scRNA-seq) allows for the analysis of the entire transcriptome from single cells. The appropriate protocol should be selected based on various parameters, such as sample size [14]. In addition to experimental steps, the analytical process requires alternative normalization methods, such as DESeq2 [61].

In transcriptomics, a significant challenge is detecting lowly expressed genes, such as transcription factors. Missing the detection of signaling intermediates during scRNA-seq can complicate the determination of whether a pathway is activated or not. Although scRNA-seq reveals individual heterogeneity that may be masked by bulk RNA studies [14], it is important to note that most gene ontology and gene enrichment databases are based on bulk studies. Therefore, assigning function to pathways may be problematic at the single-cell level [62]. Moreover, scRNA-seq introduces more technical noise and bias compared to bulk studies [63]. Validation experiments using conventional methods should be used to verify the obtained data [14].

Other protocols focus on different regulatory levels, such as epigenomics, proteomics, and metabolomics at the single-cell level. For instance, studying the proteome and lipidome using MS can provide single-cell resolution of ITH [64]. Combining multiple omics methods, such as genome-transcriptome multi-omics studies, can enhance the power of ITH studies [14].

Regardless of the method used for single-cell analysis of ITH, it is important to recognize that the behavior of a tumor is not simply the sum of its individual cell characteristics. The interaction between cell populations is a complex matter that should be considered [14].

CHALLENGES AND CLINICAL TRANSLATION OF ITH ANALYSIS

Despite substantial advances in spatial and single-cell technologies, several technical and analytical challenges continue to limit the routine clinical implementation of intratumor heterogeneity (ITH) analysis. One of the major limitations of single-cell sequencing approaches is technical noise introduced during sample preparation, amplification, and library construction. Amplification bias, allelic dropout, and low transcript capture efficiency may influence the accurate detection of rare subclonal populations and lowly expressed genes. In addition, tissue dissociation procedures required for many single-cell protocols can induce artificial transcriptional changes and disrupt native cellular interactions [42, 61, 65, 66].

Spatial methods preserve tissue architecture and microenvironmental context; however, these techniques also face important limitations. Many high-resolution spatial transcriptomic approaches remain costly, technically demanding, and computationally intensive. Optical crowding, limited multiplexing capacity in some FISH-based methods, and challenges in image registration and signal quantification may reduce analytical reproducibility. Similarly, radiomic and imaging-based analyses are highly sensitive to scanner variability, acquisition parameters, image reconstruction settings, and segmentation methods. These factors can generate substantial batch effects and reduce cross-platform reproducibility [15, 43, 67-69].

Another major challenge involves the integration and interpretation of multimodal datasets. Combining genomics, transcriptomics, proteomics, imaging, and spatial information requires extensive preprocessing, normalization, and harmonization of heterogeneous data types. Differences in sequencing depth, sample preservation, computational pipelines, and imaging platforms can significantly influence downstream analyses and complicate inter-study comparisons. Consequently, the lack of standardized analytical workflows remains a major barrier to reproducibility and clinical translation [62, 70, 71].

Artificial intelligence (AI) and machine learning methods are increasingly being utilized to address some of these challenges. Deep learning approaches have demonstrated utility in automated cell segmentation, feature extraction from histopathological and radiologic images, spatial pattern recognition, and

predictive modeling of therapeutic response. Machine learning frameworks may also improve multimodal data integration by identifying relationships between molecular, spatial, and imaging-derived features. However, many AI-based models remain limited by insufficient external validation, poor interpretability, overfitting risks, and dependence on large high-quality annotated datasets [72, 73].

For clinical implementation, future efforts should prioritize standardized quality-control metrics, reproducible computational pipelines, multicenter validation studies, and protocols compatible with routinely available clinical specimens such as formalin-fixed paraffin-embedded (FFPE) tissues. Addressing these challenges will be essential for translating ITH analysis from research settings into robust precision oncology applications.

UNIMODAL AND MULTIMODAL METHODS

Unimodal approaches analyze tumors using a single analytical platform, such as genomic sequencing, transcriptomics, proteomics, or imaging. These methods provide highly focused information regarding a specific biological dimension of intratumor heterogeneity (ITH). For example, single-cell genomic sequencing can identify subclonal mutations and copy number variations that may remain undetected in bulk sequencing studies [10]. Similarly, imaging-based unimodal approaches such as magnetic resonance imaging (MRI), computed tomography (CT), or positron emission tomography (PET) provide structural, metabolic, or physiological information regarding tumor heterogeneity. Because these techniques are optimized for a specific modality, they often achieve high analytical sensitivity and resolution within their respective domains.

Despite these advantages, unimodal methods incompletely capture the biological complexity of tumors. Genetic alterations alone may not fully explain phenotypic diversity, therapeutic resistance, or microenvironmental interactions. Likewise, imaging methods may reveal spatial heterogeneity without identifying the molecular mechanisms underlying these variations. As a result, reliance on a single modality can limit the interpretation of tumor evolution and functional heterogeneity [29, 74, 75].

Multimodal approaches aim to overcome these limitations through the integration of complementary datasets derived from multiple analytical platforms. Combining genomic, transcriptomic, proteomic, spatial, and imaging data enables a more comprehensive characterization of tumor biology and cellular interactions. For example, radiogenomic studies integrating MRI or PET imaging with genomic profiling have demonstrated correlations between imaging-derived heterogeneity patterns and molecular subclones in glioblastoma and ovarian cancer. Similarly, integration of spatial transcriptomics with single-cell RNA sequencing has improved the identification of tumor–immune niches and spatially organized cellular interactions that may not be resolved using dissociated single-cell approaches alone.

The principal advantage of multimodal strategies lies in their ability to connect molecular alterations with spatial organization and functional behavior. Integrative analyses can reveal how genomic changes influence tumor metabolism, immune infiltration, angiogenesis, and therapeutic response across different tumor regions. These approaches are therefore increasingly important for precision oncology, particularly in identifying treatment-resistant subpopulations and predicting disease progression [76, 77].

Recent advances in artificial intelligence (AI) and machine learning have further accelerated multimodal ITH analysis. Deep learning frameworks can assist in automated cell segmentation, feature extraction from pathology or radiologic images, integration of large-scale omics datasets, and prediction of treatment response patterns. AI-based methods have also been applied to identify spatial tumor architectures and infer cellular interactions from complex datasets. However, important challenges remain, including limited interpretability of some machine learning models, overfitting risks, variability in imaging acquisition protocols, and the need for large standardized datasets for external validation.

Despite their considerable potential, multimodal methods remain computationally demanding and often require extensive preprocessing, normalization, and harmonization of heterogeneous datasets. Differences in sequencing platforms, imaging protocols, sample preparation methods, and batch effects can significantly influence analytical reproducibility. Consequently, standardized computational pipelines and validation frameworks are essential before multimodal ITH analysis can be routinely translated into clinical practice [76-78].

CONCLUSION

Intratumor heterogeneity is a fundamental driver of cancer progression, treatment resistance, and

relapses, necessitating advanced analytical approaches to unravel its complexity. While traditional bulk analyses fail to capture rare but aggressive subclones, spatial and single-cell technologies , such as imaging-based transcriptomics, mass cytometry, and multi-omics integration, are reshaping our understanding of tumor biology. These methods reveal the dynamic interplay between genetic, epigenetic, and microenvironmental factors that govern ITH, providing critical insights for precision medicine.

Despite these advancements, challenges persist, including technical noise in single-cell data, the loss of spatial context in dissociated samples, and the integration of multi-modal datasets. Future research must focus on bridging these gaps, leveraging artificial intelligence and longitudinal studies to track ITH evolution during therapy. By embracing a holistic view of tumor heterogeneity, clinicians and researchers can develop more effective, adaptive treatment strategies that target not just the dominant tumor population, but also the elusive subclones responsible for recurrence and metastasis. Ultimately, overcoming ITH will require a paradigm shift in oncology from static, single-biopsy diagnostics to dynamic, multi-faceted profiling that mirrors the true complexity of cancer.

DECLARATION OF STATEMENT

The authors declare no conflicts of interest associated with the publication of this work. All authors have approved the final version of the manuscript and agree to its submission.

ETHICS STATEMENT

This study did not involve human participants, animal subjects, or clinical trials, and therefore, no ethical approval was required. The work presented is based on a hypothesis-driven conceptual framework and a review of existing scientific literature. Any referenced studies involving human or animal subjects adhered to the ethical guidelines and approvals stated in their respective publications.

CREDIT AUTHOR STATEMENT

Somaye Zareian: Conceptualization, Methodology, Writing – Original Draft Preparation, Visualization.

Soroush Sardari: Supervision, Validation, Writing – Review & Editing, Project Administration.

All authors have read and approved the final manuscript.

DECLARATION OF AI ASSISTANCE

The AI was utilized to refine the language, improve readability, and ensure consistency across the document. All scientific content, hypotheses, and ideas presented are the authors' original work, and the AI was used solely as a tool to enhance the presentation of the manuscript. The final version has been thoroughly reviewed and approved by all authors.

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