Trends in Cancer

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Review

The Spatial and Genomic Hierarchy of Tumor Ecosystems Revealed by Single-Cell Technologies

Eric A. Smith[1] and H. Courtney Hodges [1,2,3,4,*,@]

Many malignancies display heterogeneous features that support cancer progression. Emerging high-resolution methods provide a view of heterogeneity that recognizes the influence of diverse cell types and cell states of the tumor microenvironment. Here we outline a hierarchical organization of tumor heterogeneity from a genomic perspective, summarize the origins of spatially patterned metabolic features, and review recent developments in single-cell and spatially resolved techniques for genome-wide study of multicellular tissues. We also discuss how integrating these approaches can yield new insights into human cancer and emerging immune therapies. Applying these technologies for the analysis of primary tumors, patient-derived xenografts, and *in vitro* systems holds great promise for understanding the hierarchical structure and environmental influences that underlie tumor ecosystems.

Tumor Heterogeneity and Cell Plasticity

The cellular mechanisms that underlie malignancy are diverse, plastic, and adaptable. Many tumors display distinct compartments and heterogeneous phenotypes, with tumor progression manifesting aspects of ecosystems that adapt and evolve. This diversity is a key aspect of many cancers that contributes greatly to tumor progression and treatment outcomes [1]. Unfortunately, our current ability to dissect the mechanistic origins of this plasticity remains a persistent challenge. As a result, one major goal for the foreseeable future of cancer biology is to develop new high-resolution technologies capable of dissecting heterogeneous tumors to uncover the unique vulnerabilities operating within each microenvironment.

Genome-wide analysis of bulk tissues has significantly advanced our understanding of carcinogenesis [2], and emerging single-cell variants of these technologies are providing powerful opportunities to reveal new features of complex tissues containing mixed cell types and states. Single-cell and spatially resolved investigations of tumors have the capacity to reveal context-dependent mechanisms and other spatially restricted cues governing tumorigenesis, metastasis, and response to treatment. Hence, techniques capable of characterizing heterogeneous cell states and processes in malignancy have become important tools to identify previously hidden features of cancer.

In this review, we focus on exciting recent genomic and imaging-based technologies that permit high-resolution dissection of cancer processes at the single-cell level. We begin by highlighting the recurrent themes and chief contributors to phenotypic heterogeneity in tumors, and propose a hierarchy of tumor heterogeneity involving cell identities and epigenetic-metabolic states. We then review new high-resolution approaches, addressing both genomic and optical methods to characterize epigenomic and phenotypic states at the single-cell level. Together, these techniques are revealing previously unknown interactions in multicellular tissues that contribute to tumor progression and treatment response.

Highlights

Recent developments in single-cell approaches have provided an avalanche of data regarding tumor heterogeneity in many tumor settings. There remains a great need to systematize and categorize these data to yield biological insights about tumor function.

Many key features of malignancy are mediated through interactions between different cell types and the influence of the local tumor microenvironment.

We describe a conceptual framework for analyzing the hierarchy of tumor heterogeneity involving cell types and cell type-specific states.

The interactions of tumor development, progression, and varying microenvironments give rise to a spatial hierarchy of tumor heterogeneity.

New technologies for *in situ* genomics enable genome-wide study of tumor features while preserving spatial information of microenvironment features.

[1]Department of Molecular and Cellular Biology and Center for Precision Environmental Health, Baylor College of Medicine, Houston, TX 77030, USA
[2]Dan L Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA
[3]Center for Cancer Epigenetics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[4]Department of Bioengineering, Rice University, Houston, TX 77005, USA

*Correspondence: chodges@bcm.edu (H.C. Hodges).
@Twitter: @hodgeshc

The Hierarchy of Tumor Heterogeneity

Bulk tumor populations contain several cell types, including malignant cells and nonmalignant stromal cells that support or oppose their growth. Although each individual cell within a tumor adopts a gene expression pattern governed by its cell identity, these patterns adapt in response to cell-extrinsic factors and the local microenvironment. The exposure to specific local cues is therefore an important source of heterogeneity for cells sharing the same identity.

These observations led us to propose a general hierarchy describing all sources of heterogeneity operating within a tumor. At the first level, tumor populations are defined in terms of cell type or identity (Figure 1). Cells can be segmented based on characteristic inherited features (e.g., surface markers or cell-specific gene expression patterns), allowing classification of tumors into either malignant cancer cells or nonmalignant cells, such as immune cells, fibroblasts, and other stromal cells. These traits can generally be considered irreversible, based on the high epigenetic barrier to altering cell identity [3–5]. At the second tier of the hierarchy, the individual cell types can be further categorized based on cell type-specific phenotypic states, often influenced by features of the tumor microenvironment. For example, cancer cells may be grouped based on whether they are undergoing oxidative phosphorylation or Warburg metabolism [6]. The variation at this tier reflects cell-type plasticity, and in contrast to the first tier, these features are reversible based on ectopic signals and environmental conditions. Another example is the distinct set of states associated with immune cell activation, as seen in tumor associated macrophages (TAMs), which have different expression patterns and functions based on the pathway of their activation [7]. Yet another example is the transition of cancer cells into more aggressive states that promote invasion and metastasis, often referred to as epithelial-mesenchymal transition (EMT) [8,9]. These and other cell type-specific state classifications are often associated with important consequences for tumor progression and play important roles in diagnosis and treatment [9].

The ability of tumor cells to adopt cell type-specific states has been widely known for decades. A prime example of tumor heterogeneity at the level of cell state can be visualized using fluorodeoxyglucose (FDG), a glucose analog that marks malignant cells by their increased glucose uptake. Positron emission tomography imaging of FDG is widely used in the clinic to map the diverse rates of glucose utilization between and within tumors [10]. The resulting

Glossary

ATAC: a high-throughput genome-wide assay for chromatin accessibility.
CUT&RUN: a sensitive alternative to ChIP-seq that uses micrococcal nuclease (MNase) fused to protein A.
CyTOF: a mass spectrometry analog to flow cytometry that measures the single-cell abundance of many protein targets.
Multiregion sequencing: dissection of a tumor into separate discrete regions to map the variation of its features over space.
tSNE: an approach to plot high-dimensional genome-wide data that places similar cells close together on a 2D graph.
UMAP: a fast plotting procedure similar to tSNE that better preserves global structure and whose output can be used for further downstream analysis.

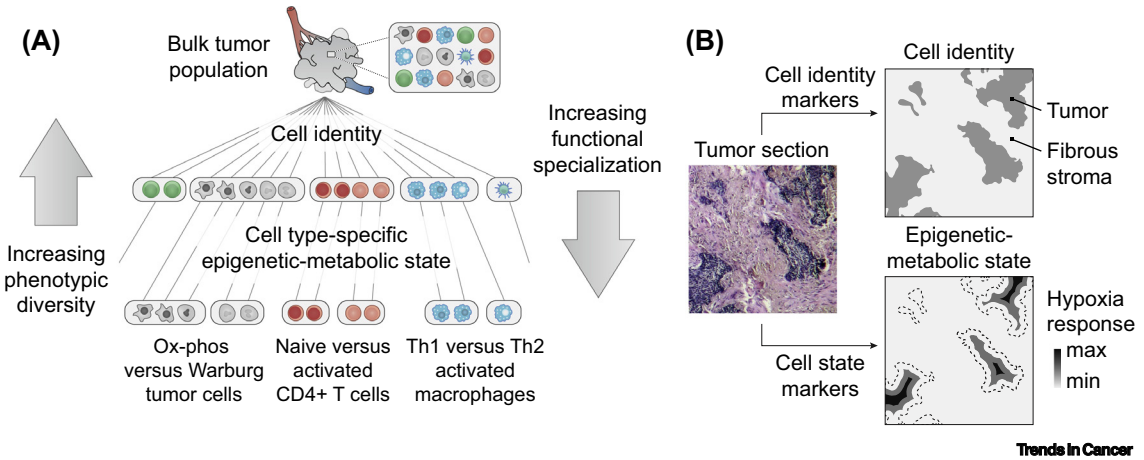

Figure 1. The Hierarchy of Tumor Heterogeneity. (A) Bulk tumor populations can be divided into distinct cell types from different lineages. Cells with similar cell identities are present in distinct microenvironments, which affect their epigenetic-metabolic states. Hence capturing the full functional specialization present in tumors requires finer classification of cells into distinct cell type-specific states. (B) Gene expression patterns vary spatially within the same cell type based on tumor microenvironment conditions.

heterogeneity is illustrative of other sources of heterogeneity that also have important functional consequences. For example, in several cancers, differences in tumor progression arise based on varied expression of multidrug transporters [11], hormone receptors [10,12], cytokines [13–16], and neoantigens [17–19]. In several of these cases, treatments focused on cellular identity alone often result in treatment-resistant phenotypes [12,20]. However, characterizing the entire spectrum of accessible cellular phenotypes provides an avenue to uncover new or shared vulnerabilities [21–23]. Elsewhere in nature, loss of a key player within an ecosystem can lead to destabilization of that ecosystem. Similarly, targeting specific vulnerabilities within portions of heterogeneous tumors may lead to destabilization of the tumor ecosystem, offering new potential therapeutic windows.

Defining the hierarchy of tumor heterogeneity in this way focuses on cell identities and cell states, however, many tumors develop distinct genetic changes through clonal or nonclonal evolution [1,24]. For example, a founding cancer cell can give rise to genetically diverse malignant cells through a series of clonal expansions [25–27]. Each of these expansions is linked to the conferral of distinct selective advantages that may vary both in time and space based on the local microenvironment. We posit that selective pressures operating on these cells are relevant to the cancer only insomuch as they alter a phenotypic property of the cells or tumor system. For example, genome-wide profiling of breast cancers [28–31] and glioblastoma [32] reveals far greater diversity at the genetic level than at the transcriptome level. These and many other cancers are routinely separated into a small number of 'expression subtypes', despite thousands of diverse genetic changes. Hence, until genetic changes confer an irreversible change in expression patterns [33–35], the genetic diversity of tumor cells can in many ways be considered a variation secondary to cell identity.

Visualizing Tumor Heterogeneity through Single-Cell Technologies

Recent advances in high-throughput sequencing methods enable measurement of the proportions of mixed cell types and cell type-specific states that shape the tumor microenvironment (Table 1). The most popular commercially available single-cell technologies currently rely on microfluidic devices that use either patterned microwells for single-cell isolation, or droplet-based barcoding of individual cells, similar to Drop-seq [36], and are extensively reviewed elsewhere [37,38]. Frequently used alternatives to these approaches are based on the clever use of split-pool barcoding schemes [39,40]. Together, these approaches have confirmed that many cancers are characterized by recurrent patterns of cell populations and states [9,19,41–43]. In many cases, the origins of this heterogeneity are thought to be influenced by cytokine production [13–16,44], variable neoantigen presentation [17–19], stromal content [9,42,45], vascularization [46], and other heterogeneous features.

In recent years, a large toolbox of single-cell epigenomic assays has emerged, based on RNA-seq [36], **ATAC**-seq [47,48] (see Glossary), and more recently, **CUT&RUN** or ChIC-seq [49,50]. The use of single-cell approaches to characterize cell states within these populations has lagged the measurement of the proportions of distinct cell types, nevertheless, several important examples of cell state changes in cancer are beginning to emerge. One of the strongest signatures is the set of changes referred to as EMT, which are associated with a pronounced change of invasiveness and metastatic potential. Using scRNA-seq of colorectal tumors, EMT-like signatures have been found in a portion of cancer-associated fibroblasts (CAFs) [42]. The increased EMT-like signature was observed only in CAFs of the tumor and not the epithelial cell population. In a separate study relying on ligand-receptor annotations, scRNA-seq data from six syngeneic mouse tumor models was used to deconvolve the complex cell–cell interactions of the tumor microenvironment [51]. This study discovered a correlation between increased tumor growth and

Table 1. High-Resolution Technologies for the Study of Tumor Ecosystems

	Technology	Applications to cancer	Strengths	Limitations/constraints	Refs
Genomic and unbiased approaches	Nanowell microfluidic devices	High-throughput single-cell analysis of subpopulations	Ease of use/established technology	Relatively expensive; loss of spatial resolution	[47]
	Droplet-based single-cell sequencing				[36,51]
	Split-pool based approaches		No complex equipment required	Protocols still in development, loss of spatial resolution	[39,40,48]
	MERFISH	Spatially resolved RNA expression in tissues and cells	Applicable to cells and tissue sections; high-resolution	Limited to 1001 unique mRNAs	[84,85]
	Slide-seq and 'spatial transcriptomics'		Applicable to tissues	Specialized oligomer arrays needed, limited resolution of 'spatial transcriptomics' is overcome in Slide-seq	[81–83]
	seqFISH		Tens of thousands of distinct transcripts detected	Specialized primary and readout probes needed	[86]
	FISSEQ		High-resolution and preserves spatial information	Expensive equipment; not ideal for low-abundance transcripts	[87]
	Multiregion sequencing	Addresses large-scale heterogeneity	Spatial information retained	Low spatial resolution	[17,19,24–27,64–69]
	LCM	Isolation of cell(s) based on microscopic visualization	Native spatial information and cell state retained	Automation required for high-throughput analyses;	[77–79]
	MALDI-IMS	Spatial proteomics, metabolomics	Label-free; preserves spatial information	Semiquantitative	[88–90]
Candidate-based approaches	Mass cytometry: CyTOF	Single-cell proteomics	Quantitative single-cell analysis of proteins	Currently limited to measurement of ~50 parameters per cell	[71–74,76,106]
	Immunohistochemistry	Visualization of heterogeneity of specific cancer markers	Retains tumor niche information	Limited by compatible antibodies and FISH probes	[76]
	CycIF	Visualization of ~60 proteins in tissue samples	Uses standard equipment and reagents	Number of cycles limited by sample integrity; limited to compatible antibodies	[92–94]
	Histocytometry	Combines microscopy and multiplexed antibody staining	Provides spatial and contextual information	Antibody availability and compatibility	[91]
	FACS	High-throughput characterization of bulk samples	Widely available commercially	Limited by antibodies available; limited to a few markers	[70,75]
	Light-sheet microscopy	Noninvasive imaging	Low phototoxicity	Expensive equipment and training required	[99,100]

tumor cell secretion of key chemokines that may signal other tumor cells or macrophages that express cognate receptors. One recurrent feature of single-cell analytical approaches is the frequent use of inference-based algorithms like SCENIC[i] [52], as well as knowledge-based annotations, for example, hallmark gene sets from MSigDB[ii] [53], to segregate cells with similar regulatory activities or phenotypic states into clusters for downstream analysis (Figure 2). Similarly, pathway activities can be derived from transcriptomics data using PROGENy[iii], which relies on pathway-responsive gene signatures to define cell states [54].

Conventional single-cell techniques currently rely on dissociated, monodisperse cells. Therefore, these approaches unfortunately require loss of spatial information. Still, such approaches allow for measurement of the cell proportions present in tumor cell populations, which has shown predictive power for classifying tumors based on their distinct microenvironments and their response to therapy (Figure 2) [55,56]. The transcriptomic patterns of cell type-specific changes are usually

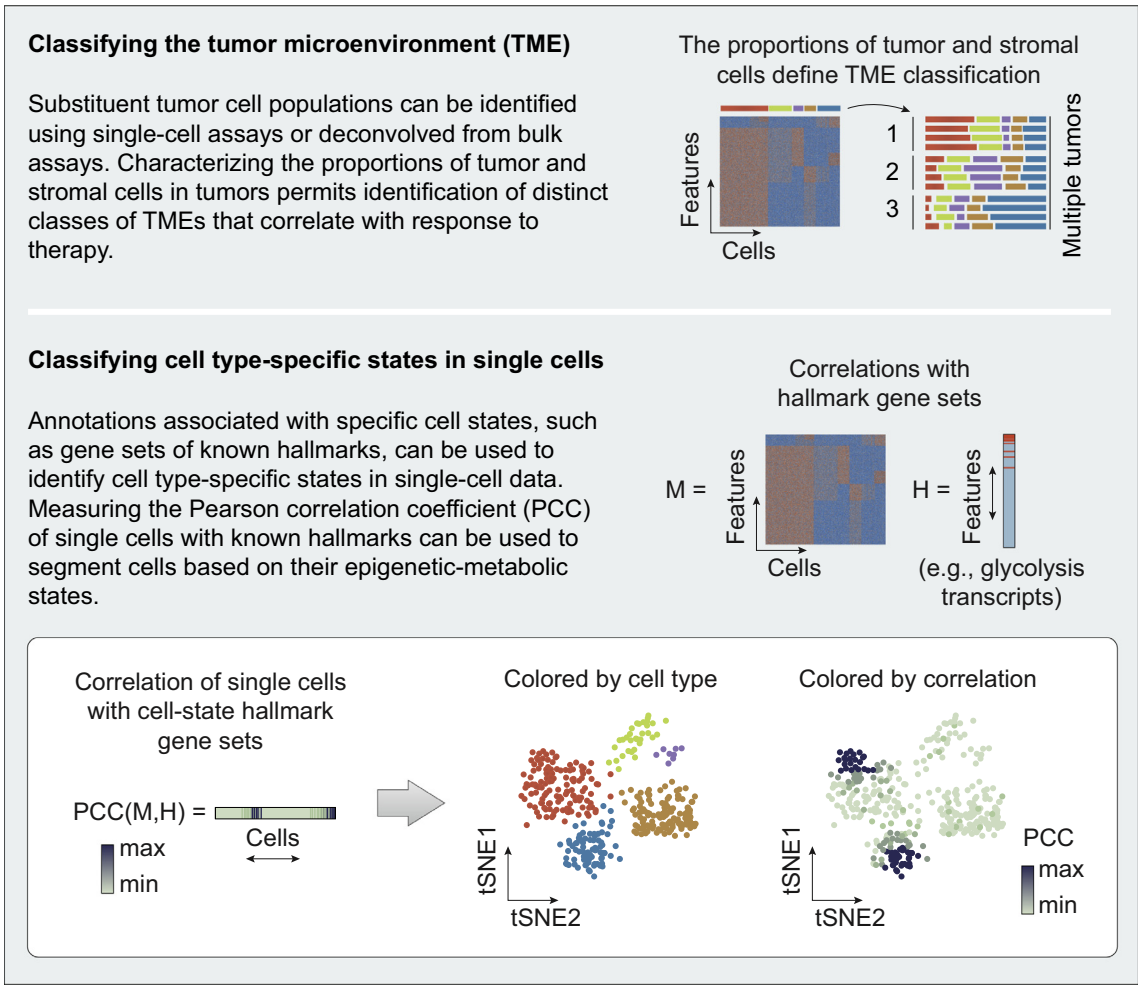

Figure 2. Visualizing Tumor Heterogeneity between and within Cell Types. Measuring the overall proportion of each cell type in mixed cell populations plays an important role in subtyping tumor microenvironments. Within and between cell types, the epigenetic-metabolic states of cells can be classified by examining correlation to known gene sets associated with specific states.

less dramatic than those separating cell identities [9], and many cell type-specific changes may be shared between multiple cell types. Therefore, analytical and visualization approaches (e.g., **tSNE** or variants like **UMAP**[iv] [57]) may benefit by being augmented to include hierarchical approaches to map cell type-specific changes within subpopulations, rather than discriminating cells based on global features. The analysis of global features has the advantage of being mathematically principled, but may fail to classify cell type-specific phenotypes, because the variations associated with them may be small compared with the higher magnitude differences associated with cell identity, and shared between different cell types. Continued application of these techniques and development of new analytical tools for refinement may yield new avenues for discovery biology and therapeutic intervention.

The Spatial Hierarchy of Tumor Heterogeneity

There is a high degree of spatial correlation within tumors because cells with similar phenotypic profiles are often contained within similar microenvironments. The phenotypic diversity of cells is shaped by diverse gradients, including those formed by systemic hormones, local diffusible factors such as TGF-β [58] and Wnt [59], as well as variable nutrient and environmental cues [6], and unknown factors that promote proliferation of cells in close proximity to adipocytes [60]. Furthermore, the heterogeneous tumor immune microenvironment substantially impacts intratumoral diversity and evolution [19] (Box 1). Altogether, spatial heterogeneity spanning from the cellular level to the tissue level impacts many physiological properties in cancer (Figure 3A). Fortunately, several technologies are ideal for clinical and experimental dissection of these changes.

Arguably one of the best models of spatial patterning in cancer is associated with hypoxia. Both mathematical modeling and experimental measures of oxygen perfusion in tumors show that hypoxic or anoxic conditions arise less than 0.5 mm from the vasculature [61]. In cancer, hypoxia arises as tumor cells become further separated from the oxygen-rich blood supply (Figure 3B). Hypoxic metabolic adaptations therefore allow tumor cells to survive low oxygen tension [62]. Inventive engineered systems have allowed researchers to experimentally mimic *in vitro* the oxygen gradients observed *in vivo* to study their influence on spatial gene expression. The metabolic microenvironment chamber (MEMIC) is a chamber for culturing cells with only a small slit through which oxygen, nutrients, and waste products can transit, inducing a spontaneous gradient of these factors (Figure 3C). Culturing TAMs in MEMIC increases

Box 1. Tumor Immune Infiltration

Tumor infiltration by immune cells is a critically important source of heterogeneity with great significance for emerging immunotherapies. Somatic mutations in tumors generate neoantigens, which influence the tumor microenvironment by recruiting immune cells and altering local cytokine concentrations [17–19,101–104]. While immune checkpoint blockade has been particularly effective against several malignancies, there remains wide variation in outcomes, with relapse occurring in a third of patients [105]. One potentially confounding issue for immune therapies is that the tumor immune microenvironment contributes both positively and negatively to oncogenic properties [56]. For this reason, identifying patient- and tumor-specific microenvironments represents a promising strategy to reveal the determinants of differential therapeutic response.

Divergent aspects of tumor immune cell heterogeneity are exemplified in breast and lung cancer. In breast cancer, tumor cells secrete cytokines, which recruit inflammatory monocytes that in turn activate gene programs that promote metastasis to the lung [106]. In the breast, positive feedback loops between cancer cells and macrophages stimulate EMT-like patterns to promote metastasis [107]. Interestingly, this effect is context-dependent, as metastasis-associated macrophages can prevent lung metastatic growth by reducing angiogenesis and influencing the extracellular matrix [108]. Altogether, the study of intercellular signaling between tumor and immune cells represents a complex area with profound implications for immune-based therapies.

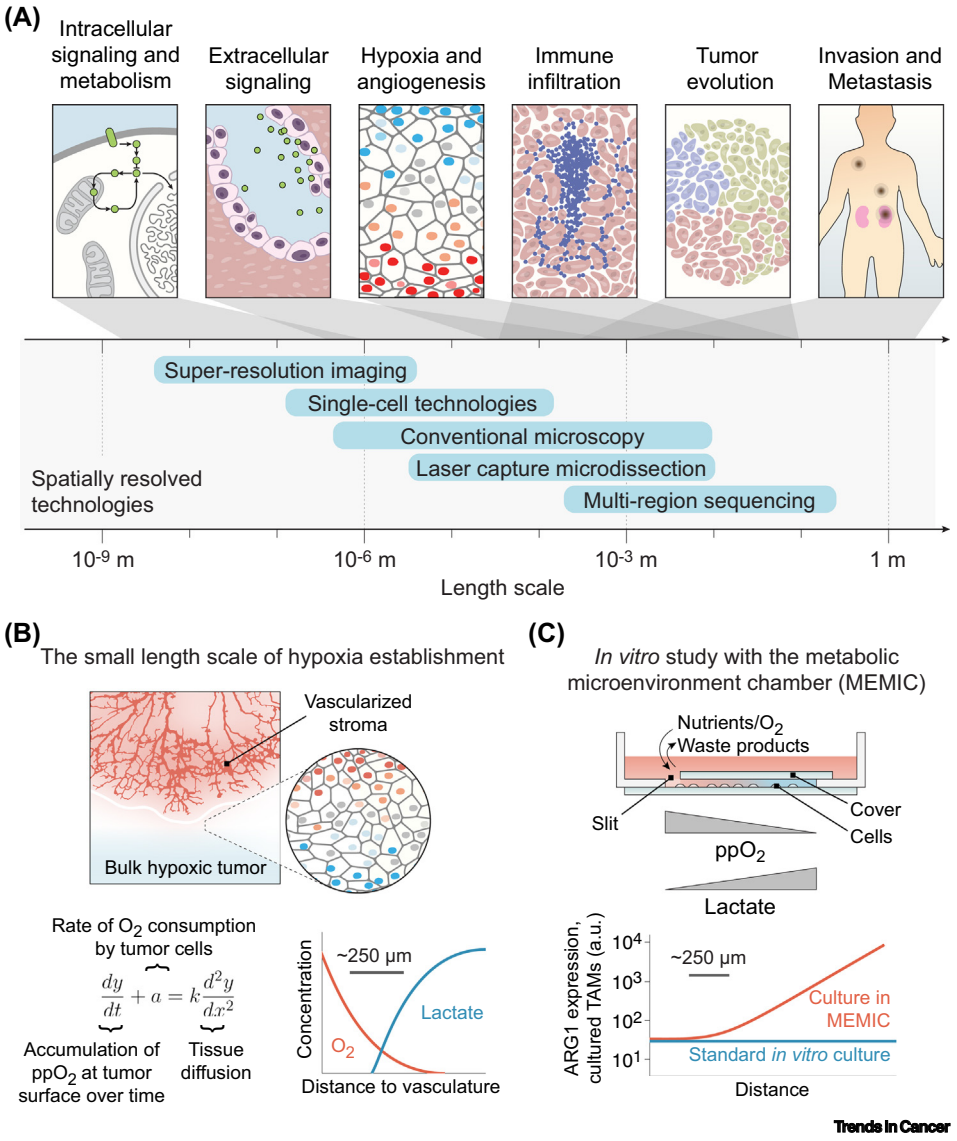

Figure 3. The Spatial Hierarchy of Tumor Heterogeneity. (A) Spatially resolved imaging- and sequence-based technologies provide insight into mechanisms that contribute to cancer biology at multiple levels of scale. (B) The establishment of hypoxia occurs over a short length scale of ~250 μm. Reaction-diffusion equations can model the spatial gradient of diffusible factors near tumor-stromal boundaries. (C) Experimental study of hypoxic gradients *in vitro* reveals that oxygen tension influences spatial gene expression patterns of tumor associated macrophages (TAMs). Abbreviation: ppO_2, partial pressure of oxygen.

arginase 1 expression in response to hypoxia over a 250–500 μm length scale [63]. This hypoxic response of TAMs is required for induction of revascularization, which allows tumors to overcome nutrient-poor regions of the tumor. Tumor revascularization in response to hypoxia is a pinnacle of tumor plasticity and survival that results in high spatial heterogeneity over relatively short distances.

At larger length scales, the effects of tumor evolution dominate the spatial hierarchy. As tumors adapt to their developing environments, intratumoral variation of gene expression arises. These

mixed cell populations can have different properties, including differential drug response, which can significantly influence therapeutic resistance and clinical management [1,64–67]. In primary clear cell renal cell carcinoma (ccRCC), approximately 75% of driver mutations characterized are subclonal, while *VHL* mutations are common to all regions of a tumor, illustrating the complex roles played by both driving mutations and tumor evolution [68]. **Multiregion sequencing** has also demonstrated that spatial limitations and tumor microenvironments cause subclonal mixing of cells, contributing to diversification of the tumor's mutational landscape. In colorectal tumors, single clonal expansions ultimately result in advanced colorectal tumors with large numbers of mutations due to later selective pressures within the tumor [69]. Altogether, the context-dependent relationships between cell identity and cell states must be investigated at both the local level and also across a tumor.

Technologies for Characterizing Multicellular Features in Malignancy

In addition to emerging single-cell technologies described above, other new technologies are also revealing key insights into malignancy. Below, we review emerging trends for high-resolution methods that have broad application in cancer research.

Advancements in Cell Sorting and Cytometry

Mixed samples can be analyzed at the single cell level in high-throughput fashion using flow cytometry and fluorescence-activated cell sorting (FACS). Modern combinations of cytometry with single-cell genomics and upgraded variations such as cytometric time-of-flight mass spectrometry (**CyTOF**) are expected to play an increasingly vital role in defining the heterogeneity of bulk samples.

Though cytometry traditionally defines cell identity through surface markers, it can be augmented with single-cell sequencing approaches to provide additional levels of information. Specifically, using FACS in combination with scATAC-seq links the expression of cell surface markers to changes of the underlying open chromatin landscape [70]. Additionally, CyTOF is a widely used commercially available tool that permits examination of heterogeneous cell states within tumors. CyTOF enables highly multiparametric studies of protein abundance, offering single-cell analysis of up to 50 parameters per cell [71]. One CyTOF study of heterogeneity within high-grade serous ovarian cancer tumors revealed rare cell populations associated with poorer outcome that were previously missed by bulk sampling. Their identification may lead to new and more specific avenues for therapeutic intervention [72]. By combining CyTOF with proximity ligation assay for RNA (PLAYR), simultaneous high-dimensional single-cell analysis of mRNA and protein can be achieved at a rate of thousands of cells per second, providing insight into the relationship between gene expression and protein abundance [73]. Furthermore, coupling of CyTOF with barcoding of normal lung, tumor tissue, and peripheral blood revealed tumor-specific states of immune cell composition and, importantly, potential immunotherapy strategies for lung adenocarcinoma [74]. Overall, paired analyses using cytometry are advancing the functionality of cell sorting and yielding a more complete picture of the heterogeneity contained within bulk samples.

Though flow cytometry is invaluable for high-throughput cell typing, its use inherently requires loss of spatial information. However, combining cytometry with other techniques can shed light on spatial heterogeneity as well. NICHE-seq preserves the cell states influenced by surrounding cells or microenvironments, allowing the characterization of these influences on tumor heterogeneity [75]. Here, after *in situ* labeling of photoactivatable fluorescent markers, FACS is coupled to high-throughput sequencing to study cellular ecosystems in live animal or *ex vivo* settings. Applying NICHE-seq to melanoma identified niche-specific changes in immune cell localization and

expression programs, where different myeloid compositions were associated with different extracellular matrix structures [75]. Spatial cell context can also be preserved when combining CyTOF with immunocytochemistry or immunohistochemistry and high-resolution laser ablation. These combination approaches have achieved spatial resolution of 1 μm in tissue sections, leading to the discovery of new subpopulations within conventional breast cancer subtypes [76]. Clever combinations of existing technologies will continue to advance discovery, providing rich resources with which to test new hypotheses.

Laser-Capture Microdissection

Laser-capture microdissection (LCM) enables the study of spatial heterogeneity while preserving spatial information. LCM allows for selection and contact-free isolation of cells of interest from distinct tumor regions with high precision [77]. Briefly, cells of interest are microscopically identified on tissue sections prior to laser-assisted isolation. Cells are then isolated using a high-intensity UV laser and collected by laser pressure catapulting. Alternatively, a near-IR laser can be used to melt a thermolabile polymer placed atop the cells of interest, enabling their removal. These regions can be based on morphological features, immunohistochemistry, or expression state. LCM can be paired with genome-wide analyses where spatial information is otherwise lost [77].

Because LCM simply enables dissection of a biological specimen, many downstream applications can be employed to reveal characteristics of cell identity and cell state, including analysis of DNA sequence or copy number, RNA profiling, and mass spectrometry. For example, topographic single cell sequencing (TSCS) combines LCM, whole-genome amplification, and single-cell DNA sequencing [78]. TSCS revealed that most mutations and copy number variations that contribute to intratumoral heterogeneity of invasive ductal carcinomas are a direct result of multiclonal invasion of local ductal carcinomas *in situ* [78]. Additionally, by pairing LCM with computer-aided microscopic isolation (CAMI), researchers created an automated high-throughput method to analyze cells from tissues or suspensions and automatically guide extraction. CAMI-LCM allows the automatic isolation and subsequent analysis of single cells based on morphology, location, or presence of specific fluorescently labeled markers [79]. Because LCM preserves cell context, inferences relating cell identity with the surrounding microenvironment can be drawn. Increased integration of LCM with automated high-content systems has great potential to identify new subpopulations that may not be readily detected by routine histopathology.

Advanced Methods That Combine Genomics with High Spatial Resolution

Several advanced technologies have been developed to perform genome-wide expression studies *in situ*, permitting genomic assessment and preservation of local tumor microenvironment landmarks. In one such plate-based assay, tissue cryosections are partitioned into a microwell chip where picoliter-scale reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) reactions take place (Figure 4A). These reactions are subsequently measured using a fluorescence plate reader to reveal spatially variable cell states based on expression patterns of key genes [80]. In another method, referred to as 'spatial transcriptomics' by the authors, tissue sections are positioned on glass slides affixed with oligo(dT) primers containing unique spatial barcodes for transcript mapping (Figure 4B). Subsequent fluorescent visualization or RNA-seq analysis demonstrated spatial transcriptomic heterogeneity while preserving histological context in breast cancer tissue [81,82]. Interestingly, this approach highlighted that only specific regions of the tumor had engaged an EMT-like program. A higher-resolution variation of this approach called Slide-seq substitutes barcoded oligo(dT) primers with barcoded 10-μm beads. Slide-seq affixes these beads to slides to provide a spatial index onto which tissue cryosections are placed [83]. Following reverse transcription, tissue digestion, and library amplification, the spatial

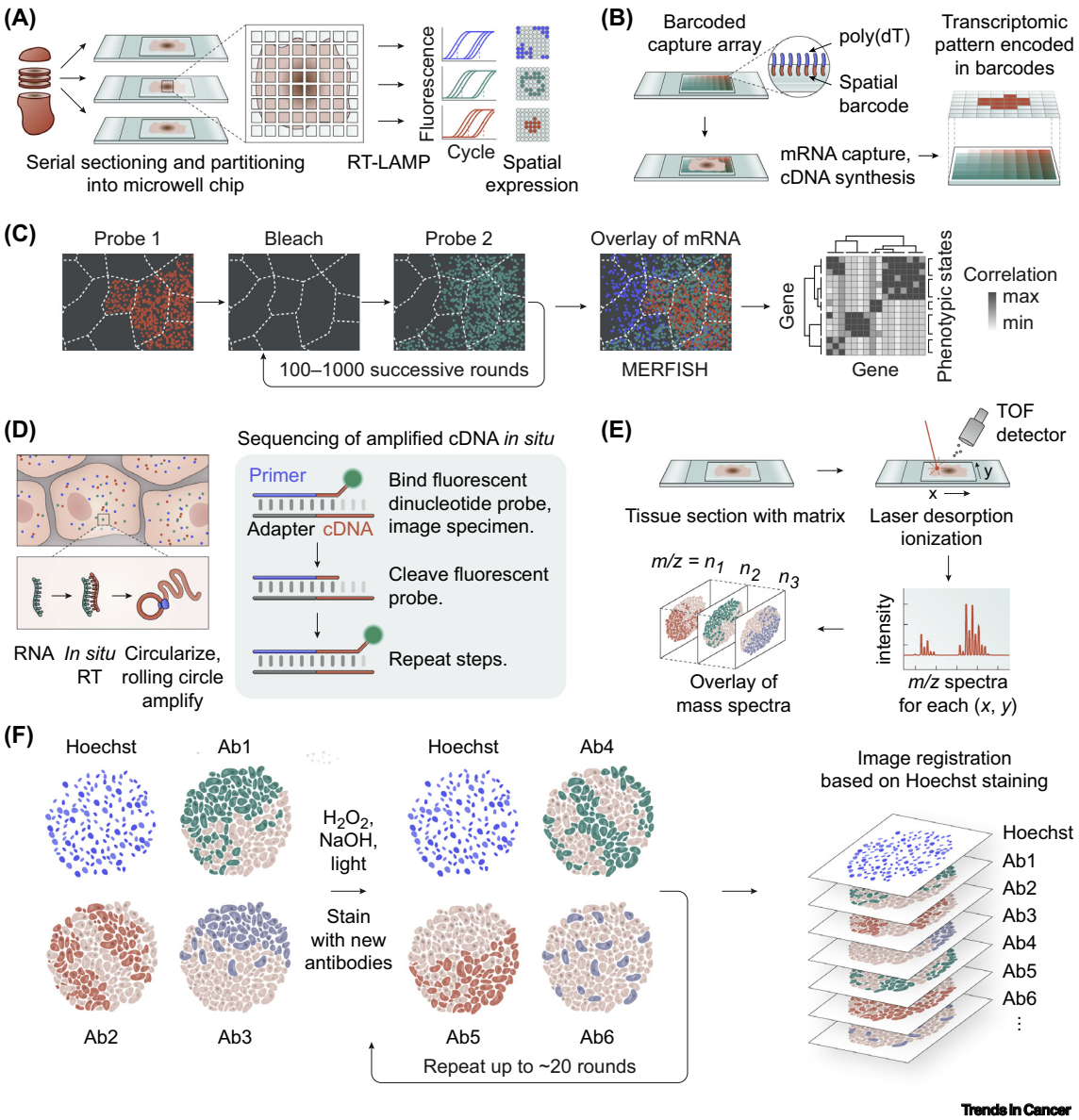

Figure 4. Advanced Technologies Enable Genomic Characterization of Malignant Processes *In Situ*. (A) Highly resolved reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay using microwells enables quantitative assessment of RNA expression changes while preserving spatial information. (B) Spatial transcriptomics using a spatially barcoded poly(dT) capture probes. (C) Multiplexed error-robust single-molecule fluorescent *in situ* hybridization (MERFISH) enables high-dimensional investigation of transcription states using error-robust single-molecule fluorescent *in situ* hybridization (FISH) counting of transcripts. (D) fluorescent *in situ* RNA sequencing (FISSEQ) enables *in situ* Sanger-like sequencing while preserving tissue structure. (E) Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) enables *in situ* mass spectrometric analysis of proteins and metabolites with better than single-cell resolution. (F) Principle of multiround, multiplexed tissue immunofluorescence [multiplexed fluorescence microscopy (MxIF) and cyclic immunofluorescence (CycIF)]. Abbreviation: TOF, time of flight.

expression profiles are computationally reconstructed. Slide-seq has been employed to spatially map individual cell types in brain cryosections, as well as the different cell states induced in response to injury [83].

Highly multiplexed single-molecule visualization of the number and distribution of transcripts has also been achieved in cells and tissue sections using successive rounds of fluorescent *in situ*

hybridization (FISH) labeling. Up to 1001 unique mRNAs can be imaged and quantified via multiplexed error-robust single-molecule fluorescent *in situ* hybridization (MERFISH). Here, cryosections or cells fixed on coverslips undergo repeated rounds of FISH labeling and imaging with a set of RNA probes, allowing for the construction of high-dimensional gene regulatory networks with intercellular and intracellular spatial resolution of individual molecules in tissues (Figure 4C) [84]. The spatial resolution of MERFISH can be increased by combining with expansion microscopy [85], a method of physical sample expansion that results in increased distances between single molecules. Furthermore, MERFISH is also compatible with immunofluorescence staining of subcellular structures, allowing mRNAs to be correlated to specific cell compartments [85]. Another sequential FISH technique, seqFISH, uses a standard confocal microscope with a fluorescent barcoding approach, enabling the detection and subcellular localization of tens of thousands of genes within single cells. Application of seqFISH to tissue sections allows the identification of cell–cell interactions such as localized expression of ligand-receptor pairs within neighboring cells [86].

Another technique, fluorescent *in situ* RNA sequencing (FISSEQ), permits RNA localization of over 8000 genes in cells and tissues through *in situ* amplification and sequencing of cDNA (Figure 4D). This method permits 3D fluorescent visualization and identification of mRNA transcripts in cells, while preserving tissue architecture [87]. By preserving tissue context, *in situ* approaches allow high-resolution characterization of cell states relative to molecular and phenotypic spatial landmarks.

Single-Cell Resolution of Protein/Metabolite Abundance

Spatially resolved visualization of proteins, lipids, metabolites, and post-translational modifications associated with tumorigenic properties can be achieved in tissue sections through matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) (Figure 4E). For example, MALDI-IMS has been used to characterize diverse metabolic states of malignancy with high spatial resolution, including components of glycolysis and the TCA cycle [88,89]. MALDI-IMS has also revealed highly vascularized regions of ccRCC tumors through detection of proteins representative of increased tumor vascularization [90]. Additionally, MALDI-IMS enabled identification of key histone modifications enriched in surrounding normal tissue relative to the tumor, thereby visualizing the underlying tumor-specific epigenetic states.

New enzymatic histochemistry approaches allow spatial characterization of metabolic signatures in complex tissue samples with single-cell resolution [91]. Using consecutive tissue slides, multiple enzymatic activities can be visualized and quantified by enzyme histochemistry and automated whole-tissue histocytometry. This approach allowed comparison of single-cell metabolic states of distinct immune cell populations in healthy versus tumor colon tissue [91]. Interestingly, the microenvironment was found to significantly influence cells of similar identity: glycolytic activity of tumor-associated macrophages is significantly decreased compared with their counterparts in normal tissue. Together, *in situ* protein and metabolic assays permits spatial characterization of heterogeneous activities otherwise inaccessible through conventional immunohistochemistry.

Conventional immunofluorescent detection of proteins in tissues has been limited by the number of fluorescent channels on a microscope. However, multiplexed fluorescence microscopy (MxIF) [92] and a related form, cyclic immunofluorescence (CycIF) [93] are imaging approaches that use multiround staining with standard reagents and equipment to achieve high-dimensional immunofluorescence images. MxIF and CycIF can detect upwards of 60 proteins through sequential antibody probing, imaging, and fluorophore bleaching to create highly multiplexed images of tumor tissue at the single-cell level (Figure 4F). In tissues, tissue-based CycIF (t-CycIF) has been used to

compare protein expression across a pancreatic tumor section, where CD45$^+$ immune cells were found to be highly infiltrated within tumor tissue compared with adjacent normal pancreas [94].

Concluding Remarks

The rapid pace of technological advances in genomics and microscopy has resulted in an explosion of new tools for cancer research. These tools are providing powerful opportunities to improve diagnoses, develop more effective therapies, and understand the complexity of cancer in new detail. In particular, the intersection of sequencing and optical methods is providing multilevel information required to investigate altered function in both space and time. Coupling these approaches with complementary tools, such as small-molecule probes [95] or environmentally selective optical reporters [96], may help to identify how conditions of the tumor microenvironment impact tumor progression and metastasis with high spatiotemporal resolution.

An important challenge is to apply these tools to uncover the recurrent patterns of cell types and states that contribute to disease progression and therapeutic response (see Outstanding Questions). Classification and systematization of tumor microenvironments is becoming increasingly valuable as a clinical diagnostic [97]. As a result, high-resolution tumor mapping may increasingly serve an important role in clinical diagnosis and treatment. Given their robustness, high scalability, and familiarity to pathologists, cyclic immunostaining approaches such as MxIF and CycIF may provide the fastest plausible path to using single-cell data in clinical trials. However, plummeting sequencing costs in the last decade have supported an avalanche of data-rich assays whose output is encoded by DNA sequence. In either case, spatially encoded and other highly multiplexed single-cell technologies are poised to reveal many new insights, as precision oncology increasingly focuses on interactions between specific cell types and states *in situ*. We therefore envision a future in which tumor biopsies are routinely examined for cell type-specific states using high-resolution techniques, in addition to screening for mutations affecting tumor suppressors and oncogenes.

As genomic methods are augmented to provide single-cell resolution, the preservation of spatial information remains a major challenge. The trend towards increased integration of genomics and microscopy suggests that their joint application will play an essential role in the study of malignancy. Several emerging super-resolution imaging techniques (e.g., single-plane illumination microscopy [98] and lattice light-sheet microscopy [99]) permit fast high-resolution 3D imaging of live samples, including immune and circulating tumor cells. These optical techniques and many variants [100] can also provide important insights into dynamic, spatially regulated processes that contribute to malignancy and tumor biology.

Our understanding of the tumor microenvironment, immune infiltration, and stemness, is becoming increasingly comprehensive thanks to many innovative quantitative techniques that can capture the heterogeneity of cell states in tumors. These technologies are expected to complement emerging immune-based and related therapies [56] by revealing currently unknown multicellular interactions that promote cancer.

Outstanding Questions

What is the best way to categorize the diverse and heterogeneous phenotypes in a population of malignant and nonmalignant cells?

Can single-cell visualization tools be improved by focusing on hierarchical cell type-specific classifications rather than global genome-wide changes?

How can single-cell assays be combined to relate single-cell phenotypes to their genetic and epigenetic-metabolic states?

What are the most important and clinically actionable features of single-cell data?

What specimen size and resolution are needed for high-resolution techniques to reveal therapeutic vulnerabilities?

Are there patterns or characteristic responses of the tumor ecosystem to perturbation?

What are the interactions between the tumor microenvironment and common genetic changes to tumor suppressors and oncogenes?

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Author Contributions

E.A.S. and H.C.H. wrote and edited the manuscript.

Disclaimer Statement

The authors declare no competing financial interests.

Resources

[i]https://scenic.aertslab.org/

[ii]http://software.broadinstitute.org/gsea/msigdb

[iii]https://saezlab.github.io/progeny/

[iv]https://umap-learn.readthedocs.io/en/latest/

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