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Decoding Cancer Biology One Cell at a Time

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Abstract

Human tumors are composed of diverse malignant and non-malignant cells, generating a complex ecosystem that governs tumor biology and response to treatments. Recent technological advances have enabled the characterization of tumors at single-cell resolution, providing a compelling strategy to dissect their intricate biology. Here we describe recent developments in single-cell expression profiling and the studies applying them in clinical settings. We highlight some of the powerful insights gleaned from these studies for tumor classification, stem cell programs, tumor micro-environment, metastasis, and response to targeted and immune therapies.

The advantage of diversity

Tumors are characterized by genomic instability, limitless replicative potential, deregulation of cellular energetics, tissue invasion and metastasis, sustained angiogenesis, tumor-promoting inflammation and avoidance of immune attack (1). While these hallmarks are shared by most tumors, there is increased recognition that within a single tumor, subsets of cells may be sufficient to enable such phenotypes. Limitless replication may be driven by a subset of undifferentiated cells known as cancer stem cells; tissue invasion and metastasis may be driven by cells that have activated an epithelial-mesenchymal transition (EMT) or other invasion programs; deregulated energetics, as well as sustained angiogenesis, may be driven by cells in particular areas of the tumor, such as those that experience hypoxia and lack of nutrients; inflammation and avoidance of immune attack may be confined to specific regions (e.g. with increased immune infiltration). Such "division of labor" between tumor subpopulations may help to explain a range of tumor phenotypes, most notably, their tendency to either endure through anti-cancer treatments or to recur months or years after

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tumor regression. The discrepancy between initial tumor response and regression (indicating that cancer cells are being eliminated by treatment) and later tumor recurrence (indicating that not all cancer cells were eliminated) is a testament to the critical significance of intra-tumoral heterogeneity (ITH). Yet, genomic and transcriptomic approaches have traditionally measured tumors as bulk, thus only revealing aggregate cellular profiles and masking important aspects of ITH. The recent emergence of single-cell "omics" profiling is paving the way for a revolution in our ability to comprehensively characterize tumors. Here we review single-cell omics methods and their application to clinical tumor samples. We focus on single cell expression profiling and the insights gained on tumor lineages, stem cell programs, tumor classification, metastasis, microenvironment composition, and response to therapies. Finally, we highlight ongoing developments in single-cell expression profiling that will help tackle unanswered questions in the field.

Profiling tumors at the single-cell level

Advances in chemistry, microfluidics, sequencing and bioinformatics have enabled the development of a number of techniques to profile multiple facets of tumors at the single-cell level (genome, transcriptome, methylome, chromatin accessibility, intracellular and cell surface proteins) (Figure 1), advancing our understanding of ITH. Initially, ITH was considered primarily at the level of the genome, and even prior to single cell approaches, genetic heterogeneity was inferred from bulk tumor profiles, based on the fraction of sequence reads that harbor each mutation (2–6). This approach was significantly empowered by profiling multiple areas of the same tumor (multi-focal sequencing), demonstrating that distinct regions of the same tumor typically harbor both shared and distinct mutations (7,8). Single-cell DNA sequencing studies have further expanded these observations in order to dissect genetic ITH (9–14). Due to the limited sensitivity in detection of individual mutations, most studies of single cell DNA sequencing are often focused on chromosomal copy number aberrations (CNAs) which are detected at much higher accuracy and are associated with considerable ITH in most tumors (15–18). The aggregation of cells sharing CNA profiles has been utilized to enhance phylogenetic analysis at single-nucleotide levels (19).

While mapping of genetic heterogeneity is extremely important, it does not reveal the functional state associated with distinct genetic events. Many cancer mutations may reflect passenger events with limited functional implications (20) and even for functional mutations, an exact functional consequence cannot be inferred from sequence information alone. Thus, single-cell RNA-sequencing (scRNA-seq) has recently become a more widely used approach to explore ITH. In principle, scRNA-seq enables analysis of both functional and genetic states, as mutations may be detected within transcripts, especially with protocols that sequence full-length transcripts (as opposed to 3′- or 5′-end sequencing). Yet, the sensitivity of such methods is highly limited (21), primarily due to the low coverage of the transcriptomes in individual cells. New methods aim to improve this coverage by adding locus-specific primers to the scRNA-seq protocol for genes mutated in a given cancer type, as first shown for the BCR-ABL fusion in leukemia (22) and more recently for CALR-mutated myeloproliferative neoplasms (23). However, while these approaches are effective for mutations in highly expressed transcripts, they require prior knowledge of the mutations

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and are limited to the amplification of few selected loci in each sample. Alternatively, DNA and RNA may both be profiled from the same single cells, and sequenced either for genome and transcriptome (24,25) or alternatively for epigenome DNA methylation and transcriptome, as recently demonstrated for chronic lymphocytic leukemia (26). While these techniques are likely to be applied in larger scale to clinical tumors in the future, integration of multi-omics modalities (27) remains rare, with most studies to date focused on single-cell tumor profiling of either RNA, DNA, or proteins, and accordingly on either the functional or the genetic states of cells.

scRNA-seq has been the most widely adopted approach leveraged for single-cell profiling of clinical tumors. Although scRNA-seq methods have until recently been limited to living cells as the starting material, characterizing individual cell transcriptomes is also possible using frozen tissue via single-nucleus RNA-sequencing (snRNA-seq/sNuc-seq) (28,29). While nuclei have less mRNA content than cells, analyses performed on live cells and nuclei isolated from frozen tissue of the same sample have demonstrated consistent transcriptomic profiles and similar clustering patterns (28). The possibility of profiling a tumor's transcriptome using frozen samples facilitates the deployment of such approaches to broader clinical contexts and enables the comparison of longitudinal samples, critical to interrogating tumor evolution and response to treatments.

Whether applied to single cells or nuclei, clinical tumor profiling by RNA-seq requires four main steps. (I) fresh or frozen samples are obtained and disaggregated into a single-cell or single-nucleus suspensions using combinations of mechanical and enzymatic digestion protocols; (II) individual cells or nuclei are separated either by flow cytometry into 96- or 384-well plates or by microfluidic devices into distinct droplets and then lysed; (III) a reverse transcription and RNA-seq protocol (either full length or the 3′ or 5'ends) is applied to individual cells or nuclei, which are barcoded and pooled; (IV) the pooled sample is sequenced and the reads mapped to the transcriptome to assess gene expression and perform additional analyses (see below). Many of these steps need to be optimized for each application, and the methods available offer a trade-off between coverage per cell and transcript (optimized by plate-based methods such as Smart-seq (30,31)) versus number of profiled cells, which is significantly higher with droplet-based platforms (32–34). Alternative methods to label individual cells by combinatorial barcoding, such as SPLiT-seq (split-pool ligation-based transcriptome sequencing), do not require the separation of individual cells into compartments but rather leverage the cells or nuclei themselves as compartments (35–38). Depending on the desired application and biological question, specific single-cell methods may thus be leveraged.

Of Cell Types and Cell States

When performing unbiased expression profiling of cells in a tumor (i.e., without pre-selecting for any particular cell type), the first step in the analysis (after quality controls and filtering of low-quality cells) is to assign the remaining cells into distinct cell types. This is typically performed by clustering the cells and annotating clusters by the identity of upregulated genes. This approach typically works well, yet the tumor introduces an additional task - the distinction between malignant (i.e. with genetic alterations) versus non-

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malignant cells (such as stromal and immune cells). Combining gene expression clustering with genetic information helps address this challenge. However, as discussed above, the ability of scRNA-seq to detect single point mutations suffers from technical limitations that recent developments are attempting to address (23–25). Importantly, malignant cells may also be identified by CNAs that can be inferred from the average expression of large sets of genes in each chromosomal region. This approach has been used by most recent studies to resolve malignant from non-malignant cells, and in some cases also to identify distinct genetic subclones among the malignant cells (21,39–41).

Relying on the identification of CNAs rather than point mutations highlights a key feature of scRNA-seq datasets that influences all computational analyses: the sensitivity of detection and the accuracy of estimating exact levels for individual genes or mutations is hindered by “dropouts” and the limited efficiency of single-cell profiling; thus, one should not rely on any individual mutation or gene to infer cell identities. Rather, the combined analysis of many related genes (i.e., adjacent genes in the case of CNAs) or a larger gene-set known to reflect a certain biological function or process, enables cells to be annotated with increased accuracy. The choice of gene-sets for such analyses is critically important, and caution should be taken when gene-sets are derived from systems distinct from the one analyzed. Ideally, a gene-set should be derived *de novo* from the data-driven analysis of a scRNA-seq dataset and then applied to the same or to an extended/related data set to derive cell scores for the corresponding biological process (17,18,21,39–42).

Analysis of the heterogeneity within tumors typically enables two layers of distinction: first, cells are grouped into cell *types* (such as malignant cells, immune cells, etc.) that typically present as very distinct clusters; each cell type is then associated with additional diversity reflective of distinct cell *states*, such as context-specific expression programs. Cell cycle phases represent the most common example of distinct cell states, with cells that are not actively cycling, and cycling cells that may be assigned to distinct phases based on relative expression of cell cycle gene-sets. “Cell states” refers here to distinct expression programs with presumed functional implications that are typically continuous and potentially dynamic (e.g., progression along the cell cycle), while cell types are more stable and unique. While these definitions are admittedly imprecise, they reflect important distinctions between two common layers of diversity. In the following section, we primarily focus on cell *states* that have been identified in scRNA/snRNAseq profiling studies (Table 1) and that exemplify the power of single-cell analysis in decoding tumor biology.

Insights into cancer biology from single-cell genomics studies

Single-cell genomics experiments have generated multiple insights into the biology of different classes of tumors. Below, we highlight different salient findings grouped by biological or clinical questions.

Cellular lineages, their differentiation and plasticity

Developmental programs play a critical role in many malignancies (43). This observation underlies the cancer stem cell model, which suggests that tumors contain subsets of self-renewing stem-like cells underlying tumor-propagation (and in some instances resistance to

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therapies), along with a majority of more differentiated cells (44,45). This model has traditionally been supported by functional approaches in animal models. Single-cell expression profiling offers an orthogonal approach to revisit the role of stemness in cancer biology and to provide a more granular definition of cellular states that encompasses the continuum of cellular programs found in tumors and their resemblance to normal development (Table 1).

Initial pioneering studies used single-cell quantitative PCR (qPCR) to target selected genes (~50-100), and identified subpopulations that recapitulate normal lineages of the gut epithelium (including LGR5+ stem-like cells) in colon adenocarcinoma (46), and differentiation of stem-like basal cells into luminal cells in triple-negative breast cancer metastases, recapitulating mammary gland architecture (47). Subsequent scRNA-seq studies in isocitrate dehydrogenase (IDH) mutant gliomas uncovered a cellular architecture reminiscent of neural development, with a neural-progenitor-like (NPC-like) compartment enriched for actively cycling cells, as well as non-proliferative compartments differentiated along astrocyte-like (AC-like) or oligodendrocyte-like (OC-like) lineages (21,48). The distinct compartments did not correlate with genetic heterogeneity of those tumors, as evaluated by CNAs, consistent with a cellular hierarchy dictated by differentiation rather than genetic changes, thereby mimicking developmental processes.

Similarly, cellular hierarchies and differentiation patterns were subsequently described in a large number of other cancers, including additional classes of glioma (39,49), melanoma (50), neuroblastoma (51), medulloblastoma (41,52,53), and multiple types of lung cancer (54,55). Importantly, although these studies demonstrate an overall similarity between normal development and cancer hierarchies, they also begin to illustrate the deviations between cancer-related hierarchies and normal differentiation.

In histone H3 lysine27-to-methionine (H3K27M)-mutant diffuse midline glioma, a cellular hierarchy similar to that of IDH-mutant was identified (39). However, in H37K27M tumors the proliferating stem-like compartment comprised the majority rather than the minority of malignant cells, suggesting a *differentiation block* and correlating with the aggressive clinical behavior observed in this type of glioma (39). Impaired differentiation trajectories may be a common feature of pediatric cancers, as they were also revealed by scRNA-seq in neuroblastoma (51), ependymoma (49) and medulloblastoma (41,52,53), where they are prognostically important and appear to underlie tumor subgroups (see section below on tumor subtypes). In IDH-wildtype glioblastoma, where numerous scRNA-seq studies demonstrated diverse cell states with similarities to neuro-development (17,40,56–60), lineage-tracing experiments demonstrate *plasticity* with malignant cells that can frequently transition between states (40). Plasticity was also highlighted in small cell lung cancer (SCLC), in a recent scRNA-seq study demonstrating that tumor cells differentiated towards a neuroendocrine lineage can dedifferentiate to a non-neuroendocrine fate via the action of Notch signaling (54). This plasticity explains transitions between previously described subtypes of SCLC (61), and may account for the ITH (54).

Cellular plasticity with transitions to earlier states in developmental lineage may also occur under therapeutic pressure. Indeed, single-cell profiling of patient-derived *BRAF* V600E-

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mutant melanoma cell lines after treatment with the BRAF inhibitor vemurafenib reveals a transition toward a primitive neural crest-like phenotype, with high expression of neural growth factor receptor (NGFR) and loss of and reduced expression of the melanocyte transcription factor *MITF*, after only three days of treatment (50). This transition towards an early developmental phenotype is thought to represent a drug-resistance adaptation (50).

Finally, even in the absence of therapeutic pressure, the cellular architecture of a given entity may drift during tumor progression: in untreated NSCLC samples, tumor cells have been noted to progressively deviate from normal differentiation towards a cancer cell state that becomes dominant as disease progresses and metastasizes (62); in IDH-mutant gliomas, higher grade lesions have decreased fractions of differentiated cells and increased pools of progenitors (48); and in IDH-wildtype glioblastoma, subsets of malignant cells adopt mesenchymal-like programs that are not anchored in neurodevelopment (40,63).

While the determinants of such deviations from normal differentiation in cancer remain difficult to address, these might be related to the frequent cancer-related alterations in chromatin regulation. Pioneering single-cell multi-omics studies that combine RNA and DNA methylation profiling (or chromatin accessibility assays) are beginning to unravel potential mechanisms. In hematological malignancies, mutations in genes involved in DNA methylation were recently shown to disrupt normal hematopoietic differentiation causing shifts in the frequencies of erythroid or myelomonocytic progenitors, due to disruption of methylation-sensitive lineage-specific transcription factors binding sites (64). Altered DNA methylation profiles were also associated with aberrant programs in chronic lymphocytic leukemia (26) and colorectal cancer (65), as suggested by concurrent single-cell DNAme and transcriptomic profiling.

Tumor Subtypes

One of the main goals of cancer genomics in the last two decades has been to identify tumor subtypes that potentially represent distinct disease subsets and may justify further patient stratification. Expression profiling of bulk tumor samples has played a major role in defining tumor classifications in many cancers, including in breast cancer, melanoma, brain tumors and others (3–6,66). Single-cell analyses are providing at least two critical insights into such tumor classification: (I) in some instances, they reveal heterogeneity of these bulk inter-tumoral classifications even among individual cells from a single sample, indicating that bulk classification reflects the relative abundance of co-existing states, rather than unique states of subsets of tumors; (II) they provide a renewed understanding of the biological underpinnings of bulk classifications, by delineating the contributions of specific cell types and their interactions.

Analysis of glioblastoma showed that distinct cells from a single tumor resemble different bulk subtypes (so-called proneural, classical, and mesenchymal glioblastoma subtypes) (17,67). More recently, scRNA-seq analysis of a much larger number of cells and samples related the subtypes to the abundance of distinct malignant cellular states and of immune cells in the micro-environment (40). In medulloblastoma and ependymoma, single-cell analyses provided key insights on the distinction between tumor subgroups by showing that they differ in the differentiation state of the malignant cells they are composed of (41,49). In

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small cell lung cancer, recently subclassified into four distinct subtypes based on expression of lineage-defining transcription factors – *ASCL1* (SCLC-A), *NEUROD1* (SCLC-N), *POU2F3* (SCLC-P) and *YAP1* (SCLC-Y) (61) – scRNA-seq identified the co-existence of several of these subtypes in distinct cells within individual tumors (54). Expression of these transcription factors might actually account for cells in different stages of tumor evolution (54). In melanoma, bulk studies classified tumors as *MITF*-high or *AXL*-high, but single-cell analysis showed that both cellular states are identified in individual tumors (18). Interestingly, AXL-high tumors are additionally confounded by the abundance of cancer-associated fibroblasts (CAFs) that highly express *AXL* and the other genes associated with the *AXL*-high signature. Similarly, bulk and single-cell studies of colorectal cancer, head-and-neck squamous cell carcinoma (HSNCC), and ovarian cancer have all suggested that mesenchymal subtypes, thought to reflect epithelial-to-mesenchymal transition of malignant cells, primarily reflect the abundance of CAFs (42,68–71).

Overall, these studies highlight the profound impact of single cell analysis on the definition and understanding of tumor classification. Existing classifications by bulk expression profiling can be interpreted as a combination of three effects: the relative frequencies of malignant cell states that co-exist within individual tumors (e.g., glioblastoma subtypes and medulloblastoma subgroups); the relative frequencies of non-malignant cell types (e.g., CAFs in head-and neck and colorectal cancer); and finally, expression programs that reflect unique genetic and epigenetic states of the malignant cells in subsets of tumors. Single cell studies are now providing the ability to distinguish between these cases and will likely serve as the basis for renewed tumor classifications.

Tumor microenvironment and response to immunotherapy

Tumor microenvironment (TME) composition impacts tumor biology and therapeutic response through various mechanisms. The most common TME cell types include T cells, macrophages, endothelial cells and fibroblasts. Each of these components, as well as less common components, contribute to tumor phenotypes through their direct functions as well as by other mechanisms such as ligand-receptor interactions influencing other cell types. Single cell profiling has been used to characterize these components, including unbiased studies that characterize the entire tumor ecosystem as well as many focused studies that zoom in on particular components such as fibroblasts, macrophages and T cells. Not surprisingly, T cells have attracted the most attention due to their critical role in the efficacy of immunotherapies (72).

Effector CD8+ T cells can lyse malignant cells, but at least two mechanisms may result in less effective responses: first, chronic antigen stimulation may result in T cell *exhaustion* and loss of effector programs; second, tumor-infiltrating lymphocytes (TILs) may be *excluded* from the tumor core through a complex set of mechanisms, including interactions with additional TME components such as CAFs, endothelial cells, and macrophages. scRNA-seq offers a compelling approach to dissect the TME components, interrogate dysfunction-specific expression signatures and identify potential mechanisms of immune exclusion. Additionally, scRNA-seq reads that cover the T cell receptor (TCR; either through full-length or V(D)J tailored protocols) enable the reconstruction of TCR sequences and the

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identification of clonal T cell subsets, and enable linking the expression state of cells to their clonal and functional properties (18,73–77). The power of such approaches to dissect immune responses is illustrated by several single-cell studies of melanoma that identified dysfunction signatures confounded by T cell activation (18,78), distinct states of CD8+ T cells associated with patient tumor regression or progression (79) and a T cell exclusion program expressed by malignant cells and that can predict response to treatment with immune checkpoint inhibitors (ICIs) (80).

Other examples include NSCLC, in which scRNA-seq studies have identified populations of pre-exhausted T cells associated with better prognosis (81), and liver cancer, where increased *LAYN* expression is associated with cytotoxic T cell exhaustion and worse overall prognosis (75). In colorectal cancer, scRNA-seq highlighted subsets of tumor-associated macrophages (TAMs) with distinct inflammatory (inhibited by anti-CSF1R antibodies) and pro-angiogenic transcriptional profiles (82). Interestingly, differentiation of colorectal cancer cells along post-absorptive (characterized by *TP53* and *APC* mutations) versus a non-absorptive lineage (characterized by *KRAS* and *BRAF* mutations) influenced the composition of the tumor microenvironment, with the post-absorptive lineage cells promoting proliferation of cytotoxic T cells immunity and the non-absorptive lineage promoting myofibroblasts and proinflammatory macrophages (83). In gliomas and brain metastases, scRNA-seq and mass cytometry efforts have refined our understanding of the immune composition of these tumors and suggested mechanisms promoting immunosuppression (84–86). These studies have shown that the TME of gliomas differs according to tumor type, with IDH-mutant tumors' TME composed primarily of activated microglia while IDH-wildtype tumors are enriched for monocyte-derived macrophages (84,85). In brain metastasis, downregulation of *Cx3cr1* and upregulation of *Cxcl10* in myeloid cells has been suggested as mechanism promoting immunosuppression (86).

Another interesting application of scRNA-seq in glioblastoma has been to leverage the approach to assess the technical success of a neoantigen vaccine trial: by coordinately interrogating the TCR sequence of vaccine-reactive T cells in blood and in the tumor, a study was able to demonstrate the presence of functionally active, neoantigen-specific T cells in a glioblastoma sample (87). Other studies have leveraged mass cytometry to describe the immune infiltration of high-grade ER+ and ER− breast cancer(88), hepatocellular carcinoma (89), renal (90) and lung carcinomas (91). Finally, in basal cell carcinoma, scATAC-seq has helped highlight key regulatory programs underlying T cell exhaustion (92). Going forward, complementing the expression state of TILs and other TME components with their spatial localization and direct physical interactions will yield additional insights into the ecosystem of tumors.

Metastasis

Metastatic progression remains a formidable challenge in the management of systemic cancers, with metastases often being treatment-refractory and accounting for most cancer-related deaths. As further described below, at least three distinct approaches are utilizing single cell profiling to improve our understanding of metastasis: (I) the characterization of

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cell states that facilitate metastasis, (II) the comparison of matched primary and metastatic samples, and (III) the analysis of circulating tumor cells.

Single cell profiling of primary tumors may uncover cellular states that facilitate metastasis and that are enriched in patients with, versus those without, metastases. In particular, epithelial-to-mesenchymal transition (EMT) is the most cited mechanism for facilitating metastasis, yet it remains controversial and extensively debated, primarily due to the focus of EMT research on model systems and the difficulty in characterizing EMT in patient samples. ScRNA-seq studies now provide an improved capacity to evaluate the exact patterns of EMT directly in clinical specimen. In HNSCC, scRNA-seq identified an EMT-like program at the invasive edge of the tumor, which was modulated by CAFs and enriched in patients with metastases (42). Notably, this program lacked certain features of traditional EMT programs, such as expression of core EMT transcription factors and loss of epithelial markers, and it was found only in two of the four HNSCC subtypes. Such context-specificity of EMT is supported by other recent studies and may help to resolve conflicting reports, thereby underscoring the significance of directly defining cellular states in diverse human tumors.

Single-cell profiling also enables the characterization of circulating tumor cells (CTCs), providing a window into early stages of the metastatic process. CTCs often express EMT programs, consistent with the role of EMT in promoting metastasis (93,94). In addition, analysis of breast cancer CTCs demonstrated an abundance of CTC clusters, with higher metastatic potential than individual CTCs (95). Profiling of the CTC clusters revealed increased expression of the adherens junction protein, plakoglobin, which maintained cell clusters and thereby increased the rate of metastases.

Finally, by comparing matched primary and metastatic samples at the single-cell level, it is possible to identify differences in genetics, cell type composition and cellular states. Genetic differences can help to trace the evolutionary process that underlie metastasis, for example by revealing which genetic clone has seeded the metastasis. Interestingly, one study demonstrated that multiple clones co-migrated and established the invasive lesions in breast cancer (9). In addition to genetics, the differences in cell type composition and cellular states between primary and metastatic samples provide insight into the adaptations of metastatic cells as they colonize different organs or anatomical compartments (42,95–99). For example, a study of leptomeningeal metastasis profiled malignant cells from cerebrospinal fluid (CSF) , and identified upregulation of LCN2 and other genes that enable iron scavenging from inflammatory cells, which promotes survival of the metastatic cells in the iron-poor CSF environment (97).

Response to targeted therapy

Response to therapy may be expected to reflect a combination of clonal selection and cellular adaptation that influence both the malignant cells and non-malignant cells in the TME. When probed in bulk post-treatment tissues, these effects are difficult to tease apart, but scRNA-seq may offer the appropriate resolution to dissect these effects. In *BRAF*-mutant melanoma, scRNA-seq identified cells in a resistance-associated state already in pre-treated samples (18). Further *in vitro* single cell studies showed that this program is

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increased upon treatment, not only due to an expected clonal selection, but also due to a drug-induced epigenetic reprogramming, which might help to explain the widespread resistance to BRAF inhibition in melanoma (100). In NSCLC, tyrosine kinase inhibitors (TKIs) were shown to induce the expression of an alveolar-regenerative cell signature in residual tumor cells, consistent with a therapy-induced cell state transition (55). In ER+/HER2− breast cancer, scRNA-seq demonstrated increased expression of HER2 after multiple courses of therapy with a phenotype that is less sensitive to targeted therapy than in primary HER2+ breast cancer (101). In prostate cancer CTCs, scRNA-seq implicated the activation of noncanonical Wnt signaling as a mechanism of resistance to antiandrogen therapy (102).

Overall, these and other studies are highlighting the formidable adaptive potential of malignant cells under therapeutic pressure. We expect that single cell profiling of post-treatment samples will dramatically expand in the next few years and may even be integrated with clinical trials in an attempt to identify the cellular responses to treatments, and the identity and mechanisms of residual malignant cells. However, in many clinical contexts, the residual cells immediately following partially successful treatments are undetected. These cells may remain dormant for some time until they resume growth and give rise to tumor relapse. Yet, by that time, the resulting tumor have undergone further genetic and epigenetic evolution, which masks the mechanisms of initial drug resistance. Hence, it is unclear to what extent resistance mechanisms can be gleaned from analysis of recurring tumors, which is motivating attempts to directly isolate and profile single cells from minimal residual disease (MRD). This can be achieved in hematological malignancies (103) or through sequential isolation of CTCs following treatments. Alternatively, this is possible with animal models in which MRD can be detected and analyzed (47,104). This approach was used initially to assess MRD in a melanoma model after response to BRAF inhibition (104), and future profiling of MRD in a range of animal models, after response to clinically-relevant treatments, may advance our understanding of resistance mechanisms.

Emerging technologies and future developments

Single-cell genomics methods are ushering a new area in the study of cancer biology, improving our understanding of tumor heterogeneity, evolution, interactions with the tumor microenvironment and response to therapy. Technological developments are enabling multiple new avenues to tackle questions of biological and clinical relevance (Figure 2). Increased throughput of scRNA-seq platforms, where thousands of cells are routinely profiled (32–34), is enabling the discovery of small but clinically significant tumor subpopulations, including those with adaptations underlying drug resistance or metastatic progression. The possibility of profiling frozen samples by sNuc-seq (28) enables the characterization of samples stored at biobanks, facilitating cross-center collaborations and the profiling at scale of rare tumor entities or matched primary and recurrent samples for the study of tumor evolution. Experiments that concurrently evaluate transcriptional and epigenomic states (DNA methylation or chromatin accessibility) in individual cells (27,105) are providing deeper insights into mechanisms governing state diversity and transitions in tumors. Spatially-resolved single-cell profiling methods, such as spatial transcriptomics of tissue sections (106,107), enable better characterization of interactions within the tumor

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microenvironment (108–110). Advances in mass spectrometry methods are also beginning to allow the characterization of thousands of proteins at single-cell resolution without the need of using tagging antibodies (111,112), as well as to quantify increasing numbers of metabolites in cancer cells (113,114). The studies we have reviewed mark the beginning of the era of single-cell profiling of human tumors, which is rapidly broadening in resolution, scale and scope. While the complexity uncovered may appear daunting, the deeper understanding gained from these approaches offers unprecedented opportunities for renewed attempts at improving the management of cancer patients.

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Significance

Intra-tumor heterogeneity (ITH) has been a major barrier to our understanding of cancer. Single-cell genomics is leading a revolution in our ability to systematically dissect ITH. In this review we focus on single cell expression profiling and insights gleaned on stem cell programs, tumor classification, metastasis, microenvironment composition, and tumor response to therapies.

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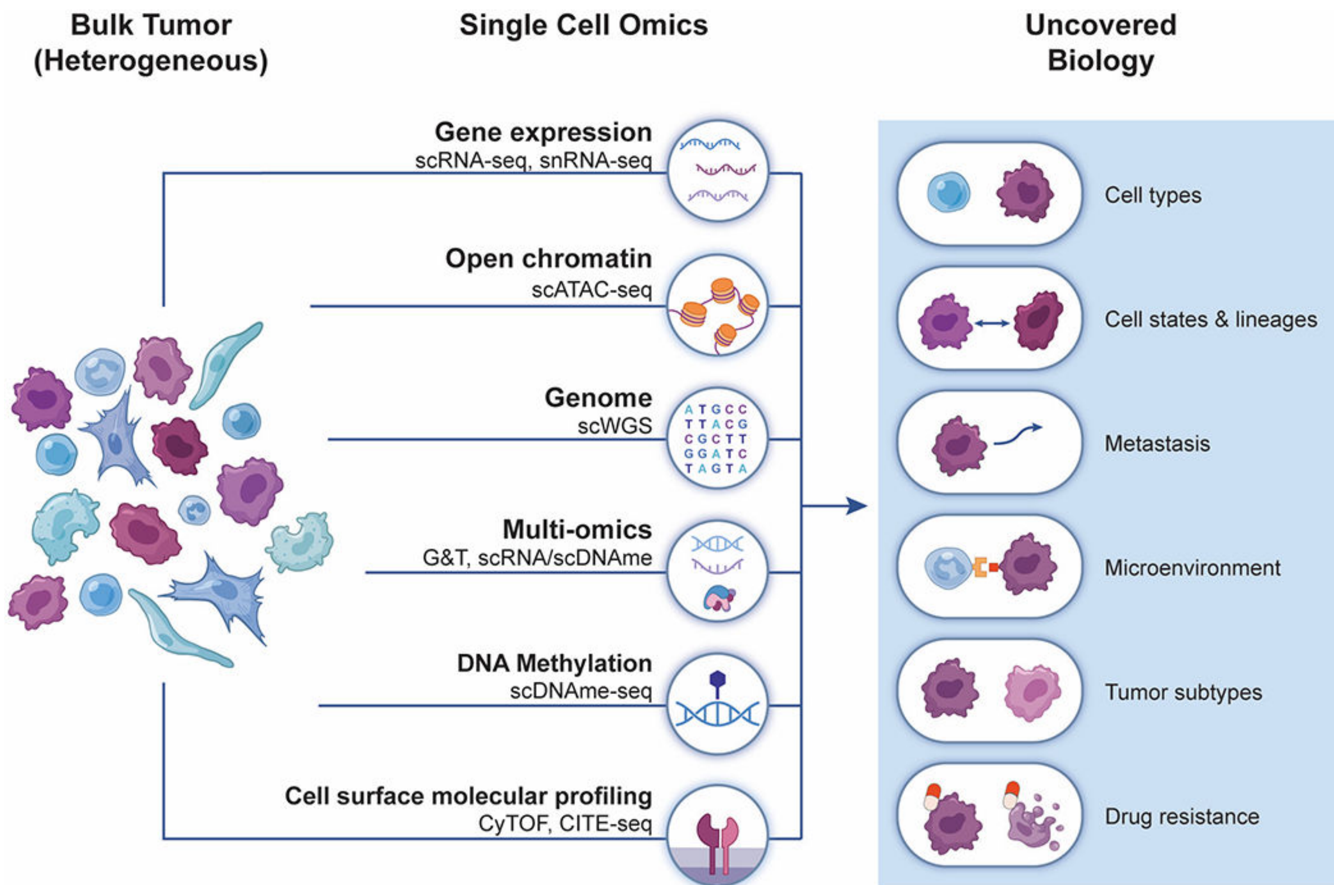

Figure 1:
Schematic of the different levels of biological information that can be profiled at single-cell resolution and examples of biology uncovered.

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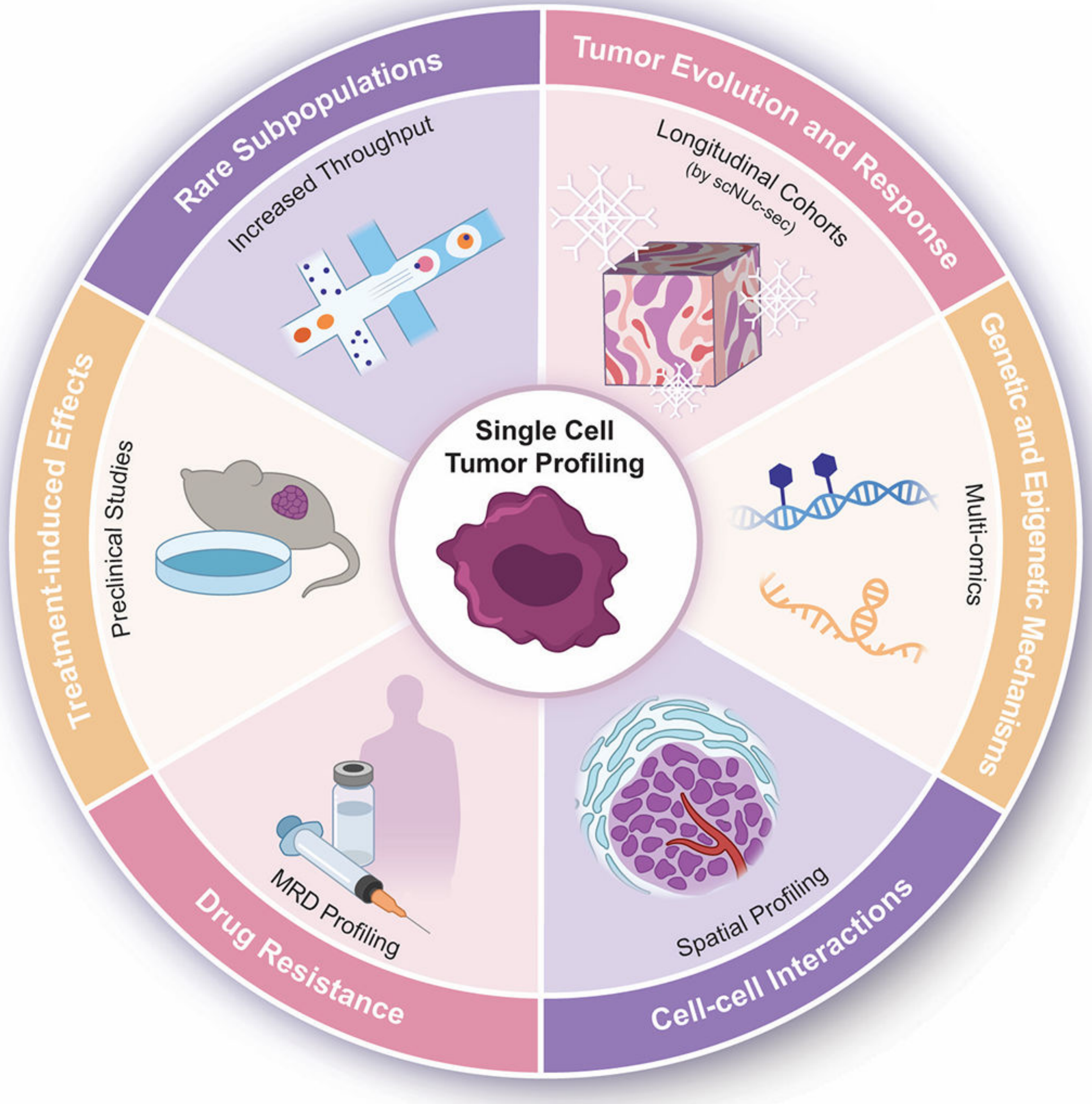

Figure 2:
Recent developments in single-cell genomics approaches and the different biological and clinical questions in oncology they will inform.

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Table 1.

Select studies using single-cell methods to study human intra-tumoral heterogeneity.

Reference	Sample / tumor type	Number of cells / nuclei (number of samples)
Miyamoto et al., 2015 (102)	Prostate CTCs	77 (13)
Jordan et al., 2016 (101)	Breast adenocarcinoma CTCs	74 (16)
Patel et al., 2014 (17)	Glioblastoma	431 (5)
Tirosh et al., 2016a (18)	Metastatic melanoma	4645 (19)
Tirosh et al., 2016b (21)	*IDH*-mutant oligodendroglioma	4347 (6)
Venteicher et al., 2017 (48)	*IDH*-mutant astrocytoma	9879 (10)
Li et al., 2017 (70)	Colorectal cancer	590 (11)
Chung et al., 2017 (115)	Breast cancer	515 (11)
Puram et al., 2017 (42)	Head and neck cancer	6000 (18)
Savage et al., 2017 (116)	Breast cancer	3483
Giustacchini et al., 2017 (22)	Chronic myeloid leukemia	2070 (20)
Brady et al., 2017 (117)	Breast cancer ascites	428 (8)
Filbin et al., 2018 (39)	*H3 K27M*-mutant glioma	3,321 (6)
Kim et al., 2018 (118)	Triple-negative breast cancer	6,862 (8)
Young et al., 2018 (119)	Wilms tumor, Clear cell renal carcinoma, Papillary renal cell carcinoma	72,501 (23)
Livnat-Jerby et al., 2018 (80)	Melanoma	7,186 (33)
Karaayvaz et al., 2018 (120)	Triple-negative breast cancer	1,189 (6)
Gaiti et al., 2019 (26)	Chronic lymphocytic leukemia	2,652 (18)
Neftel et al., 2019 (40)	Glioblastoma, *IDH*-wildtype	24,192 (28)
Peng et al., 2019 (121)	Pancreatic ductal adenocarcinoma	57,530 (35)
Nam et al., 2019 (23)	*CALR*-mutated myeloproliferative neoplasms	38,290 (5)
Hovestadt et al., 2019 (41)	Medulloblastoma	8,734 (25)
Laks et al., ,2019 (19)	Breast cancer PDXs, synovial sarcoma (mouse model), follicular lymphoma samples	51,926 (62)
Van Galen et al., 2019 (122)	Acute myeloid leukemia	38,410 (40)
Ji et al., 2020 (123)	Squamous cell carcinoma	48,164 (22)
Maynard et al., 2020 (55)	Non-small cell lung cancer	23,261 (49)
Kim et al., 2020 (62)	Primary and metastatic lung adenocarcinoma, normal lung	208,506 (44)
Lee et al., 2020 (83)	Colorectal cancer	91,103 (29)
Chen et al., 2020 (124)	Nasopharyngeal carcinoma	47,866 (16)
Izar et al., 2020 (71)	High-grade serous ovarian cancer ascites	10,906 (22)
Gojo et al., 2020 (49)	Ependymoma	74,927 (30)
Ireland et al., 2020 (54)	Small cell lung cancer	15,434 (21)

CALR: Calreticulin

CTC: Circulating tumor cell

H3 K27M: Histone H3 K27M mutation

IDH: Isocitrate-dehydrogenase

PDX: Patient-derived xenograft

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