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Deciphering Human Tumor Biology by Single-Cell Expression Profiling

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Abstract

Human tumors are complex ecosystems where diverse cancer and noncancer cells interact to determine tumor biology and response to therapies. Genomic and transcriptomic methods have traditionally profiled these intricate ecosystems as bulk samples, thereby masking individual cellular programs and the variability among them. Recent advances in single-cell profiling have paved the way for studying tumors at the resolution of individual cells, providing a compelling strategy to bridge gaps in our understanding of human tumors. Here, we review methodologies for single-cell expression profiling of tumors and the initial studies deploying them in clinical contexts. We highlight how these studies uncover new biology and provide insights into drug resistance, stem cell programs, metastasis, and tumor classifications. We also discuss areas of technology development in single-cell genomics that provide new tools to address key questions in cancer biology. These emerging studies and technologies have the potential to revolutionize our understanding and management of human malignancies.

1. INTRODUCTION

1.1. Tumor Biology is Driven by Subpopulations

Intratumoral heterogeneity (ITH) poses a significant challenge to cancer therapy (Kreso & Dick 2014, Mazor et al. 2016, Navin 2015). ITH governs key aspects of tumor biology that are driven by subpopulations of tumor cells, such as tumor growth, invasion, metastasis, and drug resistance (**Figure 1**). For example, while most tumors show measurable response to existing therapeutic efforts, subpopulations of cells survive these treatments, leading to cancer progression and recurrence. Various factors may contribute to ITH: (*a*) genetic heterogeneity, consisting of subclonal mutations that accumulate during tumor growth; (*b*) functional heterogeneity from nongenetic determinants related to developmental pathways and epigenetic programs, such as those associated with tissue stem cells and their differentiation into specialized cell types; and (*c*) spatial variability from extrinsic factors such as oxygen and nutrient availability. In addition to the heterogeneity of the malignant cells, tumors also consist of diverse nonmalignant cells, including immune, endothelial, fibroblast, and other cells in tumors that collectively form the tumor microenvironment (TME) and further influence both malignant cell function (tumor invasion, metastasis, and pharmacological resistance) and the efficacy of immunotherapies (Chen & Mellman 2017).

Together, the diverse malignant and nonmalignant cells make up the complex tumor ecosystem that ultimately governs tumor biology and clinical phenotypes. It is clearly important to precisely measure all of a tumor's cellular elements, but genomic and transcriptomic methods have traditionally profiled these intricate ecosystems as bulk samples, only revealing average cellular behavior. Thus, cancer research must develop a framework that enables a comprehensive analysis of the various cellular programs within human tumors at single-cell resolution. Here, we describe

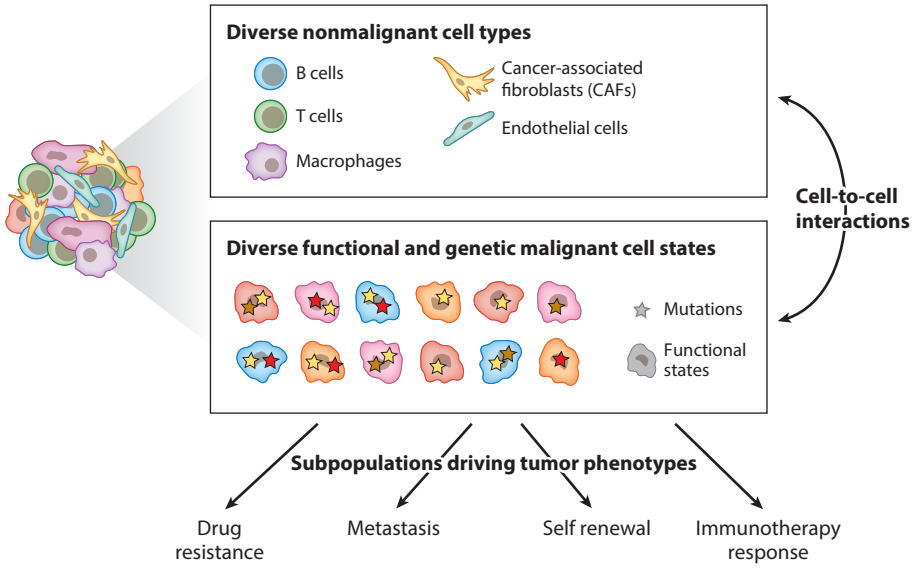

Figure 1

Subpopulations of tumor cells drive drug resistance, metastasis, self-renewal, and response to immunotherapies. Tumors (*left*) are composed of diverse malignant and nonmalignant subpopulations (*middle*) that interact through various mechanisms (*right*) and together underlie important phenotypes (*bottom*).

initial studies applying single-cell expression profiling to dissect the composition and function of tumors and discuss future directions leveraging these approaches.

1.2. Single-Cell Profiling of DNA and Gene Expression Within Tumors

Multiple methods for profiling single cells have been developed in recent years and applied to tumors. Several studies have profiled the DNA of single tumor cells in order to understand genetic ITH (Casasent et al. 2018, Francis et al. 2014, Gao et al. 2016, Leung et al. 2017, Navin & Chen 2016, Wang et al. 2014). Extending earlier studies that inferred genetic heterogeneity from individual bulk samples or from multifocal sampling, these studies demonstrated that many cancer mutations are subclonal and that distinct regions of the same tumor may harbor distinct mutations. This subclonal nature of tumors has important therapeutic implications, as described above. However, such studies have three conceptual limitations that emphasize their complementarity with single-cell expression profiling. First, characterization of genetic heterogeneity does not reveal the functional state associated with distinct genetic events. This is particularly important given that recent studies have suggested a prominent role for neutral evolution of tumors (Williams et al. 2016). Accordingly, most cancer mutations seem to reflect passenger events that may not carry functional implications. Second, functional heterogeneity of cancer cells is driven by both genetic and nongenetic determinants, such as the distinct differentiation states of tumor cells and the influence of extrinsic factors (e.g., hypoxia). Third, analysis of genetic heterogeneity is focused on cancer cells but provides no insight into the composition of the TME.

In principle, single-cell RNA sequencing (scRNA-seq) also enables the detection of mutations within transcribed regions, especially when using protocols that attempt to sequence full-length transcripts (as opposed to 3′- or 5′-end sequencing protocols). However, the sensitivity of such methods appears to be very limited (Tirosh et al. 2016b) due to the partial coverage of the transcriptomes of individual cells. New approaches have recently been proposed to improve this coverage. One such method enriches for specific genes that are likely to be mutated by adding locus-specific primers to the scRNA-seq protocol, as first demonstrated for the BCR-ABL fusion in leukemia (Giustacchini et al. 2017). However, while this approach may be effective for mutations in highly expressed transcripts, it requires prior knowledge of the mutations and is limited to amplification of only a few selected mutations in each sample. Alternatively, both DNA and RNA may be profiled from the same individual cells. Such methods have recently been published, but are currently expensive, technically challenging, and still suffer from limited sensitivity in detecting mutations (Macaulay et al. 2015, 2016). While these techniques are likely to be applied to clinical tumors in the future, studies to date have focused on single-cell tumor profiling of DNA, RNA, or proteins. Studies of single-cell DNA profiling have been recently reviewed elsewhere (Navin 2015); here, we focus primarily on scRNA-seq and on protein profiling of primary human tumors, the main goal of which is to identify the distinct functional states of tumor cells.

2. EXPERIMENTAL DESIGN FOR SINGLE-CELL TUMOR EXPRESSION PROFILING

2.1. RNA Sequencing

Single-cell RNA profiling of tumors requires four main steps (**Figure 2*a***). First, fresh tumor samples are obtained and acutely disaggregated into a single-cell suspension using combinations of mechanical and enzymatic digestion protocols. Second, individual cells are separated either by flow cytometry into 96- or 384-well plates or by microfluidic devices into distinct chambers (e.g., by fluidigm C1) or droplets (e.g., Drop-seq, inDrop, or 10X Genomics platforms) and then lysed.

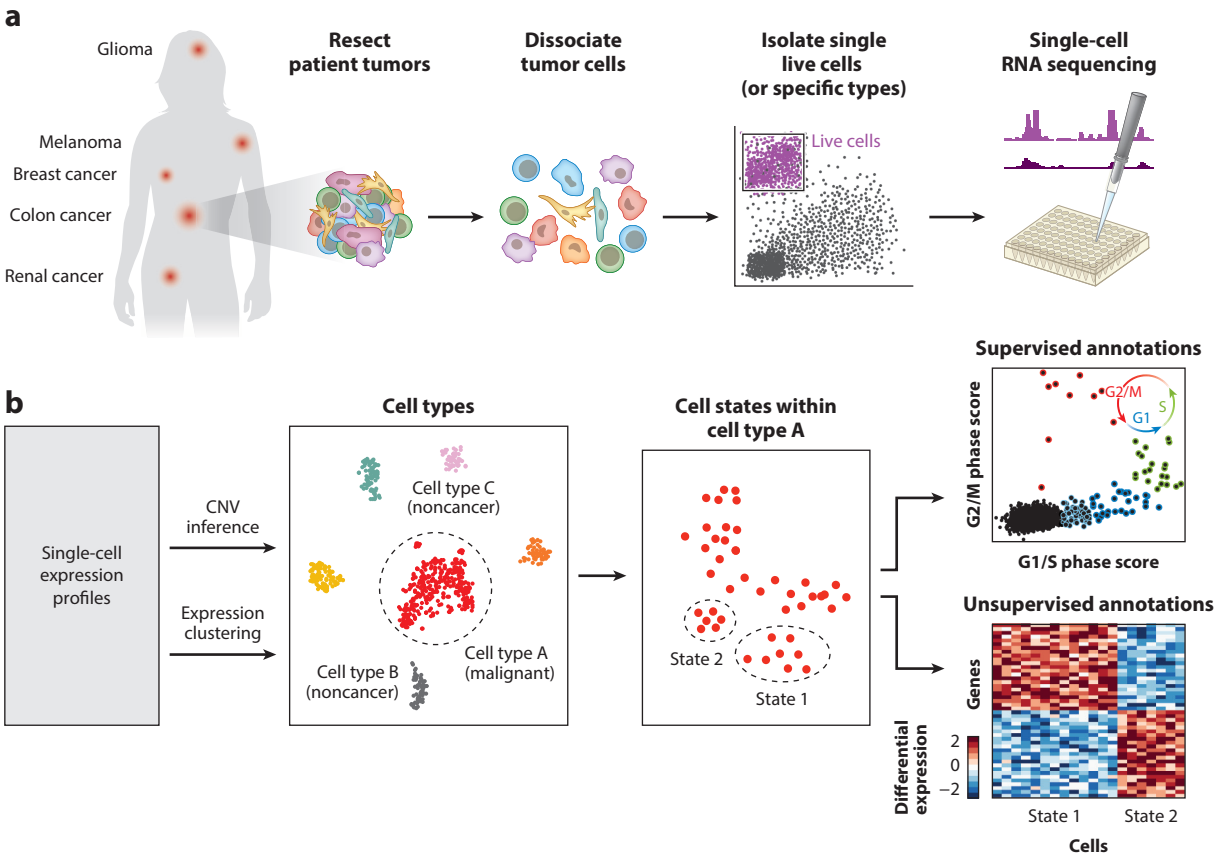

Figure 2

Experimental (*a*) and computational (*b*) workflows for single-cell RNA sequencing (scRNA-seq) analysis of human tumors. (*a*, *left to right*) Human tumors are obtained after surgical resection (shown are the tumor types studied so far; see **Table 1**); then, tumors are mechanically and enzymatically disaggregated and sorted to single live cells and potentially enriched for selected cell types; finally, individual cells are subjected to profiling by scRNA-seq. (*b*, *left to right*) Single-cell expression profiles are clustered to identify distinct cell types—shown is a t-distributed stochastic neighbor embedding (tSNE) plot, in which cells are grouped in two dimensions based on their similarity in gene expression and colored by their cluster assignment—while copy number variation (CNV) inference is used to further annotate cell types as malignant or nonmalignant; additional analysis of cells within a given cell type can define either distinct or continuous cell states; these cell states can then be annotated either by supervised analysis, i.e., scoring cells by known expression programs, such as those reflecting phases of the cell cycle (*top*), or by unsupervised analysis, in which differentially expressed genes are identified and used to derive hypotheses regarding the underlying biology (*bottom*).

Third, a reverse transcription and RNA-seq protocol (either full length or the 3′ end) is applied to individual cells, which are barcoded and then pooled. Finally, the pooled sample is sequenced and the resulting reads are mapped to the transcriptome to assess gene expression. Several of these steps need to be defined and optimized for each application: Clinical tumors require special attention to the timing of dissociation and encapsulation or sorting (to ensure that tumor cells do not die or alter their expression states); flow cytometry sorting into a 96- or 384-well plate is a method of choice for full-length RNA-seq protocols and for combined DNA and RNA extraction from the same cell, but it offers only limited scalability. Droplet-based methods are only compatible with 3′-end (or 5′-end) RNA-seq protocols but offer impressive scalability and much reduced

cost. More recently, a method to label individual cells by combinatorial barcoding was developed, SPLiT-seq (split-pool ligation-based transcriptome sequencing), that does not require the separation of individual cells into compartments but rather leverages the cells (or nuclei) themselves as compartments (Rosenberg et al. 2018). Thus, depending on the application and question, specific single-cell methods may be favored.

2.2. Protein Profiling

Cellular processes ultimately depend on the expression levels (and activity) of proteins, which can be distinct from messenger RNA (mRNA) levels. While measurement of protein expression at the single-cell level has been possible for quite some time, only a handful of proteins (typically one to four) are generally measured in most assays (e.g., by flow cytometry). CyTOF (time-of-flight mass cytometry) enables measurements of the levels of more than 100 proteins in thousands of single cells by significantly limiting the spectral overlap between antibodies labeled with heavy metal ion tags (Spitzer & Nolan 2016). CyTOF has been used to analyze leukemia samples, showing for the first time that the surface markers of leukemic blasts do not necessarily reflect their intracellular signaling (Levine et al. 2015). This approach was recently applied to analyze the malignant or immune composition of acute lymphoblastic leukemia, ovarian carcinoma, renal cancer, hepatocellular carcinoma, and melanoma (Chevrier et al. 2017, Chew et al. 2017, Gonzalez et al. 2018, Good et al. 2018, Lavin et al. 2017, Sula Karreci et al. 2017, Wei et al. 2017). Furthermore, this method has been modified to preserve spatial information when applied to tissues (see Section 4.2) (Giesen et al. 2014). An alternative approach for protein profiling tags antibodies with oligonucleotides [cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq); Stoeckius et al. 2017] and integrates both protein and mRNA measurements into a single-cell readout. While CyTOF and CITE-seq are likely to be used extensively in the future, the requirement for efficient antibodies and the need for assay optimization still limit the set of proteins that can be analyzed. Thus, to date, single-cell expression studies (as reviewed below) have primarily relied on mRNA profiling.

2.3. Distinguishing Cell Types and Cell States

The power of single-cell approaches lies in its ability to describe diverse cellular components. However, this diversity and the wealth of information obtained also introduce conceptual problems—for example, how to characterize such diverse populations and summarize a tumor's composition in ways that enable functional interpretation and comparison between tumors. One approach to reduce complexity is to focus only on a subset of cells (i.e., a specific cell type) of particular interest. These can be the malignant cells, as in studies of patient-derived xenograft (PDX) models, where only the tumor cells are of a human origin (Lawson et al. 2015), or cells in the TME, such as tumor infiltrating lymphocytes (TILs) (Singer et al. 2016). In principle, any cell type that exists within tumors can be enriched with cell type–specific markers and subsequently profiled by single-cell methods. While this makes in-depth study of particular cell types possible, other studies have taken a largely unbiased approach by attempting to profile all (or most) cell types in the tumor, including the various nonmalignant constituents of the TME. These studies require assigning cells to distinct cell types and, in particular, distinguishing malignant from nonmalignant cells. Such distinctions can typically be made by gene expression clustering, as distinct cell types typically form well-separated clusters and may be annotated based on genes with cluster-specific expression (**Figure 2*b***). However, this approach may be confounded in cases where malignant cells are very similar to their cell of origin, which may also be present in tumors, or, alternatively, in cases where malignant cells transdifferentiate and resemble other cell types (Cheng et al. 2013, Wang et al. 2010).

To address this challenge, researchers may combine gene expression clustering with classification by genetics. As noted above, the ability of scRNA-seq to detect single point mutations is limited; however, genetic profiles may also be defined by estimating chromosomal copy number variations (CNVs) based on the average expression of large sets of genes in each chromosomal region. Malignant cells typically harbor clonal large-scale CNVs that distinguish them from nonmalignant cells (**Figure 2*b***; **https://github.com/broadinstitute/inferCNV**). While this approach may have limited utility in tumors without CNVs, it performed well in studies of *IDH*-mutant glioma, glioblastoma, melanoma, head and neck squamous cell carcinoma (HNSCC), and other tumor types (Chung et al. 2017; Muller et al. 2016; Patel et al. 2014; Puram et al. 2017; Singer et al. 2016; Tirosh et al. 2016a,b; Venteicher et al. 2017).

The choice to rely on identification of CNVs instead of point mutations highlights an important feature of scRNA-seq data sets that influences all analyses: The sensitivity of detecting individual genes (or their mutations) is severely hindered by the partial efficiency of single-cell profiling (often termed dropout); thus, one cannot rely on any individual mutation or gene to infer cellular identity. By contrast, the combined analysis of many related genes (i.e., adjacent genes in the case of CNVs) produces reliable estimates of CNVs. The same approach can be applied to the analysis of pathways or biological functions, which also suffers when a single gene of particular importance is measured with limited accuracy. If a larger gene set is known to reflect a certain biological function or process, then that gene set's level may be evaluated with higher accuracy, enabling cells to be annotated by the corresponding biological processes or pathways (**Figure 2*b***). Notably, the choice of gene set for such analyses is of great importance, and caution should be taken when gene sets are derived from bulk analysis or from a biological system distinct from the one being examined. Thus, the ideal situation is when a gene set is derived from the analysis of a scRNA-seq data set and then applied to the same or to an extended data set to derive cell scores for the corresponding biological process (Filbin et al. 2018; Patel et al. 2014; Puram et al. 2017; Tirosh et al. 2016a,b).

Analysis of the diversity within tumors (or other tissues) typically allows us to distinguish between two layers: First, cells are grouped into cell types (e.g., malignant cells, T cells, fibroblasts) that often present as highly distinct clusters; second, each cell type is associated with further diversity reflective of distinct cell states, such as various context-specific expression programs (**Figure 2*b***). Phases of the cell cycle represent one prime example of distinct cell states, where gene sets may be used to reliably identify proliferating cells and further infer their cell cycle phase (e.g., S versus M phase) among cells from the same cell type (**Figure 2*b***). We use "cell states" to mean expression programs that are based on multiple genes and assumed to have a functional implication but are potentially dynamic (e.g., cells transition between phases of the cell cycle), while cell types are more stable. Although these definitions are admittedly imprecise, they reflect an important distinction between two layers of diversity in tumors. In the following section, we focus on cell states that have been identified in recent single-cell RNA profiling studies (**Table 1**) and that exemplify the power of single-cell analysis to decipher tumor biology.

3. LINKING TUMOR SUBPOPULATION PROGRAMS TO CANCER CELL PHENOTYPES

3.1. Developmental Lineages and Cancer Stem Cells

Numerous studies over the last two decades have suggested that certain tumors harbor a developmental hierarchy, with subsets of privileged cells capable of self-renewal, while other tumor cells are more differentiated and have lower tumor initiation capacity (Kreso & Dick 2014,

Table 1 Studies using single-cell expression profiling to study human intratumor heterogeneity

Reference	Technology for single-cell profiling	Sample/tumor type	Number of cells/nuclei (number of samples)	Number of genes/proteins
Dalerba et al. 2011	sc-qPCR	Colon adenocarcinoma	336 (1)	53
Lawson et al. 2015	sc-qPCR	Breast adenocarcinoma PDXs	964 (12)	116
Giesen et al. 2014	Imaging mass cytometry	Breast cancer FFPE	NA[a] (21)	32
Levine et al. 2015	Mass cytometry	Pediatric AML bone marrow	>15 million (16)	31
Ramskold et al. 2012	scRNA-seq	Melanoma CTCs	6 (1)	All
Miyamoto et al. 2015	scRNA-seq	Prostate CTCs	77 (13)	All
Jordan et al. 2016	scRNA-seq	Breast adenocarcinoma CTCs	74 (16)	All
Patel et al. 2014	scRNA-seq	Primary glioblastoma	431 (5)	All
Kim et al. 2016	scRNA-seq	Renal cell carcinoma	116 (2)	All
Tirosh et al. 2016a	scRNA-seq	Metastatic melanoma	4,645 (19)	All
Tirosh et al. 2016b	scRNA-seq	*IDH*-mutant oligodendroglioma	4,347 (6)	All
Ståhl et al. 2016	Spatial transcriptomics	Breast cancer	NA[a] (1)	All
Venteicher et al. 2017	scRNA-seq	*IDH*-mutant astrocytoma	9,879 (10)	All
Li et al. 2017	scRNA-seq	Colorectal cancer	590 (11)	All
Chung et al. 2017	scRNA-seq	Breast cancer	515 (11)	All
Puram et al. 2017	scRNA-seq	Head and neck cancer	6,000 (18)	All
Savage et al. 2017	scRNA-seq	Breast cancer	3,483 (1)	All
Giustacchini et al. 2017	scRNA-seq	Chronic myeloid leukemia	2,070 (20)	All
Chew et al. 2017	Mass cytometry	Hepatocellular carcinoma (immune cells)	>280,000 (28)	35
Chevrier et al. 2017	Mass cytometry	Renal cell carcinoma (immune cells)	3.5 million (78)	33–35
Lavin et al. 2017	Mass cytometry, scRNA-seq	Lung adenocarcinoma	NA[a] (28)	33–38
Gao et al. 2017	Single-nuclei RNA-seq	Breast cancer	416 (1)	All
Brady et al. 2017	scRNA-seq	Breast cancer ascites	428 (8)	All
Filbin et al. 2018	scRNA-seq	*H3 K27M*-mutant glioma	3,321 (6)	All

Abbreviations: AML, acute myeloid leukemia; CTCs, circulating tumor cells; FFPE, formalin-fixed, paraffin-embedded; NA, not applicable; PDX, patient-derived xenografts; sc-qPCR, single-cell quantitative polymerase chain reaction; scRNA-seq, single-cell RNA sequencing.
[a]The exact cell number either is unknown due to spatial profiling or is not reported.

Lathia et al. 2015). This model, often termed the cancer stem cell (CSC) hypothesis, has far-reaching implications but remains controversial. Single-cell expression analysis provides a novel approach to evaluate the CSC hypothesis by characterizing the diversity of cell states within tumors and their links to normal cell types, genetics, and self-renewal.

Indeed, several single-cell studies have already described the similarity of tumor subpopulations to developmental lineages. The first study to perform single-cell multiplex qPCR (quantitative polymerase chain reaction) on a human tumor analyzed colon adenocarcinoma samples and demonstrated the existence of subpopulations that recapitulate normal lineages of the gut epithelium, including LGR5$^+$ stem-like cells (Dalerba et al. 2011). A more recent study, also

utilizing a single-cell multiplex qPCR approach, leveraged PDX models to demonstrate that breast cancer metastases harbor both basal-like and luminal-like cells (Lawson et al. 2015). Importantly, early metastases (with only a few cancer cells) were dominated by basal-like cells, while more advanced metastases contained predominantly luminal cells, consistent with a model in which metastases originate from basal cells that then differentiate into luminal cells, analogous to the mammary gland architecture.

One limitation of these two pioneering studies is the use of a limited and predefined gene set (~50 genes) for qPCR analysis, which hinders a genome-wide assessment of tumor subpopulations and their functional states. Extensions of this approach to scRNA-seq revealed cellular hierarchies of *IDH*-mutant oligodendroglioma and astrocytoma (Tirosh et al. 2016b, Venteicher et al. 2017): Most cancer cells were shown to be differentiated and reminiscent of one of two glial lineages (oligodendrocyte-like and astrocyte-like cells), while a smaller subset of cells appeared undifferentiated, resembling neural stem/progenitor cells. These developmental programs were observed in all tumors analyzed and across multiple genetic subclones within the same tumor. Importantly, signatures for proliferation were highly enriched in undifferentiated cells; thus, two independent signatures (reflecting development and proliferation) point to the same model, whereby a subpopulation of stem-like cells is responsible for fueling the growth of *IDH*-mutant gliomas, while cells differentiated toward glial lineages do not cycle. Similar analysis of pediatric midline gliomas with histone H3 lysine-to-methionine mutations (*H3 K27M*-mutant glioma) uncovered a similar hierarchy, but with two important differences. First, the identity of the undifferentiated cells was different, pointing to distinct types of stem-like cells in different classes of glioma. Second, the fraction of stem-like cells was much higher in *H3 K27M*-mutant glioma than in *IDH*-mutant glioma, possibly underlying the difference in aggressiveness of these diseases (Filbin et al. 2018). Overall, these studies strongly suggest that therapeutic strategies for glioma should attempt to eradicate undifferentiated CSC-like populations or induce their differentiation. To that end, particular effort should be taken to carefully characterize the cellular states of preclinical models and understand their relationship to cellular states observed in patients, as different models might recapitulate different tumor compartments, as recently shown in *H3 K27M*-mutant glioma (Filbin et al. 2018). Future work applying similar strategies will interrogate the developmental lineages and putative CSC programs in a range of malignancies (Savage et al. 2017). In addition, identification of putative CSCs by scRNA-seq should be followed by functional studies to test the tumor initiation capacity and drug resistance of these subpopulations. While conceptually similar to previous studies in the CSC field, these renewed efforts will leverage single-cell approaches to better define putative CSC markers in a comprehensive way.

3.2. Drug Resistance

Once heterogeneous expression programs are identified by single-cell expression approaches, a central challenge is to connect these programs to specific functional features, such as sensitivity or resistance to treatments. Such associations can sometimes be made based on other studies, as recently suggested for drug resistance in melanoma (Tirosh et al. 2016a). Previous work connected the MITF transcription factor and its downstream expression program (the MITF-high program) with sensitivity of melanoma to BRAF inhibition, while an alternate program including the kinase AXL was linked to intrinsic resistance to BRAF inhibition (Konieczkowski et al. 2014, Muller et al. 2014). Interestingly, these expression programs were found to be highly variable among single cells from the same melanoma tumor such that even tumors for which bulk analysis suggested a MITF-high expression program harbored few cells with an AXL-high expression program. Thus, drug resistance may be mediated by a subpopulation of AXL-high cells that is not detected by

bulk analysis. Consistent with this possibility, the fraction of AXL-high cells appeared to increase in both tumors and cell lines upon treatment and recurrence.

Future studies should extend these approaches by comparing matched tumors before and after treatment to identify subpopulations that may represent potential drivers of drug resistance. Initial examples of this approach come from recent studies of circulating tumor cells (CTCs) in breast and prostate cancer patients that acquired drug resistance (Jordan et al. 2016, Miyamoto et al. 2015). Jordan et al. (2016) demonstrated that in a subset of advanced breast cancer patients, two dynamic subpopulations of CTCs coexist and can interconvert. Importantly, these subpopulations differ in their expression of receptor tyrosine kinases and Notch signaling activity and have distinct proliferative and drug-resistant properties. Miyamoto et al. (2015) demonstrated that CTCs from prostate cancer patients had high heterogeneity and implicated the noncanonical Wnt pathway in their resistance to antiandrogen treatments. These findings suggest a role for heterogeneous CTC populations in resistance to therapy, for which the authors propose novel combinatorial treatments.

3.3. Metastasis

Metastasis is ultimately responsible for most cancer-associated deaths, yet most tumor studies are still based on primary tumors. Single-cell studies may help bridge these gaps and deepen our understanding of the metastatic process. For example, studies of CTCs can unravel the functional state of cells that will potentially lead to metastases (Aceto et al. 2014, Jordan et al. 2016, Ramskold et al. 2012). Indeed, Aceto et al. (2014) demonstrated that breast cancer CTCs often present as multicellular clusters whose metastatic potential is higher than single CTCs. This work suggests that metastases may derive from multicellular clusters held together through plakoglobin-dependent adhesion rather than individual tumor cells, as has typically been thought.

A different approach to understand metastasis involves directly comparing matched primary and metastatic tumor samples: The first such study used scRNA-seq to compare a primary renal cell carcinoma to its lung metastasis and identified distinct activity of several drug-related pathways (Kim et al. 2016). A second study compared primary HNSCCs to their matched metastatic lymph nodes (Puram et al. 2017). Surprisingly, very limited differences were found between primary tumors and lymph node metastases, and these were patient specific, arguing against a common signature of metastasis. However, considerable heterogeneity was identified within each one of those samples, including a prominent signal for partial epithelial-mesenchymal transition (pEMT) at the invasive edge, or periphery, of those samples. Notably, pEMT signatures correlated with the presence of metastasis, suggesting that pEMT may be important for the initial phases of metastasis but is subsequently decreased within the metastatic lymph node. Future studies will extend these approaches to diverse clinical contexts.

3.4. Immunotherapy

The composition of the TME has an important impact on tumorigenesis and treatment response. In particular, tumor infiltration by cytotoxic T cells is predictive for the response to immune checkpoint inhibitors in various cancer types (Chen & Mellman 2017). Indeed, the activity of TILs has a major impact on survival in many different cancers and on the response to checkpoint blockade (Pages et al. 2009). Effector $CD8^+$ T cells can mediate lysis of malignant cells and control tumor growth, but chronic antigen stimulation and an immunosuppressive TME result in a progressive loss of T cell effector functions. Such dysfunctional T cells express several inhibitory receptors (including PD-1, CTLA-4, TIM-3, and LAG-3) (Wherry & Kurachi 2015).

Recent studies examined the diversity of TILs within human and mouse tumors and, in particular, began to characterize the dysfunctional states of TILs (Zheng et al. 2017). Two scRNA-seq studies of melanoma identified dysfunction-specific expression signatures and regulators and have shown that these signatures are often confounded by T cell activation (Singer et al. 2016, Tirosh et al. 2016a). Other studies used CyTOF to comprehensively describe the immune infiltration in renal, lung, and hepatocellular carcinoma and to assess the differential effect of anti-CTLA-4 and anti-PD-1 therapies on TILs in melanoma (Chevrier et al. 2017, Lavin et al. 2017, Wei et al. 2017). These studies will help to resolve the immunosuppressive mechanisms that operate in diverse cancer types and may lead to novel immunotherapies.

TILs that recognize tumor antigens proliferate and generate clones with identical T cell receptor (TCR) sequences. Since full-length RNA-seq reads cover the TCR, scRNA-seq enables the reconstruction of TCR sequences and the identification of clonal subpopulations, thereby linking the expression state of cells to their clonal and functional properties, as demonstrated by several recent studies (Eltahla et al. 2016, Stubbington et al. 2016, Tirosh et al. 2016a, Zheng et al. 2017).

Apart from their dysfunction, the ability of TILs to mount an effective antitumor response is often limited by their exclusion from the tumor core through a complex set of mechanisms, including interactions with additional TME components such as cancer-associated fibroblasts (CAFs), endothelial cells, and macrophages. Profiling the entire TME will help to elucidate such interactions, as recently demonstrated in melanoma (Tirosh et al. 2016a). Complementing the expression state of TILs and other TME components with their spatial localization and direct physical interactions will yield additional insights, as discussed in Section 4.2.

3.5. Classifying Tumor Subtypes

A central goal of large-scale tumor expression profiling studies, such as The Cancer Genome Atlas, has been to identify tumor subtypes that are thought to represent distinct disease entities and potentially justify patient stratification for treatments. Expression profiling has been proposed to assist in tumor classification and therapeutic decisions in many cancers, including breast cancer, glioblastoma, melanoma, medulloblastoma, and others. As single-cell analyses expand to larger numbers of samples, we expect tumor classification schemes to be redefined.

To date, single-cell studies have been based on few samples and thus were insufficient to define tumor subtypes de novo, but they have already shed new light on the subtypes defined from bulk tumors. For example, analysis of glioblastoma demonstrated that individual cells within the same tumor resemble distinct subtypes (so-called proneural, neural, classical, and mesenchymal gliobastoma subtypes) (Patel et al. 2014). Thus, bulk-level classification is driven by the most common of those programs within each tumor. This extends previous findings that distinct subtypes were represented in different areas of the same tumor (Gill et al. 2014). A similar observation was made in melanoma: While previous studies classified cell lines or tumors as MITF-high or AXL-high, both cellular states are contained within individual melanoma cell lines and tumors (Tirosh et al. 2016a). Interestingly, AXL-high tumor classification is further cofounded by the frequency of CAFs, which highly express *AXL* and the other genes associated with the AXL-high expression program. Similarly, recent bulk and single-cell studies of colorectal cancer and HNSCC have indicated that mesenchymal expression programs, which were thought to reflect the epithelial-to-mesenchymal transition of cancer cells, may instead reflect CAFs (Calon et al. 2015, Isella et al. 2015, Li et al. 2017, Puram et al. 2017). Thus, tumor classification by bulk expression profiling is governed by a combination of three effects: the relative frequencies of cancer cell states that coexist within the same tumor (e.g., the four glioblastoma subtypes); the relative frequencies of noncancer cell types (e.g., melanoma and colorectal CAFs); and finally, expression programs

that reflect the genetic and epigenetic state of the cancer cells and that are distinct across tumors. Single-cell analysis will be a promising strategy to decipher the relative contribution of these effects and to identify the underlying differences among tumor subtypes. For example, Venteicher et al. (2017) used both bulk and single-cell expression profiling to explain the global differences between two types of *IDH*-mutant gliomas, oligodendroglioma and astrocytoma, distinguishing the contributions of the TME, cancer cell genotypes, and their developmental states.

4. EMERGING TECHNOLOGIES AND FUTURE DIRECTIONS

The recent studies reviewed above provide a proof-of-concept for the utility of single-cell expression profiling of tumors, which will be extended considerably in the next few years, facilitated by continuous technology development in this rapidly growing field. Below we highlight four emerging capabilities that we anticipate will have considerable impact on future single-cell tumor analyses (**Figure 3**).

4.1. Platforms with Increased Throughput for Comprehensive Tumor Profiling

All RNA-seq and qPCR studies described above were performed with platforms that are based on chips or plates with limited cell numbers; along with cells that do not pass quality control, these approaches restrict the number of cells profiled. Over the past four years, however, several systems have emerged that are based on droplet microfluidics or other technologies (Gierahn et al. 2017) that enable an order-of-magnitude-more cells in each batch; these are gradually becoming the standard in the field (**Figure 3*a***). Droplet-based systems include Drop-seq (Macosko et al. 2015), inDrop (Klein et al. 2015), and commercial solutions such as Chromium by 10X Genomics. While the increased cell numbers come at a certain cost—droplet-based methods tend to produce data sets with reduced coverage, in terms of both the number of detected genes and the

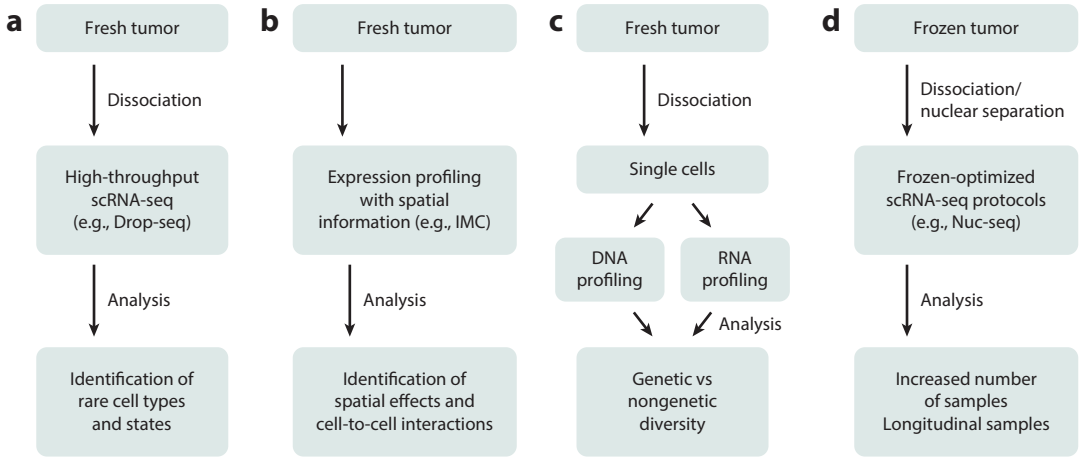

Figure 3

Emerging technologies to advance the study of human tumors by single-cell expression profiling. Schemes depicting technologies for single-cell profiling with improved throughput (*a*), added spatial information (*b*), joint RNA and DNA profiling (*c*), and the ability to analyze frozen cohorts (*d*). Readers are referred to the main text for further details; panels *a–d* correspond to Sections 4.1–4.4, respectively. Abbreviations: IMC, imaging mass cytometry; Nuc-seq, single-nucleus RNA sequencing; scRNA-seq, single-cell RNA sequencing.

proportion of the gene being sequenced—future studies will increasingly rely on such high-throughput technologies given their lower per-cell cost and the potential importance of rare subpopulations.

4.2. Exploring the Spatial Organization of Tumor Subpopulations

The state of each cell depends on its exact spatial position and physical interactions within a multicellular ecosystem. Multiple methods that can retain spatial information in single-cell expression profiling are emerging (**Figure 3*b***). Fluorescent in situ RNA sequencing was the first method to enable high-throughput single-cell expression profiling within tissue sections by in situ sequencing of stably cross-linked complementary DNA (Lee et al. 2014). Elegant studies demonstrated that sequential hybridizations can be used to quantify hundreds of RNAs by fluorescent in situ hybridization, thereby combining its advantages—high sensitivity and spatial information—with increased throughput (Chen et al. 2015, Frieda et al. 2017, Lubeck et al. 2014, Shah et al. 2016). Recently, in situ mRNA sequencing enabling the visualization and quantification of the transcriptome with spatial resolution in tissue sections was developed and applied to mouse brain and human breast cancer (Ståhl et al. 2016). An alternative approach that combines laser capture microdissection with scRNA-seq was applied to investigate the spatial transcriptome of mouse embryo and brain, as well as pathological liver and sperm tissues (Chen et al. 2017). Finally, mass cytometry has recently been coupled to high-resolution laser ablation to measure dozens of proteins while retaining spatial information (Giesen et al. 2014). While each of these technologies has specific advantages and limitations, this range of methods suggests that extensive progress will be made in the next few years in understanding the role of spatial organization within tumors.

4.3. Joint Profiling of DNA and RNA to Distinguish Genetic from Nongenetic Effects

As noted above, expression profiling is highly complementary to that of DNA sequencing. Partial genetic information may be obtained from RNA-seq data, either by inference of CNVs or by identification of mutations in RNA-seq reads (especially in the case of full-length RNA-seq protocols like Smart-seq2), yet this partial genetic information is insufficient to reconstruct a phylogenetic tree. Several methods have recently been developed that enable joint profiling of DNA and RNA from the same cell (**Figure 3*c***) (Macaulay et al. 2015, 2016). Applying these approaches to many cells in tumors is limited by the cost of whole-exome sequencing (WES) and may require cost-effective approaches such as targeted sequencing of loci that harbor mutations based on bulk WES analysis. Alternatively, microsatellites can be targeted for sequencing to derive a phylogenetic tree at a lower cost without sequencing functionally important mutations (Biezuner et al. 2016).

4.4. Profiling Frozen Samples to Overcome Limitations in Sample Acquisition

Current studies have required fresh tumor samples and a highly optimized pipeline to decrease the time from tumor resection in the operating room to cell lysis. These requirements create a severe bottleneck for sample processing, especially for rare tumor types. However, multiple approaches may enable researchers to profile frozen samples, thereby opening the door to characterizing collections of samples that are stored in hospitals and labs, including longitudinal cohorts (**Figure 3*d***). Recent work optimized RNA extraction from fixed cells and demonstrated comparable single-cell expression profiles from fresh and fixed samples of human embryonic stem cells and

neural progenitors (Thomsen et al. 2016). Alternatively, frozen or fixed samples may be analyzed by isolating and sequencing single nuclei (instead of single cells), as recently demonstrated (Gao et al. 2017; Habib et al. 2016, 2017). Although nuclei do not contain the entire cellular transcriptome, they provide sufficient information to reconstruct subpopulations of cells in tissues and to circumvent the need for enzymatic digestion, thus enabling the analysis of cellular programs in situ.

The ability to profile frozen samples will facilitate an important direction in current and future single-cell tumor studies (Brady et al. 2017, Kim et al. 2016, Puram et al. 2017): the ability to comprehensively interrogate multiple samples from the same patient—collected either from different sites (e.g., primary versus metastasis) or at different time points (e.g., initial diagnosis versus recurrence). Such studies will uncover the evolution of tumor composition during tumor progression and will help to understand the functional significance of ITH.

CONCLUDING REMARKS

Tumor heterogeneity has puzzled cancer biologists and clinicians for a long time. Yet, as long as tools were lacking to accurately measure individual cells in a tumor, researchers relied on average measurements or specific markers, masking many critical aspects of ITH and hindering our understanding of tumor biology. The studies we have reviewed here are the beginning of an exciting era of single-cell profiling of human cancer; they are paving the way for many more discoveries to come from improved technologies, resolution, and broader clinical settings. As we progress, we learn that the precise characterization of single-cell programs in tumors not only provides daunting information on tumor complexity but also offers opportunities for improved classification schemes and better understanding of tumor biology, laying the foundation for new therapeutic approaches.

DISCLOSURE STATEMENT

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