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Opinion

Tumor Functional Heterogeneity Unraveled by scRNA-seq Technologies

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Effective cancer treatment has been precluded by the presence of various forms of intratumoral complexity that drive treatment resistance and metastasis. Recent single-cell sequencing technologies are significantly facilitating the characterization of tumor internal architecture during disease progression. New applications and advances occurring at a fast pace predict an imminent broad application of these technologies in many research areas. As occurred with next-generation sequencing (NGS) technologies, once applied to clinical samples across tumor types, single-cell sequencing technologies could trigger an exponential increase in knowledge of the molecular pathways involved in cancer progression and contribute to the improvement of cancer treatment.

Highlights

Tumors are highly complex entities comprising cell populations with various transcriptional programs.

Single-cell sequencing technologies are evolving fast and have the capacity to finely characterize the huge heterogeneity inside tumors.

New single-cell sequencing protocols do not need special infrastructure and can be applied to a huge multitude of cancer sample types in many research areas.

A fine characterization of liquid biopsies, tumor functional heterogeneity, and the tumor microenvironment will be followed by an exponential increase in our knowledge on tumor progression and will significantly improve cancer treatment.

Multifaceted Heterogeneity and Its Impact on Cancer Progression

Tumors comprise various cell populations in constant evolution. Some of this complex heterogeneity derives from genetic diversification and Darwinian selection of tumor cells as they adapt to variable environments. **Next-generation sequencing** (**NGS**; see Glossary) used for the past decade had enough sensitivity to detect mutations present in minor cell populations and, combined with multisampling of human tumors (**multisampling sequencing**), fostered many studies that characterized **intratumor heterogeneity** in various cancers [1]. The level of intratumor heterogeneity is considered a main driver of therapy resistance and metastasis and is associated with poor prognosis [2].

In addition, human cancers frequently have tumor cell populations with different transcriptional programs. This functional diversity is likely associated with the **genetic heterogeneity** described above but is also the result of many other factors. First, the presence of a hierarchical structure, where a group of quiescent stem-like cells fosters the growth of a tumor comprising cells in different differentiation states, was demonstrated in various tumor types [3]. Additionally, different transcriptional programs can be activated in tumor cells as a response to stochastic factors or to a variable tumor microenvironment. This functional diversity provides tumors with a plasticity that grants a high capacity for adaptation [4].

Finally, human tumors comprise not only malignant/transformed cells but also a plethora of different cell types recruited from the surrounding tissue and the immune system. The tumor microenvironment shows also genetic and transcriptional diversity and plays important roles in tumor progression, metastasis, and treatment resistance [1,5].

Fine characterization of these levels of tumor heterogeneity is essential to the successful treatment of cancer patients. The recent development of technologies based on sequencing individual cells (single-cell sequencing technologies) opens new ways for the characterization of tumor heterogeneity. At the genetic level, single-cell DNA-seq technologies offer higher sensitivity in the detection of minority clones, the reconstruction of clone structure, and the identification of concurrent or exclusive alterations in the same cells. However, it is in the study of **functional heterogeneity** that **single-cell RNA-seq (scRNA-seq)** significantly improves on previous technologies, increasing our molecular comprehension of cancer progression. A precise cell-type annotation of complex cellular samples from primary tumors is possible thanks to the recent generation of single-cell transcriptome atlases. These comprise normal and pathological samples from human and mouse [6,7].

The Emergence of scRNA-seq Technologies

In just a few years, the ability to perform single-cell expression profiles increased from a handful of cells to thousands of cells in a single experiment [8]. After the first scRNA-seq experiment in a

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four-cell-stage blastomere [9], several studies were published based on cell isolation and individual **genomic library** preparation. These initial protocols were laborious and expensive, required RNA amplification steps that introduced bias in the data, and were characterized by reduced throughput [10,11]. The subsequent introduction of unique molecular identifiers (UMIs), which are random sequences that label individual molecules, significantly removed cDNA amplification bias [12]. Further developments in STRT-seq and CEL-seq protocols included the introduction of an individual barcoding step on isolated cells before a single retrotranscription reaction reducing batch artifacts [13,14]. In 2015, the introduction of **microfluidic** devices (Drop-seq [15] and InDrop [16]) enabled the processing of thousands of cells at once. Following this strategy, 10x Genomics automated equipment recently characterized 1.3 million cells at the single-cell level [8]. Unfortunately, microfluidics-based methods are not efficient in the removal of the abundant rRNA. Consequently, they use poly-T oligonucleotides to sequence the end of poly-A-tailed RNAs. This is useful in generating expression profiles in this group of RNAs but does not provide complete transcriptomic information. Split-seq and Sci-seq strategies avoid physical cell isolation, taking advantage of a combinatorial barcoding strategy that permits the individual labeling of more than 100 000 single-cell transcriptomes [17,18]. These techniques do not require expensive microfluidics infrastructure and permit greater control over the number of analyzed cells. Finally, **single-cell multiomics** approaches that allow the study of genetic, epigenetic, and transcriptomic profiles in the same cell have been developed [19,20]. This opens a window of opportunity for comprehensive cell characterization.

Single-cell sequencing data analysis is a great challenge, similar to the early years of the use of NGS technologies. Due to a great variety of sequencing strategies and biological questions, there are many different reported analysis workflows. Analysis tools for subpopulation identification, differential expression, functional signatures, pseudotiming modeling, and network reconstruction are publicly available for researchers with limited bioinformatics resources [21,22].

Dissecting the Tumor Ecosystem with scRNA-seq

Functional Diversity of Tumor Cells

Transcriptional heterogeneity among tumor cells has clear and direct clinical implications. First, molecular classification according to **transcriptional signatures** is commonly used for clinical management in many tumor types. Regarding this, the presence of different transcriptional programs inside the same tumor might prevent, or at least bias, molecular classification from a single biopsy. In this context, scRNA-seq experiments have demonstrated the presence within the tumor of multiple cell populations belonging to different molecular groups according to standard classifications [23–25].

Second, the presence of functional diversity within tumors likely improves their adaptation to hostile environments. Functionally diverse cell populations with symbiotic, mutually beneficial relationships have been reported in tumors [26]. This diversity can also be hierarchical, as described in several tumor types in which a minority of highly specialized cells, termed cancer stem cells (CSCs), might have special capacities to maintain tumor growth, metastasize, and resist antitumor treatments [27]. Nevertheless, the lack of universally accepted CSC markers and properties has generated controversy in these studies. scRNA-seq technologies offer an opportunity for the unbiased identification and study of those populations that supposedly are present in very low numbers and in a quiescent or dormant state, and to design more specific antitumor treatments [28]. scRNA-seq experiments recently demonstrated the presence of populations with stem-like and treatment-resistance properties in oligodendroglioma and melanoma [29,30].

Finally, single-cell technologies can detect minor treatment-resistant cell populations inside complex tumors, which can be used to select appropriate therapies. For instance, the presence of a melanoma cell population expressing high levels of AXL anticipated the occurrence of clonal selection after treatment with RAF or MEK inhibitors and the eventual development of drug resistance[29].

Glossary

Circulating tumor cells (CTCs): cancer cells that have escaped from the primary tumor and travelled through the blood vessels.
Functional heterogeneity: presence of cells with different transcriptional programs inside tumors.
Genetic heterogeneity: existence of cell clones with different genetic somatic mutations inside human tumors.
Genomic library: collection of DNA fragments with common adapters ready to be analyzed by next-generation sequencing technologies.
Intratumor heterogeneity: the presence of cell diversity inside human tumors.
Microfluidics: group of techniques that allow the manipulation of fluids in the range of microliters to picoliters.
Multisampling sequencing: comprehensive analysis of regionally distant samples from the same tumor by next-generation technologies.
Next-generation sequencing (NGS) technologies: family of applications that allow the affordable parallel sequencing of hundreds of millions of small fragments in a single reaction.
Single-cell multiomics: technologies that allow the simultaneous analysis of different cell molecular characteristics such as genomics, transcriptomics, epigenomics, or proteomics.
Single-cell RNA-sequencing (scRNA-seq): analysis of the RNA content of single cells by next-generation sequencing technologies.
Transcriptional signature: a specific set of genes expressed by a cell in a given moment under particular circumstances.

Tumor Microenvironment

Cancer-associated fibroblasts (CAFs) are present in many if not all solid tumors and participate actively in tumor development [31]. The molecular mechanisms behind CAFs' role remain largely unknown and the lack of reliable cell markers to identify CAFs prevents a clear statement of their abundance and importance in solid tumors [32]. The origin of CAFs is also under debate. They can be the result of the transformation of resident fibroblasts previously present in the normal tissue or new cells generated from special cell precursors recruited to the tumor [33]. scRNA-seq reports in the past years have provided useful information in this respect. Different types of CAFs have been reported in breast and colorectal tumors, which is likely to be associated with different cell origins [34–36]. Additionally, each group of CAFs has special functions in the recruitment of immune cells and in the induction of the epithelial–mesenchymal transition (EMT) in tumor cells [24,29,34,36].

Tumors are also frequently infiltrated by immune cells. The activation of the immune system to attack tumor cells is attractive as an antitumoral therapy [37]. Consequently, the so-called immunotherapies have become a promising tool in fighting cancer, although variable responses have been observed when they are applied to cancer patients [38]. There is a great diversity of immune cells with differing, and probably opposite, functions in tumor development. This complexity requires a correct transcriptional characterization of all the different cell types present in the tumor [39]. Here, scRNA-seq studies offer an unprecedented opportunity. A recent study demonstrated that a high proportion of active versus exhausted $CD8^+$ T lymphocytes is associated with a better outcome in non-small cell lung cancer [40]. By contrast, tumors presenting large proportions of regulatory T lymphocytes or myeloid-derived suppressor cells have a poor prognosis [41–43]. The complex relationship between the different immune cells present in the tumor will determine an overall tolerant or nontolerant environment. Finally, some studies successfully identified tumor neoantigens by single-cell characterization of the T cell receptor (TCR) repertoire, which might be useful in the diagnosis and treatment of cancer [40] (Figure 1, Key Figure).

Circulating Tumor Cells

The characterization of cells that extravasate into the blood circulation, **circulating tumor cells (CTCs)**, constitute a good and low-invasive alternative for the diagnosis and, more importantly, monitoring of tumors [44]. The utility of this strategy has been widely shown in many tumor types and the quantification of CTCs can be used as a prognostic factor [45]. Whereas many authors claim that CTCs recapitulate intratumor diversity perfectly, others have reported that they resemble metastasis more than primary tumors [46].

The low number of CTCs present in the blood circulation has forced many studies to purify CTCs according to specific epithelial surface markers. Debate about the specificity of these markers calls into question some of the reported observations [45]. Some current platforms for CTC isolation are based on physicochemical properties, but it remains unclear whether this constitutes a less biased isolation method [47]. The high throughput of modern single-cell sequencing technologies offers without doubt an opportunity to reduce the need for extensive purification, which will help to clarify the nature and the source of CTCs (Figure 1).

Relevant observations came recently from CTC single-cell sequencing studies. The presence of heterogeneous CTC populations with both epithelial and mesenchymal markers was identified, stressing that isolation methods based on epithelial markers are likely to be inadequate to capture all CTCs [48,49]. Additionally, a recent study on prostate cancer CTCs identified the activation of the noncanonical Wnt signaling pathway, anticipating the appearance of drug resistance [49]. Finally, the presence of plakoglobin in breast cancer CTCs was associated with earlier metastasis appearance [50]. This suggests that we need to include the study of CTCs in therapeutic decision-making in oncological practice.

Limitations of Single-Cell Technologies in Human Cancer

A major limitation in the application of scRNA-seq technologies to solid tumor samples is the requirement for complex dissociation protocols to obtain viable, individualized fresh cells. This limitation is especially important as several studies have raised caution on potential transcriptional changes

Key Figure

Functional Heterogeneity of Human Tumors Revealed by Single-Cell RNA-seq (scRNA-seq) Studies

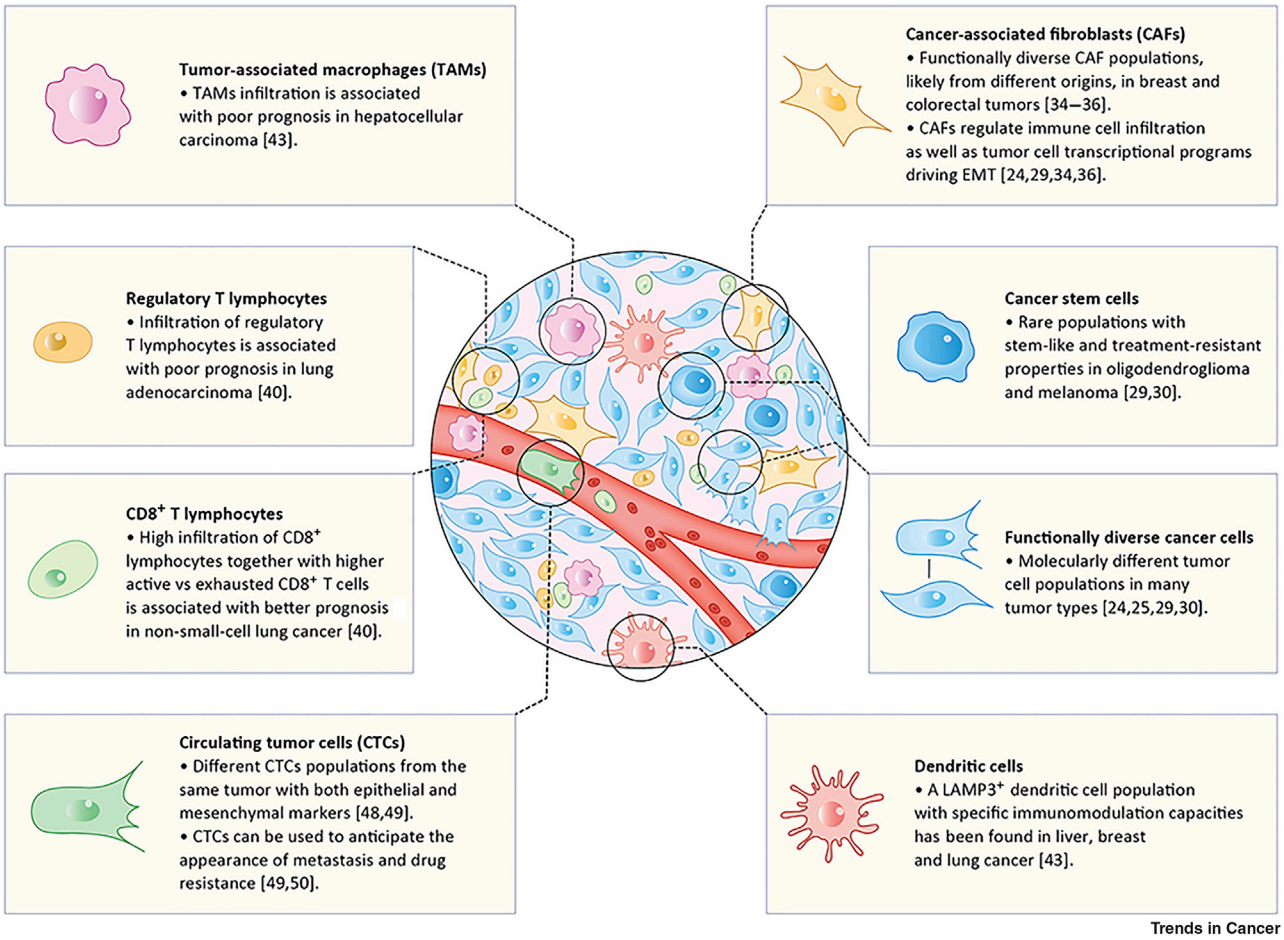

Figure 1. Graphical representation of some of the main cell types present in solid tumors. scRNA-seq studies providing useful information about potential functions in cancer progression are specified next to each cell type. Abbreviation: EMT, epithelial–mesenchymal transition. See [24,25,29,30,34–36,40,43,48–50].

arising from tissue manipulation between sample collection and processing [21]. Some authors have bypassed this limitation by working with either cell lines or organoids (Figure 2) [51]. Although these have provided useful information, they fail to mimic the complex interactions between cancer cells and the microenvironment. Additionally, to understand the molecular basis of tumor evolution, it would be important to obtain several samples, or even serial samples, from the same patient, which is not straightforward in solid tumors. Recently developed low-invasive biopsy techniques such as fine-needle aspiration (FNA) are not very practical for traditional genomic analysis due to the low amount of recovered material, but offer a window of opportunity for the application of scRNA-seq technologies in clinical research [52].

Fortunately, many platforms are compatible with cell fixation and storage protocols. Transcriptomic programs obtained from these cells seem similar to those of freshly processed cells [53,54] (Figure 2).

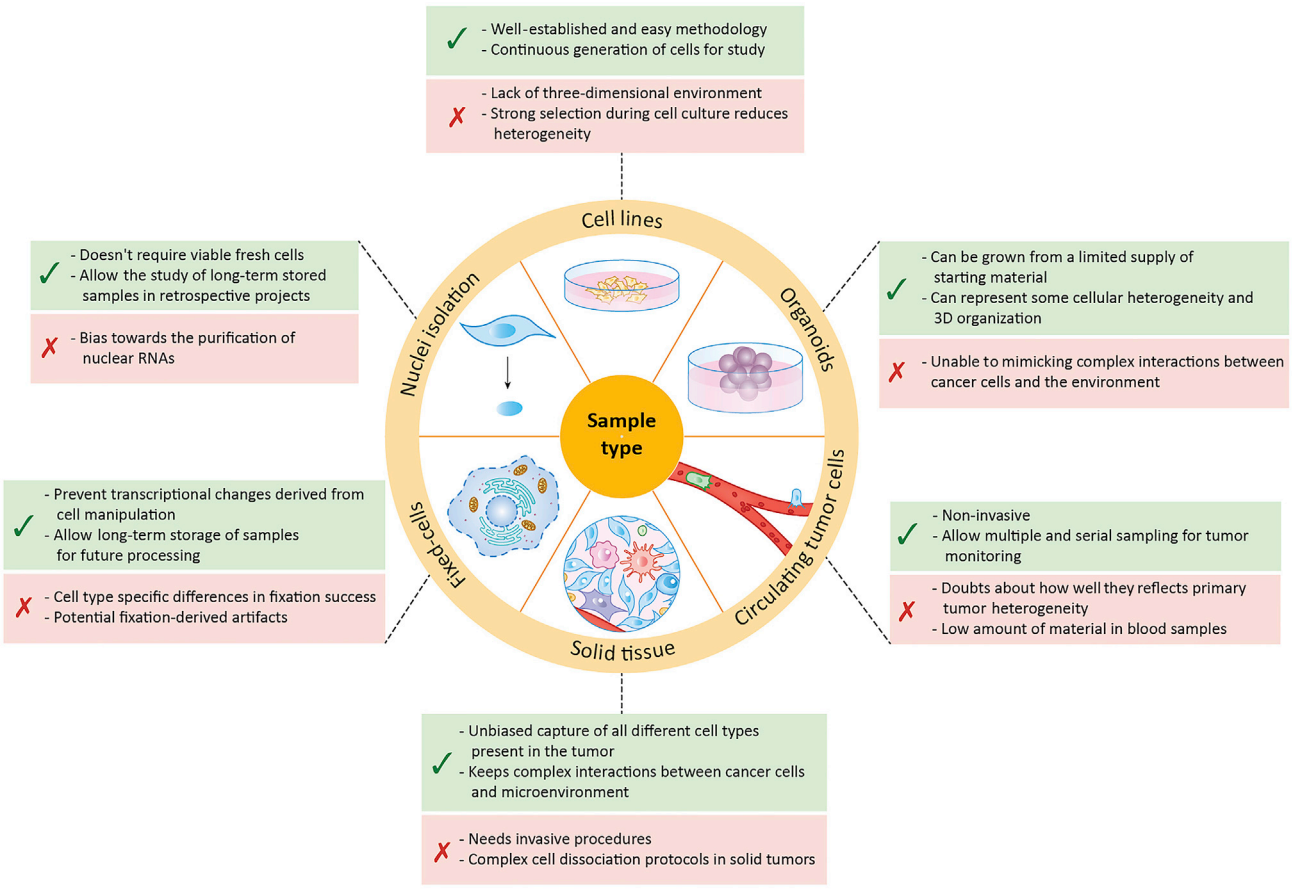

Figure 2. Different Sample Types Used for Single-Cell Sequencing.
Different types of samples currently used for single-cell sequencing are represented together with their advantages (green boxes) and disadvantages (red boxes).

If these protocols are optimized for all applications and broadly implanted, it is easy to anticipate a great increase in the application of scRNA-seq technologies in clinical research, where coordinating sample collection and processing is not always easy. Moreover, the recent development of scRNA-seq strategies for isolated single nuclei, sometimes obtained from formalin-fixed paraffin-embedded (FFPE) material, reduces the need to obtain viable cells and facilitates the study of long-stored samples in retrospective projects [11,55].

Concluding Remarks

Despite overall improvement in the treatment of cancer patients, long-term success of targeted therapies remains limited to specific tumor types. Tumor cellular complexity is likely a key factor in this failure. Consequently, tumors with huge infiltrations of different cell types, like pancreatic adenocarcinoma, have mortality rates that remained stubbornly unchanged.

The recent development of single-cell sequencing technologies brings a revolution in the characterization of tumor heterogeneity, not only at the genetic but also at the epigenetic and transcriptomic level. Despite technical problems that need to be solved, we anticipate the incorporation of these technologies in clinical research extended to many tumor types. Similar to the explosion of genetic data generated following the development of NGS, single-cell sequencing technologies will trigger

an exponential increase in knowledge about tumor architecture and evolution dynamics (see Outstanding Questions). Finally, all of this new data will be translated into better diagnosis and treatment of cancer patients.

Outstanding Questions

Do transcriptionally different cell populations collaborate during tumor progression?

Can the equilibrium inside the diverse tumor cell ecosystem be exploited to improve cancer treatment?

Is the functional diversity observed inside tumors the result of a specific hierarchical program?

What is the origin of cancer stem cells? Are they present in all tumor types?

Do circulating tumor cells faithfully recapitulate tumor functional heterogeneity? Can they be efficiently used to anticipate tumor progression, metastasis, or tumor resistance?

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