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Mini-review

Recent advances and current issues in single-cell sequencing of tumors

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ABSTRACT

Intratumoral heterogeneity is a recently recognized but important feature of cancer that underlies the various biocharacteristics of cancer tissues. The advent of next-generation sequencing technologies has facilitated large scale capture of genomic data, while the recent development of single-cell sequencing has allowed for more in-depth studies into the complex molecular mechanisms of intratumoral heterogeneity. In this review, the recent advances and current challenges in single-cell sequencing methodologies are discussed, highlighting the potential power of these data to provide insights into oncological processes, from tumorigenesis through progression to metastasis and therapy resistance.

Introduction

Cancer remains as one of the world's most lethal and debilitating diseases, despite massive research efforts to understand the pathogenic processes and underlying molecular mechanisms. While these studies have identified many features of cancer development and progression, such as genetic mutations and pathogenic phenotypes, they have also uncovered perplexing complexity in the tumor microenvironment, even the individual cells within the tumor tissue showing distinctive phenotypes [1,2]. This newly recognized feature is known as intratumoral heterogeneity and may represent differing pathogenic potentials of tumor cells, where some cells may represent a greater threat to the organism by having a greater potential for metastasis or development of resistance to chemotherapeutic drugs.

Applying single-cell sequencing technology to study the phenomenon of intratumoral heterogeneity has uncovered its role in cancer diagnosis and prognosis, as well as identifying factors that may represent clinically useful diagnostic and/or prognostic biomarkers and targets of molecular therapies [2]. The single-cell sequencing technology is powerful and has the capability of capturing dynamic genomic data for a single cancer cell under specific cancer-related conditions, such as during the early stages of tumor development or the late stages of metastasis. The resultant data may help to define condition-related genetic profiles that underlie the mechanisms of tumorigenesis, tumor progression, and development of metastasis and resistance to various therapies [3–5].

One of the major technical challenges of single-cell sequencing is the limited amount (picogram levels) of DNA and RNA in a single tumor cell, which is below the threshold of sensitivity for even the most advanced sequencing platforms. To overcome this limitation, the nucleic acids from the isolated single cell must be amplified. Thus, the current protocol for single-cell sequencing involves four main steps: isolation of a single tumor cell, cell lysis, extraction of nucleic acids, and amplification. Each step, however, carries a potential for error that limits the efficacy and sensitivity of the technology. The use of micropipetting, albeit common throughout molecular biology experimental studies, is prone to operator error and mechanical failure [6]. Isolation of single tumor cell for analysis is often carried out by fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS), both of which may alter the transcriptional state of the cells [7], confounding the subsequent genomic analysis. The methods involved in the cell lysis and extraction steps carry the risk of causing DNA or RNA degradation, sample loss, or contamination [6,8,9]. Finally, the current methods used to amplify the low concentration of nucleic acids from a single cell can yield non-uniform levels of amplification products and inconsistent coverage of the original genomic information [10,11].

The sequencing operation itself is also imperfect and even the most advanced technological platforms have limited sensitivity, with a threshold of resolution, and require operator expertise and lengthy operation times, and the design and running of algorithms to interpret the sequencing data [12,13]. Certainly, the use of single-cell sequencing technology to describe the distinctive tumor-related genetic profiles for various oncological processes will benefit

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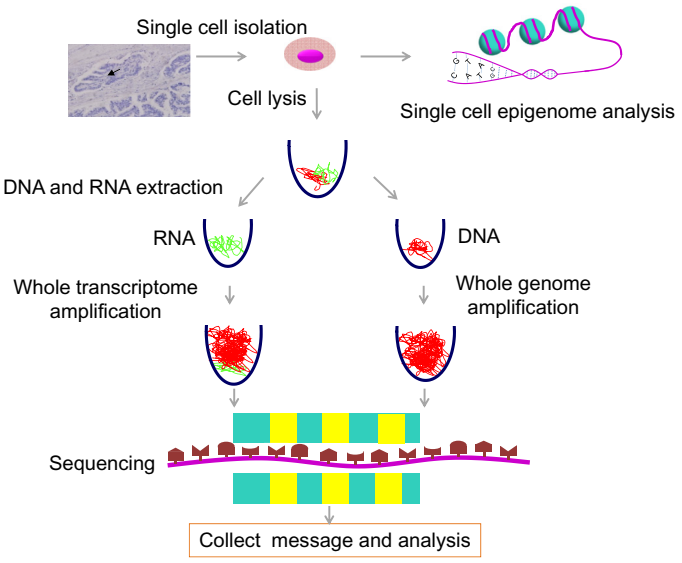

Fig. 1. Single-cell sequencing of a tumor cell. A tumor specimen is obtained by surgical excision and single cells are isolated by one of the several methods shown in Fig. 2. The individual cancer cell can be used for epigenome sequencing directly or lysed to extract the genetic material (DNA and RNA), which is in turn amplified by the methods shown in Fig. 3. Then, the amplified DNA and RNA are sequenced by single-cell sequencing technology and the result data are analyzed to provide insights into the molecular mechanisms underlying intratumor heterogeneity.

from research efforts to improve the sensitivity, efficiency and rapidity of this analytical approach.

Herein, we provide a review of the methods and procedures of single-cell sequencing (Fig. 1), including those that are more established and those that have been newly developed, and discuss their usefulness and limitations with the hope that this knowledge will support the ongoing research efforts to improve this technology and increase our knowledge in intratumor heterogeneity.

Isolation of single cells for sequencing

Isolating single cells from solid or liquid tumor tissues is the first and key step of single cancer cell sequencing. The following four methods are the most commonly used for single-cell isolation, and their primary characteristics are summarized in Table 1 [14].

Laser capture microdissection (LCM)

LCM is used to isolate individual cells from their native tissue directly and without any chemical or physical destruction. The basic procedure for LCM involves covering a section of the target tissue with a thermoplastic film and firing a laser to melt the film so that it adheres to a single tumor cell and facilitates mechanical isolation of that cell from its neighbors (Fig. 2A). Some researchers have proposed the use of immunofluorescence (i.e. fluorescent-tagged antibodies) to improve the speed and accuracy of this method [15–17]. However, the LCM laser's isolation of a single cell is still not capable of completely clearing all microenvironment materials' strong adhesion junction from a target cell's surface, which represents a source of contaminating materials from neighboring cells [18–20].

Micropipette isolation

The use of a micropipette to mechanically isolate a single cell by aspiration is a cheap and technically simple method since the only tools required are a basic micropipette and microscope with relatively lower resolution (at the individual cell level). The basic procedure for micropipette isolation involves enzymatic digestion of the tumor tissue to acquire a cell suspension, after which the suspension is diluted to approximately 10–20 cells per 1 μL solution and examined under the microscope's visual field to select a single cancer cell by micropipette-assisted aspiration (Fig. 2B). The limitations of this procedure are the laborious nature of the technique, resulting in low efficiency, and the dependence on the operator's expertise and low resolution visibility of the cells, making it highly prone to mistakes in identifying truly individual cells or contaminating non-cancer cells [6,21,22]. Some researchers have proposed the use of fluorescence *in situ* hybridization (FISH) to improve the efficiency of micropipette isolation [23].

Fluorescence-activated cell sorting (FACS)

The FACS platform is capable of both selecting and analyzing single cells from a heterogeneous tumor tissue, relying on simultaneous analysis of fluorescence signals and light scattering parameters. The operational procedure of FACS involves disruption of the cancer tissue (either by enzymatic digestion or mechanical disruption) to acquire a cell suspension, after which the target cells are immunoreacted with one or more fluorescence-tagged antibodies targeting specific antigens on the cell surface or intracellular markers; in this manner, specific cell types can be identified for isolation and analysis. After processing, the antibody-labeled cells are applied to a FACS machine in which they pass through a narrow stream of fluid with an intersecting laser beam that produces scattered light signals according to the labeled cell characteristics. The flow of the cell-containing liquid can be regulated to ensure that only one cell is present in a single droplet, and the light scattering pattern will help to identify that cell; in addition, during the FACS separation procedure, the cell will acquire temporary electrical properties that facilitate cell isolation (Fig. 2C) [24–26]. The FACS isolation approach has been modified to use a panel of isotope-tags for the immunoreactive antibodies [27].

The FACS isolation approach has several advantages over the LCM and micropipette isolation procedures, including greater rapidity and higher throughput. However, FACS detection of fluorescent signals is relatively low, and low-expression markers are difficult or impossible to detect [24], so that there is a risk of missing some specific cell types.

Table 1
Methods of isolating single cells for sequencing.

Method	Sorting principle	Single cell suspension	Cost	Throughput	Automated?	Limitation
Micropipette isolation	Microexamination	Yes	Low	Low	No	Operational bias
LCM	Laser beam microdissection	No	High	Low	No	Contamination by surrounding material
FACS	Antigen–antibody hybrid	Yes	High	High	Yes	Needs large numbers of cells
MACS	Antigen–antibody hybrid	Yes	High	High	Yes	Operational complexity

Abbreviations: LCM, laser capture microdissection; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.

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