EDITORIAL

Can single-cell RNA sequencing crack the mystery of cells?

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There is a rapid increase of evidence to address the importance of the interaction between single cells, drugs, and the response of single cells to therapies. Single-cell measurements were used to evaluate the DNA-damaging ability of the herbicide in freshly isolated human leukocytes (Villarini et al. 2000) or the ethoxyresorufin-O-deethylase activity of cytochrome P450 1A1 in single-living cells with the microspectrofluorometric technique (Taira et al. 2007). The measurements of single-cell biology and sequencing are recently considered as an important approach to investigate molecular mechanisms of drug efficacy and resistances, discovery and development of therapeutic targets, and genealogic phenotypes of cells during disease progression (Chu et al. 2017; Wang 2016; Wang et al. 2017). Single-cell sequencing is an important measure to define intercellular heterogeneity, rare cell types, cell genealogies, somatic mosaicism, microbes, and disease evolution, including single-cell DNA genome sequencing, DNA methylome sequencing, and RNA sequencing. Of those, single-cell RNA sequencing (scRNA-seq) demonstrates transcriptomic cell-to-cell variation, new cell types, developmental processes, transcriptional stochasticity, transcriptome plasticity, and genome evolution (Wang 2015) (Fig. 1). The present article aims to highlight the optimization and application of scRNA-seq to understand the

development of intercellular heterogeneity, the genealogy and evolution of cells, and key driven transcriptome networks in response to drug efficacy and toxicity.

The experimental design and technical challenges are critical in the application of scRNA-seq (Kukurba and Montgomery 2015). A number of practical protocols have been developed and validated with a great variation of RNA sequencing sensitivity and accuracy. Ziegenhain et al. (2017) made a comprehensive comparison of scRNA-seq protocols and suggested that an informed choice among six prominent scRNA-seq methods, including CEL-seq2, Drop-seq, MARS-seq, SCRB-seq, Smart-seq, and Smart-seq2, based on scRNA-seq data from mouse embryonic stem cells. Svensson et al. (2017) evaluated the protocol sensitivity and accuracy of the published data sets as well as the study designs by comparing it with 15 other protocols computationally and 4 protocols experimentally for batch-matched cell populations. Using the spike-in standards and uniform data processing, they developed a flexible tool for counting the number of unique molecular identifiers (https://github.com/vals/umis/). Such a protocol makes it possible to perform scRNA-seq and to compare gene expression, novel transcripts, alternatively spliced genes, and allele-specific expression among numerous studies and performers. Of scRNA-seq preparation procedures, cryopreserved cells using 3′-end and full-length RNA preparation methods was found to generate the same transcriptional profiles as fresh cells do (Guillaumet-Adkins et al. 2017).

Intercellular heterogeneity is a dominant element of intratumor heterogeneity, responsible for the development

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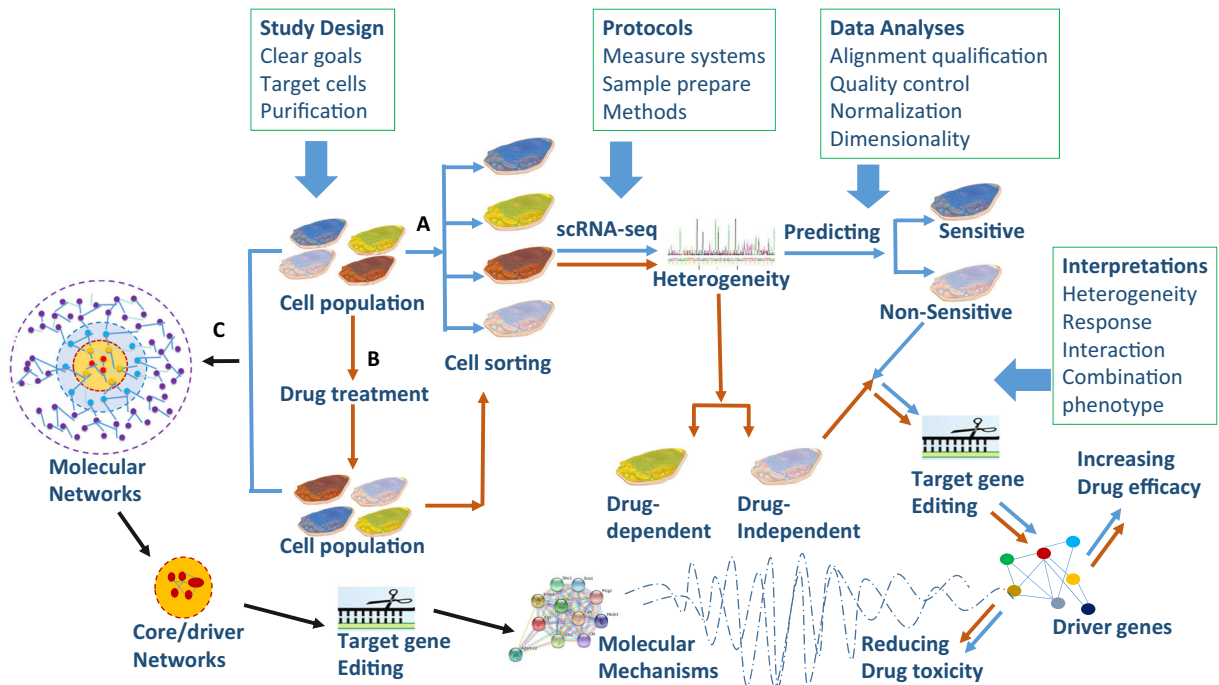e-mail: xdwang@fuccb.com

Fig. 1 Summary of processes, roles, dependent factors, and outcomes of single-cell RNA sequencing (scRNA-seq). Targeted cell populations are selected from human organs/tissues and then one type of cell population can be sorted. The selection of targeted cells is highly dependent upon the study design, goals, and cell purification **a** scRNA-seq of sorted cells can performed under the validated protocol, including measure systems, sample preparation, and methodologies. Intercellular heterogeneity identified can predict the sensitivity of targeted cells (*green arrows*) after proper data analyses, e.g., alignment qualification, quality control, normalization, and dimensionality. Drug-dependent gene mutations and drug specificity of targeted genes can be defined after selected cells are treated with targeting drugs (**b**, *brown arrows*). On the other hand, gene sequences and epigenetics of selected cells can be measured before the cell sorting (**c**, *black arrows*), in order to compare the cell population with single-cell sequences. Molecular mechanisms, drug efficacy and toxicity of identified core/driver genes and networks can be furthermore validated by editing target genes. One of the most dependent factors is how to interpret the correlation of information and outcome from scRNA-seq with cell heterogeneity, response, interaction, and phenotype as well as drug combination

of drug resistance, reoccurrence of tumor, and metastasis (Lu et al. 2017; Wang and Wang 2017a; Dong et al. 2017; Shi and Wang 2017). Intratumor heterogeneity in diverse regulatory programs can control cancer biology, prognosis, and therapy (Patel et al., 2014). The strategic method for precision medicine is highly dependent upon the degree of single-cell heterogeneity (Qian et al. 2017), which can be measured by both DNA and RNA sequencing. DNA sequencing is utilized to define the mutations within the genome, while scRNA-seq to determine the expressional differences of the entire transcriptome which is responsible for the development of drug resistance. scRNA-seq can describe the heterogeneity of gene expression within the tumor cell population which may survive as a minority after the majority was declined by therapy (Lee et al. 2014). Specific RNA variants in genes are involved in microtubule organization and stabilization, cell adhesion, and cell surface signaling in metastatic human cancer cells. Cells with specific RNA variants then became drug-tolerant after chemotherapy. Those variants driving heterogeneity were reflected by the differentiation of scRNA-seq-measured gene expression profiles among designed groups. In addition, the heterogeneity measured by scRNA-seq could also demonstrate the response of bone marrow derived dendritic cells to lipopolysaccharide (Shalek et al. 2013). A number of bimodal variations in mRNA abundance and splicing patterns could be responsible for heterogeneity between cells. The interaction between the microenvironment and the cells play a decisive role in the development of intratumor and intercellular heterogeneity. Microenvironmental components either influence the formation of cell phenotypes or result in different phenotype responses within cells due to changes within the microenvironment. Such processes can be measured by multi-dimensional analyses

of single cells, i.e., single-cell culture, live-cell imaging, target-gene editing, and scRNA-seq (Wills et al. 2017).

As host sensitivity and resistance to drugs are dependent upon the intercellular genetic and functional heterogeneity, it is crucial to identify and develop more efficient strategies for individual therapies. The comprehensive information regarding gene expression and single-nucleotide variations in individual tumor cells measured by scRNA-seq could tell the responses of lung adenocarcinoma cells to anti-cancer drugs (Kim et al. 2015). Unique tumor cell-specific gene expression profiles among subgroup cells from patient-derived xenograft tumor demonstrate the association with resistances against drugs. Of those, variations of KRAS(G12D) based on mutations and risk scores among individual cells were applied for new molecular classifications. The drug-induced rapid translocation of the regulatory transcription factor Arx to the cytoplasm reduces the production of glucagon transcriptomics in human alpha-type single cell (Li et al. 2017). The performed scRNA-seq was then integrated with identical global transcriptomic to monitor changes in specific genes related to the drug mechanism (e.g., upregulated GABRB3 and GABRG2 accompanied with elevated levels of gephyrin and GABA-receptor subunit protein) and in drug-induced cell-type-specific transcripts (e.g. EIF4A1, CRYBA2, PDK4, and MUC13).

scRNA-seq can be used to design an optimized combination of targeted agents against metastatic renal cell carcinoma on the basis of transcriptional heterogeneity during the metastatic progression (Kim et al. 2016). The intra- and inter-tumor heterogeneities, genomic architectures, and clonal evolutions were associated with spatiotemporal tumor progression. Putative therapeutic targets were selected from paired primary and metastatic renal carcinoma patient-derived xenografts. The functional heterogeneity, specific subpopulations, metastasis-, and drug response-based transcriptome signatures are defined, before functionally co-targeted subpopulations are identified by scRNA-seq. Combinational therapies can then be optimized through the application of the scRNA-seq screening. However, it should be kept in mind that dynamic phenotypes of patient lung cancer single cells may be altered during the growth of human cells in animal microenvironments.

Single-cell mRNA isoform diversity is considered as a source of stochasticity and protein diversity between cells and is critical to understand phenotypic heterogeneity responsible for the development of drug resistance. Karlsson and Linnarsson (2017) investigated single-cell oligodendrocyte transcriptomes using long-read PacBio sequencing technology and found that a considerable number of separate isoforms were present in a single cell. It proves that a gene is highly expressed with the multiple isoforms which are independent upon cell types, although it remains unclear whether a liner increase between gene expression and isoforms may only occur during evolution or in response to external challenges and drugs. Coding isoforms can influence cell phenotypes more than non-coding isoforms of coding genes. Equally important with heterogeneity of gene expression, mRNA polyadenylation isoform choice probably regulated by 3′-untranslated regions is associated with developmental state, intercellular functional heterogeneity, and classification of cell populations (Velten et al. 2015). Specific homophilic interactions and cell surface delivery of alternate isoforms depend upon *cis* interactions with other isoforms, extracellular domain. Mismatched isoforms are necessary for single-cell identity (Thu et al. 2014). It is still unclear whether drug toxicity can influence the phenotypes of mRNA isoforms, or whether drug-induced changes in mRNA isoform patterns is a key molecular mechanism by which drugs can interrupt gene function by switching mRNA isoform production. The androgen regulates a number of related genes to induce spliced mRNA isoforms, including a prostate-specific splice isoform of ST6GALNAC1 mRNA (Munkley et al. 2017). Genetic rearrangements often occur in the early stages of diseases, leading to different patterns of alternative splicing and RNA isoforms in response to drug and to abnormal protein expression, synthesis, and activation. RNA-seq can detect the alternative splicing of pre-mRNA can generate RNA isoforms by retaining the suboptimal large intron as the coding region and cellular protein diversity. However, it is also possible that a large number of RNA isoforms are hardly translated into protein isoforms, since most genes have a single dominant splice isoform and most alternative splice events are not evolutionary innovations (Tress et al. 2017).

scRNA-seq plays an important role in the monitor and confirm of gene editing. A-to-I RNA editing affects coding and non-coding transcripts at multi-sites, regulates codon identity and modifications made in splice sites, and interferes with base-pairing interactions within higher-order RNA structures. The dysregulation of RNA editing is associated with a variety of different diseases. scRNA-seq can demonstrate A-to-I patterns as

specific editing signatures to distinguish major cell types of the human brain single cells (Picardi et al. 2017). The RNA editing profiles of single cells from the human brain cortex could potentially be a new approach to understand the roles of RNA editing in neuronal plasticity and disorders and can aid to discover innovative approaches for precision medicine. Dynamic alterations in genome-wide A-to-I RNA-editing activities are measured using scRNA-seq analyses in early human embryonic development from oocyte to morula stages (Qiu et al. 2016). Five of 37 non-synonymously RNA-edited genes were frequently found in cells of multiple embryonic stages and A-to-I editing in miRNA-targeted regions of a substantial number of genes occurred in one or two sequential stages. Furthermore, Singer et al. (2016) investigated molecular mechanisms of the dysfunctional T cell state by assessing the population and single-cell RNA profiles of CD8$^+$ tumor-infiltrating lymphocytes and the distinct gene modules for T cell dysfunction by using genetic perturbations. Intracellular metallothioneins and Gata-3 were identified and their regulatory roles in the dysfunctional phenotype in CD8$^+$ tumor-infiltrating lymphocytes were confirmed with CRISPR-Cas9 genome editing.

scRNA-seq plays more critical roles in the monitoring and tracing of gene expression profiling in hereditary and somatic cell differentiations and evolution. Yang et al. (2017) measured heterogeneity within hair follicle progenitors and niches to build a complex epithelia and stem-cell plasticity as well as epithelial-mesenchymal micro-niches in multi-lineage tissue growth using scRNA-seq. They discovered that heterogeneity was present among adult tissue stem cells and short-lived transit-amplifying cells of hair follicles. This is an example where scRNA-seq can detect genealogical changes from the heterogeneity to micro-niches along epithelial-mesenchymal interfaces. It helps to define the dynamic phenotypes of cell genetics as well as to discover the responses to microenvironmental changes and the molecular signatures reflective of spatially distinct local signals and intercellular interactions. scRNA-seq is a powerful tool to scout the generational gene expression profiles of induced pluripotent stem cells before, during, and after cell therapies, especially the gene profiles of hematopoietic differentiation in clinical application for hematologic diseases (Paes et al. 2016). On the basis of transcriptional gene regulatory networks drawn from scRNA-seq data, probabilistic models and personalized dysfunctional gene network

based on differential network models can be generated to predict and define the dynamic states of early stem cell differentiation or complex diseases (Jang et al. 2017; Yu et al., 2015).

scRNA-seq is an important approach to study genotoxicity of drugs in drug discovery and development (Cristofori et al. 2015; Steinberg et al. 2016; Maguire et al. 2016). scRNA-seq was utilized to detect the first transcriptome of specific immune cell types in a non-mammalian species and to discover a new immune cell-specific gene (Carmona et al. 2017). scRNA-seq with temporal mixtures of Gaussian processes could potentially demonstrate the developmental trajectories of Th1 and Tfh cells during blood-stage Plasmodium infection and could track the clonality and Th1/Tfh bifurcation at population and single-clone levels (Lönnberg et al. 2017). Of gene expression profiles, chemokine receptors around bifurcation may orient or drive Th1/Tfh fates, by which precursor Th cells could be developed into a Th1 rather than a Tfh.

Single-cell transcriptomics on cells harvested from patient tissues are proposed as a prime examination to screen disease-specific targets and design optimal strategy of precision therapy. Transcriptomics of single oligodendrocytes, microglia, neurons, endothelial cells, and astrocytes after a 3-week culture of resected adult human brain tissue removed during neurosurgery could potentially demonstrate cell-type and patient specific transcriptional signatures (Spaethling et al. 2017). There are still a number of challenges and obstacles scRNA-seq needs to face and overcome for clinical application. The amount and accuracy of mRNA, microRNA, tiRNA, siRNA, piRNA, lncRNA, or cirRNA should be declared and confirmed in a single cell. The advances of scRNA-seq with computational and statistical methodologies are highly dependent upon the study design, question-based understanding, quality control, and expression estimation that involve raw data. Computational biology plays important roles in the design and analysis of scRNA-seq experiments and in the reliability of scRNA-seq outcomes associated with data normalization, sub-population identification, dynamic phenotypes, pseudotime reconstruction, and network inference (Grün and van Oudenaarden 2015; Bacher and Kendziorski 2016).

A few of the most critical issues in single-cell sequencing, biology, and systems biology is to ensure the same population of single cells, the same function subgroup within the population, and genetic similarity of

the subgroup. Due to the existence of intercellular heterogeneity, the appropriate and optimal number of single cells can be difficult to define for scRNA-seq. The sources of experimental noises in scRNA-seq should be closely monitored since the variations arise from genotype, technical replicates, protocols, facilities, and design. The variation of methodological analyses and models may tell different stories of cell state, circuitry, and fate decisions during development, homeostasis, and disease. scRNA-seq also has the potential to observe post-transcriptional gene regulation and expressional profiles during and after reversible mRNA methylation, metabolism, and translation by the recognition of RNA N6-methyladenosine (Zhao et al. 2017). mRNA modifications with RNA N6-methyladenosine form the epitranscriptome and collectively code a new layer of information that controls protein synthesis, where scRNA-seq may indirectly contribute to the outcome of the process.

scRNA-seq will become more practical and applicable to gene expression profiles after gene editing with CRISPR (Fang and Wang 2016) and multi-gene editing (Braff et al. 2016). Multi-genes can be edited in a single cell using a set of Cas9-guide RNA pairings to allow concurrent activation and edit genes at independent target sites in the same cell, through the identification of putatively orthogonal systems, characterization targets, and establishment of orthogonal CRISPR systems. Although there still are a number of limits and obstacles in functional phenotypes and protein biosynthesis to be overcome, scRNA-seq per se and generated data will one of major contributors to scout and crack the mystery of cells as well as establish an artificial intelligent cell with the capacities of reading, learning, analyzing, recording, and reporting (Niu et al. 2016; Wang and Wang 2017b).

In conclusion, scRNA-seq is an important approach to define the alterations of transcriptomes, development of intratumor and intercellular heterogeneity, and genotoxicity in response to drugs. The influences of single-cell RNA isoform diversity in gene, protein expression, and regulation can be dynamically monitored with scRNA-seq. However, the function of RNA isoforms remains to be explored. scRNA-seq can detect somatic mutations and epigenetic alterations in evolution, post-transcriptional RNA modifications, and RNA editing. It can also monitor the dynamics of RNA processes and responses to microenvironmental changes and drug resistances. The value of scRNA-seq is dependent upon the study design, methodology, and the scientific questions in mechanisms of RNA methylation, RNA editing and RNA splicing, and finally the functional consequences of their aberrant regulation in diseases. scRNA-seq plays a decisive role in the discovery and development of disease-specific dynamic network biomarkers and therapeutic targets.

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