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REVIEW

Single-Cell RNA Sequencing (scRNA-seq) in Cardiac Tissue: Applications and Limitations

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Abstract: Cardiovascular diseases (CVDs) are a group of disorders of the blood vessels and heart, which are considered as the leading causes of death worldwide. The pathology of CVDs could be related to the functional abnormalities of multiple cell types in the heart. Single-cell RNA sequencing (scRNA-seq) technology is a powerful method for characterizing individual cells and elucidating the molecular mechanisms by providing a high resolution of transcriptomic changes at the single-cell level. Specifically, scRNA-seq has provided novel insights into CVDs by identifying rare cardiac cell types, inferring the trajectory tree, estimating RNA velocity, elucidating the cell–cell communication, and comparing healthy and pathological heart samples. In this review, we summarize the different scRNA-seq platforms and published single-cell datasets in the cardiovascular field, and describe the utilities and limitations of this technology. Lastly, we discuss the future perspective of the application of scRNA-seq technology into cardiovascular research.

Keywords: cardiovascular diseases, clustering, trajectory inference, RNA velocity, cell–cell communication, spatial genomics

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally, taking an estimated 17.9 million (32.1%) lives in 2015, up from 12.3 million (25.8%) in 1990.[1,2] CVDs are highly heterogeneous diseases involving a group of disorders of the heart and blood vessels, which include cardiomyopathy, hypertensive heart disease, heart failure, coronary artery disease, cerebrovascular disease, rheumatic heart disease and others.[3] CVDs are complex in nature, stemming from molecular alternations at the genetic, epigenetic, transcriptomic, and even proteomic levels in various cardiac cell types.[4,5] Accurate elucidation of cellular heterogeneities is necessary for decoding the pathogenic mechanisms of CVDs, identifying novel therapeutic targets, and developing effective treatment strategies.[6]

The profiling of cellular heterogeneity at the transcriptomic level in cardiac tissues has been considered as a promising direction for measuring the global transcriptional activity dynamics, which underlie the phenotypic diversity of multiple cardiac cell types.[7,8] Over the years, next-generation sequencing (NGS) technologies have led to many discoveries in biomedical sciences, including the phenotypic consequences of molecular variation in cardiovascular research.[9–11]

Until recently, bulk RNA sequencing (RNA-seq) had been primarily used to profile the averaged gene expression from tissues that consist of various cell types.[12] Bulk profiling hence ignores the stochasticity of gene expression in each cell type and

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indicates average values from the heterogeneous population of cells, which are affected by the relative cell-type abundance and the states of each cell type within a sequencing sample.[13] Molecular differences at the transcriptional level between distinct sub-cell types are also missing. In order to measure the transcriptome of each cell, several high-throughput single-cell RNA-sequencing (scRNA-seq) technologies have been developed and commercialized (Table 1).[14–24] scRNA-seq enables the characterization and identification of transcriptionally different subpopulations at the single-cell level. This approach has the potential to identify novel directions to develop therapeutic strategies.[25]

The number of scRNA-seq studies in cardiovascular research has rapidly increased in recent years. A recent search with the keyword "(scRNA-seq or single-cell transcript*)[TIAB] AND (heart or cardiac or cardio*)[TIAB] in NCBI's PubMed database of scientific publications returned 1238 articles (Sept. 5, 2021), 257 of which were published in 2020 and 254 of which published in 2021, at the time this review was written (Figure 1). The application of scRNA-seq has transformed how we understand CVDs, with a growing recognition that cardiac cell populations are far more heterogeneous than previously expected, and that bulk population analysis is inadequate for fully characterizing the biological complexity of these various cell types (Table 2). In terms of computational processing, each particular scRNA-seq protocol, platform, and technology may require different pipelines of preprocessing of sequencing reads, quality control (QC), normalization, dimension reduction, clustering, and differentially expressed gene (DEGs) calling.[26] Along with the development of various methodologies in single-cell capture, a paradigm has been occurring for the computational methodologies for the applications in biomedical research.[27] In this review, we highlight the utilities of scRNA-seq in various analyses for cardiovascular research (Figure 2), including 1) unsupervised clustering of scRNA-seq data to identify cardiac cell types and states within both healthy and diseased conditions, 2) characterization of the dynamics of transcriptional states by trajectory inference, 3) prediction of the future transcriptional dynamic state by the estimation of RNA velocity, 4) inferring of cell–cell communication from the expression of genes encoding receptors and ligands, 5) single-cell integration of multiple datasets to identify rare cell types, 6) detecting genetic variants from scRNA-seq datasets, and 7) construction of the spatial genomic map of cardiac tissues. In the near future,

Table 1 scRNA-Seq Sequencing Methods Comparison

scRNA-Seq Protocol	Data Type	Cost	Platform	Throughput (K)	Read Depth (per Cell)	Reaction Volume	Year	Reference
Smart-seq/C1 (Fluidigm)	Full length	High	Microfluidics	0.1–1	10^6	Nanoliter	2012	[14]
Smart-seq2	Full length	High	Microfluidics	0.1–1	10^6	Microliter	2014	[15]
MATQ-seq	Full length	Moderate	Plate-based	0.1–1	10^6	Microliter	2017	[16]
MARS-seq	3′-End	Low	Plate-based	0.1–1	10^4–10^5	Microliter	2014	[17]
CEL-seq	3′-End	Moderate	Plate-based	0.1–1	10^4–10^5	Nanoliter	2016	[18]
Drop-seq	3′-End	Low	Droplet	1–10	10^4–10^5	Nanoliter	2015	[19]
msSCRB-seq	3′-End	Low	Plate-based	1–10	10^4	Nanoliter	2018	[20]
Chromium	3′-End	Low	Droplet	1–10	10^4–10^5	Nanoliter	2017	[21]
SEQ-well	3′-End	Moderate	Nanowell array	1–10	10^4–10^5	Nanoliter	2017	[22]
SPLIT-seq	3′-End	Moderate	Plate-based	1–100	10^4	Microliter	2018	[23]
ICELL8	5′-End	Moderate	Nanowell-based	1	10^4	Nanoliter	2017	[24]

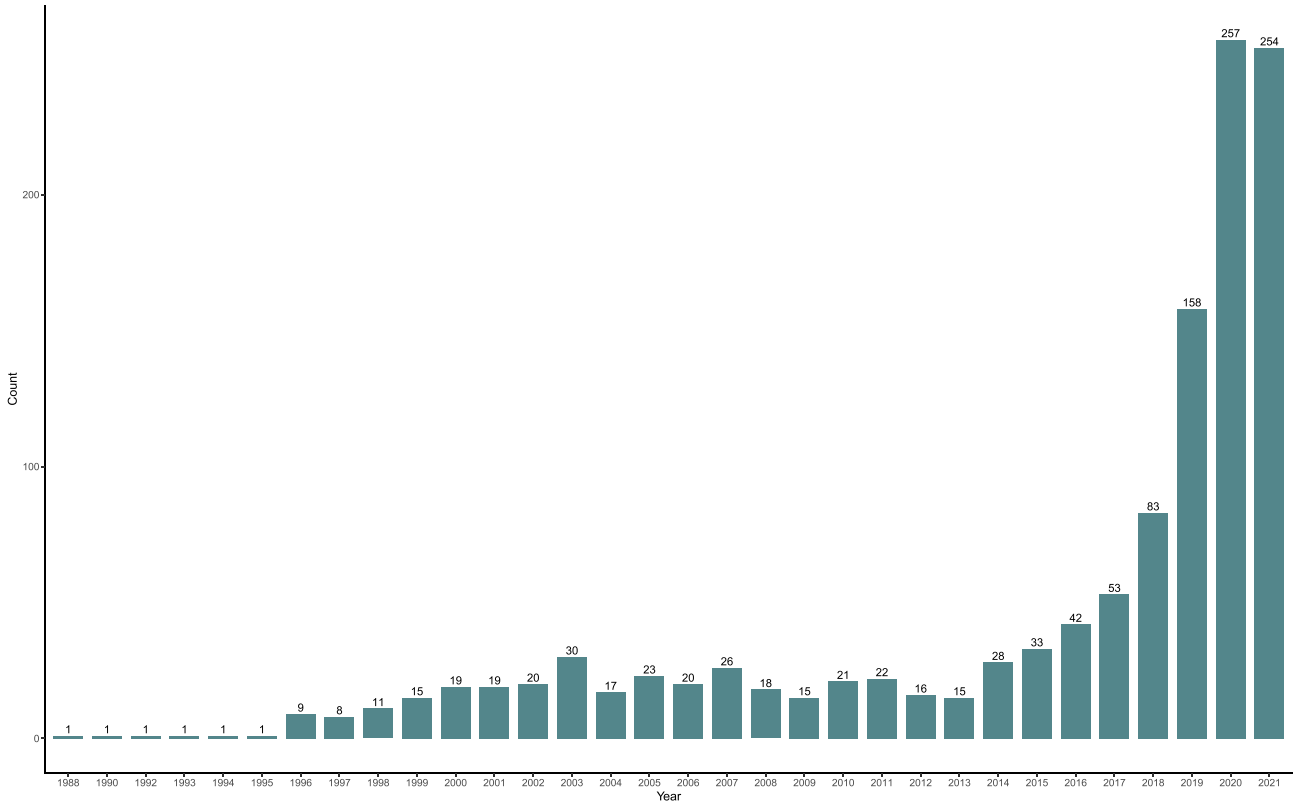

Figure 1 The number of papers published in the application of scRNA-seq in cardiovascular research in the past decades. Publications with the keyword "(scRNA-seq or single-cell transcript*)[TIAB] AND (heart or cardiac or cardio*)[TIAB]" in the NCBI PubMed database as of Aug 2021. Note the exponential growth in the number of published articles, in particular in the last 30 years.

advances in scRNA-seq research will provide further insights in better understanding the mechanisms of CVDs and in improving the diagnosis, treatment and prognosis of a broad range of CVDs.

The Workflow of scRNA-seq

A number of scRNA-seq techniques have been developed in the past decade. Different approaches of cell capture and transcript amplification result in differences in transcript length, target cell number, and read depth.[28] Despite the differences, the scRNA-seq experimental techniques have a common workflow: sample preparation, dissociation, single-cell capture, cell lysis, reverse transcription (RT) and cDNA amplification, library preparation and RNA sequencing (Figure 2).[29]

Proper sample preparation is the key to generate high-quality single-cell transcriptome data. Considering the different properties of each cell type, the protocol should be optimized based on cell size, cell viability, and culture conditions.[30] Single-cell suspensions are often achieved by a combination of enzymatic and physical dissociation. Subsequently, single cells are captured using different techniques including plate-based fluorescence-activated cell sorting (FACS) and droplet-based approaches. A broadly used droplet-based platform is the Chromium (10X Genomics) system, a microfluid device that allows rapid profiling of thousands of cells in droplets simultaneously. The Chromium system restricts the size of cells to be less than 30μm in diameter.[31] Alternatively, plate-based FACS with a larger nozzle size (up to 130μm) can be used to capture cells of large size such as adult cardiomyocytes (CMs).[32] In addition, single-nuclei RNA sequencing (snRNA-seq), a method for profiling gene expression in cells that are difficult to be dissociated, is an alternative to scRNA-seq to capture adult and large cardiomyocytes. To isolate RNA inside the nucleus, snRNA-seq uses a nuclear dissociation method that allows for minimization of technical issues.[33]

After individual cells are captured, they are lysed and processed for the first-strand cDNA synthesis by reverse transcription, followed by the second-strand synthesis and polymerase chain reaction (PCR) amplification. Single-cell system such as the Fluidigm C1 requires multiple PCR

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Table 2 Summary of Single-Cell RNA-Seq Studies on Cardiovascular Research

Species	Throughput	Heart Region/ Cell Type	Disease	Developmental Time Point	Method	Cell Number	Finding	Publishing Date	Accessibility	Ref
Human	High	Whole heart	Healthy, HF and HF recovery	Adult	FACS	21,422	CM heterogeneity, CM contractility and metabolism changes in heart function	2020	GEO ID: GSE109816, GSE121893	[46]
Human	High	Whole heart	Healthy and CHB	Fetus	10X Genomics	17,747	Heterogeneous cell population in CHB heart	2020	BioProject ID: PRJNA576243	[87]
Human	High	Whole heart	Healthy	Adult	10X Genomics	287,269	Defined the transcriptional and cellular diversity in the normal human heart	2020	SCP: SCP498	[10]
Human	High	Arterial cells	Heart failure	Adult	10X Genomics	125,253	Created a cell atlas of human nondiseased cardiac artery	2021	N/A	[88]
Human	High	hiPSC-derived endocardium	Healthy, HLHS	Fetus, hiPSC	10X Genomics	10,000	Reveal a critical role for endocardium in HLHS etiology	2020	GEO: GSE138979	[89]
Human	High	Whole heart and other organs	Healthy	Fetus	sci-RNA-seq3	101,748	A reference atlas of human fetal cell types including heart cell types	2020	GEO: GSE156793	[90]
Human	High	Whole heart and other organs	Healthy	Adult, fetus	10X Genomics	10,783	Construct a scheme for the human cell landscape including heart	2020	GEO: GSE134355	[91]
Human	High	Whole heart	Healthy	Adult	10X Genomics	486,134	Construct cells of the adult human heart	2020	HCA: ERP123138	[11]
Human	High	hiPSC-CMs	Differentiation	hiPSC	10X Genomics	43,168	Provides a key transcriptional roadmap of cardiac differentiation	2018	GEO: GSE97080	[92]
Human	High	hiPSC-CMs	Differentiation	hiPSC	Chromium + IFC system	10,376	Dissect the role of distinct cardiac transcriptional regulators associated with each cell population	2018	GEO: GSE81585	[93]
Human	Medium	Whole heart	Healthy development	Embryos	10X Genomics	3777	Spatial organization in the human embryonic heart	2019	EBI-EGA: EGAS00001003996	[43]

Human	Medium	Whole heart	Healthy development	Fetus	Mouth pipette	4948	Systematic mapping of the transcriptomic landscape of the human fetal heart	2019	GEO ID: GSE106118	[94]
Human	Low	Whole heart	Healthy development	Embryo/fetus	FACS	458	LGR5 is identified as a key regulator on congenital heart diseases	2019	BioProject ID: PRJNA510181	[95]
Human	Low	hiPSC-epi	Differentiation	hiPSC	Smart-Seq2	232	Cell heterogeneity in human epicardium regulated by BNC1	2019	GEO: GSE122714	[96]
Human	Low	hiCMs	Differentiation	hiCMs	Fluidigm	704	Molecular features of hiCM determination and cell fate conversion	2019	GEO: GSE106888	[97]
Human and mouse	Low	Left ventricles	Healthy and DCM	Adult	Smart-seq2	419	CM contractility and metabolism are prominent aspects that are correlated with changes in heart function	2018	GEO: GSE95143	[98]
Human	Low	Left ventricles, nuclei	Healthy, HF and DCM	Adult, mouse	Single molecule RNA FISH	359	Discover long intergenic lincRNA as key nodal regulators, affect dedifferentiation and cell cycle genes	2017	BioProject ID: PRJNA264588	[99]
Mice	High	Whole heart	Healthy and TAC	Adult	ICELL8	11,492	Illustrated the dynamics of all major cardiac cell types	2020	GEO: GSE120064	[50]
Mice	High	Whole heart and other organs	Healthy, aging	Neonate, adult	10X Genomics, Smart-seq2	~20,000	Mouse Ageing Cell Atlas provides how the most important hallmarks of ageing are reflected in a broad range of tissues and cell types	2020	GEO: GSE132042	[100]
Mice	High	Whole heart	Healthy, CHD	Embryo	10X Genomics	73,926	Hand2 is a specifier of outflow tract cells	2019	GEO: GSE126128	[101]
Mice	High	Whole heart	Healthy development	neonate	10X Genomics	22,462	Assess the transcriptional landscape of the entire CCS	2019	GEO: GSE132658	[102]
Mice	High	Cardiac outflow tract	Healthy development	Embryo	10X Genomics	55,611	Molecular signatures of six cell lineages and subpopulations	2019	BioProject ID: PRJNA489304	[55]
Mice	High	Whole heart, nuclei	Healthy aging	Adult	10X Genomics	27,808	Molecular changes of aging cardiac fibroblasts	2019	EBI: E-MTAB-7869	[103]

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Table 2 (Continued).

Species	Throughput	Heart Region/ Cell Type	Disease	Developmental Time Point	Method	Cell Number	Finding	Publishing Date	Accessibility	Ref
Mice	High	Ventricles, nuclei	healthy and MI	Adult	10X Genomics	31,542	Dedifferentiation may be an important prerequisite for CM proliferation	2019	GEO: GSE129175	[104]
Mice	High	Sinus node, nuclei	Healthy pacemaking	Adult	10X Genomics	5357	Unique molecular make-up of the cardiac pacemaker	2019	GEO: GSE130710	[105]
Mice	High	Non-CMs and FB	Healthy and MI	Adult	10X Genomics	13,331	Heterogeneity, dynamics and intercellular communication among immune and stromal cells	2019	EMBL-EBI: E-MTAB-7376, E-MTAB-7365	[106]
Mice	High	Endothelial cells	Healthy and MI	Adult	10X Genomics	28,598	Present cardiac specific resident ECs, and the transcriptional hierarchy underpinning endogenous vascular repair following MI	2019	Not public	[45]
Mice	High	Whole heart, nucleus	Healthy development and pediatric mitochondrial cardiomyopathy	Neonate	sNucDrop-seq	15,083	Uncovered profound cell type-specific modifications of the cardiac transcriptional landscape	2018	GEO: GSE88761	[107]
Mice	High	Whole heart and other organs	Healthy	Adult	10X Genomics, Smart-seq2	~5000	Compendium of single-cell transcriptomic data from 20 organ including heart	2018	GEO: GSE109774	[108]
Mice	Medium	Left ventricles	Healthy development	Adult	ICELL8	3717	Switching of fibroblast subtypes regulates CM maturation	2020	GEO: GSE123547	[109]
Mice	Medium	Whole heart	Healthy and Hand2os1 knock out	Embryo	FACS	3600	The regulatory complexity of the lncRNA Hand2os1 on HAND2 expression	2019	GEO: GSE102935	[110]
Mice	Medium	Aortic valve, mitral valve	Healthy development	Neonate ~ juvenile	Drop-seq	2840	Subpopulations undergo changes in gene expression during development	2019	GEO: GSE117011	[111]
Mice	Medium	Left ventricles	Healthy development	Neonate	ICELL8	4231	Transcriptomes of mono- or multi-nucleated cardiomyocytes are highly similar Interstitial cell	2019	ENA: PRJEB29049	[112]

Mice	Medium	Nkx2.5 or Isl1 expressing cardiac progenitors	Healthy development	Embryo	FACS	1231	Cxcr2 regulates chemotaxis during development	2019	GEO: GSE108963	[113]
Mice	Medium	Whole heart and other 7 organs	Healthy development	Embryo	STRT	1916	Identify mutual interactions between epithelial and mesenchymal cells	2018	GEO:GSE87038	[114]
Mice	Medium	Ventricles	Healthy, I/R and MI	Neonate, adult	CEL-Seq2	1939	Identification of CSC populations	2018	GEO: GSE102048	[115]
Mice	Medium	Nuclei from whole heart	Healthy	Fetus	IFC system	2233	Chamber-specific genes in the embryonic mouse heart	2016	GEO: GSE76118	[116]
Mice	Medium	Whole heart	Healthy development	Neonate, adult	IFC system	>1200	Reveals lineage-specific gene programs underlying normal cardiac development and congenital heart disease	2016	GEO: GSE47948, GSE62913	[117]
Mice	Low	Whole heart	Healthy and ischemia reperfusion	Adult	SORT-seq	935	Ckap4 is a modulator of fibroblasts activation in the injured heart	2018	Not public	[32]
Mice	Low	Mesp1-positive or null cardiac progenitors	Healthy development Mesp1 knock out	Embryo	SMART-Seq2	598	Mesp1 is required for the exit from the pluripotent state	2018	GEO: GSE100471	[118]
Zebrafish	Low	Epicardium	Healthy development	Embryo	SMART-Seq2	366	3 Developmental epicardial subpopulations and the functions of *tgm2b*, *sema3fb*, *cxcl12a* genes	2020	GEO: GSE121750	[119]
Zebrafish	Medium	Heart	Healthy and Pbx4-depleted	Embryo	FACS	5300	Pbx4 limits heart size and fosters arch artery formation	2020	GEO: GSE126647	[120]
Zebrafish	Medium	Heart	Cryoinjured hearts	Embryos	FACS	1536	ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart	2019	GEO: GSE139218	[121]

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Table 2 (Continued).

Species	Throughput	Heart Region/ Cell Type	Disease	Developmental Time Point	Method	Cell Number	Finding	Publishing Date	Accessibility	Ref
Zebrafish	Medium	Heart	Healthy and development	Embryos	FACS	2637	The conversion of zebrafish Etv2-deficient vascular progenitors into skeletal muscle	2020	GEO: GSE142484	[122]
Monkey	High	Heart	Healthy	Young and old ages	10X Genomics	36,210	FOXO3A loss as a key driver for arterial endothelial aging	2020	GEO: GSE117715	[123]
Monkey	High	Whole heart and other organs	Healthy	Young and old ages	10X Genomics	42,053	Depict the first transcriptomic atlas of the aged primate cardiopulmonary system	2020	NGDC: CRA002689	[124]

amplification, whereas most of the droplet-based techniques including the Chromium system allow pooled PCR using cell barcoding techniques, which significantly improves throughput.[34] The sequencing libraries of cDNA fragments are then constructed and sequenced by high-throughput next-generation sequencers such as NextSeq 500. Sequencing libraries constructed with 3′ end enrichment is more cost-effective and produces less sequencing noise, whereas libraries retained full-length transcripts often obtain increased sequencing depth.[35]

Subsequently, raw data generated from sequencers are processed to obtain the gene expression matrix based on the unique molecular identifiers (UMIs). Raw data processing software such as the Cell Ranger (10X Genomics) is capable of performing QC, assignment of reads to the corresponding barcodes, demultiplexing, genome alignment, and read count quantification.[21] The resulting count matrices, usually in the form of sparse matrices, are represented in dimensions with the row defined as the gene, and column defined as each cell. Reads assigned to unique barcodes, however, may not always originate from a single cell, as the barcodes may appear doublets, triplets, or may not tag any cells.[36]

The single-cell sparse matrix is used to normalize variance and to identify overdispersion genes. Principal component analysis (PCA) is applied to detect principal components (PCs) that capture the greatest variance among all cells. The resulting data is subjected to the graph-based Louvain clustering in high-dimensional PC space to identify cell clusters. Finally, these data are projected into 2D/3D space using dimensionality reduction methods such as t-distributed stochastic neighbor embedding (tSNE) or uniform manifold approximation and projection (UMAP) for visualization (Figure 3).[36]

Comparisons of Different scRNA-seq Platforms

Among numerous single-cell platforms varied in captured cell number and read depth per cell, the plate-based Smart-seq2 method and droplet-based 10X Genomics Chromium approach are the two frequently used scRNA-seq platforms (Table 1). The plate-based Smart-seq2 platform has a high sensitivity for gene detection, especially for transcripts with low abundance. Depending on the method used for library construction and sequencing depth, the plate-based platform can simultaneously capture the full-length transcripts and reliably quantify more than 10,000

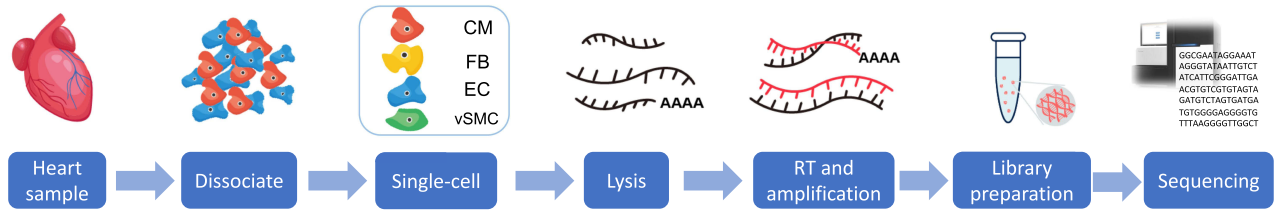

Figure 2 Experimental workflow of single-cell RNA-seq. The general experimental workflow of single-cell RNA- study begins with sample preparation. Prepared cells are captured by various single-cell methods. Reverse transcription of single-cell RNA is performed, followed by PCR amplification and library preparation of the resulting cDNA. Next-generation sequencing is performed to generate the raw reads.

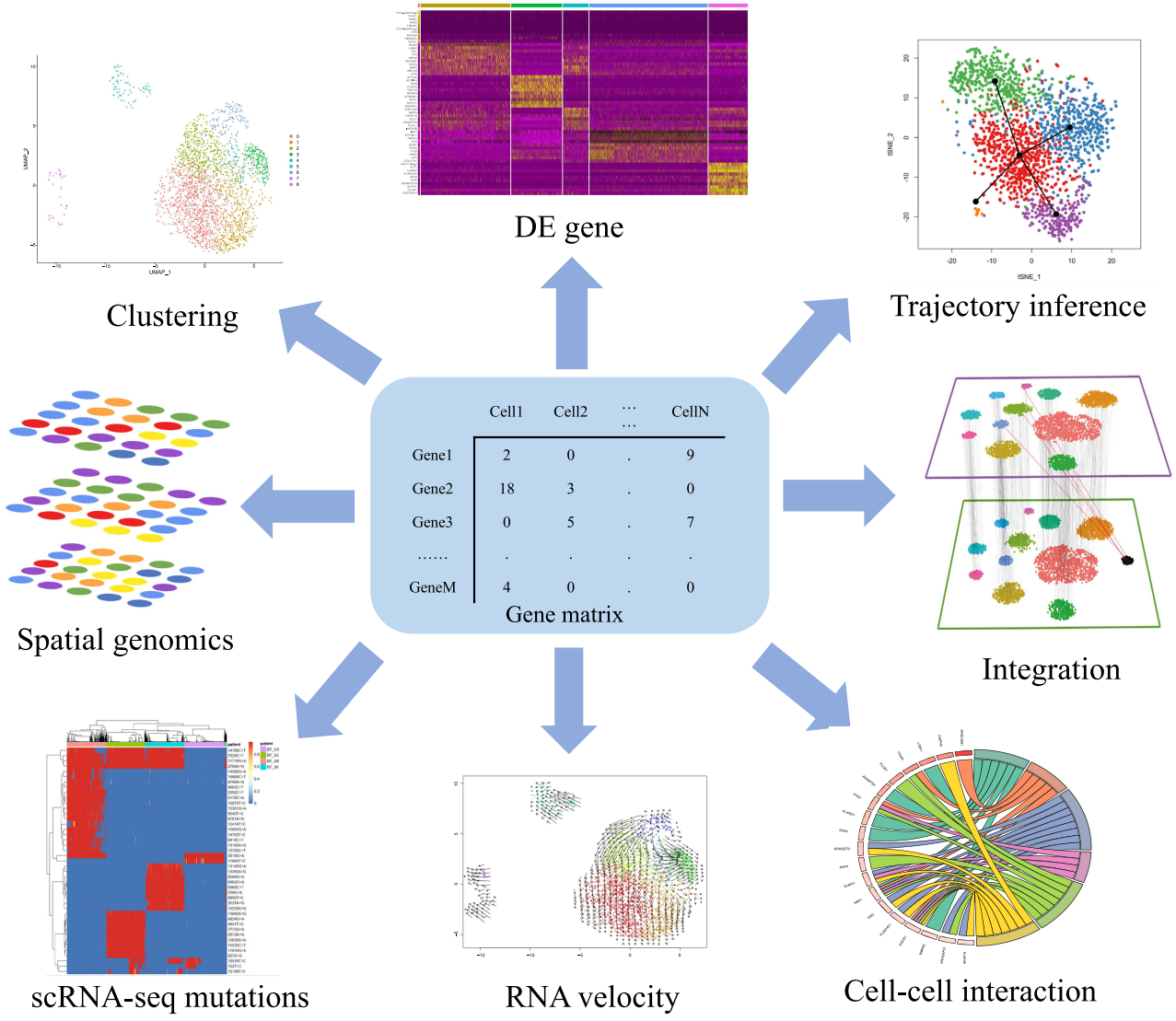

Figure 3 Application of scRNA-seq computational approach. Preprocessing steps convert the raw reads to sparse expression matrix. Downstream data analysis includes clustering, differentially expressed gene calling, cell trajectory analysis, RNA velocity, cell–cell interactions, identify mutations, integration (Reprinted from *Cell*, 177(7), Stuart T, Butler A, Hoffman P, et al. Comprehensive Integration of Single-Cell Data. 1888-1902 e182, Copyright 2019, with permission from Elsevier)[64] and spatial genomics.

genes in each cell.[37] The capacity of capturing full-length transcripts has the added advantage of facilitating the identification of splicing isoforms in single cells.[38] This approach also allows for the profiling of more cell types with a wide range of cell size, permitting the analysis of large cells such as adult cardiomyocytes that is currently

impossible to be profiled in droplet-based methods. However, reverse transcription performed in individual wells in the plate-based method prolongs the working process, limits throughput and increases noise in downstream steps.[39]

Droplet-based method such as the Chromium system from 10X Genomics enables 3′-end or 5′-end sequencing of single cells with higher throughput compared to the plate-based Smart-seq2 platform. Droplet-based methods encapsulate the single cells in oil droplets using DNA barcoding technology, substantially reducing the time and cost.[40] Meanwhile, massive parallelization profiles up to 10,000 cells per sample for a given run.[41] However, this method has a higher noise in accurately detecting the transcripts, especially for transcripts with low expression levels. The sensitivity of the protocols is expected to improve with continued protocol optimization and cost reductions. Despite the poly(A) enrichment, approximately 10–30% of all detected transcripts by both platforms are from non-coding genes, with lncRNA accounting for a higher proportion using the Chromium system.[42]

Applications of scRNA-seq in Cardiac Tissue

Unsupervised Clustering to Annotate Cardiac Cell Subtypes

scRNA-Seq can be used to annotate multiple cell types within the heart tissues based on the transcriptomic data of thousands of individual cells. In cardiovascular research, the clustered cell populations often include cardiomyocytes, fibroblasts, vascular smooth muscle cells, endothelial cells (ECs), epicardial adipocytes, immune cells and neural cells. Those identified cardiac cell clusters can also represent distinctive functional states in the different chambers such as ventricular and atrial chambers. Thus, performing unsupervised clustering to annotate cell populations represents cell types with biological relevance. Recently, scRNA-seq has been widely used to study heart development and disease. These studies utilized samples from various heart regions with or without disease, and identified multiple cardiac cell types.[11,43–46] In addition, snRNA-seq also can assess the cellular and transcriptional diversity of the human heart. For example, Tucker et al sequenced the transcriptomes of 287,269 single cardiac nuclei, which have been clustered into a total of 9 major and 20 subclusters of cardiac cell types within the human heart.[10] Cell types that were subclustered include two distinct groups of resident macrophages, four endothelial subtypes, and two fibroblasts subsets. They also identified strong enrichment for the role of cell subtypes in cardiac traits and diseases by using genetic association data.[10] The newly defined subtypes transform our understanding of human heart and may pave the way for developing new therapeutics for CVDs.

Trajectory Inference Discovers Transition States in Heart Development

Trajectory inference is a computational technique used in single-cell transcriptomic analysis to determine the pattern of the dynamic cell transitional states based on the gene expression profiles of cells in varying states. It characterizes the expression pattern of the cells and places them along a pseudotime axis, which is a time-like variable demonstrating the relative position a cell takes in a lineage representing the evolution of the process rather than placing the cells in discrete clusters.[47] By calculating a temporal dimension from the static scRNA-seq gene expression matrix, trajectory inference allows the probing of individual genes' expression dynamics along with continuous cell-state changes. If the mean expression level of a gene can be changed along pseudotime, the gene is indicated as differentially expressed which can be crucial for the underlying cellular process that generated the pseudotime.[47,48] Trajectory inference can thus illuminate the underlying biological processes by identifying key genes that play important roles in the development of particular lineages and genes differentially expressed between different lineages. Recently, Phansalkar et al applied the trajectory inference methods in developing human coronary arteries to illustrate coronary blood vessels from distinct origins can converge to equivalent states. The trajectory analysis result also suggested that artery ECs are formed by capillary ECs differentiation.[49] Ren et al used trajectory inference to reconstruct the progression trajectory to reveal intervention principles in pathological cardiac hypertrophy. The trajectory analysis also showed that activation of proinflammatory macrophages was a key event for the transition from normal to reduced ejection fraction.[50] Zhang et al used trajectory inference to illuminate the cell fate decisions and developmental origins of organ-specific cell types such as endothelial, muscle and cranial pharyngeal cell types in mesodermal progenitor cells. And they also uncovered intraembryonic progenitor from the lateral plate mesoderm (LPM) and cardiac progenitor from the late extraembryonic mesoderm can contribute to the development of cardiomyocytes.[51] Collectively, these studies have utilized

trajectory inference tools to better understand cellular transitions and intercellular communication in the early stages of human cardiac development.

RNA Velocity Predicts the Future Transcriptional Dynamic State

RNA velocity, a high-dimensional vector estimated by the ratio of unspliced and spliced mRNA reads in scRNA-seq data, is defined as the time derivative of the gene expression state.[52] RNA velocity predicts the future transcriptional dynamic state of individual cells on a timescale and enables the identification of novel cell states in a systematic and quantitative manner. It has greatly aided the analysis of developmental lineages and cellular dynamics in the human heart.[53] Recently, Wolfien et al utilized RNA velocity analysis to study the transcription kinetics and to visualize the dynamics of the transitions between mature and nascent cellular states of the cell types in the mammalian heart.[54] They found that different subgroups of mammalian cardiomyocytes have distinct marker profiles, especially for the profile of RNA velocity in cardiomyocytes. Meanwhile, via RNA velocity analysis, they identified a cell population that expressed the canonical endothelial markers that are also associated with cardiac contractile function. Thus, the RNA velocity results generated in the mammalian hearts support the hypothesis that this population is in a trans-differentiation process from an ECs-like phenotype towards a cardiomyocyte-like phenotype. In addition, Liu et al used RNA velocity analysis to identify the convergent development of the vascular smooth muscle cell (vSMC) lineage and to infer the direction and rate of the changes in vSMC state changes during heart development.[55] They found that the convergent development of vSMC lineage cell is involved in mesenchymal-to-vSMC transition or myocardial-to-vSMC transdifferentiation. Taken together, RNA velocity tools have paved new ways of studying heart development using scRNA-seq.

Identification of Unique Ligand–Receptor Interactions During Cardiac Cell–Cell Communication

Cell–cell communication is the essence of complex multicellular behaviors, of which cells communicate with one another via the binding of ligands and receptors that regulate cellular function, structure, and maintenance. The complex network of cell–cell communications among various cell types in the heart is essential to maintain the regular heart, whose disruption can lead to CVDs. These interactions underlying an intercellular network can be inferred from scRNA-seq data. Wang et al studied the cell–cell interaction networks in the human heart. The authors showed that cardiomyocytes and ECs are major cell-communication hubs and that cardiomyocytes' contractility and metabolism are the most prominent aspects that are correlated with changes in heart function.[46] For instance, Paik et al predicted the intercellular communication between ECs and other cell types in 12 major adult murine organs. This study reveals the existence of unique angiocrine ligand–receptor pairings between ECs and parenchymal cells in each major organs including heart and brain.[8] Recent studies demonstrated the unrecognized functions of the immune cell during cardiac function and diseases. In the mouse heart, macrophages were found to facilitate electrical conduction and have crucial roles in myocardial infarction and aging. These discoveries emphasize the deeper investigation of the interplay among different cardiac cell types.[56–58] Several statistical frameworks based on ligand–receptor interaction, such as CellPhoneDB, CellChat, SingleCellSignalR, have been developed to predict the enriched cellular interactions between two cell types from single-cell transcriptomic data.[59–61] Discoveries from the study of cell–cell communication have the potential to identify novel therapeutic targets for treatments of CVD patients.

scRNA-Seq Integration Discovers a Novel Subset of Cardiomyocytes Population

The broad application of scRNA-seq technologies generated an unprecedented amount of data for cardiovascular research listed in Table 2. Integrated datasets from separate studies have the potential to provide biological insights that will not be possible from analyzing individual datasets. For instance, the integration of multiple scRNA-seq datasets derived from subpopulations of cells of a particular tissue can aid in characterizing heterogeneity in these tissues under different conditions. Many powerful methods have been developed to integrate individual scRNA-seq datasets such as Seurat v3, Harmony, SIMLR, SC3.[62–65] Recently, Galow et al used the single-cell integration method to discover a minor population of cardiomyocytes characterized by proliferation markers that could not be identified by analyzing the datasets individually. The integration analysis also gave evidence that the renewal of the cardiomyocyte pool is driven by cytokinesis of resident cardiomyocytes rather than the differentiation of progenitor cells.[66] Kuppe et al used scRNA-seq, scATAC-seq

and spatial transcriptomic to profile the various physiological timepoints and zones of human health myocardium and myocardial infarction to build an integrative high-resolution map of cardiac remodeling. This integrated method increases cell-type composition spatial resolution and identifies the distinct injury, repair and remodeling cellular spatial zones.[67] In summary, single-cell integration genomics has played a fundamental role in our understanding of tissue heterogeneity and cross-species analyses may yield similar insights toward our understanding of cardiac cell diversity.

Detection of Genetic Variants from scRNAs-Seq Data

Genetic variants are generally identified from whole-genome sequencing (WGS) and whole-exon sequencing (WES) studies.[61] Detecting genetic variants from scRNA-seq data is rarely reported because of the inherited limitations of scRNA-seq platform such as low transcript abundance, allelic dropout, and incomplete transcript coverage. To overcome these limitations, SCmut has been developed to identify the cell-level recurrent variants in many single cells by controlling the false discoveries using the 2D local false discovery rate (FDR). The variants detected from scRNA-seq data can facilitate the investigation of cell-to-cell heterogeneity.[68] Compared to variants identified from WGS/WES dataset, the cell-level mutations can only be found in the exonic regions and are affected by stochastic monoallelic expression. Although the methods for detecting genetic variants from scRNA-seq data have been reported in studies of the area of cancer biology, to date, no scRNA-seq studies in cardiovascular research reported mutation analysis. However, detecting genetic variants from scRNA-seq dataset has the potential to reveal the precise mechanisms of the pathogenesis in CVDs.

Construction of Spatial Subcellular Map During Heart Development

Spatial transcriptomics has been developed to characterize the gene expression profiles simultaneously retaining spatial information in various biological contexts.[69] These methods aim to elucidate the function of individual cells in the context of their spatial organization in the tissue.[70] The methodologies in spatial transcriptomics provide important insights in cardiovascular research to explore the process of cardiac morphogenesis in humans. For example, fluorescence in situ hybridization (FISH)–based method has been developed to directly label in tissue sections to visualize each single cell, even in subcellular location.[71–73] In the latest work reporting the application of spatial genomic in the cardiovascular field, Asp et al used spatial transcriptomic method to study the transcriptional landscape of cardiac cells during the development of the embryonic heart and mapped the specific genes to the corresponding anatomical domains. They characterized the unique gene profiles in distinct anatomical regions and constructed a spatial subcellular map for the three developmental phases.[43] In a separate study, Mohenska et al used spatial transcriptomics to reconstruct a 3D gene expression pattern in the mouse adult heart. They revealed specific gene lists that displayed complex spatial expression in organ sub-compartments, and deciphered gene expression profiles of the atria and the transcriptional complexity within the ventricles, and predicted the localization of non-myocytes within the heart.[74] These spatial transcriptomics methods have greatly facilitated future studies on cardiogenesis with unprecedented resolution.

Limitations of scRNA-seq in Cardiac Tissue

Current scRNA-seq technologies are still confronting many challenges and limitations to profile the transcriptomic panorama of individual cells.[75] For instance, scRNA-seq is unable to reliably detect low-abundance transcripts. It has been reported that only approximately 10% of the transcript could be detected from a single cell and the percentage of the lost RNA content reached to 60%. Both contribute to a higher difficulty in detecting the low abundant transcripts.[76,77] The low amount of transcripts often resulted from library preparation leads to high levels of computational noise, which disturbs data analysis and may mask underlying biological variation. For example, long non-coding RNAs (lncRNAs), which have critical roles in regulatory functions, typically are presented in several copies in a cell but often could not be detected.[78] Thus, it is necessary to improve the sensitivity of scRNA-seq to detect low copy transcripts in one cell to gain a full understanding of many regulatory processes.

Furthermore, some cell types such as cardiomyocytes may not be compatible with the processing steps of popular scRNA-seq techniques. The droplet-based Chromium platform is suitable for scRNA-seq studies with cells smaller than 30μm.[31] However, adult CMs in mice and humans are relatively larger than 100μm in diameter, which deterred the use of this single-cell system.[31] Therefore, snRNA-seq, which extracted nuclei rather than intact CMs, or plate-based FACS platform could be

considered as alternative methods to profile CMs or cells from frozen specimens.[79] Future improvement in these single-cell protocols using intact cells or nuclei will help to overcome the limitations and biases.

Unsupervised clustering of scRNA-seq data is crucial for the downstream data analysis, as it annotates the cell types. In clustering, the hypothesis is that each cluster will represent one cell type. However, there is no golden standard for defining the cluster as a specific cell type.[75,80] This is partly due to the expression matrix exhibited more zero values (known as dropouts). The dropouts cause higher levels of noise to annotate the state and identity of the cells accurately.[81] The technical noise is also generated in the preparation of sequencing samples such as single-cell digestion resulted disproportionately enriches for one cell type over another.[80]

Trajectory inference can use single-cell sequencing data to infer the cells along the developmental trajectories and facilitate mapping clonal relationships onto these landscapes.[47] However, these sequencing-based lineage-tracing methods are still in their infancy. Compared with the DNA barcodes and clone analysis to reconstruct lineage relationships, sequencing-based lineage-tracing methods are sensitive to the choice of experimental platform to perform the scRNA-seq, which could affect the conclusions.[82] However, it can anticipate that lineage-tracing methods might be integrated with clonal analysis and DNA barcoding methods, which will significantly track the number of clones and establish clonal composition without requiring prior knowledge of the marker genes.[82] To accurately decipher the spatial gene expression, it is important to capture gene identity along with quantitative data. And efforts have been made to achieve higher spatial resolution due to the integrated single-cell imaging techniques.[83]

Perspective

Over the past decade, there has been an increasing interest in using scRNA-seq technologies to study cardiovascular development and disease. Imaging technologies such as FISH have been proposed to combine with scRNA-seq to study the spatial single-cell transcriptomic profiles in the cardiovascular environment.[84] In addition, long-read sequencing technology such as PacBio and Oxford Nanopore platforms can be combined with high-throughput droplet-based scRNA-Seq workflows to capture gene-expression profiles with targeted full-length mRNA sequences from a large number of cells. Such combination has the potential to achieve both high sensitivity and accuracy in capturing full-length transcripts, which could be further used for the identification of somatic mutations and the inference of clonal evolution of distinct cell types.[85] In addition, the integration of scRNA-seq and chromatin accessibility data can provide more comprehensive insights into gene regulation and cellular dynamics. As the numbers of scRNA-seq datasets are rapidly increasing due to collaborative efforts such as the Human Cell Atlas consortium,[86] it is necessary to optimize the algorithm and develop sophisticated computational methods for data analysis. Collectively, scRNA-seq technologies will greatly expand our knowledge in cardiac cell heterogeneity, CVD pathogenesis and microenvironmental interactions, and ultimately lay a foundation for precision medicine in cardiovascular diseases.

Abbreviations

CF, cardiac fibroblast; CMs, cardiomyocytes; CVDs, cardiovascular diseases; DE, differential expressed; ECs, endothelial cells; FACS, fluorescence-activated cell sorting; FDR, false discovery rate; FISH, fluorescence in situ hybridization; hiPSC, human-induced pluripotent stem cell; lncRNAs, long non-coding RNAs; LPM, lateral plate mesoderm; NGS, next-generation sequencing; PCs, principal components; PCA, principal component analysis; PCR, polymerase chain reaction; QC, quality control; RNA-seq, RNA sequencing; RT, reverse transcription; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing; tSNE, t-distributed stochastic neighbor embedding; UMAP, uniform manifold approximation and projection; UMIs, unique molecular identifiers; vSMC, vascular smooth muscle cell; WES, whole-exon sequencing; WGS, whole-genome sequencing.

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Author Contributions

All authors made a significant contribution to the manuscript, whether that is in the conception, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare in this work.

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