

## Invited Mini Review

## Single-cell and spatial transcriptomics approaches of cardiovascular development and disease

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Recent advancements in the resolution and throughput of single-cell analyses, including single-cell RNA sequencing (scRNA-seq), have achieved significant progress in biomedical research in the last decade. These techniques have been used to understand cellular heterogeneity by identifying many rare and novel cell types and characterizing subpopulations of cells that make up organs and tissues. Analysis across various datasets can elucidate temporal patterning in gene expression and developmental cues and is also employed to examine the response of cells to acute injury, damage, or disruption. Specifically, scRNA-seq and spatially resolved transcriptomics have been used to describe the identity of novel or rare cell subpopulations and transcriptional variations that are related to normal and pathological conditions in mammalian models and human tissues. These applications have critically contributed to advance basic cardiovascular research in the past decade by identifying novel cell types implicated in development and disease. In this review, we describe current scRNA-seq technologies and how current scRNA-seq and spatial transcriptomic (ST) techniques have advanced our understanding of cardiovascular development and disease. [BMB Reports 2020; 53(8): 393-399]

## INTRODUCTION

Within the field of cardiovascular biology, there have been many questions about understanding how the heart develops and how developmental cues go awry in the disease progression of congenital disorders. The mammalian heart contains many specialized cell types with differences in morphology, location, gene expression patterns, and functions. These heterogeneous cell types that comprise of the heart have been inves-

tigated at the cellular and molecular levels. However, a major drawback of these techniques is in measuring the average expression or patterns across the samples examined, resulting in the loss individual of cellular and molecular resolutions. To better understand cardiovascular development and disease, resolving the subtle differences between heterogeneous cardiac cells may have profound therapeutic implications.

scRNA-seq technologies have achieved significant progress in the last several years and have become one of the most popular tools for understanding cell heterogeneity and lineage progression (1-3). Tang *et al.* in 2009 first reported whole-transcriptome sequencing in single cells. Although technologies have advanced in throughput and resolution, the methodology of the pipeline remains similar to current techniques (4).

The scRNA-seq method involves dissociation of a tissue, which requires breaking the connection between single cells by breaking down extracellular connections, leading to a loss of individual cell localization information and can cause incomplete interpretation. Presently, there are two main scRNA-seq approaches: SMART-seq2 and 10x genomics. SMART-seq2 is used to profile the full length of transcripts but provides a relatively low throughput; 10x genomics can capture more cells but has limited ability to capture transcripts (5-8). Besides these scRNA-seq techniques, spatially resolved transcriptomics and *in situ* sequencing (ISS) also have made important progress (9, 10). Also, ISS, which achieves cell-level gene mapping in single cells *in situ*, can be used for cell-level mapping of organ development by a fluorescent-imaging-based sequencing method (Fig. 1) (11-13).

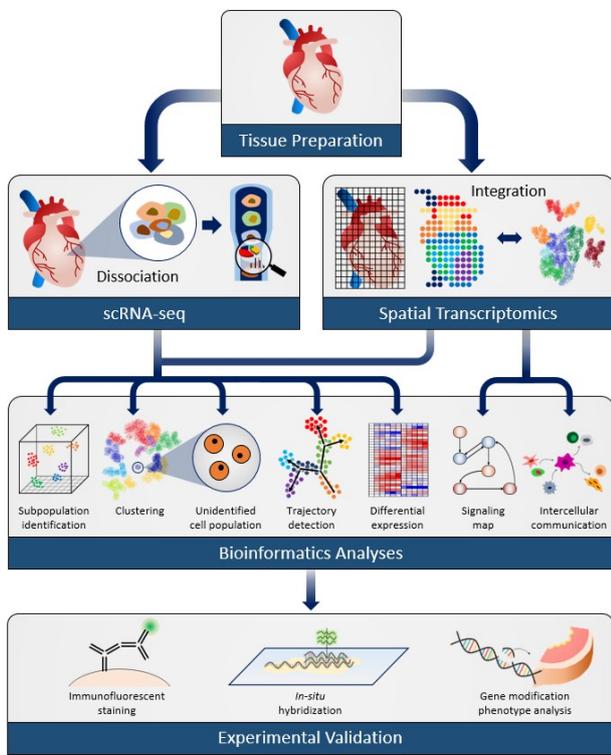
By means of scRNA-seq, researchers can map expression profiles at single-cell resolution. Since the introduction of scRNA-seq and ST, the number of single-cell experiments has greatly increased. The scRNA-seq technique has become a powerful tool for identifying and classifying cell subpopulations, characterizing rare or small subpopulations, and tracing cells along dynamic cellular stages, such as during differentiation, which has combined to understanding cardiovascular development and pathogenesis (3, 11, 14, 15). In this review, we briefly describe the history and progression of scRNA-seq and ST technologies, and summarize the recent scRNA-seq studies in cardiovascular research and their potential implications for understanding the

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**Fig. 1.** A framework of scRNA-seq and ST applications in cardiovascular research.

cardiovascular system and associated diseases (16).

## HISTORY AND DEVELOPMENT OF SINGLE-CELL-RNA AND TRANSCRIPTOMICS SEQUENCING ANALYSIS

Recent advances in biotechnology, specifically single-cell technologies, have revolutionized basic science research. In 1992, Eberwine *et al.* developed the T7 amplification technique, which can increase amounts of RNA from low concentration of starting material, and assessed expression profiles in live pyramidal neurons of the rat hippocampus (17). However, its application to molecular and cellular biology was limited to detecting only a few genes of interest by probes or RT-PCR, until the introduction of next-generation sequencing platforms. The complexity of the transcriptomes of single cells was first reported in 2009, using an mRNA-Seq assay (4). This new technique was applied to a single mouse blastomere to profile its digital gene expression and identified 1,753 previously unknown splice junctions at single-cell resolution. Since then, advances in scRNA-seq and downstream analysis tools have greatly accelerated our understanding of the complexity of gene expression, regulation, and networks.

More recently, the Chan Zuckerberg Biohub released *Tabula Muris*, a compendium of scRNA-seq data from 20 mouse organs

and tissues (18). The data provide references to cell-type composition within and between organs and tissues and identified rare or poorly characterized cell populations along with their gene expression patterns. These data represent a new resource for both cell-centric and gene-centric studies across 20 tissues. The cited study used two distinct technical approaches for most organs: 3'-end counting based on microfluidic droplets and full-length transcript analysis based on fluorescence-activated cell sorting (FACS). The differences in technical approaches will be discussed in more detail in the following section.

Detecting gene expression in single cells is challenging, as the concentration of total RNA present in a cell is on the order of picograms, and of those, polyadenylated mRNA constitutes less than 5% of total cellular RNA (19, 20). In the last decades, different high-throughput methods for scRNA-seq have been developed to isolate and capture these finite biomolecules efficiently, with different degrees of coverage, sensitivity, and multiplexing capabilities for single-cell transcriptome analysis. The key technological developments and protocol improvements that enabled large-scale scRNA-seq experiments have been previously reviewed by Svensson *et al.* (21).

Current scRNA-seq methods can be classified based on the single-cell isolation techniques and capture methods for library preparation (Table 1). Three platforms are available for sample preparation, plate-based, microfluidics, and droplets. Of those, the two most-popular approaches are plate-based SMART-seq in the Fluidigm C1 system and microdroplet-based scRNA-seq from 10X Chromium. SMART-seq2 is an improved version of SMART-seq, which relies on template switching and preamplification, with an improvement in both the cDNA library yield and average length from individual cells (22). This method has several tradeoffs, but makes possible the analysis of all exons of each transcript and detection of the different splice variants. SMART-seq2 also enables comprehensive SNP and mutation analysis, widening its field of application (23). Limitations of this approach include refining the selectivity for polyadenylated RNA, precluding analysis of polyA-RNA that does not reflect the strand-specific nature of mRNAs. In addition, this method relies on the labor of individual researchers rather than automated methods of multiplexing (19, 23).

STRT was one of the early multiplexing methods developed, although it precludes splice isoforms. This method reflects the strand-specific nature of mRNAs and identifies the transcription start site (TSS) (19). CEL-seq is another early method that implemented barcoding and pooling samples, but was the first to use linear *in vitro* transcription (IVT) amplification (24). Hashimshony *et al.* reported that CEL-seq outperforms STRT in terms of reproducibility, sensitivity, and robustness. CEL-seq does not detect miRNAs and other non-polyadenylated transcripts, which can be its strength, because the barcoded transcripts preclude rRNA, which increases the efficiency of the sequenced reads. Both methods are limited to sequencing of the different ends of the mRNA transcript (3' bias for CEL-seq and 5' bias for STRT), which severely limits their

**Table 1.** Summary of scRNA-seq techniques

Techniques	Single cell isolation techniques	Capture Platforms	cDNA ampl.	Strand specific	Multi-plexing	UMI (bp)	Features	Year / (Refs)
Full-length transcript								
Tang method	Manual	PCR tubes	PCR	X	X		Splice variant detection	2009 (4)
SMART-seq	FACS	Plate based	PCR	X	X		Template switching reaction	2012 (7)
SMART-seq2	FACS	Plate based	PCR	X	X		Improved yield, coverage and accuracy	2013 (22)
Quartz-seq	FACS	Plate based	PCR	X	X		Single PCR tube reaction without any purification	2013 (8)
MATQ-seq	FACS	Plate based	PCR	O	O		Whole gene body coverage	2017 (20)
5' End transcript								
STRT	FACS	Plate based	PCR	O	X		Highly multiplexed, TSS detection	2012 (17)
STRT-seq-2i	Limiting dilution or FACS	Microarray platform custom capture plates	PCR	O	O	6	Imaging checkpoint available	2017 (26)
WaferGen	Multi-sample nanodispenser (MSND)	Nanowell based (ICELL8)	PCR	O	O	10	Imaging software to identify nanowells	2017 (29)
3' End transcript								
CEL-seq	FACS	Plate based	IVT	O	X		First to use linear RNA amplification	2012 (22)
MARS-seq	Microfluidic, FACS	Plate based	IVT	O	O	4	Introduced automated massively	2014 (23)
CytoSeq	Recursive Poisson loading	Nanowell	PCR	O	O	8	First to present scalable mRNA cytometry	2015 (34)
Drop-seq	Double poisson loading, Microfluidic channel	Droplet based	PCR	O	O	8	Coencapsulation of single cells	2015 (29)
InDrop	Super Poisson loading	Droplet based	IVT	O	O	6	Cells are captured and barcoded	2015 (27)
CEL-seq2	Manual, Microfluidic, FACS, C1	Microfluidic chip	IVT	O	O	6	Implemented the UMI	2016 (24)
DroNC-seq	Poisson loading	Droplet based	PCR	O	O	8	High throughput	2017 (32)
Chromium 10x	Super Poisson loading	Droplet based (Single Cell A Chip)	PCR	O	O	10	High throughput, process up	2017 (33)
SPLiT-seq	No partitioning into compartments	Plate based	PCR	O	O		Can sequence fixed cells or nuclei	2018 (36)
Quartz-seq2	FACS	Plate based	PCR	O	O	8	Major improvement of Poly(A)tagging	2018 (30)
MARS-seq2	FACS	Plate based	IVT	O	O	8	Integrated pipelines for index sorting	2019 (35)

ability to distinguish splice isoforms (19, 24). CEL-seq2 is a modified version of CEL-seq. CEL-seq2 is compatible with different platforms and is the first technique to implement a single-cell, on-chip barcoding method on the Fluidigm's C1 system. CEL-seq2 outperforms both its initial version and SMART-seq on the C1 system (25). A modified and extensively reoptimized version of the 5' STRT-seq method was introduced in 2017. The STRT-seq-2i employs a dual index to allow multiplexing and 9600 microwell array capture. This method also limits dilution and eliminates the need for FACS single-cell isolation with an imaging checkpoint (26), providing an ideal platform for high throughput experiments at relatively low cost, without compromising sensitivity and accuracy.

Recent development of droplet-based, ultra-high-throughput scRNA-seq platforms, including 10X Chromium, inDrop, and Drop-seq, enable large-scale scRNA-seq studies, such as *Tabula Muris*, a compendium of scRNA-seq data, as mentioned above (18, 27). A systematic comparison of these three widely used platforms has recently been done (28). In brief, all three methods share 3' end bias mRNA digital counting, and the barcoded primer beads share a common structure, containing a PCR handle, cell barcode, UMI, and poly-T, except that inDrop beads also have a T7 promoter and a photo-cleavable moiety. Drop-seq uses small, hard resin beads, whereas both 10X and inDrop use soft and deformable hydrogel beads, which enable encapsulation to achieve a super-Poisson distribution and double Poisson distribution for Drop-seq. Thus, the cell-capture efficiency is markedly higher in the 10X and inDrop approaches (29-31). Systematic comparison showed that 10X Chromium outperforms Drop-seq and inDrop, in terms of higher molecular sensitivity and decreased technical noise (32, 33). However, high performance comes with high costs. The cost per cell is around \$0.50 for 10X Chromium and requires a Chromium controller. In comparison with compromises in sensitivity and precision, Drop-seq costs \$0.10 per cell and inDrop cost \$0.25 (28).

An automated massively parallel scRNA-seq (MARS-seq) approach was introduced in 2014. This approach is based on FACS of individual cells into 384-well plates and uses three levels of barcoding (molecular, cellular, and plate-level tags) thus facilitates molecule counting with a high degree of multiplexing (34). The second generation of MARS-seq 2.0 was published in 2019. In this generation they used longer cell barcodes (from 6 bp to 7 bp) and UMIs (from 4 bp to 8 bp), which have improved throughput, robustness, and reduced noise and costs (35).

An ideal platform should combine high throughput capabilities with flexibility in sample size to allow for profiling of increasing numbers of cells and multiple samples in parallel and identify new or rare subpopulations while maintaining sensitivity and robustness. Additional desirable features include imaging software to identify contaminating doublets and measure fluorescent reporters. Although the aforementioned methods meet most of these requirements, they are often ex-

pensive and require customized equipment, microfluidics, microwells, or cell sorters. Droplet microfluidics allow for high throughput methods at a low cost per cell, however this comes at the expense of flexibility and imaging. A more scalable single-cell profiling method with no need of customized equipment, split-pool ligation-based transcriptome sequencing (SPLiT-seq) was reported in 2018 (36). This method is compatible with both fixed cells and nuclei, allowing efficient sample multiplexing and library preparation using only basic laboratory equipment (36).

Although recent advances in scRNA-seq techniques have led to increased use of the technology, it is important to recognize the limitations of single-cell isolation including the loss of spatial information. This limitation was overcome by researchers in 2016, when ST was developed (10). The ideas behind this technique were simple; they introduced positional molecular barcodes and synthesized cDNA on a surface of the tissue sections and were captured for downstream next-generation sequencing. This technique can capture mRNA from tissues with minimal diffusion and maintain positional representation of these mRNAs (10). The barcoded reads can then be overlaid on the tissue section images. Although this method gives a single, spatially resolved whole-transcriptome read out, the resolution of barcoded regions is limited 100  $\mu\text{m}$  in diameter, which is not at the level of single cell. ST was further developed, and both resolution (from 100  $\mu\text{m}$  to 50  $\mu\text{m}$ ) and processing time were improved by 10X Genomics and were available under the name "10X Visium" (12, 37).

A higher spatial-resolution technique called Slide-seq was introduced in 2019 (12), and an improved second generation was described earlier this year (Slide-seq2) (38). Stickels et al. report that modification of barcoded bead synthesis, the sequencing pipeline, and the cDNA-processing steps have markedly improved the mRNA capture sensitivity and efficiency, approaching the level of droplet-based scRNA-seq techniques (38). For further information on the early history and currently availability of ST methods and their applications have been previously reviewed (37).

## MAJOR USE OF SCRNA-SEQ ON CARDIOVASCULAR DEVELOPMENT AND DISEASE

### Identification of cell types in tissue/organs and subpopulations of the same cell type

The scRNA-seq studies of the mammalian heart enable researchers to profile a wide range of tissues and cell types in the cardiovascular system. With advances in the resolution of scRNA-seq technologies, novel cell types of the heart have been identified and reported, along with the spatial and temporal expression patterns of various cell types along with the heterogeneity within these subpopulations of cells.

The scRNA-seq method has been used to identify mechanisms of how early cardiac cells emerge and segregate from the heart. As cells develop, their expression patterns change to

fulfill specific functions, and the expression of these specific genes mark a mature, differentiated phenotype. One can use scRNA-seq to help understand these transitional states and elucidate the activation and deactivation of specific genes that must occur in order for cells to change their profile and identity (39). In the heart, this occurs in the premature venous cells that develop an immature network of vessels, or plexus, during the development of the heart. As these cells continue to mature, some change states by downregulating specific genes, such as *CoupTF2* and *Nr2f2*, and upregulating other genes, such as *EphrinB2*, *Dll4*, and *Notch4*, which ultimately results in reprogramming of cells towards arterial identity (40).

For example, in the postnatal murine heart, valve interstitial cells cluster into two distinct subpopulations at P7 and continue to differentiate into four distinct clusters by P30, undergoing changes in the expression pattern of collagen and elastin and completing morphogenesis of the heart valves (41). In addition to capturing valve interstitial cells, Hulin *et al.* describe how endothelial-cell and immune-cell populations present at both P7 and P30 remain unchanged in their expression pattern over the course of valve stratification (41).

### Cell differentiation and cell lineage analysis in cardiac development

Using scRNA-seq to analyze the cells that constitute different regions of the heart has also allowed for insight into cell signaling, gene expression patterns and signaling gradients that contribute to the development and physiology of the heart. Employing scRNA-seq in murine embryonic hearts, Li *et al.* suggest that the expression of *Tgfβ1* by the endocardium and *Rspo1* by the epicardium may be implicated in establishing the trabecular myocardium and compact myocardium regions of the developing heart (39). Therefore, the spatial orientation of cells within the heart can affect the profile of a specific cell, and this information can be captured in scRNA-seq data. It is important to keep in mind that the cells that make up the clusters identified by means of scRNA-seq are heterogeneous, themselves, and the signaling that each individual cell receives will affect its individual expression profile.

The scRNA-seq technique has been used to reveal pathogenic mechanisms of congenital heart disease by defining local changes of subpopulations. Soysa *et al.* used scRNA-seq with a network-based computational method to predict lineage-specifying transcription factors (42). *Hand2* was specified as a biomarker for right ventricular formation during early cardiac development that was responsible for congenital heart disease. Soysa *et al.* found that *Hand2* led to dysregulation of retinoic-acid signaling and disruption of anterior-posterior patterning by using temporal single-cell-transcriptome analysis and *in situ* hybridization (42). These findings showed that scRNA-seq is critical for understanding pathological mechanisms of congenital heart disease in a single-cell resolution; it is expected that scRNA-seq can be applied to identify various diseases that have been caused by heterogeneous cell differentiation (11).

### Adult heart and cardiovascular disease models from a single-cell perspective

Similar to the use of scRNA-seq during development, the technique is also used for characterizing cell subpopulations, including rare cell type that constitute adult tissues. This enables researchers to elucidate pathological conditions or cell-type, and determine the cell trajectory in injury or disease by characterizing changes in gene expression, signaling pathways, and interaction networks in the adult cardiac cells.

The identification of different subpopulations and rare cell types has major clinical implication for cardiovascular pathogenesis. For example, Skelly *et al.* identified hybrid macrophage/fibroblast subpopulations of cells in the mouse heart that may contribute to myocardial fibrosis upon stress (43). But the exact underlying mechanism of fibrosis has been unknown. To access IL-11's role in cardiac fibrosis development, Schafer *et al.* performed bulk scRNA-seq in the fibrotic human heart and found that IL-11 RNA expression was positively correlated with myofibroblast populations. Additional scRNA-seq experiments in a fibrosis-susceptible mouse model identified a IL-11-positive subpopulation of fibroblast that shows IL-11's important role in myofibroblast differentiation from fibroblasts in cardiovascular disease (44).

The scRNA-seq technique has found greater heterogeneity across these cell types that was not determined from bulk RNA-seq. See *et al.* also discovered a heterogeneous cardiomyocytes subpopulation from both human and mouse cardiac failure that expresses non-coding RNAs that regulate the cardiomyocyte cell cycle under myocardial stress (45).

Additionally, Nomura *et al.* use SMART-seq2-based scRNA-seq to identify heterogeneous gene expressions of hypertrophic cardiomyocytes and cellular-trajectory analyses of cardiomyocytes at different timepoints from a hypertrophic cardiomyocyte model that was induced by transverse aortic constriction, this revealed the role of p53 in promoting a pathogenic gene program in a subpopulation of cardiomyocytes (46). Additional scRNA-seq data confirmed this finding from patients with cardiac failure. In addition, Nomura *et al.* used a cell-specific marker, such as a smFISH of RNA molecules, into the scRNA-seq pipeline, which is expected to provide novel biomarkers for disease analysis with a spatial map of cells-of-interest (46, 47).

### Single-cell maps of the human heart using ST

A major hurdle of transcriptome-wide scRNA-seq studies is that they don't retain spatial information of the original cells, because scRNA-seq analysis processing involves dissociation of a tissue into hundreds or thousands of individual cells, each of which is analyzed to define its gene-expression profile (48). Without local information on individual cells, accurate interpretation and application of the data can be difficult (37, 38).

To analyze cardiac gene-expression patterns and cell heterogeneity simultaneously, Asp *et al.* designed a molecular approach that uses scRNA-seq along with ST and ISS, that provide posi-

tional information of cells (37). ST allows for the quantitative spatial distribution of mRNA transcripts using barcoded oligo-dT arrays in histological sections, which can be validated using ISS, a technique that employs rolling circle amplification and padlock probes and rolling circle amplification to visualize genes which have known primers (9). In the paper, Asp et al. not only test their technical proof of concept but also uncover novel cell types, such as clusters of fibrosis-associated fibroblast-like cells and a subpopulation of cardiac muscle cells (37). They also visualized their result by integrating the spatial information into 3D transcriptional maps for other researchers. A major caveat of this combined approach is that it maximizes the efficiency of using data information by providing both cell-type and regional information from tissues, e.g., human embryo tissue, that are not easily obtained.

### LIMITATIONS AND FUTURE PERSPECTIVES

Although scRNA-seq technology has become a powerful and common place tool for researchers to understand physiological and pathological mechanisms, technical challenges and limitations still remain (21). As shown in Fig. 1, tissue dissociation is the initial step for the scRNA-seq pipeline; hence an optimal tissue dissociation protocol is essential depending on the tissue of interest. The isolation and handling of individual cells have become much simpler in recent years. However, a limitation of automated scRNA-seq platforms is the requirement for large volumes of cells compared to those needed to perform manual scRNA-seq methods. In principle, this can be overcome by expanding the pool of barcoded beads and reducing the sample capture speed, with cost timeliness as a tradeoff. Most importantly, only 5-20% transcripts are typically captured per cell; any events or conditions that may induce stress and influence the transcriptomes need to be addressed and optimized. This issue can be partially addressed by adjusting the depth of sequencing but again, increase cost.

As innovation and advancement in scRNA-seq technologies continues, deciding which scRNA-seq technique is most conducive towards addressing the goals of the study should include analysis of the benefits and drawbacks of different sequencing platforms. Currently, scRNA-seq techniques and the fundamental differences in their approach to obtain single-cell transcripts are summarized in Table 1. There is no one-size fits all; each technique has method-specific advantages and limitations. Hence researchers should choose a technique that aligns with their desired goals and sample availability. Regardless of these technical limitations, single-cell analysis has revolutionized our understanding of cardiovascular development and disease. The applications and insights that *in silico* analysis enable in the investigation of cell subpopulation variants and differentiation in cardiovascular disease and fetal heart development are invaluable to both basic and medical research and scRNA-seq technologies are expected to continue to develop rapidly with continuous improvement of experimental and analytical

methods.

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### CONFLICTS OF INTEREST

The authors have no conflicting interests.

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