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STATE-OF-THE-ART TECHNOLOGY FOR CARDIOVASCULAR RESEARCH

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Highlights

• This review highlights state-of-the-art technologies in cell and molecular biology. The directions of cardiovascular disease research where these technologies may lead to breakthrough discoveries are discussed.

Abstract

Heart and vascular diseases are responsible for tens of millions of deaths annually, underscoring an urgent need to improve the existing clinical practice in order to benefit patients. Advancement of basic science and technology enables understanding of disease etiology and pathogenesis at a deeper level of complexity. This mini-review article provides a summary of recent methods of cell and molecular biology applicable for current cardiovascular research. Widespread application of these approaches in cardiovascular disease research will be a key factor in the prolonged longevity and life expectancy of the general population.

Keywords Cardiovascular disease • Method • Atherosclerosis • Single-cell sequencing • Technology

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АНАЛИТИЧЕСКИЙ ОБЗОР

Список сокращений

ATAC-Seq	– transposase-accessible chromatin with sequencing	LDLR	– LDL receptor
CRISPR	– clustered regularly interspaced short palindromic repeats	mRNA	– messenger ribonucleic acid
		Ribo-seq	– ribosome profiling
CRISPR-Cas9	– CRISPR-associated protein 9	RNA	– ribonucleic acid
crRNAs	– CRISPR RNA	RNA-seq	– RNA sequencing
CVD	– cardiovascular disease	rRNA	– ribosomal ribonucleic acid
gRNA	– guide RNA	scRNA-seq	– single-cell RNA sequencing
IMC	– imaging mass-cytometry	TOF	– tetralogy of Fallot
LDL	– low-density lipoprotein	TTS	– takotsubo syndrome

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and invalidization in the world. Russia is a country with one of the highest CVD deaths worldwide. In 2019, Russia showed the world's highest disability-adjusted life-years due to peripheral artery disease, alcoholic cardiomyopathy, cardiomyopathy and myocarditis, and high low-density lipoprotein (LDL) cholesterol [1]. This ominous statistic could be explained by a combination of factors including social, economic, geographical, etc. On the bright side, however, the age-standardized CVD death rates in Russia have reduced by more than 20% in the last 10 years, largely due to improvements in hospital infrastructure allowing better access to healthcare for patients in dire need.

Even though the pathophysiology of CVD is generally well studied, while CVD risk factors are modifiable and can be controlled with altered behavior, more basic cardiovascular research is needed to improve current CVD treatment modalities and develop targeted therapies tailored to a patient's requirements. Twenty years ago, a protein serine protease proprotein convertase subtilisin/kexin type 9 was found to bind LDL receptor (LDLR), which induced a conformational change in the LDLR, marking it for lysosomal degradation, which eventually led to a reduced LDL-cholesterol in the blood serum [2, 3]. This fundamental discovery made in a laboratory opened a possibility of lipid-lowering therapy for patients with statin intolerance or those with familial hypercholesterolemia, therefore offering a therapeutic niche for selective high-risk patients.

The last decade has seen a major technological development leading to the emergence of novel methods of cell and molecular biology. These new approaches

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allow a deeper understanding of the occurrence and progression of diseases at unprecedented resolution. In this review article, I summarize the state-of-the-art technology applicable to current basic cardiovascular research.

Imaging Mass Cytometry

Immunostaining has been a major discovery tool for decades. This technique dates back to 1941 when Coons and colleagues developed immunofluorescence techniques for labeling antibodies [4]. While classical immunostaining is effective for both qualitative and quantitative analysis of several proteins of interest on one slide (usually 1 to 4), it does not allow the simultaneous staining of multiple (10+) antigens on the same slide. This is a major limitation in the era of big data and high-throughput technologies, and therefore immunohistochemistry and immunofluorescence (collectively termed immunostaining) are suboptimal options for use in precious clinical specimens such as tissue microarrays. In addition, conventional immunohistochemistry techniques have a number of other limitations concisely summarized by Rimm [5].

Imaging mass-cytometry (IMC) is a very recent method [6] enabling simultaneous interrogation of multiple protein markers (up to 37) on a single glass slide. Fluorescence spectra used in immunofluorescence staining have relatively broad peaks that could lead to significant signal overlaps (Figure 1A). On the contrary, the IMC technology uses metal tag spectra that are based on mass and are detected as discrete peaks of similar intensity for similar protein expression levels (Figure 1B). Once antibodies of interest are conjugated to metal tags (usually lanthanides), they can be mixed together and applied onto a tissue in a way similar to the classical immunostaining protocol, followed by a signal detection using a high-resolution laser ablation system coupled to a mass cytometer (Figure 1C). As a result, dozens of antigens can be simultaneously evaluated on a single slide. Since rare-earth-metal isotopes used as tags do not normally occur *in vivo*, IMC images produce almost no background noise; therefore, the contrast between antigens of interest and the background is perfect for image analysis.

So far, IMC application in cardiovascular research has been very limited; nonetheless, it was used to address highly relevant scientific questions. In particular, IMC was used to identify new diagnostic targets of acute cardiac infarction [7], characterize stenotic aortic valve tissues [8], and analyze the metabolic flow in an ischemic heart [9]. These studies are all observational in nature, and it would be desirable to further apply IMC for dissecting signaling networks behind poorly studied CVDs such as, for example, Takotsubo syndrome (TTS). It remains unclear how emotional stress leads to myocardial toxicity in TTS. In some patients, TTS may lead to serious complications or even death [10]. To gain a deeper understanding of TTS at a molecular level, the in-depth IMC investigation of TTS-affected left ventricles would be a feasible approach. Another

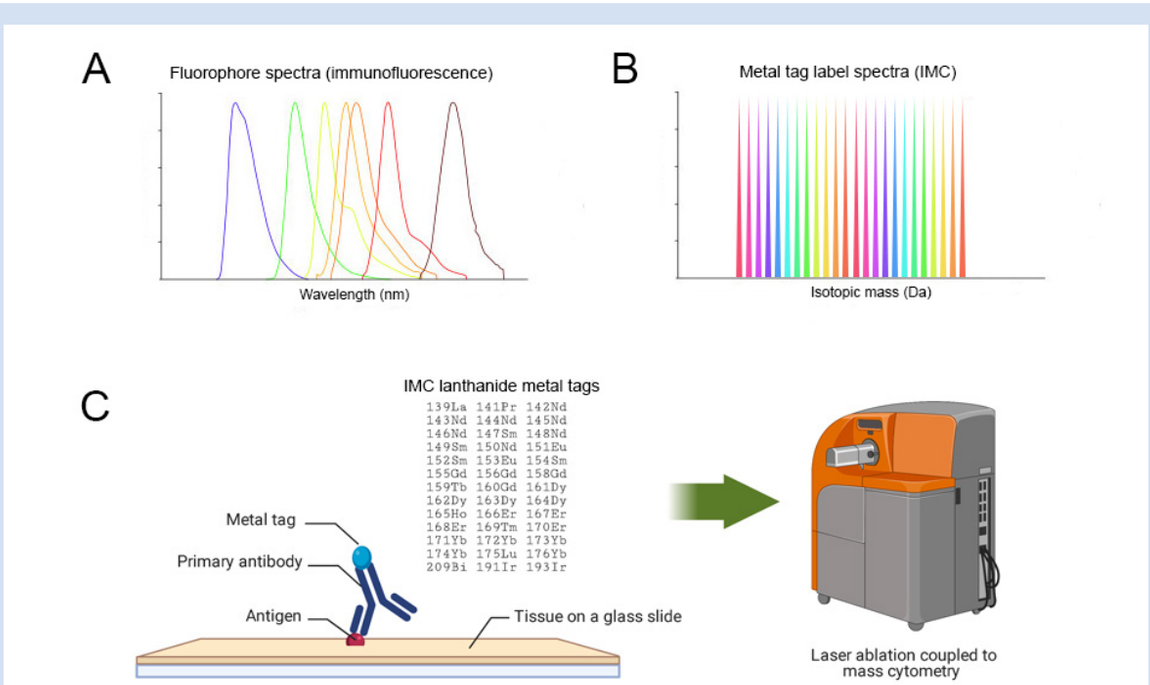

Figure 1. General principle of imaging mass cytometry. A. Typical emission spectra produced by fluorophores commonly used for immunostaining. Each peak corresponds to a fluorophore. Note a significant amount of overlap between the adjacent fluorophores' spectra. **B.** Isotopic mass spectra of metal tags used for imaging mass cytometry. Because each tag's molecular mass has a discrete value (rather than range), there is no overlap between dozens of tags used simultaneously. **C.** Schematic illustrating the principle of imaging mass cytometry and the list of 39 lanthanide metal tags commonly used for imaging mass cytometry. Created with BioRender.com (BioRender's Academic License).
Note: *IMC – imaging mass cytometry.*

example is Tetralogy of Fallot (TOF), a congenital heart defect with an unknown cause, characterized by right ventricular hypertrophy, ventricular septal defect, pulmonary stenosis, and overriding aorta [11]. Even though TOF can be treated surgically with a high rate of success, understanding molecular mechanisms of this disease at a cell level using IMC would greatly expand our knowledge about cardiovascular disorders.

Ribosome profiling

The emergence of ribonucleic acid (RNA) sequencing (RNA-seq) has revolutionized biomedical research [12]. This high-throughput method allows for counting of RNA molecules in a sample by quantifying the sequenced fragments per kilobase of the exon model per million mapped reads. RNA-seq quickly became a method of choice for those interested in understanding how particular conditions or treatments influence cells both *in vitro* and *in vivo*. Unfortunately, messenger ribonucleic acid (mRNA) concentrations do not always directly correlate with the concentration of a corresponding protein in a sample [13]. This could be due to multiple reasons, including that large amounts of mRNA are targeted for degradation and therefore never translated [14].

A more advanced RNA-seq method of ribosome profiling (Ribo-seq) represents a tool allowing to exclusively measure mRNA that is being translated at the moment [15]. The principle of Ribo-seq is the immobilization of ribosome-mRNA complexes followed by their isolation from cells. At the first step, cultured cells are pre-incubated with drugs binding to the ribosome E-site, thus blocking elongating ribosomes in their pre-translocation step. Ribosomes in the post-initiation stage can also be blocked if needed. Cells are further lysed in the presence of RNase I that digests all RNA outside the ribosome-mRNA complex (ribosomes protect mRNA from RNase I digestion). At the final step, ribosome-specific ribosomal ribonucleic acid (rRNA) is depleted and resultant mRNA fragments are converted into a deoxyribonucleic acid (DNA) library suitable for deep sequencing by a standard RNA-seq analysis protocol [16].

Ribo-seq provides an opportunity to analyze biologically meaningful transcriptomes highly relevant to a given experimental condition. In comparison with classical RNA-seq, the use of Ribo-seq may enable more accurate and reproducible results with less noise signals generally associated with irrelevant, non-translated transcripts. Cardiovascular research will greatly benefit from the widespread application of Ribo-seq technology, especially in settings where dynamic transcriptome changes in a short time period are important to gain new knowledge. For example, *in vitro* studies under flow conditions are considered a gold standard in the area of endothelial cell biology research [17–19]. However, the phenotype of endothelial cultured under flow is highly unstable owing to sensitivity to a multitude of factors such as time spent in the flow system, flow rate, cell confluency, etc. (Kutikhin, personal communications). Thus, studies under flow supplemented by Ribo-seq translatome analysis may allow the generation of more trustworthy and reproducible *in vitro* results.

Single-cell RNA sequencing

Due to the stochastic nature of biological processes, there is substantial cell-to-cell variability in the transcriptome of multicellular organisms [20]. Recent advances in microfluidics and nanotechnology have led to the boom in various single-cell sequencing techniques, including single-cell RNA sequencing (scRNA-seq). Profiling the transcriptome at a single-cell resolution has a number of advantages over classic approaches such as microarray or "bulk" RNA-seq. First, scRNA-seq reveals the diversity of phenotypes and cell states, which could provide novel insights about disease etiology or pathogenesis. Second, owing to the deconvolution of heterogeneous cell populations, scRNA-seq enables the identification of rare cell lineages within the tissue or organ of interest [21]. Third, multiple cell lineages derived from a single scRNA-seq experiment can be used to construct a trajectory inference (also called pseudotime), a quantitative measure describing how these distinct populations interact with each other in time [22].

Isolation of single cells for scRNA-seq can be achieved in a number of ways, including limiting dilution, micromanipulation, flow cytometry-activated cell sorting, microfluidic technology, negative magnetic separation, etc. These isolation methods have their own pros and cons; however, the comparison of these approaches is beyond the scope of this mini-review. Once live single cells are isolated, individual cells are lysed followed by the selection of polyadenylated mRNA molecules (e.g. mRNA) using poly[T]-primers. Thus, other RNA such as rRNA do not further proceed for sequencing. At the next step, RNA is reverse-transcribed into complementary DNA followed by its amplification by polymerase chain reaction. The resultant samples can be directly submitted for library preparation and can be further subjected to deep sequencing in accordance with standard RNA-seq protocols. During the data analysis step, single cells can be clustered into cell types and states, based on their unique gene expression signature. In addition, cells can be ordered along a predicted trajectory in pseudotime, based on computational reconstruction analysis (Figure 2). The limitation of scRNA-seq is its extremely high cost, even though it has dropped significantly in the last couple of years.

Similar to cytometry by time of flight, scRNA-seq can be used for in-depth analysis of different cell populations in a tissue. This approach could be

particularly useful to study atherosclerotic plaques that consist of multiple cell types, including endothelial cells, smooth muscle cells, infiltrating immune cells, etc. The advantage of scRNA-seq over cytometry by time of flight is that mRNA expression of each cell subpopulation can be studied in great detail. Therefore, scRNA-seq allows to study cell composition and additionally mRNA expression of each cell type. This method has proven immensely useful for a deep understanding of how the human body works both in norm and pathology; in particular, scRNAseq data is actively used to generate cardiovascular cell atlases [23].

Clustered regularly interspaced short palindromic repeats (CRISPR) functional screens

CRISPR-associated protein 9 (CRISPR-Cas9) is a gene editing technology established by 2020 Nobel Prize winners Emmanuelle Charpentier and Jennifer Doudna. The mechanism underlying CRISPR-Cas9 is a prokaryote defense system against viruses, whereby bacteria store viruses' DNA fragments in their genome in order to recognize and target these viruses during subsequent infections. During their lifespan, bacteria that survive viral infections integrate new spacers into their DNA [24]. These spacers contain DNA sequences identical to certain parts of the viral genome. If the virus happens to attack the bacteria again, the latter transcribes the aforementioned spacer sequence (precursor CRISPR RNA) that further undergoes maturation, eventually generating individual CRISPR RNA (crRNAs), each composed of a repeat portion and a virus-targeting spacer. The resultant crRNAs associate with effector complexes including Cas proteins, and crRNA guides the final complex to the virus DNA, where Cas endonucleases cut the invader's nucleic acid. Adaptation of this bacterial "adaptive immune response" machinery to the needs of gene engineering has led to the possibility of complete deletion of the gene of interest, or even replacing a gene with a customized DNA sequence [25].

For the purpose of genome editing, the bacterial system is greatly simplified and only contains two components; guide RNA (gRNA) of interest and Cas9 protein. Using the library of gRNAs, it is possible to knock out hundreds or thousands of genes in a single experiment in order to identify which genes have functional relevance in a particular *in vitro* or *in vivo* setting. In a typical experiment, a panel of gRNAs is delivered to a system in two ways: (i) positive screening, that is, selection of genes that accumulate in a population of cells due to survival advantage they confer; (ii) negative screening, which identifies genes or pathways that do not survive the selection by a drug or treatment and therefore critical for cell growth and survival. In principle, such CRISPR screens may be utilized to identify new molecular pathways associated with drug resistance and other settings. In cardiovascular research, CRISPR screening may be particularly useful for a better understanding of molecular mechanisms underlying early stages of atherosclerosis, specifically, the endothelial injury, which is a trigger of platelet aggregation and adhesion of leukocytes. Using *in vitro* models of endothelial injury, CRISPR screening may help identify genes or pathways involved in resistance to endothelial injury induced by turbulent blood flow [26] or calciprotein particles [27]. This, in turn, could lead to a better atherosclerosis prevention in the future, as subjects prone to endothelial injury or calciprotein particle formation may be subjected to tailored therapies such as potential ethylenediaminetetraacetic acid administration.

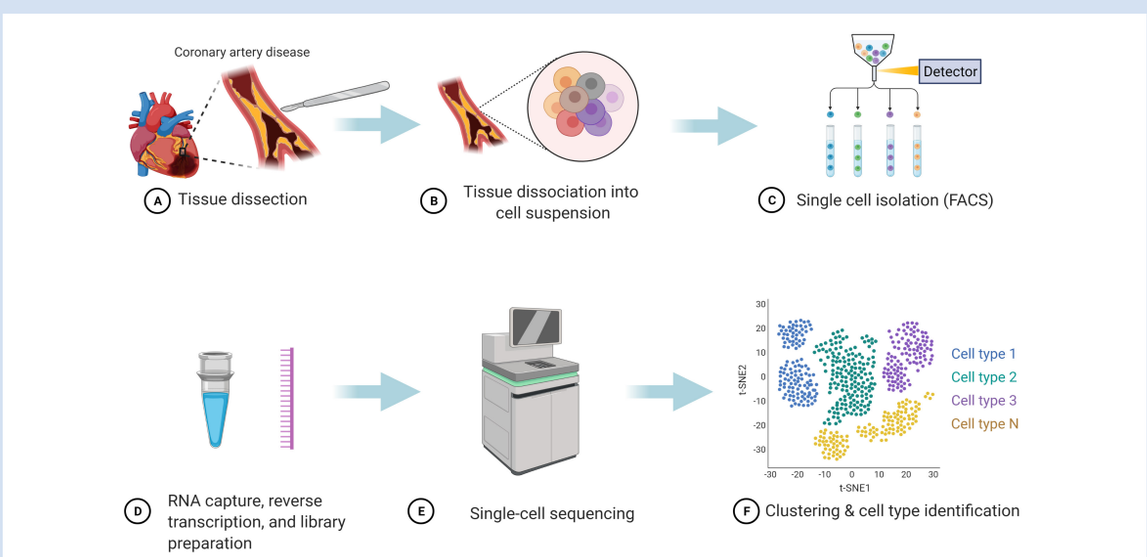

Figure 2. Single-cell RNA sequencing pipeline. Cartoon demonstrating a step-by-step protocol of single-cell RNA sequencing analysis of human atherosclerotic plaque excised from a diseased coronary artery. Adapted from "Single Cell Sequencing", by BioRender, June 2020. Retrieved from https://app.biorender.com/biorender-templates/t-5f2350bba193ae00b486136b-single-cell-sequencing. Copyright 2021 by BioRender (BioRender's Academic License).
Note: *FACS – fluorescence-activated cell sorting; RNA – ribonucleic acid.*

ATAC-sequencing

In a cell, physical compaction of DNA into nucleosomes is tightly regulated so that only certain regions of chromatin can be transcribed at a given moment; as such, only accessible parts of the chromatin are available to transcription factors, whereas closed chromatin cannot be transcribed due to tight packaging of nucleosomes. Therefore, by defining the epigenetic landscape of living cells, chromatin accessibility acts as a major regulator of cell behavior. Transposase-accessible chromatin with sequencing (ATAC-Seq) is a recently developed method for epigenetic profiling of open chromatin. In comparison with previously developed similar methods, ATAC-Seq offers a faster experiment with a lower number of cells needed for analysis.

The principle of ATAC-Seq is specific labeling of accessible chromatin by hyperactive Tn5 Transposase, an enzyme that integrates certain sequences called next generation sequencing adapters into open chromatin DNA, whereas closed chromatin remains untagged [28]. The resulting tagged DNA can be further amplified by polymerase chain reaction followed by next generation sequencing. Sample preparation for ATAC-Seq takes several hours; in contrast, other similar methods such as formaldehyde-assisted isolation of regulatory elements and sequencing of DNase I hypersensitive sites require 3–4 days of bench work and lead to extensive washout of samples. Thus, ATAC-Seq is a user-friendly method enabling high-throughput open chromatin readout without major sample loss. The emergence of combinatorial cellular indexing methods enabled to perform ATAC-Seq at a single-cell level; for this, DNA barcoding of individual cells is required before ATAC-Seq sample preparation [29].

Development of CVDs is frequently accompanied by substantial epigenetic changes; for example, human atherosclerotic lesions display strong hypomethylation of the imprinted chromatin locus 14q32 [30], while certain epigenetic programs are activated during atrial fibrillation or heart failure [31]. Nonetheless, mechanisms of transcriptional regulation remain poorly studied. Wide-spread application of ATAC-Seq technologies in the field of CVD research will greatly advance the understanding of gene expression control during disease pathogenesis.

Conclusion

Emergence of new powerful technologies provide an outstanding opportunity for exploration of CVD's pathophysiology at both cellular and molecular levels. Approaches reviewed in this paper will undoubtedly advance our understanding of CVDs and will uncover new horizons in treatment.

Conflicts of interest

A. Yuzhalin declares no conflict of interest related to this article.

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