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Evaluation of microRNAs by molecular imaging for novel diagnostics and therapeutics

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Abstract

During the past decade, microRNAs (miRNAs) have emerged both as sensitive biomarkers and promising therapeutic targets in a variety of pathophysiological processes, such as coronary artery disease, heart failure, metabolic disorders, and carcinogenesis. With conventional methods like northern blotting, real-time polymerase chain reaction (PCR), microarrays, and next-generation sequencing representing the mainstay in assessing miRNA expression, the introduction of sensitive and dynamic in vivo imaging capabilities would open avenues both for diagnostic and therapeutic purposes. Hence, recent techniques exploit fluorescent proteins, bioluminescent enzymes, molecular beacons, as well as various nanoparticles for monitoring miRNA function. In this review, we provide an overview of current state-of-the-art imaging techniques for miRNAs and point out their vital contribution to novel and more effective treatment strategies in the future. Even if we run the risk of facing some pitfalls on the long road ahead of us, the concept of miRNAs as novel diagnostic and therapeutic options remains alluring. ■ *Heart Metab*. 2014;65:15-20

Keywords: biomarker; cardiovascular disease; microRNA; molecular imaging; therapeutic target

MicroRNAs as potential biomarkers and therapies for human pathologies

The transcriptional modulation by microRNAs (miRNAs) plays a fundamental role in a variety of cardiac diseases, such as myocardial infarction, hypertrophy, and heart failure; peripheral artery disease; metabolic disorders, such as diabetes mellitus; as well as in the regulatory processes to maintain the cellular homeostasis of cell differentiation, proliferation, and apoptosis.[1-7] Importantly, miRNAs cannot only be detected in the cytoplasm of involved cell types, but also in noncellular compartments such as plasma, sputum, or urine where they remain remarkably stable.[8] Transportation of miRNAs occurs either freely, bound to proteins or lipids, or via incorporation into microvesicles or exosomes.[6] Besides the various properties of these particles, it is now crucial to not only characterize miRNAs, but also their packaging, eg, in terms of morphology.[9] Several techniques have been developed in order to reliably measure exosome size, shape, and dispersity. Among these, nanoparticle tracking analysis (NTA) is a newly developed light-scattering technique for the rapid sizing

Abbreviations

miRNA: microRNA; **NGS:** next generation sequencing; **NTA:** nanoparticle tracking analysis; **qRT-PCR:** quantitative reverse transcription polymerase chain reaction

and evaluation of extracellular vesicle concentration *(Figure 1).*[10] In this technique, microvesicles or exosomes are illuminated through a passing laser beam, with each individual particle scattering the light into the field of view of a microscope and onto the image sensor of a video camera.[10]

One of the basic principles in order to take advantage of the therapeutic potential of miRNAs is based on identifying distinctly dysregulated miRNAs in cardiac diseases; this is being extensively studied in cellular models, in various small and large animal models, and ultimately in humans. Current work includes identification of miRNA expression profiles in neonatal mouse hearts by deep sequencing,[11] study of the cargo system of circulating miRNAs in the pathophysiology of cardiac hypertrophy,[12] and reprogramming of cardiac fibroblasts toward a cardiomyocyte lineage via a single transient transfection of certain miRNAs.[13] The latter idea evolved to functional screening of the whole human miRNome, which led to the identification of miR-25 as a suppressor for intracellular calcium handling in heart muscle. Hence, high-throughput functional screening of the whole human miRNome helps identify novel targets able to restore cardiac contractility.[14]

Common to all techniques for miRNA evaluation is the development toward faster turnover of even smaller amounts of material, a higher dynamic range, and meeting excellent sensitivity and specificity criteria. Emphasis is laid on new imaging techniques enabling sensitive and nondestructive detection of miRNA expression and activity, combined with high dynamic and local resolution properties.[15]

Assessment of microRNAs by molecular imaging

Despite their huge potential, miRNAs are still in the early translational process toward clinical application. A recent publication on the role of miRNA-30c showed that transgenic mice overexpressing cardiac miRNA-30c developed a severe form of dilated cardiomyopathy.[16] This underlines that misbalanced expression of only a single miRNA can lead to a series of downstream molecular changes and severe organ dysfunction.[17] Yet our knowledge of possible harmful and off-target effects of miRNAs remains very scarce. Hence, it is crucial to be able to monitor miRNAs efficiently and reliably to further comprehend their function in regulatory pathways.

Over the years, advances have led to sophisticated assays *(Figure 2)* characterized by their ability not only to capture a single biological response, but also to meet diagnostic-grade standards of sensitivity, versatility, and fidelity. As such, miRNA expression levels can be measured at the benchside by a host of methods, eg, quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarray analysis, northern blots, and in situ hybridization. In addition, assays based on firefly luciferase, isolated from the North-American firefly *Photinus pyralis*, rank among the

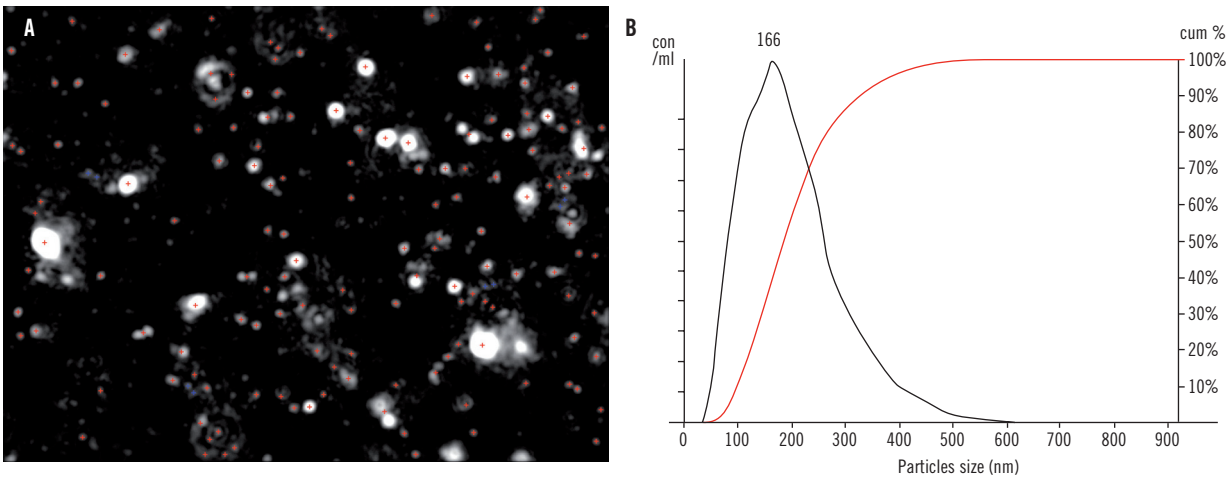

Fig. 1 *Exosome nanoparticle tracking (NTA). NTA is a light-scattering technique for rapid sizing and evaluation of extracellular vesicle concentrations, eg, of exosomes, which are visualized in* ***Panel A****. Distribution of the calculated vesicle size is shown in* ***Panel B****. Image and graph courtesy of Christian Harz, NanoSight Ltd.*
Abbreviations: *con/ml, concentration per mL; cum %, cumulative percentage.*

more and more extensively used methods due to its wide dynamic range of measurable activity. Today, it represents only a small step from analysis of a single miRNA to quantification of whole miRNomes via microarrays. Finally, the revolution of next-generation sequencing (NGS) techniques made comprehensive profiling of miRNAs possible even in a clinical setting, due to excellent detection sensitivity, a large dynamic range of detection, and high accuracy in differential expression analysis. Furthermore, NGS allows sequence motif–independent detection of completely novel miRNAs[18-20] or miRNA modifications. It was not until recently that NGS made it possible to investigate the human cardiac miRNome by quantification of miRNA expression in healthy and diseased human heart.[21] Leptidis et al[21] discovered more than 800 miRNAs expressed in the human heart, with more than 250 differentially expressed in human dilated or hypertrophic cardiomyopathy, making them potential therapeutic targets.

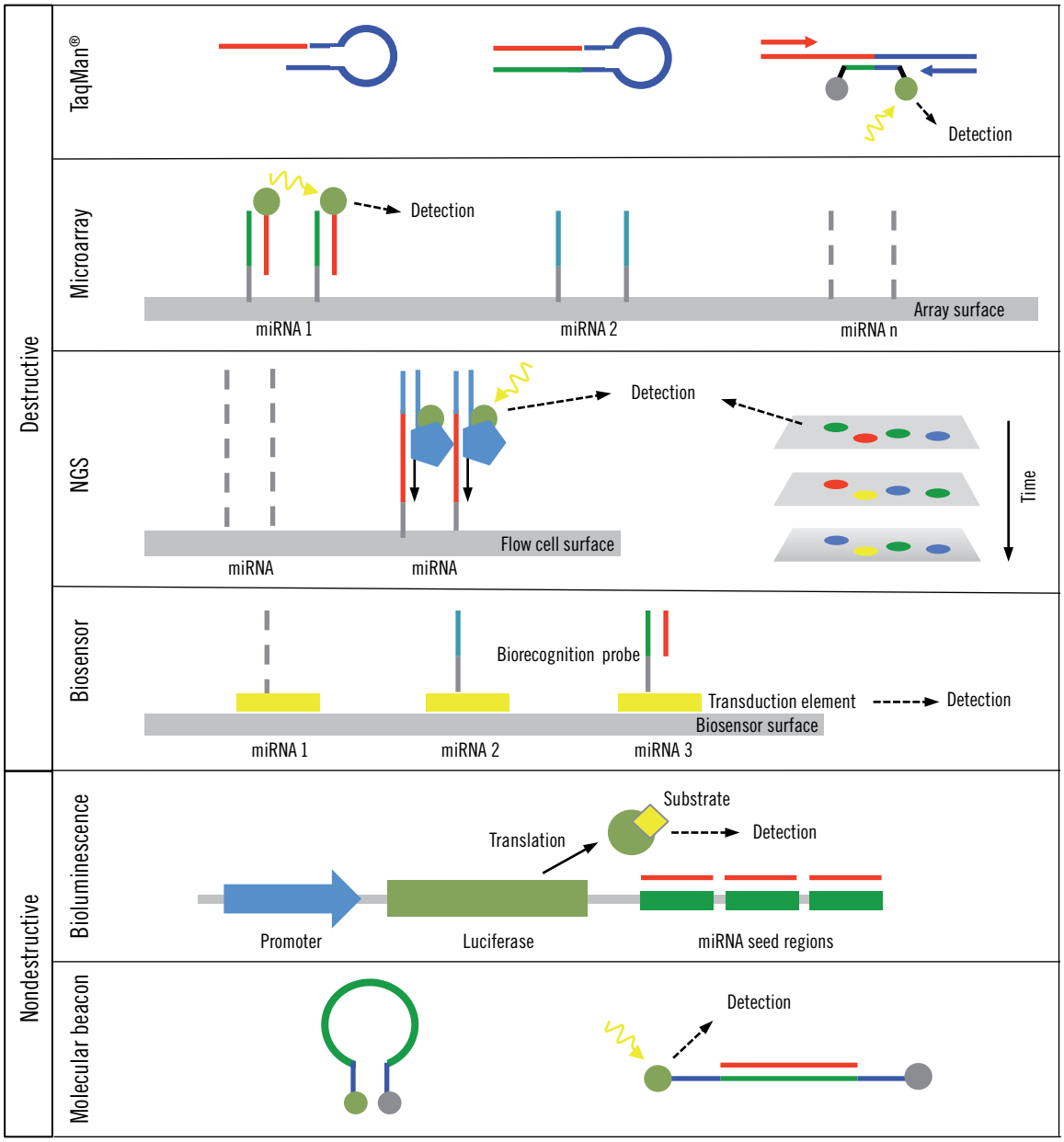

Fig. 2 *Comparison of destructive and nondestructive imaging techniques for monitoring miRNAs. miRNA expression levels can be measured with a variety of destructive methods, which means that the tissue or cell has to be lysed. Shown are real-time polymerase chain reaction, microarray, NGS, and biosensors. Fluorescence- and bioluminescence-based imaging methods and molecular beacons are representatives for nondestructive assays. Bioluminescence-based imaging depends on enzymatic reactions between luciferase and its substrate, whereas a molecular beacon employs a single-stranded and stem-looped DNA oligonucleotide complementary to its target miRNA.*
Abbreviations: *miRNA, microRNA; NGS, next-generation sequencing.*

Among the major disadvantages of the aforementioned methods is their "invasiveness," requiring the destruction of the target tissue, which hampers dynamic analysis and in vivo detection of miRNAs.[15,22] Moreover, amplification of unidentified poor-quality RNA in the initial phase of the expression profiling may yield poor-quality data and can be costly, laborious, and time consuming.[23] Thus, for example, in the field of oncology, molecular profiling of cancers has led to the development of second-generation assays based on custom-designed microarrays. Clinically applicable assays must be highly reproducible, sensitive, and specific, as well as having a comparably high dynamic range.[24] The potential implementation of a miRNA signature as a biomarker for cardiovascular diseases may not only enable patients with a high cardiovascular mortality risk to be distinguished from those at low risk, but also helps to predict poor clinical outcomes in patients suffering, eg, from heart failure.[25-27] However, current techniques using target amplification are relatively time consuming, which requires the development of assays with faster turnaround times of about 20 min, so that they are suited also for the diagnosis of acute coronary syndromes. An increasing number of studies concentrate on amplification-free, eg, biosensor-based techniques for miRNA assays, which combine a selective molecular probe with a highly sensitive transducer for rapid and extremely sensitive detection at attomolar to femtomolar levels. When compared with classic biomolecular approaches, biosensor-based techniques excel in terms of measurement reliability, reduced time-to-results, and sample preparation-free protocols,[28] together with disadvantages due to target degradation, sequence similarities, as well as variable melting temperatures.

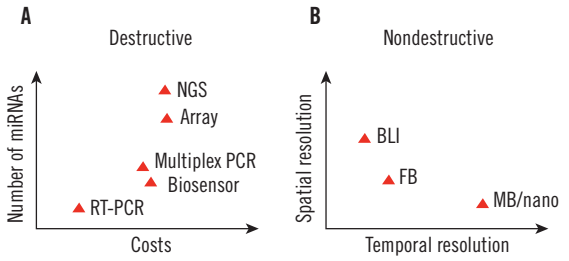

Fig. 3 *Overview of current state-of-the-art imaging techniques.* ***A.*** *Comparison of conventional invasive methods, such as qRT-PCR, multiplex PCR, microarrays, NGS, and biosensor-related techniques according to their costs and number of quantification features.* ***B.*** *Comparison of novel noninvasive technologies, such as fluorescent protein-based optical imaging (FB), bioluminescence-imaging methods (BLI), molecular beacon (MB), and nanoparticles (nano) according to their spatial and temporal resolution.*
Abbreviations: *NGS, next-generation sequencing; qRT-PCR, quantitative reverse transcription polymerase chain reaction*

In order to overcome some of the obstacles, noninvasive methods of repetitive imaging are needed for proper in vivo assessment of miRNA expression patterns. These currently comprise fluorescent proteins, bioluminescent enzymes, molecular beacons, as well as various nanoparticles, enabling visualization of miRNA biological processes in living individuals *(Figure 3)*.[15]

Back in 1962, the discovery of green fluorescent protein (GFP) in the *Aequorea victoria* jellyfish paved the way for the development of a variety of fluorescent proteins with unique emission wavelengths suitable for visualizing specific molecules within cells. As such, the DNA fragment encoding the fluorescent protein is fused to repetitive miRNA seed-targeting sites complementary to the specific miRNA.[29] In 2009, Ko et al were able to establish a miRNA reporter gene imaging system to monitor miR-124a during neurogenesis, comprising a reporter gene vector, cell transfection, in vitro luciferase assay, and in vivo bioluminescence imaging of miRNAs.[22] The group around Kato created a two-color fluorescence imaging system using a proviral vector, which allowed the more precise evaluation of miR-133 activity during myogenesis.[30] Such fluorescence-based imaging approaches allow easy and noncytotoxic expression. However, despite appealing in vitro results, there are several in vivo drawbacks, including high autofluorescence from the background as well as poor tissue penetration of the excitation light with subsequent low signal-to-noise ratios.[23,31]

Bioluminescence imaging–based methods (BLI), based on enzymatic reactions between luciferase and its substrate, provide an alternative to fluorescence imaging.[32] Besides increased sensitivity and cost effectiveness, BLI are able to overcome the much-dreaded background signal issue.[33] Tu and coworkers engineered a dual-luciferase reporter system for quantitative imaging of miR-22 in isoproterenol-induced cardiac hypertrophy for repetitive and noninvasive monitoring of dynamic changes of miR-22 expression in a real-time manner.[34] Moreover, the constructed miRNA reporter system showed that knockdown of miR-22 by antagomiR-22 is capable of silencing cardiac endogenous miR-22 expression, resulting in partial inhibition of isoproterenol-induced cardiac hypertrophy. Concerns from scientists regarding BLI methods involve decreased signal intensity in the presence of a functional miRNA as a result of the destabilized transcriptional activity of its target mRNA.[15]

A different approach to direct in vivo imaging involves molecular beacons and nanoparticles. A molecular beacon consists of a single-stranded stem-loop DNA oligonucleotide complementary to its target miRNA, and represents a simple, fast, cost-effective, and specific method for monitoring intracellular miRNAs in living cells.[35] For instance, Kang et al were able to successfully image the biogenesis of miR-206 and miR-26a during myogenesis both in vitro and in vivo by means of a molecular beacon.[36] Moreover, Baker et al could demonstrate that molecular beacons are suitable for distinguishing mature from precursor miRNAs, and therefore capable of reliably quantifying miRNA expression.[37] Limitations of molecular beacons and nanoparticles include their largely unknown toxicity, their inhomogeneous distribution in the body and tissues, and the possible requirement of a vectorization agent.[38]

To date, the vast majority of miRNA imaging strategies based on fluorescent proteins, BLI, molecular beacons, or nanoparticles is still in its infancy. All the endeavors undertaken to image intracellular targets remain more burdensome than imaging cell surface targets.

Conclusion

As every single imaging modality struggles with its own shortcomings, it has become increasingly important to find ways to combine the results of different methods in a multimodality imaging approach or optimize assays for a distinct target miRNA application. Special emphasis has to be laid on developing efficient in vivo monitoring of proper miRNA function and controlling downstream physiological effects in order to fully exploit the diagnostic and therapeutic value of miRNAs. ■

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