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Molecular Imaging Strategies for *In Vivo* Tracking of MicroRNAs: a Comprehensive Review

Reinier Hernandez¹, Hakan Orbay², and Weibo Cai^{1,2,3,*}

¹Department of Medical Physics, University of Wisconsin - Madison, WI, USA

²Department of Radiology, University of Wisconsin - Madison, WI, USA

³University of Wisconsin Carbone Cancer Center, Madison, WI, USA

Abstract

MicroRNAs (miRNAs) are single-stranded non-coding RNAs of ~22 nucleotides, which can negatively regulate gene expression through induction of mRNA degradation and/or post-transcriptional gene silencing. MiRNAs are key factors in the regulation of many biological processes such as cell proliferation, differentiation, and death. Since miRNAs are known to be in close association with cancer development, non-invasive imaging of miRNA expression and/or activity is of critical importance, for which conventional molecular biology techniques are not suitable or applicable. Over the last several years, various molecular imaging techniques have been investigated for imaging of miRNAs. In this review article, we summarize the current state-of-the-art imaging of miRNAs, which are typically based on fluorescent proteins, bioluminescent enzymes, molecular beacons, and/or various nanoparticles. Non-invasive imaging of miRNA expression and/or biological activity is still at its infancy. Future research on more clinically relevant, non-toxic techniques is required to move the field of miRNA imaging into clinical applications. Non-invasive imaging of miRNA is an invaluable method that can not only significantly advance our understandings of a wide range of human diseases, but also lead to new and more effective treatment strategies for these diseases.

Keywords

microRNA (miRNA); bioluminescence imaging (BLI); fluorescence imaging; magnetic resonance imaging (MRI); molecular imaging; cancer; nanomedicine

INTRODUCTION

Mature microRNAs (miRNAs) are single-stranded, non-coding RNAs of ~22 nucleotides that play critical roles in gene regulation in both plants and animals [1,2]. MiRNA can negatively regulate its target through two distinctive ways, based on the levels of complementarity between the miRNA and its target. The first mechanism requires perfect or near perfect base pairing of the miRNA to messenger RNAs (mRNAs), and the resulting partial RNA double strand can induce an RNA degradation process called interference (RNAi) [2-5]. This RNAi process is triggered by the formation of miRNA-induced silencing complex (miRISC) that involves several ribonucleases, which can cleave and degrade the target mRNA.

*Corresponding author: Departments of Radiology and Medical Physics, University of Wisconsin - Madison, Room 7137, 1111 Highland Ave, Madison, WI 53705-2275, USA Fax: 1-608-265-0614; Tel: 1-608-262-1749; wcai@uwhealth.org. .

CONFLICT OF INTEREST None.

Although this mechanism has been shown to occur in animals, a second process known as post-transcriptional gene silencing which does not involve degradation of the mRNA targets, seems to be dominant [6,7]. This process also involves the formation of miRISC complexes, however with several major differences that include imperfect matching of the miRNA-mRNA sequences and binding of the miRISC complex to the 3' untranslated regions on the mRNA target. The end result of this regulatory pathway is a translational repression of the target mRNA, which reduces the cellular levels of protein expression without affecting the levels of its respective mRNA [8-11].

Both of these processes require the presence of mature miRNAs, which is generated in a complex protein system that includes members of the Argonaute family and RNase III-type endonucleases (e.g. Drosha and Dicer) [12,13]. MiRNA genesis starts with the transcription of several kilobase long primary RNAs (pri-miRNAs) from intergenic regions by RNA polymerase II [14-16]. Subsequently, the pri-miRNA is processed by Drosha to form hairpin structures of ~ 70 nucleotides in size called pre-miRNA that is posteriorly transported to the cytoplasm by Exportin 5 [17]. Of note, the same pri-miRNA precursor has been found to produce several different pre-miRNAs. Once in the cytoplasm, the pre-miRNAs are excised into ~22 nucleotide miRNA duplexes by the RNase III Dicer, which subsequently unwinds it to render a principal mature miRNA strand and a complementary miRNA* strand [18-23]. The principal strand is selectively incorporated into the miRISC, whereas the miRNA* is generally believed to be rapidly degraded [24,25].

The biological roles of miRNAs have only been identified to a certain extent, however they have been reported to be key players in the regulation of several biological processes such as cell proliferation, cell cycle, differentiation, and death [2,17,26-30]. According the latest release of the miRNA database (<http://www.mirbase.org>), the number of human mature miRNAs reported to date has >2000 sequences, derived from ~1600 precursors [31-34]. Since ~50% of annotated human miRNA genes are located in “fragile” areas of the genome [35], known for their association with cancer, it is not surprising that miRNA activity has been correlated with cancer. In fact, recent genome-wide profiling of various types of cancer displayed alterations in the miRNA expression profiles of the tumor cells as compared to normal tissues, showing both down-regulated and over-expressed miRNAs [13,36-41]. The aberrant expression patterns of several miRNAs (e.g. miR-21) were found to be highly conserved among many cancer types, suggesting the existence of a fundamental signaling pathway that can be deregulated by miRNAs [38,42]. Oncogenic and tumor suppressor activities have been identified for several of those deregulated miRNAs, indicating that the same miRNA can affect multiple targets and show opposing effects depending on the cell lineage. For example, miRNAs from the cluster miR-17-92 showed tumor suppressor activity in human B-cell line and oncogenic properties in B-cell lymphomas through different interactions with the oncogene MYC [13,43].

Given the involvement of miRNAs on aforementioned cellular processes and associated diseases, the development of sensitive and robust methods for miRNA detection is imperative for potential applications of miRNAs as biomarkers in diagnostics, therapeutic agents in gene therapy, among others. Currently, conventional molecular biology techniques such as Northern blotting, microarrays analysis, or reverse transcriptase-PCR (RT-PCR) have served as the gold standard for measuring cellular levels of miRNAs [37,40,44,45]. However, these methods can be time-consuming and expensive. In addition, they also require cell fixation and/or lysis which make them unsuitable for dynamic analysis of expression level or *in vivo* detection of miRNAs. Over the last decade, the field of molecular imaging has flourished and many imaging techniques have witnessed tremendous technical advances and successful clinical translation [46-53]. Many of these imaging techniques have been investigated for sensitive and non-destructive detection of miRNA expression/activity,

including fluorescence imaging, bioluminescence imaging (BLI), and magnetic resonance imaging (MRI) [54]. In this review article, we present a comprehensive summary of the state-of-the-art for imaging of miRNAs in living cells and in animal models, which has shed invaluable biological insights on the diverse biological functions of miRNAs.

IMAGING MIRNAS WITH FLUORESCENT PROTEIN-BASED SYSTEMS

The discovery and continuous optimization of the green fluorescence protein (GFP) and other fluorescent proteins (FPs) has undoubtedly revolutionized the field of biomedical research, where expression of these FPs was used as fluorescent marker for gene activity, protein expression, subcellular components, cell status/fate, among others [55-58]. The numerous engineered variants of these FPs have also allowed scientists to scrutinize multiple cellular processes simultaneously [55,57,59,60].

Taking advantage of the replicative machinery of cells to express FPs, several miRNA-sensitive fluorescent reporters have been constructed by fusing the FP gene with several cDNA copies of specific miRNA target sequences. The presence of cDNA copies, typically located at the 3' untranslated region (3' UTR) of the FP gene, can sensitize the expression of FPs to the endogenous cellular levels of miRNA. In a pioneering study, this approach was adopted to investigate the differential miRNA expression profiles of hematopoietic cell lineages versus non-hematopoietic cells [61]. Several miRNA regulated lentiviral vectors were constructed by the insertion of four consecutive copies of a 23 base pair sequence that perfectly complemented one of three miRNA targets (miRTs) into the 3'UTR of a GFP expression cassette, driven by the ubiquitously expressed phosphoglycerate kinase promoter (Fig. (1A)). It was found that suppression of GFP expression corroborated the effectiveness of gene transfer, which provided new evidence for the robustness of miRNA regulation. The most significant differential regulation was observed for miR-142-3p, which led to >100 fold difference in GFP expression between hematopoietic cells and non-hematopoietic cells.

Subsequently, further validation of this proof-of-principle was carried out, where several miRTs were integrated into vectors that could rapidly adjust to variation on target expression levels, demonstrating the versatility of this strategy for detecting multiple endogenous miRNAs [62]. The vector was designed to express two transgenes, a GFP and a low affinity nerve growth factor receptor (NGFR). Insertion of the miRT seed sequence into the untranslated region of GFP allowed for miRNA regulation of the fluorescence signal, whereas NGFR served as a control.

In one report, combination of miRNA modulation and cytotoxic chemotherapy was shown to be effective in treating gliomas that express miR-21, and decreased levels of miR-21 was associated with increased activity of caspases [42]. Based on these findings, bimodal imaging vectors were synthesized which contain locked nucleic acid (LNA) that target miR-21, an IRES-GFP cassette, and the cDNA of an apoptosis-inducing ligand, all under a cytomegalovirus (CMV) promoter [63]. *In vitro* and *in vivo* imaging of glioma cells demonstrated the feasibility of this approach, where a decrease in cell viability and eradication of tumors in mice was observed after transfection of the viral construct.

A retroviral vector system was developed for elucidating the role of miR-133 in the proliferation of myoblasts during myogenesis [64]. Two FPs of different colors were used to monitor miR-133 activity in living cells, where a GFP reporter was fused to three copies of the target sequence of miR-133 at the 3'UTR, followed by a red fluorescent protein (RFP) gene that is independently expressed under a CMV promoter (not targeted by miR-133). The functional expression of miR-133 during myogenesis of C2C12 myoblast cells inhibited the expression of GFP, without affecting the translation of RFP (Fig. (1B)). The integration of RFP within the vector permitted internal normalization of the GFP signal, which accounted

for the fluctuations in viral expression due to random integration of the retrovirus into the cellular genome. Other miRNAs were successfully detected with this retroviral system, demonstrating that it can be a useful tool for studying various miRNA-related cellular processes.

Using a similar strategy, the expression of miRNA derived from adenovirus non-coding RNAs was also investigated [65], where a divergent transcription system was created to allow for coordinated and robust expression of two genes. An HIV-1 based lentiviral vector was engineered to provide two independent transcripts, one for RFP in the forward direction and the other for GFP in the reverse direction relative to the vector long terminal repeat. To monitor the expression of adenovirus-derived miRNAs, complementary sequences to the miRNAs were introduced between the GFP gene and a polyadenine chain. The expression of the viral RNA VA-I in cells infected with the adenovirus was successfully visualized and quantified by the reduction in GFP signal.

IMAGING MIRNAS WITH BLI

Despite the many advantages of FP-based systems, such as relatively easy and non-cytotoxic expression, this technique suffers from several drawbacks for *in vivo* imaging. Low signal to noise (S/N) ratios due to poor tissue penetration of the excitation light and, more importantly, tissue autofluorescence has restricted the utility of most FP-based systems to imaging of cultured cells or superficial tissues [56-58]. Although much effort has been devoted to overcome these issues, such as the generation of infrared FPs [66], no significant improvement has been reported to date for imaging of miRNAs with fluorescence techniques.

A convenient alternative to fluorescence imaging is BLI, which is much more sensitive for *in vivo* imaging applications due to the lack of background bioluminescence signal [67,68]. Isolated from a wide variety of insects, marine organisms, and bacteria, luciferases are photoenzymes that emit light upon interaction with a specific substrate. Firefly luciferase (Fluc), *Renilla Reniformis* luciferase (Rluc), and *Gaussian Princeps* luciferase (Gluc) are among the most common luciferases used in bioluminescence reporter systems. These luciferases differ in their optical properties: Fluc emits light at ~560 nm whereas the latter two emit light at ~480 nm. In general, Fluc is more desirable for *in vivo* imaging than Rluc and Gluc, because of better tissue penetration of photons at longer wavelengths. However, the high intensity of light emitted by Gluc as well as its small size and heat stability makes it more suitable for constructing clones and cell transfection. Specificity of these enzymes for their substrates, D-luciferin for Fluc and coelenterazine for Gluc and Rluc, is a common trait for all luciferases. Therefore, background luminescence signal is negligible, which can lead to excellent S/N ratios and sensitivity for BLI. Most of the experimental approaches for luciferase-based imaging of miRNAs resemble those FP-based systems described above.

One of the earliest efforts that described the use of luciferase reporters for the detection of miRNA activity was undertaken by Cissel *et al* [69], in which a hybridization assay was developed to detect miR-21 in breast cancer cells using Rluc as the reporter. Based on competitive binding between free miR-21 and Rluc-labeled miR-21 to an immobilized complementary DNA sequence, detection sensitivity in the order of 1 fM was obtained with the optimized assay, which was subsequently used to determine the miR-21 expression level in various cell lines. The advantages of this assay include the relatively short time required to complete the assay (1.5 h), as well as the ability to perform the assay directly on cell extracts without previous PCR amplification. In another study, gene regulation in papillary thyroid carcinoma by miR-221 was investigated using a combination of microarray analysis and a bioluminescent Gluc reporter system that is regulated by miR-221 [70]. Several

groups of mice were implanted with each of the Gluc expressing cell lines, and their responses to exogenous/endogenous miR-221 were successfully assessed with BLI.

In an interesting report, miRNA genesis and action was monitored with a dual luciferase reporter approach [71]. A miR-23-P639/Fluc plasmid carrying the Fluc sequence under the miR-23a promoter was used to determine the transcriptional levels of the primary miR-23a. In the second probe, a CMV/Gluc/3×PT-miR-23 that contains three copies of the miR-23a homing sequence on the 3' UTR region of the Gluc gene was used to interrogate the targeting activity to mature miR-23a. The *in vitro/in vivo* miR-23a activity levels of three different cell lines were analyzed using this dual reporter system, where a high Fluc bioluminescence signal was indicative of high expression level of the primary miR-23a and consequently a decrease of the Gluc signal. However, such a correlation between the Fluc and Gluc signal was not observed in some cases, which was attributed to alterations on the post-transcriptional processing of the pri-miRNA.

As mentioned earlier, the 3' end of pre-miRNA is generally considered to be the more stable strand of the duplex, which is transformed into mature miRNA [72]. To validate this aspect, one study was designed to investigate the unequal expression of the mature 3' end miRNA and its complementary 5' end strand using a luciferase reporter gene [73]. The expression levels of miR-9 and miR-9* were analyzed during retinoic acid-induced neuronal differentiation of P19 cells, and higher expression of miR-9 compared to miR-9* was observed based on BLI. Using a similar reporter system, the repression of chromosome 14 open reading frame, mediated by miR-124a during neuronal differentiation, was studied in P19 cells in another report [74]. Different expression level of miR-124a during proliferation or neurogenesis was observed.

Taking advantage of the selective expression of certain miRNAs in different tissues, Cawood *et al.* engineered the wild type DNA adenovirus 5 (Ad5, which can have significant toxicity in mice) to elicit little effect in hepatocytes, due to selective expression of miR-122 in these cells (Fig. (2)) [75]. The virus was constructed by fusing various repeats of the miR-122 binding sites within the 3' UTR of an E1A transcription cassette, to which a luciferase reporter was added after the E1A promoter for BLI. An 80 fold decrease in the *in vivo* hepatic expression of the virus was observed on the viruses bearing the miR-122 binding sites, when compared to the control, and liver toxicity was virtually abrogated. The potency of these viruses was also tested in several other cell lines, which exhibited significant viral toxicity since these cells do not express miR-122. These findings suggested that the use of a luciferase reporter together with viral engineering can be useful for future development of viral vaccines with attenuated effects in certain target tissues. In another report, a hypoxia-responsive promoter was incorporated into the viral vector, which could lead to higher levels of viral expression since tumor microenvironment is often hypoxic [76].

In a recent report, the importance of miR-26a in the regulation of hepatocyte proliferation and hence liver regeneration was investigated [77]. Adult male C57BL/6J mice undergone 70% liver resection were treated with three different adenovirus-luciferase vectors containing a miR-26a sequence, an anti-miR-26a seed, or a control. Subsequent *in vivo* BLI during liver regeneration revealed that the strongest signal was observed in mice treated with the anti-miR-26a vector, indicating a marked repression of miR-26a in hepatocytes which was validated by RT-PCR and Western blotting. Cell cycle proteins such as CCND2 and CCNE2, which were upregulated in actively proliferating cells, were identified as possible targets for miR-26a.

In another interesting study, the enhanced survival of miRNA treated cardiac progenitor cells (CPC) was evaluated using BLI after intramuscular and intramyocardial implantation [78]. The CPCs were treated with a mixture of lentiviruses encoding miR-21, miR-24, and miR-221 before injection into mice. The constitutive expression of Fluc in these CPCs enabled convenient *in vivo* tracking by BLI. Comparison of the optical signal corresponding to miRNA-treated CPCs to non-treated control showed an enhanced engraftment and survival of the former, which was suggested to be due to the inhibition of the apoptotic activator gene Bcl2111 Bim by the miRNAs.

IMAGING MIRNAS WITH NANOPARTICLE- OR MOLECULAR BEACON-BASED SYSTEMS

Different from the imaging strategies described above, where miRNA interaction with the probes typically resulted in a reduction of fluorescence or bioluminescence signal, the imaging systems described below generally offer a more sensitive and “positive contrast” readout. These systems can provide several advantages over the abovementioned approaches. For example, the use of molecular beacon (MB) with emission peak at longer wavelength can lead to better tissue penetration of signal and in the meantime keep the background signal low. Magnetic resonance imaging (MRI) has also been employed for imaging of miRNAs, where magnetic nanoparticles were used to provide a non-invasive image with better anatomical information. Multimodality systems that combine different imaging techniques have also gained popularity over the last several years, since they can provide complementary information superior than either modality alone can offer [50,52,79-82], which has also been investigated for imaging of miRNAs (e.g. optical and MRI).

To image mature miRNA expression patterns *in vivo*, a miR-124a sensitive nanoprobe was constructed using a rhodamine-coated cobalt ferrite magnetic fluorescent nanoparticle as the scaffold [83]. The nanoparticle was subsequently functionalized with a partially double-stranded oligonucleotide that contained a miR-124a seed sequence in one of the strands, and a quenching moiety in the complementary strand, which resulted in ~80% quenching of fluorescence signal when compared to the unconjugated nanoparticle. Upon exposure to mature miR-124a which binds to its complementary seed sequence, the quenching sequence was displaced to yield the probe in an “on” state with minimal fluorescence quenching. This system was successfully used to monitor miR-124a expression during induced neuronal differentiation in P19 cells, which exhibited an enhanced fluorescence signal due to increased miRNA expression level. Furthermore, the magnetic nanoparticle core also enabled *in vivo* cell tracking with MRI.

In a follow-up study, a nanoparticle-based probe was reported for imaging of miR-9 [84]. An MRI sensitive cobalt ferrite core, coated with a rhodamine B isothiocyanate-containing silica shell, was functionalized with a 12-mer peptide that is internalized after binding to the transferrin receptor (TfR). Since high expression level of TfR is critical for the success of this strategy, a CMV promoter was fused with a TfR encoding sequence containing three copies of the mature miR-9 counterpart (denoted as CMV/TfR/3XPT-miR-9), to serve as a complementary part to the nanoparticle-based system. TfR expression was promoted in cells lacking miR-9, which consequently caused enhanced internalization of the nanoparticle and image contrast. Conversely, high level of miR-9 in the transfected cells impaired viral replication and decreased internalization, which led to lower fluorescence signal. Overall, expression of miR-9 acted as an “on” switch for the optical signal, whereas the complementary MRI signal behaved as an “off” switch, which was corroborated by *in vitro* and *in vivo* imaging of miR-9 expression during neural differentiation.

Recently, an engineered MB was reported, which is composed of a stem loop DNA structure that carries a complementary sequence to the target miRNA and a fluorophore-quencher pair that is activated upon binding of the target miRNA (Fig. (3)) [85]. This MB system was employed for simultaneous monitoring of miR-206 and miR-26a biogenesis during differentiation of myocytes, where MBs containing either a miR-206 targeting sequence attached to a 6-FAM-BHQ1 pair, or a miR-26a targeting sequence coupled to a Texas Red-BHQ2 pair, were transfected into C2C12 cells. *In vitro* and *in vivo* fluorescence analysis revealed significant increase in the fluorescence signal, due to interaction of miR-206 and miR-26a with their respective MB which was only observed in differentiated cells. This imaging strategy holds great potential to be generally applicable for monitoring a wide variety of miRNA-associated cellular processes *in vivo*.

Cross-validation of this MB system has been carried out, in which it was used in combination with a BLI-based approach [86]. In this study, the expression of miR-1 was examined during myogenic differentiation of mouse myoblasts, using both luciferase- and MB-based approaches. The two strategies were found to be complementary and both were responsive to increased cellular levels of miR-1. A decrease in signal was observed for the BLI-based approach, where an increase in fluorescence signal was achieved with the MB-based system. Similarly, a MB was developed for imaging of miR-155, which is highly expressed in certain lung cancer cells, using Alexa488 and BHQ1 as the fluorophore-quencher pair [87]. *In vitro* and *in vivo* experiments were carried out using laser confocal microscopy and stereomicroscopy imaging, respectively. Studies in nude mice demonstrated good sensitivity and specificity of the probe towards miR-155, making it an attractive option for miRNA detection and potentially also as a diagnostic tool for lung cancer.

An intriguing multifunctional system was reported for theranostic applications, which was composed of magnetic fluorescent nanoparticles that are attached to tumor homing AS1411 aptamer and miR-221 sensitive MB [88]. The aptamer-conjugated nanoparticles displayed excellent target specificity in various cancer cell lines, which was termed “MFAS miR-221 MB” after further incorporation of the MB, using Cy5 and BHQ2 as the fluorophore-quencher pair. After internalization of the probe into cells, which could be detected through rhodamine fluorescence in the nanoparticle, the covalent bond holding the double-stranded oligonucleotide to the nanoparticle surface was cleaved, thereby releasing the MB for interaction with its miRNA target. The effectiveness of the probe in visualizing miR-221 biogenesis and inhibiting miR-221 associated tumor progression was investigated in C6 cells. With the use of two different fluorophores (i.e. rhodamine and Cy5) in the same probe, the signal due to nanoparticle biodistribution could be readily distinguished from the fluorescence signal generated via dequenching of the MB by endogenous miR-221. It was suggested that similar approaches could potentially be extrapolated to the detection and inhibition of other miRNA targets expressed in various cancer types.

Recently, another functionalized magnetic nanoparticle with theranostic potential was reported [89], which was aimed at the inhibition of miR-10b, a miRNA that is believed to play a significant role in the metastatic process of various cancer types including breast adenocarcinoma [90,91]. The final theranostic construct was composed of four building blocks: a dextran-coated magnetic nanoparticle as the scaffold which can be imaged with MRI, a near-infrared fluorescent dye Cy5.5 for *in vivo* optical imaging, a cyclic arginine-glycine-aspartic acid (RGD) peptide for integrin $\alpha_v\beta_3$ -targeted tumor homing, and a lock nucleic acid containing the miR-10b seed sequence as a therapeutic entity (Fig. 4(A)) [89]. Binding affinity of the nanoparticle to integrin $\alpha_v\beta_3$ -expressing tumor cells and the level of miR-10b regulation were analyzed by flow cytometry and RT-PCR, respectively, which revealed > 80% inhibition of miRNA expression in cells exposed to the multifunctional nanoparticle. To evaluate the therapeutic efficacy *in vivo*, the multifunctional nanoparticle

was intravenously injected into mice bearing orthotopic breast tumors. T2-weighted MRI and fluorescence imaging of the treated mice showed preferential accumulation of the nanoparticle in the tumor site and adjacent lymph nodes (Fig. 4(B,C)), which successfully inhibited lymph node metastasis in the pre-metastatic subjects and arrested the progression of metastases that already existed before treatment. The approach described in this study could also shed light on the elucidation of multiple context-dependent functions of miRNAs, not only for miR-10b and metastatic breast cancer, but also for many other adenocarcinomas in which aberrant expression of various miRNAs is common.

A significant improvement to the delivery of miRNA-targeting cDNA-based MB was recently reported, where PEGylated liposomes were employed to deliver a large amount of loaded MB to the target cells without undesired leakage of MB [92]. To add targeting capability, activated hyaluronic acid was conjugated for specific binding to CD44-overexpressing cancer cells. The mechanism for delivery of MB using this system involved several steps: binding of the liposomes to CD44, internalization via endocytosis, destabilization of the endosome membrane, and finally the release of MB into the cytoplasm. Once inside the cells and upon interaction with its target miRNA, the MB that is specific for miR-34a was activated. *In vitro* and *in vivo* optical imaging allowed successful measurement of miR-34a expression levels in (CD44⁺) breast cancer cells. This system was claimed to represent a major improvement over other methods for the delivery of MB, such as passive uptake, microinjection, cationic transfection, and conjugation to cell penetrating peptides.

CONCLUSION AND FUTURE PERSPECTIVES

The discovery and elucidation of the critical roles of miRNA in regulating gene expression and related biological functions has motivated the recent development of more efficient miRNA detection methods. Conventional techniques such as Northern blotting and RT-PCR are still the mainstay for *in vitro* assays. However, the lack of dynamic imaging capability and repeatability of these techniques mandate researchers to look for alternative methods that can be used for *in vivo* imaging applications, which can provide much more relevant and meaningful information in intact biological context than the “snapshots” provided by *in vitro* assays. To date, the major categories of miRNA imaging strategies are based on FPs, BLI, MB, or nanoparticles. Many of these studies were carried out in cells as proof-of-principle, and non-invasive imaging of miRNA expression and/or biological activity is still at its infancy.

Despite the many advantages over conventional methods and considerable improvement that they have provided for non-invasive imaging of miRNAs, reporter gene-based techniques (with either fluorescence or bioluminescence) have many limitations for *in vivo* studies such as high background signal, low sensitivity (especially for FP-based strategies), poor tissue penetration of signal, intense scattering hence poor spatial resolution, among others. Another concern about BLI is that the reports to date mostly provided “signal-off” imaging, in which the presence of functional miRNA decreases the signal intensity. Furthermore, imaging intracellular targets such as miRNAs is significantly more challenging than imaging cell surface targets such as receptors that are overexpressed on cancer cells. All of these drawbacks in addition to the involvement of genetic modification and toxicity of some of the reporters severely limit the potential clinical applicability of *in vivo* miRNA imaging techniques. It is likely that imaging of miRNAs will remain as a research tool in cells and small animal models in the next several years.

Multimodality imaging, which combines the strengths of different imaging techniques to provide complementary information on different aspects of the biological process under

investigation, can offer a partial solution to the technical disadvantages of individual imaging modalities. Although the multimodality approaches for miRNA imaging reported to date are exclusively based on MRI and optical imaging, such a combination may not be optimal since neither modality is highly quantitative. In addition, the many orders of magnitude difference in detection sensitivity also makes this combination not ideal. Combination of positron emission tomography and optical imaging deserves to be investigated in the future for non-invasive interrogation of miRNA expression and/or activity, which can be more clinically relevant than the MRI/optical combination. Future research on more clinically relevant, non-toxic techniques is required to move the field of miRNA imaging into clinical applications. Non-invasive imaging of miRNA is an invaluable method that can significantly advance our understandings of a wide range of human diseases, which may also lead to new and more effective treatment strategies for these diseases.

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Fig. (1). Imaging of miRNAs with fluorescent protein-based systems. **A.** Scheme of the proviral vector form of lentiviral vector modified to carry miRNA target sequences, which was used for de-targeting gene expression from human hematopoietic cells with endogenous miRNAs. **B.** Three tandem repeats with perfect complementarity to miR-133 were inserted between the region downstream of GFP and upstream of RFP. In the presence of miR-133, GFP expression is suppressed without affecting RFP expression, as evidenced by dual color imaging in differentiated myoblast C2C12 cells. Adapted with permission from [61,64].

Fig. (2).

In vivo BLI of endogenous mature miRNA expression based on a luciferase reporter system.

A. Two plasmids were constructed by fusing a luciferase reporter gene and four tandem copies of the miRNA target sequences, driven by a CMV (left) or E1A (right) promoter. **B.** BLI of mice transfected with vectors containing miR-122 targets (top) exhibited lower signal compared to the target free plasmids (bottom). Adapted with permission from [75].

Fig. (3).

A. Schematic representation of molecular beacon (MB)-based imaging of miR-206, using Texas red as the fluorophore and BHQ2 as the quencher. The fluorescence signal is activated upon hybridization of the double strand in the presence of miR-206. **B.** *In vivo* fluorescence imaging of miR-206 during differentiation of C2C12 cells, which was transfected with both the MB and the firefly luciferase gene. After differentiation induction, C2C12 cells were injected into the right thigh of a nude mouse, whereas C2C12 cells without differentiation were injected to the left thigh. BLI was performed to confirm that implanted C2C12 cells had similar cellular survival. Adapted with permission from [85].

Fig. (4).

Nanoparticle-based multimodality imaging of miR-10b. **A.** A schematic representation of the multifunctional nanoparticle (abbreviated as MN-anti-miR-10b), which contains a dextran-coated magnetic nanoparticle conjugated to the Cy5.5 dye, a tumor targeting peptide (cRGD), and an oligonucleotide targeting human miR-10b. **B.** T2-weighted MRI of MN-anti-miR-10b distribution in orthotopic MDA-MB-231-luc-D3H2LN breast tumors (dotted circle), which displayed a shortening of the T2 relaxation time within the tumor at 24 h post-injection. **C.** *In vivo* fluorescence imaging of mice before (left) and 24 h after (right) administration of MN-anti-miR-10b indicated accumulation in the primary tumor (PT) and lymph nodes. Adapted with permission from [89].