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An Image-Based Biosensor Assay Strategy to Screen for Modulators of the microRNA 21 Biogenesis Pathway

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Abstract

microRNAs (miRNAs) are evolutionary conserved, small endogenous non-coding, RNA molecules. Although their mode of action has been extensively studied, little is known about their biogenesis. As their altered expression has been implicated in many diseases, small molecules that would modulate their expression are sought after. They are generated through the concerted action of several complexes which promote their transcription, maturation, export, trafficking, and loading of mature miRNA into silencing complexes. An increasing number of studies have suggested that each of these steps serves as a regulatory junction in the process, and therefore provides an intervention point. For this purpose, we have developed a simple image-based assay strategy to screen for such modulators. Here, we describe its successful implementation which combines the use of a microRNA 21 (miR-21) synthetic mimic together with an EGFP based reporter cell line, where its expression is under the control of miR-21, to monitor EGFP expression in a format suitable for HTS. The strategy was further validated using a small panel of known gene modulators of the miRNA pathway. A screen was performed in duplicate against a library of 6,912 compounds and identified 48 initial positives exhibiting enhanced EGFP fluorescence intensity. 42 compounds were found to be inherently fluorescent in the green channel leaving the remaining 6 as potential inhibitors and with a positive rate of 0.09%. Taken together, this validated strategy offers the opportunity to discover novel and specific inhibitors of the pathway through the screening of diverse chemical libraries.

Keywords

high-throughput screening; image-based biosensor; miRNA biogenesis; modulator

Introduction

miRNAs are small, non-coding RNA molecules of 20 to 23 nucleotide length that regulate gene expression at the post-transcriptional level by causing translational repression or mRNA degradation. miRNAs are implicated in a number of diverse biological functions including cell proliferation, development, differentiation, and metabolism. According to the current biogenesis model,[1,2] the majority of miRNA are transcribed inside the nucleus into primary miRNA (pri-miRNA). The pri-miRNA undergoes endonucleolytic cleavage by the microprocessor complex[3] composed of Drosha[4,5] and DGCR8.[6] The resulting stem-loop-structure called precursor-miRNA (pre-miRNA) is exported into the cytoplasm by Exportin-5 in complex with Ran-GTP.[7] Pre-miRNA are processed by Dicer[8] into

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mature miRNAs where it gets loaded onto the RNA-induced silencing complex (RISC) along with EIF2C2[9] to target mRNA. The complementary seed region of the miRNA binds to either the upstream 3' untranslated region (UTR) or the poly A tail of the mRNA sequence resulting in translational repression or degradation.

Although the major components of the miRNA pathway have been identified, we have only begun to understand how miRNA biogenesis and expression regulates important biological processes. Gene regulation by miRNA is essential for normal development and strong evidence supports its role in underlying pathological conditions such as cancer, neurodegenerative, and hematological diseases.[10,11] For instance, studies have implicated miRNAs as oncogenes by negatively regulating tumor suppressor genes. Le Sage and co-workers were able to demonstrate through miRNA screening that miR-221 and miR-222 stimulated proliferation following inhibition of p27/Kip1 tumor suppressor.[12] Furthermore, widespread occurrences of certain miRNA signatures such as miR-21 have been shown in a variety of cancers.[13,14] Using microarray analysis, Yan and co-workers demonstrated that elevated miR-21 expression levels in human breast cancers are associated with advanced clinical stage and short overall survival.[15] These emerging miRNA-disease associations demonstrate that regulating miRNA biogenesis represent a viable target for small molecule therapeutic intervention.

The use of chemicals as tools to interrogate biological processes provides a unique strategy for dissecting the miRNA biogenesis pathways and ultimately may lead to the development of therapeutic agents. Several different assays have been described using approaches involving both *in vitro* and *in vivo* reporter methods to study the RNAi/miRNA pathway in general. Current *in vitro* approaches to measure miRNA biogenesis include a fluorescence-based assay that measures Dicer activity using FRET technology.[16] Based on the let-7 pre-miRNA sequence, a RNA hairpin was synthesized with a fluorescence molecule reporter at its 5' end and a fluorophore quencher molecule at its 3' end; hereby Dicer activity was measured by increase fluorescence signal upon binding of the labeled hairpin to its complementary target. Inhibitors are scored based on the loss of fluorescence signal; the utility of such an *in vitro* assay has not been fully evaluated in HTS and whether it could be used to identify novel modulators of the pathway with cellular activity is not known. Other methods include the use of an oligonucleotide microarray chip to profile miRNA expression in which curcumin, a flavinoid derivative was identified to up-regulate miR-22 and down-regulate miR-199a in pancreatic cancer cells.[17] Similarly, epigallocatechin gallate, a polyphenol, was found to modify the expression of several miRNAs such as up-regulating miR-16 in HepG2 cells.[18]

Another approach relies on a cell based reporter assay in HEK293 cells that employs an EGFP protein and stably expressed short hairpin RNA (shRNA) against EGFP to monitor the RNA interference (RNAi) pathway.[19] The assay was deployed in a screen of a library of 2,000 compounds where the acquired images were then visually scored, and leading to the identification of enoxacin, a fluoroquinolone antibacterial agent that enhances RNAi activity and promotes miRNA processing. Other cell based reporter assays include the use of luciferase activity as a gain of function reporter, whereby the luciferase expression is under the control of miR-21 binding sequence at its 3' UTR and acts as a negative controller of miRNA activity.[20] In a screen of a library of 1,200 compounds, several hits belonging to the diazobenzene chemical scaffold composed of two phenyl rings linked by an azo group were identified as inhibitors of miR-21 repressive activity. Recently, a similar approach in which luciferase expression is under the control of miR-122 identified several hits containing the benzothiazole motif.[21] Despite the increasing number of small chemical screens performed to date, only a handful of actives have been identified with little or no specificity towards the overall miRNA biogenesis pathway.

We reasoned that to rapidly screen for new modulators of the pathway, the assay has to be sensitive and amenable to HTS of large chemical libraries. For this purpose, we developed an image-based biosensor assay that combines the use of MISSION miRNA Mimic hsa-miR-21 as a source for an exogenous pool on miR-21 in the cell, and the HeLaS3 miR-21 EGFP cell line to produce our biosensor reporter assay of miR-21 activity, where the expression of the EGFP reporter is under the control of miR-21 molecules through complementary miR-21 sequence present at its 3'UTR. Presence of miR-21 in the reporter cell line would repress the production of EGFP resulting in little or no EGFP signal output; whereas inhibition of miR-21 production or processing would alleviate the suppression and results in an increase of EGFP fluorescence intensity signal output. In both cases, the EGFP fluorescence signal output is easily recorded through automated imaging microscopy, which is extremely advantageous to this high-content approach as it allows for a rapid and dependable evaluation of the signal output at a cellular level. Similar microscopy based screening strategies were successfully implemented by our group.[22–24] In this report, we describe the assay development and optimization in 384-well microtiter plate format, and its validation using known gene modulators of the miRNA pathway. We present the results of our screen against a diverse collection of 6,912 compounds as a proof of concept and validation of our image-based biosensor assay strategy to identify modulators of the miRNA biogenesis pathway and discuss its potential use in large scale screening of chemical libraries.

Material and Methods

Cell Culture and Materials

To generate the stable reporter cell line HeLaS3 miR-21 EGFP harboring EGFP under miRNA regulation, HeLaS3 cells were seeded into 24-well plate at 200,000 cells per well in 500 μ L of growth media containing Dulbecco's modified Eagle's medium (D-MEM), high glucose with L-glutamine, D-glucose and sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum (FBS). After 24 h, pcDNA/TO/EGFPmiR21 (Addgene, Cambridge, MA) was diluted with Opti-MEM media to 0.2 μ g/well; in which Lipofectamine 2000 transfection solution was added at a final concentration of 0.4 μ L/well, and incubated at room temperature for 15 min to promote transfection reagent complex formation. Next, 100 μ L of transfection reagent complex was added into the wells and incubated for an additional 24 h. Cells were passaged and then selected with Zeocin at a concentration of 200 μ g/mL for 6 days. Zeocin-resistant cells were harvested and HeLaS3 miR-21 EGFP cell stocks were stored at -170°C .

HeLaS3 miR-21 EGFP cells were grown at 37°C and 5% CO_2 in complete growth media containing D-MEM, high glucose with L-glutamine, D-glucose and sodium pyruvate supplemented with 10% heat inactivated FBS, and 200 μ g/mL of Zeocin. All cell culture supplies were from Life Technologies (Carlsbad, CA), and Sigma-Aldrich (St Louis, MO). Human MISSION miRNA Mimic for hsa-miR-21 (HMI0371) was purchased from Sigma-Aldrich. Human miRIDIAN miRNA Hairpin Inhibitor for hsa-miR-21 (IH-300492-05) was purchased from Thermo Scientific (Waltham, MA). Silencer Select Negative Control #1 (4390843), PLK1 (s449), and EIF2C2 (s25932) siRNAs were purchased from Life Technologies. Draq5 nuclei staining dye was purchased from Biostatus Ltd (Shepshed Leicestershire, UK).

Liquid Dispensing and Automation System

Several liquid dispensing devices were used throughout this study. Compounds, siRNAs, miR-21 mimic, and miR-21 inhibitor were plated and transferred using a 384 stainless steel head with disposable low volume polypropylene tips on a PP-384-M Personal Pipettor

(Apricot Designs, Monrovia, CA). The addition of cell suspensions and complete growth media was performed using the Multidrop 384 (Thermo Scientific). Cell fixation and staining was performed using the ELx405 automated washer (Biotek, Winooski, VT). Assay plates were incubated in the Cytomat automated incubator (Thermo Scientific) under controlled humidity at 37°C and 5% CO₂. The assay was performed on a fully automated linear track robotic platform (Thermo Scientific) using several integrated peripherals for plate handling, cell incubators, liquid dispensing, and detection systems.

Assay Development into 384-well Microtiter Plate Format for Chemical Screening

The assay was optimized for automated chemical screening. First, the assay was miniaturized to 384-well microtiter plate format by determining optimal cell growth kinetics over several days. Cell suspensions were dispensed into 384-well microtiter plates at cell densities ranging from 250, 500, to 1,000 cells per well in 50 μ L complete growth media. At 6, 8, and 10 days post-seeding, 10 μ L of 15 μ M Draq5 in PBS was dispensed into the assay plates at a final concentration of 2.5 μ M and incubated at 37°C for 20 min to complete Draq5 staining. Cells were then fixed in 4% paraformaldehyde (w/v) for 20 min followed by two washes in PBS and the assay plates were stored at 4°C. Images of cells in assay plates were acquired on the IN Cell Analyzer 3000 (INCA3000; GE Healthcare, Piscataway, NJ) for Draq5-stained nuclei. To complete development on the biosensor assay, transfection conditions for MISSION miRNA Mimic hsa-miR-21 in cells were optimized with a panel consisting of Negative Control #1 siRNA, PLK1 siRNA, EIF2C2 siRNA, miR-21 mimic, and miR-21 inhibitor. The siRNAs, miR-21 mimic, and miR-21 inhibitor were diluted from stock concentration in nuclease-free water and 5 μ L was transferred into assay plates in an arrayed format to achieve the desired final concentration of 50 nM or 100 nM. Next, 10 μ L/well Opti-MEM media was added followed by 15 μ L/well Lipofectamine RNAi Max transfection solution at a final concentration of 0.1 μ L/well and incubated at room temperature for 20 min to promote transfection reagent complex formation. Next, cell suspensions at 1,000 cells per well were dispensed into the assay plates in 50 μ L complete growth media. At 6, 8, and 10 days post-transfection, cells were stained with Draq5 and fixed as described. Images of cells in assay plates were acquired on the INCA3000 for EGFP fluorescence intensity and Draq5-stained nuclei.

Screen of Chemical Library for Modulators of miRNA Biogenesis

For the chemical screen, MISSION miRNA Mimic hsa-miR-21 were first diluted from 100 μ M stock concentration in nuclease-free water to 2 μ M concentration and 5 μ L was transferred into assay plates for a final concentration of 100 nM. For internal reference, each assay plate contained MISSION miRNA Mimic hsa-miR-21 as negative control in column 13 and miRIDIAN miRNA Hairpin Inhibitor hsa-miR-21 as positive control column 14 at a final concentration of 100 nM. Next, 10 μ L/well Opti-MEM media was added followed by 15 μ L/well Lipofectamine RNAi Max transfection solution at a final concentration of 0.1 μ L/well and incubated at room temperature for 20 min to promote transfection reagent complex formation. Cell suspensions were then dispensed into the assay plates at 1,000 cells per well in 50 μ L complete growth media. At 6 days post treatment with miR-21 mimic, media was removed from the assay plates and 30 μ L of complete growth media was added to prepare cells for compound addition. Compounds were plated in an intermediate 384-well polypropylene plate (Thermo Scientific) at 100 μ M in 10% DMSO (v/v) and 5 μ L was transferred to the assay plates at a final concentration of 10 μ M in 1% DMSO (v/v). After 4 days compound treatment, cells were stained with Draq5 and fixed using the procedure described above. Images were acquired on the INCA3000 for EGFP fluorescence intensity and Draq5-stained nuclei. The chemical screen was performed in duplicate to assess reproducibility.

Chemical Libraries

The library used for the screen combines 6,912 chemicals obtained from MicroSource, Prestwick, Tocris, Sigma-Aldrich and other commercial sources as previously described. [25–27] Briefly, the MicroSource Library contains 2,000 biologically active and structurally diverse compounds. The Prestwick Chemical Library is a unique collection of 1,119 bioactive compounds. The Tocris collection contains 1,270 compounds and the LOPAC library contained 1,280 characterized compounds. The remaining compounds were provided through internal suppliers.

Image Acquisition, Analysis, and Screening Data Management

Images were acquired on the automated laser confocal INCA3000 microscope. This laser scanning confocal imager comprises two laser light sources, three excitation lines, and three highly sensitive 12-bit charge-coupled device cameras allowing simultaneous imaging of three fluorophores with continuous laser-based autofocus. Images were acquired at the following wavelengths: 488 nm excitation / 535 nm emission in the green channel for EGFP signal and 633 nm excitation / 695 nm emission in the red channel for Draq5-stained nuclei and with an exposure time of 1.5 ms. For assay development and screening, nine images per well were collected using a 40x magnifying objective covering 90% of the well and required 20 sec per well, with a total imaging time of 135 min for a complete 384-well microtiter plate. Images were analyzed using the Raven 1.0 software's built-in object intensity analysis module. The object intensity analysis module uses a segmentation algorithm to count nuclei based on number of objects with Draq5 pixel intensities about background. To assess EGFP fluorescence intensity per well, a cell masked overlay was generated using nuclei as a marker and intensity was measured within the defined boundaries. Analysis of Draq5-stained nuclei and EGFP signal required approximately 10 min for a complete 384-well microtiter plate. Screening data files were loaded into Oncology Research Informatics System, a custom built suite of modules for compound registration, plating, and data management powered by ChemAxon Cheminformatic tools (ChemAxon).

Results

Image-based biosensor assay to identify modulators of miRNA biogenesis

To gain a better understanding and identify modulators of the miRNA biogenesis pathway, we have developed an image-based assay strategy utilizing EGFP as a reporter of miR-21 activity. In this, a stable reporter cell line expressing a gene encoding EGFP fused to a sequence with perfect complementarity to miR-21 in its 3'UTR region (HeLaS3 miR-21 EGFP); and transfected with MISSION miRNA Mimic for hsa-miR-21 to enhance the endogenous pool of mature miR-21 molecules. The miR-21 mimic binds to the fusion construct whereby endogenous miRNA processing specifically destabilizes the EGFP mRNA and results in low level expression of EGFP leading to low fluorescence intensity signal output. We hypothesize that our gain-of-function biosensor assay will identify small molecules that target proteins involved in the miRNA biogenesis pathway as they would induce varying EGFP fluorescence signal output that can be directly monitored using automated microscopy. In essence, small molecule modulators that inhibit miRNA biogenesis would lead to stabilization of the EGFP mRNA resulting in an increase production of the mature protein (Fig 1A). To identify small molecule modulators of miRNA biogenesis, we have devised a workflow with dual image acquisition for both EGFP signal and Draq5-stained nuclei (Fig 1B). Automated image analysis scored EGFP fluorescence intensity as the primary readout to identify inhibitors for miRNA biogenesis; and for secondary readout, compound cytotoxicity was scored using Draq5-stained nuclei count as leverage for loss of cell count due to toxicity.

Assay development to screen for chemical modulators of miRNA biogenesis

We have evaluated growth kinetics of the reporter cell line to determine optimal seeding density conditions in 384-well plate format. Cells were seeded at three different cell densities from 250 to 1,000 cells per well, and growth was monitored by Draq5-stained nuclei imaging at 6, 8, and 10 days post-seeding (Fig 2A). At a seeding density of 250 cells per well, cells were in subpar growth conditions as indicated by atypical growth recovery. At both 500 and 1,000 cells per well, cells recovered and showed linear growth kinetics indicating optimal seeding conditions. A seeding density of 1,000 cells per well was selected for overall consistency and cell distribution throughout the well (Fig 2B).

To establish optimal transfection conditions, we assessed the effect of siRNA duplex concentration and duration on gene silencing. Cells were seeded at 1,000 cells per well onto siRNA duplexes at both 50 nM and 100 nM final concentration, and monitored by Draq5-stained nuclei imaging at 6, 8, and 10 days post-transfection. We opted to perform reverse transfection using Lipofectamine RNAi Max at a concentration of 0.1 μ L/well as the complexing reagent, because of its amenability to automation with minimum number of reagent addition steps. For assay transfection optimization, we included the Silencer Select Negative Control #1 siRNA containing a scrambled ineffective siRNA sequence and a siRNA targeting the PLK1 gene. PLK1 is a key regulator of cell division whose knockdown results in cell death; a measureable phenotypic response.[28] Negative Control #1 siRNA showed no effects on cell viability indicating optimal transfection conditions were achieved; and as expected, PLK1 siRNA resulted in pronounced cell death consistent with a good efficiency (Fig 3). Based on these results, 100 nM was selected as the optimal concentration for the assay.

To further improve the sensitivity of our assay, we opted to transfect cells with the MISSION miRNA Mimic hsa-miR-21 in order to enhance the endogenous pool of mature miR-21 molecules and reduce the inherent background EGFP signal. To assess assay sensitivity, we included the miRIDIAN miRNA Hairpin Inhibitor hsa-miR-21 as a competitive inhibitor of miR-21 and a siRNA targeting EIF2C2 the catalytic component of the RISC complex responsible for miRNA processing.[29] Cells were seeded at 1,000 cells per well onto miR-21 mimic, miR-21 inhibitor, and siRNA at a final concentration of 100 nM and monitored by EGFP fluorescence intensity at 6, 8, and 10 days post-transfection (Fig 4). In comparison to untreated control, the miR-21 mimic reduced EGFP signal gain from 17% to 3% at day 6 and 15% to 5% at day 8, as such enhancing the assay's signal dynamic range. At day 10, the EGFP signal gain for untreated control and miR-21 mimic increased to 35% and 24%, respectively. As expected, the miR-21 inhibitor and the EIF2C2 siRNA resulted in an increased EGFP signal gain to 57% and 44%, respectively at day 6. Time course progression resulted in increasing EGFP signal gain up to 82% at day 10. Furthermore, the tested siRNA through the time course studies did not show any cytotoxic effect. Taken together, the obtained optimized conditions for the assay were a cell seeding density of 1,000 cells per well, reverse transfection of MISSION miRNA Mimic hsa-miR-21 treatment for 6 days at a concentration of 100 nM, and followed by compound treatment for 4 days.

Chemical screen for modulators of miRNA biogenesis

To identify modulators of miRNA biogenesis pathway, the image-based biosensor assay was optimized for HTS (Table 1) and screened against a library of 6,912 chemicals at a compound screening concentration of 10 μ M in 1% DMSO (v/v). The screen was performed in duplicate to assess the assay robustness and performance. The library was plated across twenty 384-well microtiter plates with columns 13 and 14 empty for negative and positive control wells. To monitor the assay's performance throughout the screen, MISSION miRNA

Mimic hsa-miR-21 was added for negative control and miRIDIAN miRNA Hairpin Inhibitor hsa-miR-21 was added for positive control. A box plot analysis shows a good separation between the negative and positive controls however, Z' factor was less than 0 due to large standard deviations (Fig 5).

To assess reproducibility, the datasets were plotted in a scatter plot to examine overall correlation for each compound. A scatter plot of EGFP signal gain (Fig 6A) showed high correlation among duplicates as depicted by the slope of the pattern and R^2 value of 0.92. A scatter plot of Nuclei count inhibition (Fig 6B) showed good overall correlation among duplicates as depicted by R^2 value of 0.75. In both readouts, a majority of compounds clustered around the control values indicating no activity with only a few outliers present. To identify modulators of miRNA biogenesis, we selected a threshold based on 3 standard deviations (3σ) from the mean of the negative control which translates to EGFP signal gain of 30% and 48 compounds were scored as positives yielding a initial positive rate of 0.69%. A number of compounds among the positives were identified several times as they were provided by multiple vendors in the screening set including doxorubicin, ellipticine, merbromin, NSC 95397, phenzopyridine hydrochloride, and quinacrine hydrochloride. In total, 41 unique compounds were identified among the positives.

High-content screening (HCS) is not immune to optical interference of compounds such as their inherent auto-fluorescence characteristics in the EGFP emission channel; though this assay involves washing and fixation steps. We compared the images of the obtained positives to those imaged in a different screen against a control NIH 3T3 cell line revealing that 42 compounds were found to be fluorescent in the green channel (Table 1S). These compounds range in EGFP signal gain from 33% to 4,899% and several were reported as false positives in previous assays using similar EGFP based readout including merbromin, NSC 95397, daunorubicin, acriflavinium, quinacrine, and sunitinib.[22,24]

Six positives remain as potential inhibitors of the miRNA biogenesis pathway yielding a final positive rate of 0.09% (Table 2). Dipropyldopamine hydrobromide[30] and 8-hydroxy-DPAT hydrobromide[31] function as an agonists on neurotransmitter receptors and 6-hydroxy-DL-DOPA[32] acts as a neurotoxin. Deoxycorticosterone[33] and flutamide[34] are corticosteroids and nonsteroidal antiandrogens, respectively. Pachyrrhizin[35] is extracted from a plant with unknown function.

DISCUSSION

Current methodologies to identify modulators of the miRNA biogenesis have been met with limited success and only a handful of small molecule modulators have been reported to date. To overcome these limitations, we developed an image-based assay that is amenable to screening of large libraries. Our strategy utilizes a stable reporter cell line that expresses a gene encoding EGFP fused to a sequence with perfect complementarity to miR-21 in its 3'UTR region and MISSION miRNA Mimic for hsa-miR-21 as a reporter of miRNA activity. To validate our assay, we demonstrated siRNA that interfere with the catalytic domain of RISC and processing of miR-21 leads to an increase EGFP fluorescence intensity. Applying this approach to chemical screening, we postulate that small molecule inhibitors of miRNA biogenesis will lead to increased fluorescence intensity as a result of EGFP mRNA stabilization.

To date, most approaches to identify miRNA modulators revolve around low-content assays that utilize end-point readout to evaluate compounds. In contrast, high-content assays rely on segmentation algorithms to extract multiple features within an image to assess the compound's biological effects. As such, HCS has a tendency to suffer from large noise as a

consequence of biological variation within any given cell population and often leads to low signal to background ratios. As a result, Z' factor is often a poor indicator of assay performance given that these data sets do not follow normal distributions.[36] A good signal window with EGFP signal gain from 1 to 100% for the negative and positive controls was observed, however and due to inherent cell-to-cell heterogeneity, it resulted in large standard deviations yielded a Z' factor of less than 0. In our case since the readout is a gain-of-function, Z' factor had no impact on performance. For hit selection, we employed a commonly used technique based on 3σ that translates to 30% threshold.[37] With this selection criteria in place, 48 initial positives were identified.

Similar to low content screens, HCS is not immune to fluorescent compound interference. As follow-up, we recalled images from a previous screen that did not rely on EGFP as a reporter and found that 87% of our initial positives were false due to compound auto-fluorescence in the green channel. Few of these compounds were previously identified including merbromin, NSC 95397, daunorubicin, acriflavinium, quinacrine, and sunitinib. [22,24] Among the false positives, several classes of compounds were found to be enriched including anthracyclines, fluorescein derivatives, and acridines. The complete list of compounds that exhibit auto-fluorescence in the green channel are summarized in Table 1S. Library quality also remains a key concern for screening regardless of reporter technology. We observed considerable variation between compounds from different vendors including doxorubicin, ellipticine, merbromin, NSC 95397, phenzopyridine, and quinacrine. For instance, quinacrine was identified as positive on three separate occasions with EGFP signal gain of 255% from MicroSource, 85% with Prestwick Chemicals, and 42% with Sigma-Aldrich. As such, these results emphasize the importance of validating screening positives and thorough follow-up analysis.

One of the earliest screens by Shan and co-workers utilized a cell based reporter assay with EGFP and stably expressing shRNA against EGFP as a monitor of RNAi/miRNA processing.[19] They screened 2,000 compounds at a concentration of 50 μ M and identified the fluoroquinone antibacterial compound enoxacin as a promoter of miRNA biogenesis. Enoxacin was present in our library and in duplicate as it was provided in two libraries and scored as inactive at a screening concentration of 10 μ M (Table 2S). Notably, another difference between the two screens is the manner in which hits were scored; Shan and co-workers visually scored for modulators whereas we extracted data using segmentation algorithms allowing for greater sensitivity to detect small changes that are not discernible to the eye.

Gumireddy and co-workers were the first to report a luciferase based reporter assay under the control of miR-21, and was screened 1,200 compounds leading to the identification of diazobenzene containing compounds as hits.[20] Furthermore, their assay was progressed to large chemical screening campaign at the NCGC, one of the NIH's Molecular Libraries Screening Centers, where a qHTS screen was performed against a library of 333,523 compounds. 3,282 positives were identified and displaying a dose response curve with an initial positive rate of 0.98%.[38–40] A follow up counter screen using a purified firefly luciferase identified a substantial number of compounds as false positives and interfering with the luciferase function. Overall, 79 hits were confirmed. An overlap analysis did not identify any common scaffolds among our 6 identified positives. The screens performed to date have yielded low hit rates and the scarcity of hits seems to suggest that the miRNA biogenesis pathway is tightly regulated.

In summary, we have developed and validated a simple image-based assay to screen for modulators of the miRNA biogenesis pathway with the identification of 6 positives from a screen of approximately 7,000 compounds. HCS as an approach significantly decreases

follow-up time by allowing quick assessment of compound activity, monitoring cytotoxicity, and eliminating false positives. This strategy offers a new opportunity to identify novel and specific inhibitors of the miRNA biogenesis pathway with potential use to treat disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

miRNA	microRNA
miR-21	microRNA 21
HCS	high-content screening
HTS	high-throughput screening
INCA3000	IN Cell Analyzer 3000
EGFP	enhanced green fluorescent protein

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Figure 1. Image-based biosensor assay strategy to identify modulators of miRNA biogenesis pathway

A) Principle of biosensor assay. HeLaS3 miR-21 EGFP cells are transfected with MISSION miRNA Mimic hsa-miR-21 for 6 days and then treated with compound for 4 days. Compounds that inhibit miRNA biogenesis results in an increased EGFP signal output. Inactive compounds do not change EGFP signal output. **B)** Workflow of biosensor assay and imaging analysis. HeLaS3 miR-21 EGFP cells (1,000 cells per well) are reverse transfected with 100 nM MISSION miRNA Mimic hsa-miR-21 followed by incubation for 6 days. Cells are then treated with 10 μ M of compound for 4 days. Automated image analysis scored EGFP fluorescence intensity as the primary readout for modulators of miRNA biogenesis and Draq5-stained nuclei as secondary readout for cytotoxicity.

Figure 2. Assay development in 384-well microtiter plates

Growth kinetics of stable reporter cell line. **A)** Cells were seeded at cell densities ranging from 250 to 1,000 cells per well. For each cell density range, average and standard deviations are calculated from triplicate wells. **B)** Images of Draq5-stained nuclei at 1,000 cells per well at 6, 8, and 10 days post-seeding (left to right). Images were acquired using the INCA3000 with red channel for Draq5-stained nuclei.

Figure 3. Transfection optimization for image-based biosensor assay

Cells (1,000 cells per well) were tested against a panel of siRNA using reverse transfection procedure and evaluated at 6, 8, and 10 days post-seeding. **A)** Transfection with Silencer Select Negative Control #1 and PLK1 siRNA at 50 nM and 100 nM followed by assessment of nuclei count. For each condition, average and standard deviations are calculated from quadruplicate wells. **B)** Images of Untreated Control, Negative Control #1 siRNA, and PLK1 siRNA at 100 nM and 6 days post-seeding (left to right). Images were acquired using the INCA3000 with red channel for Draq5-stained nuclei and green channel for EGFP signal.

Figure 4. Validation of image-based biosensor assay

Cells (1,000 cells per well) were tested against a panel of RNAi using reverse transfection procedure and evaluated at 6, 8, and 10 days post-seeding. **A)** Transfection with MISSION miRNA Mimic hsa-miR-21, miRIDIAN miRNA Hairpin Inhibitor hsa-miR-21, EIF2C2 siRNA at 100 nM followed by assessment of EGFP fluorescence intensity. For each condition, average and standard deviations are calculated from quadruplicate wells. **B)** Images at 100 nM at 6, 8, and 10 days post-seeding. Images were acquired using the INCA3000 with red channel for Draq5-stained nuclei and green channel for EGFP signal.

Figure 5. Performance of control wells in image-based biosensor assay

Cells (1,000 cells per well) were transfected with either 100 nM of MISSION miRNA Mimic hsa-miR-21 as the negative control or miRIDIAN miRNA Hairpin Inhibitor hsa-miR-21 as the positive control for 6 days followed by treatment with 1% DMSO (v/v) for 4 days. **A.** Box plot analysis of control wells. **B.** Images of control wells from Set 1 miR-21 Mimic, Set 1 miR-21 Inhibitor, Set 2 miR-21 Mimic, and Set 2 miR-21 Inhibitor (left to right). Images were acquired using the INCA3000 with red channel for Draq5-stained nuclei and green channel for EGFP signal.

Figure 6. Assessment of the performance of the assay during screening

Assay robustness and performance were evaluated against a library of 6,912 compounds screened in duplicates to assess reproducibility. **A)** Scatter plot analysis of EGFP Signal gain in Set 1 versus Set 2. Compound modulators that inhibit miRNA biogenesis are depicted in green. Positive threshold for Set 1 and Set 2 at 30% is depicted in grey dashed lines. Linear regression fit is shown in solid blue line. **B)** Scatter plot analysis of Nuclei Count inhibition in Set 1 versus Set 2. Cytotoxic compounds are depicted in red. Cytotoxicity threshold for Set 1 and Set 2 at 20% is depicted in grey dashed lines. Linear regression fit is shown in solid blue line.

Table 1

Workflow of the image-based biosensor assay.

Step	Parameter	Value	Description
1	miRNA Mimic plating – arrayed format	5 μ L	miR-21 Mimic diluted in water at 2 μ M concentration
2	miRNA Mimic dilution	10 μ L	Opti-MEM media
3	Transfection reagent	15 μ L	Lipofectamine RNAi Max at 0.1 μ L/well in Opti-MEM media
4	Complex formation	20 min	Complex formation for miR-21 Mimic delivery into cells
5	Cell plating	50 μ L	1,000 HeLaS3 miR-21 EGFP cells in complete growth media
6	Incubation time	6 day	37°C, 5% CO ₂
7	Growth media	30 μ L	Remove media and addition of complete growth media
8	Library compounds	5 μ L	100 μ M in 10% DMSO (v/v)
9	Incubation time	4 day	37°C, 5% CO ₂
10	Nuclear staining	10 μ L	Draq5 diluted in PBS at 15 μ M
11	Incubation time	15 min	37°C, 5% CO ₂
12	Fix	50 μ L	4% PFA (w/v) for 20 min
13	Assay readout	488nm/535nm & 633nm/695nm (ex/em)	INCA3000 automated microscope
14	Image analysis	--	Multiparametric analysis using Raven 1.0 software

Step	Notes
1	Dispensing on the PP-384-M Personal Pipettor using a custom 384 head; 30 sec per 384-well microtiter plate
2	Dispensing into assay plate with Multidrop 384
3	Dispensing into assay plate with Multidrop 384
4	Assay plates at room temperature
5	Cells prepared in media and dispensed into assay plate with Multidrop 384
6	Assay plates stored in the Cytomat; an automated incubator
7	Aspirating on the ELx405 automated washer and dispensing with Multidrop 384; 1 min per 384-well microtiter plate
8	Dispensing on the PP-384-M Personal Pipettor using a custom 384 head; 30 sec per 384-well microtiter plate
9	Assay plates stored in the Cytomat
10	Dispensing into assay plate with Multidrop 384

Step	Notes
11	Assay plates stored in the Cytomat
12	Aspirating on the ELx405 and dispensing with Multidrop 384; 1 min per 384-well microtiter plate
13	For assay development and screening, nine images per well for 20 sec per well with total imaging time of 135 min per 384-well microtiter plate.
14	Analysis of EGFP ⁺ signal and Draq5-stained nuclei, 10 min per 384-well microtiter plate

Table 2

Chemical structures and images for 6 inhibitors identified in image-based biosensor assay.

Structure	Compound Name	Supplier	ID	Image	EGFP Signal % Gain	Reference
6-Hydroxy-DL-DOPA	Sigma-Aldrich	H 2380		40	[32]
Deoxycorticosterone	Prestwick Chemicals	957		37	[33]
Dipropylamine Hydrobromide	Sigma-Aldrich	D 031		35	[30]
8-Hydroxy-DPAT Hydrobromide	Sigma-Aldrich	H 140		33	[31]
Pachyrhizin	MicroSource	201602		32	[35]

Structure	Compound Name	Supplier	ID	Image	EGFP Signal % Gain	Reference
Flutamide	Sigma-Aldrich	F 9397		30	[34]