Electronic Supplementary Information

An Emulsion-Free Digital Flow Cytometric Platform for the Precise Quantification of microRNA Based on Single Molecule Extension-Illuminated Microbeads (dFlowSeim)

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1. Reagents and Materials

Dynabeads® M-270 Epoxy (Epoxy MBs, 2.8 μm in diameter) and terminal deoxynucleotidyl transferase (TdT) were purchased from Thermo Fisher Scientific (Invitrogen, Oslo, Norway). All of the nucleic acid sequences were custom synthesized and purified by Takara Biotechnology (Dalian, China), and the detailed sequences are listed in Table S1. All the other reagents used in this work were of analytical grade and were used as received without further purification.

Table S1. The DNA sequences used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>ath-miR156a</td>
<td>UGACAGAAGAGAGUGAGCAGC-2’mec</td>
</tr>
<tr>
<td>Probe A</td>
<td>NH2-CCCCCCCCCC(spacer)-GTGCTCACTC-DBCO</td>
</tr>
<tr>
<td>Probe B</td>
<td>N3-TCTTCTGTCA</td>
</tr>
<tr>
<td>Probe C</td>
<td>AGTCTAGGATTTCGGCGTGGGTTAAAACTGTGAAAAAAANAAAAA-FAM</td>
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<td>Let-7a</td>
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<tr>
<td>Probe A-let-7a</td>
<td>NH2-CCCCCCCCC(spacer)-AACTATACAAC-DBCO</td>
</tr>
<tr>
<td>Probe B-let-7a</td>
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<td>ODN1</td>
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<td>ODN2</td>
<td>CCCCCCCCCGGTCTCAGCCTCTCCTCCTCTGC</td>
</tr>
<tr>
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</tr>
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<td></td>
<td>TG-FAM</td>
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<td>miR21</td>
<td>TG</td>
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<td>miR24</td>
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<td>miR92a</td>
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<td>miR155</td>
<td>UAUUGCACUUGUCGCGCGACCU</td>
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<tr>
<td>miR143</td>
<td>UUAUGCUAAUCGAGAUAGGGGU</td>
</tr>
<tr>
<td>SFODN</td>
<td>UGAGAUGAAGAGACUGUAGCUC</td>
</tr>
</tbody>
</table>
P1  AF488-AACTATAAACCCTACTACCTCATTTTTTTTTT-biotin
P2  Aza-DBCO-TCTTCTGTCATTTTTTTTTT-biotin
AF488-GTGCTCACTC-N$_3$

**Note:** “2’me” at the 3’-terminus indicates 2’-O-methylation in plant miRNA, and AF488 represents AlexaFluor 488. The solid underlined sequences of the probes are complementary to the corresponding solid underlined sequences in the target miRNAs, while the dot underlined sequences in the probes are complementary to the dot underlined sequences in target miRNAs. The bold sequences in Probe C represent the HCR trigger.

2. **Standard procedures of the dFlowSeim platform for the detection of ath-miR156a**

Probe A (5’-NH$_2$/3’-aza-DBCO, ~6 × 10$^5$ molecules) was firstly immobilized on the Epoxy MBs (~1.45 × 10$^5$ beads) by incubating at 37 °C for 24 h to form the Probe A-MBs complexes according to the instruction of Epoxy MBs. Then the MBs were rotated in 200 mM Tris for 4 h at 37 °C to block the vacant surface area. After magnetic purification, the Probe A-MBs complexes were further mixed with Probe B (5’-N$_3$/3’-OH, ~6 × 10$^5$ molecules) and various amounts of target ath-miR156a in 10 µL of 10 mM phosphate buffer (PBS, containing 0.3 M NaCl and 0.01% Tween-20, pH 7.4). The mixture was incubated for 2 h at room temperature with gentle rotation to conduct the target-mediated CNAL. Bridged by the target ath-miR156a, each Probe B with 3’-OH end would be immobilized on an MB.

The MBs anchored with ligation product were further dispersed in 10 µL of TdT reaction buffer containing 0.5 U of TdT and 0.5 mM dTTP. The mixture was incubated at 37 °C for 1 h under gentle shaking to conduct the on-bead TdT-catalyzed terminal DNA polymerization. Then, 10 nM of 5’-FAM-labeled Probe C whose sequence contains both a poly(A) sequence and HCR trigger domain was introduced followed by a further incubation at 4 °C for 1 h. Afterward, the MBs were magnetically separated and incubated with 10 nM H1 and 10 nM H2 in PBS buffer (50 mM PB containing 0.75 M NaCl, pH 7.4) for 4 h at room temperature with rotation to conduct the branched HCR. Finally, the fluorescence signals of MBs in each sample were analyzed one-by-one on an FCM (FACSCalibur, BD Biosciences, analyzed in FL1 channel), and the fluorescence-positive/negative MBs were thus counted respectively based on a pre-set threshold for the digital analysis of miRNA.
3. The Poisson distribution of Probe A on the MBs with a 1:4 molar ratio of MBs/Probe A

According to the dFlowSeim mechanism, the number of Probe A molecules conjugated to the beads follows the Poisson distribution:

\[
P(\kappa) = \frac{\lambda^\kappa e^{-\lambda}}{\kappa!}
\]

where \( P(\kappa) \) and \( \lambda \) represent the probability of conjugating \( \kappa \) molecules to beads and the average number of Probe A on the beads. When the molar ratio of MBs/Probe A is controlled at 1:4, \( \lambda = 4 \).

According to the Poisson equation, \( P(\kappa=0) = 0.0183 \); \( P(\kappa=1) = 0.0733 \); \( P(\kappa=2) = 0.1465 \); \( P(\kappa=3) = 0.1954 \); \( P(\kappa=4) = 0.1954 \), therefore, \( P(\kappa \geq 1) = 0.9817 \). This result indicates that more than 98% MBs are conjugated with at least one Probe A with an extremely low density.

4. Determining the percentage of singlets

In the proposed dFlowSeim strategy, the quantification of the target molecule is based on counting the number of the singlet fluorescence-positive MBs. To expel the potential interference from the small amount of inevitable aggregated MBs, only the signal of singlets is gated for analysis. Fig. S1 shows the SSC vs FSC plots of the pure M-270 Epoxy MBs, the MBs involved in the blank control, as well as the MBs treated with ath-miR156a in the dFlowSeim system. It can be seen that the percentage of singlets in the three kinds of beads are identical. This result indicates that the single molecule-induced terminal extension and branched amplification will not affect the monodispersity of the MBs.
**Fig. S1.** The SSC vs FSC scattering plots of the pure M-270 Epoxy MBs, the MBs involved in the blank control as well as the MBs treated with ath-miR156a in the dFlowSeim system.

**5. Investigation on the efficiency of miRNA-templated click nucleic acid ligation (CNAL)**

Besides the results shown in Fig. 2, another set of experiments are also conducted to determine the efficiency of miRNA-templated chemical nucleic acid ligation. First, a series concentrations (0.5~7.5 nM in the 10 μL reaction system, much lower than the maximum biotin-ODN loading capacity of the used M-270 Streptavidin MBs) of standard fluorescent ODN (SFODN, 5′-AF488-AACTATAACCTACTACCTCATTTTTTTTTTTTTTTTTTTT-biotin-3′) were incubated with ~1.45 ×10⁵ STV-MBs at room temperature for 1h. Then the fluorescent signals were analyzed with a flow cytometer (Fig. S2a). The linear calibration curve between the mean fluorescence intensity (MFI) and the concentrations of SFODNs can be adopted to quantitatively determine the amount of ath-miR156a-templated chemical ligation products (Fig. S2b).

To evaluate the ligation efficiency, two probes that can hybridize with the two moieties of ath-miR156a were designed. Probe 1 (P1) was functionalized with Aza-DBCO at 5′-end and biotin at 3′-terminus, Probe 2 (P2) was functionalized with AF488 and N₃ at 5′ and 3′-termini, respectively. At first, 5 nM P1 was immobilized on ~1.45 ×10⁵ STV-MBs under room temperature. Then ath-miR156a samples of 1 nM and 2 nM were separately introduced to conduct the chemical ligation with the concentration of P2 kept at 5 nM. Afterward, the fluorescent signals were analyzed with a flow cytometer (Fig. S2c). We can see that the fluorescence signals of MBs treated with 1 nM and 2 nM ath-miR156a approximate to those of MBs immobilized with 1 nM and 2 nM SFODNs. According to the standard calibration curve obtained from Fig. S2b, the ligated products templated by 1 nM and 2 nM ath-miR156a are determined to be 0.96 nM and 1.88 nM, suggesting that the miRNA-templated ligation efficiency is ~96% and ~94% respectively. Such results clearly indicate the high efficiency of the miRNA-templated chemical ligation.
Fig. S2  (a) Fluorescence histograms of the STV-MBs loaded with different concentrations of SFODNs. (b) The standard calibration curve between the mean fluorescence intensity (MFI) of the MBs and the concentrations of immobilized SFODNs. (c) Fluorescence histograms of the STV-MBs loaded with ath-miR156a-templated ligation product in the presence of 1 nM and 2 nM ath-miR156a, respectively.

6. Illustration of the amplification efficiency of TdT-HCR

The rational design of the single target molecule-induced signal amplification strategy is the key to introducing sufficient fluorescence on one single MB to make it bright enough. To illustrate the amplification efficiency of the dFlowSeim, the experiment to compare the amplification efficiencies of TdT-HCR and TdT was conducted. In this experiment, except for the HCR process, other conditions were all identical to the standard procedure of the dFlowSeim. From Fig. S3a and b, it can be seen that just a neglectable number of MBs are fluorescence-positive when only TdT is conducted without HCR, whereas considerable positive MBs can be identified (Fig. S3c and d) when the MBs were treated with standard TdT-HCR procedures of dFlowSeim. Therefore, the TdT-HCR amplification is capable of introducing sufficient fluorescent molecules to the surface of the MB. In this way, the single MB can be illuminated by a single target miRNA.
Fig. S3 (a-b) FSC vs FL1 scattering plots of the MBs in the absence (a, blank control) and the presence of 20 fM target (b) with only TdT-based amplification (without HCR). (c-d) FSC vs FL1 scattering plots of the MBs in the absence (a, blank control) and the presence of 20 fM target (b) with standard TdT-HCR procedures of dFlowSeim.

7. Optimization of the experimental conditions in the dFlowSeim assay

To obtain the best assay performance, the concentration of Probe B and the amount of TdT were optimized by using ath-miR156a as the model target.

Fig. S4 Effect of the concentration of Probe B on the proposed dFlowSeim platform for ath-miR156a analysis. (a) FSC vs FL1 scattering plot of singlet Probe A-MBs. The vertical line represents the threshold set for distinguishing the positive and negative MBs, which completely isolates the singlet Probe A-MBs as fluorescence-negative ones. (b) FSC vs FL1 scattering plots of the MBs in the presence of different concentrations of Probe B: (I) 20 fM; (II) 40 fM; (III) 100 fM; (IV) 150 fM; (V) 200 fM. Top panel: blank control samples. Bottom panel: samples with 20 fM ath-miR156a added. (c) The relationship between the positive MBs produced by the blank controls and the samples, and the
concentration of Probe B. Other conditions: TdT, 1 U; dTTP, 0.5 mM. FL1 Voltage for the flow cytometer measurement, 515V.

The concentration of Probe B has a crucial effect on ligation efficiency. Therefore, the optimization of Probe B was firstly conducted. The results are shown in Fig. S4. According to the FSC vs FL1 scattering plot of pure MBs displayed in Fig. S4a, a threshold was rationally set to isolate all Probe A-MBs as negative ones. From Fig. S4b and c, it can be seen that the number of positive MBs produced by 20 fM miRNA targets increases gradually as the concentration of Probe B increases from 20 fM to 100 fM, and then keeps almost invariable. Meanwhile, the positive MBs’ number of the blank control (without ath-miR156a) also displays a slow increasing tendency with the increase of Probe B, probably because the increased Probe B may lead to some nonspecific ligation. Considering both the low background and high signal-to-background ratio, 100 fM Probe B (with a ratio of ~4:1 to the number of the Probe A-MBs) was used in the subsequent experiments.

The amplification efficiency relies on the TdT-mediated extension, which would also affect the fluorescence signal and the discrimination of positive/negative MBs. Thus, the dosage of TdT was also optimized in the dFlowSeim. As shown in Fig. S5, the numbers of positive MBs produced by 20 fM target miRNA increase sharply with the increase of TdT dosage from 0.2 U to 5 U. Meanwhile, the fluorescence-positive MBs proportion of the blank controls also displays an increasing trend with the increasing amount of TdT. Considering both the low nonspecific background and the highest target-induced signal-to-background ratio, 0.5 U TdT was selected as the optimum in this dFlowSeim assay for miRNA analysis.

**Fig. S5.** Effect of the dosage of TdT on the proposed dFlowSeim strategy for ath-miR156a analysis. (a) FSC vs FL1 scattering plot of singlet pure MBs. The vertical line represents the threshold set for distinguishing positive and negative MBs, which completely isolates the pure MBs as fluorescence-negative. (b) FSC vs FL1 scattering plots of the MBs with different dosage of TdT: (I) 0.2 U; (II) 0.5 U;
(III) 1 U; (IV) 2 U; (V) 5 U. Top panel: blank control samples. Bottom panel: samples with 20 fM ath-miR156a added. (c) The plot between positive MBs produced by the blank and the samples, and the dosages of TdT. Other conditions: Probe B, 100 fM; dTTP, 0.5 mM. FL1 Voltage for the flow cytometer measurement, 515V.

8. The linear relationship between the proportion of positive MBs ($P_{\text{exp}}$) and the ath-miR156a concentration ($C_{\text{ath-miR156a}}$)

As shown in Fig. S6, there is a good linear relationship between the $P_{\text{exp}}$ and $C_{\text{ath-miR156a}}$, and the corresponding calibration is $P_{\text{exp}} (\times 100) = 3.75C_{\text{ath-miR156a}}$ (fM) + 0.48 with the correlation coefficient $R^2=0.9937$. Therefore, the ath-miR156a in real samples can also be quantitatively determined following the obtained standard calibration curve.

![Graph showing the relationship between proportion of positive MBs and ath-miR156a concentration](image)

**Fig. S6** The relationship between the proportion of positive MBs and ath-miR156a concentration by using the dFlowSeim. The error bars represent the standard deviation of three replicates for each data point.

9. Fluorescence imaging by a confocal microscope to support the results of dFlowSeim

The digital detection mode of the dFlowSeim is also verified with fluorescence imaging results acquired on a confocal microscope. For the fluorescence imaging test, after the TdT-HCR amplification, the MBs are directly dropped onto a glass cover slide for imaging. The fluorescence images are obtained on an Olympus FV1200 confocal laser scanning microscope. Then, all the images are treated with ImageJ based on a pre-set threshold to discriminate the
positive and negative MBs. The threshold value of 22 is employed on the basis that all MBs of the blank control are just undetectable. Finally, for each image, the ratio of fluorescence-positive (bright) MBs to that of the total MBs (in bright filed image) can be recorded. As shown in Fig. S7, it is evident that the more ath-miR156a is added, the more fluorescent MBs can be observed in the fluorescence images. The ratios of positive MBs/total MBs obtained from fluorescence images agree well with those obtained from the FCM analysis at different concentrations of ath-miR156a.

**Fig. S7** Fluorescence imaging results of the MBs after single target molecule-initiated TdT-HCR amplification in the presence of 0 (blank), 5 fM, and 20 fM ath-miR156a. It can be seen that no MB can be illuminated for the blank control (a), while 19% MBs become fluorescently observed when 5 fM ath-miR156a is added (b). When ath-miR156a concentration is further increased to 20 fM, ~78% MBs are lightened up (c).

10. The original FSC vs FL1 scattering plot for selectivity evaluation
11. Quantification of ath-miR156a in total *Arabidopsis thaliana* RNA by dFlowSeim and qRT-PCR

We implement the dFlowSeim platform for ath-miR156a quantification in the total RNA sample extracted from *Arabidopsis thaliana*. Following the standard assaying procedures established, 20.8% positive MBs are identified when 0.5 ng total RNA is introduced to the reaction system. The content of ath-miR156a in the 0.5 ng total RNA is directly determined to be 5.28 fM based on the dFlowSeim. To test the accuracy of the dFlowSeim platform, 10 fM of standard synthetic ath-miR156a was spiked into 0.5 ng of total *Arabidopsis thaliana* RNA. The ath-miR156a in the spiked sample is determined to be 15.81 fM, showing a recovery rate of 105.3%.

Furthermore, the ath-miR156a amount in the same batch of total *Arabidopsis thaliana* RNA sample is also detected by using a standard real-time qPCR method. First, the synthetic standard ath-miR156a was detected with a commercial qRT-PCR kit (TaqMan® MicroRNA Assays Kit, Applied Biosystems, U.S.A.), following the recommended procedures of the manufacturer. Then the calibration curve was constructed between $C_t$ values obtained from the StepOne Real-Time PCR system (Applied Biosystems, U.S.A.) and the logarithm (lg) of synthetic standard ath-miR156a concentrations (Fig. S9a). Finally, the amount of ath-miR156a in the same batch of total RNA samples can be determined by the qRT-PCR.
according to the constructed calibration curve. From Fig. S9b, it can be seen that the result agrees well with the dFlowSeim strategy, suggesting the dFlowSeim is reliable for quantification of nucleic acid target in complex biological samples.

![Fig. S9](image)

**Fig. S9** (a) Standard calibration curve of the qRT-PCR protocol for the detection of ath-miR156a, which is constructed by using series dilutions of synthetic ath-miR156a standard; The relative standard deviation (RSD) values calculated from three parallel measurements for each data point are all less than 10%. (b) The determined amount of ath-miR156a in 0.5 ng total *Arabidopsis thaliana* RNA by using the qRT-PCR protocol and the dFlowSeim assay, respectively.

### 12. Analytical performance of the dFlowSeim platform for the digital detection of let-7a

According to the working principle, the proposed dFlowSeim platform is universally applicable for the detection of both plant and human miRNAs. To test this point, this platform is further applied to the detection of let-7a miRNA. All the reaction conditions are the same as those for detecting ath-miR156a, except that the let-7a-specific ligation probes are employed. As can be seen from Fig. S10, the number of positive MBs gradually increases with increasing amounts of let-7a in a good linear relationship (Fig. S10h). Moreover, as low as 500 aM let-7a can be distinctly detected. These results well support the generality of the dFlowSeim platform for precisely assaying different miRNA species.
Fig. S10 (a-g) FSC vs FL1 scattering plots of the MBs in the presence of different concentrations of let-7a. From (a) to (g): 0 (blank control), 500 aM, 1 fM, 5 fM, 10 fM, 15 fM, and 20 fM. (h) The relationship between the proportion of positive MBs and let-7a concentrations. The error bars represent the standard deviation of three replicates for each data point.

We have also evaluated the let-7a concentration in the total RNA extracted from HCT-116 cells. The let-7a in 50 pg of total RNA sample was quantitatively determined to be 6.65 fM (in 10 μL) by the dFlowSeim strategy, while the let-7a level in 50 pg of the same batch of RNA extract determined by a commercial qRT-PCR Kit (Applied Biosystems) was 6.35 fM. The detection results are consistent.