MicroRNA detection with flow cytometry and fluorescent beads

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Abstract

MicroRNAs (miRNA) are small non-coding RNA that regulate translation. The presence of miRNA in human blood can be indicative of certain diseases. This study aimed to detect miRNA at low concentrations (1nM to 1 pM) through the use of fluorescent beads and flow cytometry. If successful, the method can provide a sensitive technique to detect miRNA, with possible applications in diagnostics. Two biotinylated RNA probes, each with half of the complimentary sequence of the target miRNA, were bound to two streptavidin-coated beads that fluoresce at different wavelengths. The probes were then placed in a sample solution containing miRNA and allowed to hybridize with the target. The sample was analyzed using flow cytometry by detecting fluorescence signal. When both probes are hybridized to the target miRNA, the flow cytometer will detect a signal that fluoresces at two different wavelengths. This signal indicates that miRNA is present in the solution. A titration with synthetic miRNAs was performed in order to determine the limit of detection (LOD) of the developed method, but miRNA was not detected in these samples.

Introduction

miRNA

Micro ribonucleic acids (miRNA) are short, non-coding RNA that function in post-transcriptional regulation of gene expression. The miRNA will bind to target mRNA and prevent the translation of this mRNA. The presence of specific miRNA in the blood serum may serve as a biomarker for various disease, including cancer and cardiovascular diseases (Reid *et al*., 2011).

Flow Cytometry

Flow cytometers are instruments used to examine properties of biological particles. They use laser-based technology that can exploit the properties of fluorescent molecules and allows thousands of measurements to be made within a few seconds. Flow cytometry can be used to examine whole cells, cell constituents, beads, and other particles. A solution containing the particle to be examined is passed through a tube in such a way that the particles pass through the tube one at a time. A laser (the excitation light source) is shone at a point on this tube, and it will strike the particles as they pass through the tube. This causes light scattering and possibly florescence, which are measured by detectors. Light scattering provides information on the size of the particle, while florescent data allows the detection of fluorescent markers (Jaroszeski and Radcliff, 1999).

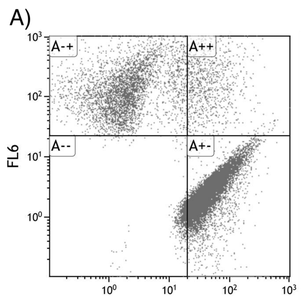
miRNA detection

A flow cytometer cannot detect miRNA due to its extremely small size, but can detect beads. Theoretically, if the beads are attached to the miRNA, the miRNA can be detected using the flow cytometer. In order to attach the beads to miRNA, RNA probes and the extremely stable bond between streptavidin and biotin are used. Two biotinylated RNA probes, each with half of the complimentary sequence of the target miRNA, will each be attached to separate streptavidin-coated beads that fluoresce at specific wavelengths. When miRNA is in the sample, the two probes will bind to the miRNA. Because both these probes are attached to fluorescent beads, when the sample is passed through the flow cytometer, there will be an event with fluorescence at two different wavelengths if the probes are both bound to miRNA. A surfactant is added to the solution to prevent the probes from interacting with each other when not bound to miRNA.

Methods

Green and red ScreenCORE streptavidin-coated beads (from Chemicell) that are 1µm in size were mixed with separate biotinylated RNA probes to allow the streptavidin-biotin bond to form. The resulting bead and probe complexes were then added to a solution containing miRNA and a control solution that did not contain miRNA. These solutions were incubated on a shaker for 20 minutes at 37°C to allow hybridization to take place. The miRNA solution was then divided into smaller portion to test three different surfactants (triton, BSA, and tween) at different concentrations. After the appropriate surfactant had been chosen, a miRNA titration was performed. The bead and probe complex was added to solutions containing various amounts of miRNA and a control solution containing no miRNA. The solutions were then incubated on a shaker for 20 minutes at 37°C to allow hybridization to take place, and the samples were analyzed with flow cytometry.

Results



Gate	Number	%Total	%Gated
All	20,000	100.00	100.00
A--	770	3.85	3.85
A-+	6,888	34.44	34.44
A+-	11,401	57.01	57.01
A++	941	4.71	4.71

Gate	Number	%Total	%Gated
All	20,000	100.00	100.00
A--	763	3.82	3.82
A-+	5,566	27.83	27.83
A+-	10,822	54.11	54.11
A++	2,849	14.25	14.25

Figure 1. A) Flow cytometry results for a sample containing the fluorescent beads, 0.01% triton and no miRNA. **B)** Flow cytometry results for a sample containing the fluorescent beads, 0.01% triton and miRNA.

The experiments performed to detect miRNA using flow cytometry failed to consistently detect miRNA in solution. To indicate that miRNA was present, there should be a significantly higher amount of events that fluoresced at both wavelengths (a double positive) in a sample containing miRNA compared to a sample containing no miRNA (a control). This result was only obtained when testing which surfactant would best suit these experiments. In these experiments, 100nM miRNA was present in the solutions. The results obtained from flow cytometry analysis for the sample using 0.01% triton as surfactant are presented in **figure 1**. In this case, it is evident that the portion of events in the double positive quadrant for the miRNA sample (14.25%) is higher than the events in the double positive quadrant for the control sample (4.71%). Similar results were obtained for each surfactant and are presented in **figure 2**. Using 0.01% triton as the surfactant caused the largest difference in double positive events between the miRNA sample and control sample.

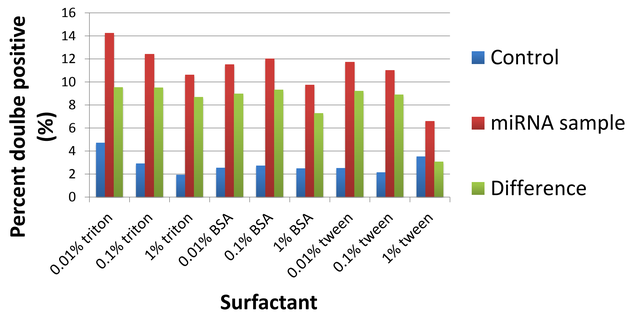


Figure 2. Flow cytometry results for surfactant testing: percentage of double positive events versus surfactant used.

After the surfactant was chosen, the miRNA titration was performed, and during this phase of the experiment the detection of miRNA failed. In the results obtained there was no significant difference between the amount of double positive events in the miRNA containing samples and the control samples, with the biggest difference between the two at 0.8 (**figure 3**). No noticeable trend was observed, and the amount of double positive events increased as the concentration of miRNA decreased. An additional miRNA titration was performed, but the results obtained were similarly inconclusive.

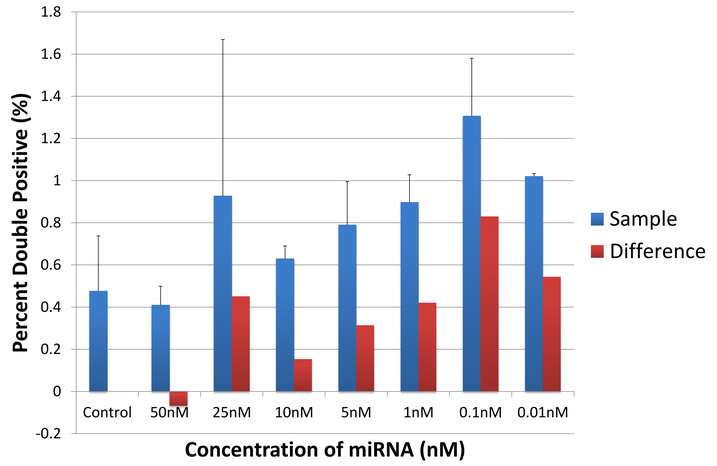


Figure 2. Flow cytometry results for miRNA titration: percentage of double positive events versus concentration of miRNA. The difference column represents the difference in the percentage of double positive events between the sample and the control.

Conclusions

The consistent detection of miRNA using fluorescent beads and flow cytometry was not achieved through these experiments. While testing various surfactants, the method proved successful in detecting miRNA. However, during the miRNA titration performed to find a limit of detection, the method failed in detecting miRNA even in samples with high miRNA concentrations (50nM). The concentration of miRNA in the solutions for surfactant testing (100nM) was higher than the concentration of miRNA in any of the solutions for the titration, which may indicate that this method requires a high concentration of miRNA. If this were the case, it would greatly limit the utility of the method. However, experiments performed in this lab by Nasrin Khan, which were identical to these except that they used magnetic fluorescent beads, have been successful. This indicates that detection at concentrations lower than 50nM should be possible, and that the ScreenCORE beads may have caused the problem. To discover were the problem is occurring, further miRNA titrations with varying conditions would be necessary. If the detection of synthetic miRNA were successful, the next steps would involve applying this method to detect miRNA in blood serum samples to verify that the method could be applied to a biological sample.

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References

Jaroszeski, M.J. and Radcliff, G. (1999). Fundamentals of Flow Cytometry. Molecular Biotechnology 11:37-53.

Reid, G., Kirschner, M.B. and van Zandwijk, N. (2011). Circulating microRNAs: Association with disease and potential use as biomarkers. Critical Reviews in Oncology/Hematology 80:193-208.