Video Article

The Visual Colorimetric Detection of Multi-nucleotide Polymorphisms on a Pneumatic Droplet Manipulation Platform

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Abstract

A simple and visual method to detect multi-nucleotide polymorphism (MNP) was performed on a pneumatic droplet manipulation platform on an open surface. This approach to colorimetric DNA detection was based on the hybridization-mediated growth of gold nanoparticle probes (AuNP probes). The growth size and configuration of the AuNP are dominated by the number of DNA samples hybridized with the probes. Based on the specific size- and shape-dependent optical properties of the nanoparticles, the number of mismatches in a sample DNA fragment to the probes is able to be discriminated. The tests were conducted via droplets containing reagents and DNA samples respectively, and were transported and mixed on the pneumatic platform with the controlled pneumatic suction of the flexible PDMS-based superhydrophobic membrane. Droplets can be delivered simultaneously and precisely on an open-surface on the proposed pneumatic platform that is highly biocompatible with no side effect of DNA samples inside the droplets. Combining the two proposed methods, the multi-nucleotide polymorphism can be detected at sight on the pneumatic droplet manipulation platform; no additional instrument is required. The procedure from installing the droplets on the platform to the final result takes less than 5 min, much less than with existing methods. Moreover, this combined MNP detection approach requires a sample volume of only 10 μ l in each operation, which is remarkably less than that of a macro system.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54424/>

Introduction

Single-nucleotide polymorphism (SNP), which is a single base-pair difference in a DNA sequence, is one of the most common genetic variations. Current studies report that SNPs are associated with disease risk, drug efficacy and side-effects of individuals by affecting gene function.^{1,2} Recent studies also revealed that two- or multi-point mutations (multi-nucleotide polymorphism) cause particular diseases and individual differences in the effects of disease.^{3,4} The detection of nucleotide polymorphism is therefore imperative in prescreening disease. Simple and efficient methods for the rapid detection of sequence-specific oligonucleotides were highly developed in the past two decades.^{1,5} Current approaches to identify DNA mutations typically involve procedures including probe immobilization, fluorescence labeling, gel electrophoresis, etc.^{6,7} but those methods generally require a long analytical process, expensive equipment, well trained technicians, and significant consumption of samples and reagents.

A nanoparticle with a large ratio of surface area to volume and unique physicochemical properties is an ideal material as a highly sensitive and cheap detection platform for specific biomarkers. Gold nanoparticles (AuNP) are widely used for DNA detection because of their great ability to be modified with oligonucleotide probes.⁸⁻¹⁰ SNP detection techniques were also developed using AuNP.¹¹⁻¹³ In this work we adopted a novel colorimetric approach to detect the multi-nucleotide polymorphism (MNP) through DNA hybridization-mediated growth of AuNP probes.¹⁴ This simple and rapid probing method is based on the theory that varied lengths of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) conjugated to AuNP influence the growth size and shape of the AuNP (see **Figure 1**).¹⁵ This method of DNA detection features a small consumption of reagents, a small assay duration (a few minutes), and a simple procedure without thermal control that is prospectively applicable for clinical diagnosis and domestic medical screening.

Several microfluidic systems to detect the DNA sequence have been developed;¹⁶ those microfluidic systems, evolved from traditional experimental protocols, required fewer pieces of large-scale equipment and simplified the experimental protocols so as to improve the sensitivity, detection limit and specificity of the DNA biosensor. DNA detection methods in the microfluidic systems still require, however, instruments of a subsequent process such as a PCR (polymerase chain reaction) machine for signal amplification and a fluorescence reader for a single readout to identify the heterogeneous SNP.^{17,18} Developing a simple platform without the subsequent processing to directly read out the results of multi-nucleotide polymorphism is highly desirable. Compared to well used, conventional, closed microfluidic systems, the open-surface microfluidic devices promisingly offer several advantages, such as a clear optical path, an easy way to access the sample, a direct environmental accessibility and no easily formed cavitation or interfacial obstruction in the channel.¹⁹ Our previous work introduced a simple pneumatic platform for open-surface droplet manipulation (see **Figure 2**).²⁰ On this platform, droplets can be simultaneously transported and manipulated without

interference from a driving energy using a suction force, which has a great potential in biological and chemical applications. This pneumatic platform was thus utilized to execute the manipulation of DNA samples for MNP detection in combination with the colorimetric approach using the concept of DNA hybridization-mediated growth of AuNP probes.

The protocol presented in this paper describes a simple visual detection of multi-nucleotide polymorphisms on the pneumatic droplet manipulation platform on an open surface. This work confirms that multi-nucleotide polymorphism is detectable with the naked eye; the proposed pneumatic platform is suitable for biological and chemical applications.

Protocol

1. Method to Detect MNP

Note: This section describes the procedure to detect the MNP based on the hybridization-mediated growth of gold nanoparticles.

1. Prepare the probe DNA (5'-thiol-GAGCTGGTGGCGTAGGCAAG-3') solution at a concentration 100 μ M.
2. Prepare the probe DNA-modified AuNP (AuNP probe) particles.²¹
Note: The volume used here depends on the requirement of probe DNA-modified AuNP (AuNP probe) particles. It is hence dependent on the number of experiments to be conducted. We typically prepare excess AuNP probe particles as spares. The volume used in these steps is adjustable and flexible.
 1. Add probe DNA (100 μ M, prepared in step 1.1) to a solution of gold nanoparticles (13 nm, 17 nm) at concentration 1 OD/ml.
 2. Bring the final concentrations of sodium dodecyl sulfate ($\text{NaC}_{12}\text{H}_{25}\text{SO}_4$, SDS) and phosphate buffer (PBS) to 0.01% and 0.01 M, respectively.
 3. After 20 min, bring the concentration of sodium chloride (NaCl) to 0.05 M with a solution of NaCl (2 M) and PBS (0.01 M) while maintaining SDS at 0.01% and incubate for 20 min.
 4. Increase the NaCl concentration in increments of 0.1 M to a final concentration of 1 M over 20 min intervals.
 5. Incubate overnight with shaking on a vortex mixer at 23 °C. The shaking speed does not affect the experiment as long as the DNA samples and AuNP probes become hybridized.
 6. Centrifuge the gold nanoparticles at 7,000 x g for 30 sec and remove the supernatant.
3. Add AuNP probe particles into deionized water (DI water) at a concentration of 25 nM (AuNP probe solution).
4. Prepare target DNA samples (fully complementary: 5'-CTTGCCTACGCCACCAGCTC-3'; three base-pair mismatched: 5'-CTTGCCTACTTTACCAGCTC-3'; six base-pair mismatched: 5'-CTTGCCTTTTTTCCAGCTC-3') at concentrations of 0.06, 0.11, 0.17, 0.20, 0.30, 0.50 μ M, respectively.
5. Add AuNP probe solution (6 μ l, 25 nM) into the DNA samples (350 μ l) with NaCl (14 μ M).
6. Shake the mixture of DNA samples and AuNP probes with a vortex mixer at 23 °C for 5 min for DNA hybridization.
7. For AuNP growth, add NH_2OH (6 μ l, 400 mM) and HAuCl_4 (6 μ l, 25.4 mM) to the solution (the mixture of DNA samples and AuNP probes). The growth of AuNP takes approximately 30 sec for completion. The steady change of coloration maintains more than 1 hr.
CAUTION: Skin contact with HAuCl_4 might produce severe toxic effects. Please consult the material safety data sheets (MSDS) before use. Please wear gloves and use a fume cupboard when using HAuCl_4 .

2. Fabrication of a Pneumatic Droplet Manipulation Platform

Note: The PDMS-based droplet manipulation platform comprises two components: a PDMS membrane (100 μ m) with a super-hydrophobic surface and an air-channel layer (5 mm). Without a MEMS process, the common machining processes were utilized to fabricate this device, which includes computer-numerical control (CNC) micromachining for making a mold, PDMS casting and replication for rapid prototyping of the microfluidic components, and laser micromachining for the fabrication of a super-hydrophobic surface (see **Figure 3**).

1. Use the CNC machine equipped with a drill bit (0.5 mm) to produce PMMA-based master molds (see **Figure 3**) with microstructures. Use a feed speed of the drill bit 7 mm/sec and a rate of rotation 26,000 rpm. Use an air blower and DI water to remove the PMMA scrap and to clean the surface of the master molds.
2. Prepare a mixture of the PDMS base and the curing agent in ratio 10:1. Degas the mixture inside a desiccator for 30 min, or until all air bubbles are removed.
3. Pour the PDMS into the master mold and bake the PDMS for 3 hr at 60 °C in the oven to obtain the inverse microstructures of the air chambers (PDMS membrane) and air channels (PDMS layer).
4. Remove the PDMS layer from the master mold (by hand). Punch holes on the PDMS layer for air-suction inlets.
5. Put the PDMS membrane, the PDMS layer and the glass substrate into the chamber of the oxygen-plasma treatment system. After oxygen-plasma treatment, bond the PDMS membrane, the PDMS layer and the glass substrate together on a hotplate (90 °C) for 10 min.
6. Put the composite chip into the laser-cutting machine (PDMS membrane side up). Import the .dwg file to define the superhydrophobic area (15 mm x 12 mm). See **Figure 3**. Directly engrave the PDMS membrane with a CO_2 -laser machine to create the superhydrophobic area on the PDMS membrane (power 12 W, scanning velocity 106.68 mm/sec).
7. Clean the superhydrophobic surface with DI water to wash away the carbonized residues on the surface.
8. Connect the air-suction inlet and vacuum pump through a solenoid valve (see **Figure 4**). Connect the solenoid valve to the computer and control with a digital I/O USB module.

3. Operation for Detection of MNP on the Pneumatic Platform

Note: This section describes the operation to identify colorimetrically and rapidly the MNP on the pneumatic droplet manipulation platform (see **Figure 5**). All steps take place at 23 °C (ambient temperature) and relative humidity 85%.

1. Place the target DNA sample droplet (10 μ l, 0.5 μ M) on the superhydrophobic area of the pneumatic droplet manipulation platform.
2. Place the AuNP probe droplet (10 μ l, 25 nM) on the superhydrophobic area of the pneumatic droplet manipulation platform.
3. Control the air suction to cause the deflection of the PDMS membrane with a solenoid valve and a simple program (e.g., Labview). Provide air suction in each air chamber along the migration path of the droplets (containing target DNA and AuNP probes respectively) on the PDMS membrane such that the droplets collide with each other and coalesce. Use a working pressure of the vacuum pump of -80 kPa (gauge pressure).
 1. Alternatively, control the air suction manually as long as the droplet is fully mixed. By hand, connect or disconnect the vacuum pump and inlets of air chamber with tubes in the early stages of testing.
4. Control the air suction to roll the coalesced droplet forward and backward to enhance the mixing efficiency for 3 min using the controlling system of the solenoid valve. The target DNA and AuNP probes become well mixed and hybridized within 3 min. Use a driving frequency and a working pressure of 5 Hz and -80 kPa (gauge pressure), respectively.
5. Place a droplet containing NH_2OH (2 μ l, 400 mM) and HAuCl_4 (2 μ l, 25.4 mM) on the superhydrophobic area of the pneumatic droplet manipulation platform.
6. Control the air suction (-80 kPa gauge pressure) to let the droplets collide with each other and coalesce. Then control the air suction (5 Hz and -80 kPa gauge pressure) to let the coalesced droplet roll forward and backward to enhance the mixing efficiency for 1 min.
7. Measure and record the color of the coalesced droplet with the naked eye.

Representative Results

In this work, three DNA samples were tested using a simple and novel method of detection through the DNA hybridization-mediated growth of the AuNP probes. The sequences of probe DNA and DNA samples of three kinds, specifically, CDNA (fully complementary to probe DNA), TMDNA (three base-pair mismatched DNA), and SixMDNA (six base-pair mismatched DNA) are listed in Protocol step 1. The mismatches to the probe of the DNA samples tested here are both in the middle segments of the DNA samples. **Figure 6** shows the colors of the growth AuNP solutions for the DNA samples at varied concentration. For the perfectly matched DNA (CDNA), the hues of solutions vary from pink to indigo and then to transparent with increasing DNA concentration. For TMDNA, the color of solutions goes from pink to dark purple and then to indigo, and for SixMDNA, the color goes from pink to dark pink with increasing DNA concentration. The separate DNA samples possess varied hybridization affinities to the probe DNA that caused varied growth size of AuNP and varied color of solutions. The TMDNA sample, with three base-pair mutation from the probe, has less hybridization affinity than the CDNA sample, which caused a smaller growth size of the AuNP. As the results in **Figure 6** show, there are distinctions of color between CDNA and TMDNA samples, especially at a large DNA concentration. Through many mismatches with the probe, SixMDNA sample has a weak hybridization affinity to the probe, which resulted in a few dsDNA conjugated to AuNP, even with the DNA concentration greater than 0.2 μ M. The growth size of AuNP is consequently small in this SixMDNA sample, causing the AuNP solution to change from pink to amaranth, which is not obviously measured with a naked eye. Based on the color images, the CDNA, TMDNA and SixMDNA are roughly differentiated for a DNA concentration from 0.11 to 0.50 μ M.

With the protocols and methods presented here, a simple and novel droplet-based manipulation platform for open-surface microfluidics was demonstrated. Using pneumatic suction as a force to generate the surface deformation of the PDMS membrane, the facile transport and manipulation of droplets can be achieved without interruption to the bio-sample, DNA in this case, in the droplets (see **Figure 7A**). The new and visual detection of MNP is demonstrated on this pneumatic open-surface droplet manipulation platform. As a result as shown in **Figure 7B**, the detection of a DNA mismatch is readily observable with the naked eye. In the original state, the AuNP probe droplet exhibited a red color. The maximum optical absorption of AuNP probes occurs at 520 nm, which is attributed to a surface-plasmon resonance (SPR). After hybridization with the various DNA sample droplets, the target-probe DNA duplex of AuNP with a fully complementary DNA (CDNA) sample droplet became transparent as the SPR feature of the increased AuNP disappeared. In contrast, AuNP hybridized with the three base-pair mismatched DNA (TMDNA) and six base-pair mismatched DNA (SixMDNA) were blue and purple respectively. With this approach, the proposed platform and method of DNA detection are combined and achieved in a simple way.

Figure 1. Principle of colorimetric detection of MNP. The thiol-modified single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) from the hybridization of the thiol-modified ssDNA on AuNP influenced the growth size and shape of the AuNP, and caused varied optical properties of the AuNP. [Please click here to view a larger version of this figure.](#)

Figure 2. Schematic diagram of the pneumatic droplet manipulation platform. With a pneumatic suction as a force to cause a deflection of the flexible PDMS-based superhydrophobic membrane, the droplet on the membrane becomes thereby activated. [Please click here to view a larger version of this figure.](#)

Figure 3. Fabrication of a pneumatic droplet manipulation platform. The fabrication included computer-numerical-control (CNC) micromachining, PDMS casting or replication techniques, and laser micromachining. [Please click here to view a larger version of this figure.](#)

Figure 4. Experimental setup of the pneumatic droplet manipulation platform. The vacuum pump generated an air suction to provide a diminished pressure in the air chamber and to cause the deflection of the PDMS membrane for droplet manipulation. The vacuum pump and the inlet of the air channel are connected through the solenoid valve. [Please click here to view a larger version of this figure.](#)

Figure 5. Schematic illustration of the operation of this MNP detection technique. The droplets containing target DNA and the AuNP probes are loaded on the platform, mixed and hybridized. The droplet containing both NH_2OH and HAuCl_4 is loaded on the platform and mixed for AuNP growth. Finally, the target-probe DNA duplex of AuNP shifted the absorption maximum and altered the color. [Please click here to view a larger version of this figure.](#)

Figure 6. Variation of color of various DNA samples with concentration. For the fully complementary DNA (CDNA), the hues of solutions vary from pink to indigo with increasing DNA concentration. For TMDNA, the color of solutions goes from pink to dark purple, and for SixMDNA, the color goes from pink to dark pink with increasing DNA concentration. [Please click here to view a larger version of this figure.](#)

Figure 7. Images of droplet on the pneumatic platform. **A)** Serial images of droplet transport on the pneumatic platform with control by air suction. **B)** Images of the corresponding target-probe DNA duplex of AuNP on the proposed pneumatic droplet manipulation platform. The operating conditions of the driving frequency and the working pressure were 5 Hz and -80 kPa (gauge pressure), respectively. In the original state, the AuNP probe droplet exhibits a red color. The target-probe DNA duplex of AuNP in the presence of the CDNA was transparent. The AuNP probes hybridized with the TMDNA and SixMDNA were blue and purple respectively. [Please click here to view a larger version of this figure.](#)

Discussion

In this protocol, a simple colorimetric method to detect MNP can be implemented at concentrations ranging from 0.11-0.50 μM in microcentrifuge tubes. Furthermore, the proposed MNP detection method is conducted on a pneumatic droplet manipulation platform that has a high potential for DNA screening and other bio-medical applications. In practice, the detectable range of the sample DNA concentration depends on the mixing efficiency of the operating platforms. To ensure that the coalesced droplet is fully mixed, the critical step in this protocol is the control of the air suction (pressure and frequency) that depends on the size of the coalesced droplet. The discrepancy of hybridization affinity between the fully complementary sample DNA and the mismatched sample DNA is difficult to distinguish with the naked eye for a long DNA sequence fragment. The limitation of this detection technique is hence the fragment length of the sample DNA. For samples with long DNA sequences, the sizes or conformation of the growth AuNP are similar to those of varied DNA samples containing various mismatches. The mismatches of DNA samples to the probe appear in the middle segment in this protocol. With identical lengths and homomeric mismatches of sample DNA, the position of the mismatches could also affect the hybridization affinity and growth size of AuNP. Many illnesses are currently being traced to particular defective genes. The genome databases for particular illnesses are still expanding and improving. Once the DNA sequence variation of the specific DNA fragment is confirmed to be responsible for a specific disease, the specifically designed probe (with an appropriate length and mismatch position of the DNA) can be used to acquire the number of mismatches of sample DNA to the probe within the detectable concentration range on the basis already tested in this work.

The proposed pneumatic droplet manipulation platform is performed in an open environment. The evaporation of droplets increases the concentration of DNA samples and affects the accuracy of the colorimetric readout. To improve the accuracy of MNP analysis, a controlled environmental temperature and humidity are required. Laser micromachining was applied to fabricate a superhydrophobic surface in the presented platform. The superhydrophobicity of the PDMS membrane does not persist under many iterations of the droplet manipulation (>20 times). The loss of superhydrophobicity decreases the mobility and maneuverability of this droplet manipulation platform. Once the droplet manipulation platform loses the superhydrophobicity on the surface, remodifying the superhydrophobicity of the PDMS membrane (repeat steps 2.6-2.8) is required. The simple fabrication of a more durable superhydrophobic surface is under future consideration. In this protocol, we used a solenoid valve and a simple controlling program (with data acquisition equipment) to control the air suction. The control of air suction can be utilized in other ways or with equipment including but not limited to use of a solenoid valve.

In this manuscript, we introduced the combination of a simple colorimetric method of DNA detection and a pneumatic droplet manipulation platform. Both techniques are unique and highly prospective; we found no alternative DNA detection method (that is both rapid and visual) to achieve the same results. Several droplet manipulation techniques on an open surface including optowetting,²² dielectrophoresis,²³ electrowetting,²⁴ vibration²⁵ and thermos-capillary actuation²⁶ have been reported. The drawbacks of all these droplet manipulation techniques were discussed in our previous work.²⁰ The proposed pneumatic platform is more biocompatible than the aforementioned methods.

This technique to detect multi-nucleotide polymorphisms on a pneumatic droplet manipulation platform is straightforward for researchers in the biochemical field, which means that samples and reagents can be directly loaded and collected with pipettes. The integration of the presented platform is being undertaken with an automatic control system and a design of a user interface for improved and wide use in the future.

Disclosures

The authors declare that they have no competing financial interests.

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