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Pneumatic nano-sieve for CRISPR-based detection of drug-resistant bacteria

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Experimental methods

Device fabrication

The pneumatically-regulated nano-sieve device consists of a channel-patterned glass substrate and a PDMS topping with a pneumatic chamber. Briefly, a cleaned 4-inch glass wafer (University wafer, D263, 550 µm, double side polished) was deposited by a layer of TEOS (PECVD, AME P5000) with a thickness of 200 nm. After spin-coating a thin layer (1 µm in thickness) of positive photoresist (AZ Mir 701), a plastic photomask (Fineline Imaging, CO, USA) was applied to transfer the channel pattern onto the photoresist layer. Followed by buffer oxide etching (BOE), the nano-sieve channel was created on the glass substrate, with a width of 5 mm and a length of 15 mm. To fabricate a pneumatic chamber of 2 mm in height and 2 mm in width, a PDMS mixture with one part of curing agent and ten parts of base polymer (SYLGARDTM 184, Krayden Inc., CO, USA) was poured into a three-dimensional (3-D) printed mold (Fictiv, CA, USA). After the curing process of PDMS in the oven at 60 °C overnight, the chamber layer was punched by a 1 mm puncher (INTEGRATM MiltexTM), for the pneumatic regulation by the air pump (Precigenome LLC, CA, USA). This chamber layer was bonded onto a thin layer of cured PDMS thin film (200 µm in thickness) via plasma treatment (Electro-Technic Products, IL, USA). Then the entire part was baked on the hot plate at 100 °C for 2 hrs to obtain the robust bonding in between. To finalize the fabrication of a functional device, the nano-sieve channel on the substrate was sealed by bonding with the pneumatic layer via plasma treatment and baking process on a hot plate. Before using the device for experiments, the holes of inlet and outlet were punched by a 1 mm puncher, so that the microfluidic tubing (Scientific Commodities,Inc., BB31695-PE/3) can connect the device to the sample sources in a syringe (BD 1 mL, NJ, USA).

Beads stacking functionalization

A strategy of creating the 3-D microstructure of stacked beads (Alpha Nanotech Inc., NPSSMX1, 10 mg/mL, 10 µm, 10 mL) was applied to physically capture the target bacteria from the initial sample. In this study, the nano-sieve channel was first rinsed and cleaned by isopropyl alcohol (IPA) solution. Before introducing the magnetic beads into the channel, the air pump was used for ensuring the pressure applied in the pneumatic layer consistently at 12 Psi. Then, a 50 µL of 10 µm magnetic beads with a concentration of 10 mg/mL was injected into the channel to form a "coarse filter" by employing a syringe pump with a flow rate of 30 µL/min. The injection of another 50 µL of 5 µm magnetic beads with a concentration of 10 mg/mL into the channel was followed, to form a "fine filter" under a flow rate of 20 µL/min. Finally, this functionalized nano-sieve device is ready for separating the target bacteria from the introduced sample solution.

Bacterial culture

Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus aureus* (ATCC 25923) were purchased from Fisher Scientific, Kanamycin-resistant *E. coli* 10798 and *E. coli* MG1655 were from lab stock. The bacteria were cultured in tryptic soy broth medium (MilliporeSigma) and maintained on the tryptic soy agar plate. After the overnight culture at 37 ℃ under 200 rpm, 1 mL of bacterial cells was centrifuged at 8000g for 5 min to form a pellet. The pellet was then resuspended in 1 mL of phosphate buffer saline (PBS) or a mixture of 1:4 of human plasma (MilliporeSigma) and PBS. This process was repeated twice to completely remove culture media. The concentration of the bacterial cells was determined by counting the colony-forming unit (CFU) on standard TSA plates. The harvested cells were diluted in PBS or a 1:4 mixture of plasma and PBS using a 10-fold dilution for future use.

Bacterial staining

Two microliters of *BacLight* green dye (MilliporeSigma) was mixed with 1 mL PBS-based bacteria solution, and the mixture was incubated at room temperature for 20 min by following the manufacturer's instruction. Then the solution was centrifuged at 8000g for 5 min to discard the PBS that consists of extra dye. The bacteria were resuspended in 1 mL fresh PBS for the further use.

Bacteria capture and concentration

The 600 µL of prepared sample solutions, in which the bacteria with a certain concentration were spiked in 1:4 diluted plasma solution, was first loaded into several 1 mm sterile syringes. A multi-channel

syringe pump was used to simultaneously introduce the sample solutions into the functionalized nano-sieve channels through the microfluidic tubing, under a flow rate of 4 µL/min. After completing the separation process, the air pump was stopped and a 30 µL of fresh PBS solution was applied for rinsing the entire channel to retrieve the magnetic beads and the captured targets, from the channel to a sterile centrifuge tube. A simple and direct separation with an external magnet was used for extracting the bacteria-involved PBS solution, which is ready for the further detection based on RPA/CRISPR technique. The concentration factor in this case is expected to be 20-fold.

Fluorescence microscopy imaging

After the completion of bacteria separation, 10 µL of each retrieved sample and initial sample were placed onto a glass slide for the measurement of cell density. Under the microscope equipped with a high-speed camera and the fluorescent light resource, the fluorescent images were captured and analyzed by using Leica LAS X software.

TEM Characterization

A JEOL 2100 transmission electron microscope (TEM) operating at 200 kV was used to image the samples. An aqueous solution of magnetic beads +/- DNA was deposited onto a carbon-coated copper TEM grid and left to allow evaporation of the water. Then, the dried grids were dipped into 18 Mega Ohm water for 30 s to remove excess salts. The images were then captured with a Gatan Orius camera.

Nucleic acid extraction

The nucleic acid preparation involves bacterial lysis and DNA purification, which was carried out at room temperature and completed in less than 30 min. To prepare the enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, and 1.2% Triton X-100), lysozyme (Thermo Fisher) was added at a concentration of 20 mg/mL immediately before use. Next, 18 µL of the enzymatic lysis buffer was added to 10 µL of the pathogens and incubated for 10 min. Following this, 2 µL of proteinase K (Thermo Fisher) was added, and the mixture was incubated for 5 min. For DNA purification, AMPure XP beads (Beckman Coulter) were used following the vendor's instruction with slight modification. Briefly, 54 µL of magnet beads were added to the lysate and incubated for 5 min. The tube was then placed on the magnet for 2 min to separate beads from the solution. Next, the beads were washed twice with 70 µL of 5 M Guanidinium chloride (MilliporeSigma), and the DNA was eluted with 20 µL of nuclease-free water (Thermo Fisher).

RPA amplification and CRISPR-Cas12a detection

TwistAmp$^{@}$ Basic kit was purchased from TwistDx^{TM}. The RPA primers, crRNA, AsCas12a, and fluorophore–quencher probes were all obtained from Integrated DNA Technologies, and detailed information about the synthetic oligonucleotides are listed in Table S1. The RPA primer sets were designed using PrimerQuest^{TM} Tool. Additionally, NEBuffer^{TM} r2.1 was purchased from New England Biolabs. The RPA reaction was conducted based on the instructions: A mixture of 29.5 µL of rehydration buffer, 11.2 µL of nuclease-free water, and 2.4 µL each of forward and reverse primers (10 µM) was added to the enzyme pellet. Then, 2 µL of purified DNA and 2.5 µL of MgOAc (280 mM) were added and mixed to achieve a total volume of 50 µL. The mixture was incubated at 37 °C for 20 min. Following the incubation, 2 µL of RPA amplicons were added to a pre-assembled CRISPR-Cas12a mixture comprising 50 nM of AsCas12a, 62.5 nM of crRNA , 10× buffer, and 2.5 µM of ssDNA-FQ probe, resulting in a final reaction volume of 20 µL. The reaction solution was incubated at 37 °C for 30 min. After the incubation, the mixture was excited by a blue light transilluminator (brand: SmartBlue, Part number: NEB-E4100, excitation wavelength of 465 nm) for naked-eye observation. Finally, 20 µL of nuclease-free water was added to 5 µL of the mixture, which was then characterized by an Agilent BioTek Cytation 5 imaging reader.

Table S1: List of synthetic oligos sequence used in this study

Name	Sequence (5′-3′)
crRNA 1	UAAUUUCUACUAAGUGUAGAUAGUUCUGCAGUACCGGAUUUG
Target 1	CAAATCCGGTACTGCAGAACT
F1	GATTAACCCAGTACAGATCCTTTCAATCTA
R1	ATAGCCATCATCATGTTTGGATTATCTTTATC
F2	TATGCAACAAGTCGTAAATAAAACACATAAAG
R2	TCATATGATATAAACCACCCAATTTGTCTGCC
crRNA 2	UAAUUUCUACUAAGUGUAGAUCUAGAGUAGCACUCGAAUUAG
Target 2	CTAGAGTAGCACTCGAATTAG
F3	AAACAAGCAATAGAATCATCAGATAACATTT
R3	TATAGATTGAAAGGATCTGTACTGGGTTAAT
F4	AAACAAGCAATAGAATCATCAGATAACATTT
R4	AAGGATCTGTACTGGGTTAATCAGTATTTC
ssDNA-FQ Probe	/56-FAM/TTATT/3IABkFQ