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Droplet based Microfluidic Approach and Microsphere-PCR Amplification for single stranded DNA Amplicons

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Dear Editor:

Enclosed please find the revised manuscript (Manuscript ID: 57703-R1) plus figure and table, submitted to JoVE. The revised manuscript that includes table and figures is prepared according to the guideline suggested by JoVE. It is the original work of the authors and all authors mutually agreed that it should be submitted to JoVE. This manuscript has not been published elsewhere and it is not being submitted for publication elsewhere.

We thank you for Editor' helpful comments. We carefully revised the manuscript on the basis of the editor' comments. We have improved our manuscript and believe that we have fully addressed the editor' recommendations.

Very truly yours,

Ok Chan Jeong, Ph.D.

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TITLE:

A Droplet-Based Microfluidic Approach and Microsphere-PCR Amplification for Single-Stranded DNA Amplicons

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KEYWORDS:

Copolymerization, Microfluidic channel, Flow-focusing Microsphere-PCR, ssDNA Amplification, Asymmetric PCR

SUMMARY:

This work provides a method for the fabrication of droplet-based microfluidic platforms and the application of polyacrylamide microspheres for microsphere-PCR amplification. The microsphere-PCR method makes it possible to obtain single-stranded DNA amplicons without separating double-stranded DNA.

ABSTRACT:

Droplet-based microfluidics enable the reliable production of homogeneous microspheres in the microfluidic channel, providing controlled size and morphology of the obtained microsphere. A microsphere copolymerized with an acrydite-DNA probe was successfully fabricated. Different methods such as asymmetric PCR, exonuclease digestion, and isolation on streptavidin-coated magnetic beads can be used to synthesize single-stranded DNA (ssDNA). However, these methods

cannot efficiently use large amounts of highly purified ssDNA. Here, we describe a microsphere-PCR protocol detailing how ssDNA can be efficiently amplified and separated from dsDNA simply by pipetting from a PCR reaction tube. The amplification of ssDNA can be applied as potential reagents for the DNA microarray and DNA-SELEX (Systematic evolution of ligands by exponential enrichment) processes.

INTRODUCTION:

Single-stranded DNA (ssDNA) has been extensively considered as a molecular recognition element (MRE) due to its intrinsic properties for DNA-DNA hybridization[1,2]. The development of ssDNA synthetic systems can lead to biological applications such as DNA microarrays[3], oligotherapeutics, diagnostics, and integrated molecular sensing based on complementary interactions[4,5].

To date, micrometer-scale polymer particles have been successfully demonstrated using microfluidic devices. Several microfluidic techniques have been proven to be powerful for producing highly homogenous microspheres on continuous flow in the microchannel environment[6,7].

In the study of Lee *et al.*[8], a droplet-based microfluidic platform for the microfluidic synthesis of copolymerizable oligo-microsphere and ssDNA amplification was reported. The microfluidic platform consists of two PDMS (polydimethylsiloxane) layers: an upper part with a microfluidic channel network for generating microsphere and a bottom flat part. These consist of three kinds of PDMS fluidic channels: 1) a flow focusing channel for droplet generation, 2) a serpentine channel for mixing two solutions, and 3) a sequential polymerization channel for microsphere solidification. Once two immiscible flows are introduced into a single PDMS fluidic channel, the flows can be forced through the narrow orifice structure. The flow behaviors such as channel geometry, flow-rate, and viscosity affect the size and morphology of the microsphere. Therefore, the main liquid stream can be divided into microscale monospheres[9,10].

Here, a detailed microsphere-PCR protocol is provided for the amplification of ssDNA. First, a droplet-based microfluidic device design process is described. Then, the way in which polyacrylamide microspheres can be functionalized with random DNA template in a complementary manner is explained. Finally, a microsphere-PCR protocol for amplifying ssDNA is shown.

PROTOCOL:

1. Fabrication of a PDMS Microfluidic Platform

1.1. Prepare 20 mL of liquid PDMS prepolymer by mixing base polymer and catalyst in a volume ratio of 10:1. Pour 10 mL of the liquid PDMS onto a prepared SU-8 mold on a silicon wafer for the upper part of the microfluidic network. For the bottom flat part, pour the same volume of liquid

PDMS on the silicon wafer without a mold structure.

Note: The microfluidic network is designed in a CAD program and then converted into a photomask in order to fabricate a master using the typical photolithography process (See **Supplemental Figures**). This master is comprised of the negative photoresist SU-8 mold on the silicon wafer[11].

1.2. Place two silicon wafers coated with liquid PDMS prepolymer on the hot plate and cure at 75 °C for 30 min.

1.2.1. Manually peel off the cured PDMS layer from the SU-8 mold. Align a 1.5 mm diameter round hole punching tool to the oil port on the replicated microfluidic network for interfacing micron-scale flow channels with the macro fluid samples. Punch out the through-hole manually.

1.2.2. Repeat this punching process three times for the formation of the two solutions and the outlet port.

1.3. Perform hydrophilic surface treatment on both the upper and bottom PDMS layers using a hand-held corona treater[12] for several seconds per sample.

1.3.1. Stack two plasma-treated PDMS layers and heat at 90 °C for 30 minutes using a hot plate for the PDMS-to-PDMS bonding process. Supply the pressurized water using the syringe pump into three inlet ports for the structural bonding and leakage testing of the fabricated device.

2. Production of Polyacrylamide Oligo-Microspheres

2.1. Prepare bead-mixture detailed in **Table 1**.

2.2. Vortex and briefly centrifuge the standard ssDNA acrydite labeled probe (Ap, 100 μM) and acrylamide:bis (19:1) stock solution.

2.3. Prepare solution I by mixing 25 μL of 40% acrylamide bis solution, 10 μL of ssDNA (acrydite probe), 10 μL of 5x TBE buffer (1.1 M Tris; 900 mM borate; 25 mM EDTA), and 5 μL of water. Prepare solution II with 50 μL of 20% ammonium persulfate.

2.4. Prepare two syringes individually filled with solution I and solution II, and mount them onto the pump to introduce solution flows into the microfluidic platform. Prepare mineral oil mixed with 0.4% TEMED for surface solidification of the microsphere.

Note: TEMED is well-known as a free radical stabilizer. Free radicals can accelerate the rate of polymer formation with ammonium persulfate (APS) in order to catalyze acrylamide

polymerization.

2.5. Fill a glass bottle with 4 mL of the mineral oil to generate a continuous flow.

2.6. Insert two tubes to two ports in the cap of the glass bottle as a microfluidic reservoir: the pneumatic port for applying the compressed air into the glass bottle from the air compressor and the fluid port for supplying the pressurized oil to the micro channel network from the glass bottle. Connect tubes between the glass bottle and the oil port in the microfluidic device.

2.6.1. Connect the tubes to the two solution ports in the microfluidic device in order to supply the two solutions from the syringe pumps. Insert tubes to the outlet port in order to transfer the generated microsphere into the beaker.

2.7. Set the flow rate of the syringe pump to 0.4 - 0.7 mL/h. Adjust compressed air pressure of the compressor using a regulator (82 - 116 kPa). Set the rotational speed (500 rpm) of the magnetic stirrer bar in the glass beaker on a hot plate. Operate the syringe pump and supply the compressed air generated by an external compressor into the glass bottle using the ON/OFF control of an electromagnetic valve[13].

2.8. Observe the formation of microspheres in the flow-focusing geometry and solidification of generated microspheres in the glass beaker with a digital microscope.

Note: The size and production speed of the microsphere depend on the flowrate of solutions and the pressure applied for the mineral oil flow **(Table 2).**

3. Performing Polyacrylamide Oligo-Microspheres Counts

3.1. For hemocytometer quantification, take a small amount (approximately 100 µL) of aqueous solution of polyacrylamide oligo-microspheres and place on the glass hemocytometer, and gently fill the microsphere suspension up the well of the counting chamber.

Note: For further details, see http://www.abcam.com/protocols/counting-cells-using-a-haemocytometer.

3.2. Use a microscope and hand tally counter to count microspheres in one set of 16 squares. Then, move the hemocytometer to the next set of the chamber and carry on counting until all four sets of 16 corners are counted.

3.3. Determine the average microsphere count and calculate the number of microspheres in the original bead suspension.

3.4. Transfer 100 µL of microspheres to a 1.5 mL microcentrifuge tube. Remove the supernatant

through gentle centrifuging (400 x g) and pipetting.

4. Performing DNA Hybridization on the Surface of Polyacrylamide Oligomicrosphere

Note: An identical DNA probe with a 5'-NH_2-group instead of 5'-acrytide modification is added into solution I and tested for Ap-containing microspheres in parallel. DNA hybridization results are shown in **Figure 2**. The Cy3-labeled complementary oligonucleotide probes (cAp) solution should be placed in a dark room.

4.1. Resuspend Cy3-cAp using 100 μL of 1xTE buffer (TE buffer: 10 mM Tris and 1mM EDTA, pH 8.0) in order to achieve a final concentration of 100 μM. For example, resuspend 1 pmol of cAp in 100 μL of TE buffer and transfer to a microcentrifuge tube covered with aluminum foil.

Note: For strand sequences, see **Table 3**.

4.2. Add 100 μM of Cy3-cAp to a sterile 1.5 mL microcentrifuge tube containing Ap-copolymerized microspheres.

4.3. Tap the tube a few times to mix and incubate at room temperature in the dark for 1 h.

4.4. Discard the supernatant and remove residual buffer through pipetting.

4.5. Rinse three times with 500 μL of TE buffer.

4.6. Resuspend microspheres by gently tapping the microcentrifuge tube. Then, repeat step 4.4.

4.7. Place microspheres on the glass slide (75 mm x 50 mm) and cover with aluminum foil prior to imaging.

5. Asymmetric PCR for Amplifying ssDNA

5.1. Prepare an asymmetric PCR reagent mix for amplifying ssDNA to be analyzed. Thaw the reagents in **Table 4** on ice. Do not keep the Taq polymerase enzyme (50 U/μL) on ice, but rather store it at -20 °C until needed.

5.2. Gently vortex all reagents and then briefly centrifuge tubes at 10,000 x g for 10 s.

5.3. Combine all reagents as described in **Table 4.**

5.4. Place samples into a thermocycler and start the asymmetric PCR under the following conditions: 25 cycles (95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s), 85 °C for 5 min, hold at

4 °C.

6. Microsphere-PCR for Amplifying ssDNA

Note: This section describes the protocol for amplifying ssDNA in a PCR reaction tube. Microsphere-PCR reactions were performed in 50 µL of reaction volume. The detailed sequences used to amplify ssDNA are listed in **Table 5**. In this case, Ap on the surface of microspheres can anneal to random DNA templates in a complementary manner. This is a very important step for producing complementary DNA strands (antisense DNA strand, **Figure 3**). The DNA extended is used as a template for microsphere-PCR amplification.

6.1. Prepare microsphere-PCR reagent mix. Thaw the reagents in **Table 6** on ice; however, do not keep the Taq polymerase enzyme on ice. Store it at -20 °C until needed.

6.2. Gently vortex all reagents and then briefly centrifuge tubes at 10,000 x g for 10 s.

6.3. Obtain approximately ~25 microspheres through microscopic counting.

Note: The number of microspheres in one reaction tube are calculated using a light microscope with 40X magnification. About ~25 microspheres are used for microsphere-PCR amplification. More detailed information is in Step 3.

6.4. Combine all reagents as described in **Table 6.**

6.5. Place samples into a thermocycler and start the asymmetric PCR under the following conditions: 25 cycles (95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s), 85 °C for 5 min, hold at 4 °C.

6.6. Following amplification, add 8 µL of 6x loading buffer and load 15 µL of each sample into 2% agarose gel. Then, perform electrophoresis at 100 V for 35 min in 1x TAE (Tris-acetate-EDTA, 40 mM Tris acetate, 1 mM EDTA, pH 8.2) buffer.

7. Confocal Microscopy Acquisition

Note: The results of microsphere-DNA probe hybridization are imaged under a confocal microscope. Image analysis is performed using ImageJ.

7.1. Fix hybridized microspheres to the stage of the microscope in a holder.

7.2. Select the laser (Helium/Neon laser, 543 nm line) and turn it on in laser control.

7.3. Select the objective lens in microscope control.

7.4. Select the desired filter for Cy3 and channel in configuration control.

7.5. Start the experiment and observe the sample. Settings for the confocal microscope are summarized in **Table 7**.

REPRESENTATIVE RESULTS:

The fabricated polymeric droplet-based microfluidic platform consists of two PDMS layers (**Figure 1a**). Three kinds of microfluidic channel networks are used for generating microspheres: 1) Flow-focusing geometry as shown in **Figure 1b**, 2) a serpentine channel for mixing solution I and solution II, and 3) a polymerization channel for microsphere solidification. The height of all channels was 60 µm. The channel length for mixing and polymerization were 74.35 mm and 94.45 mm, respectively. The widths of the microchannel for two immiscible fluid flows and the one for mineral oil flow were 100 µm and 200 µm, respectively. The orifice structure used for microsphere formation was of 50 µm length and 25 µm width. The angle of the diffuser structure was 37°. A lab-based pneumatic control system for continuous flow of the mineral oil and two syringe-pumps for the solution flow (**Figure 1c**) were used for generating microspheres in the microfluidic platform (**Figure 1d**). The production speed of microspheres was about 30 microspheres per second when the flow rate of solutions and applied pressure were at 0.6 mL/h and 108 kPa, respectively (**Table 2**). Its diameter in the micro channel is 78.7 ± 2.5 µm. The on-flow synthesis of microspheres can be successfully manipulated using the microfluidic device. Bead sizes were measured after swelling. The average diameter of the resulting microspheres was 150.4 ± 12.8 µm. The size variations were about 8.5%. The microspheres undergo swelling in water, resulting in an enormous size increase.

The copolymerizable property of microsphere can be varied. We incorporated a 5'-acrydite-DNA probe into the microsphere solution (solution I, see **Table 1**). Following co-polymerization, a complementary DNA probe is labeled with fluorescent dye, Cy3, at room temperature for 1 h. Confocal microscopy can be used to prove the synthesis of copolymerizable oligomicrospheres as well as functional hybridization on the surface of microspheres. Fluorescent images of the microsphere are shown in **Figure 2**. If microspheres are correctly functionalized with the 5'-acrydite-DNA probe, they should result in coverage of fluorescent activity on the surface during the hybridization experiment. If copolymerization did not occur correctly, the optical microsphere image would exhibit internal-contamination inside the microspheres. As shown in **Figure 2**, there is no interference of random-interior orientation. This result allowed us to carry out the DNA probe presentation in a 3-dimensional (3-D) arrangement and microsphere-PCR. It should be noted that an identical DNA probe with a 5'-NH_2-group instead of a 5'-acrytide modification did not copolymerize during on-flow synthesis of microsphere[8].

When working with microspheres, manipulating the 3-D surface with a DNA oligo-probe is a much faster process[14]. Therefore, an on-flow microsphere synthesis platform can provide a tool for ssDNA amplification and purification, as outlined in **Figure 3**. The anti-sense DNA template (-,

complementary template) can be extended by adding a random DNA template (+, template). In this case, the initially copolymerized 5'-Ap provides a 3'-OH end for DNA polymerization after annealing to its complementary template (76 nt). Microsphere-PCR can be performed in a single PCR microtube using functionalized microspheres, a random DNA template, and a Tag-forward primer. In order to distinguish the resultant ssDNA amplicons from random DNA template (+ template, 76 nt), the forward primer has an additional 24 nucleotide tag. If the forward primer does not have an additional tag sequence, it is very hard to recognize between ssDNA amplicons and the initial random DNA template.

An asymmetric PCR experiment was performed. We were able to observe dsDNA contaminants as shown in **Figure 4**. In most cases, it is necessary to isolate ssDNA using streptavidin-coated magnetic beads and exonuclease digestion. However, microsphere-PCR makes efficient use of a single primer (Tag-forward primer) to accumulate ssDNA in aqueous phase. It is expected that dsDNA contaminants will attach to the surface of microspheres. Therefore, ssDNA amplicons can be obtained through pipetting without the need for centrifugation. The resultant ssDNA was demonstrated by comparing it to the synthetic size markers (76 nt ssDNA and 100 nt ssDNA) through gel electrophoresis analysis.

Figure Legends:

Figure 1. The microfluidic platform. (a) Fabricated microfluidic platform, (b) enlarged view of the flow-focusing geometry, (c) experimental set-up, and (d) Captured images showing continuous generation of microspheres. 1: Micro channel structure for flow focusing geometry, 2: for mixing solutions, 3: for microsphere solidification.

Figure 2. Fluorescent readout of DNA hybridization on the surface of microspheres. 5'-Acrydite-modified DNA probes (Ap) were capable of hybridizing with complementary Cy3 labeled DNA probes (cAp).

Figure 3. Illustration of the microsphere-PCR protocol.

Figure 4. **Comparison between conventional asymmetric PCR and microsphere-PCR.** M; ssDNA marker (76mer and 100mer), Lane 1; Asymmetric PCR, Lane 2; Microbeads-PCR. Reprinted with permission from previous work[8].

Table 1. Reagent mix components for on-flow polyacrylamide microsphere synthesis.

Table 2. Summary of operational conditions and produced microsphere.

Table 3. Sequence information of DNA probes.

Table 4. Asymmetric PCR reagent mix components in 20 μL reaction.

Table 5. Sequence information for Microsphere-PCR.

Table 6. Microsphere-PCR reagent mix components in 50 μL reaction.

Table 7. Settings for the confocal microscope.

DISCUSSION:

Contaminants of dsDNA are a major issue in ssDNA amplification. It remains difficult to minimize dsDNA amplification in conventional asymmetric PCR amplification[15]. In addition, although technical improvements for generating ssDNA have enabled us to increase the efficiency of sample throughput, ssDNA isolation is still problematic due to its high costs and incomplete purification yields.

Asymmetric PCR is one of the most challenging methods used when working with ssDNA. This method applies unequal amounts of primer (*e.g.*, 20: 1 ratio) in order to generate large amounts of ssDNA. However, it is very difficult to optimize every amplification reaction to yield ssDNA. Thus, the byproducts (ds-DNA) must be eliminated from the resultants[4].

In order to generate ssDNA without additional separation steps, we produced polyacrylamide microspheres to expose DNA probes based on the acrydite copolymerization method. Our DNA attachment method was easily adapted by using a droplet-based microfluidic platform. Copolymerized oligomicrospheres having microscale diameters were successfully produced. Consequently, microsphere-PCR was used to amplify the ssDNA in a single-tube reaction. Of course, to adapt this procedure for other PCR experiments, commonly necessary adjustments (*e.g.*, changing the amplification cycle, annealing temperature, and template sequences) are required. In conclusion, microsphere-PCR has been detailed here, making it available for bioassay development, DNA sequencing, and DNA microarray analysis.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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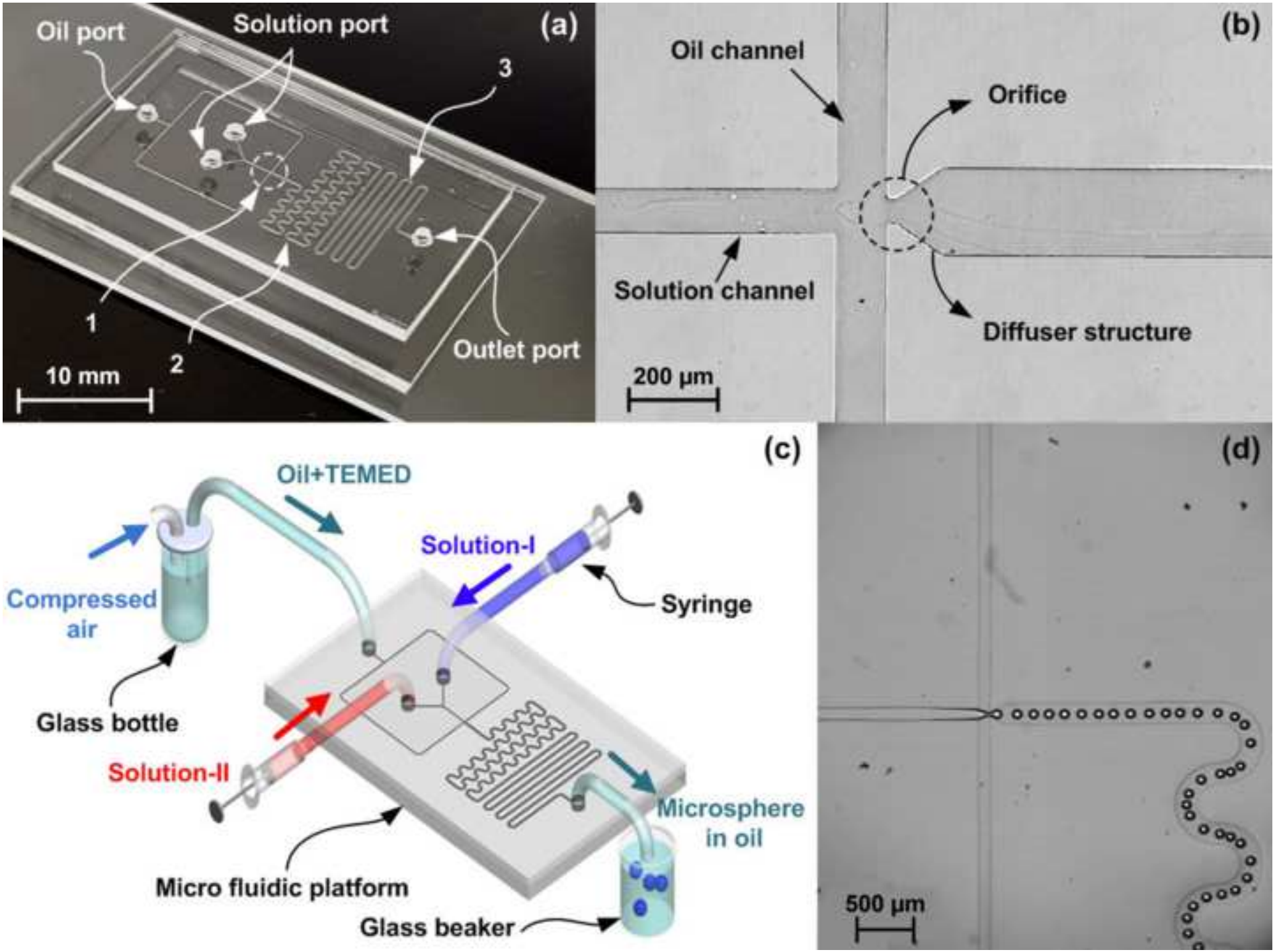

Figure 2

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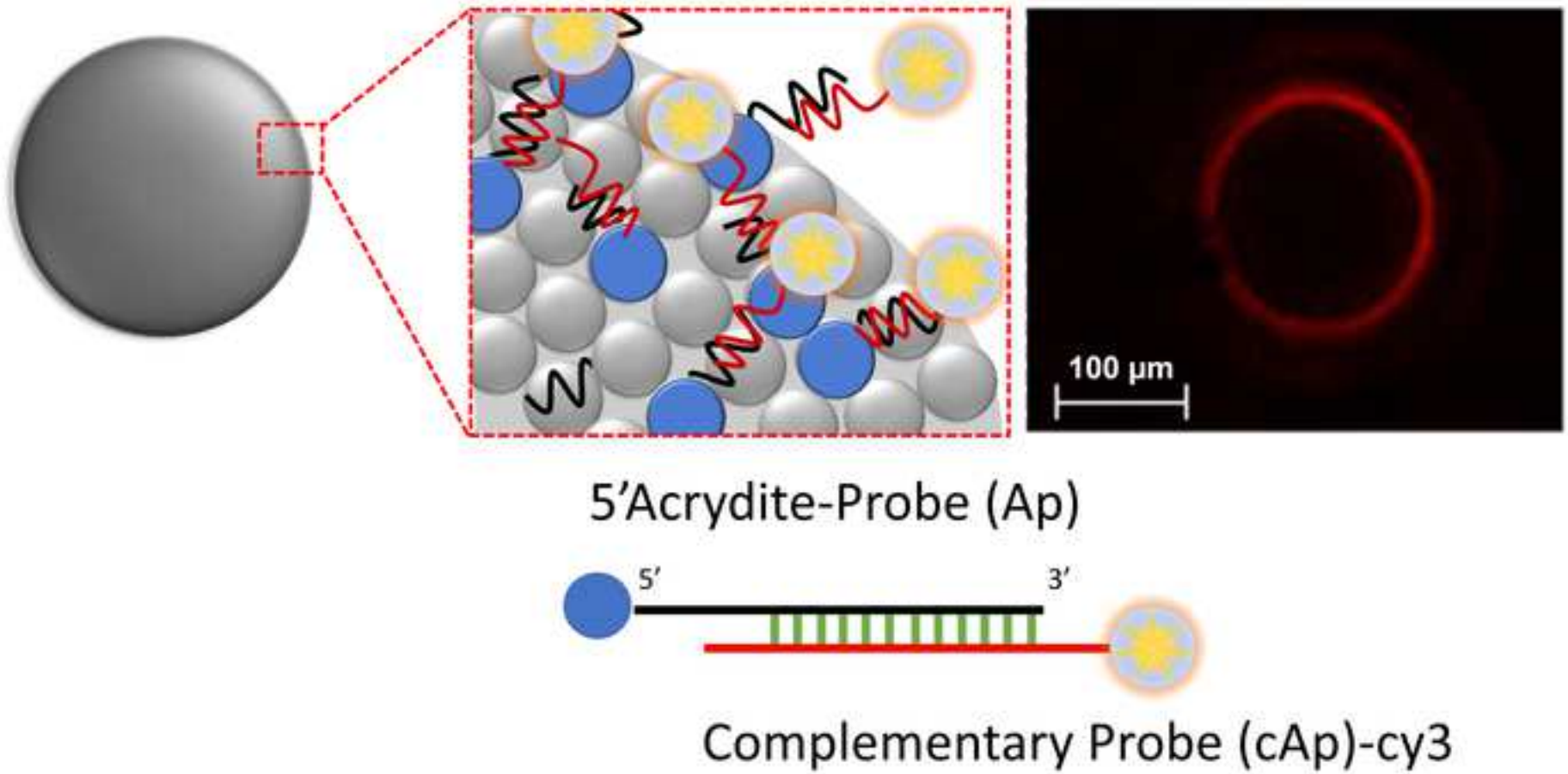

Figure 3

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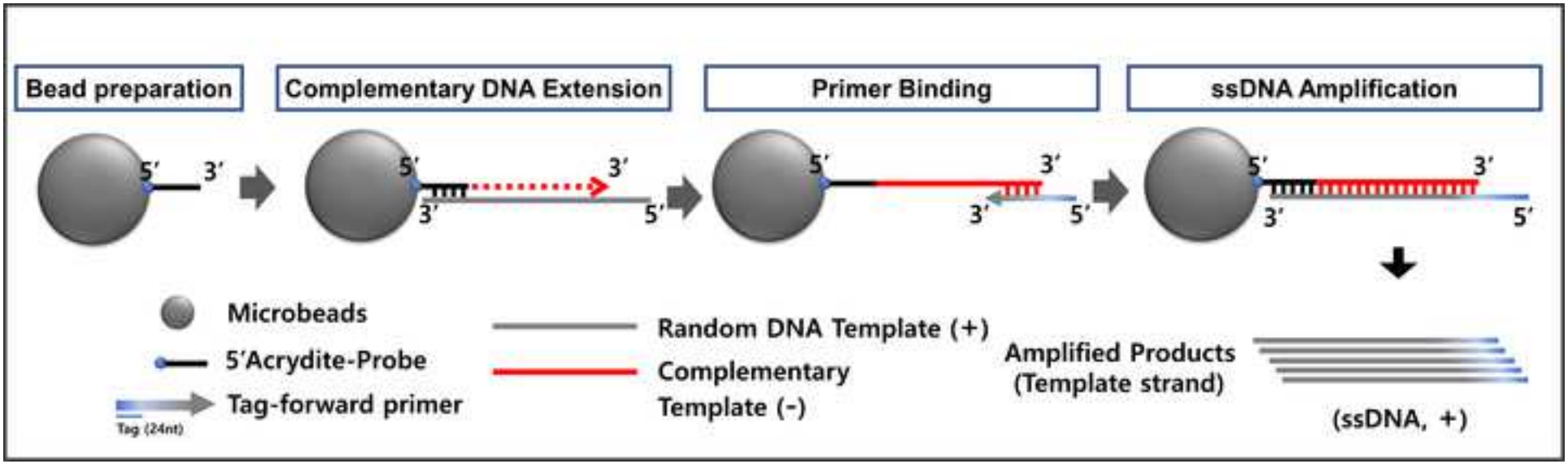

Figure 4

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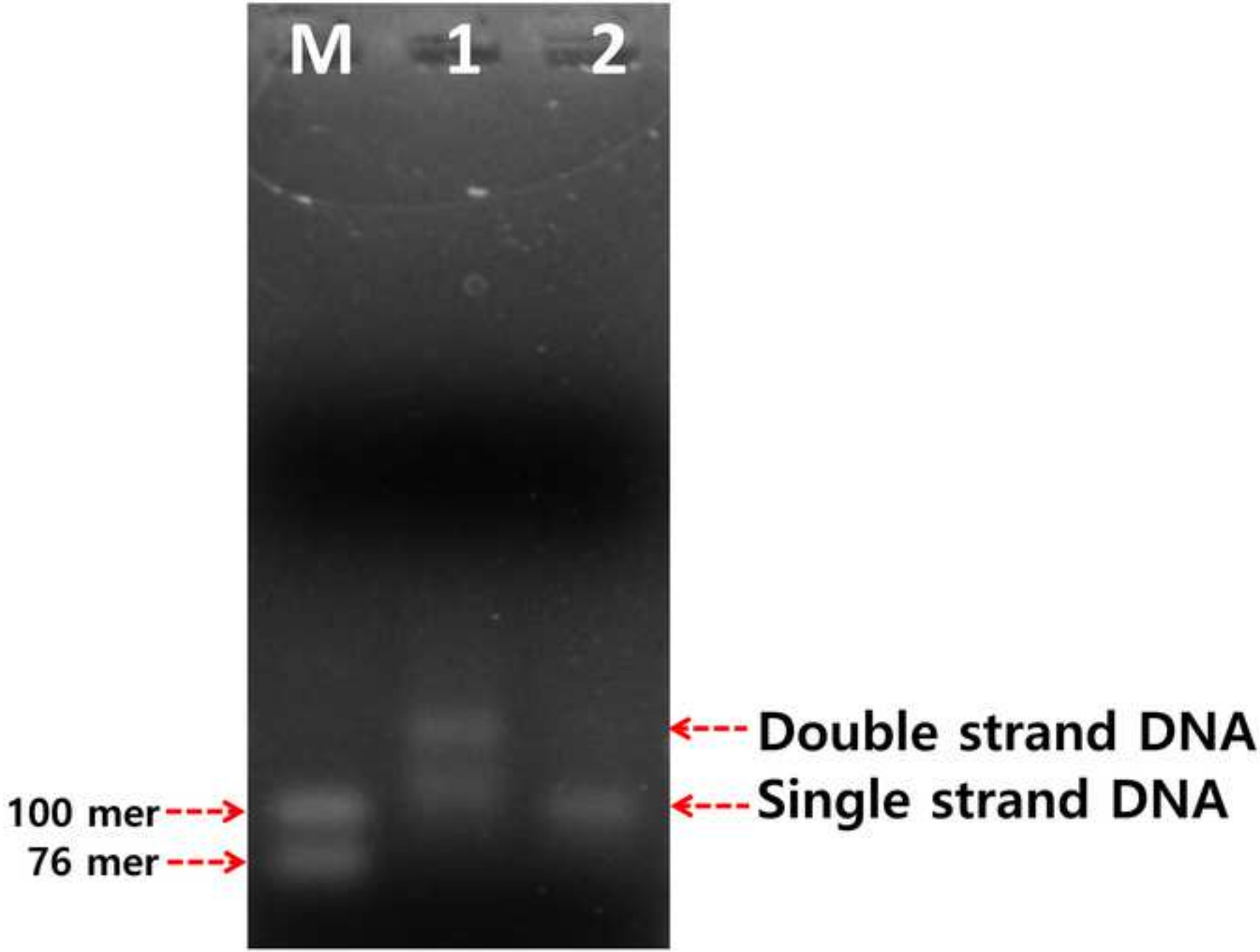

Table 1

	Reagent	Volume in mix (μL)	Final Concentration
Solution I	40% Acrylamide:bis solution (19:1)	25	10%
	100 μM Acrydite probe (Ap, 5'-Acrydite-(TTTTTTT, linker sequence) AGA TTG CAC TTA CTA TCT-3')	10	10 μM
	5X TBE buffer (Tris-base-EDTA)	10	0.5X
	Water	5	-
Solution II	20% Ammonium persulfate	50	10%
Solution III	TEMED (*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine)	8	0.40%
	Mineral oil	1000	-

Table 2

Operational condition		Microsphere production speed
Solution flowrate	**Oil pressure**	
(mL/h)	(kPa)	(/ sec)
0.4	82	7.1
0.5	94	13.3
0.6	108	28.9
0.7	116	36.5

Table 3

DNA probe	Sequences
Cy3 labeled complementary oligonucleotide probe (cAp)	5’-Cy3-AGATAGTAAGTGCAATCT-3’
Tag (first 24 nt)-forward primer	5’- GGT AAT ACG ACT CAC TAT AGG GAG ATA CCA GCT TAT TCA ATT-3’

Table 4

Reagent	Volume for 1x reaction (μL)	Final concentration
Random DNA template	1	1 ng/μL
Tag-forward primer	1	0.4 μM
Reverse primer	1	0.02 μM
10X Taq buffer	5	1X
10 mM of dNTP	4	2.5 mM
Ex taq (1000U)	0.2	1 U
Water	37.8	-
Total	50	-

Table 5 Click here to download Table Table 5 073018.xlsx

Template	Sequence	Length (nt)
Random DNA template	5'- ATA CCA GCT TAT TCA ATT (Random sequence, 40 mer) AGA TAG TAA GTG CAA TCT-3'	76
Tag-Forward primer (Tag-F)	5'- GGT AAT ACG ACT CAC TAT AGG GAG ATA CCA GCT TAT TCA ATT-3'	42
Reverse primer	5'- AGA TTG CAC TTA CTA TCT-3'	18

Table 6 Click here to download Table Table 6 073018.xlsx

Reagent	Volume for 1x reaction (μL)	Final concentration
Ap-Microspheres	~25 microspheres	-
Random DNA template	1	1 ng/μL
Tag-Forward primer	1	0.4 μM
10X Taq buffer	5	1X
10 mM dNTP	4	2.5 mM
Ex taq (1000U)	0.2	1 U
Water	37.8	-
Total	50	-

Table 7

Scan mode	Plane
Scaling	X: 2.49 µm, Y: 2.49 µm
Stack size	X: 1272.79 µm, Y: 1272.79 µm
Scan zoom	0.7
objective	EC Plan-Neofluar 10 x / 0.3 M27
Average	1
Pinhole	Ch2: 104 um
Filters	Ch3: LP 420
Beam splitters	MBS: HFT 488 / 543, DBS1: mirror, DBS2: NFT 515, FW1: None
Wavelength	543 nm 100.0 %

Name of Material/ Equipment	Company
liquid polydimethylsiloxane, PDMS	Dow Corning Inc.
40% Acrylamide:bis solution (19:1)	Bio-rad
Ammonium persulfate, APS	Sigma Aldrich
N,N,N',N'-Tetramethylethylenediamine, TEMED	Sigma Aldrich
Mineral oil	Sigma Aldrich
Cy3 labeled complementary oligonucleotide probes	Bioneer
ssDNA acrydite labeled probe	Bioneer
Tris	Biosesang
EDTA	Sigma Aldrich
Ex taq	Takara
Confocal microscope	Carl Zeiss
Light Microscope	Nikon Instruments Inc.
T100 Thermal Cycler	Bio-rad
Hand-held Corona Treater	Electro-Technic
Hot plate	As one
Syringe pump	kd Scientific
Compressor	Kohands
Bright-Line Hemacytometer	Sigma Aldrich

Catalog Number

Sylgard 184

1610140

A3678

T9281

M5904

synthesized

synthesized

T1016

EDS

RR001A

LSM 510

eclipse 80i

1861096

BD-20AC Laboratory Corona Treater

HI-1000

78-1100

KC-250A

Z359629

Comments/Description

Components of chip

Components of Copolymerizable oligo-microsphere

Hardener of acrylamide:bis solution

Catalyst of ammonium persulfate

Table 1. Solution III. Component of microsphere reagents

Table 3. Sequence information

Table 1. Solution I. Component of microsphere reagents

Components of TE buffer, pH buffer solution

Components of TE buffer, removal of ion (Ca^{2+})

ssDNA amplification

Identifying oligonucleotides expossure of microsphere surface

Caculating number of microspheres

ssDNA amplification

Hydrophilic surface treatment

heating plate for curing of liquid PDMS

Uniform flow of Solution I and Solution II

Flow control of Solution III

Caculating number of microspheres

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Title of Article: Droplet based Microfluidic Approach and Microsphere-PCR Amplification for single stranded DNA Amplificons

Author(s): Se Hee Lee, Ho Won Lee, Da Som Kim, Hyuck Gi Kwon, Jong Hyun Lee, Yang-Hoon Kim, Ok Chan Jeong, and Ji-Young Ahn

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Title: "Droplet based Microfluidic Approach and Microsphere-PCR Amplification for single stranded DNA Amplicons"

Author(s): Se Hee Lee, Ho Won Lee, Da Som Kim, Hyuck Gi Kwon, Jong Hyun Lee, Yang-Hoon Kim, Ok Chan Jeong, and Ji-Young Ahn

Editorial comments:

C1. Please proofread; there are still some grammar and usage errors.

[Response] English edit certificate is attached.

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Manuscript Title :

Droplet based Microfluidic Approach and Microsphere-PCR Amplification for single stranded DNA Amplicons

Manuscript Authors :

Ji-Young Ahn

Date of Issue: :

July 20, 2018

Yours truly,

HARRISCO

C2. Abstract: “Corrected by simple pipetting” isn’t clear; are you referring to the centrifuging/removal of supernatant step to get rid of dsDNA? This is also unclear in the last paragraph of the Results.

[Response]

This sentence is now changed as shown in below (Line 30-31):

- “~~corrected by pipetting from”

The result part (Line 289~290) is now modified as shown in below:

- Therefore, ssDNA amplicons can be obtained by pipetting without centrifugation step.

C3. 1.1: Can you provide the CAD file as supplemental information, or at least provide a schematic with more details/dimensions? You mentioned a reference in your response to Reviewer 2 ("Integrated Microfluidic Selex Using Free Solution Electrokinetics"), but that does not appear to describe the same device.

[Response] We provided the CAD file. See supporting information.

C4. 5.1/6.1: By the following reagents, do you mean the reagents in Tables 4/6 (respectively)?

[Response] we put the Note (Line 181) information. Table 4 shows the reagent information for the asymmetric PCR. Table 6 describes the reagent for the microsphere-PCR.

C5. Please highlight 2.75 pages or less of the Protocol that you want filmed; this will enable us to write a script that will guide the filming of your procedure. 2.75 pages is our limit due to filming time and video length limitations.

[Response] The part of protocol is highlighted with yellow color.

C6. Figure 1: Please separate numbers and units in the Figure itself (e.g., 10 mm instead of 10mm).

[Response] We separate the numbers and units in the Figures.

C7. Figure 2: Please explain the schematics in the legend. What excitation/emission wavelengths are used for the microscopy here?

[Response] The figure legend for Figure 2 is now modified. Cy3 wavelength is described in 7.2 (Line 231).

- Figure 2. Fluorescent readout of DNA hybridization on the surface of microsphere. 5’-Acrydite-modified DNA probe (Ap) were capable of hybridizing with complementary Cy3 labeled DNA probes (cAp).

C8. Figures: ‘Figure 1/Figure 2/etc.’ are still in the Figures themselves; please remove and additionally remove excess whitespace.

[Response] Figures are now changed and attached with TIFF format.

C9. Tables: Please remove all embedded tables from the manuscript and instead upload each individually as .xls/.xlsx files to your Editorial Manager account (there is an option to upload a ‘Table’, distinct from the Table of Materials).

[Response] All tables are now removed from the manuscript and they are now upload each individually as xlsx files.

C10. Discussion: Please include more information to help future readers decide whether this protocol fits their needs and to help them replicate it--critical steps, common troubleshooting procedures, limitations (of the protocol presented here, not other ones), and future directions.

[Response] Discussion is now improved.

In addition to the editorial comments, we corrected SI units according to the Jove author instructions. For example,

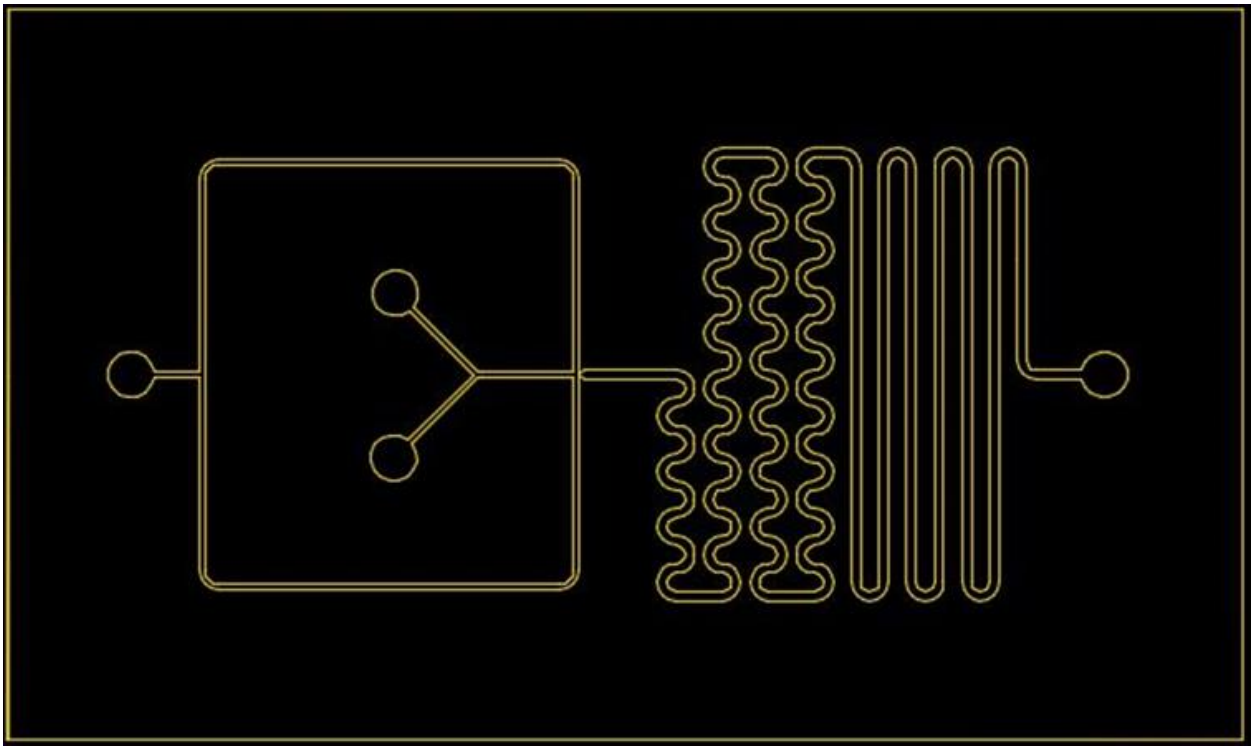
- ml → mL
- µl → µL
- hr → h

[Supporting Information]

Title: "Droplet based Microfluidic Approach and Microsphere-PCR Amplification for single stranded DNA Amplicons"

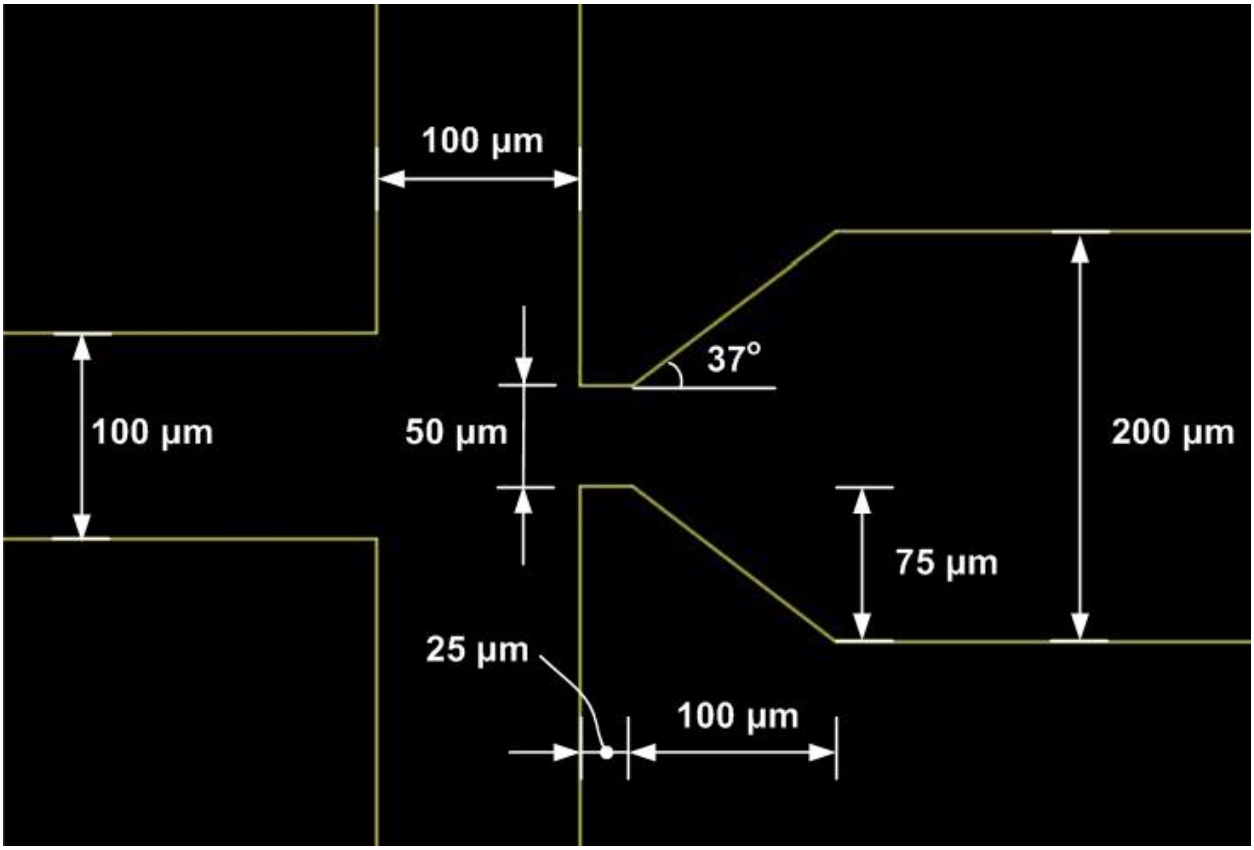
Author(s): Se Hee Lee, Ho Won Lee, Da Som Kim, Hyuck Gi Kwon, Jong Hyun Lee, Yang-Hoon Kim, Ok Chan Jeong, and Ji-Young Ahn

Supplementary Figure 1 illustrates the mask layout of the microfluidic network designed in CAD program. The length of the serpentine channel for mixing two solutions and the sequential polymerization channel for microsphere solidification are 59.29 mm and 66.25 mm, respectively.



Supplementary Figure 1. Mask layout of the microfluidic network.

Supplementary Figure 2 shows the magnified view of the flow focusing geometry in the microfluidic network.



Supplementary Figure 2. Magnified view of the flow focusing geometry.