Dot PCR fluorescence quantification method searching

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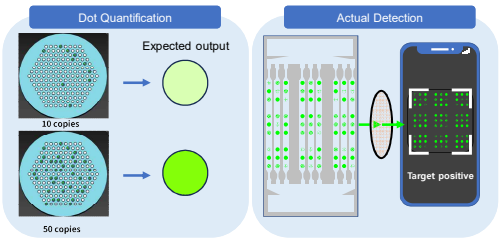
Introduction

In December 2019, the COVID-19 outbreak hit. The emergence of new strains of viruses had not been uncommon, but this one, with its high infectivity and lethality, posed an unparalleled threat to humanity. Diagnostics, let alone a cure, were not quickly established, and polymerase chain reaction (PCR), a method of massively replicating DNA, became synonymous with coronavirus testing. However, the coronavirus tests that we had been diligently swabbing our noses were limited by the fact that PCR requires specialized equipment and takes a long time. We focused on point-of-care testing (POCT) device to solve these limitations. The biggest challenge was the need for precise temperature control in a non-laboratory environment. Our key material to facilitate PCR diagnosis was Fluorescence.

For 8 weeks, Dot PCR quantification proof of concept was performed. The goal of dot PCR quantification is to observe difference in fluorescence signal intensity among multiple hydrogel points where each point has varying number of targets which will lead to various signal intensities according to it.

The principle is that each point will work as a single PCR pot where PCR happens separately. Thus, dot PCR will provide a platform that can measure the amount of initial target number by fluorescence intensity which is not provided by conventional PCR steps.

The advantage of this is that first, user can verify patients' severity according to the signal intensity. Second, signal depends on normalized quantification of each points which prohibit opportunity for false detection that will bias the result.

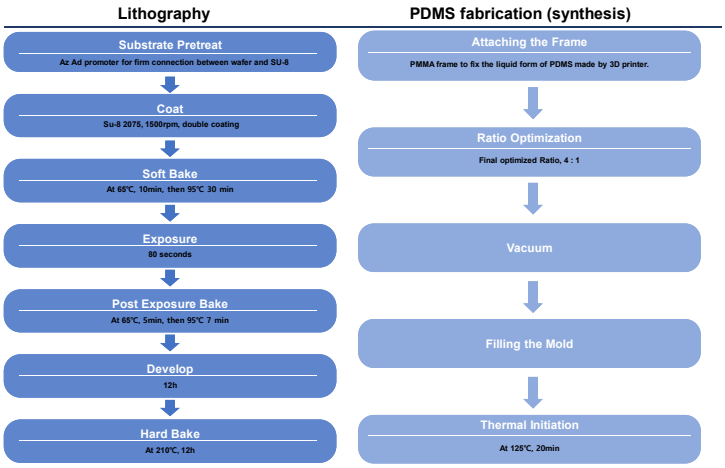


Experimental Method

1. Dot Quantification Mold

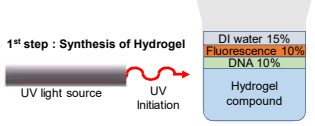
For the dot, we designed hexagonal pillars.
To achieve target volume, 1.7ml, Lithography was inevitable.

To Fabricate mold, PDMS were used.
The ratio and temperature were optimized for desired mechanical property.

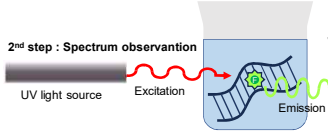


2. Dot Fluorescence Test

Multiple hydrogels will become Detection points.
We needed to guarantee that when DNA is successfully duplicated, Fluorescence shows enough signal intensity.
Therefore, we conducted a preliminary experiment to observe the intensity of fluorescence in the hydrogel.
We used PEG,PEGDA and Darocur for Hydrogel compound, SyBr safe, SyBr Green I, EtBr for Fluorescence



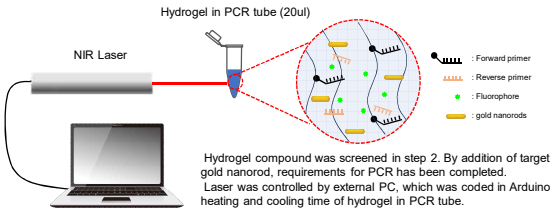
Hydrogel compound : PEG, PEGDA, Darocur 1173
1. PEG : porogen for target DNA
2. PEGDA : Scaffold for Hydrogel
3. Darocur 1173 : Photo - initiator
4. Target : PCR amplified genes
5. Fluorescence : double strand specific fluorescence



Visible Fluoresence by intercalated Fluorophore
1. Excitation spectrum specific for each fluorophore
2. 2 second exposure
3. Observation by Chemi-doc

3. Dot PCR Test

Photothermal heating of nanoparticle was employed as a heat source for PCR in the dot.
To achieve this, point separated PCR is required and point separation could be achieved by confining resources in hydrogels
By using NIR wavelength specifically heated AuNR and imbedding in hydrogel, we could observe heat gradient in hydrogel.



Hydrogel compound was screened in step 2. By addition of target DNA's primers, fluorophore and gold nanorod, requirements for PCR has been completed.
Laser was controlled by external PC, which was coded in Arduino that user can monitor the heating and cooling time of hydrogel in PCR tube.

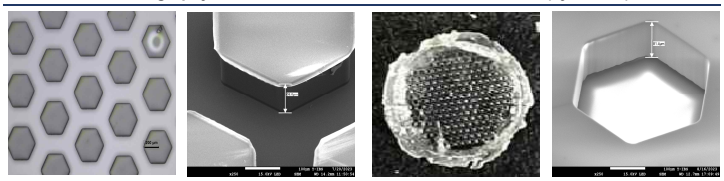
Results

1. Dot Quantification Mold

The target was a hexagon pillars with a length of 200um on each side.
Target thickness was achieved, over 100um but pillar showed a hat which has top part being swelled up as shown in SEM image. We speculated that the height imbalance between the center and ends due to viscosity was causing the problem.
We tried Edge Bead Removal (EBR) to solve the imbalance, but the result was disappointing.

Fortunately, when we proceed to molding step, the hat did not affect quality of PDMS fabrication. From the beginning, we tried to get great resolution of PDMS as a mold.
SEM image showed that the resolution that we desired has satisfied well beyond our requirements.
The single problem was that PDMS itself did not harden well. Therefore, we kept increasing initiative temperature and concluded 125℃ is optimal.

Lithography | **PDMS fabrication (synthesis)**



Light Microscope image of Su-8 lithography. Could observe target resolution with each edge with 200um

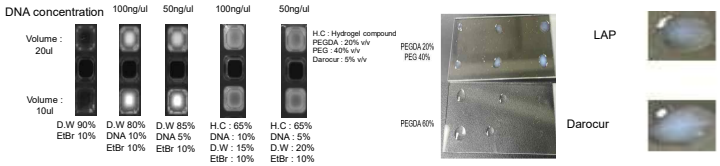
SEM image of Su-8 lithography showing that height is 69° on 45° tilted dimension. Proving that thickness is over 100um

PDMS object separated from mold.

SEM image of one hexagonal pillar of PDMS. Could observe the depth of each well is over 100um

2. Dot Fluorescence Test

Multiple fluorescence were screened.
First, fluorescence of SyBr safe in water dissolved form showed not enough intensity to be detected.
Second, fluorescence of EtBr in water dissolved form showed enough intensity to be differentiated in 100ul volume.
However, signal of EtBr in hydrogel form was not detected. Opaqueness of hydrogel compound was suggested as core factor that this problem happens.
To make hydrogel compound more transparent, photo-initiator was changed. It showed a small improvement, yet not enough.
Second, hydrogel compound mixture was changed to PEGDA only. It showed great transparency, yet DNA penetration was not guaranteed due to exclusion of porogen which provide space for dsDNAs to pass.



fluorescence captured by Chemidoc.

Comparing opaqueness of whether PEG is mixed or only PEGDA

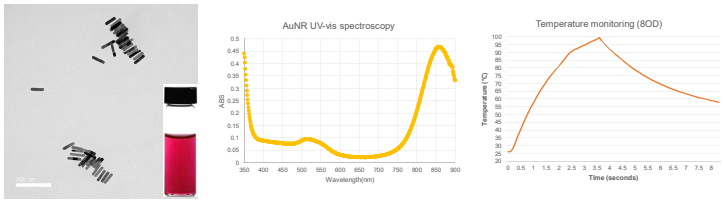
Each particle initiated by LAP and Darocur

Thirdly, the dryness of PEG+PEGDA hydrogel was suspected for the opaqueness of the compound where the inner space of hydrogel was too dry that it void between PEGDA are filled with air leading to cloudy property of hydrogel.
By re-moisturizing PEG+PEGDA hydrogel, the cloudy property has disappeared.

3. Dot PCR Test

Au nanorods which are photothermal convertor worked very well.
It was designed to be specific at NIR spectrum where it is expected to has least amount of interference to fluorescence signal. Aspect ratio was about 5, which will have its longitutional peak around 850nm, specific for NIR laser amplification

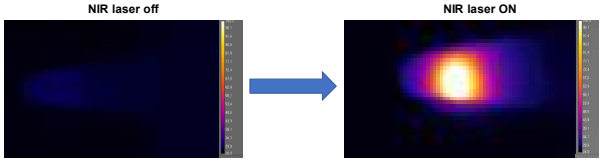
By using specified AuNR, thermal rate monitoring was done.
At 8 OD, target temperature, 95℃, was achieved in 4 seconds which is enough time scale to be used in nanoPCR.



TEM image of AuNR. Aspect ratio at around 5.

UV-vis spectroscopy of AuNR. Shows strong peak at 850nm

Temperature monitoring of AuNR at 8OD in water dissolved form. Could achieve target under 4 seconds.



AuNR showed its performance in hydrogel form. Proving that temperature gradient can re replicated in hydrogel form

Conclusion & Further Study

We've optimized Dot PCR fluorescence quantification methods.
First, Mold was prepared with great resolution. Second, The performance of our key material Au NR was outstanding. However, lastly, fluorescence with hydrogel did not shine as we expected.
Further fluorescence signal screening seems to be required as changing the fluorophore for stronger than EtBr such as SyBr Green I. If not enough, fluorescence signal amplification might be implemented.

After proving third step by optimizing the compound of hydrogel, integration of hydrogel onto PDMS mold where PCR will happen under NIR laser will take place for final proof of concept for dot PCR quantification.

Reference

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