BIT-iGEM2017

〔2017〕07

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Protocol for microfluidic-chip and device

Synopsis of instruments:

The instruments used by the iGEM_BIT team at the project in 2017 are made up of the microfluidic chip, optical and temperature control system, single chip microcomputer control system and external mechanical frame structure,which are made by ourselves. The purpose of this project is to develop a miniaturized testing instrument for innovative biological detection methods, which can provide good and suitable detection conditions. Microfluidic chip provides two reaction chambers for the whole biological detection methods, and the two chambers are used for detection of magnetic bead aptamers and fluorescence expression of engineering bacteria respectively. The detection speed will be greatly improved by making the aptamers and engineering bacteria freeze-dried in the chip by vacuum freeze-drying in advance. In addition, the design of the instrument used a confocal optical path which can detect red and green light at the same time (the name of optical devices from the company name), the use of the confocal optical path not only reduce the space occupied by the optical instrument effectively, but also have no effect on the accuracy of optical detection. The peristaltic pump (From Baoding Sinuo Fluid Technology Co., Ltd.), which is equipped with microfluidic chip for negative pressure injection, has the lowest pump speed of 6ul/min and gets the best accuracy at 39ul/min (+ 2%).

During the project development process, we have set up and carried out the following experiments:

1. Determine the growth curve of the engineering bacteria inside and outside the microfluidic chip respectively.
2. Determine the expression of reporter gene under the condition of the engineering bacteria containing Arabia sugar inducible promoter inside and outside the microfluidic chip respectively(The off-chip test involves the effects of anaerobic conditions).
3. The experiments of microfluidic chip negative pressure injection.
4. In the microfluidic chip, the magnetic beads will be injected by negative pressure method, and study whether the magnetic beads will transferred to the next chamber as the liquid was transferred under the adsorption of the magnet.
5. Determine the separation of magnetic bead aptamers in microfluidic chip
6. Carrying out bacteria freeze-drying experiment in microfluidic chips (including

lyophilization and resuscitation outside the chip).
7. Carrying out freeze dried experiments of magnetic beads in microfluidic chips (including lyophilization and their reaction situation with alpha-fetoprotein outside the chip).
8. Optical detection of microfluidic chip (Qualitative).
9. Thermal detection of microfluidic chips (Quantitative).
10. Simulation of optical instruments.

Experimental display:

1. Determine the growth curve of the engineering bacteria inside and outside the microfluidic chip respectively.

Experimental objective: to characterize the growth situation of the bacterial on the chip, and evaluate the ability of cultivating bacterial on the chip.
Design idea: Through OD value and the fluorescence intensity to characterize the growth conditions of bacteria and campare it with the situations off-chip (cultured in the tube) to reflect the chip's ability to grow bacteria . The microplate reader used for the determination of these two parameters does not support direct detection of the chip, so it is necessary to remove the bacterial fluid from the chip. In view of the problem of volume quantification and residual fluid in the chip, we adopted the method of discharge liquid quantitative liquid dilution to remove the bacteria from the chip and put it on the 96 hole plate for detection.
Experimental design:

Design variables: time (measured once every 2 hours).

Control experiment design: places of bacterial growth: chip and culture in tube.

Results Indicators: the results of OD- index by time curve and fluorescence intensity time curve.

Experimental materials: 7ml LB non-resistant medium, sterile water, chloride antibiotics (related to bacterial resistance), -20℃.

Strains preserved by glycerol ,syringes, microfluidic chip (using 12mm diameter circular culture cavity, height of chamber is 0.4mm,the number of preparation was determined by estimated time and measurement interval), 96 orifice plate, and 1.5ml centrifuge tube (2 for each turn).
Experimental steps:

a. Pretreatment of the chip

Under 100 μl/min flow rate inject 5ml ethanol and 3ml PBS buffer using a syringe respectively, dry overnight.
Be careful：

① The pretreatment of chip has a great influence on the bacteria chip cultured on the chip.

② Ethanol can rinse off the immature cured glue.

③ The role of PBS is to rinse off the residual ethanol and deal with the chip

chamber surface,to make a better environment for bacterial survival.

b. resuscitation strains J-D-P(plux-RBS-LuxR-RBS-Luxl-RBS-LacI-RBS-GFP-T) overnight (the strains can express GFP)

7 ml of non-resistant medium was added 3 ‰ of chlorinease (21 μl) and 100μl of the strain which preserved at -20 ° C in glycerol and incubated at 37 ° C overnight.

c. Transfer strains

7ml of non-resistant medium was added with 3‰ of chlorinease (21 μl) and 100 μl of the strain which cultured overnight, culture 1h at shaker at 37℃.

d. Began to detect, inject the bacteria liquid

In the culture tube evenly take 100μl bacteria point in the 96-well plate, point three times, detection of OD value and fluorescence intensity.

In the culture tube evenly take 100μl bacteria into the clean 1.5ml centrifuge tube, with a syringe to absorb the bacteria into the chip, the chip placed in the gun box, culture into the shaker in 37 ℃.

Be careful：

Because during the experiment,we did not use the pump for injection, so the injection method is manual injection, requiring evenly and slightly tilted chip as much as possible to prevent the injection of bubbles.

e. Detection of effluent effluent

After two hours, remove the chip, inject the air with a syringe, drain the bacteria to a 1.5 ml clean centrifuge tube.Drawing 70μl of bacteria to another clean 1.5ml centrifuge tube from the centrifuge tube and culture tube respectively, and absorb 280μl sterile water diluted bacteria. And then take 100μl from the diluted bacteria to point on the 96-well plate, point three times the sample, detect OD value and fluorescence intensity.

Be careful：

The tilting chip allows the liquid to discharge smoothly.

f. Repeating step e and detect once every 2 hours.

g. Draw the OD-time curve and the fluorescence intensity-time curve.

Experimental results:

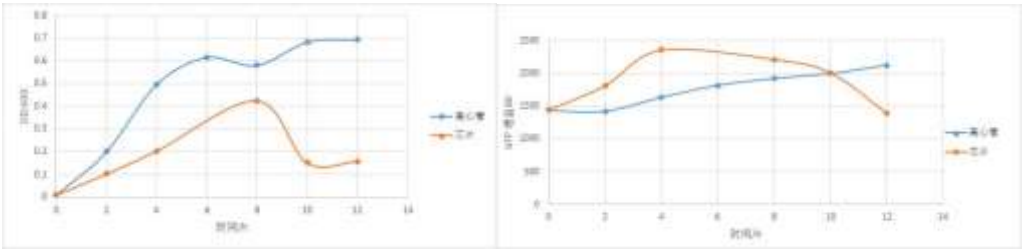


Figure 1. OD-time curve and fluorescence intensity-time curve of Escherichia coli obtained after 12 hours.

Result analysis:

It can be seen from the curve, the OD curve in-chip has a more obvious decline after 8 h, the fluorescence intensity in-chip also shows a downward trend in the 4-12h, and the in-chip OD value below the centrifuge tube OD value. We analyze the possible reasons are:

①: the chip environment relative to the test tube is more narrow, have a certain impact to the metabolism of bacteria.

②:It is lack of permeability of the chip, the number of flora to a certain extent, faced with hypoxia.

2. Determine the expression of reporter gene under the condition of the engineering bacteria containing Arabia sugar inducible promoter inside and outside the microfluidic chip respectively(The off-chip test involves the effects of anaerobic conditions).

Module 1: Explore the effect of intracellular hypoxia on bacterial growth in-chip.

Experimental design: Since the results of the culture of the bacteria in the chip were very different from those in the centrifuge tube, we analyzed that the hypoxic environment in the chip might affect the growth and expression of the bacteria. So we designed a simulated hypoxic environment outside the chip to explore this effect. And using arabinose-induced expression of E.coli as an experimental strain, setting different concentrations of arabinose concentration（10g/L、5g/L、1g/L、0.1g/L、0.01g/L、0g/L）.

Experimental materials: Escherichia coli induced by arabinose （pbad-RBS-LacI-RBS-GFP-T-plac-RBS-RFP-T）, ampicillin (AMP), 100 mg / ml arabinose solution, 7 ml non-anti-LB liquid medium, sterile water, PCR tube, 1.5 ml centrifuge tube, 96 well plate.

Experimental steps:

① add the materials, resuscitation bacteria

Adding 100μL strains preserved by glycerol and 1 AMP (7 μL) into the 7ml non-resistant LB medium,and then added 0.7μl、7μl、70μl、350μl、700μl 100mg/ml arabinose solutions respectively. Cultured at 37℃about 1h for resuscitation.

② Divided into PCR tube and centrifugal tube

Remove the bacteria,added 100μL bacteria liquid to 1.5ml centrifuge tubes (each gradient arabinose solutions for four tubes), 250μL bacteria liquid to PCR tube (basically fill, each gradient arabinose solutions for four tubes). Resting on the placing plate, placed 37 degrees shaking culture. And then draw 100μl bacteria solution in the 96 hole plate sample, three times each gradient,detect OD value and fluorescence intensity.

③ Dilution detection

Every two hours draw each concentration of PCR tube and centrifuge tubes for

detecting,and take out 70 μL in another clean 1.5ml centrifuge tube, diluted 5 times,take out 100μL bacteria solution into the 96 hole point sample, points three times,detect OD value and fluorescence intensity.

④ Copy out the data and draw the growth expression curve.

Experimental results:

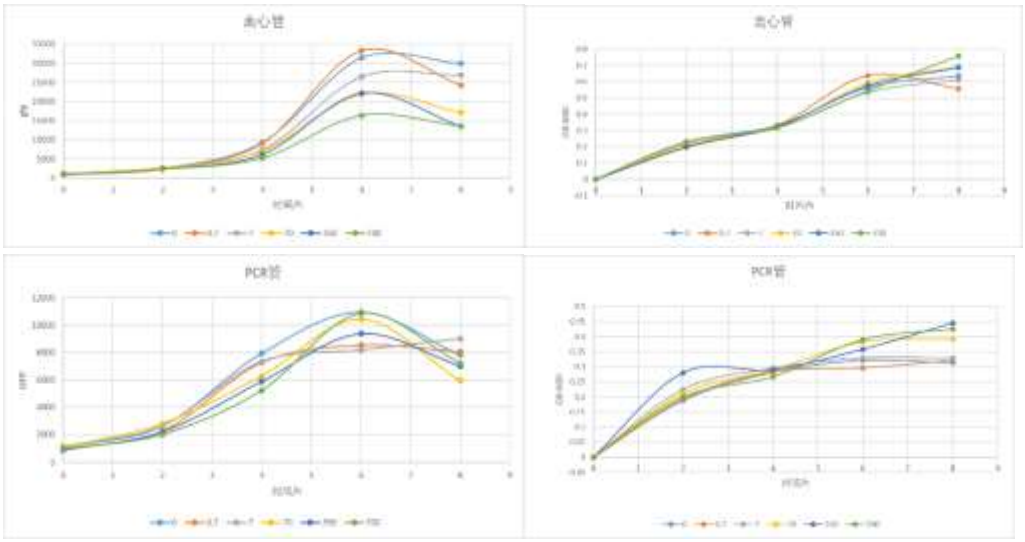


Figure 2. The induction experiment of arabinose: the growth curve and fluorescence curve of Escherichia coli in centrifuge tube and PCR tube.

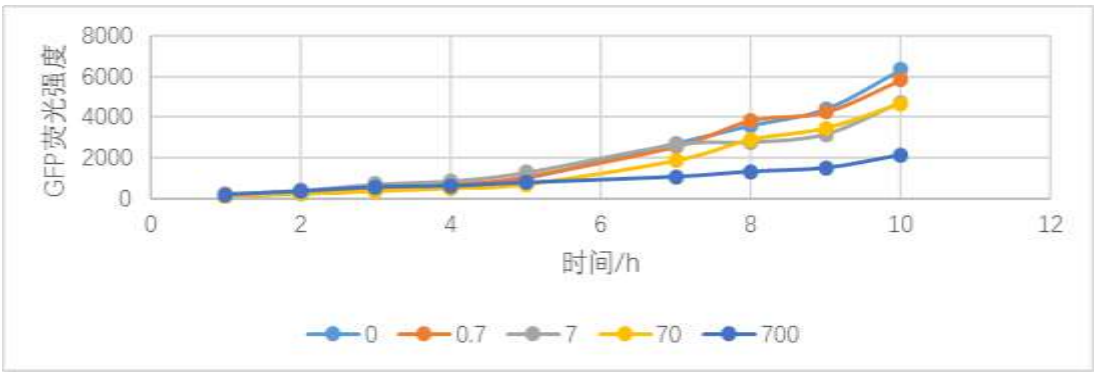


Figure 3. Arabinose induction induced experiment: fluorescence curve in 96 pore plate of Escherichia coli.

The experimental results show that under the hypoxia environment the growth and expression of bacterial are in a certain decline, which is indeed a current difference between culture inside the chip and outside the chip, talking about the arabinose concentration induced Escherichia coli,compared with the experiment we did before（study the effect of arabinose on the fluorescence expression of engineering bacteria），we found that the induction of arabinose is not as high as the higher the concentration.

3. The experiments of microfluidic chip negative pressure injection.

Experimental Objective: To verify whether the peristaltic pump can complete the task of injecting beads and bacteria into the chip.

Design ideas: We simulate the injection of the peristaltic pump by fixing the flow rate of the syringe pump. Magnetic bead suspension has been injecting into the chamber of the chip, observing the size and number of bubbles in the chamber and the magnetic beads in the chamber. Similarly, bacteria was injected into the chamber two, observing the size of the cavity two miles and the number of bubbles.
Experimental design: Variable design: liquid type.
Results Indicators: chamber 1: bubble size and number, and the case of magnetic beads; chamber two: bubble size and number.
Experimental material: one pump (two injections), 30 microliters of magnetic beads, 50 microliters of bacteria, one with a microvalve, one on a magnet, one in 100 microliters, two in a centrifuge tube The
Experimental steps:

①: injection of magnetic beads suspension

Spray 30 μl of the magnetic beads in a centrifuge tube with a pipette, and use a syringe to aspirate the magnetic beads in the centrifuge tube and inject it into the first chamber with 100 μl per minute with a syringe pump.

Note: Shake the liquid before shaking the magnetic beads.

②: place the magnet, adsorption beads

Place the magnet in the chamber and place it for 5 minutes until the supernatant in the chamber is not in turbidity and proceed to the next step.

Note: The position of the magnet should be just below the chamber, not too close to the injection port.

③: into the bacteria liquid

Take 50 microliters of bacteria in a test tube with a pipette and inject it with a syringe pump at 100 μl per minute from a sample injection port in the second chamber.

Note: the liquid before pumping the liquid shake.

Experimental results: No.
Results Analysis: No.

4. microfluidic chip through the negative pressure method for magnetic bead fitness sample injection, and study in the magnet adsorption, whether the magnetic beacon will flow with the liquid flow to the next chamber

Experimental Objective: To find the optimal flow rate of the liquid injection chip, reduce the liquid injection time, while reducing the number of subsequent injection of liquid washed away the number of beads.
Design ideas: first in the chip into a certain amount of magnetic beads suspension, then the magnet A on the bottom of the chip adsorption beads. Set a set of flow gradient, with a syringe pump at different flow rates into the chip into the water. Finally, the magnetic beads of the liquid are sampled by magnet B, and the optimum

flow rate is judged by observing the number of magnetic beads adsorbed on the magnet.

Experimental design: Variable design: flow rate.

Control design: chips at different flow rates are controlled by each other.

Result: The number of magnetic beads adsorbed on magnet B.

Experimental material: syringe pump, magnetic beads liquid 100 microliters, two magnets, pipettes, centrifuge tube six, the first cavity is the oval-shaped three new chips.

Experimental steps:

① : injection of magnetic beads suspension

Twenty microliters of the magnetic suspension were pipetted in three centrifuge tubes with a pipette, followed by injecting 20 microliters of the suspension into the chambers of the three chips with a syringe.

Note

1. Remove the magnetic beads before the first shake the liquid to control the three concentric tubes within the same concentration of beads.

2. When using the syringe, pay attention to the injection rate, too fast to easily damage the chip, and prone to air bubbles.

②: place the magnet, adsorption beads

Place the magnet in the chamber of the three chips and let stand for 5 minutes until the supernatant in the chamber is not in turbidity and proceed to the next step.

Note: The position of the magnet should be just below the chamber, not too close to the injection port.

③: into the water

The three chips were labeled as No. 1, No. 2 and No. 3, and 300 μl of water was injected into the injection holes of the first chamber of the first and third chips at a flow rate of 200, 300, 400 μl per minute using a syringe pump , With another centrifuge tube, then out of the liquid, and then put the other magnetic block were placed in the liquid full contact, take pictures of each magnet on the record of the amount of beads.

Experimental results: No.

Results Analysis: No.

5.microfluidic chip in the bacteria freeze-drying experiments (including chip-free freeze and recovery)

Module 1:

Purpose:

(1) Compare the difference between sterilized skimmed milk powder and unsterilized skim milk powder

(2) observe the dry powder form

Experimental content:

(1). Configure 10% skimmed milk powder, 0.3% glycerol aqueous solution as a protective agent, not sterilized. The other group was 10% skimmed milk powder

solution, 115 ℃ sterilization 15min.

(2). Take the day before the shaker, OD up to about 1.0 of wild-type E. coli 1mL and 1.5mL centrifuge tube, 4 ℃ refrigerator 7000rpm centrifuge 30s. With 1000uL pipettes to absorb the supernatant, leaving the bacteria The

(3) with 1000uL pipetting gun to the centrifuge tube by adding 1mL protective agent, blowing mixed evenly, made of bacteria suspension.

(4). Take 4 10mL centrifuge tubes, each loaded 500uL bacteria suspension.

(5). Cut off the centrifuge tube cover, covered with a hole hole sealing film.

(6). Pre-freeze: 4 ℃ 5min, -20 ℃ 10min, -80 ℃ 45min gradient freeze. At the same time open the vacuum freeze dryer, -53 ℃ pre-freeze 1h.

(7). Place the material in, open the vacuum pump, vacuum dry for 7.5h.

(8). Remove the sample and store it in a refrigerator at 4 ° C.

Experimental results and analysis:

(1). Sample morphology:

Figure 4 freeze-dried bacteria picture (2017.09.23 21:23, not sterile protective agent) freeze-dried bacteria picture (2017.09.14 21:29, sterilization protective agent)

Summary: The sample less, from -80 ℃ refrigerator transfer to the dryer speed, the material temperature is displayed at 4 ℃, not the formation of foam, the effect is good. Unsterilized protective agent compared with the sterilization agent, due to the addition of glycerol, viscosity, dry powder sticky pipe wall is not easy to fall.

(2). Bacteria:

Due to the original bacteria liquid was drained, can not compare the number of viable bacteria before and after freezing, so this experiment has not yet done. The effect of different protective agents on bacteria can be compared with the method of calculating the number of colonies. And because of flat plate problems (agar distribution is not uniform, too soft, can not be coated), can only reassemble LB solid medium, and then coated compared.

The dry powder was resuscitated with LB liquid medium, and the bacteria were diluted with E2, E4, E5 times, 100uL per plate and three plates. The number of colonies was calculated after 24 h or 48 h after application

Dilution factor	0	E2	E4	E5

Protection agent type				
Not sterilized				
sterilized				

Table 1 Relationship between the dilution factor and the type of protective agent

Module 2:

Purpose:

(1). Comparison of bacterial activity before and after freeze-drying

(2) Compare the effects of different factors on the morphology of dry powder

Experimental content:

(1). Configure 10% skimmed milk powder as a protective agent.

(2). Take the night before into the shaker in the bacteria solution, measuring its OD value, about 0.8. Take 6 1.5mL centrifuge tube, each transferred 1mL bacteria, 4 ℃ centrifuge 7000rpm centrifuge 30s. Carefully aspirate the supernatant with a 1000 μL pipetting gun, leaving the bacteria.

(3). Add 1mL of the protective agent, blowing mixed evenly, made of bacteria suspension.

(4). Take 14 10mL test tube, according to the following table classification:

Bacteria volume Frozen way	1mL	500uL	200uL
Gradient frozen	one, open the lid	three	three
Direct frozen	one, open the lid	three	three

Table 2 the relationship between the amount of liquid and freeze-drying table

A total of 14 test tubes, which did not mark the cover, that cut off the tube cover, covered with hole hole sealing film. Gradient freezing method is still 4 ℃ 5min, -20 ℃ 10min, -80 ℃ 45min. Directly frozen for direct storage into -80 ℃ refrigerator pre-freeze 1h.

(5). Put in freeze dryer and dry. Due to freeze dryer Saturday and Sunday working hours for the 9: 00-17: 00, so this failed to dry once, divided into two.

(6) .After each tube, add equal volume of liquid medium, resuscitation, remove 200uL post-resuscitation bacteria, add 0.3% CAM liquid medium, add 37 ℃ shaker culture, by measuring the growth curve and fluorescence curve comparison freeze-drying before and after Bacteria change.

Experimental results and analysis:

Figure 5 freeze dried bacteria picture (2017.09.24 17:13)

As shown, many dry powders are foamy. (The material temperature is raised to 8 ℃, or even higher), part of the melting, resulting in the drying process is not directly from the ice sublimation of water vapor for the water vapor, the temperature is higher than the temperature, , But by water vaporization caused by water vapor. The formation of foam-like reasons to confirm the amount of bacteria and liquid, whether to seal and other factors have nothing to do. Experiments show that the less the sample in the cold trap, the lower the material temperature.

Figure 6 freeze-dried bacteria show the parameters set

(2). Before and after freeze-drying and different pre-freezing method of bacteria live comparison: (still in the experiment)

6.microfluidic chip within the beads of aptamer freeze-drying experiments (including chip-free freeze-dried and alpha-fetoprotein reaction)

7. microfluidic chip optical detection (qualitative)

Experiment purpose: View the optical distribution of the chip
Experimental equipment: 6 chips in 3 chips, 1 mL syringe, 4 centrifuge tubes.
Experimental materials: fluorescent microglobulin solution, fluorescein sodium solution
Experimental steps: the experiment is divided into two parts: First, the qualitative part of the two, quantitative part

First, Qualitative part

1, the first fluorescence compared to fluorescein sodium fluorescent microglobulin 50uL solution into the chip.

2, the chip in the injection process to keep tilted 30 ° angle, the experimental people in the injection process as far as possible to keep the injection process slow and uniform.

3, in order to do comparison, choose the same concentration of fluorescent microspheres directly onto the slide, observe the fluorescence distribution.

4, in the original slide on the cover glass slides, and then observe the fluorescence distribution.

Second, Quantitative distribution

1, weighed 0.3763g of sodium fluorescein added to 10mL of distilled water, dubbed 0.1mol / L sodium fluorescein solution.

2, 0.1mol / L sodium fluorescein solution were diluted 7000 times, 10000 times, 20000 times, respectively, fluorescein sodium solution ①, ②, ③.

3, the extraction of fluorescein sodium solution ①, ②, ③ 100uL added to the 96-well plate to measure the fluorescence value (gain election 50).

3, the fluorescein sodium solution ①, ②, ③ were added to the chip, observe the fluorescence distribution.

Experimental results:

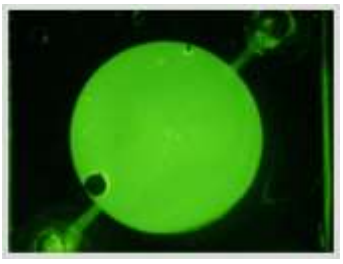


Figure 7 Fluorescent microspheres in the chip

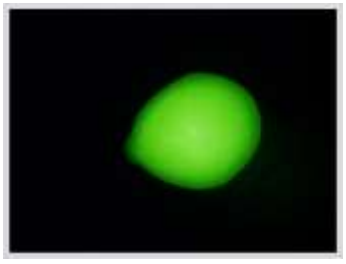
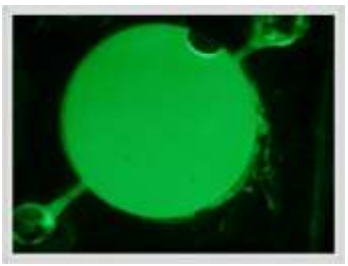
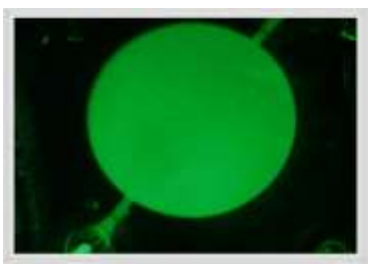


Figure 8 Fluorescent microspheres on glass slides

Figure 9 Cover the slides





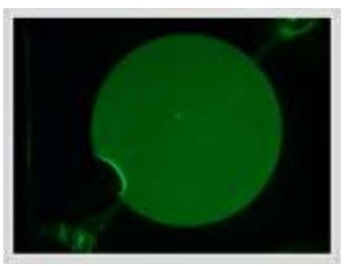


Figure 10 diluted 70,000 times the amount of fluorescein sodium	Figure 11 diluted one hundred thousand times	Figure 12 diluted two hundred thousand times
6	4	4
5	10549	5
5	5786	3
4	3298	8
11	12	0

Table 3 Fluorescence values of fluorescein sodium solution

Experimental results:

First, we compare Figure 1, Figure 2 We found that our chip is very good light transmission, in the chip and on the slide on the fluorescence is almost the same.

Second, through Figure 3, that is covered with coverslips, we found that the intensity of fluorescence greatly reduced, had to do in order to do, you should find a 50uL slot can be mounted on the slide to observe. But because of the more stringent conditions, failed to experiment, but we combined with the first conclusion, it can be concluded that the photoresist does not affect the distribution of light too much

Third, through Figure 4, Figure 5, Figure 6, we can find the chip's light transmission is still possible. Combined with Figure 7 of our fluorescence measurement, the quantitative description of our chip's light transmission is good

8. microfluidic chip thermal testing (qualitative / quantitative)

9.simulation of optical instruments

Experimental Objective: To verify the optical path we designed to optimize the optical path according to the verification results

Design ideas: the use of the actual components of the optical path verification there are many inconvenience: First, the higher price of optical components, blind experiments may cause unnecessary losses, and secondly, according to the verification results continue to adjust the location of the process is more time-consuming and laborious. In order to avoid the waste of time and money, we use the optical simulation method for our follow-up optical path to provide guidance and reference.

Experimental design: The Zemax software was used to simulate the optical path construction process according to the component parameters provided on the thorlabs website and to evaluate the effect of our optical path by checking the aperture distribution of the detection surface in the software and to improve our optical path.

Use components:

flat convex lens (2) Model: LA1304, focal length: 4mm, radius of curvature: 20.6mm

Dichroic mirror (reflected in the actual simulation process)

Experiment procedure:

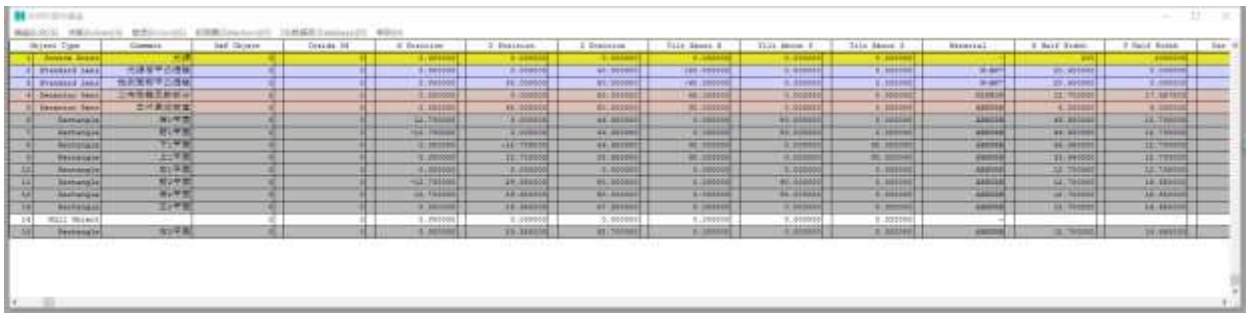


Figure 13 Optical simulation using the ZEMAX interface

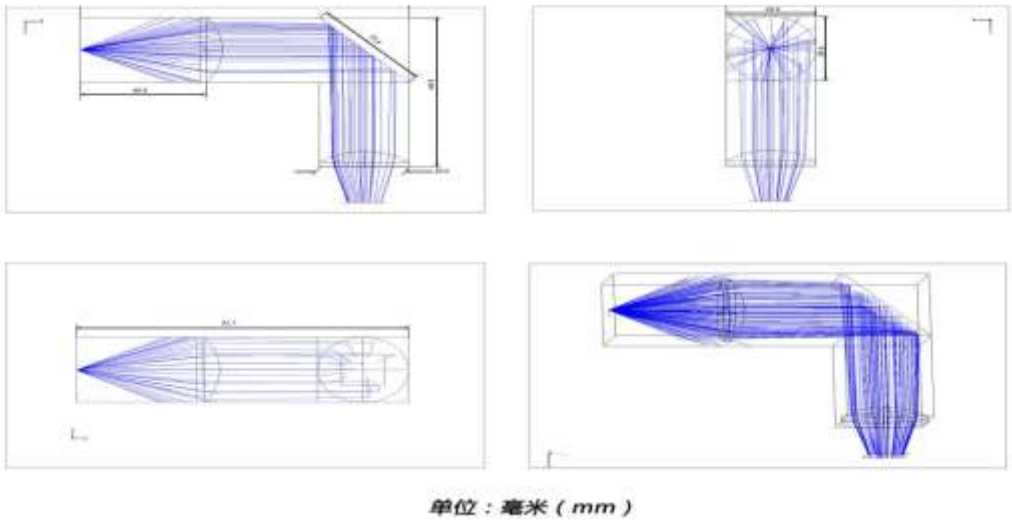


Figure 14 Optical path diagram in optical simulation

The simulation process mainly presents two problems:

1, the aperture is too small

Solution: the aperture is too small, the first thought is two aspects: First, the lens refractive index is too large, two, the detection surface and dichroism mirror too close, so the most direct is the detection surface Y value (ie Vertical coordinates) from 30 to 40.

2, the aperture is too dark

Solution: From the above figure we found that the light divergence angle is too large, resulting in a lot of light are shining on the wall, so reduce the divergence angle or increase the aperture can be, so get the final result.

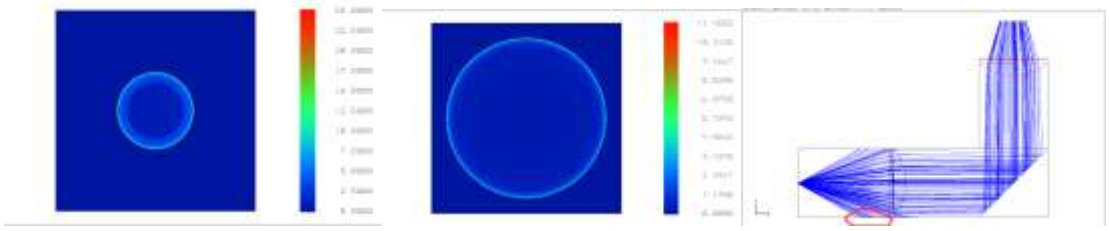


Figure 15 Problems encountered in optical simulation

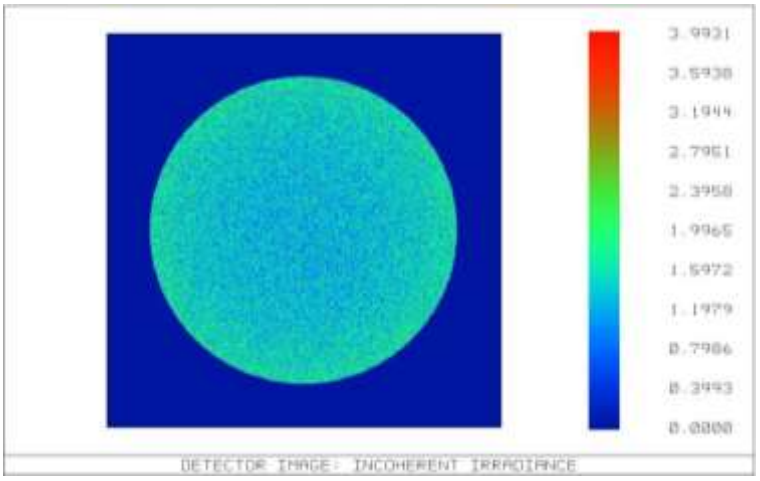


Figure 16 Results of the detectors

From Figure 2 we can see that the light path simulation results are better, the light distribution is more uniform, light intensity is weak, because the light source of the selected power is only 1W, simply increase the intensity of the light can be, not repeat here.

Conclusion:

We chose the LA1304 lens in line with our basic requirements, to achieve our ideal results, simulation success.