

Unexpected Growth of *E. coli* under Ag (I) Pulse in Microfluidic Device

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1. Introduction

Microfluidic device is a newly developed technique that enabled a large spectrum of experiment be performed in an unprecedented microscopic level. Some examples include microbiological chemotaxis, mechanism of anti-freezing protein, single way protein encounter, unfolding of bacteria chromosome, behavior of bacteria colony in high concentration, even measurements of elastic moduli of silicone gel.

One of the central properties that enabled these variety of experiments to be performed is the ability of delicate control over microscopic environment by switching incoming fluids. In this experiment, this property is utilized to produce a short pulse of growth media containing Ag (I), which is expected to inhibit the growth of bacteria either temporarily or permanently.

The bacteria *E. coli* was used in this experiment. The bacteria used was a transgene strain that has GFP (Green Fluorescent Protein) gene incorporated in its chromosome. After bacteria has been trapped in the device, a constant flow of growth media (LB) was provided, and the area of fluorescent, as the indicator of bacteria number, was monitored by fluorescent microscope. After two hours of constant growth, 20 minutes of growth media flow containing 100 μ g/L Ag (I) was flown through the device. Then the flow was switched back to LB and the bacteria was allowed to grow until 3 hours was reached.

The picture taken was then analyzed and the growth curve drawn, an unexpected “delayed inhibition” phenomenon was observed, the mechanism of this phenomenon requires further investigation.

The microfluidics device used in this experiment is shown in the following diagram. The device was made by sticking a piece of PDME gel with microscopic structure on a piece of clean cover glass, this procedure should be repeated every time the device is reused (see appendix for more information). Note the different length of channels, arrays of chambers and different definition of holes.

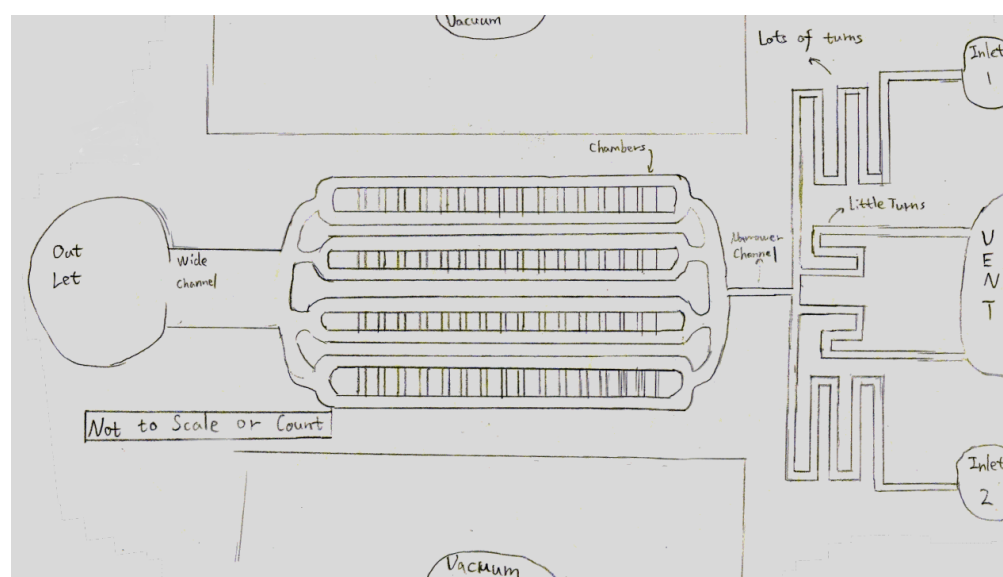


Figure 1 Schematic of Microfluidics Device

Chambers: Chambers are the place where bacteria live, openness on the two side of the chamber allows rapid exchange of growth media, keeping the environment in the chamber fresh all the time, it also allows rapid switch of growth media.

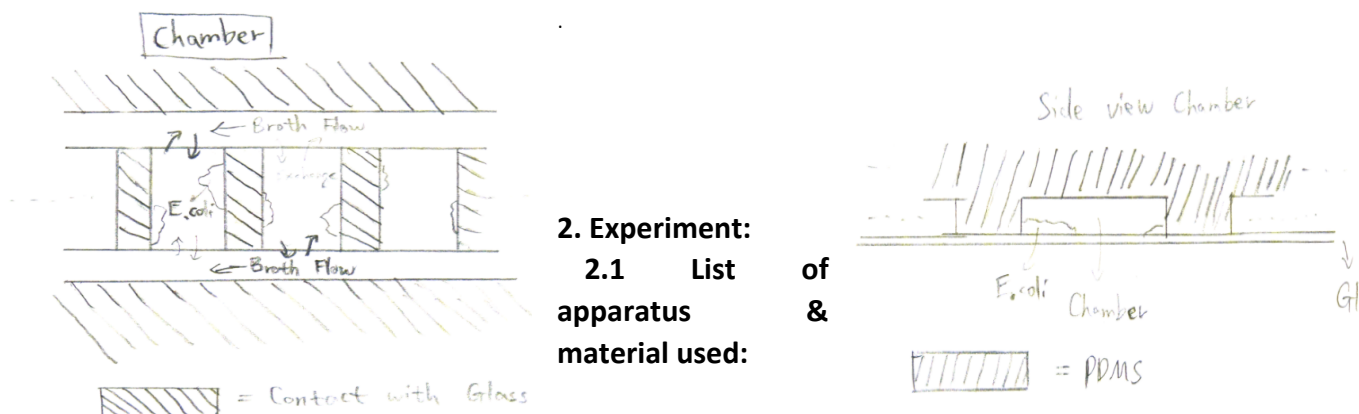


Figure 2 Schematic of Chamber, Seen From Above and Side

| Apparatus& material | Notes |
|-------------------------|---|
| Invert Microscope | Olympus IX 50 with Andor iXon X-2335 EMCCD Camera. |
| Microfluidic Device | Reused from one used by Winter 2013 microfluidic group. |
| Orange Straw tube | Reused from Winter 2013 microfluidic device. Extra provided by Hwa Lab. |
| Computer | The one next to invert microscope, used for CCD Camera imaging. |
| E. coli | Strain NCM 3722 chr: km ptet-GFP (Resistance and transgene information) |
| Syringes | 5 ml |
| Tubes | Transparent and soft. |
| Tapes | For setting up |
| Car defreezer | For temperature control, an air heater. |
| Temperature controller. | For temperature monitoring. |
| Acrodisc Syringe filter | Filter BSA solution to prevent clogging. |
| LB | Made in Hwa Lab |
| BSA | 1% solution. Prepared from solid obtained from Hwa Lab. |

2.3 Experimental procedure

Using a tip, some E. coli from a single colony on the plate was transferred to several ml of LB. The bacteria in LB was then incubated in a shaker under 37°C, 180rpm overnight to allow the bacteria to saturate. The saturated bacteria solution was then diluted by the factor of 2 using LB and was ready to use.

Microfluidic device was fixed on a glass slide and then attached to the invert microscope on a proper position. First, BSA was pushed through from vent using gravitational potential provided by fixing the syringe containing the solution on a relative high position (all other solutions were pushed though using the same method). Clogging of channel on the vent side was observed immediately because of the solid contained in the BSA solution, so BSA was disconnected and inserted in the outlet after putting a syringe filter between syringe and tube. No other clogging was observed and the initial clogging substance was also washed away. Gradually, drops of liquid can be seen on other openings, vent and the two inlets, it is the sign that the channels are

through and the device is healthy. Drops can also help avoiding bubbles in subsequent operations. Now, under microscope, you can see that bubbles in the device was being squeezed out from the air-permeable walls of the device. Constantly check the device under microscope, after about an hour (it can be faster if air pressure is used to drive BSA through), the device is completely filled up, and is ready to use.

After disconnecting BSA tube and pushing some LB solution from vent to form a drop on outlet, clipped bacteria tube was plugged into outlet, and bacteria solution was allowed to flow until some cells are confirmed to have entered some chambers, confirming can be really hard under microscope. Ideally there should be two to three individual or groups of cells in one chamber. After the confirming, apply vacuum to the vacuum pad to confine them in their chambers. Then, after disconnecting bacteria tube from outlet, more LB was flown through the device from one of the inlet to provide fresh nutrient and wash away as much extra bacteria as possible.

Finally, waste collecting tube was connected to outlet and vent, and clipped LB solution with 100 μ g/L Ag (I) was inserted in one of the inlet, waiting to provide the pulse of Ag (I). The car defreezer, simply a heating fan, was turned on to blow 37°C air to the device to provide optimized constant temperature for the bacteria. The temperature is monitored by a temperature controller with sensor attached to the air outlet of the defreezer.

2.4 Growth and Measurement.

Using 10 \times Objective, a certain area was monitored using EMCCD camera attached to the microscope. Picture was taken every 10 minutes, the exposure time was set to be 2 seconds, and the exposure of bacteria under excitation light was controlled to be less than 10s to avoid bleaching. After two hours, the clip on the LB with Ag (I) was released and the normal LB tube was clipped. All other conditions are kept unchanged, pictures are still taken every 10 minutes. After 20 minutes, the growth media was switched back to normal LB by clipping the LB with Ag (I) tube and relaxing normal LB tube. Some picture taken is shown below:

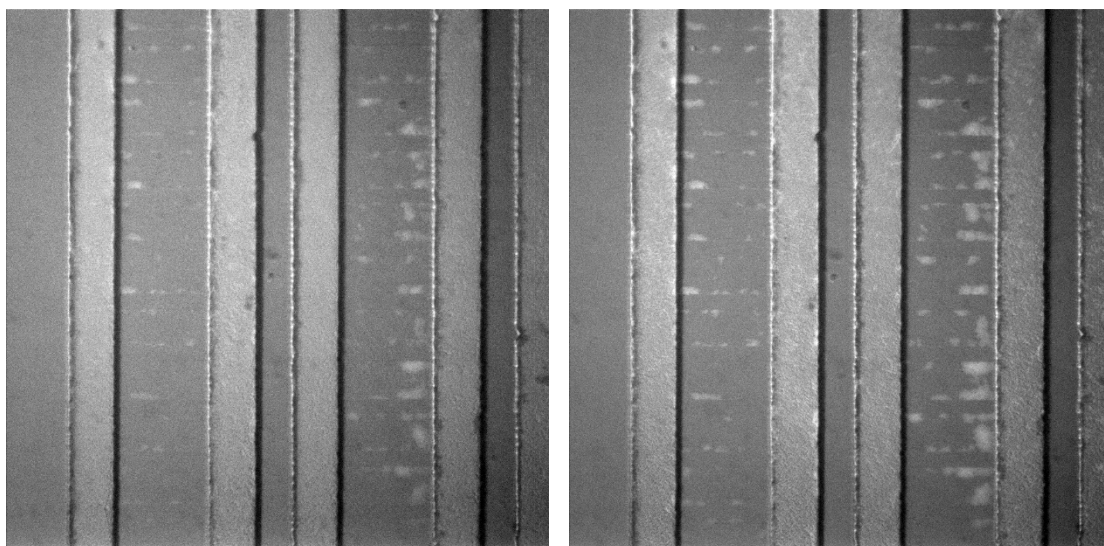


Figure 3 Picture Taken During Experiment. Left: t= 120min Right: t= 180min

3 Data Analysis

3.1 Model

In this experiment, the chambers are pulled down by atmosphere pressure due to the vacuum in the two vacuum pads, so if we assume that bacteria can only grow horizontally, the area of the image of bacteria colony is thus representative of the total mass of the colony. In this experiment, before Ag (I) was applied, due to fast exchange of growth media between chamber and channel nearby, a chemostat is maintained, so the bacteria will divide robustly, that simply means every cell has the same odd to divide in a certain amount of time, using area for number of cells and making the model continuous, we have the simple classic growth model:

$$\frac{dA}{dx} = A * \sigma$$

Where σ is growth rate. Its solution is also well known:

$$A = A_0 e^{\sigma * t}$$

Doubling time is also a good indicator for the growth rate, it can be obtained as follows:

$$T = \frac{\ln(2)}{\sigma}$$

Note exponential growth only apply to data taken before Ag (I) was introduced.

3.2 Image Analysis

Using ImageJ, it's possible to setup a threshold to turn all pixels brighter than a certain value directly to white and all that is dimmer to black, than, extra noise can be removed using median filter and the number of white pixels obtained from mean value of color over the entire image.

This process is not as easy as it seems, because the nonuniformness of illumination, a very high back ground noise, and different brightness between pictures, separation of bacteria colony from noise in image is very hard, only very small region in the photo can be analyzed at a time, and the threshold should sometimes be manually changed between images in order to obtain the best representative picture of colony size. There's inevitable subjectivity involved in this process, and that's one of the major problem encountered.

However, one set of nearly satisfactory picture was obtained. No further attempts was made on other colonies due to time limit. Other colonies can of course analyzed and compared, but the main observation can be obtained with this one.

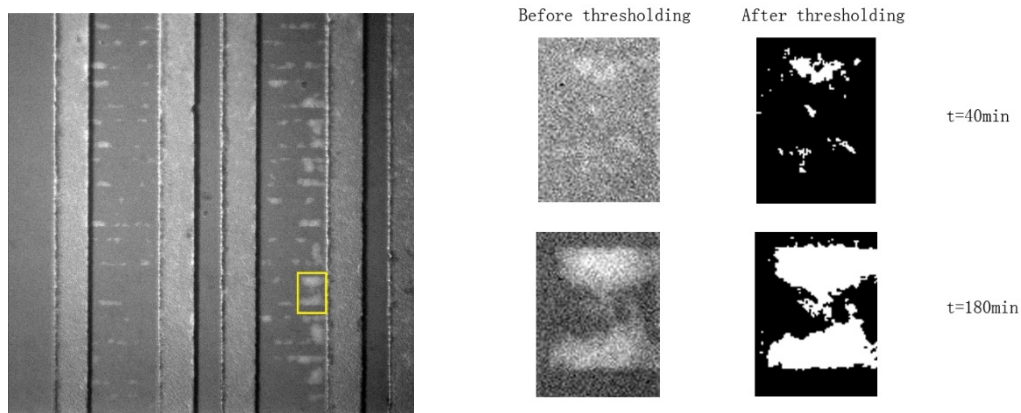


Figure 4 Area Under Analysis (Shown in Yellow Rectangle) And Example of increasing contrast, noise is very significant in the first picture.

3.3 Plotting and Fitting

The area of Brightness is then plotted in Matlab and fitted with exponential model. The last five points were excluded because they are data taken after Ag (I) has been introduced, so they may not follow the exponential model given above. The fitting of first 13 data points are shown below (Point thereafter is shown but not included in the fitting):

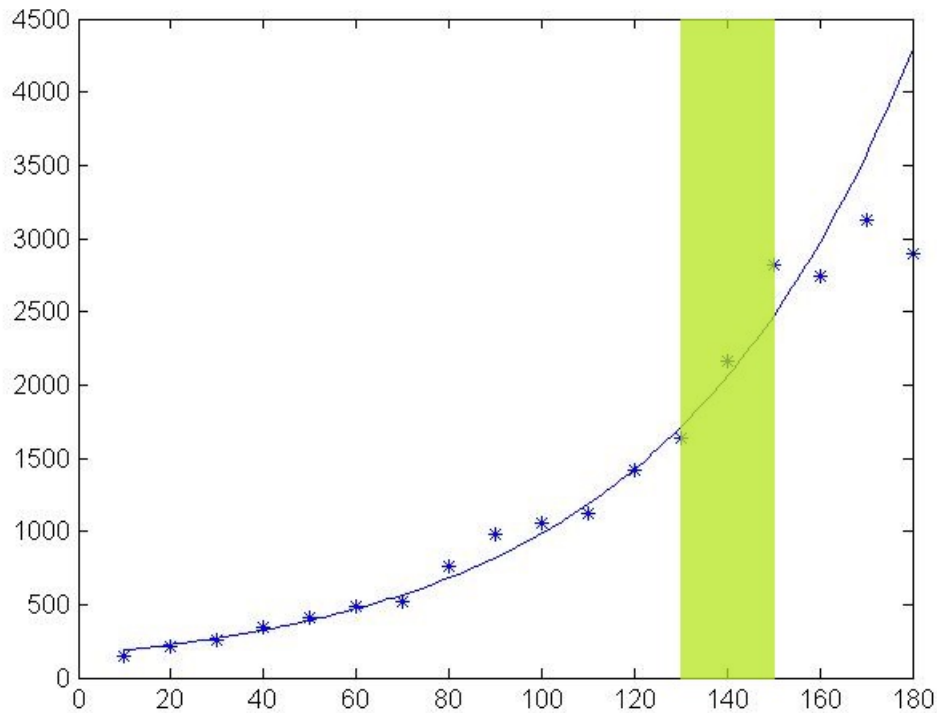


Figure 5 Fitting of first 13 point X axis: Time (in minutes) Y axis: Area (in pixels).

Best approximation: $A=160*\exp(0.0184*t)$ Period when Ag (I) is applied is marked green.

3.4 Analysis and conclusion

From the picture one can see that before the Ag (I) was applied, the colony follows exponential growth nicely, the two points after Ag (I) is applied also follow this trend, the effect of Ag (I) is only apparent after 30 min when it is applied, where data no longer follow the exponential curve. Doubling time is determined to be 38 min.

An interesting thing happens here, for some reason the effect of Ag ion is delayed by 30 min, and the inhibiting effect actually happened after “the growth media no longer contain Ag.” This may due to a variety of reasons, firstly one will guess that the delivery of growth media containing Ag maybe delayed due to long channel, this is not possible because the effect happened after Ag is no longer applied. Thus, it is very likely that a not learned resisting or absorbing process is happening when Ag is used to inhibit growth of E. coli. However, definite confirmation of this effect requires carefully repeating this experiment and further investigation.

4. Discussion

A lot can be improved in this experiment. Although invert microscope makes setting up process a little bit easier, the imaging quality of this microscope is poor, fluorescent light is often very weak, and there is very high back ground noise, that makes data analysis a very painful

process. The illumination on fluorescent mode can also be made more even to avoid different brightness over the image, which will also help data analysis. The choice of filter is also important, after the experiment is done, I found a much better filter in the lab, if that is found earlier the data analysis would have been much easier.

One major uncertain factor in this experiment is the concentration of bacteria solution when trying to trap bacteria in the device. From the data pictures one can see that the trapping is far from perfect, with a little bit too much cells on one side and too little on the other side. More trials should be done to optimize the concentration being used.

When it comes to the experiment set up, air pressure can be used in order to make the filling up process faster and the pressure easier to control. Temperature control should be improved, maybe by making a container that can enclose the microscope and the device to utilize the feedback control ability. Automatic shutter can be connected to the computer to make it possible for the experiment to run without having to wait for hours.

5 Acknowledgment (Name in Alphabetical Order)

| Thanks | For |
|----------------|--|
| Grant Allen | Being TA, helping me and encourage me. |
| Jerome Wong Ng | Giving me help on microbiology, providing me materials |
| ManLu Zhu. | Giving me help on microbiology and giving me BSA. |
| Mya Warren | Teaching me about reusing microfluidic device and giving me another E. coli plate. |
| Philbert Tsai | Helping me fix microscope, instructing me about ImageJ and encourage me. |
| Tomoyo Honda | Providing me orange straw tube. |

Link to my presentation slide:

<https://drive.google.com/file/d/0Bx7Vomdgp-0ecnpqYU5KNU80VmM/edit?usp=sharing>

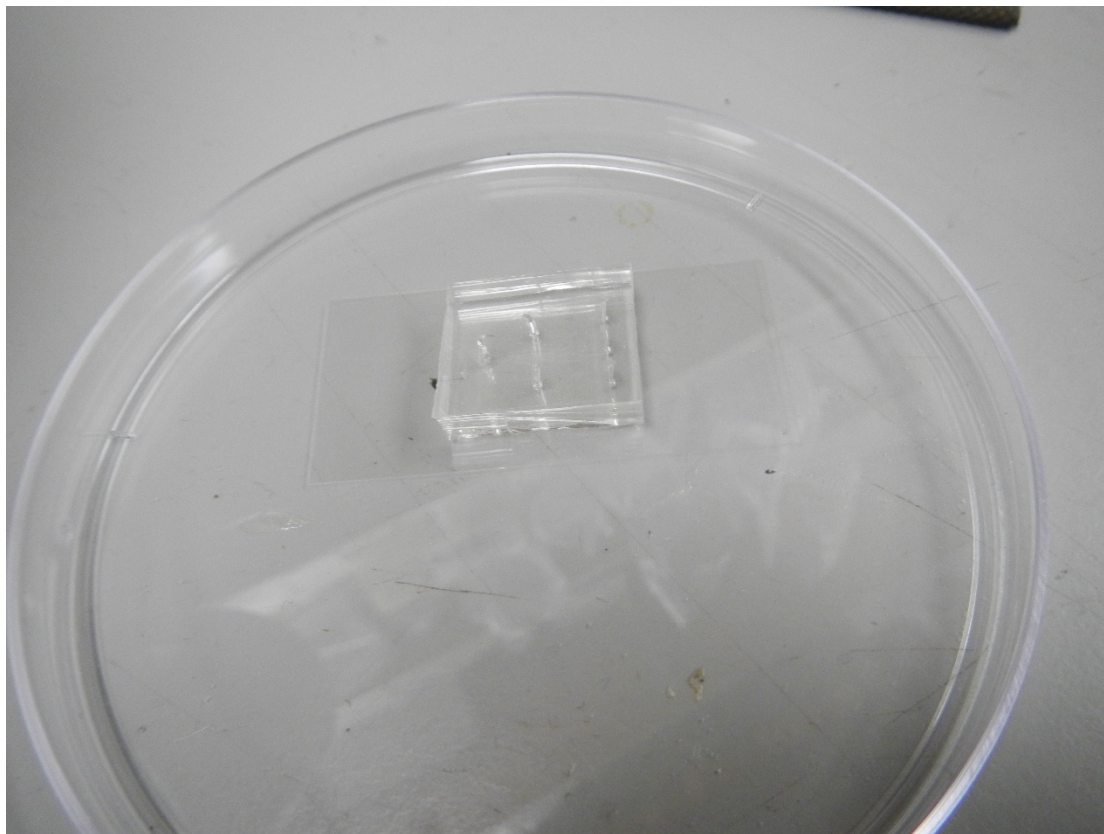
The appendix is the summary of knowledge that I learned this quarter about microfluidic device, if you are the group planning to work on this device, hope that can save you some pain and more importantly, time.

Thank you for reading, any problem? Contact: watercooling250@163.com

Appendix:

On the use and reuse of microfluidic device.

This is a guide about how to use and reuse microfluidic device. Hope this can save you some time and pain. First, a real picture of the device:



The device consists of a piece of glass cover glass and a block of flexible gel stick together, the definition of the five holes are shown in the diagram in the beginning of the report.

To understand why holes are used in the way described in the report, one principle should be remembered: The flow rate of liquid under low speed and confined channel is analogous to electricity (refer to Hagen–Poiseuille equation). The current is the flow rate of water, voltage is the pressure, and resistance the resistance of the channel.

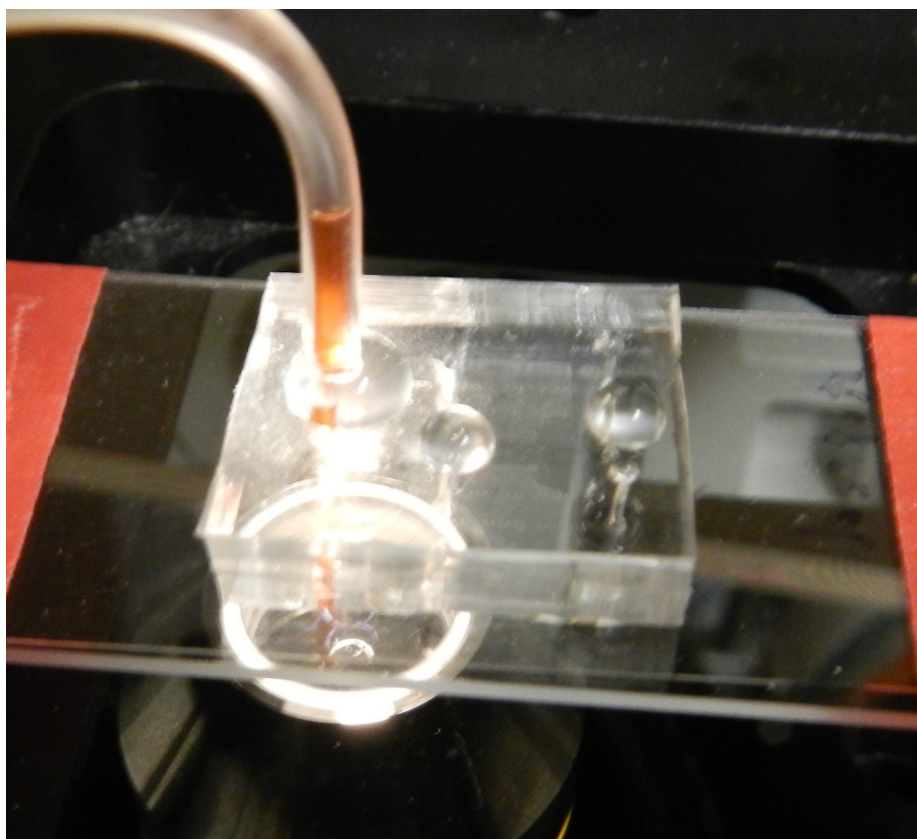
The resistance of the channel depends on their length and width, what's exactly the way this device is designed. After the two inlets there is the longest and smallest channels, so there is most resistance from inlet. That's why inlet was not used in the preparation process because it will make the filling up process even longer. Then comes the vent, the purpose of vent is to let some flow from inlet to get out immediately to allow fast switching of content flowing to the chamber array (refer to <http://scitation.aip.org/content/aip/journal/bmf/6/2/10.1063/1.3687379>), so it has narrow channel and second longest channel in total. This fancy method of switching flow by pressure was not utilized in this experiment because it's redundancy in this application. However, vent provide a low resistance inlet that can be used when trying to fill up the device. In this experiment, outlet was used for filling up purpose in the end because of the accident happened about BSA solution. The outlet is the widest channel in this device, and it's on the other side of

the device from vent and inlets, so this hole is used to push bacteria into the device to avoid clogging the small channels on the inlet and vent side.

Several points should be noticed when using microfluidic device.

First: NEVER PUT TOO MUCH PRESSURE!

Syringe should not be used in any case to push liquid inside, since your own hand is too unpredictable. Use syringe as container to use gravity to push liquid in. This is what happens when you push too much pressure to the device:



Yes, liquid is flowing out from the vacuum out let, that means the vacuum pad and the device is already connected, device is damaged and should be reused in this case. Also note the drop on the place where is tube is plugged in. That is liquid leaking, a sign of too much pressure and device damaging.

You should see two devices in the room (if you are winter 2015 group) they are the same, but one of them, the one not on the microscope is already damaged by me.....Sorry about that. The vent and out let are abused too much that leaking became very serious. One of the holes is expanded and the other one has a crack near it. It is possible to fix it by putting some PDMS around the hole, go to Gorisman lab for help if necessary.

Next thing to keep in mind is that, try your best to avoid clogging, the BSA from Hwa lab has some crystals in it naturally, I don't know the reason for it, but it is necessary to use syringe filter to solved this problem, also, don't leave anything overnight in the device except water, anything dried inside will clog the device immediately.

Surprisingly, microfluidic device can be reused, and the entire process can be performed in our Phys 173 lab. The detailed procedure is as follows:

First, separate the gel from cover glass carefully and wash it with ethanol. Detergent and

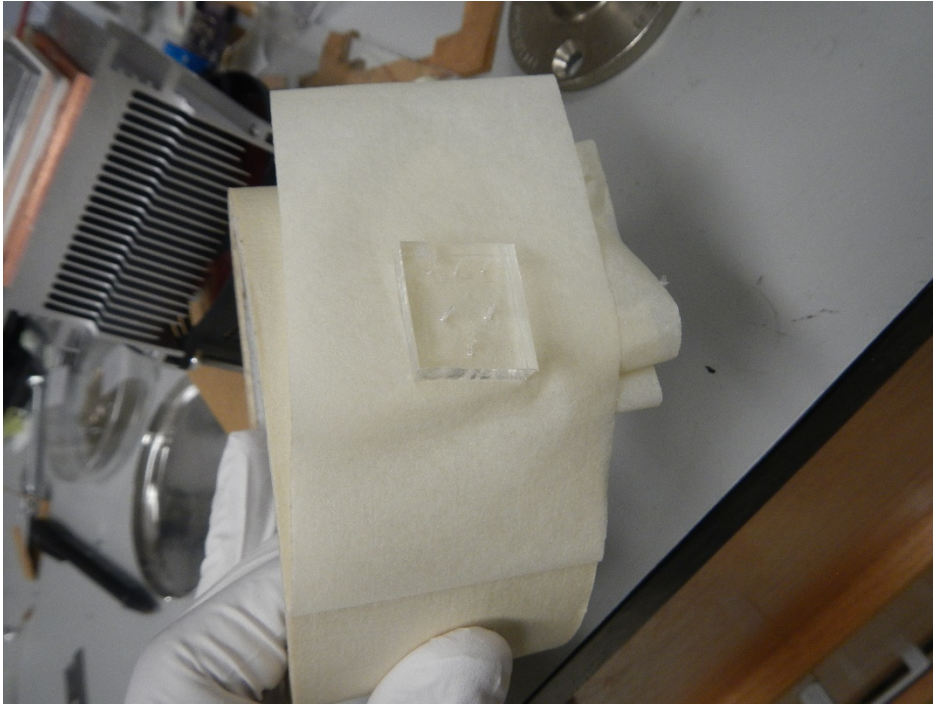
ultrasound is necessary if there's too much dirt in the device.



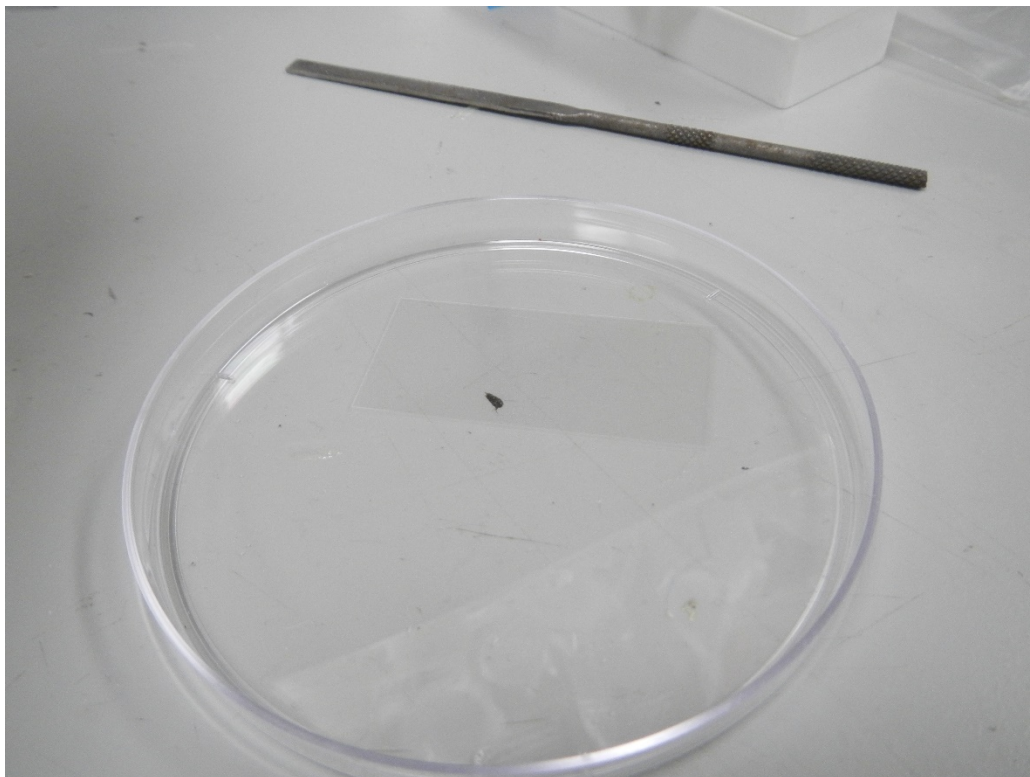
Then dry it with air flow.



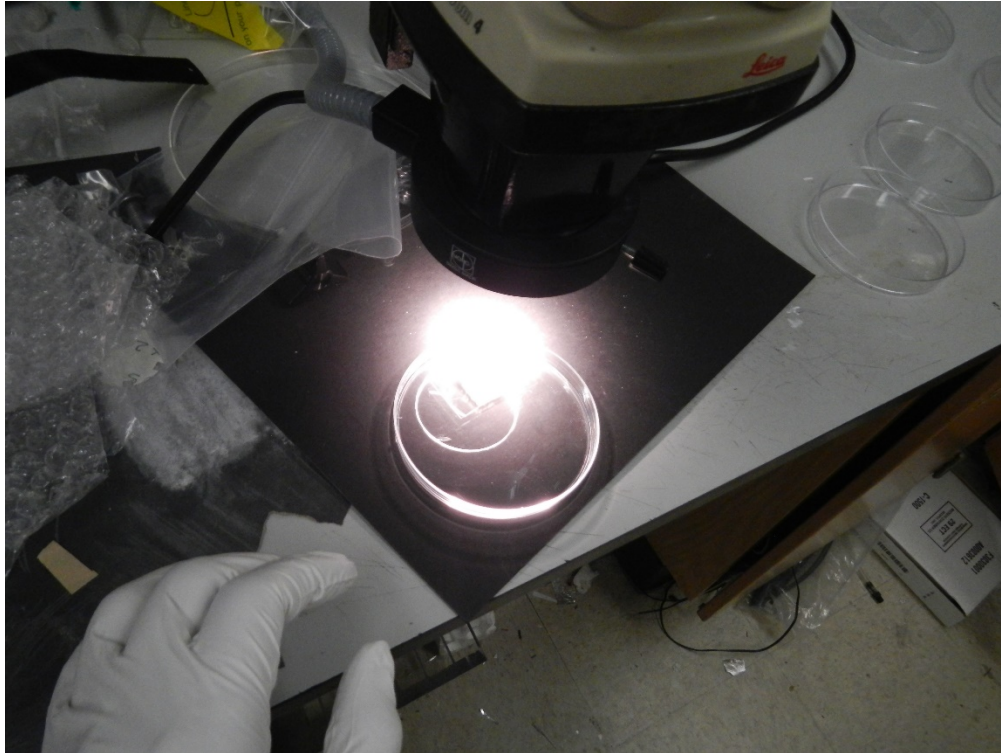
Thirdly, stick the microstructure face of the device on a tape, then remove the device and repeat several times, this is to remove any dust and waste from the channels and chambers.



Find a piece of cover glass, clean it using ethanol if necessary to remove any dust from the surface.



Put the device on the cover glass, tap it slightly to make contact. This is like putting a protection film on your cell phone screen. After you think the contact is fine, check it under microscope



This is to see where the device has contact with the glass and where it doesn't to check if the microscopic structure is intact, also you can see any dust between the gel and the glass, they should make a bubble. The way to check is by holding the device on your hand, and tilt it a little bit to let it reflect the light source while keeping it in focus, the contact of gel with glass will make it look more transparent, and in the place where it should be channels, you will be able to see reflection from the lower surface of the cover glass.

If the desired contact is achieved and no dust is present, go ahead to put the device in an incubator under 80°C overnight and the device will be ready to use. If there's dust present, separate the device from cover glass and clean the device and cover glass again, put them together and check until the desired contact is achieved and there's no dust between cover glass and device.

This is the tips I can provide about using and reusing microfluidic device, hope that helps. Good luck on your project.