Facultad
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Ciencias

Montaje de un dispositivo experimental para el estudio de biofilms en cámaras de flujo

Assembling of an experimental setup to study biofilms in flow chambers

Trabajo de Fin de Grado
para acceder al

GRADO EN FÍSICA

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Abstract

A new experiment was developed in which the growth rate of Escherichia coli bacterial colonies (biofilms) were studied under the effect of different flow velocities of growth medium. The phases of the experiment consisted of: i) the creation of the chip (microfabrication), ii) preparation of the bacteria inoculum and iii) the monitoring of their growth. All of the three phases exhibited difficulties, in which the sealing and the air bubbles can be singled out. Despite these problems, the simulation of the bacterial growth under the velocities of 0.02, 0.03, 0.04, 0.05, 0.07 and 0.10 mL/ min were recorded once and the bacterial growth of the 0.02, 0.04 and 0.10 mL/min chips were analyzed by measuring the density of the biofilms observing the grey scale throughout the trial and the statistical analysis in the last image that was recorded. This gave a general idea of what could be expected: high biofilm growth rates at low flows and low growth rate at large velocities. It was also noted that at large flows, the biofilms grew homogeneously while it was less homogeneous at low velocities.

Keywords: Microfludics, setup, biofilm, flow.

Resumen

Se ha montado un experimento nuevo en el que se ha estudiado el crecimiento de las colonias de bacterias (biofilms) de Escherichia coli en un chip creado a partir de PDMS dependiendo del flujo del liquido nutritivo que se le inyectaba. Se expondrán las fases de i) microfabricación para crear el chip, ii) la preparación del inoculo de bacterias y de iii) la parte de seguimento del crecimiento en los chips (microfluidica). Se explicará el dispositivo experimental de cada fase, y los problemas que surgieron en las mismas, en especial el sellado del chip al cubreobjetos de cristal y las burbujas de aire. Pese a estos problemas se simularon una única vez corrientes de 0.02, 0.03, 0.04, 0.05, 0.07 y a 0.10 mL/ min. De estos resultados, el crecimiento de los biofilms creados en los chips a 0.02, 0.04 y 0.10 mL/min fueron analizados midiendo la densidad de éstos mediante la observación de la escala de grises a lo largo de todo el proceso y el análisis estadístico de la última imagen tomada.
Los resultados obtenidos dieron una idea general de lo que ocurre: gran crecimiento de los biofilms a flujos bajos y bajo crecimiento en flujos altos. También se observó gran homogeniedad de crecimiento en flujos altos y poca en flujos bajos.

Palabras clave: Microfluidica, dispositivo experimental, biofilm, flujo.

Contents

1 Introduction

1.1 Objectives

The objectives of this project are:

- Assemble a functional experimental setup where we can determine the effect of flow velocity on the growth rate of bacterial biofilms.
- Develop a method to quantify the growth of bacterial biofilms from time-lapse image data.

1.2 What is microfluidics?

Microfluidics is a relatively new field that deals with the dynamics and manipulation of fluids at microscale. It is a multidisciplinary field that involves physics, chemistry, biology and engineering. It may be used for medical and pharmaceutical purposes, but also in engineering fields in energy generation and control systems. Here we use it to study the bacteria colonies (biofilms) in a *chip* created from a polymer with millimetric resolution. The process of fabrication of this kind of microfluidic chips is defined as microfabrication.

For years, microorganism growth has been studied through animal and in vitro (Petri dishes) testing. These two methods have been successful but both have important disadvantages. Microorganisms have short lifespans when grown in Petri dishes due to the lack of new nutrients, typically only lasting three days. These volumes are usually too big to observe the individual growth and it is very hard to control all aspects of the environment, which does not always favor the bacterial growth. On the other hand, animal testing is associated to high costs and slow data recollection. Most importantly sometimes it fails to give a viable prediction of the response on humans, and may lead to moral and ethical issues. [1]
The advantages of using microfluidics to study microorganisms are:

1. The human body can be better simulated since it is easier to imitate the conditions and variables of the human body (flow velocity, temperature, nutrient concentration...). The fluids and nutrients that are injected can be controlled on a submilimetric scale precisely and be manipulated by controlling the fluid pressures.
2. Low cost relative to other methods due to the chips being fabricated in a similar fashion.
3. The transparency of the chips helps the observation and can be recorded in real time.
4. The method can mimic the environment, the bacteria live longer, and enables independent 3D bacterial growth.
5. It can simulate high and low throughput analysis.

The experiments that have been done until now have proved various effects and events that were not considered or even knew that they existed. Microfluidis has opened new channels and methods of investigation in the fields of medicine, biology and pharmaceuticals that will prove to be very beneficial.

1.3 Mechanics of fluids at microscales[2]

It is important to point out that the physics of microscopic fluids differ from that of macroscopic fluids due to the importance of surface tension, low energy dissipation and extremely low Reynolds numbers.

In fluid mechanics, discrete quantities like mass transform themselves to continuous fields like density and force density. The velocity field for a Newtonian fluid obeys the Navier-Stokes equation:

$$\rho(\frac{\delta \mathbf{u}}{\delta t} + \mathbf{u} \cdot \nabla\ \mathbf{u}) = \nabla\sigma\ + \mathbf{f} = -\nabla p + \eta\nabla^2\mathbf{u} + \mathbf{f} \tag{1}$$

where u is the velocity, f is the force density, σ is the fluid stresses, ρ is the density of the fluid, p is the pressure and η is the viscosity. In microfluidics, the inertial forces are small compared to viscous forces leaving the equation as:

$$\rho\frac{\delta \mathbf{u}}{\delta t} = \nabla\sigma \ + \mathbf{f} = -\nabla p + \eta\nabla^2\mathbf{u} + \mathbf{f} \tag{2}$$

In any case, the condition for mass conservation is:

$$\frac{\delta\rho}{\delta t} + \nabla\cdot(\rho\mathbf{u}) = 0 \tag{3}$$

given the incompressibility condition $\nabla\cdot\mathbf{u} = 0$ for slow flowing fluids with $\rho \approx 1$g/mL.

There are various dimensionless quantities that indicate the change between macroscopic and microscopic scales. One of the examples is the Reynolds number. This is often mentioned in microfluidics, though its mostly irrelevant since the inertial forces can be neglected in these scales. The Reynolds number is defined as:

$$R = \frac{f_{inertia}}{f_{viscosity}} = \frac{\rho U_0 L_0}{\eta} \tag{4}$$

where L_0 sets the scale. In the macroscopic world we are used to having inertia, and as the Reynolds number increases, the non linear inertial term produces an irregular turbulent flow. The turbulences start at a Reynolds number around 2500. On the other hand, in ranges from 1-100 μm and velocities from 1μm-1cm / s the range of the Reynolds number is 10^{-6}-10. Implying that the viscous forces overwhelm the inertial forces, with which the non linear terms are neglected form eq. (1) resulting in eq. (2), defining the flow as laminar and predictable.

The impossibility of having turbulence makes it extremely difficult, for instance, to mix two fluids at microscale. Similar caveats occur when trying to apply our macroscopic intuition about fluid dynamics at micrometer scales related to the behaviour of surface tension, capillary forces, viscosity, vorticity, etc.

1.4 Bacteria growth

Bacterium growth is the continuous reproduction of a single bacteria. It depends on two basic things: the environment and their source of energy.

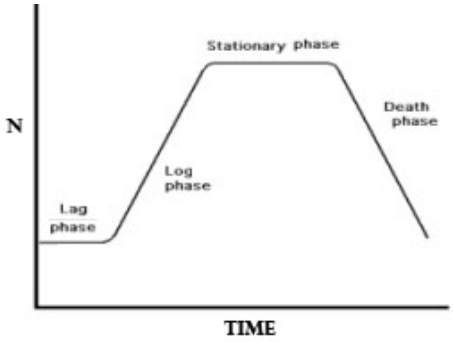


Figure 1: Graph representing the phases of the bacterial growth. N is the bacteria population.

Bacteria reproduce by a process named binary fission in which the cell divides itself into two identical bacteria. This leads to an exponential process and it takes little time to obtain a big colony. The time it takes to divide itself is called the generation time G and can be obtained with:

$$G = \frac{t}{n} = \frac{t}{\frac{log(b)-log(B)}{log2}} = \frac{t}{3\log(\mathrm{b/B})} \tag{5}$$

where B is the number bacteria present at t=0, b is the number of bacteria after a given time t and n is the number of generations.

This approximation is only valid for the first phase of the growth, when the biofilm grow at a constant speed hence the biofilm grows exponentially. There are 4 phases in bacterial growth, defined as in Fig. 1:[3],[4]

1. Lag phase: the bacteria settle in the environment and grow in size. They generate enzymes to reproduce in the new environment. The duration of this phase depends on the size of the inoculum, adaption time to create the enzymes.

2. Exponential phase: the bacteria reproduce in an logarithmic, constant manner, with the same interval of time between generation. It depends on the external conditions, like temperature and oxygen concentration. This phase continues until nutrients run off or toxins pile up.
3. Stationary phase: in which the size of the colony remains constant, due to equal reproduction and death rates of the bacteria. Due to lack of nutrients, space or too many toxins.
4. Death phase: when the rate of dying bacteria overcomes the rate of division. It is an exponential or logarithmic decay like the log phase.

Under constant flow of nutrients cells do not enter into stationary phase. Growth continues until the biofilm occupies the entire space, or the rate of cell division equals the rate of cells detaching from the biofilm. In the results section, we can see the first two phases since the nutrients are under constant flow. These two phases can be divided int three sub-phases: [5]

1. The cells/ bacteria stick to a surface and it begins to grow and reproduce according to the external conditions.
2. The various bacteria join together forming a colony.
3. The colony is too big so it detaches itself from the surface. Single bacteria also break from the colony and land on a surface, restarting the cycle.

This is due to the continuous flow of nutrients and the volume of growth medium is constant throughout the experiment. Here the rate of entrance of the medium is important because it has a limiting quantity of nutrients and eliminates the toxins and excess of bacteria at that given rate. [4]

A biofilm is a combination of microorganisms, like a bacteria colony, which stick on a surface. These cells are covered by a matrix formed by extracelular polymeric substances (EPS) that protects the cells from toxic substances and the environment. It also creates a stable environment inside where enzymes, nutrients and other molecules are concentrated, helping the growth of the bacteria.[6] The microorganisms may form biofilms due to environmental conditions, such as nutrition or toxic substances such as antibiotics, to protect their growth. When this happens, the phenotype of the bacteria shift.

There are four stages in the formation of biofilm: i)initial attachment, ii) early development of biofilm structure, iii) maturation of the biofilm and iv) dispersion of the cells.[6]

There are various ways in which one can measure the bacterial and biofilm growth: [4]

1. Physical measurement: measuring the mass, volume, etc.
2. Chemical measurement: measuring DNA, proteins, etc.
3. Measuring chemical activity for example O_2 CO_2 ...
4. Turbidity measurement: using setup to see how light scatters by the cells. The optical density is proportional to the number of cells.

The first two are direct measurements while the other two are indirect. This last method is the one we have used in the analysis of the results to see in a qualitative manner the evolution of our bacteria.

1.5 Possible applications of this experiment

Microfluidics has a bright future ahead. The field of medicine, biology and pharmaceutical experiments are the ones that stand out more, but they have also have a place in engineering fields for energy generation, regulators, control panels... In the biomedicine fields, cells from animals or humans and bacteria can be tested on, using only a millimetric surface, minimizing costs. This is a huge step for the investigation on diseases such as cancer, drug trials, etc. [7]

Biofilms are a huge medical problem, because once they develop they are very difficult to eliminate. Bacteria form biofilms con catheters and prosthetic replacements, leading to infections that can be life-threatening. Biofilms form in any aqueous flow is essential factor in their development.[8] Our experimental setup is thus of use to study not only how flow velocities impact biofilm formation. It can also be employed to study the effect of other factors like, for example, chemical compounds which could inhibit biofilm formation.

Also, a possible continuation of this experiment is the opposite procedure: have a bacterial colony inside the chip and insert antibiotics on with a different concentration or flow velocity and observe how the biofilms start to die. This could be an optimal way to treat many patients with prosthesis.

2 Experimental setup

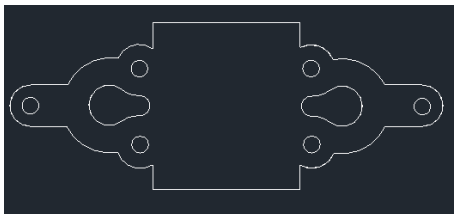
This experiment has passed through various phases which were: microfabrication, preparation of the bacteria and microfluidics. A different branch of science was involved in each of the phases: engineering, biology and physics, respectively , in that order, thus a comprehensive approach was required to develop the entire assay successfully. .

2.1 Microfabrication

This phase deals with the fabrication of the chips where the bacteria will grow. This process included i) design of the chip in AutoCAD ii) generation of the mold by 3D printing, iii) fabrication of PDMS chips and iv) a standard quality test to validate the chip.

2.1.1 Design

Our design was based on the CAD prototype by Song et al.[9] We modified this design to a smaller scale with dimensions 3.8mm x 9.81 mm x 0.24 mm (width, length, height). For microfabrication, we design using CAD the mold that will be used to generate the PDMS chips. Our mold design is shown in Fig.2. The resulting chip obtained from that mold is shown in Fig. 4. Table 1 shows the the dimensions of the most important features of the chip. Fig. 3 is the design that was 3D printed and cured with isopropyl alcohol, in order to obtain a fair number of chips in each 3D printing process.



(a)

(b)

Figure 2: Design used for the chips that was obtained by the article from [9] and adapted to a 3.8mm x 9.81 mmx 0.24 mm format. (a) Top view of the chip, (b) side view of the chip.

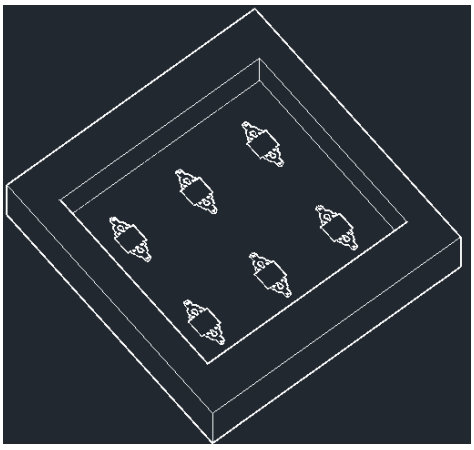


Figure 3: Final mold to create the chips. Here 6 chip designs were engraved to optimize the procedure and a resin wall was attached to ease the cutting process in the fabrication phase.

In the article, the values of the measurements of the chip were too big for this experiment, that is why the design was reduced by a scale of 1:0.29 as seen in Fig. 4.

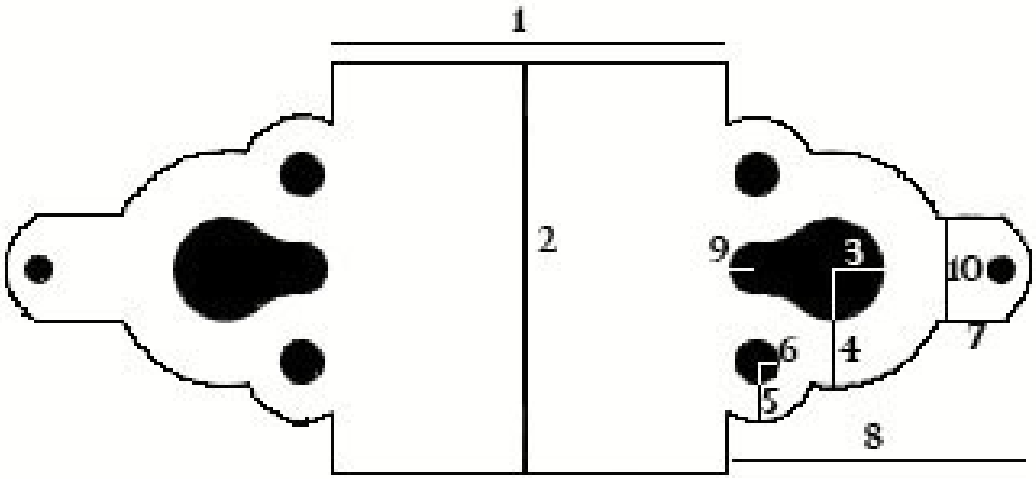


Figure 4: Diagram of the chip used for the experiment with the most important measurements labeled. The values of these dimensions are seen in Table 1.

n°	length/ mm
1	1.760
2	3.800
3	0.470
4	1.090
5	0.550
6	0.190
7	0.260
8	3.090
9	0.230
10	1.010

Table 1: Values of the sizes of the sides and radius in Fig. 4. The error of the length is ±0.005 mm.

With these dimensions it is simple to approximate the area of the chip:

$$A_{chip} \approx 12.330 \pm 0.005 \text{ mm}^2 \tag{6}$$

and its volume is:

$$V_{chip} = A_{chip} \cdot 0.24 = \approx 2.959 \pm 0.005 \text{ mm}^3 \tag{7}$$

2.1.2 Fabrication

Fabrication involves two steps. First the CAD file is transformed into the STL (Standard Tessellation Language) format used by 3D printers. For 3D printing, we used a FormLabs 1+ laser 3D printer (www.formlabs.com). This printer uses a 405 nm violet laser (120mW) to induce the polymerization of a viscous resin. For our molds, we used GPBK03 black resin, which has a resolution up to 25 microns. After 3D printing, molds were washed with isopropanol twice to remove unpolymerized resine, and post-cured under a UV lamp for at least 3 hours.

PDMS chips were generated from these molds. Microfabrication took place in a semi-white room, where chips and molds were kept in a laminar flow hood to avoid interference from dust or any debris. In order to avoid any fibers from contaminating the chip manufacture, we had to wear full body covers, gloves and a protection screen.

In the semi-white room, the PDMS (polydimethylsiloxane) was mixed in a laminar-flow hood with a curing agent in a 10:1g proportion and put in a vacuum chamber for 30 min to remove the bubbles created during the mixing. Afterwards, the mix was poured in the 3D printed mold, seen in Fig. 3 and was put in a vacuum oven (Fig.6a) for another 30 min at room temperature. This process eliminated the rest of air bubbles in the PDMS mix that would influence negatively in the next phase. Once this was finished, the mold was put into a normal oven at 50°C overnight. An outline of the fabrication process is shown in Fig. 5.1.

The following day the PDMS had solidified into a flexible polimer surface. It was peeled off from the mold, and each chip was cut individually. Each chip was then punctured with a 0.3 mm punch, in the at the two distant points along the longest axis of the chip, creating an input and an output hole from the chip chamber. Next, the chips were cleaned with isopropyl alcohol, dried with an air pistol and put on a laboratory slide, as shown in Fig. 5.2. Also a crystal coverslip was cleaned with ethanol, isopropyl alcohol, water and isopropyl alcohol, in that order and then dried with the air pistol as well. After cleaning the crystal, one of the corners was marked with a pen so it was known which of the faces was going to be "glued" to the chip. Both the cover and the slide with the PDMS chip were put into an O_2 plasma generator (Fig.6b), with the marked surface facing the ceiling, seen in Fig. 5.3. In order to operate the generator, first vacuum was needed. The pressure had to drop to 0.3 mbar. At this pressure, plasma generation was ignited. A bright purple glowing in the chamber was indicative of plasma generation. Chips and coverslides were subjected to plasma for 3 minutes. Finally, after taking both objects out of the plasma generator, they were taken to the hood where the chip and crystal cover slip were sealed together by the marked surface, Fig.5.4 and put into the oven at 50°C overnight. The full process of fabrication of the chips of a single mold took over 48 h.

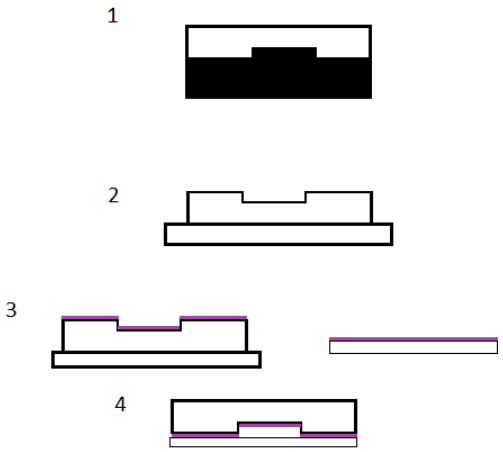
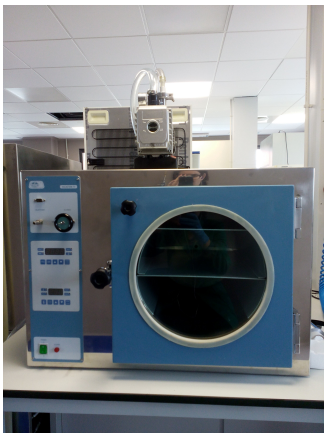
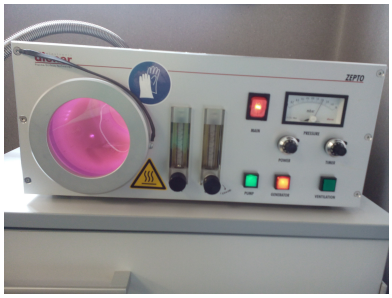


Figure 5: Outline of the microfabrication process. In 1- The process in which the mold (seen in black) was covered by the PDMS (in white) and this took its form, i.e all of the process of the first day. 2- The PDMS chip, once it was cut, was placed on the coverslip and cleaned. 3- The PDMS chip and the crystal slip were inserted into the O_2 plasma generator and the plasma (the purple line) was perched on its surfaces 4- Both objects were placed and glued together with the plasma creating a small vacuum where the bacteria could grow.

The last step in this phase was the first quality check of the chip. For this part, a syringe with sterile H_2O and two plastic tubes of 0.3mm diameter with adapters were needed, shown in Fig. 7. Both adapters were put on the extremities of the chip, one as the input and the other as the output. The input tube was connected to the syringe and the output end directed to a plastic cup. The water from the syringe was then pushed through the tube, into the PDMS chip and out the tube into the cup. If there were no water leaks in the PDMS chip it meant that it could be used for the biofilm



(a)



(b)

Figure 6: (a)The oven with vacuum used on the first day of the microfabrication process. (b) The O_2 generator used on the second day.

growth experiment, and if there were leaks then it was useless. Approximately 50% of the chips failed to pass this control and had to be disposed of.

Figure 7: Image of the first quality check of a 33mm x 13mm x 0.24 mm PDMS chip.

2.2 Preparation of bacteria inoculum

For the experiment two basic elements were needed: the PDMS chips and the bacteria. The chips were produced during the microfabrication process described above. The bacteria inoculum creation process went as follows.

The preparation of the bacterial culture used in the microfluidic experiment went as follows.

On the first day, in a laminar hood cabinet, to ensure sterile conditions, E.coli cells (0.1 ml) were inoculated into 10 mL Erlenmeyer flask filled with Luria-Bertani rich medium (LB). Bacteria were allowed to grow overnight by incubating the flask at 37C under gentle agitation.

This bacterial culture was then used in the microfluidic experiment, but first the depeted growth medium was refreshed with new one. For this purpose, the overnight culture was poured into a Falcon tube and centrifuged at 4000 rpms in an Eppendorf 5810 centrifuge for 10 minutes. This made bacteria to precipitate to the bottom of the Falcon tube, so the old growth medium could be poured out and an equivalent volume of LB was poured in. Cells were then resuspended in this medium. This culture was used in the experiment as the bacteria injection on the chip to create the biofilm.

2.2.1 Growth medium preparation for the experiment

The growth medium is a sterile liquid solution with nutrients on which the bacteria feed on. The medium we employed is Luria-Bertani (LB), a rich medium based on yeast extract, which contains the,inorganic salts and organic compounds required for vigorous, fast growth of E.coli. [10]

The biofilms could not be seen by themselves since they were transparent. So the medium was tinted with a purple dye so the nutrients were coloured, making the bacteria darker. For this purpose, we used the triarylmethane dye crystal violet. 0.01%. w/v proportion. This percentage did not interfere with bacterial growth, but staining was intense enough for biofilms to be monitored.

After dying the liquid, it was necessary to put it into a syringe to be used for the next phase. This was done in a clean extraction chamber. There, a 50 mL syringe was opened from behind, in other words, from the plunger top and the dyed medium was poured in slowly (after confirming that the adapter was blocked). This method of filling the syringe was useful to avoid air bubbles, which were a main source of problem in the process downstream. Afterwards, the syringe was closed and the little air inside was pushed out. Finally, a filter was put on the adapter, to avoid bacteria from the chip to swim back into the syringe with the reservoir medium.

2.3 Microfluidics

The experimental setup for this experiment was made of 2 pumps, a microscope, a photo camera, a heat chamber, a computer, various syringes, a filter and various 0.3 mm diameter tubes and adapters, as shown in Fig 8.

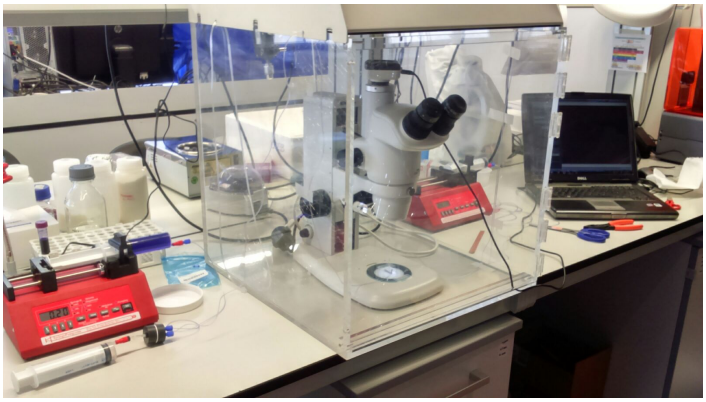


Figure 8: Setup for the microfluidics phase. On the side the computer in which the photos from the camera were recorded.

First, tubing was cleansed with ethanol and water. For this purpose, a syringe was filled with a solution of ethanol at 0,7 M and put into the input syringe. The pump was turned on and tuned in a flow speed of 1 mL/ min. It was left for 10 minutes to eliminate previous substances or bacteria that might be left in the tubes. Next, with the same syringe, the same process was done with the output tubes. Afterwards, with a different syringe, sterile water was pushed through the tubes, to eliminate any rests of ethanol. After this process, the experimental setup was ready to be used.

Once the tubes were cleaned, the chip was connected to the input and the output tubes in the middle of the microscope inside a Petri dish.

Then 10 mL of sterile water was inserted into a syringe and put into the input pump, also an empty syringe of the same volume was put into the output pump. Both were set to a constant flow of 0.14 mL/min, one to insert and the other to withdraw the water. This double pumping/withdrawing flux allowed a more stable flow inside the device. The setup was then left for 30 min to work and checked every 10 minutes. The first quality check was done in the microfabrication phase, testing if the PDMS

chip was well sealed. This tested if the chip could cope with a continuous liquid flow for a long period of time. If there are no leaks, the chip can be used, the chip could be used in the following procedures, if there were leaks, it was discarded.

If the chip had passed both tests, it was strained in the Petri dish and the dish was then fixed, making sure that most of the chip was in the field of the camera so the growth could be properly recorded. Also the thermal chamber was closed and turned on to 37°C.

A new syringe, loaded with LB+Crystal violet (0.01% w/v) was inserted into the syringe pump. Then flow was programed to the appropriate flow velocity (0.02, 0.03, 0.04, 0.05, 0.07 or 0.10 mL/ min). The output pump was changed and reprogrammed to the same withdrawing speed. The setup was then left alone for ten minutes so all of the spaces were filled with the liquid and avoid possible formation of bubbles. After those ten minutes, a small 1 mL syringe was filled with the bacteria dissolution mentioned in Section 2.2 and both of the pumps were stopped. The connecting tube from the input was divided into two and the bacteria syringe was connected to the chip.Bacteria were injected into the chip and left for 15 minutes without any flow, so they could settle in the floor of the chip. Just before the 15 minutes were done, the input pump was restarted, but not connected to the chip, to make sure that the were not any air bubbles in the tube. Once the liquid dropped from the tube, the bacteria syringe was disconnected from the chip, and the tube was reconnected to it. Also, the withdraw pump was restarted, creating the continuous flow. Finally the camera was turned on and programed to take a photo every 20 minutes, to record the growth of the biofilm.

Depending on the pump velocity the recording of images lasted between 24 and 48 hours. In each trial the volume of the medium inserted for the experiment was 55 mL approximately.

2.4 Data analysis

After obtaining the photographs, an image program was used to enhance the contrast of the grey scale of the images by 0.5%, to see the differences between the background and the bacteria colonies better. These photographs were then put together in videos, which can be seen in Section 4 in Table 3. Three experiments were chosen to see the how low (0.02 mL/min), medium (0.04 ml/min) and high (0.1 mL/min) flow velocities affected the biofilm growth. For these videos, the analysis had to be more thorough.

The background was eliminated by cutting out just the chip from each photograph and placing it in a white background, and the grey scale of these new images were obtained, represented as histograms, thus seeing the density of the biofilm throughout all the time laps for each of the three videos. From these images, the last photo was singled out and its grey scale was analyzed neglecting the last 3 channels from the histogram that came from the background. Also, each biofilm was singled out in these images and analyzed as well though this process. From the analysis, the statistical data, i.e. mean, median, mode, σ, σ^2 etc. were obtained. This data is shown in Table 4.

3 Troubleshooting throughout the experiment

This experiment was new, with no previous references or setup, so throughout all the phases that were explained on the previous chapter, various problems were encountered and had to be fixed in order to go forward and obtain results. In this chapter these problems and their solutions will be explained.

3.1 Microfabrication phase

This phase of the experiment could be considered the most important one because without the PDMS chips, there would be no recipient in which the bacteria would grow. For that, the chips had to be totally sealed and sterile.

The main problems in this phase were:

- Size
- Sealing

3.1.1 Creation of the mold

First of all, the design for the chip was redone three times due to the scaling problem. At first the measurements for the chip were the ones in the article by Song et al[9]: 45mm x 13mm x 0.24mm (width, length, height) shown in Fig.9a. When trying them in the last phase, it was seen that they were too big to be seen entirely though the microscope, so the rectangular chamber was reduced to half so the new measurements of the chip were 33mm x 13mm x 0.24mm in Fig. 9b. These chips could be seen entirely through the microscope, but not the camera eye, so the mold had to be rescaled to 9.8mm x 3.8mm x0.24mm seen in Fig. 9c and 9d, which could barely appear as a whole on the image of the camera, as seen in the results chapter.

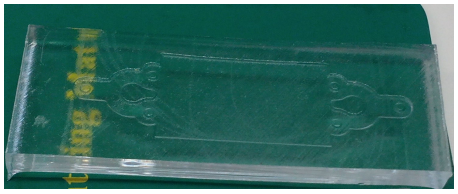
Another factor related to the size was the sealing problem. One way it affected the sealing was because of the pattern. The larger the chip, the more stripes it had, thus making it more probable to leak water. The other way was having too much area which affected how it handled the water flow, specially in the border of the chip . As the chip got smaller, less water was in the chip so it had less force against the sealing, making it less probable to create a leak.

Also, the type of resin used for the 3D mold had repercussions. With a good resin the 3D mold would hardly have a striped pattern, thus the sealing would be better. The first mold was done with a low quality mold and the chips that did stick to the crystal coverslip were not totally sealed so very few passed the first quality check. This was mostly because of the striped pattern on the surface of the mold due to the low resolution and quality of the resin. This was fixed on the second mold by using a high quality resin which made the striped pattern undetectable and thus the chips were sealed with double the success rate than the previous ones. Unfortunately, the high quality resin could not be used for the final mold because of external factors and a medium quality resin was used for the final 3D impression, but with a higher resolution. With this mold, the striped pattern was detectable but less than in the first mold and a large number of chips were sealed.

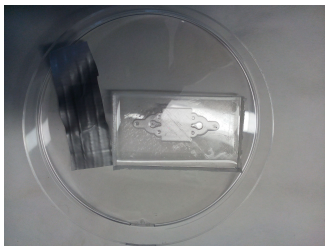
The last complication in the mold was its overuse. After using the mold too much, the PDMS would not stick to the crystal, losing the possibility of obtaining any chips.

3.1.2 Sealing

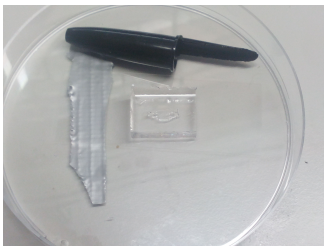
Sealing was the main problem which persisted until the end. This limited everything in the experiment, because, as stated earlier, the fabrication of the chips were the most important part and it took two days to obtain the chips. The mold had 6 chips in its design, i.e. a maximum of 6 chips were obtained per day, by doing the first and second phase every day. Unfortunately, on average only half of them passed the first quality check.



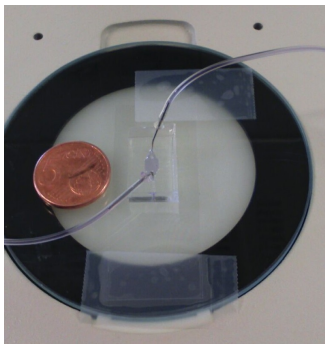
(a)



(b)



(c)



(d)

Figure 9: Images of the three different sizes of the chips. (a) The original size that was estimated from article [8], (b) The chip after reducing the chamber to half. Images (c) and (d) are the final chip that was used throughout the experiment.

In the beginning, the scale of pressure where the O_2 plasma was created was 0.2-0.4 mbar. Any pressure in that range should have worked to perfectly seal the chip to the crystal. After numerous tries it became clear that if the pressure is 0.30±0.02 mbars for 3 minutes the probability of the chip being stuck on the crystal increased by more than 50%. Also if the pressure was higher or lower than that, there was a 60% chance it would not be useful and the chip had to be thrown away. In the generator only one chip and one crystal were put in to avoid problems, i.e. chips sticking together or the crystals sticking together.

Inside the generator, once air was let in, the crystal would fly towards the end of the generator. This caused in some cases for the crystal to break (partially or entirely) or to stick on the chip in an unhelpful manner. The time that the objects had to be inside the generator was tested. The chip would not be sealed if they were less than 1.5 minutes. Between 1.5 minutes and 3 minutes, the chip would be sealed but with less probability than if it were in the generator for over 3 minutes.

In every case, if the chip could not get sealed to the crystal on the first attempt, putting it back in the generator, with or without re-cleaning it, it would not stick properly. So once it was proved inviable, it was thrown away. This reduced the number of viable chips that could have been used for the experiment significantly. Also, if the chip was sealed wrong, i.e permitting air bubbles between the PDMS and the coverslip or if it was stuck with too much force, creating a fine fissure in the chip, it was problematic and the chip could not pass the quality tests.

3.2 Microfluidics phase

In this phase the problems that were faced had more repercussion experimentally, because they directly affected the results.

3.2.1 Leakage and second quality test

Starting with the low percentage of chips that passed the first quality check at the end of the microfabrication phase, a significant percentage of the chips that did pass could not stand the continuous

flow of water for a long time, thus minimizing the number experimental chips that could be used. This was due to a small sealing error that would not be seen in a small amount of time but, with an ongoing liquid, it broke into a leakage.

Also, too much fluid pressure could break the chip even if it was sealed. This proved that a minimum error in the previous process could not be neglected. Since there were no previous papers explaining this experiment, all of the trials to deduce the maximum flow that the chip could support, had to be done experimentally by us. It was proven that a flow higher than 1.4 mL/min was harmful for the chip, so it was established that this flow velocity would be the testing speed for the quality control.

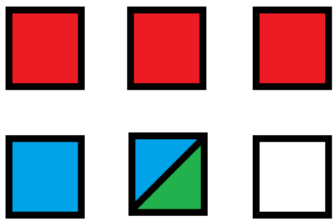


Figure 10: General outline of the effectiveness of the microfabrication process in which six chips were made but only one (in white) could be used to cultivate biofilms. Here in red are the statistical number of chips that could not be sealed o had a problem with the sealing. In blue is the statistical number of chips that did not pass the first quality check. Finally in green is the number of chips that passed the first quality check but not the second.

The leaks in the unions of the tubes and in the syringes presented another technical problem. When there were leaks, the flow would be altered and the quantity of medium was affected thus the attempt failed. Even if there were no leaks during the second quality exam, it still could present leaks during the biofilm growth time, so it was custom to check randomly if the setup was correct even during this phase.

3.2.2 Air bubbles

Another big problem that was encountered were the air bubbles because of the following reasons:

- They occupied space where the biofilm would grow.
- They would make the growth medium create flow movements that would affect the nearby biofilm.
- If the biofilm had already started growing, the air bubbles it would stop it.

The main reasons bubbles were created were:

1. In the quality checks due to handling of the chips.
2. In the transfer of the growth medium to the syringe.
3. In the injection of the bacteria inoculum into the chip.

In the first case, bubbles occurred after passing either of the chip quality checks. If the test was made one day and used another, it was possible that there would be leftovers of the water used, making it very probable that it would form a path for the water or medium to pass the chip, not occupying the whole of it. The best way to avoid these bubbles was to do the quality check the same day as the bacterial growth. This action largely minimized the probability of obtaining this kind of bubbles.

The bubbles created in the second case were due to the vacuum in the unused syringes. At first the growth medium would be poured into a clean Petri dish and then it was sucked into the syringe. Since the syringe had vacuum inside it, the action of pulling the medium in it, created air bubbles, specially when the liquid in the Petri dish was low. Most of the air could be expelled by making sure the air bubble was in the exit point of the syringe and pushing the air out, but not all of it, so there were little air bubbles that got through the tube and into the chip in the last stage.
The best way to avoid these bubbles was to change the way of inserting the liquid into the syringe. The method changed to the one explained in the previous chapter, i.e. taking off the plunger from the syringe and pouring the medium into it, after covering the adapter.

Finally, the other main reason was the air going through the tube while the bacteria inocule was connected to the chip. In this case, the air bubble was due to the separation of the main tube from the chip, thus making the growth medium to pour out of the setup (in a controlled manner) because of gravity and inertia. When this was not considered, there was a huge air bubble after the bacteria injection that compromised the experiment. When this was taken into account, the way to avoid the bubble was to restart the pump without connecting the tube to the chip, and wait until the medium began to leak from it. Once this happened, the syringe with the bacteria inocule was disconnected from the chip and the tube with the fluid was reconnected to the setup.

Even when these three causes were considered and properly handled, there was a small chance of getting an air bubble in the chip. Depending on the phase in which the PDMS chip was, there were two ways to get rid of them. If it was before injecting the bacteria, the chip could be disconnected from the setup and air was injected with a sterile syringe until the bubble was gone. Then, sterile water was injected to confirm that no more bubbles would be formed. If it was already being used for bacterial growth, the only possible thing to do was to wait for the bubble to move towards the output hole, which did not happen often. Tampering with the experiment at that phase would alter the results.

3.2.3 Camera focus

The final problem, which was not as big as the rest, was the resolution and focus of the camera on the biofilm. When the chip had the bacteria inoculum injected, the camera was turned on and it began to take pictures. Since the chip is transparent and the dye only affected the bacteria, in the first photos it was hardly possible to distinguish the chip with the background and thus, made it impossible to focus the camera on the colonies. This made the data recollection imprecise but still valuable.

In Table 2 the problems summarized above are listed with their effects and solutions.

Problem	**Reasons**	**Effects**	**Solutions**
Bad sealing / Leaks	-Striped pattern in the mold -Mold too big -Air bubbles between PDMS and crystal -Overuse of mold -Others	Chips useable due to water leaks	-Change the mold to one with better resolution -Sand down the mold
Bubbles	Bubbles are created in chamber	-Alter course of the fluid -Misuse space for biofilm growth	-Introduce liquid under more pressure -Leave the pumps on longer
Size	-Mold design too large	- Chip unusable -Cannot record all of the chip	Make smaller design
Camera resolution	Inability to focus due to transperancy of chip	Blurry image of biofilms	-Focus once biofilm grows -Leave previous focus

Table 2: Summary of the problems explained throughout this section, with their reasons, effects and possible solutions. They are organized by importance and the frequency.

4 Results and Analysis

In this section, the videos for each fluid flow will be shown and the analysis of the photographs of the experiments at 0.02, 0.04 and 0.1 mL/ min will be explained and be observed. In Table 3 the compilation of videos can be observed through hyperlinks in PDF version and in the Appendix the hyperlink directions are written.

Number	F ml/min	Original Video	Edited video
1	0.02	0.02.1	0.02.2
2	0.03	0.03.1	0.03.2
3	0.04	0.04.1	0.04.2
4	0.05	0.05.1	0.05.2
5	0.07	0.07.1	0.07.2
6	0.10	0.10.1	0.10.2

Table 3: Videos of the recompiled data of each flow F of each flow. In the last column, contrast between greys was enhanced to see the difference between the background and the biofilms. Also in the edited videos of the 0.02, 0.04 and 0.10 mL/min, the background was cut out and their grey scale was analyzed in all of the images, seen as a histogram on the top left corner.

Our study of the biofilms, as stated previously, has focused on their density. For that a grey scale was considered, this is, the darker the photo, the more bacterial colonies were formed. The grey scale has been represented in histograms where the channels go from 0 (black) to 255 (white). For the considered chips (0.02, 0.04 and 0.10 mL/min) the grey scale histograms of the total chip and each biofilm are shown in pairs. On the left the original histogram is shown and on the right a modified version of it. In the modified histogram, the background pixels (the last three channels) and the first channels until the first registered pixel were eliminated since they did not contribute to the analysis. From this second graph, the statistical data was obtained and is shown in Table 4.

4.1 Analysis of the 0.02 mL/min chip

Here, the last image obtained form the experiment with the flow velocity of 0.02 mL/min will be examined in a more qualitative way by observing the density of each of the biofilms with the grey scale histograms and obtain the statistical data from them.

In Fig. 11 a final image from Video 0.02.2 is shown. Here, six biofilms with different forms and sizes are have been selected and will be examined as well as the density of total chip.

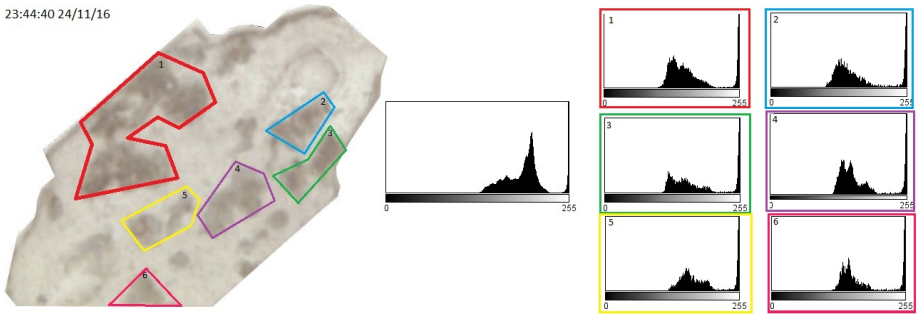
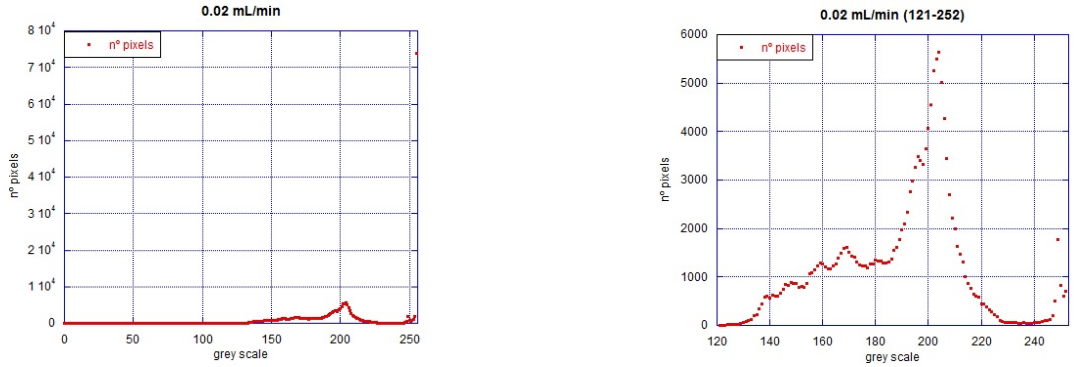


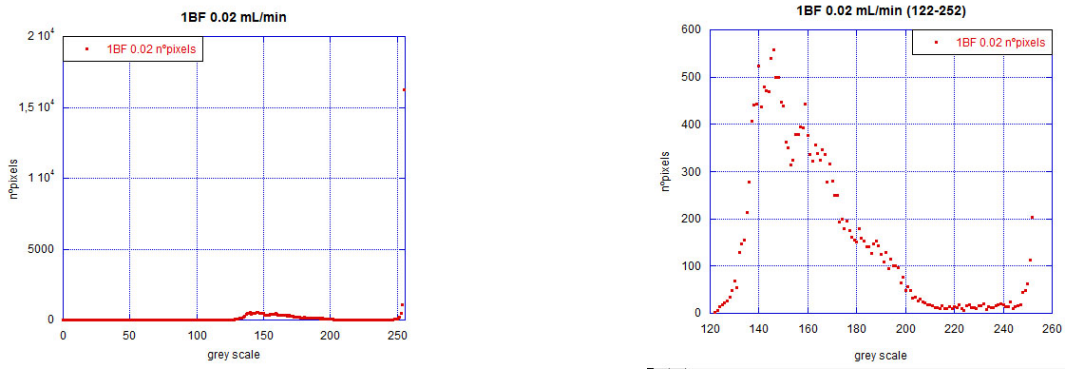
Figure 11: Final image in Video 0.02.2 in which six bacterial colonies have been selected (1-6) and isolated in the coloured perimeters (red, blue, green, yellow, purple and pink) with each of the histograms corresponding to each biofilm on the right side of the image (each with the color that corresponds to the perimeter). In the middle, the total density of the chip is displayed without colour.

In the following figures, Fig. 12- Fig18, the grey scale histograms of the chip and the biofilms, seen in Fig. 11, are shown in order.



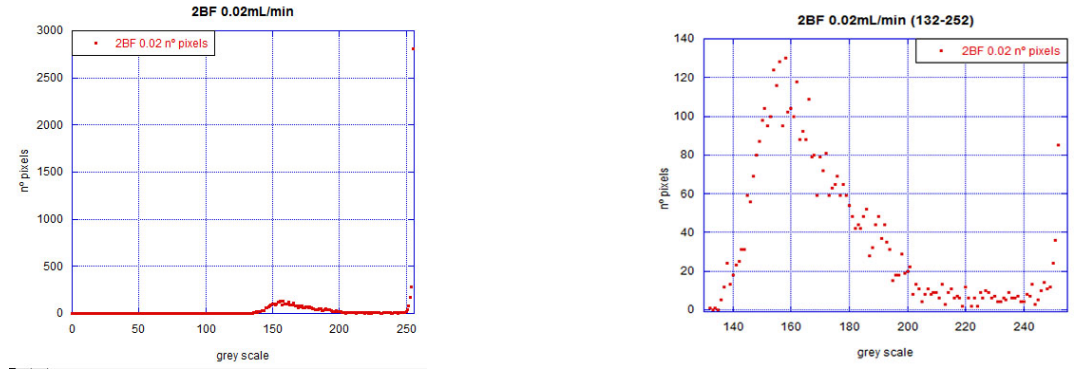
(a) (b)

Figure 12: Histograms of the 0.02 mL/min of the chip. (a) is the total histogram seen in the center of Fig. 11 and (b) the same, removing the first 120 channels.



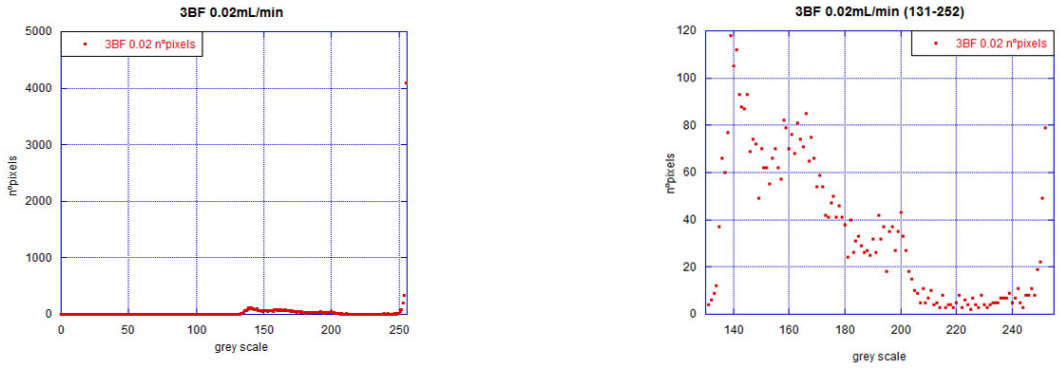
(a) (b)

Figure 13: Grey scale histogram of the first biofilm (1) in the 0.02 mL/min chip, surrounded in red in Fig. 11. (a) Is the total histogram enclosed in red and (b) the histogram starting at channel 122.



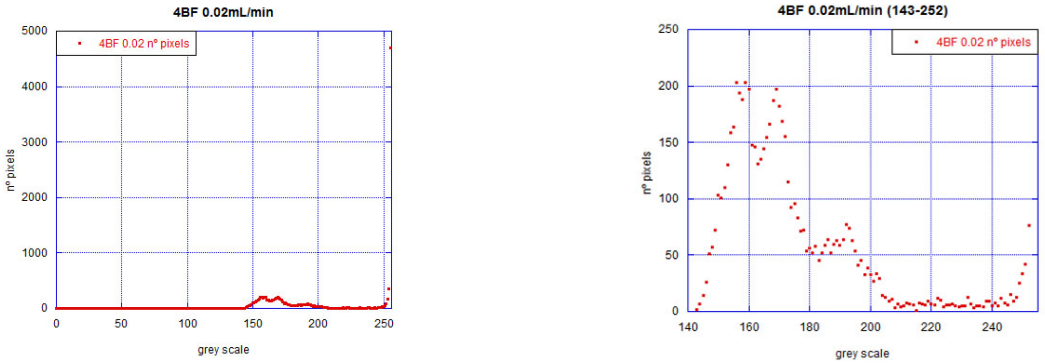
(a) (b)

Figure 14: Grey scale histogram of the second biofilm (2) in the 0.02 mL/min chip, surrounded in blue in Fig. 11. (a) Is the total histogram enveloped in blue and (b) is the histogram starting at 132.



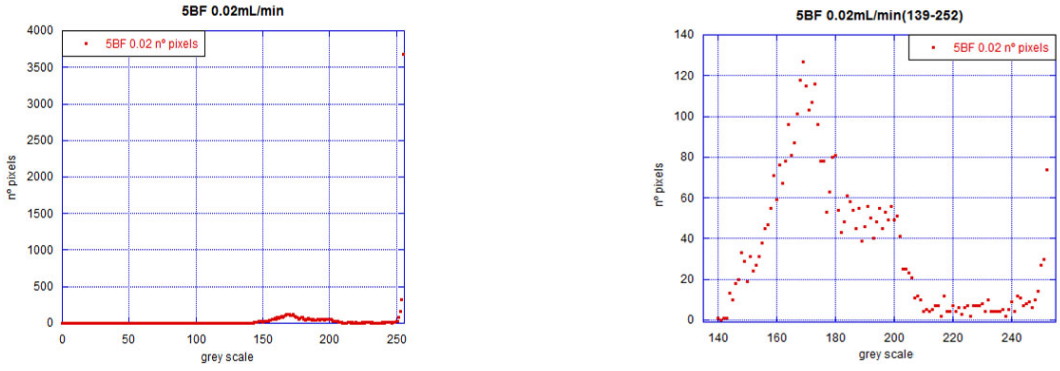
(a) (b)

Figure 15: Grey scale histogram of the third biofilm (3) in the 0.02 mL/min chip, surrounded in green in Fig. 11. (a) Is the total histogram and (b) is the histogram starting at 131.



(a) (b)

Figure 16: Grey scale histogram of the fourth biofilm (4) in the 0.02 mL/min chip, surrounded in purple in Fig. 11. (a) Total histogram and (b) is the histogram starting in channel 143.



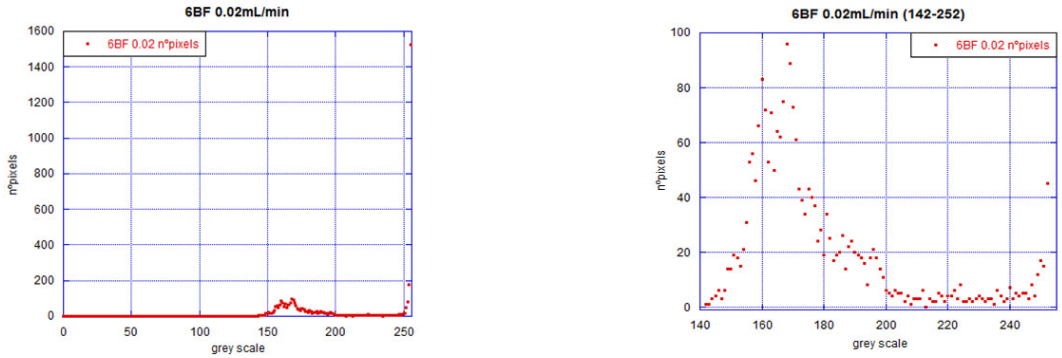
(a) (b)

Figure 17: Grey scale histogram of the fifth biofilm (5) in the 0.02 mL/min chip, surrounded in yellow in Fig. 11. (a) Total histogram of the biofilm and (b) is the histogram starting in bin 139.

4.2 Analysis of the 0.04 mL/min chip

Next, the same procedure was carried for the 0.04 mL/min chip. In Fig. 19, the last photo from Video 0.04.2 is shown, and three biofilms are distinguished.

The four histograms seen in the last image are represented in pairs in Figures 20 to 23.



(a) (b)

Figure 18: Grey scale histogram of the sixth biofilm (6) in the 0.02 mL/min chip, surrounded in pink in Fig. 11. (a) Total histogram and (b) is the histogram starting in channel 142.

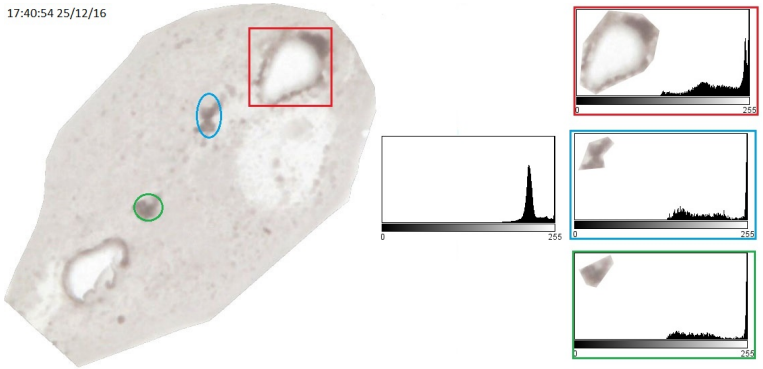
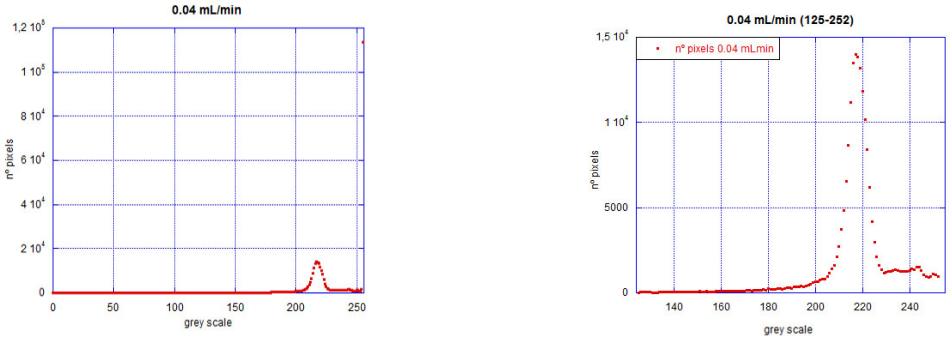


Figure 19: Final photo in Video 0.04.2 in which the three bacterial colonies have been selected and isolated in the coloured perimeters (red, blue and green) with each of the histograms corresponding to each biofilm on the right side of the image (each with the colony and colour that corresponds to the perimeter). In the middle the total density of the chip is displayed without colour.

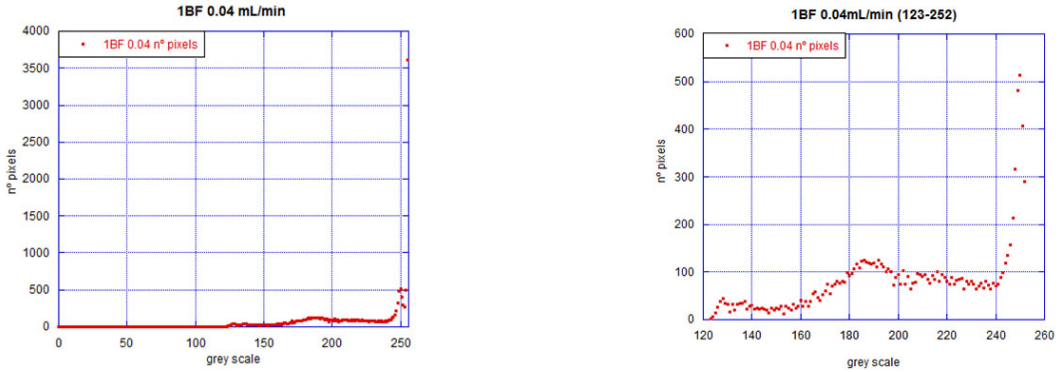


(a) (b)

Figure 20: Histograms of the 0.04 mL/min chip. (a) The total histogram seen in the center of Fig. 19 and (b) the histogram cutting off the first 124 channels.

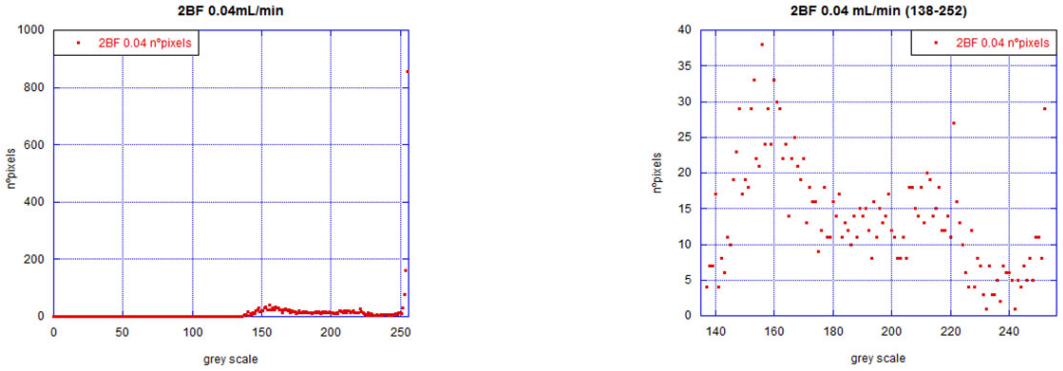
4.3 Analysis of the 0.10 mL/min chip

Finally, the last chip considered was the chip with a flow velocity of 0.10 mL/min. The same analysis method was done as in the previous chips. In Fig. 24 the last image from Video 0.10.2 can be observed, with each biofilm defined and circled. In Figures 25 to 28 the paired histograms of the chip and the biofilms are seen.



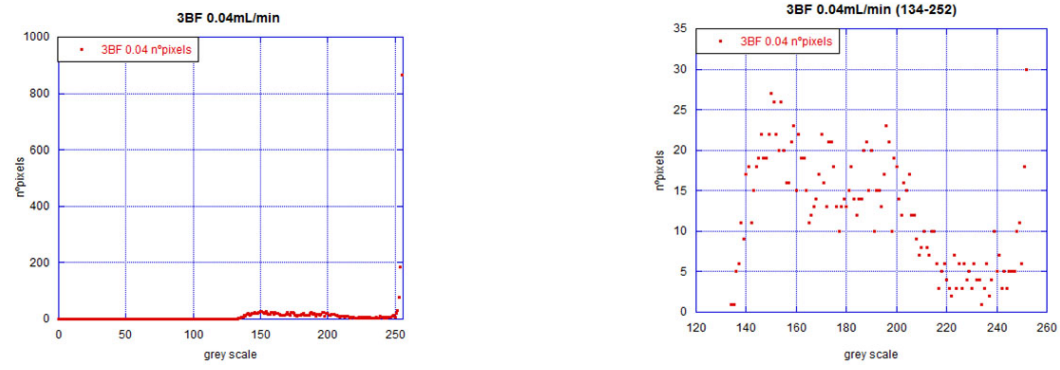
(a) (b)

Figure 21: Grey scale histogram of the first biofilm in the 0.04 mL/min chip, on the top right corner in Fig. 11 surrounded in red. (a) Total histogram seen in that figure and (b) the histogram starting in channel 123.



(a) (b)

Figure 22: Grey scale histogram of the second biofilm in the 0.04 mL/min chip, in the middle of the right column in Fig. 11 surrounded in blue. (a) Is the total histogram seen in that figure and (b) the histogram starting in channel 138.



(a) (b)

Figure 23: Grey scale histogram of the last biofilm in the 0.04 mL/min chip, on the bottom right corner in Fig. 11 surrounded in green. (a) Is the total histogram seen in that figure and (b) the histogram starting in channel 134.

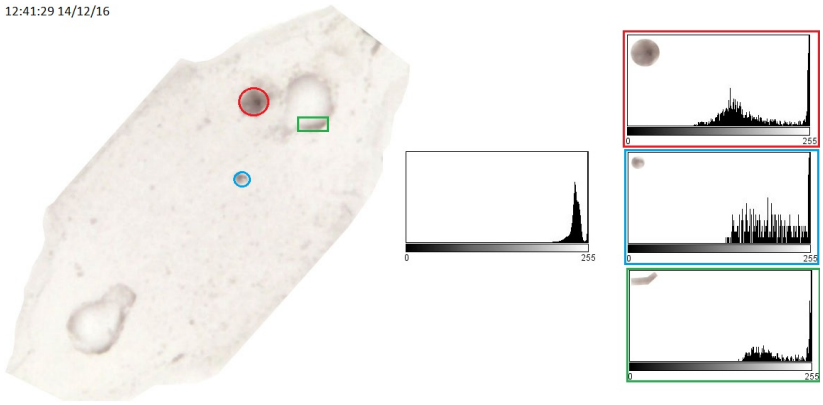
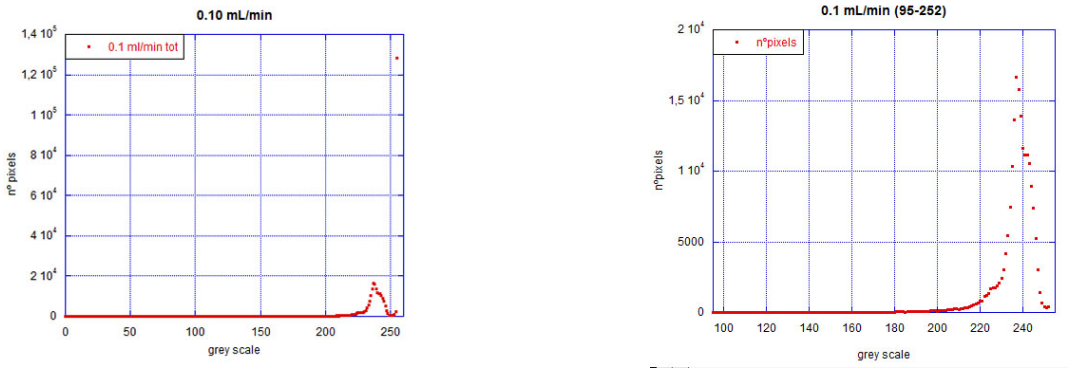
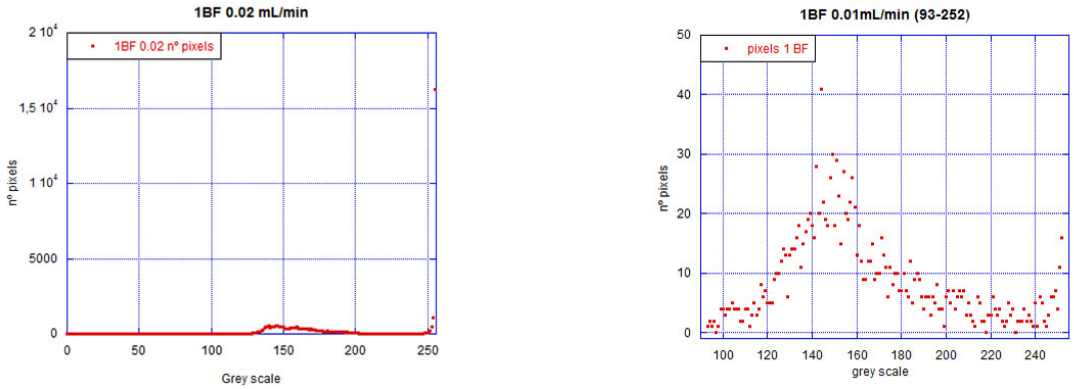


Figure 24: Final photo obtained in Video 0.10.2 in which the three bacterial colonies have been selected and isolated in the coloured perimeters (red, blue and green) with each of the histograms corresponding to each biofilm on the right side of the image (each with the colony and colour that corresponds to the perimeter). In the middle the total density of the chip is displayed without colour.



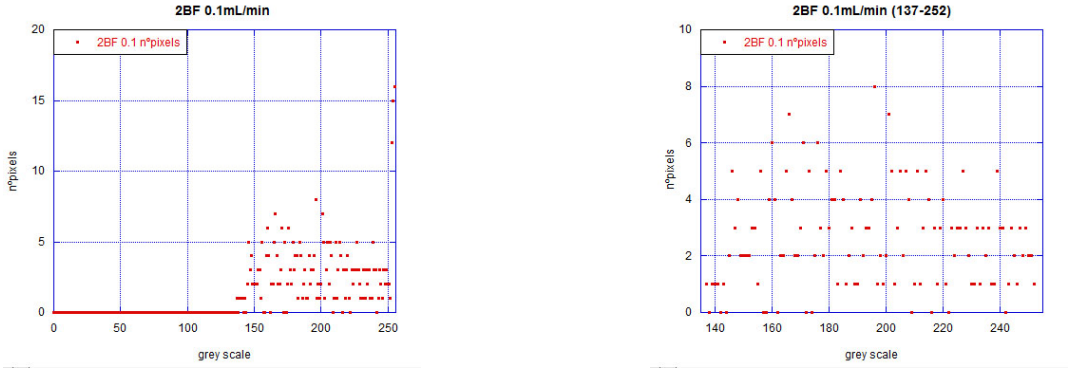
(a) (b)

Figure 25: Histograms of the 0.10 mL/min chip. (a) Total histogram seen in the center of Fig. 24 and (b) the histogram cutting off the first 94 channels.



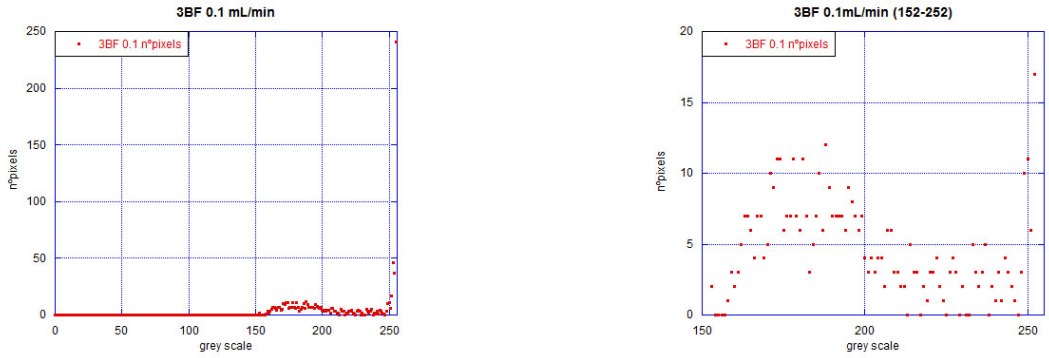
(a) (b)

Figure 26: Grey scale histogram of the first biofilm in the 0.10 mL/min chip, on the bottom right corner in Fig. 24 surrounded in red. (a) Total histogram seen in that figure and (b) the histogram starting in channel 93.



(a) (b)

Figure 27: Grey scale histogram of the second biofilm in the 0.10 mL/min chip, on the bottom right corner in Fig. 24 surrounded in blue. (a) Total histogram seen in that figure and (b) the histogram starting in channel 137.



(a) (b)

Figure 28: Grey scale histogram of the last biofilm in the 0.10 mL/min chip, on the bottom right corner in Fig. 24 surrounded in green. (a) Total histogram seen in that figure and (b) the histogram starting in channel 156.

4.4 Statistical Data

Flow	**0.02 mL/min**							**0.04 mL/min**				**0.10 mL/min**			
	Total	***1st BF***	***2nd BF***	***3rd BF***	***4th BF***	***5th BF***	***6th BF***	***Total***	***1st BF***	***2nd BF***	***3rd BF***	***Total***	***1st BF***	***2nd BF***	***3rd BF***
Figure	12b	13b	14b	15b	16b	17b	18b	20b	21b	22b	23b	25b	26b	27b	28b
Limit	121	120	130	129	141	138	140	123	121	135	132	95	93	135	151
Points	131	132	122	123	111	114	112	129	131	117	120	157	159	117	101
Min	1	3	0	2	1	0	0	17	2	1	1	0	0	0	0
Max	5642	558	130	118	203	127	96	14012	513	38	30	16621	41	8	17
Mean	190	163	179	170	174	181	178	218	209	185	183	236	162	194	198
Median	196	155	195	191	218	211	180	179	195	157	166	163	168	176	170
Mode	204	146	158	139	159	169	168	217	250	156	252	237	161	196	252
σ	24	33	30	27	92	34	31	31	22	25	26	29	23	24	25
σ^2	563	1063	924	752	8393	1175	944	961	489	618	680	842	529	565	606

Table 4: Data obtained from the cropped histograms where each column represents a total chip (*Total*) or a biofilm (*BF*). The first row is the minimum channel considered, where pixels were registered. The following two rows indicate the minimum and maximum values of pixels registered in the histograms. Next is the number of channels taken into account. Following the mean, median and mode of the histogram, and finally the standard deviation σ and variance σ^2. The last 5 rows have an error of ± 0.5.

5 Discussion and conclusion

Even though it was a procedure plagued with difficulties in each phase, we were able to obtain reasonably good results in this first experiment.

By looking at Fig. 11, 19 and 24 it is clear that the lower the flow, the more biofilms are produced and the larger they are. This is confirmed because the image is darker, thus the biofilms are more dense than in high flows, as we expected. This is probably due to the fact that the bacteria is subject to lower dragging force, so it settles better on the crystal floor and it obtains more nutrients for its growth. Also, looking at Table 4, we can see that the mean and the mode are generally smaller in the low flow cases than in the large flow chips, increasing proportionally to the flow.

In video 0.03.2 it is observed that the chip is not centered in the image frame, instead it is on the top left corner. This is an example of one of the problems explained in Section 3, since the chip is transparent, it is hard to fully confirm that it is centered and focused. Fortunately, most of the chip can be seen and it is possible to distinguish various and dense biofilms, apart from the base biofilm. These colonies are as dense as in the 0.02 mL/min chip, so not such a big difference between chips with ΔF= 0.01mL/min.

In video 0.05.2, which was the first one to be recorded, it is obvious the problems created by the air bubbles in the chip. In this video we see that the biofilm formed only goes where the growth medium is, the bubbles took up too much space for it to grow freely. Also, once there is no more medium, the air bubbles move because of the vacuum and so does the biofilm. Fortunately, the method was improved as the trials went forward so the mistakes in each video we avoided as well as they could but we were unable to repeat the round of experiments.

In video 0.07.2 there are no biofilms formed, as in other videos. One could initially think that bacteria cannot be settled at that high velocity but in video 0.10.1 and 0.10.2 we see that 3 biofilms were formed, so it is possible to have bacterial colonies, even if they are small. So the more probable explanation is that there was an error in the procedure while doing the experiment at this flow. Unfortunately, it was not possible to obtain more videos or redo the experiments, so this particular example cannot be repeated.

A curious fact that was observed was in large flows (seen in videos 0.10.2 and 0.07.2) was that there is a base growth throughout the chip of bacterial colonies. We did not expect a fine colonial growth all through the chip, since the current would have been too fast for the bacteria to settle in the chip.

Going to the analyzed videos, in video 0.02.2 we only chose six biofilms to study with different sized because they were sufficient material to study. In this video one can actually see the detachment of the biofilms due to their size and the flow as in video 0.03.2. These videos are the only ones where the biofilms grew all throughout the chip and not in specific places.

Most of the biofilms formed in the 0.04 mL and 0.10 mL/ min chips are in the top half of the chip, from where the nutrient enters the chip. It could be that the bacteria settled in that top half has better access to the nutrients thus favoring the growth and depriving it from the bottom half. Apart from the nutrients the medium also transports the toxins created from the top bacteria to the output. This also affects the growth in the bottom half of the chip. This could be the reason that the peaks seen in Fig. 12 to 28 are not symmetrical.

A notable thing that we see in video 0.10.2, is that 2 of the biofilms are formed almost immediately after the experiment starts: the first biofilm is formed at 12.01 and the second is formed at 13.01, 2 hours after the medium starts flowing into the chip.

According to Table 4 we see the absolute maximum is in the 0.10 mL/min chip at channel 237. This is a very close channel to the maximum white colour, with a difference of 15 channels between the whitest range selected and this channel. This indicates, together with the video, that the chip is much more homogeneous in the growth of the bacteria colonies, with small biofilms. On the other

hand comparing the three total data sets, the smallest maximum is in the 0.02 mL/min, confirming that there is more diversity in the growth as there is less homogeneus base colonies and larger biofilm formation. A strange fact is that generally the limit for the pixel count (first data row) in the total chip is higher than the lowest limit, the first biofilm. This indicates that it is not a total sum of the histograms of all of the chip, but it is pondered.

5.1 Conclusion

The setup for this experiment consists of easy phases with different aspects of science (engineering, biology and physics) that work together for a common goal with a bright future.

There were a lot of problems in this experiments since there are no previous papers in which we could have based it on but they were relatively easy to overcome and obtain good results to analyze. Unfortunately we could only obtain one video for each flow, so we have formed a general idea of what happens instead of a statistical view.

At low rates, the biofilms grow larger and more dense than in high flow conditions and less homogeneous. There is always a non-dense biofilm in the surface of the crystal in every chip, which acquires importance as the flow increases.

Microfabrication and microfluidics is still being used in the IBBTEC, in which this procedure is being perfected and setting path for future studies.

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7 Appendix: Videos

In this appendix, the hyperlinks of the videos that are able to be seen in Table 3 are written down.

Number	F mL/min	Original Video	Edited Video
1	0.02	$https://youtu.be/ElvXxJJLtqU$	https://youtu.be/K5xFJlEBXqI
2	0.03	$https://youtu.be/TZQuOeAK7cM$	$https://youtu.be/hbYLyzL_rFM$
3	0.04	$https://youtu.be/NbTW-gbfjcE$	$https://youtu.be/TKgJAKXKS48$
4	0.05	$https://youtu.be/EXoaCBn1vqc$	$https://youtu.be/DXt57r8dyCkI$
5	0.07	$https://youtu.be/kXgO7o6Ay9M$	$https://youtu.be/6vjbCvwpoBY$
6	0.10	$https://youtu.be/35FJtFszx2c$	$https://youtu.be/0LLUxEesyVg$

Table 5: Hyperlinks to the videos of the results that were obtained.