The influence of fluid flow on trapped protoplast in a microfluidic device

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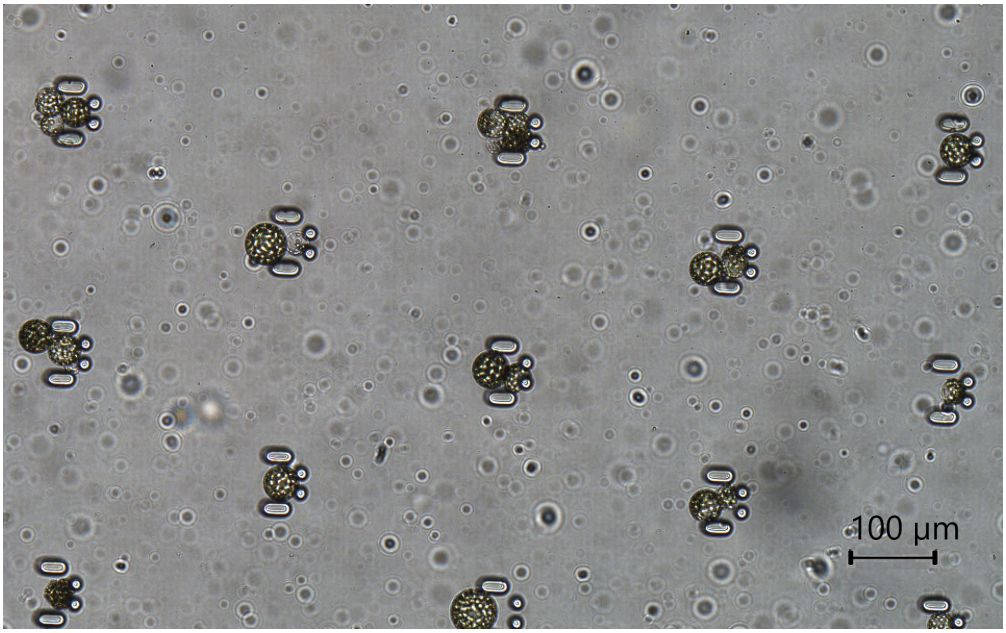

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Front page picture: trapped protoplasts in the for this project designed microfluidic device.

Contents

1 Abstract

Protoplasts are plant cells with their cell wall enzymatically removed, and therefore quite fragile. In this report, a protocol developed for trapping protoplasts (isolated of the leaves of Nicotiana Tabacum) inside a microfluidic device will be discussed. A chip is made of polydimethylsiloxane (PDMS) with U-shaped trapping structures, bonded to a microscope glass slide. No air bubbles on chip were established after filling the chip with fluid directly after bonding (using an oxygen plasma oven). When varying the flow speed, the most efficient trapping was reached with a flow speed of 2.0 $\mu L/min$.
A protocol was made for the research into the influence of continuous flow on trapped protoplasts. Due to unforeseen external interference, these experiments failed. Thence, the influence of penicillin-streptomycin (pen/strep) on the viability of protoplasts was tested. Based on the morphology of the protoplasts, it seems that pen/strep does not harm the protoplast. However, more research is needed into the influence on cell wall regrowth and viability over a longer time. The protocol for continuous flow on trapped protoplasts seems promising based on morphology during the monitoring of the experiments.
To quantify the viability of the protoplasts, the use of live/dead staining Fluorescein Diacetate (FDA) and propidium iodide (PI) is evaluated. On day 0, FDA staining evaluates the viability of the protoplasts, but harms the protoplasts, resulting in dead protoplasts after 24 hours. The results of this project can be used to trap protoplasts on chip and evaluate the viability for several days, but further research is needed to optimize the used protocols.

2 Introduction

Microfluidics is the science of fluid behavior through micro-channels and the technology of manufacturing microminiaturized devices containing chambers and tunnels through which fluid flows or is confined to. [1] Some advantages of microfluidic devices are their compactness, real-time processing/monitoring, increased sensitivity, and low cost. [2] The use of microfluidic devices grows in the research areas. An important application in biology is to study and replicate human organ systems, using microfluidic devices. [3] A part of this field focuses on single-cell analysis. Understanding cellular molecular mechanisms or drug delivery are only two examples of this broad field. [4, 5] Next to recreating human organs, these techniques are used for researching plant cells. After the first reports of using microfluidic devices for plant cell research, a ongoing interest was developed rapidly. [6] The advantages of using microfluidic devices can help the research into plant development.

Protoplasts are plant cells with their cell wall enzymatically removed. Protoplasts are potentially totipotent: they can form tissue, which can be used as the foundation for the regeneration into complete plants, depending on the correct chemical and physical stimuli. [7, 8] The process of the protoplast entering cell division is known as proliferation. [9]

Where there has been research into the influence of pH [10], temperature [11] and light [12], this project focuses on the influence of fluid flow on the protoplasts. Since the protoplasts are quite fragile because of the enzymatic removal of the cell wall, the goal is to investigate the influence of fluid flow on the protoplast. For this, a single-cell trapping chip is developed. Mammalian cells, like endothelial cells, react strongly to the direction of fluid flow. With this project, we want to investigate if the proliferation and differentiation of protoplasts are influenced by fluid flow in the chip. [13, 14]

2.1 Problem description

A project within the minor Lab-on-a-chip from the BIOS-group of the University of Twente showed that we could trap protoplasts of the *Nicotania Tabacum* on chip. From the project results, the researchers were curious about the influence of fluid flow on protoplasts. To gather information, two sub-goals are created:

- Create a protocol for efficient trapping of protoplasts on chip
- Apply continuous fluid flow on trapped protoplast on chip and monitor the cell to see if this influences the division

To create a protocol for efficient trapping of protoplasts on chip, the effect of the flow speed during trapping will be investigated. The method, concentration of the protoplasts, and duration of trapping will remain constant. To determine the trapping efficiency, the amount of traps containing one alive protoplast will be counted directly after the flow is stopped. Alongside, the viability after 24 hours will be determined based on morphology, using a microscope.

Protoplasts show cell proliferation after around five days, therefore the duration of the long-term experiment is set at five days. [15] The established protocol will be used to trap the protoplasts for the experiment with the continuous fluid flow on trapped protoplasts. A culture media, K8P, will be used as fluid flow, where the flow rate will vary. Other conditions like temperature and concentration will remain as constant as possible. A computer-controllable syringe pump (Nemesys syringe pump) will be used to create the constant flow rate, and the protoplasts will be monitored with a microscope (Olympus IX51).

2.2 Goal and expectations

The main goal of this research is to create a protocol for trapping protoplasts on chip. Since the design has already been tested in the minor Lab-on-a-chip, the expectation is that trapping of the protoplasts on chip will be successful. After trapping the protoplast on chip, the expectation is that the protoplasts will stay alive on chip after the proliferation, which can be monitored using a microscope. When this is established, the expectation is that the influence of fluid flow on the proliferation of protoplasts can be monitored.

3 Theoretical Background

In this section, an overview of the theoretical background will be given. This includes information about the protoplasts, chip design, and the products that are involved in the project.

3.1 Protoplast

As mentioned in section 2, protoplasts are plant cells with their cell wall enzymatic removed. The protoplasts can be isolated from different parts of the plant, though common parts are leaves, nodes, and roots. [16] In this project the leaves of *Nicotiana Tabacum* are used. The *Nicotiana Tabacum* is a frequently used plant and is known as the 'white mouse' of the plant world. [17] The protoplast will start with rebuilding its cell wall, which is necessary for cell survival and division, and goes into cell division by mitosis. This process is called proliferation. The time it takes to go into cell division by mitosis is influenced by multiple factors. [9] Based on the morphology of the protoplasts, an estimation of the viability of the protoplast can be made. A freshly isolated protoplast is alive if it's ideally spherical, with an even distribution of the chloroplasts. When the protoplast starts losing one of these criteria, it is dying. When both criteria are not visible, the protoplast is dead. An overview of the difference between life and dead cells can be seen in Figure 1.

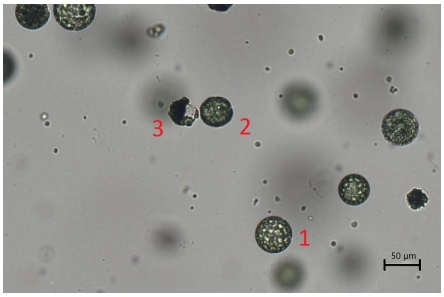


Figure 1: Overview of the morphology of the protoplast with 1: alive, 2: dying, and 3: dead.

3.2 Chip design

The design for the trapping structures is based on a microchip design of Kaori Sakai et al. [18] In our design the U-shape is altered to two walls with two pillars at the bottom. An overview of the trap structure can be seen in Figure 2. The width of the trap is based on the average size of the protoplasts, after using a sieve of 50 μm in the isolation process.

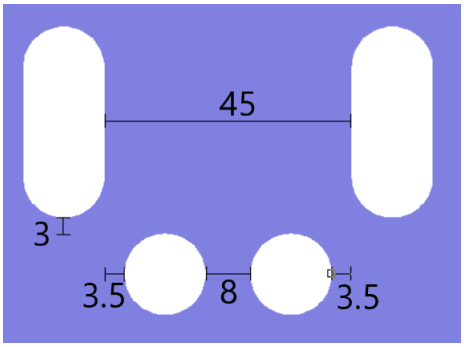


Figure 2: Overview of a trap structure with dimensions in micrometer.

The traps are placed in a row next to each other, with a space of 200 microns between the two traps. The row below is altered so that the trap is placed in the middle of the space between the two traps above. In total, the chip contains seventeen rows of traps, with a total of 196 traps. The chamber containing all the traps is connected with an inlet and outlet. An overview of the chip design can be seen in Figure 3.

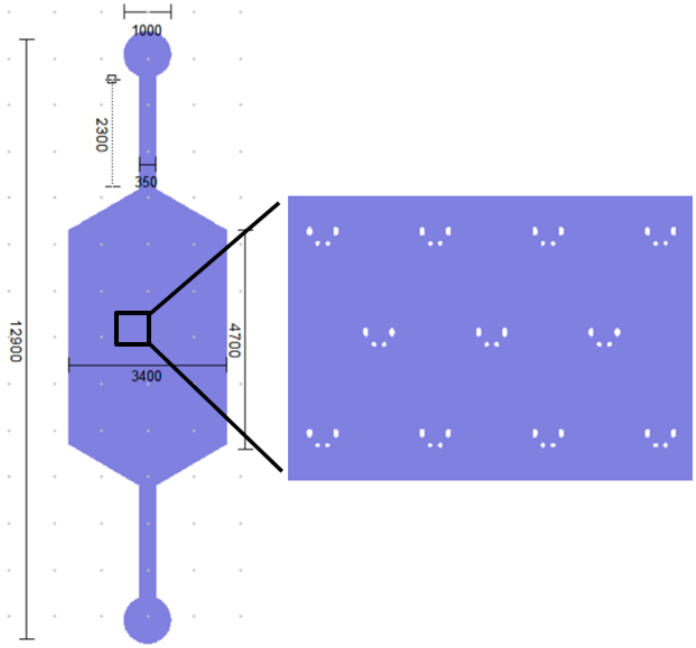


Figure 3: Design of the chip, dimensions in micrometers.

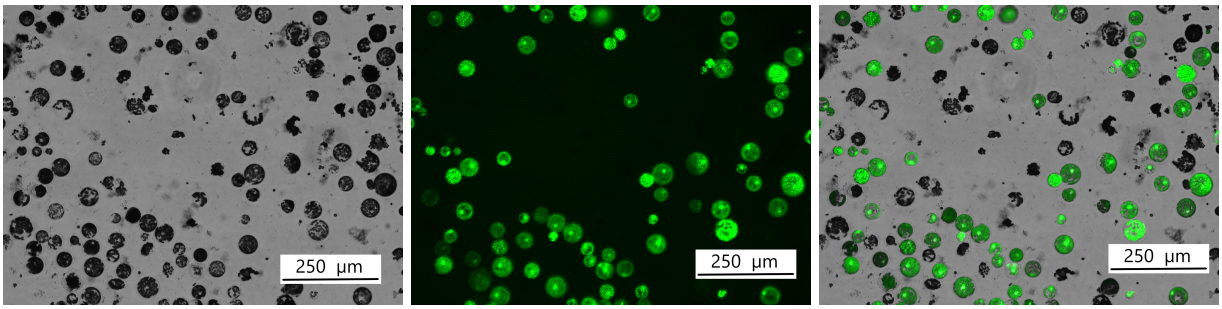
3.3 Hydrophilic coating

For this project, ensuring proper wetting of the channel is crucial to remove any air bubbles inside the chip. Air bubbles can disrupt the flow, and create shear on protoplasts on chip. [19] Shear stress is explained in Appendix A. Pluronic is a triblock co-polymer, this determines that it will migrate towards the surface of an aqueous solution inside a microfluidic device. Pluronic F-127 is a commonly used coating since it is commercially available and has low toxicity. The main purpose of using pluronic in a microfluidic device is to reduce the biomolecule absorption and enhance the wetting of the channel. [20]

3.4 Live/dead staining

3.4.1 FDA staining

Fluorescein Diacetate (FDA) is a cell-permeant esterase substrate that can be used as a viability probe of protoplast. The intact plasma membrane is permeable to the FDA, and the FDA is converted into a green fluorescent dye, fluorescein, by internal esterases. This results in a display of green fluorescence in viable cells. [21] In combination with a bright-field picture of the same spot, the viability of the protoplasts can be determined, see Figure 4.

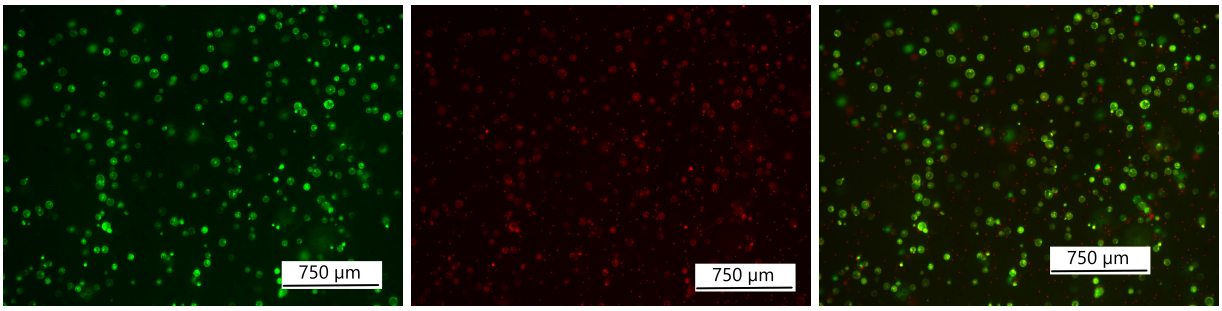


(a) Bright-field picture **(b)** GFP picture **(c)** Combined picture of a and b

Figure 4: Overview of staining protoplasts with FDA. When overlaying (a) and (b), the result is (c). Alive protoplasts light up green.

3.4.2 Propidium iodide

Propidium iodide (PI) is a fluorescent dye that is known only to pass through the membrane of a dying or dead cell. The viability of the cells can be determined in combination with the FDA staining. This will result in alive cells showing up in green and dead/dying cells as red. [21] This is visible in Figure 5.



(a) GFP picture **(b)** RFP picture **(c)** Combined picture of a and b

Figure 5: Overview of staining protoplasts with FDA and PI. When overlaying (a) and (b), the result is (c). Alive protoplasts light up green and dead/dying cells red.

4 Materials and Methods

In this section, the materials and methods of the experiments will be explained. This includes the fabrication of the chips and protocols of the experiments.

4.1 Chip fabrication

The chip design explained in section 3.2 is created in CleWin5 (WieWeb software, NL). Due to the size limitations of other fabrication methods, maskless photolithography was used as an SU-8 mold fabrication method. Polydimethylsiloxane (PDMS) replica molding was used for chip fabrication.
PDMS is a silicon-based elastomer with many advantages. One of the main features of PDMS is biocompatibility, which is necessary to grow protoplasts on chip. [22] Based on the settings for the SU-8 mold, the channel has a height of 43 micrometers in the wafer. The PDMS (mixed in a ratio of 10:1 with the curing agent and degassed) is poured on the wafer and baked in the oven at 60 °C. After the PDMS is cured, it is carefully removed from the wafer and cut into pieces containing the channel. A biopsy needle of one millimeter is used to punch an inlet and outlet.
To complete the chip, the PDMS is bonded to a microscope glass slide. In order to bond, the PDMS and glass slide are treated with oxygen plasma in the vacuum plasma cleaner (Femto Science Inc. KR) for one minute at 50 watts power. After the treatment, the treated side of the PDMS is carefully aligned with the treated side of the glass slide. A step-by-step guide to the fabrication of the chip is explained in Appendix B.

4.2 Protoplast isolation protocol

The protoplasts of the leaves of the *Nicotiana Tabacum* have been enzymatically isolated. The step-by-step protocol can be seen in Appendix C.
The culturing medium used is K8P, containing mainly glucose and vitamins.[23, 24]

4.3 Hydrophilic coating

Make a solution with a pluronic-F127 (Sigma-Aldrich, USA) concentration of 0.1% in DI water. Sterilize this solution with a 0.2 micrometer PES filter (Cytiva, Whatman, USA).
Directly after bonding, flush the chips with the pluronic solution until there are no air bubbles in the chamber and leave a droplet on the inlet and outlet of the chip. After the incubation time of an hour, flush the chip three times with DI water to remove all the pluronic solution. Afterward, flush the chips with K8P to prepare the chips for the protoplasts. To prevent air trapping when connecting the tubes, ensure a droplet of fluid is on the inlet and outlet of the chip.

4.4 Fluid flow on protoplasts

Based on the available syringes with the corresponding minimum flow of the syringe pump and volume capacity for six days, the following speed range has been determined to test the fluid flow on protoplasts:

- 500 μL syringe - 0.0 $\mu L/min$
- 500 μL syringe - 0.035 $\mu L/min$
- 1000 μL syringe - 0.11 $\mu L/min$
- 5000 μL syringe - 0.57 $\mu L/min$

The calculation is based on six days. If necessary, the experiment can continue for one extra day.

Using the computer-controlled syringe pump (Nemesys syringe pump, NEM-B101-02 B), load the freshly isolated protoplasts (with an average concentration of $4.0*10^5$ $cells/mL$) into the 500 μL syringes (Hamilton Company, NV, USA) with a speed of 5.0 $\mu L/min$ until a volume of 220 μL is reached.
Fill the outlet tube (Tygon, outer diameter: 1.2 mm, inner diameter: 1.0 mm, connected with needles of diameter 1.0 mm) with K8P and connect these between the outlet of the chip and the waste beaker. Load all the other syringes with K8P.
When a bubble is formed at the end of the tube, connect the syringe with the protoplasts to the inlet of the chip. Load the protoplasts with a fluid flow of 5.0 $\mu L/min$ for 10 minutes. Stop the flow, start the fluid flow of the syringe with K8P, and wait until a droplet is formed at the end of the tube before connecting the tube to the inlet of the chip. Let the flow run for five days and monitor the chip daily with the microscope (Olympus IX51 microscope). Load the next chips with the same speed of 5.0 $\mu L/min$. After loading the last chip, stop the flow and let the syringe attached to the chip. This is the syringe without fluid flow. An overview of the set-up can be seen in Figure 6.

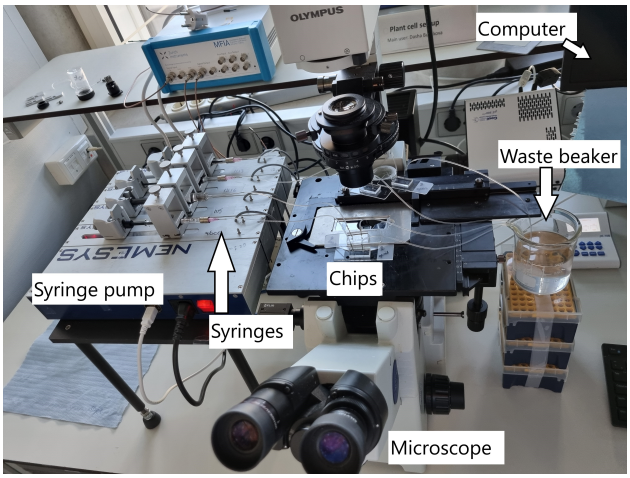


Figure 6: Overview of the set-up of the experiment for continuous fluid flow on protoplasts.

As an external control, make a cell dilution of a total of 2 mL with a concentration of around $7.5 * 10^4$ protoplasts/mL in K8P. Divide over two Petri dishes, and seal with parafilm M. Place one in the incubator (25 °C in the dark) and one next to the experimental set-up. Take daily pictures of the control groups with a microscope.

4.4.1 Updated protocol

To prevent any contamination, 1% pen/strep was added to the K8P, and the waste beaker was emptied. The outlet tubes were placed with tape at the top of the waste beaker and the top was sealed with parafilm M.

4.5 Penicillin-streptomycin test

Penicillin-streptomycin (pen/strep) is the most commonly used antibiotic in mammalian cell cultures. [25] Generally known is that penicillin does not inhibit the protoplast cell wall regrowth. [26] The influence of streptomycin on the proliferation of protoplasts is yet unknown.
The following experiment was performed for five days to test if pen/strep had a negative influence on the viability of the protoplasts. This experiment consisted of adding two different concentrations of pen/strep (Thermo Fisher Scientific Inc. USA) to the protoplasts in K8P, and a control group.
Work in a sterile environment, using sterile equipment to make the following solutions:

- The control group: 3 mL of freshly isolated protoplasts in K8P with a concentration of around $4.5 * 10^4$ protoplasts/mL.
- 1% pen/strep: 3 mL of freshly isolated protoplasts in K8P with a concentration of around $4.5 * 10^4$ protoplasts/mL, and 30 μL pen/strep [$5.0 mg/mL$].
- 5% pen/strep: 3 mL of freshly isolated protoplasts in K8P with a concentration of around $4.5 * 10^4$ protoplasts/mL, and 150 μL pen/strep [$5.0 mg/mL$].

In a 6-well plate: add 1.5 mL of the control group to wells A1 and B1, add 1.5 mL of the 1% pen/strep solution to wells A2 and B2, and add 1.5 mL of the 5% pen/strep concentration to wells A3 and B3. Cover the sides of the well plate with parafilm M and make control pictures with the microscope. Put the plate in the incubator (at 25°C in the dark). Take pictures the following days around the same time as the start of the experiment.

4.6 Live/dead staining

The live/dead staining experiment has been split up into two experiments. The first experiment with FDA staining. The main goal of this experiment is to investigate if FDA influences the viability of the protoplasts after 24 hours. The second experiment is with FDA in combination with PI staining, combining live and dead staining.

4.6.1 FDA staining

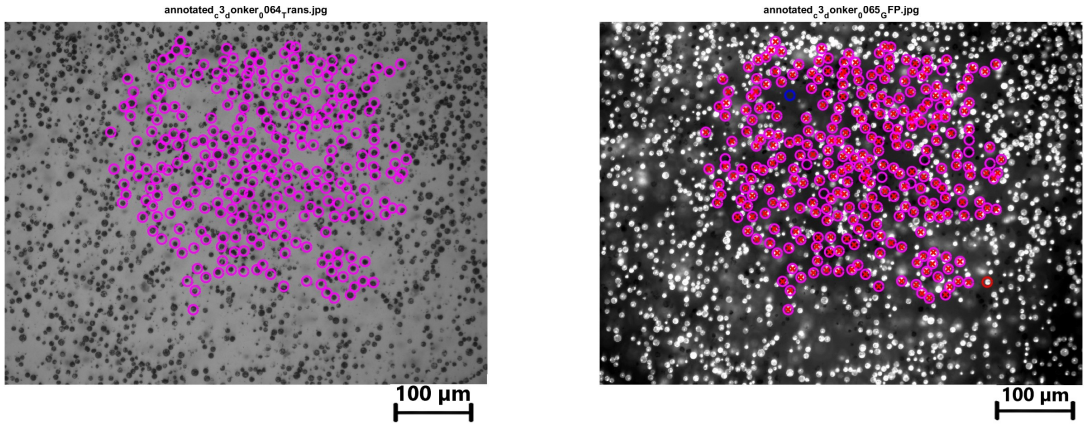
For the first experiment, two 12-well plates will be used. Work in a sterile environment, using sterile equipment and solutions. Make two times a cell dilution in K8P of in total 7.5 mL with a concentration of freshly isolated protoplasts of around $7.5 * 10^4$ protoplasts/mL. Use one of the dilutions as the control group, and pipette 1 mL in row 1 of both well plates. Add 75 μL of FDA [5 mg/mL](Invitrogen, Thermo Fisher Scientific Corporation, USA) to the other dilution, and after carefully mixing, pipette 1 mL in row 3 of both well plates. Seal both well plates with Parafilm M, and take pictures of all the wells with the EVOS (Invitrogen, Thermo Fisher Scientific Corporation, USA) (bright-field and GFP light cube). Place one well plate in the incubator (at 25 °C in the dark) and one at the desk. Take control pictures with the EVOS on day one. After three days, add 10 μL of FDA staining to all the wells, and take pictures with the EVOS (bright-field and GFP light cube).

4.6.2 FDA + PI staining

For the second experiment, one 12-well plate is used. Work in a sterile environment, using sterile equipment and solutions. Make two times a cell dilution of freshly isolated protoplasts in K8P of in total of 3 mL with a concentration of around $7.5 * 10^4$ protoplasts/mL in K8P. Use one of the dilutions to fill row 1 in the wells plate as a control group. Add to the other dilution 30 µL of FDA [5 mg/mL]. After carefully mixing, fill row 4 on the well plate. Take pictures with the EVOS (bright-field and GFP light cube) and place the well plate in the incubator. After 24 hours, add 10 μL FDA staining and 6.25 μL PI [10 $\mu g/mL$] (Invitrogen, Thermo Fisher Scientific Corporation, USA) to each well. After carefully mixing, take pictures with the EVOS (GFP and RFP light cubes).

4.6.3 Determine the viability of the protoplasts

The average viability percentage of the protoplasts is determined using the MatLab (MathWorks, MA, USA) script written by J.T. Loessberg-Zahl. Therefore, around 50 % of the protoplasts in the bright-field picture are selected, by clicking on the center of the cell. The GFP picture is loaded, where the background and one live protoplast need to be set as reference points. The program counts how many protoplasts are alive based on a threshold, for this project 0.2. From those results, the average viability can be determined. An overview of one counting can be seen in Figure 7. The script can be seen in Appendix G.



(a) Bright-field picture with in-focus selected protoplasts

(b) GFP picture, where the background is selected by the blue circle and alive protoplast by red circle

Figure 7: Result of the viability determination. A cross in the circle means alive cell and no cross means dead cell.

4.7 Trapping protoplast on chip

The syringe pump is connected to the outlet of the chip, to create a negative pressure. Make a cell dilution of freshly isolated protoplasts in K8P of a total of 4 mL with a concentration of around $7.5*10^4$ protoplasts/mL. Fill a 1000 μL pipette tip (Eppendorf, DE) with the cell dilution. Apply careful force to form a bubble and place the pipette tip in the channel's inlet. Wait ten minutes to allow protoplasts to sink towards the inlet of the chamber. Run the syringe pump for ten minutes with the specific flow rate. Count the amount of traps per row that contain at least one alive protoplast, by eye using the microscope. Monitor the protoplasts the following day. The set-up for one chip is shown in Figure 8. The range for the first experiment is based on the trapping speed used in the project of the minor Lab-on-a-chip. They used a flow speed of 5.0 $\mu L/min$, therefore the following four different flow speeds up to 5.0 $\mu L/min$ have been determined:

- 500 μL syringe - 0.5 $\mu L/min$
- 500 μL syringe - 1.0 $\mu L/min$
- 1000 μL syringe - 2.5 $\mu L/min$
- 5000 μL syringe - 5.0 $\mu L/min$

Based on the results of the first experiment, the following range was determined:

- 500 μL syringe - 1.0 $\mu L/min$
- 500 μL syringe - 2.0 $\mu L/min$
- 1000 μL syringe - 3.0 $\mu L/min$
- 5000 μL syringe - 4.0 $\mu L/min$

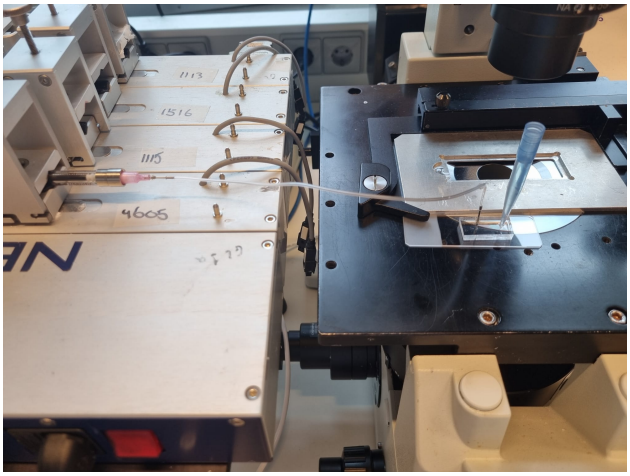


Figure 8: Overview of the set-up of the experiment for determination of the trapping efficiency.

5 Results

Here, the results of the experiments mentioned in the prior section will be shown and discussed.

5.1 No air bubbles on chip

Filling the chip directly after bonding with the pluronic solution resulted in the absence of air bubbles in the chip. After spotting some debris in the chip with the microscope, see Figure 9, the experiment was altered to fill the chip directly after bonding with K8P. The reason for this was to determine whether the debris was coming from the pluronic solution. Filling the chip with K8P resulted in the absence of any air bubbles, which is preferred since air bubbles disrupt the flow and can create shear stress on protoplasts, yet contain occasional debris. Therefore, using the pluronic solution to ensure wetting of the channel became unnecessary. The debris results from punching the inlet and outlet using a biopsy needle, which failed to make a clean cut. Going forward, before the bonding the chips will be carefully cleaned with tape (magic tape Scotch, 3M) before placing the chip parts in the oxygen plasma oven.

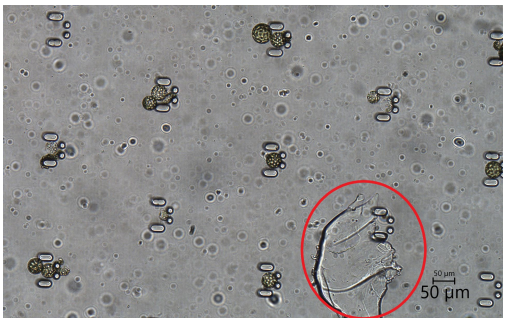


Figure 9: Picture of debris (inside of the red circle) in the chip.

5.2 Continuous flow test 1

The results of the continuous fluid flow speeds seemed promising on day 1, but when the chips were monitored on day 4 (after the weekend), there was a fungus infection in the chips. A picture of the fungus infection can be seen in Figure 10. The experiment was stopped and evaluated. The tubes were not properly cleaned with 70% ethanol, only with water and DI-water, which could have led to the fungus infection. Going forward, further experiments will have their equipment cleaned with 70 % ethanol in a sterile environment (cross-flow cabinet). Besides better cleaning of the equipment, the influence of the antibiotic pen/strep on protoplasts was tested. Since the experiment of continuous flow was not performed in a sterile environment, the use of pen/strep could help prevent contamination and the top of the waste beaker will be sealed.

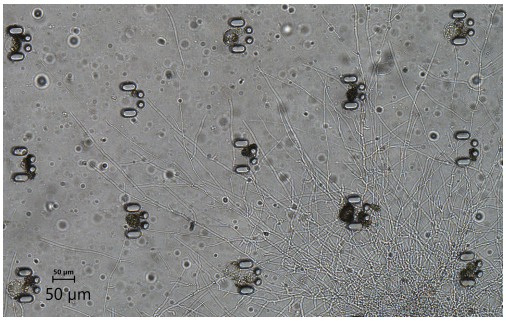
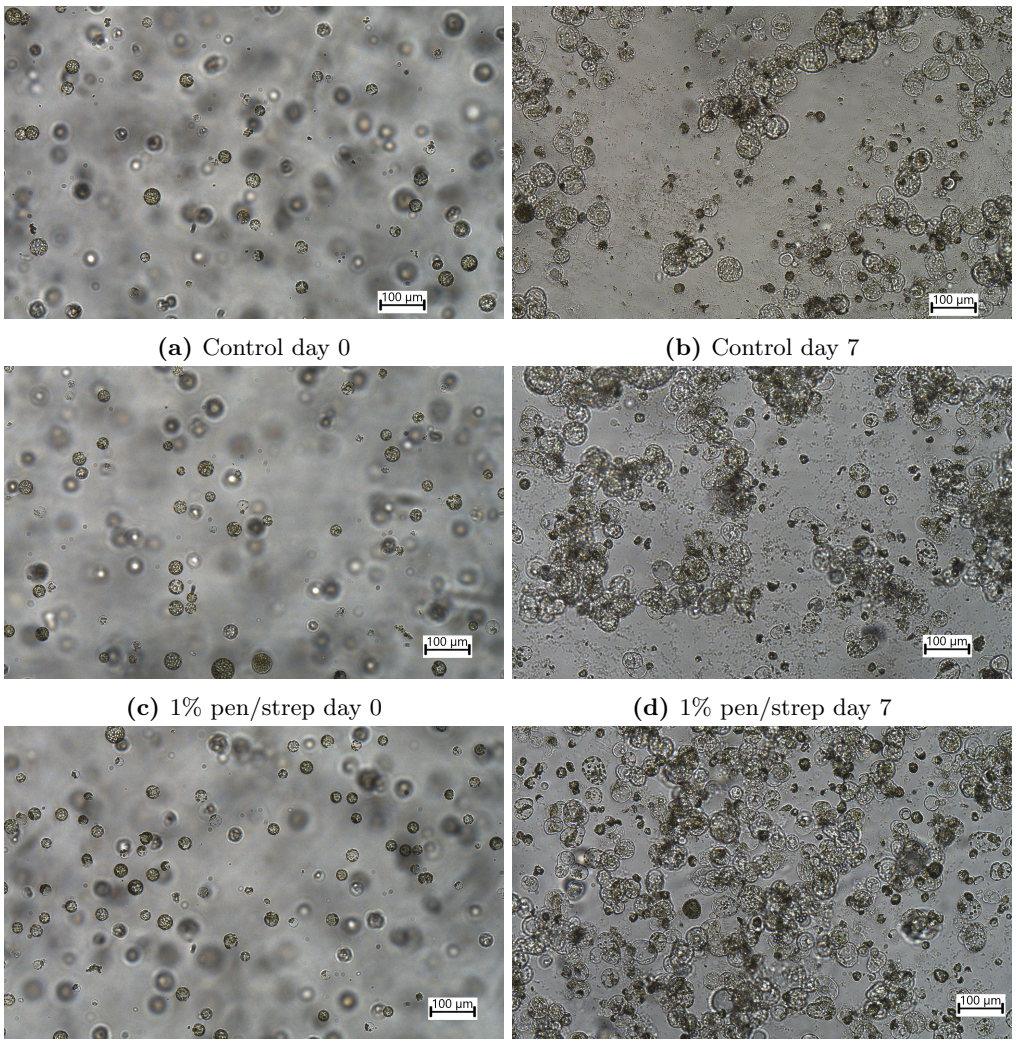


Figure 10: Fungus infection in the chip on day 4. Spores are visible as thin white lines.

5.3 Penicillin-streptomycin

In Figure 11 the results of the pen/strep experiment are shown. Based on the morphology of the protoplasts we conclude that pen/strep does not influence the viability of the protoplasts. The protoplasts are a little swollen, which is expected when they go into proliferation. The pictures show no difference in the reaction of the protoplasts to a higher concentration of pen/strep. The images of all the wells, taken on days 0, 1, 6, and 7 are displayed in Appendix D.



(a) Control day 0 **(b)** Control day 7

(c) 1% pen/strep day 0 **(d)** 1% pen/strep day 7

(e) 5% pen/strep day 0 **(f)** 5% pen/strep day 7

Figure 11: Experiment with control, 1% pen/strep and 5% pen/strep concentration. Pictures were taken on days 0 and 7, at random locations in the Petri dish. On day 7, all the conditions show viable protoplasts based on morphology.

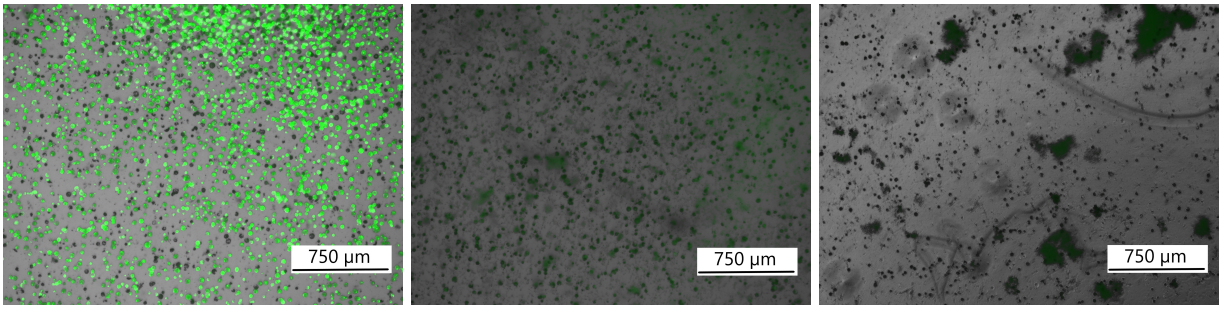
For the cell culture of mammalian cells, it is normal to use a concentration of 1% pen/strep. Since there is no visible indication that pen/strep influences the viability of the protoplasts, it is recommended to use 1% pen/strep in the culture media K8P when culturing protoplasts for five days.

5.4 Continuous flow test 2

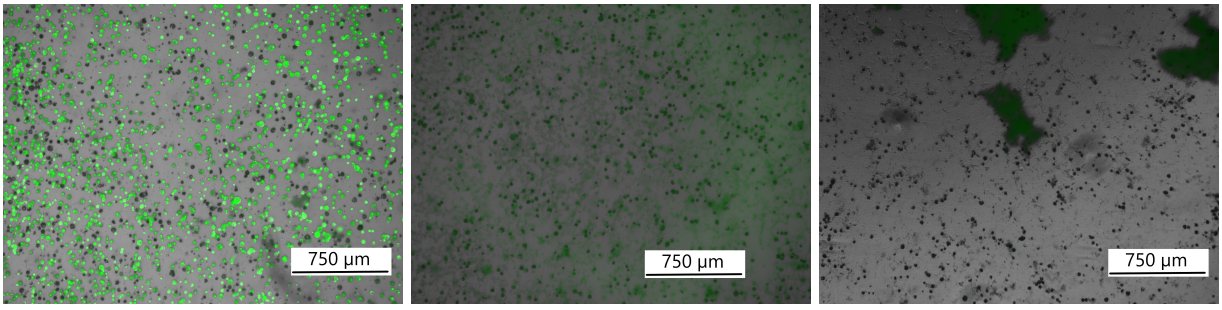
After updating the protocol for the continuous flow on protoplasts by improving the cleaning of the equipment and adding 1% pen/strep to K8P, the second experiment with the same range of continuous flow speeds as test 1 was performed. This experiment was cut short due to unforeseen external interference with the experiment's equipment. With the remaining time left for the project, the focus shifted to optimizing the efficient trapping of protoplasts. This includes using live/dead staining to quantify the results.
To quantify if the protoplasts were still alive after trapping and survived for one day on chip, the use of live/dead staining was investigated.

5.5 Live/dead staining test 1

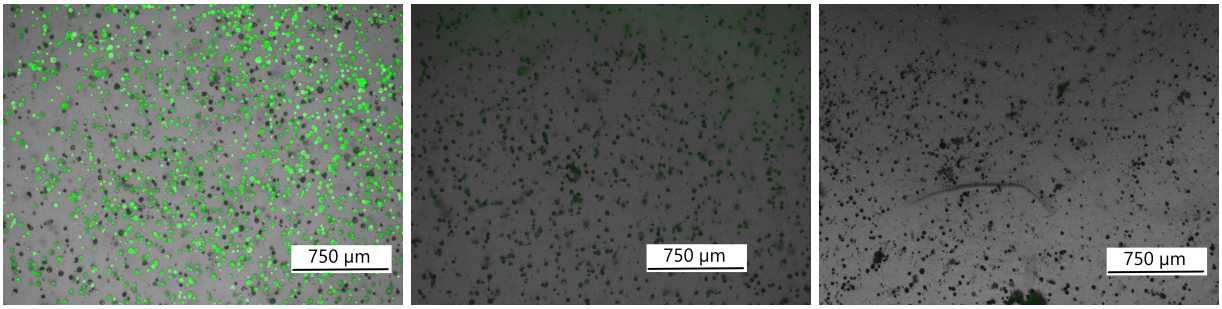
The results of the first FDA staining experiment can be seen in Figure 12. These results show the wells where the FDA was added on day 0. Most protoplasts are alive on day 0, as the protoplasts show green fluorescence. On day 1 there is a display of green fluorescence, but not with the same intensity shown on day 0. When increasing the fluorescent light intensity to obtain a higher fluorescent signal, a lot of background noise is observed. FDA is converted by the protoplast and during the process of dying, the fluorescein is released in the medium which creates the background noise. As a control, all the wells were stained with 1 % FDA on day 3. The reason for this was to check if the control group was still alive, and if adding more FDA influenced the fluorescence of the experimental group.
Once 1% FDA was added to every well on day 3, there was still no display of green fluorescence in alive protoplasts at the wells where the FDA was added on day 0. The control group, which only got 1% FDA staining on day 3, showed green fluorescence, as seen in Figure 13, resulting in alive protoplasts in the control group.



(a) Well A3 captured at day 0 **(b)** Well A3 captured at day 1 **(c)** Well A3 captured at day 3

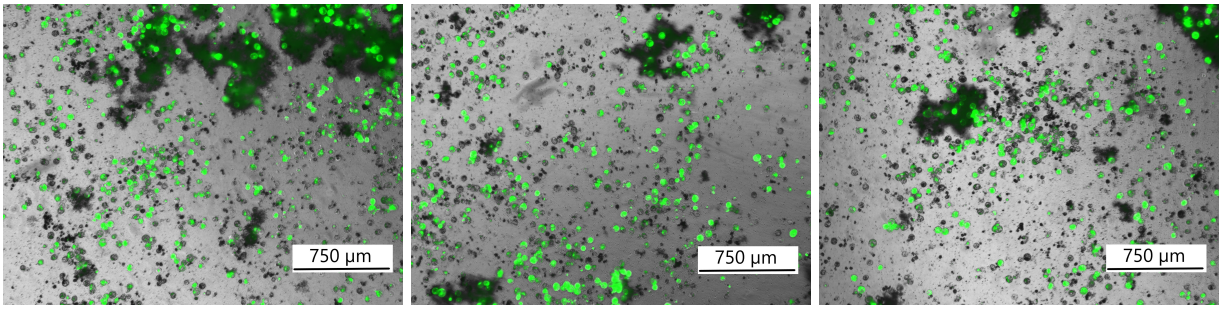


(d) Well B3 captured at day 0 **(e)** Well B3 captured at day 1 **(f)** Well B3 captured at day 3



(g) Well C3 captured at day 0 **(h)** Well C3 captured at day 1 **(i)** Well C3 captured at day 3

Figure 12: Capture on days 0, 1, and 3 of the protoplasts with 1% FDA staining on day 0 and day 3. Made with the EVOS (bright-field and GFP light cube combined).



(a) Well A1 captured at day 3 **(b)** Well B1 captured at day 3 **(c)** Well C1 captured at day 3

Figure 13: Control group after adding 1% FDA staining on day three. Pictures made with EVOS (bright-field and GFP light cube combined)

The average viability of the protoplasts was determined to see if there was a difference in the viability of protoplasts placed in light or dark. In Table 1, the results of average viability are shown. These results show no difference in viability on day 3 between protoplasts placed in the dark or light. The viability of the protoplasts is decreased by 10 % in three days, however this can not be said with certainty. The viability per picture of the wells can be seen in Appendix E.

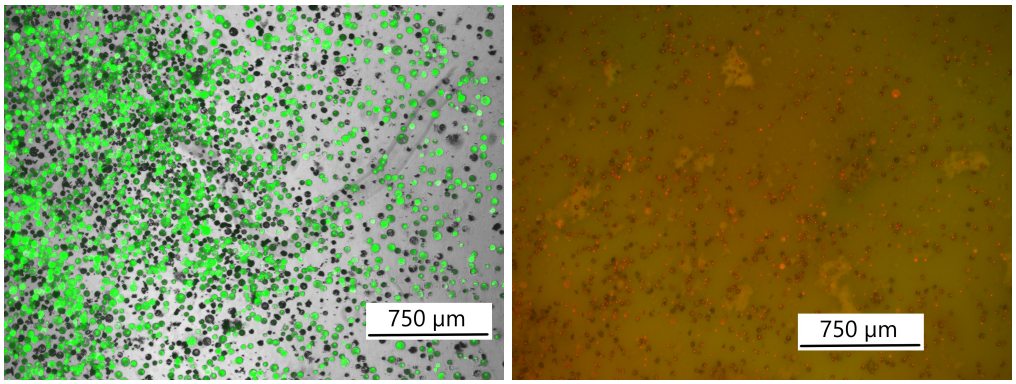
Table 1: Average viability of protoplasts per condition. Control on day 0 lacked FDA staining, therefore, no viability could be determined.

	Day 0 (%)	Day 3 (%)
Average viability dark FDA	91.67	0
Average viability dark Control	-	79.87
Average viability light FDA	90.54	0
Average viability light Control	-	79.86

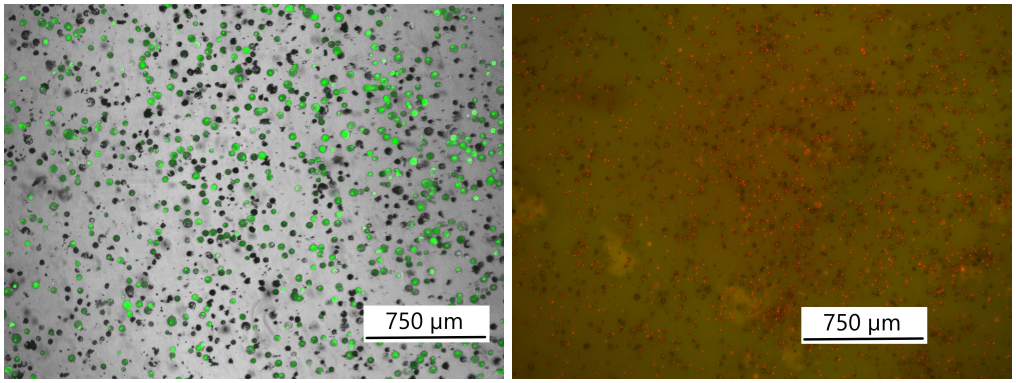
Based on these results, it is concluded that the isolation of protoplasts is successful since high viability is reached after isolation. After staining the protoplasts with the FDA, the viability of the protoplasts can be determined, however, there are no live protoplasts after 24 hours. Therefore, the FDA should be used as an endpoint measurement, but the exact influence of the FDA on the protoplasts should be investigated further.

5.6 Live/dead staining test 2

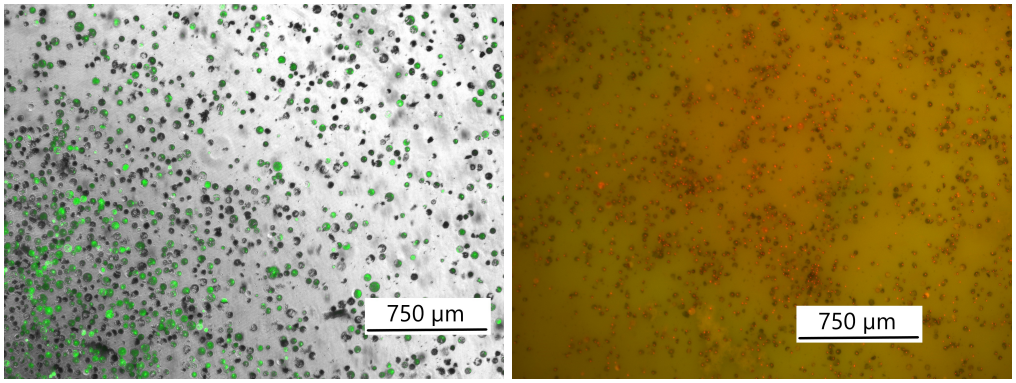
To confirm that the protoplasts with FDA staining on day 0 were dead after 24 hours, a second live/dead staining experiment was performed. The reason for this second experiment was to quantify dead protoplasts, using PI. The results are shown in Figure 14, which were taken with the EVOS (GFP and RFP light cubes).



(a) Well A4 captured at day 0 **(b)** Well A4 captured at day 1



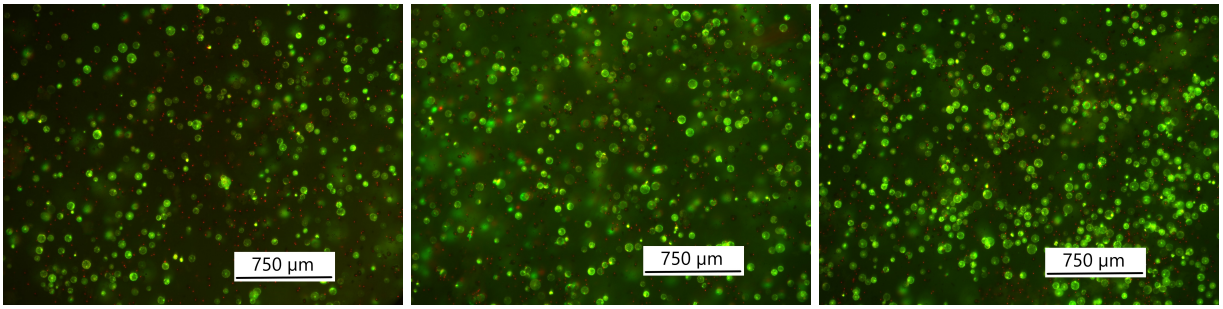
(c) Well B4 captured at day 0 **(d)** Well B4 captured at day 1



(e) Well C4 captured at day 0 **(f)** Well C4 captured at day 1

Figure 14: Overview of protoplasts stained with FDA on day 0 and stained with FDA and PI on day 1. Captured with the EVOS on day 0 (bright-field and GFP light cube combined), and day 1 (GFP and RFP light cubes combined).

The control group has also been stained with FDA and PI on day 1. The results are shown in Figure 15. The results show that the control group is still alive after day 1, yet it contains some dead protoplasts. The protoplasts stained with FDA on day 0 show lots of dead protoplasts. However, the fluorescein in the K8P, as explained in section 5.5, introduces noise, therefore the results are inconclusive.



(a) Well A1 **(b)** Well B1 **(c)** Well C1

Figure 15: Overview of the control group stained with FDA and PI. Captured on day 1 with the EVOS (GFP and RFP light cubes combined).

5.7 Trap efficiency

The trapping efficiency was determined by counting how many traps per row were filled with at least one living protoplast, directly after the fluid flow was cut off. The results can be seen in Table 2. The 2.5 $\mu L/min$ measurement failed due to a technical error. Therefore, its result is inconclusive. Monitoring this condition, the trapping efficiency seemed promising. In the traps of the flow speed of 5.0 $\mu L/min$, there were a significant amount of dead protoplasts, based on morphology. On day 1, the protoplasts in the chips were monitored and no significant difference in the viability of the protoplasts was spotted (based on morphology). Based on these results, the $1.0 - 4.0$ $\mu L/min$, range was determined, with steps of 1.0 $\mu L/min$ to be further investigated in the second experiment.

Table 2: Trapping efficiency of four different flow speeds, directly after the fluid flow is cut-off, counted once by eye using the microscope.

	Trapping efficiency (%)
0.5 $\mu L/min$	43.9
1.0 $\mu L/min$	84.2
2.5 $\mu L/min$	24.5
5.0 $\mu L/min$	73.0

The trapping efficiency of the range $1.0 - 4.0$ $\mu L/min$ is shown in Table 3. The trapping efficiency rate was determined by counting how many traps per row were filled with at least one alive protoplast, directly after the fluid flow was cut off. On day 1, the protoplasts were monitored using a microscope, and based on their morphology the protoplasts survived the speed for trapping. Since the FDA staining killed the protoplasts after 24 hours, the FDA staining was not initially added to the solution. The FDA could have been added at the end, but the absence of an initial measurement of protoplast viability renders this approach non-representative. An overview of traps filled per row of both experiments is shown in Appendix F.

Table 3: Trapping efficiency of a specific range of four different flow speeds, directly after the fluid flow is cut-off, counted once by eye using the microscope.

	Trapping efficiency (%)
1.0 $\mu L/min$	61.2
2.0 $\mu L/min$	91.3
3.0 $\mu L/min$	87.8
4.0 $\mu L/min$	80.1

6 Discussion

In this section, the results of the experiments will be discussed and recommendations will be given.

The debris in Figure 9 is probably the result of a blunt biopsy needle. This resulted in loose pieces of PDMS in the inlet/outlet. When the chip was filled with fluid, the pieces went into the trapping chamber and got stuck. Before bonding, cleaning the inlet and outlet with tape (magic tape Scotch, 3M) by multiple times carefully placing and removing on the chamber, seemed to remove some of the pieces. However, a sharp biopsy needle would help to reduce the loose pieces of PDMS to keep the chip as clean as possible. To remove all air bubbles on chip, directly using K8P medium is the preference, since this makes the more chips suitable for cell culture, in comparison with the pluronic solution.

Unfortunately, the experiments for the continuous fluid flow were not successful. Preliminary results showed promising results about the influence of continuous fluid flow on the protoplasts, so further research is needed. A technical improvement would be to not switch between two tubes. When disconnecting the tube of the syringe with the protoplasts, a lot of the protoplasts left the traps. After connecting the culturing media syringe and applying the fluid flow, some protoplasts got trapped again, but not all. To prevent contamination, a recommendation for further experiments is to work in a sterile environment.

The results gathered in this project look promising, but a lot of the results are not quantified. For further research, quantification is strongly recommended. The pen/strep test results gave a good impression that pen/strep does not harm the protoplasts, but this conclusion is based on the morphology of the protoplasts. A recommendation is to use live/dead staining (FDA) to quantify the survival percentage and use staining for cellulose to investigate the influence of pen/strep on cell wall regrowth. In Figure 11 it seems that the residue of dead protoplasts in the K8P reduces by a higher concentration of pen/strep. An explanation can be that the pen/strep helps to keep the media clean, but this requires further research.

The FDA staining is an appropriate method to determine the viability of the protoplasts, however, the results show that it should be used as an endpoint measurement. This means that after the FDA is added to the growing media with protoplast, the protoplasts will die after 24 hours. Further experiments need to take this into account when using FDA staining for the viability of the protoplasts. Otherwise, there should be more research into live/dead staining of the protoplasts without harming them.
Using FDA staining in combination with PI staining gives results when both are added simultaneously. From the results where the FDA was already added on day 0, the background created noise and therefore gave inconclusive results. From the second experiment of live/dead staining, the viability of the protoplasts could not be determined, since the bright-field image was not taken. Next to including the bright-field image, further research into the determination of the viability using FDA in combination with PI is needed.
The results of a decrease in viability of 10 %, with no difference between dark and light incubation of three days, the results from Table 1, are based on the different wells. The protoplasts are from the same isolation, but further research is needed to confirm that there is no difference between light and dark.
The threshold of 0.2, mentioned in section 4.6.3, is not evaluated. For further investigation into the viability of the protoplasts based on the MatLab script, this threshold value needs to be investigated. Next to the threshold value, the bright-field and fluorescent light intensity needs to stay as constant as possible for better comparison between the results of the viability and can be used to investigate if there is a change in the intensity of the fluorescein in the K8P.

The quantification of the results of the trapping efficiency can improve. The counting is done once by eye, and the viability of the protoplasts is based on morphology. The FDA staining can be used here to determine the viability after the experiment, but more research is needed to quantify the viability directly after trapping.

This result holds that for loading the protoplasts for 10 minutes, the highest trapping efficiency is reached with a fluid flow of 2.0 $\mu L/min$. For further research into the trapping efficiency, more samples need to be used to make a more accurate statement about the results. In addition, other conditions like concentration or loading time can be investigated.
A recommendation for continuous fluid flow experiments is to use the method of creating negative pressure for trapping. Since the tubes do not need to be switched, the protoplasts will stay inside the traps.
The control groups were not on chip, but in a Petri dish. These protoplasts do not experience the shear/-compressive stress from the chip. Therefore, additional research is needed to create a control group on chip.

The chip design works ideal when there is a continuous flow. Changing the tubes of the inlet or even lightly moving the tubes, influences the fluid inside the chip. A recommendation is to build a fixed set-up, that does not require to be moved (only carefully for monitoring using the microscope) Another option could be to design a closed trapping structure. The trapping structure height was 43 micrometers, which is smaller than the used sieve for the isolation. In this project, the shear/compressive stress resulting from the chip height is neglected. For further experiments, the height of the trapping structure should be at least higher than 50 micrometers, to prevent extra shear/compressive stress on the protoplasts.

The focus of this project has been on the experimental side. Therefore, the deep theoretical background has not been investigated. The laminar flow profile has been determined but needs further investigation to be calculated precisely. An important research element of the theoretical background should be the probability of trapping the protoplasts in this design. With the probability of trapping, the trapping efficiency can be better determined. Furthermore, the probability of trapping can help optimize the trapping structure. Due to the time limit, the probability distribution of this design is not established.

7 Conclusion

In this research, a protocol has been established to trap protoplasts on chip. The chip contained no air bubbles when filled with fluid directly after bonding using an oxygen plasma oven. The highest trapping efficiency, directly measured after flow cut-off, was reached with a flow speed of 2.0 $\mu L/min$ while keeping the other conditions constant.
The preliminary results of the continuous fluid flow seemed promising, however, the experiments for the influence of continuous fluid flow on trapped protoplasts failed. The use of the antibiotic penicillin-streptomycin (pen/strep) seems not to influence the viability of the protoplasts, yet additional research is needed to investigate the influence of pen/strep on cell regrowth. FDA staining is an endpoint measurement, as all the protoplasts die after 24 hours. The results of this project can be used to trap protoplasts on chip and evaluate the viability, but further research is needed to optimize the used protocols.

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A Shear stress

Fluid shear stress is the tangential stress generated by the fluid flow. The fluid shear stress depends on the type of flow (laminar or turbulent flow). [27] The type of flow can be determined with Reynolds' number. Reynolds' number is the ratio of inertial forces to viscous forces and can be calculated with Equation 1. [28]

$$Re = \frac{\rho u L}{\mu} \tag{1}$$

Where:
Re is the Reynolds number
ρ is the density of the fluid $[kg/m^3]$
u is the fluid velocity $[m/s]$
L is a characteristic length $[m]$
μ is the dynamic viscosity of the fluid $[Pa * s]$

After the Reynolds number is determined, the type of flow can be determined with:
Re $<$ 10; Laminar flow
10 $<$ Re $<$ 2000; Intermediate regime, flow depends on system geometry
2000 $<$ Re; Turbulent flow

The exact Reynolds number can not be determined because of the unknown values of the density and the dynamic viscosity of the culturing media, which will be used for the fluid. Therefore with the following assumptions an estimated Reynolds number can be calculated:

- The density and dynamic viscosity of the fluid are equal to water at 25 °C since the main substance is water
- Fluid velocity is based on the fastest velocity used in this experiment
- The height of the channel is the characteristic length

The density of water at 25 °C is 997 kg/m^3. [29] The characteristic length is 43 micrometers = $43 * 10^{-3}$ m. The dynamic viscosity of water at 25 °C is $1.0005 * 10^{-3}$ $Pa * s$. [29] The highest flow speed used in the project is 0.57 $microliter/min$, and the dimensions of the chip are $43\mu m$ by $3400\mu m$. This results in an area of $1.462 * 10^{-7}$ m^2. The fluid velocity is calculated by dividing the flow speed by the area. This results in a fluid velocity of $6.498 * 10^{-5}$ m/s. Using formula 1, results in a Reynolds number of 0.00278, which indicates that there is laminar flow inside the chamber.

B Fabrication steps

The chip design has been sent to the cleanroom. Here SU-8 is added on the wafer. The design on the wafer is made using maskless photolithography. After exposure, the wafer is baked again. After this, the wafer is put in a developer solution. After the development is done, the wafer is cleaned with isopropyl alcohol (IPA) and dried in a spin dryer.
The PDMS is mixed with a curing agent in a ratio of 10:1. The fluids are mixed for approximately one minute to get a homogeneous mixture. This mixture is put in the vacuum to degas. After all the bubbles are gone, the mixture is ready to put on the wafer. The wafer is placed in a glass Petri dish and the mixture is slowly poured on the wafer and put back in the vacuum. After thirty minutes in the vacuum, the wafer is put in the oven at sixty degrees to start curing. This is for at least three hours. After the PDMS is fully cured, the PDMS can be cut with a scalpel and carefully removed from the wafer with tweezers. To keep the channel clean, a piece of tape is carefully placed on the channel side of the PDMS. A biopsy puncher of one millimeter is used to remove the material, to create an inlet and outlet for the chip.

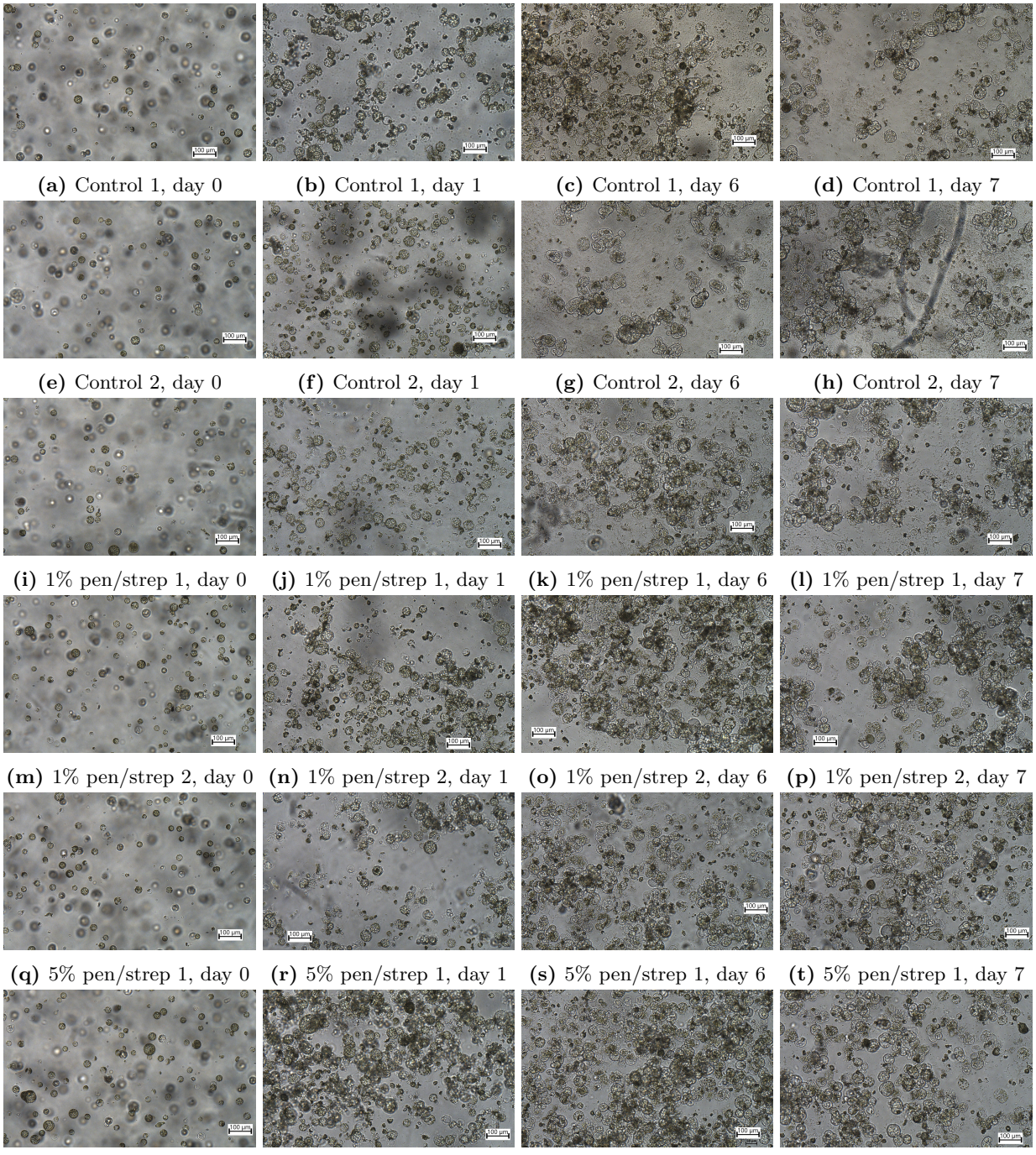
Both surfaces will be treated with oxygen plasma for activation to ensure a direct bonding of the glass slide with the PDMS. This will happen in a plasma oven, with the set time for oxygen plasma treatment at one minute. Directly after the activation, the PDMS is carefully placed on the glass slide.

C Protocol of isolation protoplast

Here the protocol of isolating protoplast from the tobacco plant is described in detail.[30].

1. In vitro shoot cultures of Nicotiana Tabacum leaf protoplasts are maintained on a medium complemented with IBA (indole-3-butyric acid), in high jars at 16/8 h photoperiod of 2000 lux at 25°C and 60-70% RH (relative humility).
2. Prepare sterile media and equipment at least one day before use.
3. Harvest young, fresh-looking leaves into a square Petri dish containing a sterile medium.
4. Holding the leaf upside down, gently scarify/slice the lower epidermis, perpendicular to the main vein, every mm using a fresh scalpel blade.
5. Transfer the scarified/sliced leaves, floating upside up, into a 15 cm Petri dish containing 10 mL Osmoticum [9.1 g mannitol and 140 mg calcium chloride dissolved in 100 mL double distilled water].
6. Incubate the material in enzyme mixture [1.5% celluclast + 0.5% Pectinex] and gently swirl the dish to distribute the enzyme. Incubate overnight at 25°C in the dark.
7. The next morning, gently swirl the dish to help release the protoplasts.
8. Filter the digest through a 50 µm pore size stainless steel sieve.
9. Mount one drop of the filtrate on a slide and observe using an inverted microscope, this gives an idea about the efficiency of digestion.
10. Distribute the filtrate into screw cap tubes and centrifuge for 10 minutes at about 660 rpm.
11. The protoplasts collect as a pellet at the bottom of the tube. Decant the enzyme solution using a pipette without disturbing the pellet.
12. Suspend the pellet of protoplasts in wash medium (osmoticum 550 mOsm).
13. Repeat centrifugation and re-suspend in fresh medium twice to remove the traces of enzymes.
14. Further suspend the protoplasts in 2 mL of wash medium.
15. Layer 2 mL of the concentrated protoplast suspension on top of about 9 mL of floating medium (17% sucrose) in screw cap tubes and centrifuge for 10 minutes at 600 rpm.
16. The healthy protoplasts will be concentrated as a band at the interface of the wash medium and floating medium. Carefully collect this with a pipette to a screw cap tube.

D Results pen/strep test



(a) Control 1, day 0 **(b)** Control 1, day 1 **(c)** Control 1, day 6 **(d)** Control 1, day 7

(e) Control 2, day 0 **(f)** Control 2, day 1 **(g)** Control 2, day 6 **(h)** Control 2, day 7

(i) 1% pen/strep 1, day 0 **(j)** 1% pen/strep 1, day 1 **(k)** 1% pen/strep 1, day 6 **(l)** 1% pen/strep 1, day 7

(m) 1% pen/strep 2, day 0 **(n)** 1% pen/strep 2, day 1 **(o)** 1% pen/strep 2, day 6 **(p)** 1% pen/strep 2, day 7

(q) 5% pen/strep 1, day 0 **(r)** 5% pen/strep 1, day 1 **(s)** 5% pen/strep 1, day 6 **(t)** 5% pen/strep 1, day 7

(u) 5% pen/strep 2, day 0 **(v)** 5% pen/strep 2, day 1 **(w)** 5% pen/strep 2, day 6 **(x)** 5% pen/strep 2, day 7

Figure 16: Experiment with two times: a control group, 1 % pen/strep and 5% pen/strep. Pictures were taken at random places, on days 0, 1, 6, and 7 with the Olympus IX51 microscope.

E Viability results

The results of the viability of day 0 can be seen in Table 4, and day 3 can be seen in Table 5.

Table 4: Results of the viability of the protoplasts on day 0. Gathered by using the MatLab script of J.T. Loessberg-Zahl.

Image numbers (trans + GFP)	Total cells	Live cells	Dead cells	Viability (%)
a3_donker_0038_Trans.jpg,a3_donker_0039_GFP.jpg	194	182	12	93.81
a3_donker_0041_Trans.jpg,a3_donker_0042_GFP.jpg	206	178	28	86.41
a3_donker_0044_Trans.jpg,a3_donker_0045_GFP.jpg	168	154	14	91.67
b3_donker_0047_Trans.jpg,b3_donker_0048_GFP.jpg	202	186	16	92.08
b3_donker_0052_Trans.jpg,b3_donker_0053_GFP.jpg	241	229	12	95.02
b3_donker_0055_Trans.jpg,b3_donker_0056_GFP.jpg	180	164	16	91.11
c3_donker_0058_Trans.jpg,c3_donker_0059_GFP.jpg	249	228	21	91.57
c3_donker_0061_Trans.jpg,c3_donker_0062_GFP.jpg	243	212	31	87.24
c3_donker_0064_Trans.jpg,c3_donker_0065_GFP.jpg	284	273	11	96.13
a3_licht_0088_Trans.jpg,a3_licht_0089_GFP.jpg	166	149	17	89.76
a3_licht_0091_Trans.jpg,a3_licht_0092_GFP.jpg	141	124	17	87.94
a3_licht_0094_Trans.jpg,a3_licht_0095_GFP.jpg	147	130	17	88.44
b3_licht_0076_Trans.jpg,b3_licht_0077_GFP.jpg	142	136	6	95.77
b3_licht_0079_Trans.jpg,b3_licht_0080_GFP.jpg	168	149	19	88.17
b3_licht_0082_Trans.jpg,b3_licht_0083_GFP.jpg	199	180	19	90.45
b3_licht_0085_Trans.jpg,b3_licht_0086_GFP.jpg	233	218	15	93.56
c3_licht_0067_Trans.jpg,c3_licht_0068_GFP.jpg	198	181	17	91.41
c3_licht_0073_Trans.jpg,c3_licht_0074_GFP.jpg	197	176	21	89.34

Table 5: Results of the viability of the protoplasts on day 3. Gathered by using the MatLab script of J.T. Loessberg-Zahl.

Image numbers (trans + GFP)	Total cells	Live cells	Dead cells	Viability (%)
a1_donker_dag3_0582_Trans.jpg,a1_donker_dag3_0583_GFP.jpg	150	125	25	83.33
a1_donker_dag3_0585_Trans.jpg,a1_donker_dag3_0586_GFP.jpg	129	114	15	88.37
a1_donker_dag3_0588_Trans.jpg,a1_donker_dag3_0589_GFP.jpg	129	107	22	82.95
a1_licht_dag3_0423_Trans.jpg,a1_licht_dag3_0425_GFP.jpg	160	129	31	80.63
a1_licht_dag3_0427_Trans.jpg,a1_licht_dag3_0428_GFP.jpg	174	132	42	75.86
a1_licht_dag3_0430_Trans.jpg,a1_licht_dag3_0431_GFP.jpg	197	156	41	79.19
b1_donker_dag3_0563_Trans.jpg,b1_donker_dag3_0564_GFP.jpg	158	122	36	77.22
b1_donker_dag3_0566_Trans.jpg,b1_donker_dag3_0567_GFP.jpg	152	124	28	81.58
b1_donker_dag3_0569_Trans.jpg,b1_donker_dag3_0570_GFP.jpg,	170	139	31	81.76
b1_licht_dag3_0436_Trans.jpg,b1_licht_dag3_0437_GFP.jpg	162	132	30	81.48
b1_licht_dag3_0439_Trans.jpg,b1_licht_dag3_0440_GFP.jpg	132	104	28	78.79
b1_licht_dag3_0442_Trans.jpg,b1_licht_dag3_0443_GFP.jpg	144	115	29	79.86
c1_licht_dag3_0454_Trans.jpg,c1_licht_dag3_0455_GFP.jpg	128	99	29	77.34
c1_licht_dag3_0457_Trans.jpg,c1_licht_dag3_0458_GFP.jpg	143	122	21	85.31
c1_licht_dag3_0461_Trans.jpg,c1_licht_dag3_0462_GFP.jpg	127	102	25	80.31
c1_donker_dag3_0552_Trans.jpg,c1_donker_dag3_0553_GFP.jpg	135	97	38	71.85
c1_donker_dag3_0554_Trans.jpg,c1_donker_dag3_0555_GFP.jpg	117	96	21	82.05
c1_donker_dag3_0560_Trans.jpg,c1_donker_dag3_0561_GFP.jpg	109	76	33	69.72

F Trapping efficiency

In Table 6 the results of counting how many traps in a row contain at least one alive protoplast. The trapping efficiency was calculated by the total amount of traps containing at least one alive protoplast divided by the total amount of traps (196 traps) multiplied by 100 %, rounded to one decimal. As example:

$$trappingefficiency = \frac{86}{196} * 100\% = 43.9\% \tag{2}$$

During the first time loading the chip with the flow speed of 2.5 $\mu L/min$, the inlet and outlet were switched. After loading, the inlet and outlet were switched and loaded again. The loading of this chip seemed promising, but a lot of dead cells got loaded. The chip loaded with 5.0 $\mu L/min$ contained a lot of dead protoplasts. With these results, the decision was made to do more research into the range of 1.0 to 4.0 $\mu L/min$. With steps of 1.0 $\mu L/min$, from the reason that there are four syringes available.

Table 6: Overview of how many traps contained at least one alive protoplast per row. Counted once by eye using the microscope, directly after stopping the fluid flow.

	0.5 $\mu L/min$	1.0 $\mu L/min$	2.5 $\mu L/min$	5.0 $\mu L/min$
Row 1	9	9	4	10
Row 2	11	9	2	8
Row 3	9	10	7	7
Row 4	9	8	4	7
Row 5	10	10	4	10
Row 6	8	10	6	9
Row 7	4	10	3	9
Row 8	4	9	4	9
Row 9	4	8	0	7
Row 10	2	10	5	7
Row 11	1	11	1	10
Row 12	1	9	1	9
Row 13	3	11	3	9
Row 14	3	10	1	9
Row 15	4	12	1	8
Row 16	3	8	1	8
Row 17	1	11	1	7
Total	86	165	48	143

The results from the second experiment can be seen in Table 7. This gives an overview of how many traps per row contained at least one alive protoplast. The trapping efficiency was calculated by the same formula mentioned above.

Table 7: Overview of how many traps contained at least one alive protoplast per row. Counted once by eye using the microscope, directly after stopping the fluid flow.

	1.0 $\mu L/min$	2.0 $\mu L/min$	3.0 $\mu L/min$	4.0 $\mu L/min$
Row 1	7	12	9	12
Row 2	8	11	8	11
Row 3	9	11	11	12
Row 4	6	8	11	8
Row 5	7	12	10	10
Row 6	6	8	11	8
Row 7	7	12	9	10
Row 8	8	10	11	9
Row 9	7	12	11	10
Row 10	9	10	10	8
Row 11	7	12	10	11
Row 12	7	10	10	10
Row 13	6	10	12	8
Row 14	6	10	11	7
Row 15	9	10	9	8
Row 16	7	11	8	7
Row 17	4	10	11	8
Total	120	179	172	157

G Matlab script for viability of the protoplasts

```
path=[''];              %path to the starting folder can be added here
radius = 20;            %cell radius in pixels
threshold = 0.2;        %fraction of "high" signal that qualifies as live

live=0;
dead=0;
total=0;

%finite state machine for LcD counting
%figure titles have user instructions

%state initialization
running = 1;
opening_white = 1;
clicking_white = 0;
opening_gfp = 0;
getting_min = 0;
getting_max = 0;
checking_live = 0;
outputing_data = 0;

%open the working directory
savePath=uigetdir(path,'Pick Directory to Save In | Close to Quit');
if isa(savePath,'double')
    reading_white = 0;
    running = 0;
end

savePath = strcat(savePath,'\');
disp(path)
```

```
31
32 saveFile=strcat(savePath,'LDData.csv');
33
34 listing = dir(saveFile);
35 disp(listing)
36
37 if isempty(listing)
38     fid = fopen(saveFile,'wt');
39     fprintf(fid,'BImage,FImage,Total,Live,Dead\n');
40     fclose(fid);
41 end
42
43
44 %main loop
45 while running == 1
46
47     if opening_white == 1
48         %either gets a filename or quits
49         [quit,bFile,path] = open_or_quit(path,'Pick Brightfield Image | Close to Quit');
50         if quit == 1
51             reading_white = 0;
52             running = 0;
53         end
54
55         I1=imread([path bFile]);
56         imshow(I1);
57         title(strcat('Pick in-focus, single cells     |     Enter to continue     |     ...
               File: ', bFile))
58         hold on;
59
60         coords1 = [];
61         coords2 = [];
62         circles =[];
63         crosses = [];
64
65         %changing state to clicking_white
66         clicking_white = 1;
67         opening_white = 0;
68     end
69
70     if clicking_white==1
71         [x,y,button]=myginput(1,'custom');
72         if isempty(button)      %if nothing is clicked moves onto opening gfp image
73             title(['annotated_' bFile]);
74             saveas(gcf,[path 'annotated_' bFile])
75             clicking_white = 0;
76             opening_gfp = 1;
77         elseif button==26       %if ctrl+z is pressed, either deletes the last pick or ...
               allows choice of a new file
78             if isempty(circles)
79                 clicking_white = 0;
80                 opening_white = 1;
81             else
82                 delete(circles(end));
83                 circles=circles(1:end-1);
84                 coords1=coords1(1:end-1,:);
85             end
86         elseif button == 1
87             circles=[circles;circle(x,y,radius,'m')];
88             coord=[x,y];
89             coords1=[coords1;coord];
90         end
91     end
92
93     %opens the FDA stained image
```

```
94      if opening_gfp == 1
95          %either gets a filename or quits
96          [quit,fFile,path] = open_or_quit(path,'Pick Fluorescence Image | Close to Quit');
97          if quit == 1
98              opening_gfp = 0;
99              running = 0;
100             break;
101         end
102
103         I2=imread([path fFile]);
104         I2Green=I2(:,:,2);
105         I2GreenNorm=imadjust(I2Green);
106
107         imshow(I2GreenNorm);
108         title(strcat('Pick Background     |     File: ', fFile))
109
110         for i=1:length(circles)
111             circle(coords1(i,1),coords1(i,2),radius,'m');
112         end
113
114         bkg_coord = [];
115         bkg_circ = [];
116         coords2 = [];
117         crosses= [];
118         opening_gfp = 0;
119         getting_min = 1;
120     end
121
122     %asks the user to select the background
123     if getting_min==1
124         [x,y,button]=myginput(1,'custom');
125         if button==26 %if ctrl+z is pressed, either deletes the last pick or allows choice ...
                of a new file
126             getting_min = 0;
127             opening_gfp = 1;
128         elseif button == 1
129             bkg_circ = circle(x,y,radius,'b');
130             bkg_coord = [x,y];
131             getting_min = 0;
132             getting_max = 1;
133         end
134     end
135
136     %asks the user to select the brightest protoplast
137     if getting_max == 1
138         title(strcat('Pick Foreground     |     File: ', fFile))
139         [x,y,button]=myginput(1,'custom');
140         if button==26 %if ctrl+z is pressed, either deletes the last pick or allows choice ...
                of a new file
141             getting_max = 0;
142             getting_min = 1;
143             delete(bkg_circ);
144         elseif button == 1
145             fore_circ = circle(x,y,radius,'r');
146             fore_coord = [x,y];
147             getting_max = 0;
148             checking_live = 1;
149             pause(0.01);
150         end
151     end
152
153     if checking_live == 1
154         title(strcat('Counting....     |     File: ', fFile))
155         pause(0.1)
156         live_level = 0;
```

```
157         dead_level = 0;
158         min_live = 0;
159         %despeckles the image
160         deSpec=speckle_filter(I2GreenNorm,0.02);
161 
162         %calculates the minimum signal that qualifies for being alive
163         inner=circle_inner_coords(bkg_coord,radius);
164         max_val = double(0);
165         for j=1:length(inner)
166             if double(deSpec(inner(j,2),inner(j,1)))>max_val
167                 max_val=double(deSpec(inner(j,2),inner(j,1)));
168             end
169         end
170         dead_level = max_val;
171 
172         inner=circle_inner_coords(fore_coord,radius);
173         max_val = double(0);
174         for j=1:length(inner)
175             if double(deSpec(inner(j,2),inner(j,1)))>max_val
176                 max_val=(deSpec(inner(j,2),inner(j,1)));
177             end
178         end
179         live_level = max_val;
180 
181         min_live = dead_level+threshold*(live_level-dead_level);
182 
183         for i = 1:length(coords1)
184             inner=circle_inner_coords(coords1(i,:),radius);
185             max_val = double(0);
186             for j=1:length(inner)
187                 if double(deSpec(inner(j,2),inner(j,1)))>max_val
188                     max_val=double(deSpec(inner(j,2),inner(j,1)));
189                 end
190             end
191 
192             if max_val≥min_live
193                 coords2=[coords2;coords1(i,:)];
194                 crosses=[crosses;plot(coords1(i,1),coords1(i,2),'rx','MarkerSize',7,'LineWidth',2)];
195             end
196         end
197         pause(0.1)
198         title(strcat('Counted. Enter to continue     |     File: ', fFile))
199         [x,y,button]=myginput(1,'custom');
200         if button==26 %if ctrl+z is pressed, either deletes the last pick or allows choice ...
                of a new file
201             getting_max = 1;
202             checking_live = 0;
203             coords2 = [];
204             for i = 1:length(crosses)
205                 delete(crosses(i));
206             end
207             crosses = [];
208             delete(fore_circ);
209             pause(0.01);
210         elseif isempty(button)
211             outputing_data = 1;
212             checking_live = 0;
213             title(['annotated_' fFile]);
214             saveas(gcf,[path 'annotated_' fFile])
215         end
216     end
217 
218     %saves the marked images and saves the data to file
219     if outputing_data == 1
220         live=length(crosses);
```

```
221           dead=length(circles)-length(crosses);
222           total=length(circles);
223
224           fid = fopen(saveFile,'a+');
225           fprintf(fid,[bFile,',',fFile,',',num2str(total),',',num2str(live),',',num2str(dead),'\n']);
226           fclose(fid);
227
228           outputing_data = 0;
229           opening_white = 1;
230       end
231   end
```

Figure 17: Chrome Dino found in one of the chips.