Testing the applicability of the Omi perfusion device for research into circulating tumour cells.

Research into the possibility of circulating tumour cells and the effect of flow on cell culture on chip models using the Omi perfusion device.

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Abstract

In this study the Omi automated perfusion system by Fluigent will be beta-tested for use in cancer research. Omi is designed to be able to recirculate culture medium over organs-on-chip, using small amounts of fluid at flow rates from 1 µl/min up to 2000 µl/min.

Two experiments were designed to test the suitability of Omi for its use in cancer research. The first experiment aimed to emulate circulating tumour clusters and had Omi recirculate cells to determine the effect of circulation on the viability of the perfused cells. Aside from that some Omi components were analysed for aggregation of cells after circulation. The second experiment had Omi recirculate medium over blood vessel-on-chips seeded at ($2 \cdot 10^6$ cells/ml and $0.5 \cdot 10^6$ cells/ml) to investigate the effect of flow on the cells.

The results concluded that circulation of cells did not impact the viability, but lowered the concentration in the suspension. Analysis of Omi components under a fluorescence microscope showed no evident aggregation of cells had occurred. The second experiment concluded that flow caused endothelial cells seeded at a low density in the microfluidic chip to align in the direction of the flow. At a high seeding density cells did not show this behaviour.

Due to time constraints and encountered with Omi prototype the first experiment could only be performed once and should be repeated to establish a more reliable view of the cell circulating capabilities of Omi. If these tests are complete Omi could prove useful for research into the effects of circulating tumour cells on blood vessel on microfluidic chip models.

1 Introduction

In this study the Omi perfusion device by Fluigent, shown in Figure 2, has been beta-tested for its use in cancer research. Omi is designed to optimally support organ-on-chip applications by perfusing culture medium over cells. It can do so using a multitude of flow profiles at flow rates from 1 µl/min up to 2000 µl/min to mimic the in vivo environment.

Omi is relatively small compared to alternatives at 190mm x 120mm x 60 mm and has been designed to withstand the humidity conditions found in an incubator. On top of that it can recirculate small volumes of fluid, potentially making it ideal for longer tests. This is especially the case when recirculating cells, where large amounts of cell suspension can be difficult to acquire.

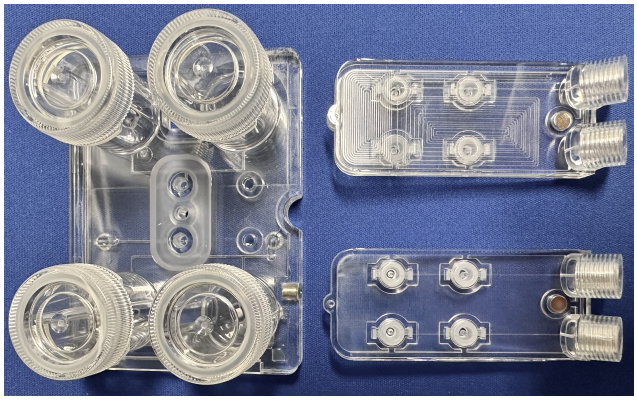

Figure 1: *the Omi cartridge is displayed on the left and the high and low resistance adaptors are shown on the top and bottom right respectively.*

Aside from that, Omi comes with cartridges

and both low and high resistance adaptors, shown in Figure 1. They all come sterile packed for easy use in environments like LAF cabinets. Each cartridge contains four reservoirs with a capacity of 4ml each.

Figure 2: *A picture of Omi. [1]*

Omi can be controlled via the accompanying Omi app. Here protocols can be created and the current status of Omi can be seen. The app also contains animations detailing how to perform steps like cleaning, loading, and sterilisation. Aside from that, the app contains steps like perfusion, recirculation, sampling, and injection. These steps are explained in Figure 3.

Furthermore, Omi can calculate the shear stresses that the flow exerts on the walls of the chip. It does so by using a version of Poiseuille's law, equation 1. Here Q is the flow rate in $[m^3/s]$, τ is the shear stress in $[Pa]$, d is the diameter of the vessel in $[m]$, and η is the dynamic viscosity of the medium used. [2, 3]

$$Q = \frac{\pi \tau d^3}{32\eta} \tag{1}$$

The Omi app allows for the values of d and η to be changed and it assumes a shear stress of 1 Pa. However, the in vivo situation is more complex, with shear stresses varying significantly between vessels from ≤ 1 to ≥ 60 Pa. [3, 4] As a result, in order to simulate the in vivo situation accurately the use of other shear stresses might be desired, in which case these would have to be manually calculated and the resulting flow rate filled in to the Omi app.

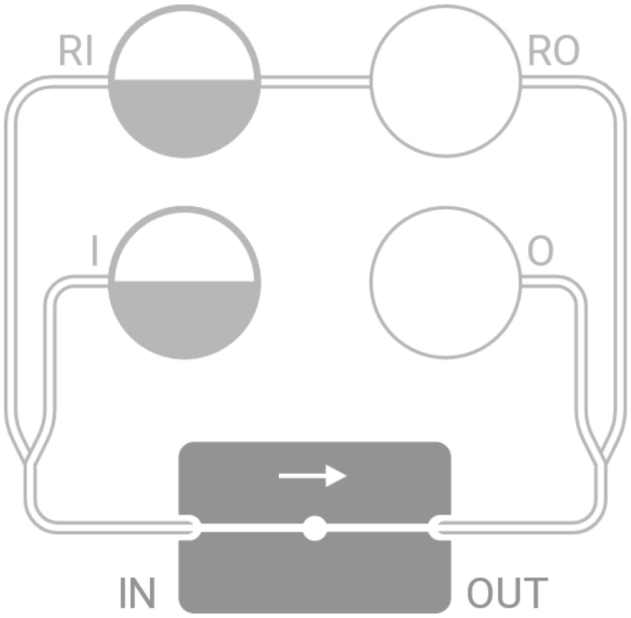

Figure 3: *A schematic representation of the Omi cartridge. Here fluids are pumped counterclockwise. Perfusion pumps fluids from RI to RO. Recirculation works the same way, but will pump fluid from RO back to RI when RI is empty. Sampling will pump fluid from RI to O, and injection will pump fluid from I to RO. IN and OUT represent the places where the perfused fluid enters and leaves the attached chip respectively.*

1.1 Cancer and metastasis

Breast cancer is the most common type of cancer in women. [5] Around ninety percent of cancer related deaths are caused by metastases. [6, 7] As a result inhibiting the occurrence of metastases could potentially reduce the mortality and morbidity. However, in order to achieve this a better understanding of the mechanisms behind metastasis is necessary.

Tumour cells can spread more rapidly due to epithelial mesenchymal transition (EMT). In this process epithelial tumour cells will transition into a mesenchymal phenotype with fewer intercellular bonds, allowing for increased potential for migration into other tissues.[5, 8, 9] When the tumour cells have reached the blood vessels they eventually enter the blood stream. Cells, but also clumps of tumour cells called cell clusters, can enter circulation, creating circulating tumour cells and clusters. Out of these, metastases are most

likely to arise from circulating tumour cell clusters as these are more likely to survive circulation. [5] As a result, This research will focus more on circulating clusters as opposed to single cells.

Before metastasis occurs the future site of metastasis is made more suitable for tumour invasion through the interaction of tumour secreted factors with the vessel endothelium. This environment is called the pre-metastatic-niche. The first step in pre-metastatic niche formation is the increase in permeability of the endothelial walls allowing circulating tumour cells to extravasate. [6, 8, 10, 11]

The effects of circulating clusters and on blood vessel endothelium can be studied by exposing blood vessels to different tumour clusters and determining how they affect the vessel permeability.

1.2 Setup

In this paper Omi is tested in two experiments. The first experiment will aims to perfuse circulating tumour clusters and determine the suitability of Omi for this task. The second experiment will circulate medium over blood vessel-on-chip models to determine the effect of flow on cell morphology.

1.3 Microfluidic chips

In order to effectively research the blood vessels need to be emulated. A way to achieve this is by using blood vessels in microfluidic chips. This allows for a more tunable environment than an in vivo study while also allowing for easier upscaling. [12]

One way to make blood vessels on chips is by using the Viscous Finger Patterning (VFP) technique. [2, 13] This yields more complex and realistic vessels than a 2D cell culture. This can be expanded upon by making double layer VFP vessels to simulate the in vivo condition even more accurately. [14] However, use of the VFP technique complicates both the production and imaging processes. Therefore, a simpler 2D vessel is used in this study.

1.4 Cell types

Two different tumour cell lines were considered, namely the MCF-7 and MDA-MB-231 cell lines. Both of these cell lines are derived from breast cancer, but they present different characteristics.

The MCF-7 cell line is positive for both oestrogen and progesterone receptors and has an epithelial-like morphology. [15–17]

MDA-MB-231 is an aggressive triple negative breast cancer cell line. This means it is negative to oestrogen, progesterone, and her2. [18, 19] The cells are spindle-shaped and although of epithelial origin have more mesemchymal properties. [19, 20]

Beside tumour cells, other cell types are used in the experiments. HL-60 cells are used in circulation. These cells were isolated from leukocytes from a patient with leukaemia and they most closely resemble neutrophilic promyelocytes. [21] The main reason they are used in this study is because they are non-adherent.

Lastly, for making the vessels-on-chips, human Mesenchymal Stem Cells (hMSCs) were used. hMSCs can be differentiated into a variety of cell types like adipocytes, osteocytes, and chondrocytes, but have also been shown to be able to differentiate into epithelial-like cells. [22–24]

2 Troubleshooting

2.1 General approach

Since Omi was being beta-tested during these experiments the device had to be thoroughly troubleshooted when errors occurred. Fluigent had already notified us that the quality of the reservoir cartridges was not yet optimal and that they might not all function as expected. As a result, before any other tests were conducted, two cartridges were validated and confirmed to be functional. This was done by running the 'CheckFunctionality' and 'MaxFlow' protocols on Omi. The protocols and motivations behind them are described in detail in Appendix A. Along with that the cartridges were used for overnight tests to ensure these were fit for longer runs, as some errors only occurred during longer tests. However, the overnight test were conducted later, after the the experiment described in Chapter 3.1.

Every experiment used a new sterile cartridge and low resistance adaptor. Because of this the 'CheckFunctionality' protocol was run every time a new cartridge was used. This ensured that the new cartridges did not leak and would help minimise any tests failing due to faulty cartridges.

If any errors did occur and could be reproduced the cartridge, adaptor, and attached chip were first checked for leaks again by testing whether the errors recurred with the validated cartridges.

Lastly, if an error persisted between the replaceable components it was reported back to Fluigent who provided technical support.

2.2 Major troubleshooting

During the course of the experiments there were some occasions where experiments were halted and Fluigent was contacted for technical support.

When the first major error occurred Omi could only run specific protocols. With most protocols Omi would show the 'MissingFluidicComponent' error, indicating that either the cartridge or adaptor was not inserted. However, this error occurred only for specific protocols and while all components were inserted properly. Some protocols would run without this issue. Newly made identical protocols would also run, but small changes would yield an error message. There did not appear to be a clear link between what protocols worked based on the steps they contained as protocols containing different flow rates and steps would run, while rearranging the steps could stop them working entirely.

This turned out to be the result of sensors that had become decalibrated. The exact sensors that were malfunctioning were not clearly identified for some proprietary reasons on the specifics of Omi hardware and software. After scheduling a meeting with Fluigent technical support the issue was quickly fixed by running a special calibration programme, which was sent to Omi over Wi-Fi by the support team. Although, at the time of writing this issue cannot be fixed without contacting Fluigent a future update should prevent Omi from losing its calibration.

The second time that Omi could not be fixed without support the flow rate delivered by Omi had deteriorated significantly, with maximum flow rate reaching up to 200µl/min and eventually falling further down to below 10µl/min. To fix this Fluigent support suggested manually pumping distilled water and 70% ethanol through the Flow sensor within Omi using a syringe in order to get rid of any potential blockage. This temporarily fixed the problem, but maximum flow rates remained around 200µl/min. On top of that the Flow sensor had to be cleaned again with every couple hours of use.

The fifth time that the flow sensor was cleaned no fluids were able to pass through, even when more pressure was applied than was necessary the previous time the flow sensor was cleaned. When running protocols Omi also did not get the flow rate above 0µl/min even when applying its maximum pressure of 800mbar. After more contact with Fluigent support, they concluded that Omi would have to be sent back for analysis.

A new Omi was sent and used for the rest of the experiments and no significant issues were encountered.

2.3 Small Inconveniences

Beside the major troubleshooting there were smaller issues and inconveniences which did not halt the continuation of experiments. These have all been reported back to Fluigent. The inconveniences that persisted between the two Omis are listed below.

- The MAC address of Wi-Fi chip was not listed on the label making MAC-address based Wi-Fi passwords more inconvenient to acquire.
- Sometimes the app froze on the start-up screen.
- The battery indicator was not very accurate.
- After a programme is paused Omi occasionally unpauses by itself.

Lastly, one error occurred with the newly sent Omi where it would not pump liquid from RO to RI claiming that reservoir RI was full, even when it was not. This problem was easily fixed by unscrewing the cap on the RI reservoir and tightening it again. As a result experiments were not hindered. According to Fluigent support this error can be prevented by running the calibration step at the start of every protocol.

3 Materials & Methods

3.1 Determining the effects of circulation

Omi was tested in two different experiments. The first experiment was meant to determine the effects of the recirculation on cell health and to locate places in Omi where cell aggregation occurs. This was done without a chip attached to Omi and instead with the inlet and outlet connected by tubing. The HL-60, MCF-7, and MDA-MB-231 cells used in this test were cultured using the same culture medium of DMEM (Gibco) with 10% FBS (Sigma), 1% Glutamax (Gibco), and 1% pen/strep (Gibco).

3.1.1 CellTracker

HL-60 cells, A kind gift from AOT group UT-wente, were cultured and stained using CellTracker (Invitrogen C7025). This was done by centrifuging the cell suspension for three minutes at 200g and resuspending the cell pellet in 1ml

of serum free medium. One vial containing CellTracker powder was dissolved in 20µl DMSO (Sigma D2650) after which 10µl of this solution was added to the cell suspension. This was placed in an incubator, at 37°C with 5% CO_2, for 30 minutes after which the suspension was centrifuged and resuspended in 5ml of culture medium.

3.1.2 Clusters

The tumour clusters were created as follows. First 3.0g of agarose (Invitrogen 16500500) was added to 110ml of PBS (Sigma) after which is was mixed and heated until all agarose was dissolved. 100µm diameter microwell moulds were 3d-printed, using the Form 3B+ (Formlabs), and placed in two 6-wells plates. The agarose solution was poured over the moulds such that a 1.5-2.5mm thick layer remained on top of the moulds. After that the plates were centrifuged at 300g and at room temperature for 2 minutes with a slow acceleration and deceleration. The wells plates were subsequently refrigerated for 20 minutes. Following this the moulds were removed and the resulting microwell chips were punched out and placed in a 12-wells plate.

The tumour clusters were created by carefully pipetting 1ml of cell suspension, containing 10^5 cells/ml, over the microwells after which the 12 wells plate was centrifuged for three minutes at 300g with a slow acceleration and deceleration. Half of the wells were seeded with MCF-7 cells and the other half was seeded with MDA-MB-231 cells. After that 2ml of culture medium was carefully pipetted onto the side of each well to ensure cells were minimally disturbed and displaced by the flow of medium. Lastly, the 12-wells plate containing the microwells was placed in an incubator for approximately 48 hours.

3.1.3 Circulation

The circulation experiments were conducted as follows. First, a sterile cartridge and low resistance adaptor were inserted into Omi. To ensure no leaks were present the 'Check Functionality' programme was run with 2ml of distilled water in reservoirs RI and I. After this the system was sterilised and the 'Perfusion 2 Samples' programme was run with 2ml of cell suspension with a concentration of approximately $9 \cdot 10^5$ cells/ml. As a control measurement 2ml of the same suspension was pipetted into an Eppendorf tube and placed next to Omi to keep the conditions aside from flow as similar as possible. The viability and concentration was checked after perfusion using an EVE Automated Cell Counter (NanoEntek). A small amount of PBS remained in the cartridge from the last step of sterilisation. The amount left was determined in a separate experiment shown in Appendix B. The remaining amount of PBS was calculated and 33 µl would end up in the RO reservoir, while 20 µl would end up in the O reservoir. These volumes were used to offset the resulting dilution. In between tests Omi was cleaned using the cleaning step from Omi app. This test was repeated three times to ensure the results acquired were properly representative of the situation. Based on the results the test times were varied in order to establish a function for the concentration and viability of the cells over time. When the experiments were finished Omi was cleaned by running the cleaning step from Omi app.

The protocol for the circulation of tumour clusters was the same as described above, but some extra steps were taken. To more accurately determine the concentrations and viability before and after circulation of the tumour clusters the circulation samples were trypsinized, to break down the clusters into single cells, and a live/dead (Sigma) staining was performed in parallel with the EVE tests. This was done by adding 0.75µl live stain and 3µl dead stain to 3ml of PBS to make the staining solution. After that the samples were centrifuged, aspirated, and resuspended in 500µl of the staining solution. This was placed in an incubator for 15 minutes after which pictures were taken using the EVOS.

3.1.4 Imaging

To check whether any aggregation of cells occurred within Omi the cartridge and low resistance adaptor were imaged after the circulation of the HL-60 cells. This was done using an EVOS fluorescence microscope (Thermofisher). The pictures taken were analysed for any visible difference in concentration to determine if and where most aggregation of cells would occur.

3.2 Recirculating culture medium over cells

The second experiment done was circulating culture medium over cells on chips. This was done to determine the effects of flow on cell orientation. For this test Mesenchymal Stem Cells (ATCC PCS-500-012) were cultured in α-MEM (Gibco) containing 10% FBS and 1% pen/strep.

3.2.1 Chip production

To make the chips, moulds were designed in SOLIDWORKS, shown in Figure 4. These were

3D-printed after which 1:10 curing agent to PDMS (Sylgard 184) was poured into them. After degassing and curing the chips were removed from the mould taking care not to damage the channels. After that the PDMS chips and microscope slides were treated with the CUTE Plasma System by Femto Science ensuring that the channels on the PDMS chips faced up. Here a generation time of 40 seconds was used. Immediately after treatment the PDMS chips were firmly pressed onto the slides.

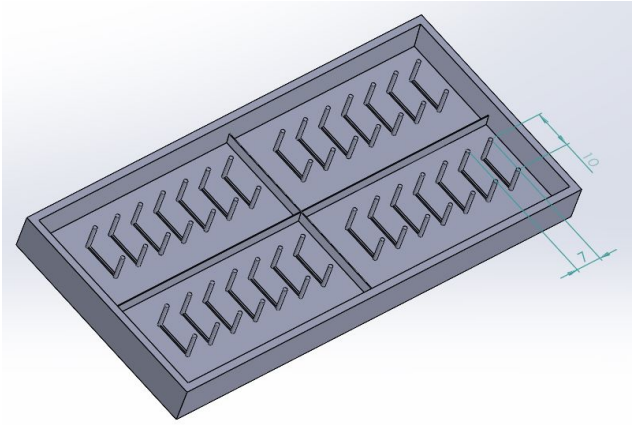

Figure 4: *The mould that was used to make the chips. This mould was made to accommodate four chips containing seven channels each. The distance between the channels is 7mm and the length of the channels is 10mm. The pillars are 1000μm in diameter and the width and height of the channel are both 500μm.*

3.2.2 Preparation for cell culture

The channels were treated with Polydopamine (Sigma H8502) in 10mM Tris buffer with a pH of 8.5 at a concentration of 2mg per ml of buffer and left to incubate at room temperature for an hour. After that they were washed four times with water followed by one hour of incubation in an oven at 65°C. Before cells were seeded the chips were also treated with collagen (Cellmatrix Type I-P). A solution of 1% Cellmatrix in PBS was injected into the canals on the chips and placed in an incubator for half an hour. After that they was washed with PBS.

3.2.3 Seeding cells in chips

The MSCs were first washed with PBS and subsequently trypsinized (Gibco) at room temperature for three minutes after which culture medium was added to neutralise the trypsin. The resulting suspension was collected and centrifuged at 300g for 3 minutes. The supernatant was aspirated and the cell pellet was resuspended in 0.5ml of culture medium. This cell suspension was used to create suspensions of $10 \cdot 10^6$ cells/ml, $2 \cdot 10^6$ cells/ml, and $1 \cdot 10^6$ cells/ml. Three chips were used, one for each concentration. The chips were seeded by pipetting $10\mu l$ of cell suspension into each channel. For this experiment five channels were seeded per chip. After seeding the chips were placed in an incubator for 3 hours, whereafter 100µl of medium was added. The chips were further incubated for 48 hours with 100µl more medium added around the 24 hour mark.

3.2.4 Circulation

Omi was sterilised and a new cartridge and low resistance adaptor were inserted. First the device was tested for leaks by running the 'CheckFunctionality' protocol. After that the system was sterilised by running the sterilisation step from Omi app.

During the sterilisation cells in the chips were stained using Hoechst (Thermofisher 62249). This was done by diluting the Hoechst 1:200 in PBS. All channels on the chips were washed with PBS after which they were filled with the Hoechst 1:200 in PBS and left to incubate at room temperature for five minutes. After that they were again washed with PBS and pictures were taken using the EVOS microscope to ensure the staining was successful and to compare against pictures made after the experiment. When the experiments were finished Omi was cleaned by running the cleaning step from Omi app.

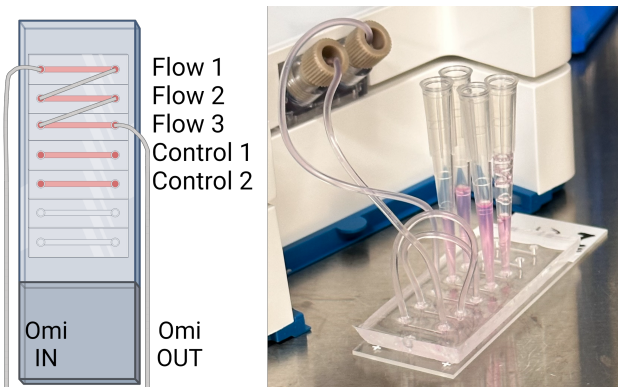

Figure 5: *A schematic representation of the setup is shown on the left. On the right a picture is shown of the setup during the experiment. The top three channels of the chips were the flow channels. These had medium recirculated through them by Omi. The two channels below had pipette tips with medium attached to them to create a static control measurement to compare to the flow conditions. The bottom two channels were left empty.*

Tubing was connected to the top three channels on the chips, see Figure 5, while the bottom two channels were left as control measurements. The test was done by running the 'Recirculate' programme. Between tests Omi was cleaned by filling reservoir RI with 3ml of PBS and running the 'PBS run' protocol, shown in Appendix A. The used chip was then viewed under the EVOS to generally check if differences were visible compared to the situation before recirculation.

3.2.5 Fixation and staining

After each circulation test the used chips were fixed using 4% PFA (Sigma) in PBS. The channels were washed with PBS and subsequently filled with the PFA solution after which they were incubated at room temperature for 10 minutes. Following this they were washed three times with PBS and placed in a refrigerator at 5°C.

Later, the chips were stained with Texas Red™-X Phalloidin (Invitrogen) and Hoechst. They were first washed with 1% Triton X100 (Sigma T8787) in PBS to make the cell membranes more permeable and increase the effectiveness of the staining. After that they were washed with PBS after which the staining solution was inserted into the channels. The chips were then left to incubate at room temperature for half an hour. Following this they were washed with PBS three times.

3.2.6 Imaging

The channels were imaged using the Zeiss LSM880 confocal microscope. Three pictures were taken per channel such that one picture represented the left, middle, and right of the channel.

4 Results

4.1 Challenges encountered

As a result of the problems encountered, described in Chapter 2, not all tests described in Chapter 3 could be conducted within the designated time frame. This was mainly the case when determining the effects of circulation on cells. Here the clusters were not used and only HL-60 cells have been recirculated. Additionally, only one run could be conducted with the HL-60 cells as opposed to the three times that were planned, and the test times were not varied. Furthermore, a suspension containing $6 \cdot 10^5$ cells/ml was used instead of the intended $9 \cdot 10^5$ cells/ml and the flow rate was lowered to 200 µl/min.

The second experiment was not hindered by the troubleshooting process. There were however a couple other changes. The hMSCs did not yield enough cells when replating in order to achieve the desired seeding densities. As a result the chips were seeded with $2 \cdot 10^6$ cells/ml, $1 \cdot 10^6$ cells/ml, and $0.5 \cdot 10^6$ cells/ml. Due to time constraints only the chips containing the seeding densities of $2 \cdot 10^6$ cells/ml and $0.5 \cdot 10^6$ cells/ml had medium recirculated through them. Despite this the remaining chip was stained, fixed, and imaged as described in Chapter 3.2. Lastly, The first flow channel on the $0.5 \cdot 10^6$ cells/ml chip was leaking after the start of the recirculation protocol. As a result this channel was disconnected and the test was resumed with only the second and third flow channels attached to Omi. The first channel was still fixed, stained, and imaged as described.

4.2 Determining the effects of circulation

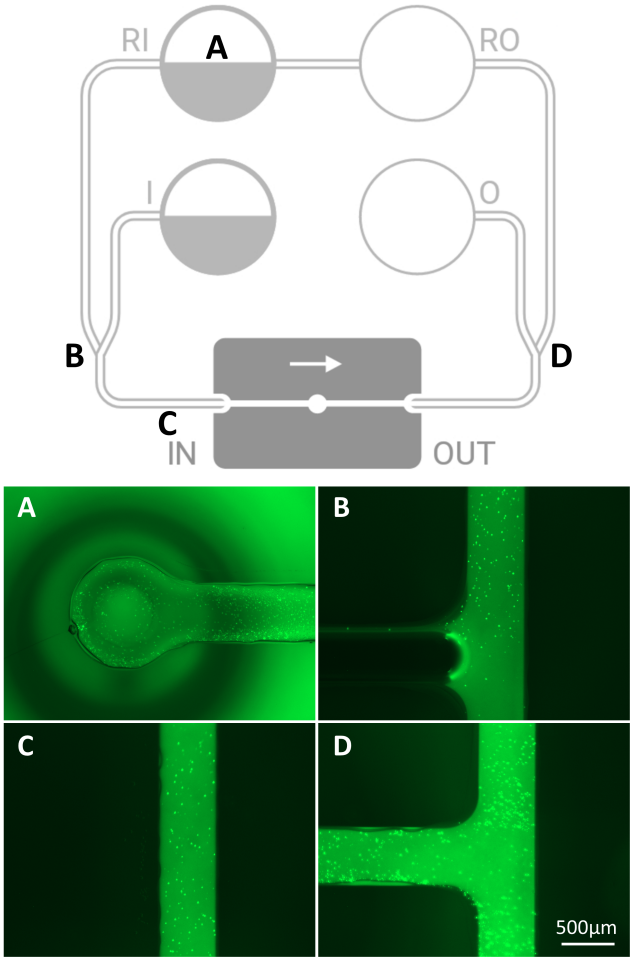

Figure 6: *The cells stained with CellTracker are visible as green dots in the pictures A through D. The figure indicates the locations on the cartridge where the pictures have been taken. The layout of the Omi cartridge differs slightly from the schematic representation shown. The pictures show were taken using the EVOS fluorescence microscope using a 4x objective.*

The effect of the circulation on the cell concentration was measured using the EVE cell counter. The amount of cells in the control group was $7.1 \cdot 10^5$ cells/ml, reservoir RO yielded $6.6 \cdot 10^5$ cells/ml, and the sampling reservoir yielded $4.5 \cdot 10^5$ cells/ml. The amount of fluids remaining in reservoirs RO and O was estimated to be 900 µl and 70 µl respectively. Offsetting the results for RO and O with the values found in Appendix B yields concentrations of $6.9 \cdot 10^5$ cells/ml and $6.3 \cdot 10^5$ cells/ml respectively. The calculations are shown in equation 2.

$$\begin{aligned} 6.6 \cdot 10^5 \cdot \frac{900\mu l}{900\mu l - 33\mu l} &= 6.9 \cdot 10^5 \\ 4.5 \cdot 10^5 \cdot \frac{70\mu l}{70\mu l - 20\mu l} &= 6.3 \cdot 10^5 \end{aligned} \tag{2}$$

Interestingly, the cell suspension in the O reservoir yielded a higher cell loss than that of the RO reservoir despite it having spent less time circulating in Omi. No significant decline in viability was detected using the EVE cell counter. The average results for the control, RO, and O suspensions were 79%, 81%, and 79%, respectively.

The pictures taken of the aggregation within the Omi cartridge are shown in Figure 6. The adaptor could not be imaged as no fluorescent signal was detected. Differences in the concentration of cells are visible within the photographed channels. The biggest changes in concentration are visible in the junctions visible in Figure 6 B and D.

4.3 Recirculating culture medium over cells

Before and after the recirculation experiment pictures were taken with the EVOS microscope. representative pictures are shown in Figure 7. A reduction in cells is visible for the 0.5 million cells/ml chip after the circulation experiment. The 2 million cells/ml chip does not show this behaviour. Of all channels only flow channel 2 of the 0.5 million cells/ml chips appeared to show this behaviour. Other channels did not present such a visible change, though flow channel 1 and the first control channel of the same chip appeared to be largely empty even before the recirculation test was conducted.

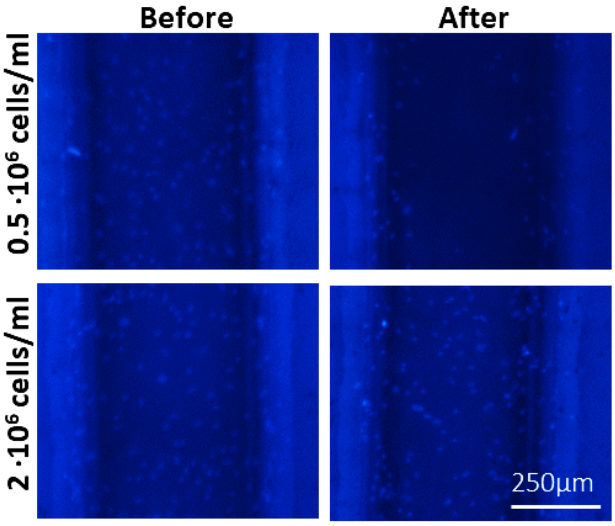

Figure 7: *Pictures of the channels before and after the recirculation experiments. The Hoechst stained nuclei of the cells are visible in blue. The top row contains pictures from flow channel 2 of the 0.5 million cells/ml seeded chip and the bottom row contains the pictures from flow channel 1 of the 2 million cells/ml chip. A reduction in cell density is visible for the 0.5 million cells/ml chip after the experiment. These pictures were taken using the EVOS microscope using a 2x objective.*

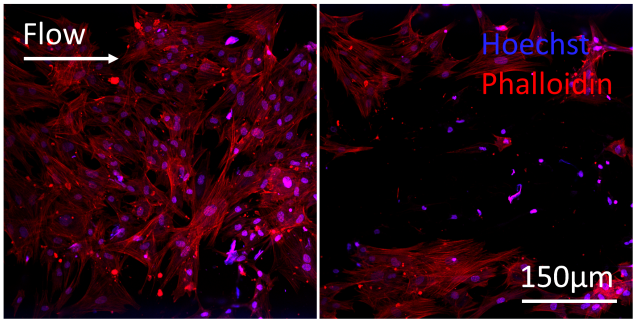

Figure 8: *The Hoechst stained nuclei of the cells are visible in blue and the Phalloidin stained actin filaments are visible in red. The direction of flow was from left to right. These pictures are of the chip seeded with a suspension containing 1 million cells/ml. The right picture contains fewer cells than the left image. These pictures were taken with the Zeiss LSM880 confocal microscope using a 10x objective.*

The confocal microscope pictures of the chips that had medium recirculated over them are shown in Figure 9. The Flow channels 3 and to some extent 2 of the 0.5 million cells/ml chip show that the cells inside the channels appear to be aligned in the direction of the flow. Such a phenomenon does not appear to be present in the 2 million cells/ml chip, where no specific directional alignment is visible.

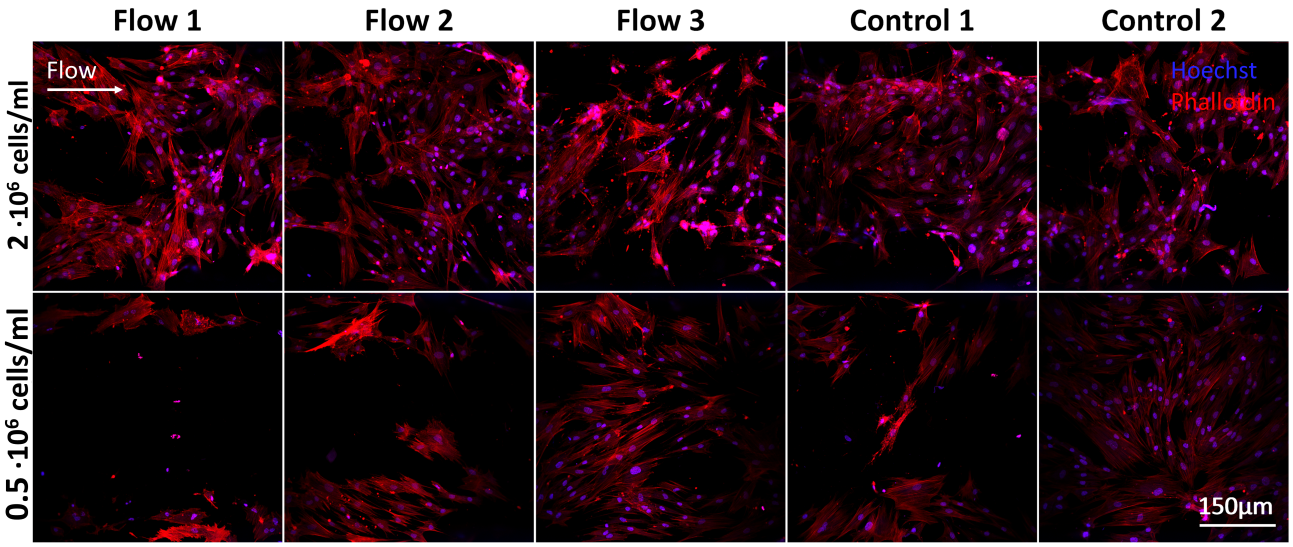

Figure 9: *The Hoechst stained nuclei of the cells are visible in blue and the Phalloidin stained actin filaments are visible in red. The direction of flow was from left to right. The top row of pictures shows the channels of the chip which was seeded with the suspension containing 2 million cells/ml. The bottom row of pictures is of the chip which was seeded with the 0.5 million cells/ml suspension. For the 0.5 million cells/ml chip the Flow 2 and Control 1 conditions show a reduction in cell density in the centre of the channel. This phenomenon is also, but not as clearly, visible in the 2 million cells/ml chip for the Flow 1 and Control 2 conditions. There is also a visible reduction in cell density in the Flow 1 channel of the 0.5 million cells/ml chip possibly as a result of the leak. These pictures were taken using the EVOS microscope using a 2x objective.*

The confocal microscope pictures of the 1 million cells/ml chip are shown in Figure 8. Since this chip did not have any medium recirculated over it the situation is comparable to the control measurements of the other two chips, shown in Figure 9. Comparing the controls of the 0.5 and 2 million cells/ml chips to the 1 million cells/ml chip shows that the latter appears to be closer in confluence to the 2 million cells/ml chip. Noteworthy is that a cell concentration loss similar to what can be seen for the 0.5 million cells/ml chip is visible.

5 Discussion

5.1 Determining the effects of circulation

The results for the circulation tests mostly look promising, with no significant decline viability after five minutes of circulation. Additionally, though some fluctuations in concentration were present, no aggregation of cells appeared to be present within Omi. There was however a decline in cell concentration present. At most the decline was 10% of the concentration of the control group. Interestingly the decline was the highest in the solution from reservoir O, which had circulated for half the time of the solution from reservoir RO. The reason for this lower value could be pipetting mistakes during the preparation of slides that were to be analysed by the EVE. Therefore, it is possible that the actual concentration of the solution of O was higher, but also that the concentration of the solution in RO was lower.

Aside form that, there are multiple places where this experiment did not end up going as intended, making the results less reliable.

The first and most important point is that the test was only conducted once, which prevents from drawing definintive conclusions.

A suspension containing $6 \cdot 10^5$ cells/ml was used for this test. This is lower than intended and introduces a risk of inaccuracy if the concentration and viability were to decline as it is nearer to the accurate range of the EVE cell counter of $1 \cdot 10^5$ cells/ml. This did however not influence the acquired results as after the test the solutions still had concentrations above this value.

The flow rate was lower than intended. As a result the shear stress was lower at 0.24 Pa. Although lower, this is still a physiologically relevant shear stress. [3, 4]

Another point of note is that the test time was short, at five minutes in total. This time was meant to be lengthened based on the results of the first test in order to establish the concentration and viability as a function of time. However, due to time constraints this could not be done. Based on the results the test time of a following

test would have been a test of 30 minutes with a sample taken at 15 minutes.

Lastly, the tumour clusters were not circulated through Omi. Since the HL-60 cells used are by themselves non-adherent, and the tumour cells are adherent, conducting this experiment with the tumour clusters might have yielded very different results both in concentration loss and aggregation.

5.2 Recirculating culture medium over cells

There were fewer complications during this test compared to the previous one. However, lower seeding concentrations were used than initially planned. This did not impact the rest of the experiment as all further steps were still conducted.

Another difference is that only two conditions were tested as opposed to the three that were planned. Despite this, the lowest and the highest seeding densities were tested and, by comparing the control measurements between chips, it appears that the $1 \cdot 10^6$ cells/ml has a confluence in between that of the other two chips and most closely resembling the situation of the $2 \cdot 10^6$ cells/ml chip. This could mean that the results of the flow test would have also looked similar to those of the $2 \cdot 10^6$ cells/ml chip, but further experimentation is needed.

An interesting phenomenon that occurred in this test is that some channels were empty in the centre, only containing cells at the channel walls. This happened on all chips but the lower seeding densities appear to have been affected most. Since this phenomenon also occurred in the control measurements it cannot solely be caused by Omi. A possible explanation is that the pipetting of PBS and Hoechst solution before the experiment was not done with enough care causing shear stresses within the chips that resulted in cells being displaced. However, there was an isolated example where channel 2 on the $0.5 \cdot 10^6$ cells/ml chip was full of cells after the Hoechst staining, but largely empty after the recirculation tests, meaning Omi might have been partially to blame for the loss of cells. Even so, this only clearly occurred once within the tests making it an isolated event and further experiments would be necessary to determine whether Omi can cause this phenomenon to occur at the used flow rate.

The $0.5 \cdot 10^6$ cells/ml chip showed some alignment of cells in the direction of the flow. The $2 \cdot 10^6$ cells/ml chip however did not appear to display this behaviour. A possible explanation for this is that the more confluent layer of cells in the $2 \cdot 10^6$ cells/ml condition was not as affected by the shear stresses exerted upon them due to the higher amount of intercellular connections with surrounding cells.

5.3 Recommendations

For future research into the suitability of Omi for recirculating cells, clusters, and medium over cell cultures on chips more tests are necessary. Firstly the intended tests should be performed again multiple times to establish an idea of the effect of time on concentration and viability when recirculating at 250 µl/min. Beside that it would be useful to repeat the tests for multiple shear stresses. This way the results could be used to estimate the expected viability and concentration decline for a given shear stress and test time. This would make setting up experiments for circulating cells with Omi easier as the maximum experiment time could be determined before any tests are conducted.

Aside from that, the channels used for recirculating medium over cells were not very representative of in vivo vessels. To improve upon this the single or double layer VFP channels, described in Chapter 1.3, could be used in future research. Furthermore, varying the shear stresses exerted on the channel walls could yield insight into what stresses the created channels could endure and how these would compare to the viability and concentration of the circulating tumour clusters over time.

When recirculating culture medium over cells the flow conditions presented a change in the directionality of the cells. The exact change could be determined using software like ImageJ and could yield insights into the relation between seeding density and change of directionality. Since most conditions in this study had two channels assigned to them, and several channels had lost cells, no analysis of this kind was performed. For future studies that aim to perform an analysis of directionality larger sample sizes should be considered.

When all aforementioned tests have been conducted the two experiments could be combined. Here the effect of circulating tumour clusters on the permeability of blood vessel on chips could be studied.

During the experiments conducted in this study a number of stainings and treatments were performed on the produced microfluidic channels. As discussed earlier it is likely that pipetting mistakes made during one of the stainings caused a loss of cells in some of the used channels. Considering that the protocols used most often used only two different solutions and low shear stresses are

desired Omi might be well suited to these kinds of tasks. An example would be the Hoechst staining conducted in this study. The RI reservoir could be filled with PBS while the I reservoir could be filled with the Hoechst in PBS solution. The flow rate could be set low so as not to induce shear stresses that are too high and the programme would consist of Perfusion, to wash with PBS, Injection, to add the staining solution, Wait, to incubate, and Perfusion to wash the channels again. Although this would not likely take long to set up it would take longer than if the protocol were to be followed by a human. The main advantage is the precise control of shear stresses exerted. Therefore, this would be an option for staining very fragile samples.

6 Conclusion

To conclude, two experiments were conducted to beta-test Omi. Although Omi shows potential according to the results that were gathered by circulating cells, not many tests were conducted due to troubleshooting taking up a significant amount of the planned experiment time. The tests that were conducted should be repeated and longer tests should be conducted in order to determine definitively whether Omi is suitable for circulating cells and cell clusters. Perfusing medium over cells on chips resulted in the alignment of low density seeded cells with flow, while a higher seeding density did not yield these results. If the described tests are repeated Omi could prove useful for research into the effects of circulating tumour cells on blood vessel on microfluidic chip models.

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A Omi protocols used

Different protocols were designed for Omi. In this study a relatively low shear stress of 0.3 Pa was chosen to be used, corresponding to 250 µl/min of flow, to give the cells used in this study an optimal chance of survival making any significant impact likely the result of Omi. The first protocol is the 'CheckFunctionality' protocol, shown in Table 1. This protocol contains every major function that Omi will need to perform during the experiments except for the sterilisation and cleaning steps which were conducted separately. The flow rate is set slightly higher than what is used for the experiments to ensure both the Omi and cartridge can handle the resulting stresses.

Table 1: *Protocol for the CheckFunctionality protocol used for validating Omi and cartridges.*

CheckFunctionality		
Function	**Time [min]**	**Flow [µl/min]**
Calibration	-	-
Loading	-	-
Perfusion	2	300
Sampling	2	300
Injection	2	300
Recirculation	2	300

The 'Maxflow' protocol is shown in Table 2. This protocol runs steps connected to every reservoir at the maximum flow rate that the Omi app allows. Omi is not built to consistently reach this rate and in most cases it will not, but this protocol does give an insight into what the maximum flow rate is. If the maximum flow rate turns out to be lower than 1000 µl/min then the device would have to be troubleshooted.

Table 2: *Protocol for the MaxFlow protocol used for validating the Omi and cartridges.*

MaxFlow		
Function	**Time [min]**	**Flow [µl/min]**
Recirculation	2	2000
Injection	2	2000
Sampling	2	2000

The 'Perfusion 2 Samples' protocol is shown in Table 3. This protocol is meant as the first short test for circulating cells. Notably this protocol does not use the Recirculation step and instead uses the Perfusion step. Since this is a short test with a low flow rate the RI reservoir should not be empty before the end of the test, making recirculation unnecessary. To avoid having Omi accidentally start recirculating due to errors the Perfusion step was used instead of the Recirculation step. For tests that last longer than 6 minutes in total the Perfusion step would be replaced with the Recirculation step.

Table 3: *Protocol for the Perfusion 2 Samples protocol used for circulating cells.*

Perfusion 2 Samples		
Function	**Time [min]**	**Flow [µl/min]**
Perfusion	2	250
Sampling	0.5	250
Perfusion	2.5	250

The 'Recirculate' protocol is shown in Table 4. This protocol first fills the tubing with medium so that the cells will not be exposed to air for too long. After that medium is recirculated over the cells on chips for a duration of one hour.

Table 4: *Protocol for the Recirculate protocol used in the second experiment.*

Recirculate		
Function	**Time [min]**	**Flow [µl/min]**
Loading	-	-
Recirculation	60	250

The 'PBS run' protocol is shown in Table 5. This protocol was created to quickly clean Omi with PBS in between tests running the 'Recirculate' protocol. This is because the cleaning step could take 45 minutes to complete during testing, which would make running multiple tests on the same day difficult. This shorter programme was created as an alternative to make running multiple tests take up less time.

Table 5: *Protocol for the PBS run protocol used in the second experiment.*

PBS run		
Function	**Time [min]**	**Flow [µl/min]**
Recirculation	5	1000

B Determining Omi cartridge volume

The loading step fills Omi with medium from the RI reservoir until IN, as shown in Figure 10. As a result, between OUT and the RO and O reservoirs liquid used in previous steps still remains. When perfusing larger amounts of fluid, or when small changes in concentration are acceptable, this does not pose a problem. However, when determining the effects of circulation on cells in suspension the liquid still left in the cartridge will lower the concentration of cells ending up in the reservoirs. Because of this, the volume of remaining liquid must be determined in order to properly determine whether the effect of circulation on the concentration of cells ending up in the reservoirs.

B.1 Methods

Omi was set to run a perfusion programme followed by a sampling programme, both of which at 100 µl/min lasting one hour. The time it took for the distilled water to travel between OUT and RO was measured using a stopwatch. After that the perfusion step was skipped and the time between the Omi screen displaying the sampling had started and the water entering reservoir O was measured using a stopwatch. This experiment was repeated three times.

B.2 Results

The results are that it takes 33µl for fluids to fill the cartridge between OUT and RO. The amount of time it took for fluid to be visible in reservoir O after the sampling started was on average 12 seconds equating to 20 µl.

B.3 Discussion

One point of note is that determining by eye whether fluid has entered a reservoir proved difficult as the amount of fluid entering the reservoir was very small. To more accurately determine the volume of the channels within the Omi cartridge a more precise way to determine whether fluid has entered the cartridge is necessary.

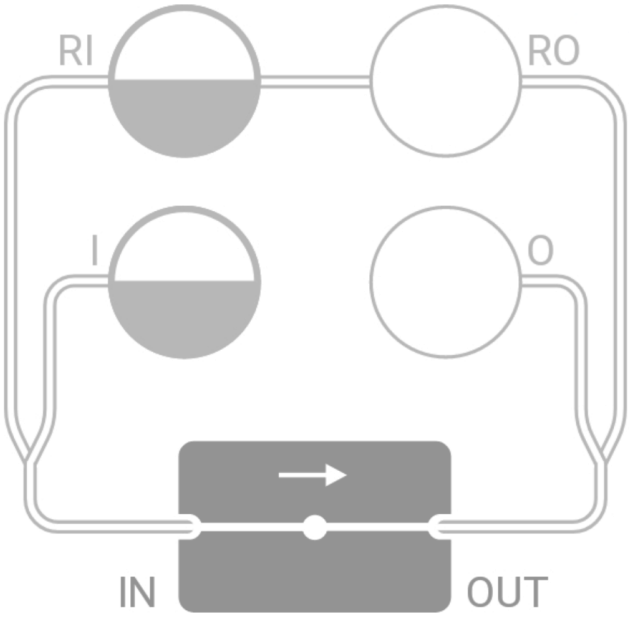

Figure 10: *A schematic representation of the Omi cartridge. Here fluids are pumped counterclockwise. Perfusion pumps fluids from RI to RO. Recirculation works the same way, but will pump fluid from RO back to RI when RI is empty. Sampling will pump fluid from RI to O, and injection will pump fluid from I to RO. IN and OUT represent the places where the perfused fluid enters and leaves the attached chip respectively.*