Design and Setup of a Sample Collection Method for a Fully Automated Microfluidic Cell Culturing System

Gunnar Sigvaldi Hilmarsson

Faculty of Industrial,- Mechanical Engineering and Computer Science
University of Iceland

Design and Setup of a Sample Collection Method for a Fully Automated Microfluidic Cell Culturing System

Gunnar Sigvaldi Hilmarsson

30 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in Mechanical Engineering

Advisor(s)
Sigurður Brynjólfsson, Ph.D
Ronan M.T. Fleming, Ph.D

Faculty Representative
Fjóla Jónsdóttir, Ph.D

Faculty of Industrial, Mechanical Engineering and Computer Science
School of Engineering and Natural Sciences
University of Iceland
Reykjavik, January 2012

Design and Setup of a Sample Collection Method for a Fully Automated Microfluidic Cell Culturing System
30 ECTS thesis submitted in partial fulfillment of a *Magister Scientiarum* degree in Mechanical Engineering

Faculty of Industrial, - Mechanical Engineering and Computer Science
School of Engineering and Natural Sciences
University of Iceland
VRII, Hjardarhagi 2-6
107, Reykjavik
Iceland

Telephone: +354 525 4000

Bibliographic information:
Gunnar Sigvaldi Hilmarsson, 2012, Design and Setup of a Sample Collection Method for a Fully Automated Microfluidic Cell Culturing System, Master's thesis, Faculty of Industrial, - Mechanical Engineering and Computer Science, University of Iceland, pp. 43.

Reykjavik, Iceland, February 2012

Abstract

Automated cell culturing experimental systems are crucial to further development of medicine and the understanding of cell inner workings. Their potential for throughput and high level of standardization and reproducibility enables researchers to analyze cell behavior with much greater efficiency than conventional means. The fully automated microfluidic cell culture system designed and built by the Quake Labs at the Department of Bioengineering, Stanford has been setup at the Center for System Biology at University of Iceland.

This project is concerned with adding to the system ability to monitor cell behavior by designing a sample gathering system that collects spent cell culture media and delivers them into a standardized 96-well sample plate. The samples will then be analyzed using mass spectrometry to gather data for cellular metabolic modeling.

The design uses the previously present system controls to extract samples from cell culturing chambers within the chip and deliver them to point of extraction. Pressurized air forces the samples into predetermined wells. A fraction collector guides each sample into a corresponding well. When the samples are inserted into a well, a pneumatic jack pierces through the rubber mat that seals each well off from the surroundings.

The process is fully automated and the sample extraction scheduling is incorporated into the feeding schedule.

Útdráttur

Sjálfvirk frumuræktunarkerfi eru mikilvæg fyrir þróun í læknisfræði og auðvelda skilning á innri gerð frumna. Með notkun þeirra er unnt að auka afköst til mikilla muna, bæta stöðlun og nákvæmni við greiningar á þróun frumna frá því sem áður var.
Sjálfvirka “microfluidic” frumuræktunarkerfið, sem hannað var af Quake tilraunastofunni hjá lífverkfræðideild Stanford Háskóla, hefur verið sett upp hjá kerfislíffræðisetri Háskóla Íslands.

Markmið verkefnisins er að bæta núverandi notagildi kerfisins til að unnt sé að fylgjast sjónrænt með hegðan frumnanna. Hannað var kerfi til sýnatöku sem safnar saman útskilnaði frumna og skilar honum í staðlaðan 96-brunna tilraunabakka. Sýnin eru síðan rannsökuð með massagreini til að afla upplýsinga sem notaðar eru í frumulíkanasmíði.

Hönnunin notast við stýringar frumuræktunarkerfisins til að sækja sýni úr frumuræktunarklefum og staðsetja þau í kerfinu þannig að þau séu tilbúin fyrir flutning. Þrýstioft er notað til að færa sýnin í fyrirfram ákveðinn brunn. Sjálfvirkur sýnasafnari sér um að beina sýninu ofan tiltekinn brunn. Áður en að sýninu er þrýst í brunninn sér loftknúinn tjakkur um að ýta sýnaslöngunni í gegn um gúmímottu sem einangrar sýnið frá umhverfinu.

Ferlið er sjálfvirkt og tímasetningar sýnatöku eru samhæfðar við fóðrun á frumunum.

Table of Contents

List of Figures

List of Tables

Abbreviations

CHIP: Microfluidic cell culturing chip designed and built at Stanford's Department of Bioengineering

CSB: Center for Systems Biology at University of Iceland

MUX: On-chip multiplexer

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDMS: Polydimethylsiloxane (polymeric organosilicon compounds)

SU8: Commonly used epoxy-based negative photoresist polymer.

Acknowledgements

First of all, I would like to thank the Center for System Biology, University of Iceland for the support and accommodation for this project. The level of professionalism practiced there will influence and inspire me for times to come. Particularly I would like to thank Skarpi for his tireless attitude towards experimental work and keeping an open mind for any possible solutions to tasks that were at hand.

I would like to thank Rafael Gómes-Sjöberg for his input during the design process which came as a validation of the projects progress and a source of confidence in the work that had been done.

I would like to express gratitude to my supervisors, Sigurður Brynjólfsson and Ronan M.T. Fleming for their guidance through the process.

At last, I want to thank my family for all their support and especially my father Hilmar Sigvaldason, who with his continued support and design related discussions, was a great inspiration.

Introduction

With the increasing demand for high throughput cell culture experiments and the inventive usage of polymeric organosilicon compounds such as Polydimethylsiloxane (PDMS), the field of microfluidics has taken off quickly. The ability to create thin layers of PDMS with microstructures and an ingenious method of creating miniature control valves has made it possible to produce cell culture labs contained within a matchbox sized chip.

The layers are created by printing a computer aided designs onto a mold using photolithographic techniques and then allowing PDMS to form within the mold. This creates microscopic lines within the layer where the flow of liquid can be controlled. These layers can then be molded together in such a way that a cell culturing experiment can be executed on a microscopic scale [1].

In the very same way as electronic microchips are created, the method of creating a complex network of binary valves and flow lines allows for automated cell culturing to be performed. The microscopic scale of the cell culturing systems enables researchers to run high throughput experiments with very low amount of culture reagents. In addition to these benefits, the system benefits from being automated and thus experiments are reproducible and less error prone than manually run experiments [2].

The main objective of the system designers was to be able to photograph each chamber within the system periodically and thus gain visual information about cell behavior and growth. The cells can be stained with coloring agents for visual conformation that certain chemical reactions have occurred and a variety of filters can be used to analyze cell inner workings as well as the cells outer layers [3].

The objective of this project is to build upon the functionality already present within the system by adding a mechanism to retrieve fluid containing cell excretion from within culturing chambers and extract them into sample wells. These samples will then be analyzed using mass spectrometry to determine the chemical compounds of cell excretion and their density. This enhances researcher's ability to analyze which chemical reactions that occur within the cell.

The designing and building of the sample gathering system was performed between May and August of 2011 at the University Iceland's Center for System Biology.

Objective

The projects main objective is to add an automated method of gathering samples from a microfluidic cell culturing chip and preparing them for mass spectrometry analysis. In order to accomplish the objective, it must first be clearly defined and its requirements presented in a measurable manner.

1.1 Requirement analysis

The requirement analysis are split up in two categories, inner and outer requirements. The first category contains requirements needed in order to create a solution to the problem. Secondly, outer requirements handle the needs of the user or researcher and are a list of tasks that a feasible design accomplishes.

1.1.1 Inner Requirements

• In order to perform a sample extraction from the chamber, the automated sample gathering process needs to use the built in controls of the microfluidic chip.

• Since the chip is controlled via MATLAB® scripts, the process must be able to trigger MATLAB® functions and receive confirmation that the requested tasks have been completed.

• The mechanism needed must be available for purchase, rental or borrowing within the timeframe May to August of 2011.

1.1.2 Outer Requirements

• As researchers lay down a plan for an experiment, the option must be available to them to add a sample gathering process at a set point in time. As MATLAB® scripts are used to create these schedules, the sample gathering process must be triggered from these scripts as well.

• The process then needs to extract samples from a number of chambers previously decided by the researchers.

• As the chip is mounted on a motorized stage and scheduled for regular photographing, it cannot be moved. This entails that samples need to be delivered to an extraction point on the chip and then transported to the 96-well tray.

• The samples must be placed in previously determined wells, as planned in the researchers schedule.

• As cell cultivation experiments can last over a number of days, preservation of the sample is important. It needs to be kept as cool as possible and with minimum contact with atmosphere as possible.

1.2 Functional Analysis

The basic function of the sample gathering method is to retrieve samples from individual chambers of the chip and deliver them into a standardized 96-well PRC plate. The samples on the plate are then treated by researchers and analyzed using mass spectrometry. The treatment of the samples after gathering is dependent on the experimental setup.

1.2.1 Design Constraints

There are a number of constraints that the sample gathering method must satisfy in order to become a feasible solution. These are often referred to as hard constrains, as the successful solution of the problem depends on them being fulfilled.

The design must be able to finish the fundamental task of retrieving fluid stored in a on-chip chambers and deliver it in to a well on a 96-well tray. One or more chambers may be selected for sampling at a time.
As cell culture experiment duration can be a number of days, it is important that the sample gathering method is fully automated. By the time researchers have prepared the sample gathering mechanism and gone over the pre experimental checklist, no further manual assistance should be needed in order to complete the experiment.

Cell culture experiments in the microfluidic chip are setup using MATLAB® software, supplied from the Stanford labs. The script allows researchers to create a number of schedules and assign chambers to schedules. This way the researcher is able to control flow of inputs into each chamber individually, or assign the same feeding schedule to a group of chambers in order to increase experimental accuracy. While creating a schedule, the researchers will have the option to add sample gathering at any time, specify from which chambers samples should be gathered from, set sample size and declare which of the 96 wells should be used to store the sample.

1.2.2 Terms of Evaluation

For the design to be considered to be valid, all the design constraints must be met. Designs that fulfill all requirements can then be evaluated by comparing effectiveness parameters. Although not crucial to the success of the experiment, these parameters enhance the experimental reliability and accuracy.

Sample purity is highly important to these experiments as a very small amount of substance in the sample may be measurable and of interest. Since the chambers are sealed off from their surroundings by the chip, the only exterior contamination may be introduced

to the sample while it is being transported into the well. Cross contamination between chambers may occur during the sample extraction process, while chambers are emptied or transported via flow lines on the chip to one of the ports. Cross contamination may be hindered by taking proper care while designing the chip control protocol used in sample collection.

During a cell culture experiment the cells within chip chambers need to be fed at regular intervals. These intervals can vary from roughly 30 minutes up to several hours. Feeding is done by either flowing or pumping inputs into the chambers with a low flow rate so the adhered cells will remain within the chamber. This task is time consuming and can leave little time between feeding schedules for sample extraction. In this respect it is important to keep the time needed for sample gathering to a minimum.

The goal of the system is to be able to perform regular cell culture experiments and test various aspects of how cells behave and especially what their inputs and outputs are. Automation makes the setup a splendid platform to perform such experiments. However it is important that the sample gathering method is stable and regular. Reproducibility is very important to researchers, so the design needs to be reliable and not prone to malfunctions.

Analyzing samples from this microfluidic chip via mass spectrometry has not publicly been published before. Thus the technology is in its infant stages and hard to determine the various kinds of experiments that will be run using this setup. It is therefore important for the sample gathering design to be as flexible as possible and to allow for changes, both in mechanical setup and in control of when and how to gather samples. Functional flexibility can save researchers time and help to make the experiments more reliable and reproducible.

A number of researchers need to be able to use the sample gathering mechanism, so it is of value that it is easy to operate. Clean instruments are crucial to cell culture experiments, so the mechanism must be as modular as possible in order to ease the sanitation process. In addition to this, every part in contact with samples must be easily cleanable and sterilized.

The Center for System Biology is funded by grants so the costs of the project should be kept low. Although this term may apply to every laboratory or company, it should be noted that gathering liquid samples of cell excrements from the microfluidic chip had not been tested before. The project might have been unfeasible and therefore hard to supply funding before any proof of concept had been seen.

It is quite frequent that cell culture experiments last several days and thus it is important that the samples gathered during the experiment stay preserved while sitting in the well. The sample should be kept as cold as possible to minimize evaporation and contact with external environment should be kept to a minimum.

2 Theory

In the experimental setup for the microfluidic cell culture system, the pathways fluid flows through are very small and during all circumstances the flow is strictly laminar. The following chapter goes over some fundamental laws of physics that are very important to have in mind when designing solutions on the microscopic scale. When the flow within a microfluidic chip is compared with that within pipes of the scale used by in common devices, vast differences can be observed in its nature.

2.1 Reynolds Numbers

A Reynolds number is a dimensionless entity that measures the ratio of inertial forces to viscous forces. It is derived from the conditions in which flow occurs and is a deciding variable in determining what types of flow are possible within the system.

$$Re = \frac{\rho v L}{\mu}$$

where:

- L, is a characteristic linear dimension $[m]$
- ρ, is the density of the fluid $\left[\frac{kg}{m3}\right]$
- v, is the kinematic viscosity $\left[\frac{m^2}{s}\right]$
- μ, is the dynamic viscosity of the fluid $\left[\frac{kg}{m*s}\right]$.

For small values of *Re* the flow is laminar as can be seen in *Figure 11*. This is characterized by fluid flowing in parallel layers, with no disruption between the layers. In laminar flow the motion of the particles of fluid is orderly with all particles moving in straight lines parallel to the pipe walls [4].

2.2 Hagen–Poiseuille

By assuming that the liquid is not compressible, the speed of laminar flow can be derived from the Navier–Stokes equations. These are the Hagen–Poiseuille equation where the pressure drop in a fluid flowing through a long cylindrical pipe is correlated with the volume flow through it:

$$\Delta P = \frac{128 \mu L Q}{\pi d^4}$$

where:

- ΔP, is the pressure difference $[Pa]$
- Q, is the volumatic flow through the tube $\left[\frac{m^3}{s}\right]$
- d, is the tubes diameter $[m]$.

By applying a boundary condition eliminating slip at the tube walls, the following equation can be derived to describe velocity (V) of flow as a function of distance from center of tube:

$$V = -\frac{1}{4\mu}\frac{\Delta P}{\Delta x} * (R^2 - r^2)$$

where:

- $\frac{\Delta P}{\Delta x}$, is the pressure difference over a distance Δx along the tube
- R, is the tubes radius $[m]$
- r, is the distance from the center of the tube $[m]$.

The maximum velocity in the tube is therefore at the center:

$$V_{max} = -\frac{1}{4\mu}\frac{\Delta P}{\Delta x}R^2$$

When the velocity v is integrated over the tubes diameter and divided by total area of the cross section, the average flow is found to be the half of the maximum:

$$V_{Avg} = \frac{1}{2}V_{max}$$

Integration is used to add up contributions from each lamina, thereby getting the total volume flow:

$$\Phi = \frac{\pi}{2\mu}\frac{|\Delta \mathrm{P}|}{\Delta x}\int_0^R (rR^2 - r^3)dr = \frac{|\Delta \mathrm{P}|\pi \mathrm{R}^4}{8\mu\Delta x}$$

2.3 Bernoulli's principle

For incompressible liquid the Bernoulli's principle states that $\frac{V^2}{2} + gz + \frac{p}{\rho}$ is constant, where g is the gravity constant, p is the pressure within the system, z is the elevation and ρ

is the fluid density. In the microfluidic chip the channels lie horizontally and therefore it can be concluded that in the current system the following equation is valid:

$$\frac{V^2}{2} + \frac{p}{\rho} = Constant$$

2.4 Surface tension

Surface tension of water is caused by the molecular dipole of the water molecule. Even though the total charge on a molecule is zero, the nature of chemical bonds is such that the positive and negative charges do not completely overlap in most molecules (Figure 1). Such molecules are said to be polar because they possess a permanent dipole moment.

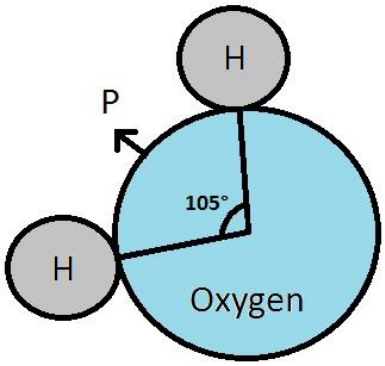

Figure 1. A schematic diagram of the dipole produced by the asymmetric structure of the water molecule

This dipole moment is important factor to waters high specific heat, as well as providing a method of interacting with each molecule, used in microwave ovens.

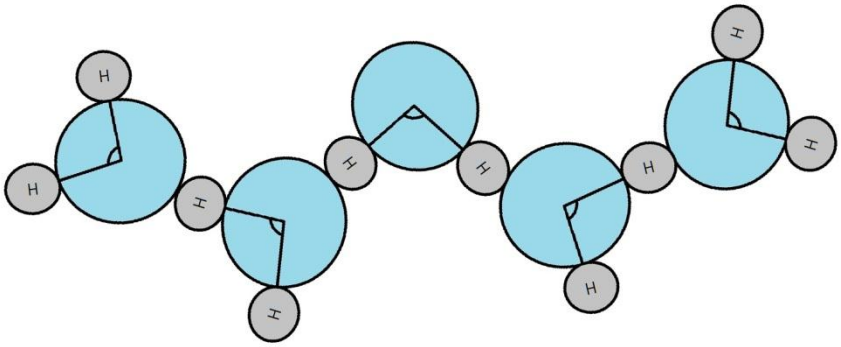

Figure 2. A chain of water molecules attach themselves together via their dipole.

The nature of the water molecules bonds them together in groups. A molecule inside a droplet is pulled equally by its neighboring molecules resulting in a net force of zero, while a molecule at the surface has a net force pointing inwards, into the droplet (Figure 2). It is these cohesive forces that are the driving force in waters high surface tension [5].

The surface tension is measured in Newtons per meter and therefore becomes relatively more important when dealing with systems on the microscopic level.

2.5 Slug bubbles

In a multiphase-fluid flow a Slug bubble is characterized by occupying almost the entire cross-sectional area of the tubing in which they are created. Slug bubbles (often referred to as Taylor bubbles [6, 7]) have been a popular topic for experimentation due to the inherent unique change in flow behavior they cause within pipes [8].

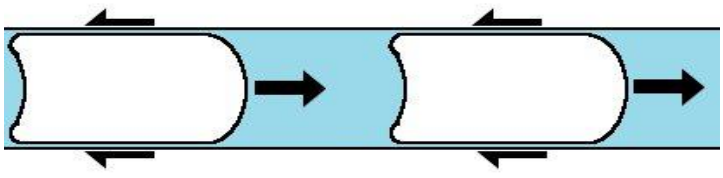

Figure 3. A slug bubble travels through a tube by allowing a thin film of liquid to pass by its sides. The bubble transforms the traditionally laminar flow by flattening out the velocity gradient over the tubes diagonal.

These bubbles travel through tubing by allowing liquid to pass between them and the tube in a thin layer (Figure 3). The relative width of this exchanging layer and the traveling speed of slug bubbles are influenced by the force driving the bubble, liquid viscosity, tube diameter and bubble length [9, 10].

3 The Chip

As the fields of system biology and stem cell research continue to grow the demand of automated cell culture technology becomes larger by the day. The Department of Bioengineering Quake group at Stanford University have for several years worked on further developing methods of cell cultivation. The researchers at Stanford used Multilayer Soft Lithography to create a fully automatic cell culture system, based on a microfluidic chip (Figure 4.).

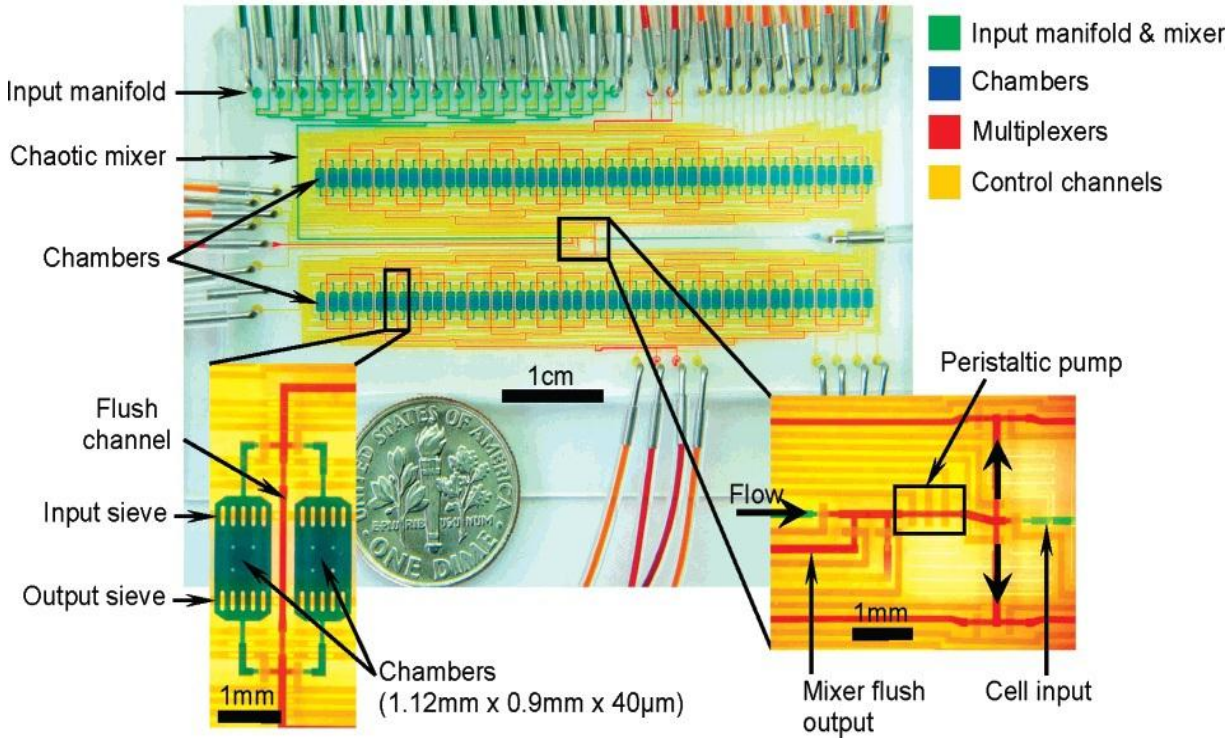

Figure 4. The microfluidic chip is shown next to a one dime coin. The chips flow lines have been colored red, the culture chambers blue and the control lines have a yellow color. To the left and right are enhanced images of cell culture chambers (left) and the on-chip peristaltic pump (right) [3].

The chip houses 96 isolated culture chambers designed to house cells and maintain their viability. The PDMS permeability to air allows for the cells to be supplied with a carbon dioxide enhanced air so that a viable condition for the cells to grow in can be maintained. Fluid can be guided from 16 different inputs to be mixed and delivered into any chosen chamber.

3.1 Chip Design

The 96 chambers of the chip have a footprint of 0.9 *mm* by 1.12 *mm* and a height of 35-40 *µm*. This allows for a typical rounded human cell (ca. 25 *µm* in diameter) to flow in to and out of the chambers unrestricted. Both entrance and exit of culture chambers have controllable valves, so flow can be precisely controlled in and out of each chamber.

There are 16 ports on the chip dedicated for supplying the chip with feeding medium, staining reagents, surface treatment reagents, etc. Any combination of these inputs can be blended together and then directed into any of the chips 96 chambers.

The inputs are connected into a binary tree manifold, with equal fluidic resistance in each branch, where they all come together before proceeding along the feedlines of the chip. Next the fluids go through a chaotic mixer (4.8 cm long) with a herringbone pattern (Figure 5), which mixes the fluids together and delivers it to the multiplexer (MUX). The chaotic mixer ensures an even distribution of the mixed inputs. It is equipped with a staggered herringbone pattern which is ideal for mixing continuous microscopic flow as it does not depend on inertia [11]. The pattern is repeated over a hundred times along the mixer.

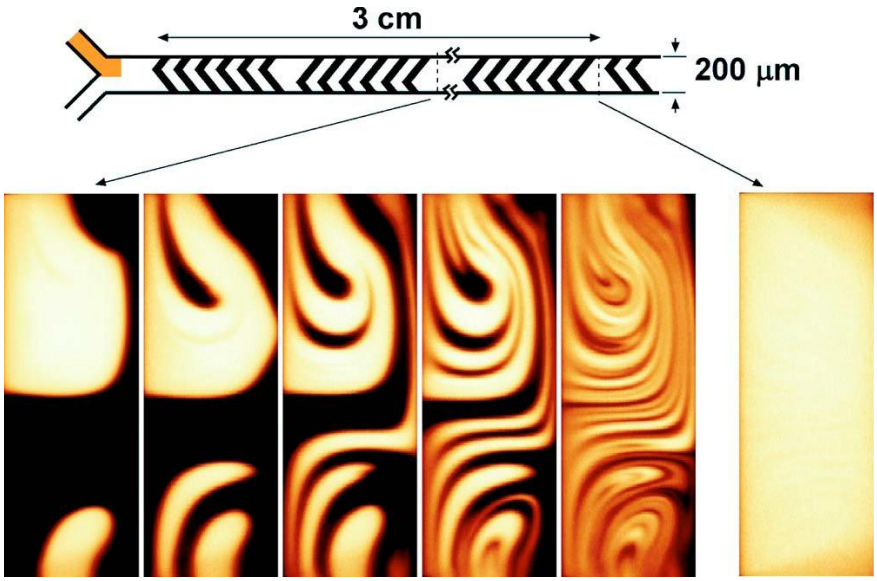

Figure 5. Schematic figure of the herringbone structure. The structure forces the flow to be folded onto itself as seen below. This enables diffusive mixing to take place [11].

This causes the liquid from each input to be dispersed over the diagonal of the flow line as each cycle cuts the distance between input flow layers in half. Thus the distance between layers is lowered exponentially. Diffusive mixing occurs when molecular diffusion can be carried from one layer to the next [12].

This procedure is required since the flow is strictly laminar and will not distribute through the width of the flow line when left to flow unhindered. The procedure ensures effective blending of all active inputs and ensures that all chambers receive identical mixtures.

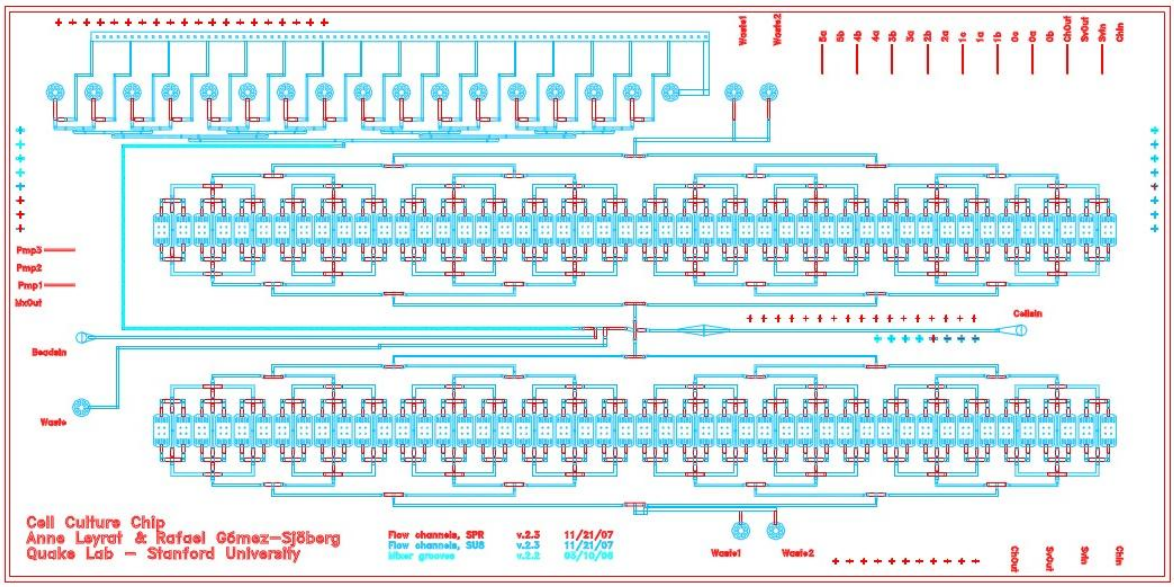

Figure 6. A CAD drawing of the chips flow lines and chambers. Both flow lines and chambers are colored blue and control valves have a red color (Image is shown with consent from owner Rafael Gómez-Sjöberg).

In addition to the 16 inputs there is a dedicated purging input that is used to clean input lines, the manifold and chaotic mixer in order to minimize cross-contamination(Figure 6).

The multiplexer distributes the inputs to selected chambers via its tree of fluidic pathways and valves controlling flow to each new branch. An identical multiplexer on the output side routes the flow from each chamber to the waste. The output multiplexer has the same valve status combination as the input multiplexer and such hinders any back-flow and cross-contamination issues.

Between each pair of chambers is a flushing line that directly connects the input and output multiplexers and is used in order to clear the multiplexer between input changes.

In order to precisely control the amount of fluid inserted into each chamber the chip utilizes an on-chip peristaltic pump.

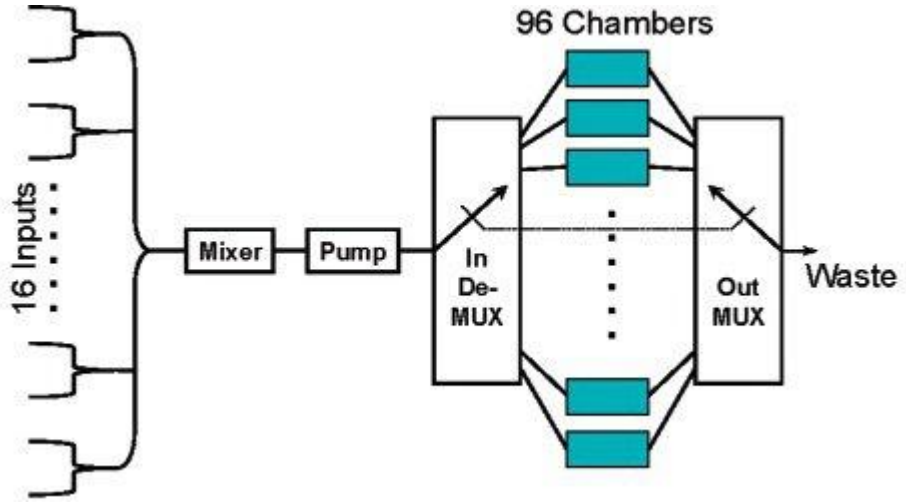

Figure 7. Schematic illustration of the on-chip flow lines. The inputs first travel through a chaotic mixer before passing through the Pump. The MUX then delivers the liquid to predetermined chambers. From there it is moved to a waste port (image adopted from [3]).

The pump is located at the root of the multiplexer (Figure 7) and is composed of three valves in series. The consecutive valves open and close in such a manner that roughly *1-3%* of the chamber volume is pumped each cycle.

The on chip valves are controlled by inflating or pressurizing control lines that lie on top of flow lines.

The chip infrastructure is setup in two layers that are placed on top of each other. The flow layer holds flow lines that reach from inputs, into chambers and back out the waste ports. The second layer, which sits on top of the flow layer, is called the control layer and houses control lines. One end of these lines is connected to the control ports of the chip, they then lead along the chip to some junction of the flow lines in the lower layer. There they cross over the flow line at a specific junction and end inside the chip. When these lines are pressurized they expand and hinder all flow through the flow lines in the flow layer at the crossover point.

3.2 Chip Manufacturing

The chip is made out of Polydimethylsiloxane (PDMS) by a process of Multilayer Soft Lithography. The PDMS layers are casted using photoresist-based photolithographic techniques. In order to create the pattern of the flow lines a mask is printed on the mold at 20,000*dpi* from an AutoCAD design file. The mold is then hard baked to obtain rounded patterns with a maximum height of *35-40 μm*. To create a layer with uniform wetting properties to the PDMS the mold is spin-coated with a layer of SU8 photoresist polymer, baked at 95°*C*, exposed to 160 $\frac{\mathrm{mJ}}{\mathrm{cm}^2}$ and rebaked at 95°*C*.

Each chip consists of two layers of PDMS, one houses the flow channels and the other that houses the control valves. The flow channels all lead from the inputs, into the chambers and out of the chip via waste exits as described in the previous chapter. However the

control layer houses lines that are dead-ended. This way lines in the control layer can be pressurized and used to close off the flow lines of the layer sitting beneath it.

The flow layer is created by pouring uncured PDMS into the mold to the thickness of *3-5 mm*. When creating the control layer however the PDMS is spin-coated onto the mold to ensure a uniform thickness of *35-40 μm*. The PDMS is then cured by baking at 80°*C*, the control layer pealed from its mold and both layers aligned together. The second round of baking binds them together before pealing both layers together from the control layer mold. The layers are bonded to a glass slide using a thin layer of PDMS and a third baking. Input and output ports are punched into the PDMS layers using a 20 gauge round hole cutter.

Input/output ports for the channels are created by punching the PDMS layers, on predefined locations, using a 20-gauge round hole cutter (Technical Innovations Inc., Brazoria, Texas). These ports permit the connection of 1/16" Tygon tubing to the chip using 23-gauge stainless steel tubes (New England Small Tube Corp., Litchfieeld, New Hampshire). A tight fit is achieved both into the Tygon tubing and into the chips ports, both control and flow ports. It is the flexibility of the PDMS that allows for the stainless tubes to be connectable despite having a larger outer diameter than the gauge of the ports. The inputs and output ports of the chip must be sealed with tape before the final baking to ensure sterility.

3.3 Lab Setup

All vales in the chip are driven by solenoid valves, which are in turn controlled by custom electronics. The electronics are a link between the solenoids and the USB port of the computer. Each solenoid valve switches one or more valves in the chip by applying roughly *240 kPa* pressure. The laboratory facilities supply an indoor connection to pressurized air, which first is set to an exact value via air pressure controls and then used to pressurize the solenoids. Attached to the solenoids is a bracket of water filled cylinders. When pressure is allowed through one of the valves on a solenoid it is lead to a cylinder where the air pressure is converted into water pressure. From the bottom of each cylinder the water is then lead to one of the control lines (see Figure 8.). This exchange of air to water pressure is needed since the PDMS is permeable to air, but not to liquid and so a fluid is needed to fill the control lines of the chip.

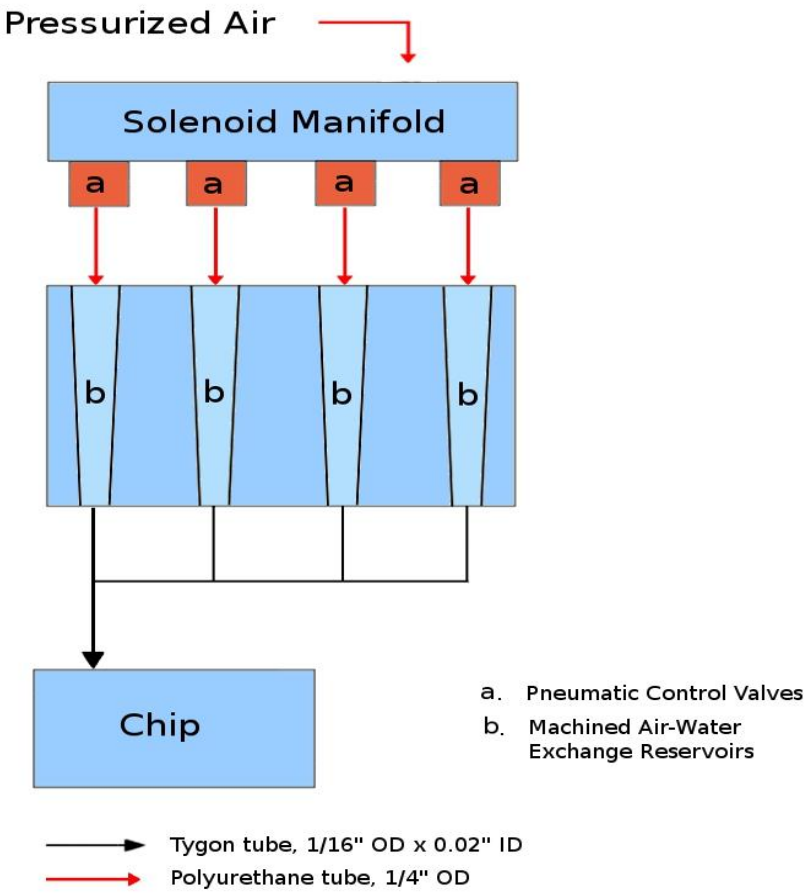

Figure 8. Because of the PDMS's permeability to air, the pressure must be a water/air pressure exchange. This is performed in machined reservoirs. The pressurized air is controlled via valves on the solenoid manifold. The air pressure is transformed into water pressure and used to inflate the on-chip control lines.

The chip is mounted on an automated microscope using a XY-motorized stage and an environmental chamber for temperature and atmosphere composition control.

The chip is controlled using custom software developed by the system designers using MATLAB®. It is also used in controlling the microscope, camera and to perform data analysis. The software includes a user interface that allows researchers to control each valve on the chip and thus perform any tasks needed for the preparation for each experiment. Experiments are run by scripts and are fully automated and unattended during the whole experiment.

3.4 Technology Benefits

The revolutionary design of the chip has several mayor benefits when compared with the common cell culturing methods. The feeding and imaging is completely automated and isolated from exterior influences. This lowers the risk of both experimental failures due to researcher's errors and contamination of cells and samples.

Cells are constantly interacting with their surrounding microenvironment and neighboring cells. These interactions allow for example the change of nutrients and cell waste, maintenance of cell homeostasis and regulation of cell behavior. Regulation occurs via different signaling mechanisms, which include growth factors, hormones and juxtacrine signals. In in vitro studies it is of interest to reproduce these in vivo conditions as well as possible.

The microfluidic cell culture technology enables the creation of cell culture conditions that are more physiological than those found in other in vitro systems (e.g. standard cell culture) when measured in terms of exchange rates of nutrients and tunable mechanical stimulation. Advantages are gained by the minimizing the system size and the ability to do real-time microscopic observations. In addition cell culture substrates can be patterned and the composition of culture medium varied over space using gradient generators.

Cells consume components and introduce new factors into the culture medium, which changes the medium over time. The automation within the cell culture chip allows for studies of the culture medium exchange rates, the conditions of fluid flow through the culture chambers and the exploration of different types of temporal gradients in the environmental conditions, which is not possible using a standard cell culture setup.

In a living organism, cells are often cooperatively regulated by numerous signals in their microenvironment [13]. Regulators such as diffusive growth factors, the extracellular matrix and juxtacrine signals from neighboring cells can be studied in vitro using a microscopic experimental setup such as micro arrays. However, because these arrays are not a cell culturing system any time dependent stimulations need to be pre applied simultaneously.

In most culture systems cell seeding density is controlled by the concentration of the cell suspension, which leads to a scarce distributions of cells across culture chambers. However cell numbers in the chip can independently adjusted in each chamber. The cells are loaded into each chamber using a sequential loading procedure. This ensures that cell numbers in each chamber can be precisely and independently adjusted around a set range.

4 Modeling

A model based on the Hagen - Poiseuille equations was constructed with two major objectives in mind. To give researchers a visual indication of which of the chips flow lines are open and to obtain an estimation on flow speeds within the chip.

It is not intended of the model to give an exact value for the flow speed, but rather to supply researchers an idea of scale.

The model assumes the flow lines are tube shaped and clear of any dirt or other obstacles hindering flow. Furthermore it is assumed that the system has reached equilibrium with constant flow speed through the chip.

4.1 Model Description

Open Office was used during the modeling process because of its ease of use and relative simplicity of the model. In addition to those benefits is the software free and can be installed onto both Microsoft and Linux based systems.

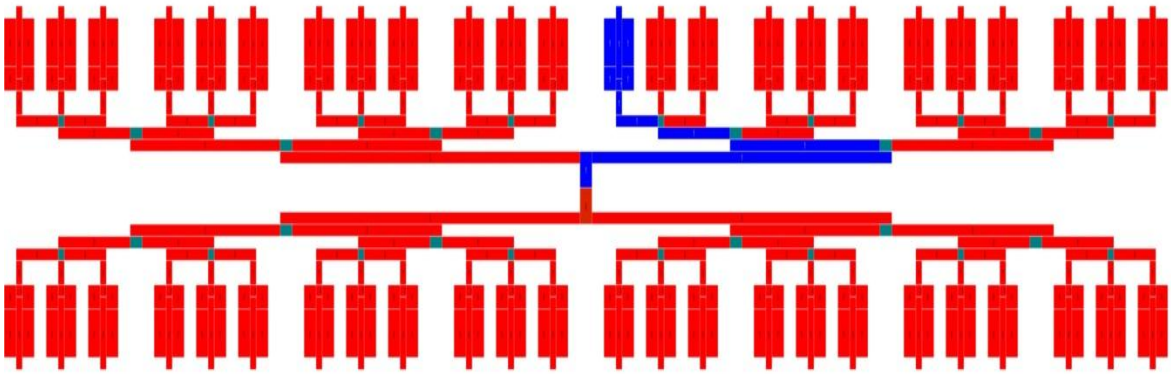

Figure 9, The graphical interface of the model allows the researcher to visualize effects of valve setup, before applying them to the chip. Both valve settings and pressure can be adjusted by adding values into skin colored fields. The graphical interface then illustrates open channels with a blue color. Red color represents closed channels.

The model simulates the chips control valves by taking in binary values that represent whether the vale is open or not. These values then influence the schematic picture of the chip, in the same way as the corresponding valves influence the flow within the chip. This enables the user to see graphically which pathways are open within the chip (Figure 9.), if the same settings are applied to the chip. These values can be altered at any point and the model will update both the graphical interface and its calculated values. This is useful since the graphical interface used to control the chip only displays which chambers are open to flow, at any given valve settings.

Q = dP*pi/(128*mu*sum(Number*L/d^4)) =>	5,25E-10	m^3/s
V = sum(Volume*Number) =>	2,89E-10	m^3
T = V/Q =>	5,50E-01	sec
Length =>	1,01E-01	m
Velocity =>	1,83E-01	m/s
Volume going through chip for set Time =>	6,30E-08	m^3
Reynolds Number =>	9,16E-03	

Figure 10. A screenshot of the values displayed within the models user interface. The model supplies the researchers with values relevant to the specific system setup. These values include volumetric flow, total volume of open channels, average time liquid spends within the chip, on-chip traveling distance, average flow velocity, etc.

In addition to supplying the graphical interface, the model provides estimations for expected flow speed for a given valve and pressure setup. Although the model relies on relatively large assumptions and approximations, the values forecasted by the model give a rough estimation of the scale of speed the researcher should expect during experiments.

4.2 Effectiveness of Modeling

During experimentation on sample extraction method the model was used to give an estimate on flow speed and to evaluate the time needed to keep on-chip valves open, in order to collect sufficient amount of sample fluid. Since the flow speed and volume is dependent on the chip valve setup, the model would serve to give an idea of how to set time parameters.

A direct measurement of flow speed via pipette proved that the model could predict flow speeds within a *20%* error margin. However this was not tested thoroughly as the model was used in order to obtain a scale of reference rather than exact numbers.

Although the systems Reynolds number is dependent on the controller setup within the chip, both modeling and experimental results indicate that it lies well within the boundary for laminar flow (see Figure 11), for all relevant system settings and pressure variances.

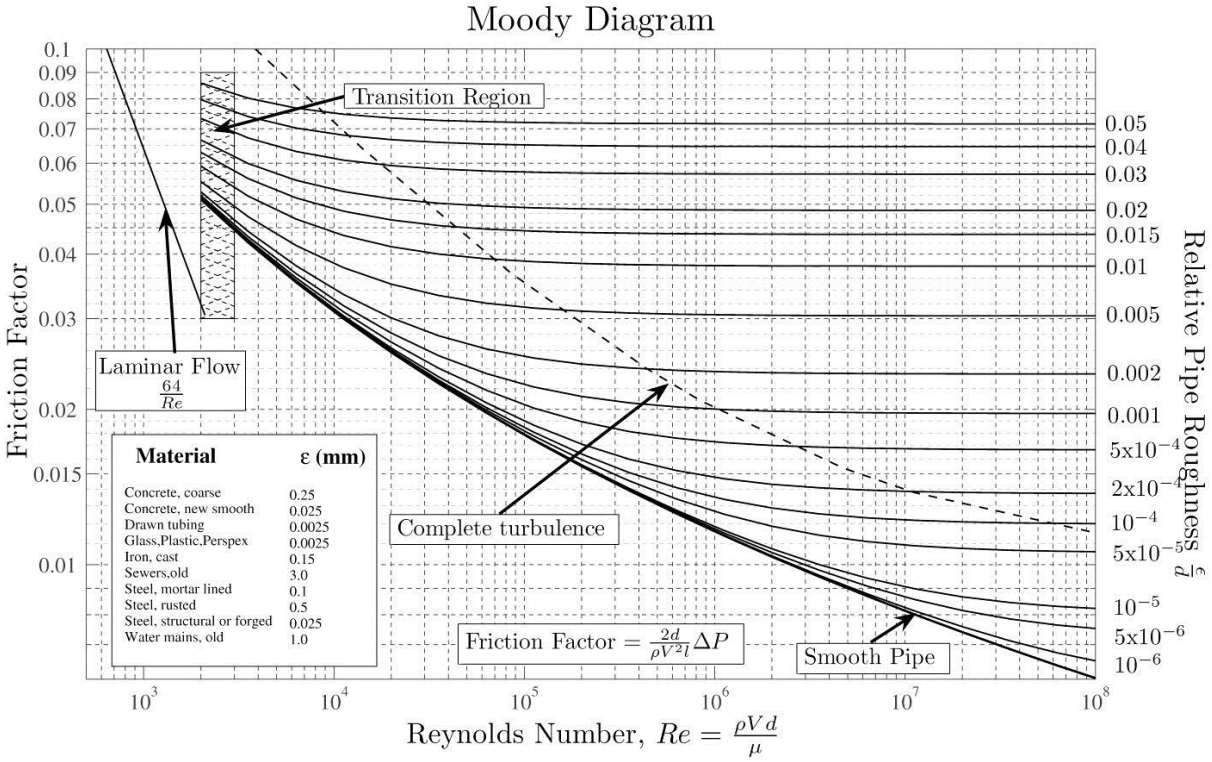

Figure 11. Moody Diagram showing various regions of laminar and turbulent flow [4].

Both PDMS and Teflon tubes have a Friction Factor lower than 0.01 and can therefore be considered to lie well within the laminar flow section of the Moody Diagram (Figure 11.). Thus the flow within the chip and in the external tubing can safely be considered as strictly laminar [14].

5 Design Process

The design process is divided into subcategories, previously described in the requirement analysis.

These categories are

- Extraction of samples from chambers
- On-Chip transportation of samples towards extraction location
- External transportation of samples from Chip towards Sampling tray
- Well selection
- Sample handling
- Programming
- Scheduling

5.1 Extraction of samples from chambers

Once cells have been seeded into their chambers and have fixed themselves to the chamber walls, fluid can be pumped slowly through the chamber without rinsing the cells out. This way the cells are fed with fluid from the inputs regularly. This same method of pumping liquid through the chamber is used during the sample gathering process in order to extract samples.

The pumping is performed by a miniaturized peristaltic pump located at the root of the multiplexer. The pump is composed of 3 control lines that cross a flow line perpendicularly consecutively (Figure 4.). The liquid within the flow line is pumped by running a control sequence that resembles a milking motion, as seen on in Figure 12.

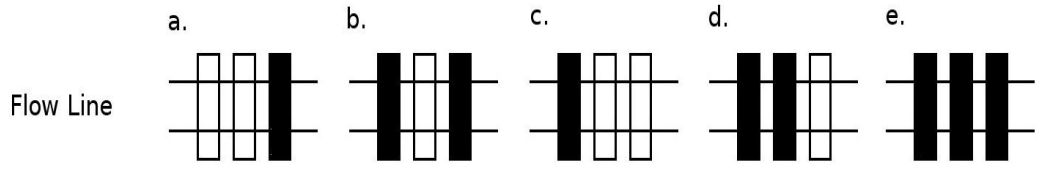

Figure 12. The 5 stages of valve settings that the pump goes through in order to perform one pumping cycle. The flow line is horizontal and is crossed over by the vertical control lines. The black coloring indicates that the corresponding control line is closed. In the first

stage (a) liquid is allowed to flow into the pump. It is then trapped (b) and pushed out again (steps c, d and e).

When the controls loop through the sequence they change stages roughly every *20-50 ms* and a whole sequence takes up to half a second. The pumping frequency is limited by the mechanical design of the controls. When a valve is closed, a manifold valve opens to allow air to flow into the pressurized cylinder and from there through Teflon tubing (1/16" OD x 0.02" ID) to the chip control layer. One pumping cycle delivers roughly *1%* of chamber volume into the chamber or *0.6 nl*.

When creating an experimental schedule, the researcher can decide how many pumping rounds should be used to extract the sample. Since samples will be flushed out using saline or a PBS (Phosphate Buffered Saline) solution, sensitive cells might take ill to too much exposure. Thus the researchers are given the option to customize each sample gathering to the specification of the experiment in question.

5.2 On-Chip transportation of samples towards extraction location

When the sample has been pumped into the flushing line between the chamber pair it needs to be transported to a chip exit or port. These are 20-gauge holes that reach from the surface of the chip down into the flow layer. As there are a number of these on the chip, many possible solutions to this problem exist. Perhaps the most obvious exit would be the one of the "Waste" ports. At each side of the chip there is a pair of waste ports named "Waste1_Up", "Waste2_Up" and "Waste1_Dn", "Waste2_Dn" (Figure 13).

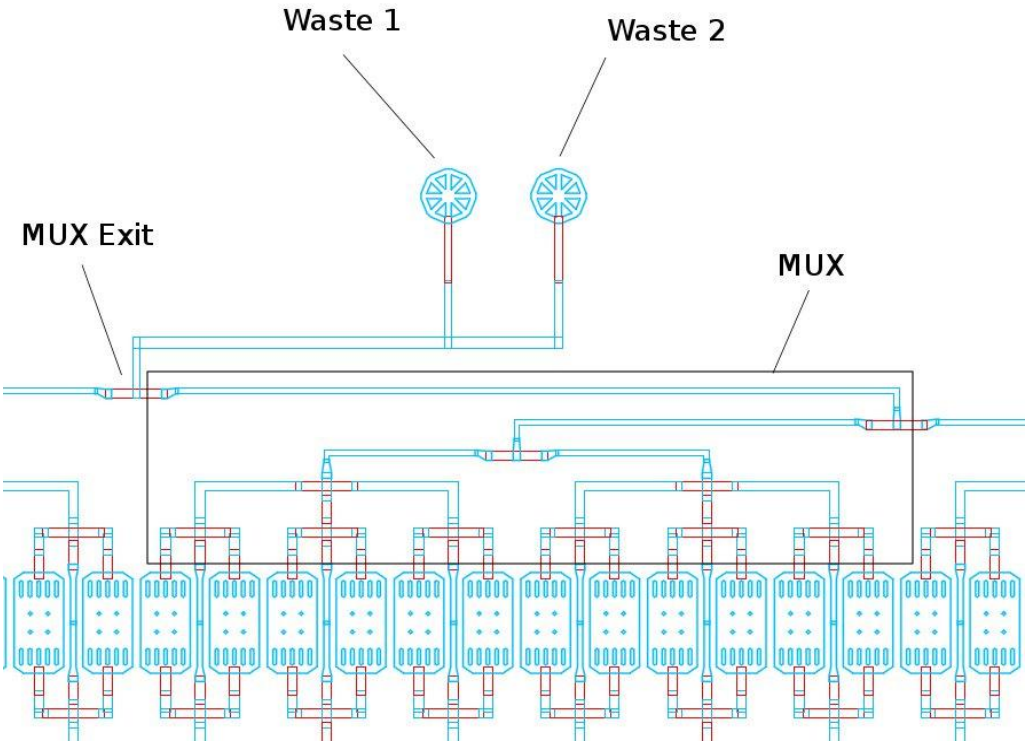

Figure 13. An enlarged view of the chips CAD illustration focusing on the waste ports. Above the chambers is the outgoing MUX which leads into a waste channel with 2 ports. To enable sample extraction, the ports named Waste 2 are pressurized and used as inputs for flushing liquid (Image is shown with consent from owner Rafael Gómez-Sjöberg).

The ports named Waste1 are connected to a bottle that collects waste during normal procedures. However the ports named Waste2 are not used in experiments and are therefore a strong candidate for a suitable port for sample collection.

The mayor issue with these ports is that there are two of them, on each side of the chip. Extracting samples from them would mean that samples exit the chip on two locations. This would greatly enhance the complexity of external transportation mechanism and increase probability of mechanical issues arising.

The ports that lie between the MUX and the pump are great candidates for sample extraction. The port named "BeadsIn" is not used during normal experimental procedure and can therefore be used for sample extraction. It was designed to be an input for microscopic beads, that are flooded into the chambers and serve to fill up the chips inner filter. This is done to trap cells that cannot fix themselves to the chamber walls and would otherwise be allowed to flow out of the chamber during feeding. However, this procedure is not frequently performed and can be programmed to utilize other nearby ports when needed.

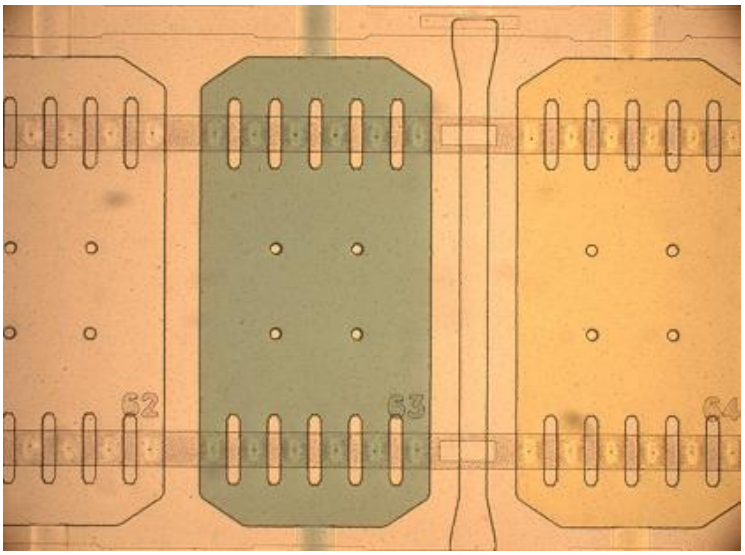

Figure 14. A picture taken through a microscope of three chambers. Chamber 63 has been filled with blue solution and chamber 64 with yellow. The filters used to trap cells can be seen as the horizontal strips at the top and bottom part of the chambers.

The port is easily sealed both by its control valves and nearby valves (MUX valve and pump). It lies at the left hand side of the chip, which makes connections easy with the current lab setup and will not cause any blocking of stage movement. In addition, cables connected to the exit do not cause risk of obscuring the camera. The port lies in the middle of the chip and thus the travel distance from chamber to the port is uniform throughout all chambers, which is crucial to ensure reproducibility during sample collection.

Experiments using a blue dye solution were used in order to evaluate the duration of flow time needed for the sample to exit the chip (Figure 14). Chambers were filled with the dyed liquid and sealed off. The chip was flushed out with clear water and the blue color pumped out of the chambers. By observing the chips flow lines it was visually determined when the last traces of blue color had left the chip. These times were documented and stored within the sample collection process, depending on number of chambers open at any time.

5.3 External transportation of samples from Chip towards Sampling tray

The chips ports are designed to fit a 23-gauge stainless steel tubing (New England Small Tubes Inc.), which in return can be threaded into any tube with inner diameter of 0.02".

During initial experiments with gathering samples from the chip, it became clear that sample purity suffered greatly from the time it exited the chip and until delivered into a well. The experiment was performed by filling the chips chambers up with a solution which contains a known chemical composition. The chemicals relative strengths were analyzed using a mass spectrometry (MS) device at the CSB. Samples were taken from 1, 2, 6, 12, 24 and 48 chambers at a time and each one transported by opening a pressurized input and allowing it to flow through the chip, the 30cm of Tygon tubing and into a well.

Between samples, the chip and Tygon tubing were rinsed and a new well was put in place for the next sample. The water used for rinsing was collected in a separate well.

The MS analysis concluded that although a trend of proportional increase of strength to number of chambers could be seen in samples with a high number of chambers per sample, the samples containing liquid from 2 or fewer chambers could not be distinguished from the flushwater.

Number of chambers	Alanyl-glutamine	Arginine	Glucose	Lysine
	(µg/ml)	(µg/ml)	(µg/ml)	(µg/ml)
1	0,93	0,44	2,19	0,06
2	0,87	0,40	4,69	0,05
6	2,47	1,03	14,11	0,22
12	4,71	1,72	29,98	0,38
24	10,29	3,65	59,52	0,80
48	16,65	6,04	88,49	1,20
Flushwater	1,05	0,48	3,89	0,07

Table 1. The results from the MS analysis. Each column represents a specific solvent that was present in the solution stored in each chamber. Rows represent each sample depending on the number of chambers sampled from. When the solution strength of each row is compared with that of the flushwater, the level of cross contamination can be estimated.

Table 1. shows the strength of a few chemicals within the samples taken and in comparison the strength of the same solvents within the flushwater. The concentration of the chemicals within the flushwater shows that a large portion of the samples is not being delivered to the well. More importantly, it accounts for cross contamination between samples.

Thus for any experiments to produce meaningful results using this method of sample extraction, samples would need to be collected from a group of chambers at a time. This can be visually evaluated from Figure 15, where the concentration of the solvents in flushwater is shown compared to the strength within the individual samples.

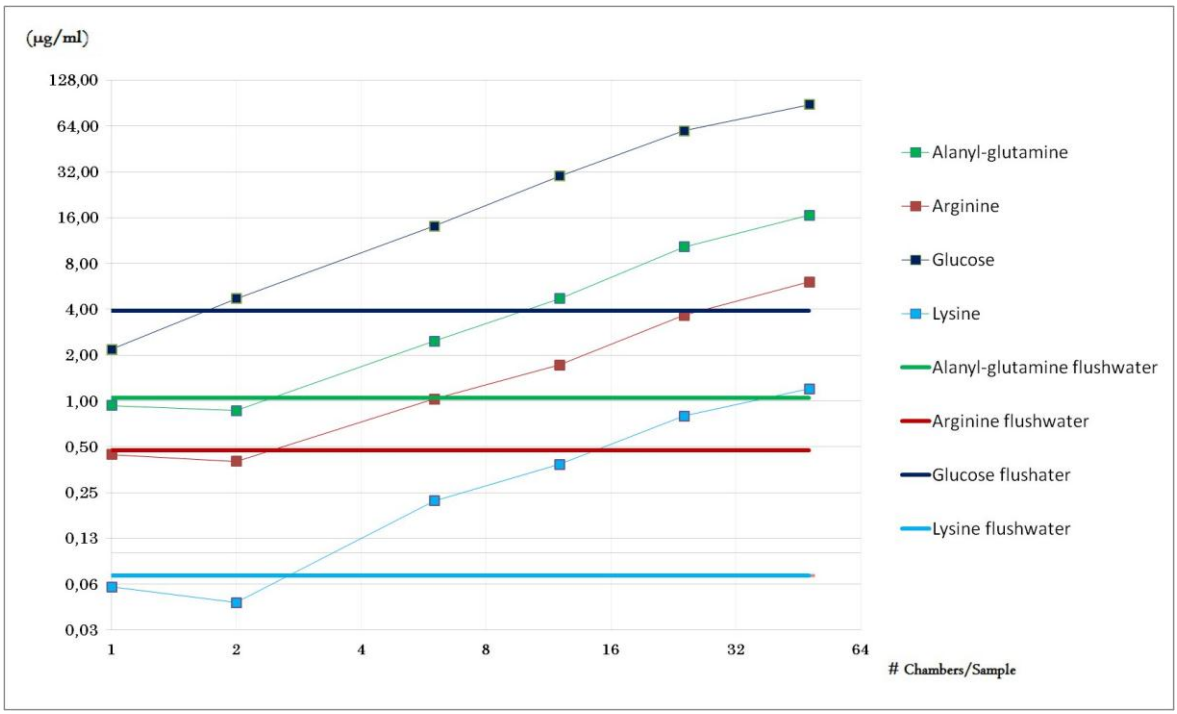

Figure 15. The concentration of measured solvents as a function of number of chambers sampled from. The horizontal lines show the concentration of the corresponding solvents within flushwater. The concentration of the solvents within the flushwater is comparable with samples with liquid from 1 or 2 chambers.

This lack of sample purity was due to the gradient of flow speed in the tube, that is characteristic of laminar flow. As described in chapter 2.2, the flow speed along the tube is a function of the distance from its center.

$$v = -\frac{1}{4\mu}\frac{\Delta P}{\Delta x} * (R^2 - r^2)$$

This describes how the liquid at the center of the tube travels at twice the average flow speed, while the liquid at the outer diameter hardly moves at all. This effect of this can be seen in Figure 16.

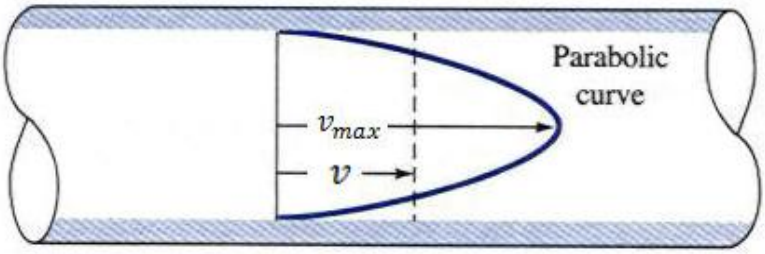

Figure 16. The parabolic velocity gradient along the diagonal of a tube. The flow velocity is greatest in the middle of the tube and dissipates towards the edges, where the flow comes to a halt (grey area) [15].

The parabolic velocity gradient during laminar flow causes the sample to be spread out along the tube during sample transportation. This makes achieving perfect sample purity literally impossible. In the experiments this effect was clearly noticed, even though they used only a 30cm long Tygon tube (the final tubing would need to be at least 1 meter long). Even after several minutes of rinsing, any parts of sample that entered the tube by its side would still linger on within the tubing.

This experiment made it clear that the sample gathering process was in dire need for another method of transferring samples from chip into the wells. In order to reduce lateral sample diffusion along the sample transferring tube, experiments were conducted using a slug bubbles. As described in chapter 2.5 a slug bubble can travel along the small tube without leaving a large amount of liquid behind, so they can be used to separate samples from other liquid flowing in the tube. Essentially such a bubble works as a piston making its way through the tube and therefore flattens out the diagonal velocity gradient. Rather than the sample being stretched out along the tube, it stays packed together, only leaving a thin layer of liquid along the side of the pipe. The layer thickness is dependent on liquid viscosity, pressure and whether or not the tube is hydrophobic. These parameters can be set so the layer thickness does not contribute to a measurable sample loss.

Although microfluidics is a relatively new field of research and thus the method of using bubbles in order to separate samples from such systems is equally unexplored [2]. However a method has been designed to insert bubbles within a microfluidic system itself by other research facilities [16, 17]. Bubbles are added before and after sample extraction using flood lines on a microfluidic chip. The valves within the microfluidic chip allow for precise control of bubble size and sample isolation (Figure 17).

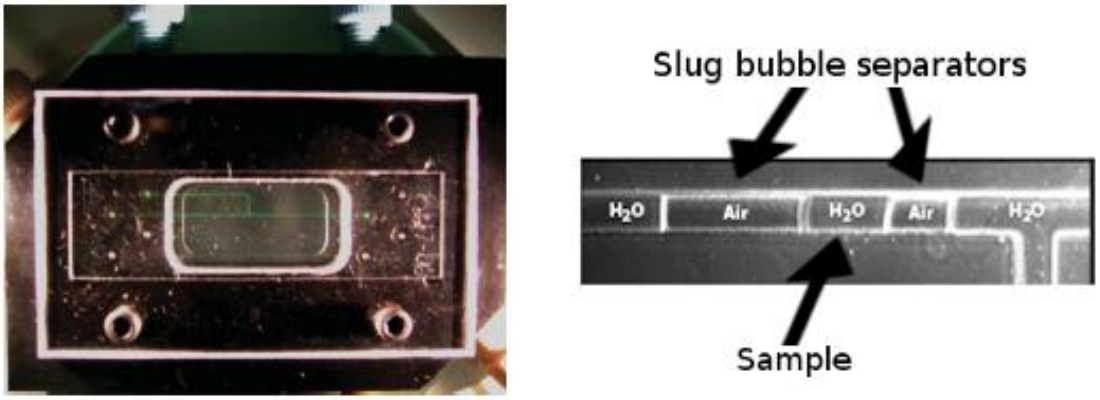

Figure 17. A microfluidic channel designed for inserting slug bubbles into the flowline (left). Samples are isolated by adding a bubble both prior and after sample extraction (right [17]).

As the cell culture microfluidic chip is very permeable to air, adding a bubble within the chip and using such fine-tuned control valve is not an option. Any leakage of air into control or flow lines can hinder regular fluid flow and risk ruining entire experiments.

An exterior solution was constructed using a three way 0.02" inner diameter stainless steel tube (New England Small Tubes Inc.) referred to as a Y-intersection. It allow for bubbles to be inserted into the sample transportation tube. Since the lateral diffusion becomes a far greater problem as the diameter of the flow line grows, it is important that the sample is isolated as soon as possible after it exits the chip. Thus the three way pipe is inserted straight into the chip, allowing the sample to travel less than *10 mm* within the tube, before it is separated by a bubble.

The bubbles are created by flowing air into the air input port of the three way tube as seen in Figure 18.

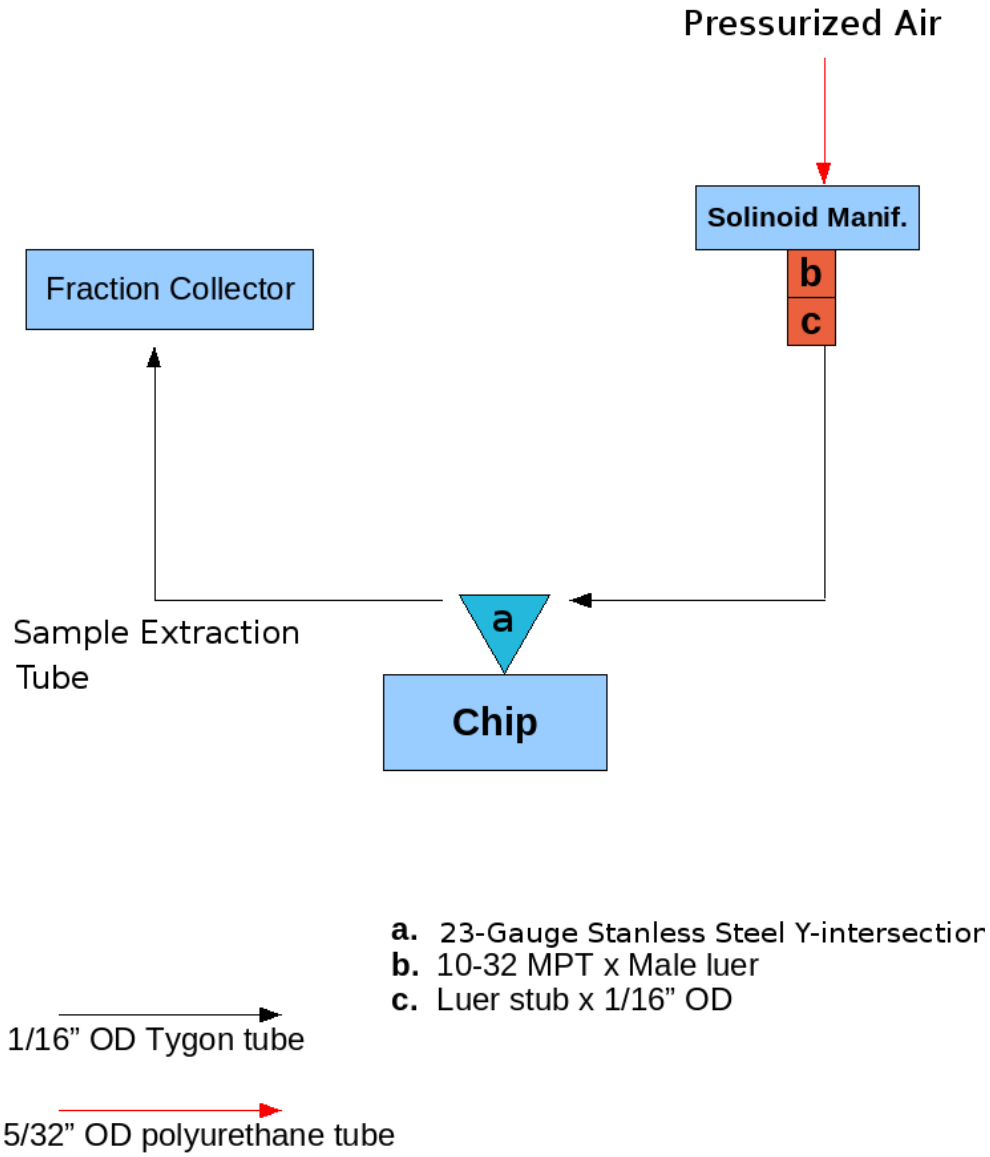

Figure 18. A schematic figure showing the mechanical setup used to transport samples from chip to sampling tray. The pressurized air is controlled via control valves in the solenoid manifold. When sample has passed from the chip and into the sample extraction tube, the air is allowed to force the sample towards the fraction collector.

Manifold valves control when a bubble is added into the sample transport tube, the same valves as are used to close on-chip control valves. The manifold is connected by roughly a meter long Teflon Tubing (1/16" OD x 0.02" ID), since it is more hydrophobic than the Tygon Tubing (1/16" OD x 0.02" ID) used for inputs and exits of the chip. The tube type selection and treatment process was performed in collaboration with colleagues at Stanford university. They have performed numerous experiments using similar microfluidic system and have expertise in the field.

The Teflon Tubing will be passivated before use by either pluronic or bovine serum albumin, to reduce absorption of hydrophobic molecules to the tube surface.

Initial experiments gave hope of good results, however there were several difficulties. Since the valve controlling bubble creation was located relatively far away from the Y-intersection, sample fluid is not guaranteed to flow towards the well, but can just as well crawl up the Teflon tubing used to insert the bubbles. This causes irregularity affects bubble creation and general speed of flow towards the sample tray. Since bubble size is hard to measure, the experimental setup used a fixed time for the air input valve to be open. The conclusion of these tests was that in order to guarantee sufficient bubble size, the valves needed to be open for a relatively long time. In most cases though this long exposure to pressurized airflow into the sample transportation tube resulted in irregular flow. In addition the irregular flow created small non-slug bubbles along the pipe, greatly increasing pressure needed to propel liquid through the tube.

Another issue concerning transportation of the sample arises when the sample separation is achieved. The matter of pushing the sample from the Y-intersection to the sample tray is very time consuming when using flow that first has to travel through the chip. While using full allowable input pressure of *15 psi* and using as many flow lines within the chip as possible to minimize friction, the transportation of a single sample takes from 40 seconds up to 2 minutes depending on fluid viscosity.

When the sample reaches the end of the transportation tube, it is important that the tube end should be placed over the correct well. In order to accomplish this, it must be clear when the sample is at the end of tube. The miniature diameter of the tube and the issue that most samples collected from the chip are bound to have no color, make detecting when the sample is at the end of the tube very difficult. A fixed time interval can be set from the sample extraction to the point of placing the tube end over the preferred well. This time period will have to be adjusted according to fluid viscosity, input pressure and number of flow lines open during the transportation. If flow becomes unstable due to bubble creation the time interval can become invalid and result in spilling and/or contaminating samples.

Because of the many issues arising when separating samples by inserting bubbles in the stream, other methods of separation were examined. Experiments were run where rather than using the air input to create bubbles, it was used to force the sample towards the sample tray. The disadvantage of this method is that some droplets of sample will still be clinging to the tubes walls, even after air has been forced through the tube for a considerable amount of time.

This method using air pressure to force the sample towards the fraction collector has a considerable amount of benefits compared to the previously described. The mayor benefit is the time required to transport the sample from the chip to the chip. Forcing the sample takes anywhere from 10 to 20 seconds, but when compared to 40 sec up to 2 minutes it takes to push the sample via chip inputs, it is clear that this is a major improvement.

In addition this method does not require detection or estimation of when the sample reaches the tube end. No excess liquid is introduced into the well since only air is used to push the sample through the tube.

Between sampling, the sample transfer tube is filled with flushwater and emptied using the air input to clear the tube of any remaining liquid. This is done to ensure that the microscopic droplets that form in the tube during sample transportation do not contaminate other samples.

5.4 Well selection

An automated method is required to place the end of the sample transportation tubing into the previously determined well on the sample tray. A standardized solution to this problem is a fraction collector. A stepper motor controlled machine with an attached dripper that has two horizontal degrees of freedom. The dripper holds the far end of the transportation tube in place, and is moved over a specified well, whenever a sample extraction is requested.

Since the CSB does not own a fraction collector, a wide variety was evaluated before settling on a used Transgenommic FCW-200 fraction collector.

Figure 19. The Transgenomic FCW-200 fraction collector [18].

The fraction collector was originally not sold as a separate unit, but as an accessory to a Wave System Model 4500, used for sequence variant analysis and nucleic acid fragments.

As such, the software controlling the fraction collector was specified for the Wave System and could not be run without its complete setup. Aside from the mechanical difficulties of running the software, the fact that it can only be run in a Windows environment was the last straw in deeming the software unfeasible.

Inquiries were sent out to Transgenomic Inc. concerning alternative means of controlling the fraction collector and were meet with a document listing a command protocol allowing the fraction collector to be controlled via serial communication.

In addition to the automated dripper the fraction collector is fitted with a peristaltic pump used for rinsing the dripper between sample taking. This function lowers risk of cross contamination between samples and keeps sample material from drying out and hindering flow through the dripper.

The tray seats are equipped with a cooling mechanism that lowers the sample temperature below 10°C [18]. This serves both to preserve the samples and keeps them from drying out over the duration of the experiment.

5.5 Sample handling

In order to hinder sample contamination and evaporation, a 96-well PCR standardized rubber mat is placed on top of the sample tray (Figure 20). The mat is designed to seal the wells, while still allowing inserts to puncture the surface and place samples into them.

Figure 20. The rubber mat seals the wells and hinders evaporation, but allows the end of the transportation tubing to penetrate into the well.

This setup requires the additional mechanical property of the fraction collector to allow for Z-axis automated movement of the dripper, as it needs to puncture the rubber mat surface. The FCW-200 fraction does not come with this possibility so an external device had to be designed and manufactured that would allow for the Z-axis movement.

The design requirements are that the mechanism does not hinder movement of the fraction collector, supplies a controllable vertical movement between the sample try and the dripper.

The selected design is a pneumatic jack controlled by the same sort of manifold valve as is used in the chip control (Figure 21). When the manifold valve is open, roughly *15 psi* pressure is allowed to pass into the jack, pushing it down. The dripper is attached at the plunger part of the jack and is moved down through the rubber mat when the control valve is open. When the valve is closed a spring within the jack pushes the piston back up to top position, removing the dripper from the well and up through the rubber mat. Once the dripper has been pulled up through the mat, the mat again works to seal off the well in question.

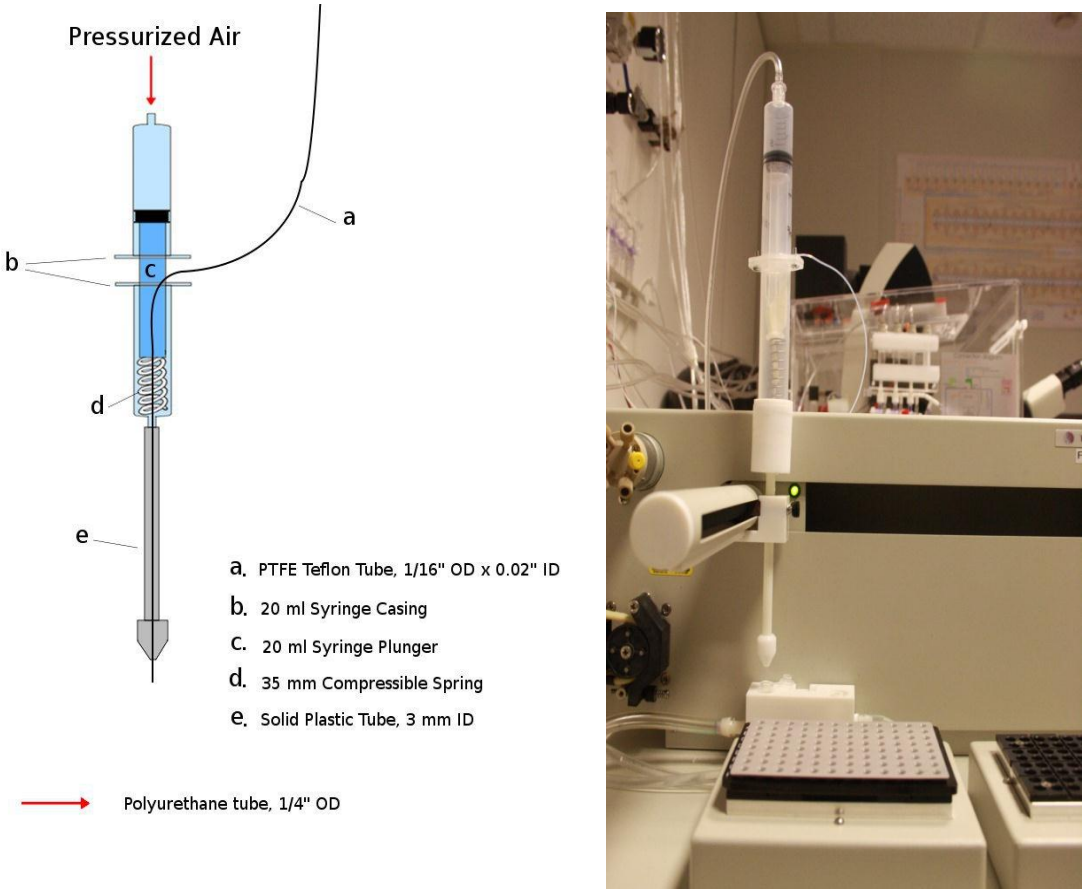

Figure 21. The pneumatic jack enables the transportation tube to penetrate the rubber mat and enter the well. To the left is a schematic picture of the jacks design. To the right, the jack has been mounted onto the fraction collector.

This design has the benefits of being very light weight and low building cost. There is only one moving part and each part is easily replaced. A silicon based lube is applied to the plunger to hinder any stiffness and thus lack of performance of the jack. All control mechanism and software are already in place and in use by the cell culturing system, so both setup cost and construction duration are minimal.

5.6 Control Programming

After installation of the system, the lab had control manifolds, solenoid valves and ports on a electrical circuit to accommodate the control mechanism needed for sample collection.

As is previously described, the cell culturing system is controlled using MATLAB®. The automation software was therefore written using the same software, which allowed for reuse software used to control the chips solenoid valves.

Contained in a document sent from Transgenomic Inc. were a set of commands recognized by the fraction collector. By sending these commands via serial port, all of the fraction collectors automated functions can be controlled.

Two functions were are used to control the sample gathering process.

The function ***fcollect(method, param1, param2, param3, param4)*** handles all these communication with the fraction collector. The function allows the researcher to schedule sample dripping into any of the 196 wells available on the two sample trays. Besides controlling the position of the dripper, the function can also call for rinsing of the dripper by controlling the fraction collectors peristaltic pump.

The ***gathersample([chambers], pumpcycles, wellnumber, firstsample)*** function handles the entire sample collection process and puts on hold the cell culturing regular schedule while it is running. It gathers samples from a set of chambers (***[chambers]***) and pumps a set amount of cycles ***pumpcycles*** from each chamber. The extracted sample is then delivered to a predetermined well ***wellnumber***. Since a small sampling duration is a key to a successful design, the sampling control code has been optimized for fast gathering. The ***firstsample*** variable is used to decide whether or not the flow lines of the chip should be flushed with water before sample is taken. Often a sequence of samples will be taken and thus only need to clean the flow lines when the first sample is taken.

5.7 Scheduling

The controls for the sample gathering process were designed in order to allow flexibility with their usage. The MATLAB® functions are stored within the chips control script and can be referenced from any script researchers run on the designated experiment computer.

During normal cell culturing experiments a scripts containing a feeding schedule takes care of inputting fresh medium into chambers and photographing them with a fixed interval. The experiment is setup in a schedule matrix where feeding schedules are designated for groups of chambers at a time.

This feeding schedule has been modified in order to accommodate for sample collection. In the same manner as researchers could preset a certain input to be pumped into a set of chambers, they can now add sample collection to the very same schedule.

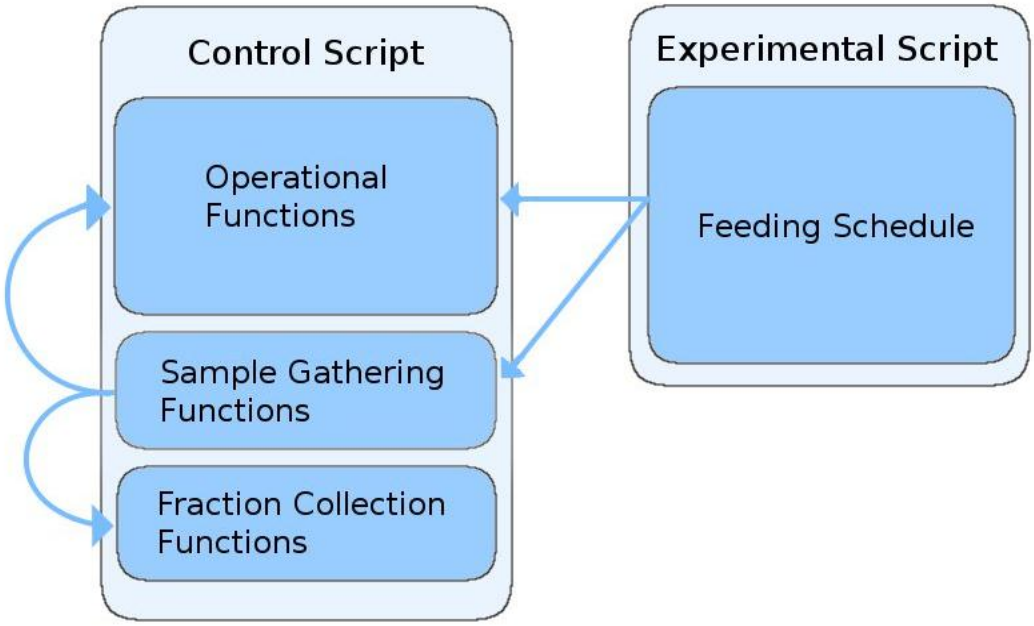

Figure 22. The experimental script uses both the chips operational functions as well as sample collection functions to perform experiments. When the sample gathering function is activated it uses both the operational functions for the chip and the function created to communicate with the fraction collector.

The experimental script calls on both control functions dedicated to controlling flow within the chip and the sample gathering function (***gathersample***). When requested, the sample gathering function then utilizes the chips control function and the fraction collector function (***fcollect***), as described in Figure 22. Both functions are available in the Appendix section of this paper.

6 Functionality

The designed system of sample collection has been built and setup at the CSB, where it is connected to the microfluidic system. Experiments have been performed in order to test functionality of the sample gathering process. The chips chambers were split into groups of six, where every other group was filled with a solution containing a blue concentrated food coloring liquid (Reyners BLUE) and the remaining groups filled with clear water. The strength of the dye solution was a tenth of the total solution volume. The solution strength was chosen to be as dark as possible, while still allowing for the solution to be inserted into chambers without visual contamination to adjacent flow lines.

A script was written to use the ***gathersample*** to gather samples from every group. The wells were selected sequentially so the expected outcome is that every other well would have a blue color, while the remaining do not. The pattern is reversed at the 8th sample caused by the symmetric nature of the chip's layout.

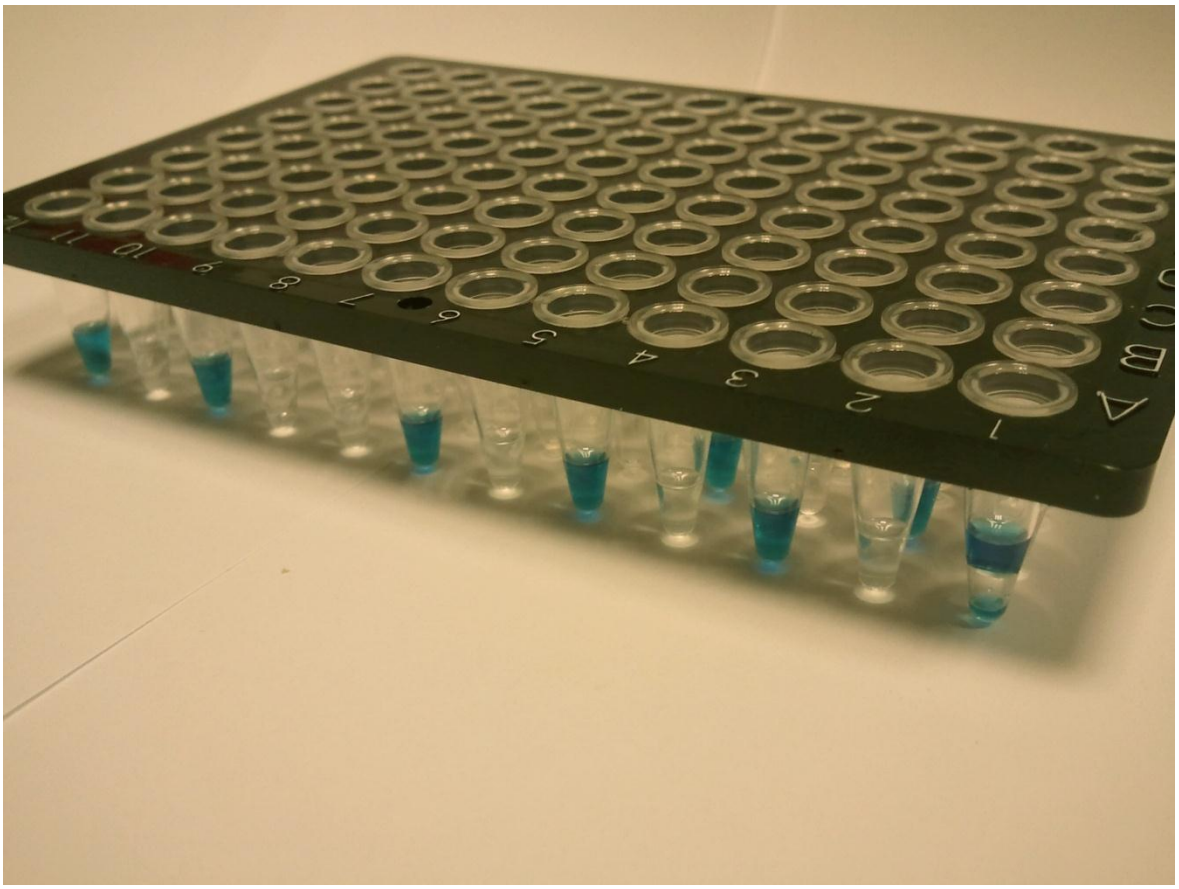

Figure 23. A picture taken of the sample tray, showing no signs of cross contamination.

Figure 23 shows the 16 samples gathered in the experiment. No contamination can be seen within any well and sample size is stable within a *5%* error margin. Although these results are only visible, some conclusions may be made concerning sample purity.

Experiments show that the blue color of the solution becomes visible when the strength of the dye is stronger than 1^{-4} part of the solution volume. Given that the sample size is roughly $29 - 32\ mg$ or $29{,}000 - 32{,}000\ nl$ and the extracted volume for each sample is $6 * 60\ nl = 360\ nl$, it is clear that less than 10% of the chamber volume of one sample can be transferred into another.

This observation places an upper boundary to cross contamination and further research is required to evaluate this more precisely. Repeating the MS experiment, now that the design is in place would determine the sample purity with greater precision.

No constraints were assigned to sample purity during requirement analysis. A sample taken from a microfluidic system will never be free of cross contamination. The cross contamination can be further minimized by increasing time used to rinse the flow lines of the chip and the sample gathering mechanism, however this will increase sampling time.

The duration of the sample gathering process is both dependent on the number of samples and how many chambers should be extracted from in each sample. Using the parameters from the experiment described above, the sampling time for all 96 chambers of the chip is 81 minutes. Thus the entire chip can be sampled from during the time interval between feeding schedules, given that feeding occurs on a two hour interval. The duration can be shortened, but at the expense of higher amount of cross contamination between samples.

As researchers start using the cell cultivation system, the sample collections time factors that define the gathering duration will be refined and adjusted to suit individual experiments.

7 Remarks on chip design

The chip design has several attributes that are beneficial to the design of a sample gathering mechanism. The distance from one end of the multiplexer to the other changes only very slightly from one chamber to the next. Thus the time used to flush a sample from chamber to the extraction point can be set, independently of chamber. Total chamber volume is small enough so that samples can be extracted from the chambers and stored entirely within the chip. This allows for a sample to be pumped towards the Waste ports and then to be pushed back through the multiplexer towards the BeadsIn port, without ever exiting the chip.

However during the design process it became clear that some modifications to the chip design would help simplify the sample gathering process. Following is a list of ideas that could be useful in further development of the chip and other developers designing a microfluidic cell culturing system that wish to incorporate sample extractions.

During experiments with sample transportation, it became clear that using air to blow samples to the well would be more feasible than water. However since the PDMS is permeable to air, adding air into the system had to be done externally. If a section of the chip could be formed out of a polymer that was not permeable to air, the air flow could be added internally and controlled using on chip valves. This would lower the number of mechanical parts and give researchers greater control over sample size.

The BeadsIn port was chosen for sample extraction over the Waste2 ports because of the difficulties that using two separate ports presents. If the lines leading to the Waste ports from each side of the chip would be connected, resulting in only a pair of Waste ports, they would be the most feasible option for sample extraction.

It is beneficial to the sample quality to use water when extracting the sample from the chip, rather than flushing samples out with medium or PBS. Flowing water through chambers is not viable option however, in a continued cell culturing experiment.

To address this, a “sample holding reservoir” can be built into the chip. Thus samples could be pumped out of the chambers and into the reservoir with minimum amount of medium required. The samples could then be extracted from the reservoir with water, without compromising the cells.

8 Closing words

The main objective of this project was to design and build an automated sample gathering mechanism for the microfluidic cell culture chip setup at the CSB. Although testing and development will continue as more and more experiments are performed, the objective has been fulfilled. After a general idea of the basic layout of the project, the functions were broken down into subcategories and tackled separately. For each category a feasible solution was found and implemented, although some were more trivial than others.

Costs of machines and material played a large role when the general outlines were laid out. The project had not formally been funded and thus options that required a large sum of financing were very limited. Generally the largest costs with setting up a sample collection process is purchase of a fraction collector.

During first experiments and the design of outer tubing, keeping cross contamination at a minimum was a major relevance. It became clear that transportation of the sample could not be achieved by the same way as input and output transportation is handled. The sample would have to be separated from flushwater before transportation to the sample tray.

In testing and code development, time factors became the most influential. As the samples had to be extracted within the time period between changing of medium within chambers. In addition to influencing flushing durations and other time factors, the method of using slug bubbles for sample separation was deemed unfeasible due to extensive transportation duration.

As the cell culturing setup is still in its infancy, the future developments of the sample extraction method are hard to predict. However, development is underway to make the sample collection more flexible and easily adjusted to various experimental setups. Methods that extract samples by flowing flushwater straight through chambers, rather than pumping the liquid through, have been developed and are currently being tested by researchers.

9 Bibliography

[1] D. C. Duffy, J. C. McDonald, O. J. Schueller, and G. M. Whitesides, "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)," *Anal Chem,* vol. 70, pp. 4974-84, 1998.

[2] R. Mariella, "Sample preparation: the weak link in microfluidics-based biodetection," *Biomed Microdevices,* vol. 10, pp. 777-84, 2008.

[3] R. Gomez-Sjoberg, A. A. Leyrat, D. M. Pirone, C. S. Chen, and S. R. Quake, "Versatile, fully automated, microfluidic cell culture system," *Analytical Chemistry,* vol. 79, pp. 8557-8563, 2007.

[4] C. T. Crowe, D. F. Elger, and J. A. Roberson, *Engineering fluid mechanics*, 8th ed. Hoboken, NJ: Wiley, 2005.

[5] M. Morra, *Water in biomaterials surface science*. Chichester ; New York: Wiley, 2001.

[6] K. Mack, J. D. Bugg, and J. S. Gabriel, "A numerical study of periodic slug flow at zero gravity and normal gravity," *Microgravity Science and Technology,* vol. 17, pp. 41-48, 2005.

[7] R. Gupta, D. F. Fletcher, and B. S. Haynes, "On the CFD modelling of Taylor flow in microchannels," *Chemical Engineering Science,* vol. 64, pp. 2941-2950, 2009.

[8] F. Guo and B. Chen, "Numerical Study on Taylor Bubble Formation in a Micro-channel T-Junction Using VOF Method," *Microgravity Sci. Technol.,* vol. 21, pp. 51-58, 2009.

[9] T. Fu, Y. Ma, D. Funfschilling, and H. Z. Li, "Bubble formation and breakup mechanism in a microfluidic flow-focusing device," *Chemical EngineeringScience,* vol. 64, pp. 2392-2400, 2009.

[10] S. Polonsky, L. Shemer, and D. Barnea, "The relation between the Taylor bubble motion and the velocity field ahead of it," *International Journal of Multiphase Flow,* vol. 25, pp. 957-975, 1999.

[11] A. D. Stroock, S. K. Dertinger, A. Ajdari, I. Mezic, H. A. Stone, and G. M. Whitesides, "Chaotic mixer for microchannels," *Science,* vol. 295, pp. 647-51, 2002.

[12] T. M. Squires and S. R. Quake, "Microfluidics: Fluid physics at the nanoliter scale," *Reviews of Modern Physics,* vol. 77, pp. 977-1026, 2005.

[13] D. T. Scadden, "The stem-cell niche as an entity of action," *Nature,* vol. 441, pp. 1075-1079, 2006.

[14] E. M. Purcell, "Life at Low Reynolds-Number," *American Journal of Physics,* vol. 45, pp. 3-11, 1977.

[15] F. M. White, *Fluid mechanics*, Fifth Edition ed. Boston: McGraw-Hill Companies, 2008.

[16] J. D. Greenwood, Y. Liu, D. E. Busacker, D. M. Cheng, and H. R. Jiang, "Collection of Gaseous and Aerosolized Samples Using Microfluidic Devices With Gas-Liquid Interfaces," *Ieee Sensors Journal,* vol. 10, pp. 952-959, 2010.

[17] K. A. Rose, "Precision Sample Control and Extraction Component," Lawrence Livermore National Laboratory (LLNL), Livermore, CA, 2007.

[18] Transgenomic, *FCW 200 WAVE Fragment Collector Manual*, 1997.

Appendix

```
%  Function:   fcollect(method,param1,param2,param3,param4)


% Function that is used to control the FCW-200 Fraction Collector.
% Method parameter is used to define action to be performed
%
% Methods:
%     moveto    Can be used to move the drop dispencer to a
specific
%               vial (param1). Specific vial can also be targetet
using
%               param2,param3 and param4 signifying column,line
and tray
%               respectfully.
%               Example:
%               fcollect(moveto,15);  --Will move to vial number
15
%               fcollect(moveto,[],3,2,2);  --Will move to column
3 and
%               line 2 in tray number 2.
%
%     rinse     Will move dispenser to rinsing position
%
%     reset     Set the fragment collector to a state consistent
with being power cycled
%
%     firstvial Moves the dispenser to the position of the first
vial (1,1,1)
%
%     pulse     Used for turning on the flow controllerï¿½s air
pulses.  Pulsing will continue until it is aborted with an <esc>
character.
%
%     home      Returns dispenser to home position
%
%     esc       Stops the machine pulsing


function [out] = fcollect(method,param1,param2,param3,param4)

global CCChs CCCdata;

% Reset all serials in buffer
% delete(instrfindall);

% Setting communication parameters
serial_port_com1 = serial('COM8');
serial_port_com1.BaudRate = 9600;
serial_port_com1.DataBits = 8;
serial_port_com1.Parity = 'none';
```

```
serial_port_com1.StopBits = 1;
serial_port_com1.FlowControl = 'none';
serial_port_com1.Terminator ='CR';

% Set timeout for all responces to 10 sec
set(serial_port_com1,'Timeout',10);

% Open the port, and establish connection
fopen(serial_port_com1);

switch method
    case 'moveto'
        if isempty(param1)
         serialstring = ['Tube ',int2str(param2),'
',int2str(param3),' ',int2str(param4)];
         fprintf(serial_port_com1,serialstring);
         pause(.1);

        else
         FCTray = (param1 -1 - mod(param1 -1,96))/96+1;
         FCLine = (param1 -1 - mod(param1 -1 - (FCTray-
1)*96,12))/12 - (FCTray-1)*8+1;
         FCColumn = param1 -1 - (FCTray - 1)*96 - (FCLine -
1)*12+1;
         serialstring = ['Tube ',int2str(FCColumn),'
',int2str(FCLine),' ',int2str(FCTray)];
         fprintf(serial_port_com1,serialstring);
         pause(.1);
         end

    case 'rinse'
        fprintf(serial_port_com1,'RINSE');
    case 'reset'
        fprintf(serial_port_com1,'RESET');
    case 'firstvial'
        fprintf(serial_port_com1,'FIRSTVIAL');
    case 'pulse'
        fprintf(serial_port_com1,'PULSE');
    case 'home'
        fprintf(serial_port_com1,'HOME');
    case 'esc'
        fprintf(serial_port_com1,char(27));
    otherwise
        warning('Please select a supported method');
end

%   out = fscanf(serial_port_com1);
fclose(serial_port_com1);
delete(serial_port_com1);


clear CCChs CCCdata;
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%


function gathersample(chamber,pumpcycles,vialnr,first_sample)

%   Gathers samples from chambers designated by vector chamber.
%   When collecting from more than one chamber at a time, chambers
need to
%   be in ascending order and paired, e.g. [1,2,5,6,9,10].
%   A number pumpcycles will be performed by the pump and samples
delivered
%   to a place on the tray, specified by vialnr.


global CCChs CCCdata;



%-----------------PARAMETERS-----------------------------------------
-------

%   Setting fixed parameters
imax = size(chamber);
xoff = 0;
yoff = 0;
zoff = 0;

%   Setting measured parameters
initialflushtime1 = 40; %Initial cleansing of the chip flow lines
from "Purge" through "Waste1"
initialflushtime2 = 30; %Secondary cleansing of the chip from
"Waste2" through "BeadsIn"

traveltime = 20; % Time used to blow air and move sample to plate
well
rinsetime = 5; % Time where nossle is over rinse sink
pumpperiod = 0.04; %speed of peristaltic pump

total_flush_time = 90; % time in seconds. delivers ca 30 µL
sample. can be reduced but must exceed "flushtime1" x 3
flushtime1 = 20; %Flushes past the second junction, counting from
chamber. 20 sec is optimal for up to 6 chambers, must be reduced
if collecting from more chambers.
flushtime2 = 0; %Flushes past the third junction. Only used if
sample is to exit chip via "Waste2"
flushtime3 = 0; %Flushes past the fourth junction. Only used if
sample is to exit chip via "Waste2"
flushtime4 = 0; %Flushes past the last junction. Only used if
sample is to exit chip via "Waste2"
flushtime5 = total_flush_time -flushtime1*imax(2)/2 -
flushtime2*imax(2)/6 - flushtime3*imax(2)/12 -
flushtime4*imax(2)/24;
%"flushtime5" must have a positive value, "total_flush_time" and
%"flushtime1" must be set accordingly
```

```
%------------------CLEAN OUT TUBES AND FLOW LINES-----------------
--------


%   Begin by flushing out the flush lines through Waste1
close_inputs;
open_valve('SvIn'); % relaxes the pneumatic jack
open_valve('SvOut'); % closes the airflow in the sample collection
pipe
open_pump;
open_valve('MixerOut');
open_valve('InPurge');

% set_mux(chamber(1), 1, 1, 1, 0, 0)  % To rinse only first
flushway
set_mux(101,1,1,1,0,0); %   Open all flushways

if first_sample
    open_valve('Waste1Up');
    open_valve('Waste1Dn');

    pause(initialflushtime1);

    close_valve('Waste1Up');
    close_valve('Waste1Dn');
    close_valve('MixerOut');
    close_valve('InPurge');
end

%   Swich to flushing through Waste2
open_valve('Waste2Up');
open_valve('Waste2Dn');
open_valve('BeadsIn');


pause(initialflushtime2);

close_pump;


%   Close the outputs to make ready for air blow
close_valve('Waste2Up');
close_valve('Waste2Dn');
close_valve('BeadsIn');


%   Make sure the valve closes
pause(2);


%   Start pushing liquid with air

close_valve('SvOut');
```

```
%   Wait until all liquid is out of tube
pause(traveltime/2);

    %   Push liquid out of chip in order to clean Y-junction
    open_valve('InPurge');
    open_valve('MixerOut');
    open_valve('BeadsIn');
    pause(1);
    close_valve('InPurge');
    close_valve('MixerOut');
    close_valve('BeadsIn');


pause(traveltime/2);


%   Stop pushing liquid with air

open_valve('SvOut');


%   Make sure the valve closes
pause(2);




%---------------------GATHER SAMPLES-------------------------------
-------

if ~CCCdata.Scr.Stop  % Checks if stop button has been pressed
   if   pumpcycles > 0
        %   Move dispenser to correct vial
        fcollect('moveto',vialnr);


        %   Loop through all chambers that should be emptied
        for i = 1:imax(2)
            %   Open Waste1 to be ready for sample extraction
            open_valve('Waste1Up');
            open_valve('Waste1Dn');
            open_valve('InPurge');
            open_valve('MixerOut');
            %   Pump out of chambers towards Waste1 from InPurge
            set_mux(chamber(i), 1, 1, 0, 0, 0);
            if CCCdata.XY.Tuned || CCCdata.XY.Calibrated  % Checks
if stage is calibrated
                moveto(chamber(i), [xoff; yoff; zoff], 0); % Moves
stage to chamber
            end
            open_valve('ChOut');
            open_valve('ChIn');
            pump(pumpcycles, pumpperiod, 1);    % pumps out of
chamber
            close_valve('ChOut');
```

```
            close_valve('ChIn');
            close_pump;
            %   Push the sample back to BeadsIn using Waste2
            close_valve('MixerOut');
            close_valve('Waste1Up');
            close_valve('Waste1Dn');
            pause(1);
            %   Open pathway to flush sample out of chip and into
tube
            open_valve('Waste2Up');
            open_valve('Waste2Dn');
            open_valve('BeadsIn');
            open_pump;
            set_mux(chamber(i), 1, 1, 1, 0, 0);
            pause(flushtime1/2);
            close_valve('Waste2Up');
            close_valve('Waste2Dn');
            close_valve('BeadsIn');
            close_pump;
            pause(1);
        end
   else
        pumpcycles = -1 * pumpcycles;
        %   Move dispenser to correct vial
        fcollect('moveto',vialnr);

        open_valve('InPurge');
        open_valve('MixerOut');
        open_pump;
        set_mux(101,1,1,1,0,0); %   Open all flushways
        open_valve('Waste1UP');
        open_valve('Waste1Dn');

        pause(20); % Fill waste1 with PBS

        close_valve('InPurge');
        close_valve('MixerOut');
        close_pump;

        %   Open BeadsIn to be ready for sample extraction
        open_valve('BeadsIn');

        %   Loop through all chambers that should be emptied
        for i = 1:imax(2)

            %   Flush out of chambers towards BeadsIn from Waste1
            set_mux(chamber(i), 1, 1, 0, 0, 0);
            pause(0.2);

            open_pump;

            if CCCdata.XY.Tuned || CCCdata.XY.Calibrated  % Checks
if stage is calibrated
                moveto(chamber(i), [xoff; yoff; zoff], 0); % Moves
stage to chamber
```

```
            end
            open_valve('ChOut');
            open_valve('ChIn');
            pause(pumpcycles);    % flushes out of chamber
            close_valve('ChOut');
            close_valve('ChIn');

            close_pump;

            pause(0.5);
        end

    close_valve('Waste1Up');
    close_valve('Waste1Dn');
    end

end


open_valve('Waste2Up');
open_valve('Waste2Dn');
open_valve('BeadsIn');
open_pump;

%   Flush the samples out

if ~CCCdata.Scr.Stop    % Checks if stop button has been pressed

    for j2 = 1:6:imax(2)    % Pushes samples past third junction
    set_mux(chamber(j2), 1, 1, 1, 1, 1);
    pause(flushtime2);
    end

    for j3 = 1:12:imax(2)   % Pushes samples past fourth junction
    set_mux(chamber(j3), 1, 1, 1, 1, 1);
    pause(flushtime3);
    end

    for j4 = 1:24:imax(2)   % Pushes samples past fifth junction
    set_mux(chamber(j4), 1, 1, 1, 1, 1);
    pause(flushtime4);
    end

    pause(flushtime5);  %   Moves sample from the last junction
and past the Y-split

end

close_pump;

%   Close the outputs to make ready for air blow
close_valve('Waste2Up');
close_valve('Waste2Dn');
close_valve('BeadsIn');
```

```
%-------------------MOVE SAMPLES TO FRACTION COLLECTOR------------
-------
if ~CCCdata.Scr.Stop    % Checks if stop button has been pressed

    %   Make sure the valve closes
    pause(2);
    % Wait for tube of pneumatic jack to enter vial

    close_valve('SvIn');
    pause(4); %   Start pushing sample with air
    close_valve('SvOut');

    %   Wait until all sample is out of tube
    pause(traveltime);



    open_valve('SvOut');    %   Stop air flow
    pause(4);   %   Wait for tube of pneumatic jack to leave vial
    open_valve('SvIn');     %   Lift tube from vial

    %   Make sure the valve closes
    pause(5);

    %--------------------CLEAN TUBE WITH FLUSHWATER---------------
-----------
    % open_pump;
    %
    % open_valve('Waste2Up');
    % open_valve('Waste2Dn');
    %
    % pause(initialflushtime2);
    %
    % close_pump;
    %
    %
    % %   Close the outputs to make ready for air blow
    % close_valve('Waste2Up');
    % close_valve('Waste2Dn');
    %
    % %   Start pushing sample with air
    % open_valve('SvIn');
    % open_valve('SvOut');
    %
    % %   Wait until all sample is in vial
    % pause(traveltime);
    %
    % %   Stop blowing air, and stop sample gathering
    % close_valve('SvIn');
    % close_valve('SvOut');
    %

    %--------------------CLEAN EQUIPMENT AND RESET CHIP-----------
------------
```

```
    %   Rinse the tip of sample collector


    fcollect('rinse');

    pause(3); %Waits for the sample collector to reach rinse area.

            %   Push tube down and start blowing air
    close_valve('SvIn');
    pause(1); %  Wait for tube to descend
    close_valve('SvOut');


    pause(rinsetime);


    open_valve('SvOut');    %   Stop air flow
    open_valve('SvIn');     %   Lift tube from vial
    pause(3);  %  Wait for tube to lift

    %   Reset the dispenser to home position
    fcollect('reset');
end

%   Close valves on chip
set_mux(99, 1, 1, 1, 1, 1);

close_valve('MixerOut');
close_valve('InPurge');
pause(1);
% Open the Waste 1 valves in order to continue feed cycle.
open_valve('Waste1Up');
open_valve('Waste1Dn');


clear CCChs CCCdata;
```