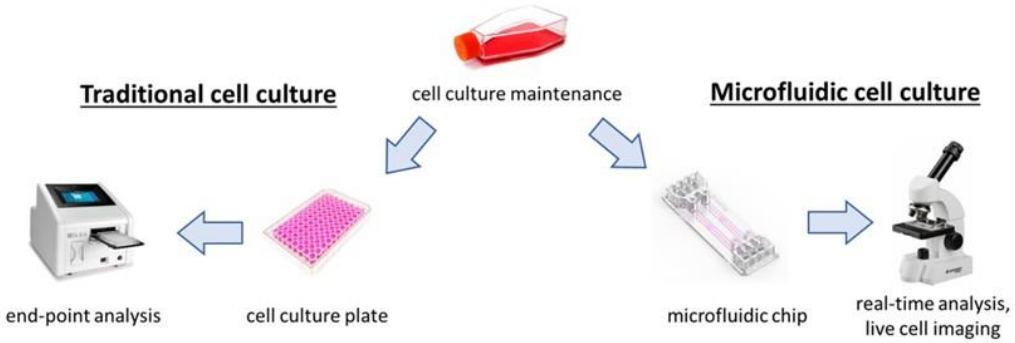
PRESSURE AS A TOOL TO EVALUATE CELL GROWTH

Summary
This application presents a simple method to monitor cell proliferation in microfluidic chips in real time. This is demonstrated experimentally using a custom microfluidic chip. Cell morphology was studied under flowing and static culture conditions.

INTRODUCTION

Microfluidic cell culture has significant advantages over macroscopic culture in flasks, dishes and well-plates[1]. The microfluidic chip fabrication process allows a great flexibility in the design of microfluidic devices, permitting one to control interactions between cells, substrates, and the surrounding medium, physically as well as biochemically[2]. This technology offers new possibilities to accurately reproduce the cellular environment and enables the analysis of biological processes that were not accessible before. Morphology wise, chips can be structured at the cell scale to reproduce the mechanical constraints experienced by cells. Biochemically, stable gradients can be implemented with high spatial resolution (typically, micrometer resolution). Finally, constant perfusion enables the continuous renewal of nutrients and oxygen to promote cell growth and maintain optimal activity during long term cell culture. Cost reduction due to volume reduction is also a major benefit of microfluidics as many reagents for bioassays or cell culture studies can be very expensive[2].



advantages and challenges of microfluidic cell culture

advantages	*challenges*
- Flexibility of device design	- Non-standard culture protocols
- Real time, on chip analysis	- Novel culture surface
- Perfusion culture	- Complex operational control and chip design
- Reduced reagent consumption	
- Single cell handling	
- Automation possible	

Despite these advantages, moving cells from a macroscopic culture environment to microfluidic cell culture often requires a revision of culture protocols. Several unique factors distinguish microfluidic from macroscopic cell culture, such as different culture materials, oxygen, osmolarity, pH and nutrient consumption. It is important to compare cell behavior in macroscopic and microfluidic devices before experiments are translated from one to another.

Cell proliferation assays or live/dead assays are very common for controlling cell viability[3,4]. Performing these assays can however be expensive and time consuming. The two methods most commonly used are the following:

- *Cell counting using a microscope:* cells are fixed and stained with Dapi. Several areas of the chip are selected on a microscope and nuclei in these areas are counted. The main drawback of this method is that its accuracy depends on the cell type considered and its density. Some cells grow in 3D, are small and dense which can make counting difficult compared to 2D sparse and widely spread cells.
- *Cell counting using a counting chamber:* As it is difficult to perform cell counting on adherent cells, trypsinization is usually required and cells in suspension are counted on counting chambers.

These two methods are endpoint assays: only one time point measurement can be performed as cells are either fixed in the chip or retrieved from it. As a result, it is not possible to repeat experiments on the same sample. As a consequence, if one wants to monitor cell proliferation over time, one sample per time point is needed.

Unlike endpoint approaches, real-time assay systems allow for the tracking of cellular growth over the entire time course of an experiment. Real-time assays are typically performed using equipment capable of capturing images at regular intervals and quantifying cellular surface area coverage as a measure of proliferation[5]. Although highly accurate, these systems are very expensive, cumbersome to use, and often not appropriate for microfluidic systems.

We present an alternative method to monitor cell growth in real time while controlling the flow rate in the microfluidic chip. This method is based on the calculation of the hydrodynamic resistance. It requires the use of pressure controllers associated with flow sensors to control flow rate. Briefly, in microfluidic chips the pressure drop between the input and the output of a system and flow rate are related as follows:

$$\Delta P = R_h . Q$$

With ΔP the pressure difference across the flow conduit, R_h the hydrodynamic resistance of a microfluidic system and Q the flow rate. The hydrodynamic resistance can thus be thought of as the resistance to fluid flow at a given driving pressure. At a constant pressure drop, the higher the resistance, the lower the flow rate. R_h is proportional to the channel length and inversely proportional to the channel inner dimensions (as a power of four)[6]. Cells growing inside the chip change the resistance through time. In fact, as cell division occurs within the microfluidic chip, cell density increases and the channel size of the microfluidic chip is reduced. The channel size reduction directly affects the microfluidic chip resistance, and this change can be detected using a pressure controller coupled to a flow sensor. If a flow rate is set to be constant and if the resistance increases, the applied pressure must increase in order to keep the flow rate constant. Monitoring the pressure increases gives access to cell proliferation information.

METHOD FOR DETERMINING CELL PROLIFERATION

The method determines cell proliferation by measuring the pressure increase using a pressure-based microfluidic system coupled to a flow sensor and using the equation $\Delta P = R_h . Q$.

Generic system setup and protocol

Setup

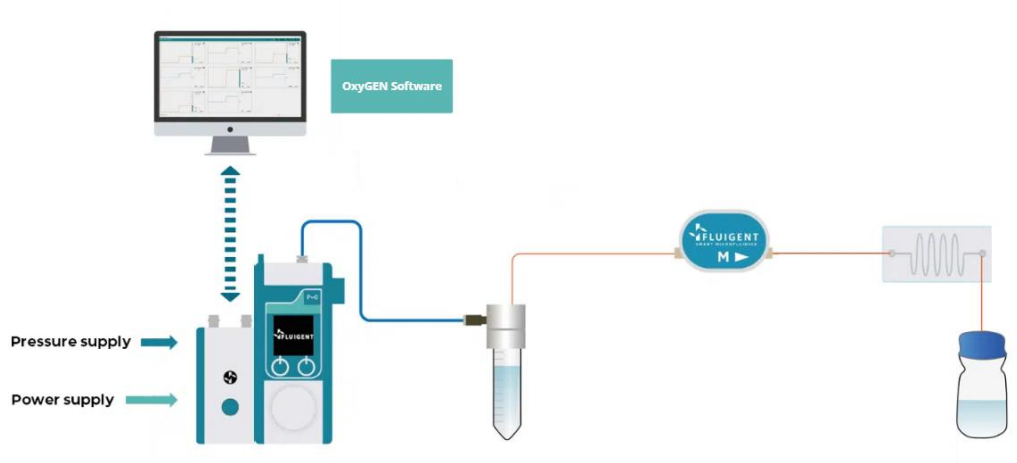


Figure 2: System setup. The Flow-EZ pressure controller is connected to a reservoir to control the pressure difference ΔP. The tubing passes through the microfluidic chip and a Flow Unit to measure flow rate.

The setup includes the following material:

- *Flow-EZ:* The Flow EZ is the most advanced flow controller for pressure-based fluid control. It can be combined with a Flow Unit to control pressure or flow rate. A range of 10 – 40 mbar was used during the experiments.
- *Flow unit M:* A flow sensor that allows real time flow rate measurement up to 80 µL/min. By combining a Flow Unit with the Flow EZ, it is possible to switch from pressure control to flow rate control. (more details on www.fluigent.com/products)
- Your microfluidic cell culture chamber chip. At least 2 chips should be used for the first calibration
- Your tubing
- Cell culture media
- Your cell line

Steps for determining cell proliferation within a microfluidic chip in real-time:

1. Connect all your elements as described in figure 2. The Flow EZ is connected to one reservoir to set the system pressure. The reservoir contains the media to be injected. The tubing passes through the microfluidic chip and a Flow Unit is added to allow flow rate measurement and subsequent control.
2. Inject your cell suspension to the microfluidic chip using a suitable flow rate. Wait overnight or the appropriate time for your cells to adhere on the microfluidic chip.
3. For the first experiment to set the standard ratio of adherent cells over injected cells (some cells remain in the tubing or swept volume of the system), the number of adherent cells should be counted. This will allow estimating the number of adherent cells after injection for the next experiments. After waiting the corresponding time for your cell line to adhere,

cells can be retrieved using trypsin and counted on a counting chamber. We advise to use new tubing when using trypsin to ensure to count adherent cells within the chip only. (Example: if 200k cells are injected and 150k adherent cells are subsequently counted, the system standard ratio is 150/200 = 0,75)

4. Use the OxyGEN software to inject media using a constant flow rate Q for 3 days. The flow rate used should be appropriate for cell cultivation. The microfluidic chip resistance will increase as cell division occurs. Because $\Delta P = R.Q$ and as the flow rate is kept constant, the pressure difference ΔP must increase to counteract the resistance increase. Warning: You need to record this step with OxyGEN software.
5. Plot the pressure difference ΔP (it is measured and displayed on OxyGEN) as a function of thetime t on a graph.
6. For the first experiment to set the relationship between pressure increase and the number of new cells in the system, some chips need to be set apart to quantify the total number of cells within them. The cells can be retrieved using trypsin and counted on a counting chamber.
7. Plot the pressure required to keep the flow rate constant as a function of the cell number within the microfluidic chip for t = 0 (no cells injected), after cell injection, and after 3 days injection. Plot the corresponding linear trend, as shown in the theoretical plot from figure 3. It is now possible to estimate the number of cells as a function of the applied pressure for maintaining a specific flow rate. Example: In our theoretical plot, we get: $\Delta P = 4.10^{-5}.N_{cells} + 10$ and thus: $N_{cells} = (\Delta P - 10)/4.10^{-5}$

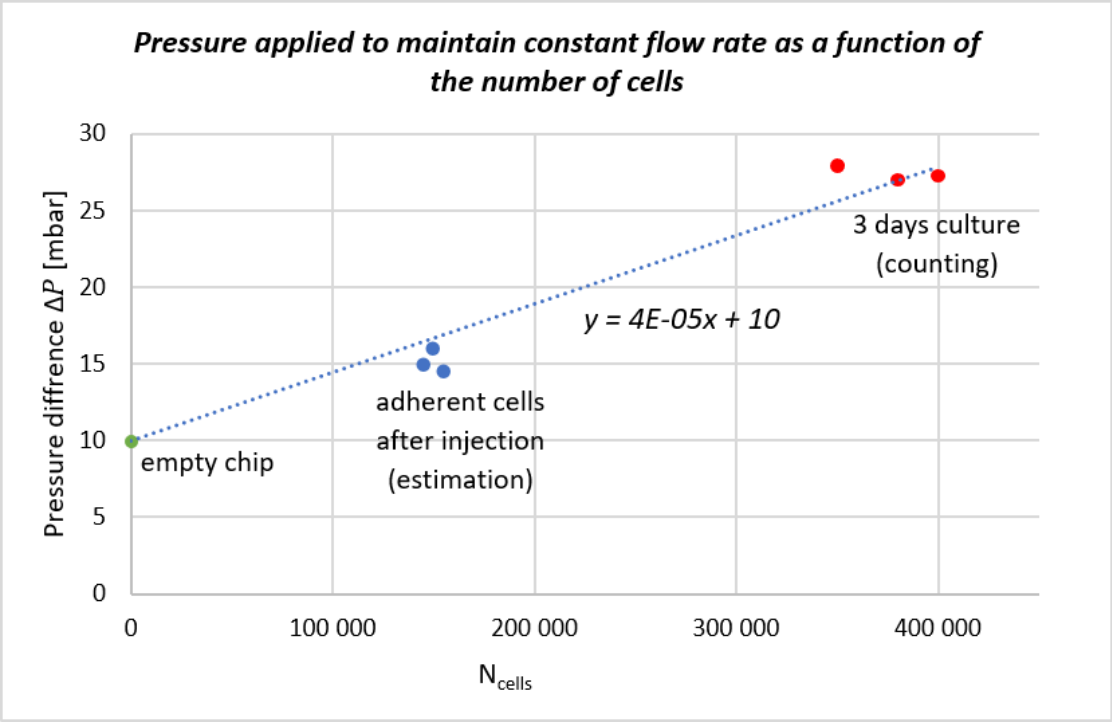


Figure 3: Pressure applied to maintain constant flow rate as a function of the number of cells within the microfluidic chip

You can repeat the process to get a better estimation of the pressure – cell growth correlation, as shown in figure 3. This process should be repeated 3 times to be statistically significant.

Generic system setup and protocol

As a demonstration of the generic setup, we followed the proposed method using a custom microfluidic chip and tubing. We used here a MCFS^{TM}-EZ pressure controller, with tubing passing through the microfluidic chip and a Flow Unit M. A constant flow rate of 10 µL/min was used. The microfluidic chip and tubing are described below:

- *Microfluidic chip:* "Biochip" The laboratory Biomechanics - Bioengineering (UMR CNRS 7338) of Compiegne University of Technology developed a 3D microfluidic devices to promote cell culture. This device is made of a biocompatible material and allows a culture in dynamic condition through a continuous renewal of the culture medium.
- *Tubing:* 1/32 in. PEEK tubing of 254 µm and 157 µm inner diameter (ID).
- *Cell line:* HepG2/C3A cell line, derived from a human hepatocarcinoma (ATCC-CRL-10741, USA).

Protocol for cell injection in the microfluidic chip and counting

To increase cell adhesion inside the microfluidic biochip, a collagen coating was first applied. Cell counting was performed to estimate the number of cells injected within the microfluidic chip. ~250k cells were injected to the microfluidic chip, corresponding to ~150k adherent cells on the chip culture chamber. After 3 days of perfusion with media at constant flow rate, cells were detached using trypsin-EDTA and counted using a Malassez cell counting chamber.

Actin and nucleus staining

To determine if flow rate has an effect on cell proliferation and on cell morphology, actin and nucleus staining were performed. The chip was rinsed with PBS. Paraformaldehyde was injected to fix cells and 0,5% Triton X-100 was injected to permeabilize cells. Hoechst was used to stain cell nucleus and phalloidin was used to stain F-actin (an extracellular protein that helps maintaining the extracellular matrix). The staining solution was injected into the biochip and incubated for 30 minutes. Visualization was performed under optical microscope in fluorescent mode.

RESULTS

Determining cell proliferation within the biochip in real-time

To determine cell proliferation within our microfluidic chip, cell culture and cell injection was performed following steps 1 to 5 described in part 2 a) from the "Method" part. The pressure as a function of the time is then plotted. Figure 4 shows fixed flow rate injection using 10 µL/min as a function of time over 3 days and figure 5 shows ΔP as a function of the time for the same period (on the horizontal axis, 1 unit = 10 seconds).

We observe in figure 4 that the flow rate remained constant at 10 µL/min during the experiment and from figure 5 that the applied pressure was 12 mbar initially and reached 24 mbar after 3 days of perfusion. This increase is due to cell division and proliferation within the microfluidic chip.

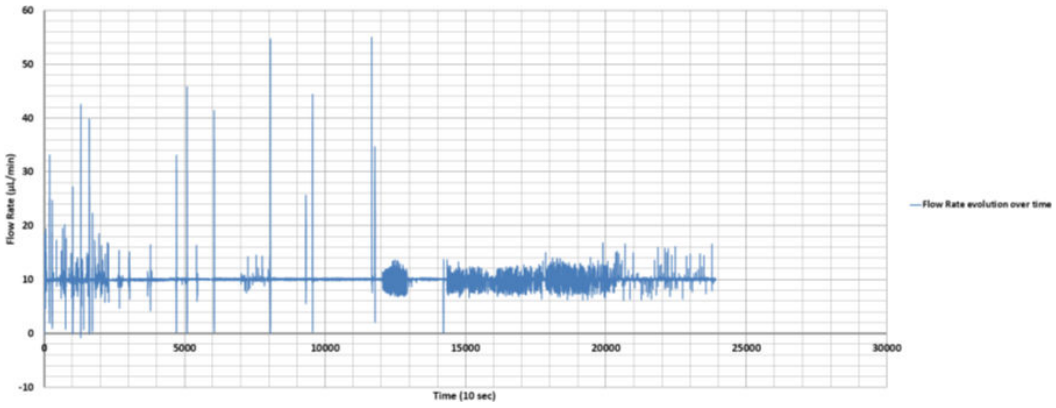


Figure 4: Constant flow rate as a function of time

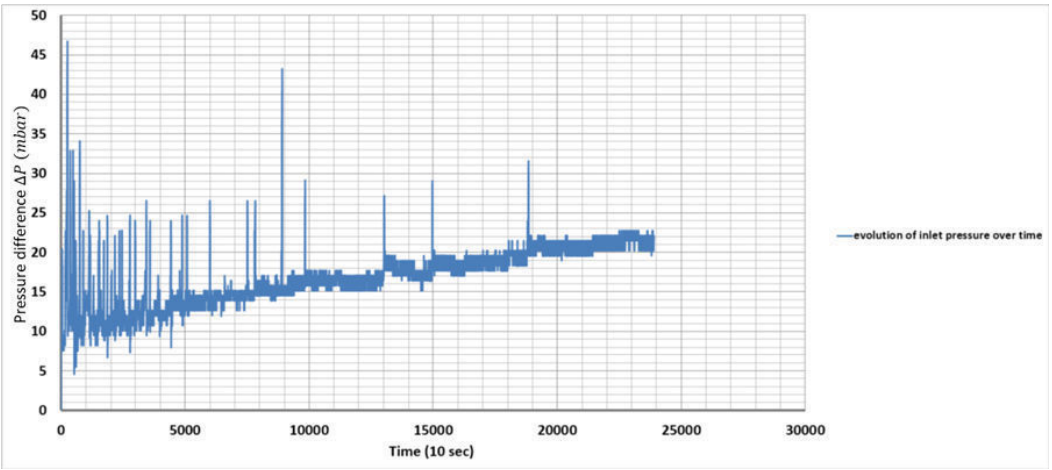
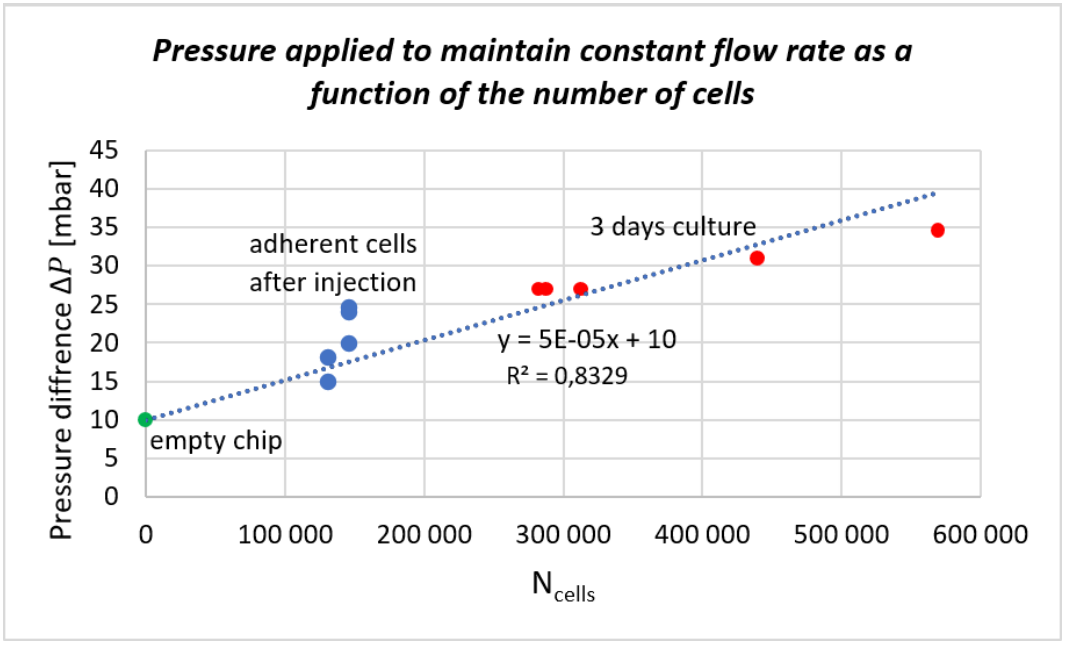


Figure 5: Pressure applied to maintain constant flow rate as a function of time

To find the correlation between cell proliferation and the pressure increase for maintaining a steady flow rate, we followed steps 6 and 7 from the protocol. The experiment was repeated on 5 biochips to increase statistical significance. The pressure applied to maintain a flow rate of 10 µL/min as a function of the number of cells (estimated after injection, and counted after 3 days of perfusion) is shown in figure 6.



We can observe a good correlation between the pressure applied and cell number for cells cultured during 3 days and counted afterwards. The different pressures determined for the injected cells might arise from the fact that injected cell quantity was only estimated and not counted. The slope from the curve was determined, and the following linear function is given below:

$$\Delta P = 5.10^{-5}.N_{cells} + 10$$
$$\Rightarrow N_{cells} = (\Delta P - 10)/5.10^{-5}$$

Using the equation above, it is thus possible to estimate the number of cells as a function of the applied pressure using the same system and under identical conditions. The user can monitor and estimate cell proliferation in real time within a microfluidic chip in parallel with a main experiment.

Cell viability under steady and dynamic flow conditions

To assess the influence of flow rate on cells, cell morphology of cells cultured under dynamic and static conditions was compared, as shown in figure 7.

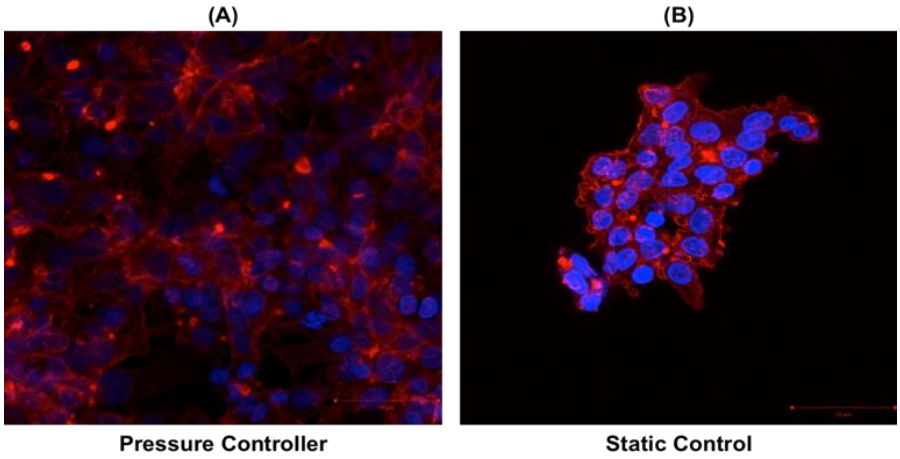


Figure 7: Pressure applied to maintain constant flow rate as a function of time (actin labelled in red and nucleus in blue)

We observe in figure 7 that the actin network is more developed under dynamic conditions compared to static conditions. In fact, under flowing (dynamic) conditions, a low shear is applied on cells. This shear stress tends to elongate cells, and as consequence 2-dimensional cell growth is favored. Under static conditions, 3-dimensional cell growth is favored as no shear is applied. Cells growing 3-dimensionally could lead to increased cellular heterogeneity as they do not have access the same amount of nutrients or oxygen within the microfluidic chamber. Under dynamic conditions, cells are in a favorable growth environment, that is a continuous and homogeneous perfusion culture with a steady and low shear stress applied on cells.

CONCLUSION

We here demonstrated the use of pressure controllers coupled with flow sensors for determining and estimating cell proliferation within a microfluidic chip in real time. The user can track in real time cell proliferation by simply monitoring pressure increase. This method allows one to estimate cell proliferation kinetics within a chip in an inexpensive fashion. This system shows great advantages as it offers real time information on pressure and flow rate, without requiring the preparation of additional replicates dedicated to monitor proliferation at different time points, hence making it a strong and versatile tool.

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