Automated Microfluidic System for Suspension Cell Culture Using Lab-on-Chip Devices

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Automated microfluidic system for suspension cell culture using Lab-on-Chip devices

For the degree of Master of Science (M.Sc.)

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Master of Science in Biomedical Sciences

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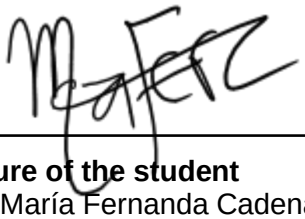

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Table of Contents

Abstract

Microfluidic systems, known as the miniaturization of the laboratory, allow the development of experiments on a minimal scale and the automatization of the process, minimizing experiment and personnel costs and maximizing reproducibility. These systems work with Lab-on-Chip (LOC) devices, which allow the constant renewal of fresh medium making long-term experiments feasible. Despite this, working with suspension cells is complicated as they do not attach to surfaces. Therefore, this project implements a suspension cell culture in LOC devices using a pressure-driven flow-controlled microfluidic system. Yeast (Saccharomyces cerevisiae) and Jurkat cells (T-cell line) were used as a representative of suspension cells. Two different LOC devices were tested and then the microfluidic system was validated by comparing the expansion of cells and the action of poly-L-lysine and etoposide with a 96-well plate. The system proved to be not only as useful but more robust than the conventional 96-well plate. As almost all the assays can be automated, human intervention was reduced, decreasing the reproducibility issues. Likewise, as it is possible to image the whole well using an automated microscope, the results obtained using the microfluidic system are more reliable. The results obtained from these experiments can be potentially applied to optimize the expansion and activation of CAR T cells, allowing the reduction of the cost of this new promising treatment.

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Abbreviations and Symbols

µL/min	Microliters per minute
µM	Micromolar
µm	Micrometer
ACT	Adoptive cell therapy
CAR	Chimeric antigen receptor
DNA	Deoxyribonucleic acid
ESI	Elveflow Smart Interface (ESI) software
FBS	fetal bovine serum
h	hours
LOC	Lab-on-Chip
ms	milliseconds
PDMS	Polydimethylsiloxane
PI	Propidium Iodide

INTRODUCTION

Cell culture

Cell culture is a standard laboratory method by which cells are maintained outside their natural environment under controlled physiological conditions. Using this method, many types of eukaryotic and prokaryotic cells can be kept in a laboratory to perform all kinds of research (Kapałczyńska et al., 2018; Segeritz & Vallier, 2017).

This methodology has been used since the 1900s, being first employed for studying nervous tissue growth. Subsequently, it has been applied for research in virology and vaccine production, model systems in health and disease, drug development and drug testing, tissue regeneration and transplantation, genetic engineering, and gene therapy; in general, many in vitro studies on cell behavior that simulates in vivo conditions. In essence, cell culture includes studies on cell differentiation, migration, growth, and mechanics and their relation to their biochemical and biomechanical microenvironment (Duval et al., 2017; Segeritz & Vallier, 2017).

Advantages and Disadvantages of cell culture

Cell culture is one of the bases of laboratory protocols, especially in biomedical research, healthcare, and the pharmaceutical sector, which is why it is seen as the future of clinical testing. One of the main advantages of this technique is that it is an alternative to animal testing. The use of cell culture reduces ethical problems and costs of experiments and simplifies complex tests compared to animal models. Also, as research based on cell culture is developed in earlier stages, it saves time before clinical trials. Moreover, by having the capacity of working with specific types of cells in a controlled microenvironment, this technic has opened the door to research at the genetic and molecular level, allowing access to data not possible when studying a whole organ, system, or individual (Kapałczyńska et al., 2018; Ravi et al., 2015; Segeritz & Vallier, 2017).

The general approach to performing cell culture is manual handling, resulting in time- consuming and error-prone processes, leading to high variability of outcomes, reagent consumption, and low reproducibility. In addition, the development of long-term experiments that requires many reagents, for example, the research of new drugs to treat different conditions, is difficult to perform manually. As denoted in an analysis by Nature, from 1,576 researchers that took part in the questionnaire, more than 70% have tried and failed to reproduce another scientist’s experiments, and almost half of them have failed to reproduce their own (Allison et al., 2016; Baker & Penny, 2016; Segeritz & Vallier, 2017).

Adherent vs. non-adherent cells culture

There are two basic systems of cell growth in culture: adherent cells, which grow in a monolayer on an artificial surface; and non-adherent cells, which grow in suspension in the culture medium (Coluccio et al., 2019; faCellitate, 2022; Hacking & Khademhosseini, 2013).

Adherent cells need to attach and interact with the surface to proliferate, differentiate, and maintain viability. Therefore, the vessel surface has to be treated to make it suitable for cell adhesion. Being anchorage-dependent, the quantity of cells is limited by the surface area of the vessel. Also, to dissociate the cells, special protocols, such as enzymatic or mechanical actions, are required. Most cell types belong to this system, including most vertebrate-derived cells that are used for many research applications. On the contrary, non-adherent cells are in suspension in the culture medium. They may require agitation to ensure gas exchange but treated surfaces or protocols for dissociation are not needed. The quantity of cells is limited by their concentration in the medium. In this system, one of the most widely cultured cell lines are hematopoietic cells (Coluccio et al., 2019; faCellitate, 2022; Hacking & Khademhosseini, 2013).

Cell lines

When working with cell cultures, there is the possibility to work with two major types of cells, primary cells or established cell lines. Although both are used for research, each type has its own characteristics (Montano, 2014; Segeritz & Vallier, 2017).

Primary cells are isolated directly from the tissue and processed to adapt to *in vitro* conditions. In biomedical and translational research, these are the cells of choice because they maintain the morphological and physiological conditions and keep the genetic integrity of the original tissue. This allows us to avoid ethical objections that come with animal testing and to perform experiments on human tissues. Therefore, the use of primary cells has more biological relevance since they provide more significant results as they simulate better in vivo environments. However, there could be a difference in responses (due to proinflammatory cytokines) as they are donor-dependent. Another drawback is that these cells have a limited lifespan, have finite proliferation, take more time to divide, and need special optimized culture conditions (Montano, 2014; Pastor et al., 2010; Segeritz & Vallier, 2017).

In contrast, established cell lines are immortalized cells that are generated by naturally occurring mutations, as in cancer, or by genetic manipulation. These cells can proliferate indefinitely, as they have an unlimited lifespan. Since the culture conditions and

protocols are well established, they grow faster, and the maintenance costs are lower. With time they, however, acquire genetic mutations resulting in the loss of the genetic integrity, producing changes that distance them from the characteristics of the original tissues (Geraghty et al., 2014; Montano, 2014).

Cancer

Cancer is defined by the uncontrolled division of abnormal cells. Normally, damaged cells die, but due to certain mutations in their DNA, especially in genes that control growth, division and survival, these abnormal cells can proliferate and survive indefinitely. Cancerous cells grow and proliferate in the absence of growth factors and other signaling molecules, and avoid programmed cell death, like apoptosis (National Cancer Institute, 2021; World Health Organization, 2022).

When adherent cells accumulate, they become tumors, which can be benign or malignant. In the case of malignant tumors, they can spread and invade other parts of the body (a process called metastasis), and even when removed, they can grow back. In the case of non-adherent cells, they keep dividing in body fluids, like leukemia in the blood (National Cancer Institute, 2021; World Health Organization, 2022).

Cancerous cells can hide from or manipulate the immune system, so they are protected and not eliminated. Additionally, they have different metabolism and the capacity to induce the growth of blood vessels, allowing them to grow faster than normal cells (National Cancer Institute, 2021; World Health Organization, 2022).

Treatment

There are more than 100 types of cancer, as this disease can affect any cell. The available treatments for cancer range from surgery, radiotherapy to systemic therapy (chemotherapy, hormonal treatments, targeted drugs, and immunotherapy), depending on the type of cancer. (Liu et al., 2021; National Cancer Institute, 2021).

Generally speaking, in the case of solid tumors, the first approach is surgery. By removing the damaged cells, metastasis is prevented. Another option is radiotherapy, which uses high-energy radiation to kill cancerous cells and shrink tumors. The effect of the therapy depends on the radiosensitivity and radioresistance of cancerous cells. Normal cells tend to be insensitive to radiation and do not get affected by this therapy (Liu et al., 2021).

Systemic therapies are based on the use of different drugs that help shrink tumors, and ensure all cancerous cells are eliminated. This treatment can be given before or after the previously discussed ones or applied to non-solid tumors, for example, hematological malignancies (Liu et al., 2021; Palumbo et al., 2013).

Chemotherapy is based on the use of drugs to interrupt the growth and spread of cancer through the body. The effectiveness of the therapy depends on the dose, schedule, and the use of appropriate drug combinations. As in other cases of drug-based treatments, chemoresistance is one of the drawbacks of this therapy. The use of drugs can produce resistance to one or more substances used, decreasing the possibilities of successful therapy. Similarly, in hormonal therapy, medicines that control the production of certain hormones are used because some types of cancer are generated by and depend on them (Liu et al., 2021; National Cancer Institute, 2021; Palumbo et al., 2013).

In the case of targeted drugs, they are considered the foundation of precision medicine, as it involves specialized medicines that target specific proteins that regulate the growth, division, and spread of cancer cells but exert little effects on normal cells. They can also stop signals that aid angiogenesis in tumors and deliver killing substances to cancer cells (Aggarwal, 2010; Palumbo et al., 2013).

The latest advance in cancer treatment is immunotherapy. As the name implies, the goal of this therapy is to use the patient's immune system to attack cancer cells. Although cancer suppresses the action of the immune system, several methodologies have been developed to overcome this drawback (Farkona et al., 2016; Palumbo et al., 2013).

One strategy is the application of cytokines (IL-2 or IFN-α) to boost the immune system; albeit it shows a low response rate and risks of serious systemic inflammation. Another strategy is the use of specialized antibodies to enhance T-cell activity (by targeting families of receptors such as the tumor necrosis factor receptor to produce co-stimulatory signals) or downregulating regulatory T cells (by targeting directly CD25). Antibodies can be used against immune checkpoint molecules such as CTLA-4 and PD1. Despite the benefits, antibody therapy still has a low-efficiency rate and can generate severe immune-related adverse events such as autoimmune diseases. Another option to increase the patient's immune response against cancer cells is vaccination with tumor antigens or augmentation of antigen presentations, but unfortunately, there is no universal cancer antigen (Farkona et al., 2016; Palumbo et al., 2013).

One of the most promising immunotherapy strategies is adoptive cell therapy (ACT) because as a personalized treatment, it is more likely to be effective. This therapy consists of isolating the patient's immune cells, genetically modify them to produce proteins that recognize and eliminate cancer cells, expand them, and inject them back into the patient (Bajgain et al., 2014; Buechner et al., 2018; Grosskopf et al., 2022; Levine, 2007).

CAR T cells

Among the variety of ACT treatments, chimeric antigen receptor (CAR) - T cells have proved to be one of the most effective therapies, especially for treating hematological malignancies. This cell therapy focuses on genetically modifying a patient's T cells to produce receptors composed of an antigen recognition domain derived from a monoclonal antibody and an intracellular signaling and co-stimulation domain, enabling them to specifically identify antigens present in cancer cells and eliminate them. This therapy has already been used not only for research but also in clinical studies. The most common type of cancer treated with this therapy is the one affecting B cells (B cell lymphomas), as there are already approved therapies. The European Hematology Association predicts that this type of therapy will improve prognosis and reduce morbidity for patients carrying such malignancies (Buechner et al., 2018; Grosskopf et al., 2022; Levine et al., 2017; Prasad, 2018; Tyagarajan et al., 2020).

The production of CAR-T cells consists of several steps. First, leukocytes are isolated from the patient's blood by leukapheresis. Then, T cells are separated and specific subsets of T cells, such as CD4+ and CD8+, are enriched and selected using antibodies bead conjugates or markers. The selected cells are activated using antigen-presenting cells, beads coated with antibodies, and/or using growth factors like IL-2. The activated T cells are transduced to express the chimeric antigen receptor (CAR) recognizing a particular tumor antigen (for example CD19 on B cell lymphomas). Lastly, cells expressing CAR are expanded in static cultures or bioreactors until the intended dose is achieved (10^6– 10^7 cells/kg body weight, although lower doses have been effective), and finally injected back into the patient (Grosskopf et al., 2022; Tyagarajan et al., 2020; Wang & Rivière, 2016; Zhang et al., 2017).

Although this is a promising therapy, there are still some challenges to overcome to improve it, since the current methods used for this process present some drawbacks. Taking as an example, the activation of CAR-T cells with excessive IL-2 can lead to exhausted T cells, which become non-functional; or the use of different combinations of interleukins results in a subset of cells that are not effector cells (Vormittag et al., 2018; Zhang et al., 2017).

In the case of expansion of T cells, one major drawback is that the bioreactors or flasks where the cells are kept, have to be opened in several steps, increasing the probability of culture contamination. In addition, the available options that work with perfusion, need large volumes of culture medium, up to 25 L. In addition, there are new platforms that can influence the kinetics of cells if disturbed during the process. Likewise, the price of implementing this therapy is exorbitant, costing up to $475,000 for a one-time infusion (Bajgain et al., 2014; Huang et al., 2020; Prasad, 2018; Somerville et al., 2012; Vormittag et al., 2018; Wang & Rivière, 2016).

Microfluidics

Microfluidics is the technology used for the miniaturization of the laboratory utilizing microminiaturized devices containing chambers and channels that allow the application of flow. The capacity of perfusing fresh cell culture media carrying nutrients while simultaneously removing waste products at a regulated flow rate leads to the establishment of controlled microenvironments, long-term cell cultures, and analysis of the outlet medium. Moreover, perfusion provides a more realistic environment that includes physical shear stress by exposure to fluid flow (Kerk et al., 2021; Peñaherrera et al., 2016).

Lab-on-Chip (LOC) devices

The main components used for microfluidics are LOC devices. The material used for their manufacture is biocompatible, so they can be used as a microreactor where prokaryotic and eukaryotic cells can grow, allowing the co-culture of different cell types. Also, the small size of the devices allows for experimentation on a minimal scale, using a small sample volume and reduction of reagents usage. In addition, the design of the microchannels can be customized, allowing adaptation to each experimental objective and multiplexed assays to be performed on these platforms. Finally, the integration of various experimental steps in the same device provides the advantage of having parallelization of experiments and reproducible results (Bourguignon, Attallah, et al., 2018; Bourguignon, Olmos, et al., 2018; Kerk et al., 2021; Ladner et al., 2017; Luo et al., 2009; Peñaherrera et al., 2016).

Pumps

There are different types of pumps used to perfuse liquids into the LOC devices and to maintain a stable and adaptable flow during experimentations. The type of pump that is selected is very important, they can manipulate from nanoliters to liters with different accuracy, which will affect the reproducibility of the results (Varma & Voldman, 2018).

Peristaltic pumps are positive displacement pumps which transport liquids using a rotary motion. As the motor rotates, it squeezes the tubing that contains the liquid and creates a vacuum that allows the liquid to move. The advantage of this system is that it does not need to be in direct contact with the liquids and can produce bidirectional flow. One disadvantage is that the flow pattern is pulsatile, which can damage the cells in the culture (Byun et al., 2014; Coluccio et al., 2019; Varma & Voldman, 2018).

In the case of syringe pumps, they work with a piston that applies positive pressure to push liquids, avoiding the pulsating flow. The disadvantage of this system is that it only works with a fixed volume (Byun et al., 2014).

Microfluidic pumps, for example, the system offered by Elveflow®**,** are the most accurate ones. This microfluidic system uses pressure-driven flow control technology that offers precise and pulseless flow control with a response time of 35ms, giving it the advantage over other pumps. In addition, this system can handle flow rates from 0.07 to 5000 µL/min with a 5% accuracy and a low dead volume down to 1 µL. It is operated by software capable of controlling and monitoring the pressure and/or flow conditions of an experiment automatically (Elveflow, 2022b).

Automatization

Another advantage of microfluidics is the capacity to integrate automatic systems to control the experiment, avoiding variations during manual handling. This also allows real-time monitoring, and rapid analysis, and improves the evaluation of the acquired data. All in all, microfluidics maximizes reproducibility and minimizes experiment and personnel costs (Peñaherrera et al., 2016; Xiao et al., 2017).

Live cell imaging

Live cell imaging refers to the capacity of visualizing cells under the microscope in vitro in real-time. New technologies now allow us to monitor cells with access to high-quality images using time-lapse microscopy (Ibidi GmbH, n.d.).

To perform long-term experiments, a basic characteristic of these systems is the availability of adjustable microenvironments. To reach this goal, many microscopes adapted incubator chambers to control temperature, percentage of CO_2, and humidity; crucial factors to maintain mammalian cells in culture. Also, access to auto-focus or fixed-focus programs has facilitated data acquisition (ibidi GmbH, n.d.; Ibidi GmbH, 2019; Kim et al., 2019).

Applications and problems

Microfluidic technology has been used for different purposes, such us simulating the in-vivo environment by generating channels to test the shear stress in cells, allowing co- culture of different cells to test different drug reactions in the body, using LOC devices as bioreactors for the production of different components, even the recreation of entire organs inside the devices (Armistead et al., 2019; Bourguignon, Attallah, et al., 2018; Chong et al., 2022; Ladner et al., 2017; Xiao et al., 2017).

As seen, microfluidics has been applied to a variety of experiments, but most of them were carried out with adherent cells. Working with non-adherent cells in microfluidic devices, especially with perfusion, is difficult since it poses the risk of the cells getting washed out during the exchange. This makes long-term cell culture somewhat complicated, but not impossible. One of the solutions to ease this problem is selecting a suitable design of the LOC device to culture suspension cells, like the ones used in this study (Castiaux et al., 2019; Luo et al., 2009; Peñaherrera et al., 2016).

OBJECTIVES

Developing a good strategy to culture suspension cells has become of great importance, as it is part of the process of manufacturing CAR-T cells. Therefore, the need to optimize CAR-T cell manufacturing and make this therapy more affordable is urgent. Here we propose to implement a long-term suspension cell culture in LOC devices using a pressure- driven flow-controlled microfluidic system. To fulfill the aim, the project has been divided into objectives, as follows:

1. **Assemble the microfluidic system**
   - 1.1. Set up the microfluidic system
   - 1.2. Achieve a steady flow with minimal fluctuation of ± 1µl/min
2. **Achieve long-term suspension cell cultures in LOC devices using the microfluidic system**
   - 2.1. Establish the imaging parameters
   - 2.2. Compare LOC devices
   - 2.3. Determine flow conditions that prevent the loss of cells as well as the drying of the channels over the imaging period.
   - 2.4. Ascertain variations in seeding protocols
     - 2.4.1. Compare well-by-well seeding versus seeding with flow protocols
     - 2.4.2. Identify the minimal initial seeding cell density
     - 2.4.3. Determine the time taken by the cells to fill a well using the minimum initial seeding density.
     - 2.4.4. Evaluate the cell density and viability at the end of the experiment.
   - 2.5. Evaluate the action of poly-L-lysine and poly-D-lysine coating in the LOC devices.
3. **Establish an adequate medium to cultivate Jurkat T cells using a 96-well plate**
   - 3.1. Test different types of media (RPMI and advanced RPMI) with different serum concentrations.
   - 3.2. Analyze the effect of poly-L-lysine coating.
4. **Validate the system by comparing the microfluidic system with 96-well plates**
   - 4.1. Compare culture growth between the microfluidic system and the 96-well plates using the medium selected in objective 3.
   - 4.2. Compare the action of etoposide in cells and the poly-L-lysine coating between the microfluidic system and a 96- well plate.

MATERIALS AND METHODS

Microfluidic system assembly

The microfluidic system (Figure 1) operates with pressure-driven flow control. Thus, the principal component of the system is the pressure controller, called OB1. This instrument is attached to an external pressure source (air compressor) from one side, and a splitter to connect different reservoirs (Falcon® tubes of 15 ml and 50 ml filled with different liquids) from the other side. The OB1 is protected against the accidental introduction of liquids, which can damage its adequate functioning, by adding an air dryer in between the connection to the air compressor and an anti-backflow filter in between the connection to the splitter (Elveflow, 2022d).

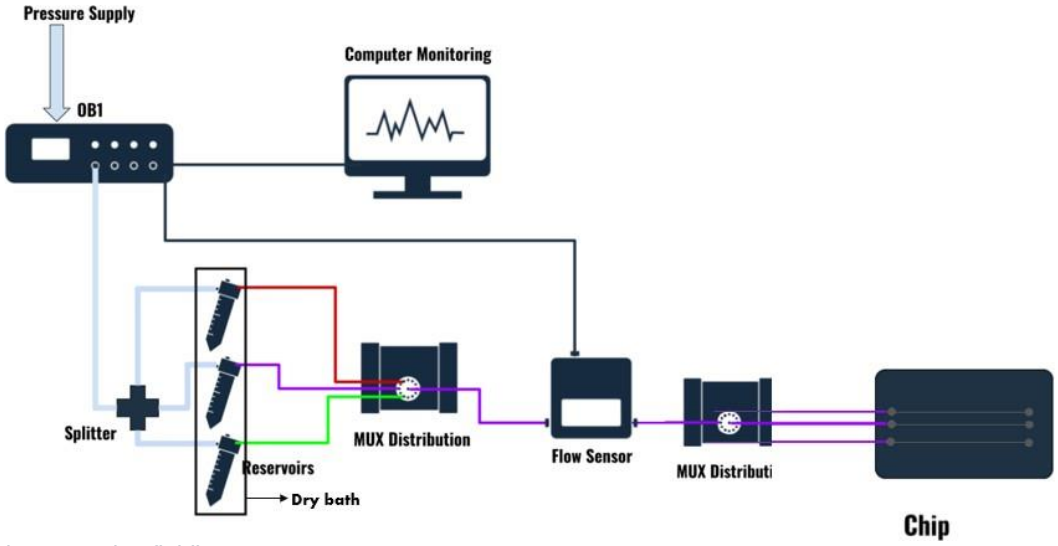

Figure 1. Microfluidic system

The principle of the system is based on pressurizing the reservoirs to create a pressure difference inside them, which causes the liquid to flow out of the outlet (Figure 2). To control the flow rate, the OB1 regulates the pressure applied to the system based on the feedback given by the MFS flow sensor. To maintain the reservoir's temperature, a dry bath was added to the system (Elveflow, 2022d; Klenck, 2015).

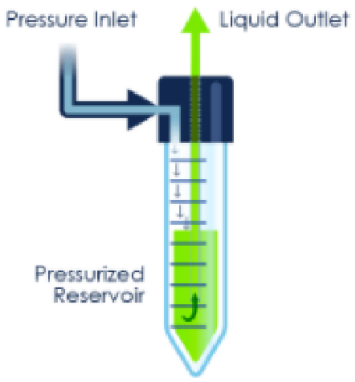

Figure 2. System functioning

The liquids are transported by tubing attached to distribution valves, called MUX. For an adequate distribution of liquids, two valves were used, one to distribute the different liquids from the reservoirs and a second one to distribute them to the labware, i.e., LOC device (Elveflow, 2022b, 2022c).

The equipment was calibrated before use to ensure the proper functioning of the system. All pressure outlets were closed with the appropriate Luer Locks and the sensor was disconnected before running the calibration mode. This step was repeated when the equipment or the flow circuit was modified to ensure that the conditions of the system are consistent (Elveflow, 2022d).

Cell Lines

To develop the experiments, budding yeast (*Saccharomyces cerevisiae*) and the immortalized human T lymphocyte cell line Jurkat were used as models for non-adherent cells.

Saccharomyces cerevisiae is used as a model organism due to its simple growth requirements biological similarity to other eukaryotes, ability to produce many by-products, and its use in the study of various diseases (Montano, 2014; Nielsen, 2019; Totaro et al., 2020)**.**

The leukemic cell line Jurkat is used as a typical T cell line to study multiple events like signaling and molecular events during infection. The cells are cultivated in T-25 flasks using advanced (adv.) RPMI 1640 medium (Gibco) supplemented with 4% fetal bovine serum (FBS), 1% Pen Strep (10,000 Units/ml penicillin, 10,000 µg/ml streptomycin; Gibco) and 1% L-glutamine. The cells are incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells are passaged every three days (Abraham & Weiss, 2004).

Cell Culture Conditions

To establish an adequate medium to cultivate Jurkat T cells, three variables were tested, (i) a poly-L-lysine coat, (ii) RPMI or adv. RPMI medium, and (iii) different FBS concentrations, in a 96-well plate. Half of the plate (columns 7 to 12) were coated with 0.1mg/ml poly-L-lysine. Then, one row for each of the following conditions was prepared: RPMI with 0% and 10% FBS, and adv. RPMI with 1%, 2%, 3%, 4%, and 5% FBS. Both, RPMI, and adv. RPMI medium were supplemented with 1% Pen Strep (10,000 Units/ml penicillin, 10,000 µg/ml streptomycin; Gibco) and 0.25 µg/ml Propidium Iodide (PI). Adv. RPMI was additionally supplemented with 1% L-glutamine. The cell density used was 1 x 10^5 cells/ml.

LOC devices

To establish flow conditions, a single channel LOC device (µ-Slide I 0.4 Luer, ibidi ®) and budding yeast (*Saccharomyces cerevisiae*) were employed. The LOC device was seeded with 100 µl of yeast cells suspended in sterile water. Then, each luer was filled with 60 µl of pure water and the microfluidic system was connected.

For the main assays, two different types of multi-welled LOC devices were used, the PDMS custom-made and the commercial-made by ibidi ® (µ-Slide Spheroid Perfusion). The design of the multi-welled LOC devices is based on microchambers that allow the cells to settle at the bottom of the micro-wells so the renewal of media can be performed without flushing the cells out. The device's dimensions are smaller than the conventional methods for cultivating suspension cells, which saves reagent consumption. The optimal nutrition of the cells is ensured by diffusion, which will move nutrients and oxygen to the cells in the niche (ibidi GmbH, 2021; Luo et al., 2009).

LOC devices seeding and coating protocol

For both multi-welled LOC devices, the cell density was set before seeding. In the case of the ibidi ® LOC device, two seeding protocols were tested. For the first protocol, the coverslip was placed, and the cell suspension was injected directly into each channel twice to ensure well-to-well cell homogeneity. After incubating for 1 h, cell-free medium was injected to remove any remaining bubbles and the luers were filled. For the second protocol, 2 µl of cell suspension was dispensed to each well, ensuring no bubbles were left in the bottom and the coverslip was placed. After 1 h of incubation, cell-free medium was injected to fill each channel and to take out the remaining bubbles, and also the luers were filled. At the end of both protocols, the microfluidic system was connected to the LOC device. For coating, 3.5 µl of poly-D-lysine (channel A) and poly-L-lysine (channel B) were applied per well and the LOC device was left at room temperature for at least 30 min before washing out the coating with PBS and seeding.

For the PDMS LOC device, the cell suspension was injected directly into the channel slowly using a pipet, ensuring all the area was covered without generating bubbles and it was incubated for 1 h.

Setting flow experiments

The LOC devices were placed in a stage-top incubator and connected to the microfluidic system. The reservoirs were filled with the chosen culture medium. The Elveflow Smart Interface (ESI) software controls the system. With the help of the ESI software, various dispensing schedules were tested by applying a range of flow rates at

set time intervals to determine the most suitable conditions. After each experiment, cells were retrieved by removing the coverslip, and cell density and viability was determined applying the trypan blue protocol using the Bio- Rad TC10TM (Bio-Rad, USA) automated cell counter.

Etoposide experiments

Half of the 96-well plate (columns 7 to 12) was coated with 0.1 mg/ml poly-L-lysine and allowed to air dry for at least 30 min. The cell density was set at 1 x 10^5 cells/ml using adv. RPMI + 4% FBS, 1% Pen Strep (10,000 units/ml penicillin, 10,000 µg/ml streptomycin; Gibco), 1% L-glutamine and 0.25µg/ml propidium Iodide (PI). Different concentrations of etoposide were prepared (0 µM, 15 µM and 50 µM).

For the LOC device, the cell density was set at 5 x 10^5 cells/ml using adv. RPMI + 4% FBS, 1% Pen Strep (10,000 units/ml penicillin, 10,000 µg/ml streptomycin; Gibco), 1% L-glutamine and 0.25µg/ml PI. Cells were seeded by well and the LOC device was incubated for 1h. Using the microfluidic system, 2 different concentrations of etoposide (15 µM and 50 µM) were applied every 3 h in different channels. One channel was used as a control (0 µM).

Image acquisition and data analysis

To analyze the 96-well plates, the Incucyte ® Live-Cell Analysis System was used. Images were taken every three hours for a total period of 72 h and the etoposide assay´s images were taken every two hours until cell death was visualized. Four positions per well were imaged at 20 x. From the Incucyte ®, the area covered by cells per well (µm²/well) and the area covered by cells marked with PI per well (µm²/well) over time (h) were obtained. Using these values, the percentage of area covered by dead cells were calculated and graphed in GraphPad Software (2019).

For the microfluidic assays, images were acquired with the Lionheart FX automated microscope. This microscope allows a broad range of imaging workflows. It contains fluorescence, brightfield, and phase contrast imaging channels and objectives of 4 x, 10 x, and 20 x magnifications. The microscope works with the Gen5 software for configuration of imaging parameters across different channels and magnifications during the same assay, which makes it possible to follow cells in a long-term experiment over time (Agilent, 2022).

First, the labware definition was performed in the Gen5 software. Here, the dimensions of the LOC device including the size of the vessel, the location and size of the wells, and the bottom elevation, were uploaded to take adequate images. As each LOC device design has different dimensions, this process was performed for each one. Also, to

maintain cell viability during long-term experiments, a stage-top incubator was coupled to the microscope to keep temperature at 37°C, CO_2 at 5%, and a humidified atmosphere during the whole assay.

To establish flow conditions the microscope was set up to take pictures at 4 x. The protocol for the main assay was to acquire brightfield images at 4 x every 3 hs. The assay was run until the wells were filled with cells. The protocol for the etoposide assay was to acquire brightfield images at 4 x every 2 hs. Images were analyzed using the Trainable WEKA Segmentation plugin (Arganda-Carreras et al., 2017) in Fiji (Image J), the parameter analyzed was the area covered by cells per well (µm2/well) over time (h). Results were graphed in GraphPad Software (2019).

RESULTS AND DISCUSSION

Microfluidic system

The microfluidic system (Figure 3) uses pressure-driven flow control technology. The OB1 measures the pressure applied to the system and regulates it from the feedback given by the MFS flow sensor. The ESI software was used to modify the Proportional (Fast/Stable) and Integral (Sensitive/Smooth) values, which using a standardized algorithm, serve to adapt the pressure depending on the value set at the sensor. To achieve a steady flow of ±1µl/min, the standardized values were P: 0,04 and I: 0,01. Flow stability was confirmed by visualization of moving cells (*Saccharomyces cerevisiae*) within the µ-Slide I Luer form ibidi ® (Figure 4) (Elveflow, 2022a; ibidi GmbH, 2020).

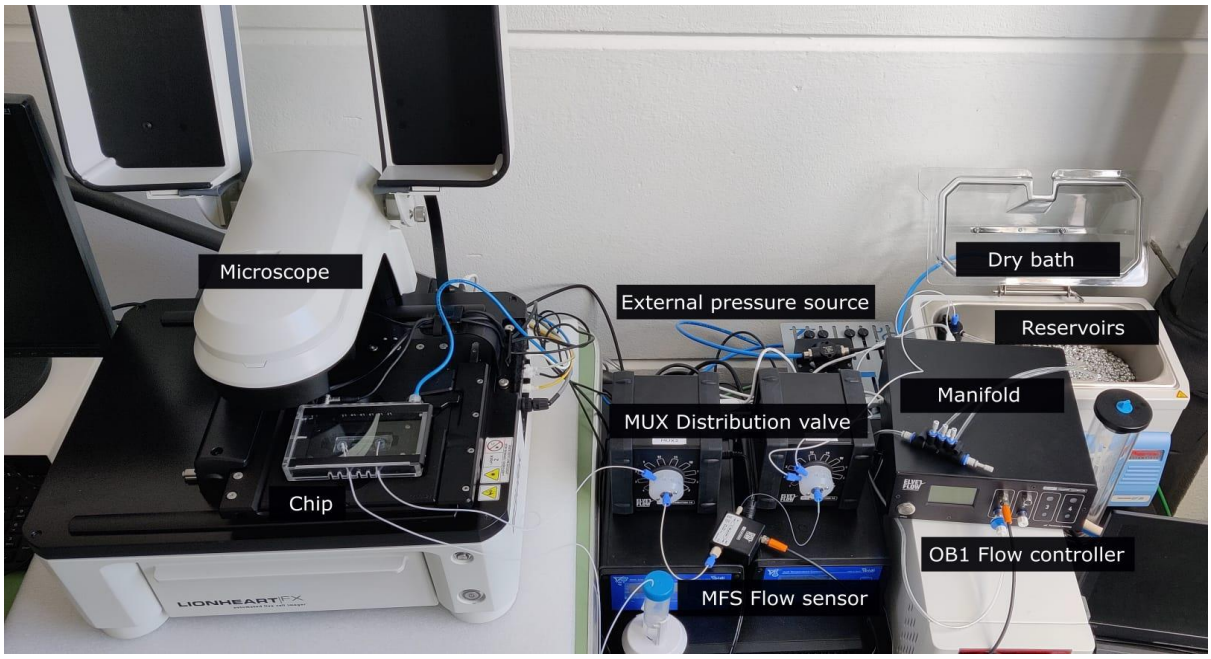

Figure 3. Assembled microfluidic and live cell imaging setup

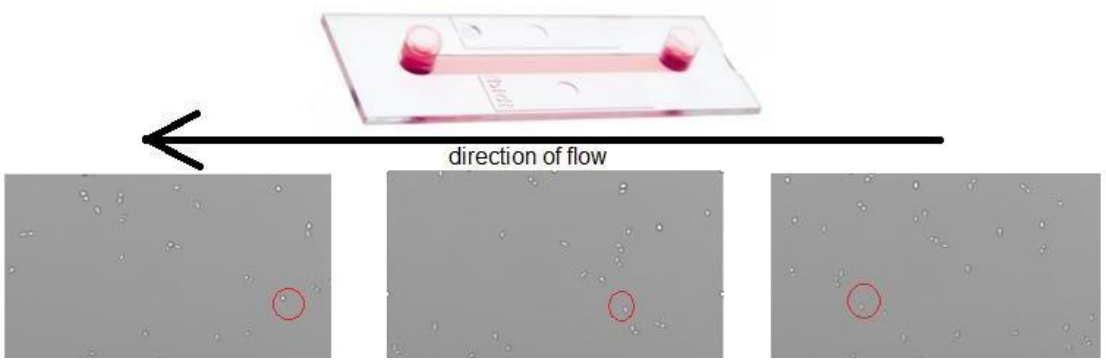

Figure 4. µ-Slide I Luer form ibidi® and movement of cells during time. Images are taken at the same position in different moments (a, first, b second a c third), so it is possible to see the cells changing positions.

In addition, when the MFS flow sensor showed an unsteady flow, it indicated the formation of bubbles or clots in the tubbing, which helped to keep the system bubble-free before starting the experiments. Other measures taken to avoid the formation of bubbles, a major inconvenience when working with microfluidics, were leaving the LOC devices, medium, and tubbing overnight at 37°C with CO_2 and maintaining the medium warm during the whole experiment by keeping the reservoirs in the dry bath. These measures help to avoid temperature gradients, that change the solubility of gases in liquids and plastics (Elveflow, 2022b; Ibidi GmbH, 2019).

The microfluidic system presented here when compared with other equipment like the syringe or the peristaltic pump has several advantages. One of them is that it can pump small amounts of liquids with a flow rate of 1 µl/min maintaining a precise and pulse-free flow control with a response time of up to 35 ms. Furthermore, adding the MUX valves to the setup allows for injection into microfluidic experiments, programmed perfusion, and sequential delivery of different reagents. It is possible to inject twelve different liquid samples from the reservoirs into one microfluidic line or, vice versa, inject one liquid sample into twelve different microfluidic lines in less than 156ms (Elveflow, 2022c, 2022d; Wong et al., 2018).

LOC devices

The design of the LOC devices allows the cells to settle at the bottom of the wells and for the renewal of media without flushing the cells out (Figure 5 and Figure 7). Delivery of nutrients and oxygen to the cells is accomplished via diffusion. These designs also reduce shear forces that can affect cells when flow is applied (Armistead et al., 2019; ibidi GmbH, 2021).

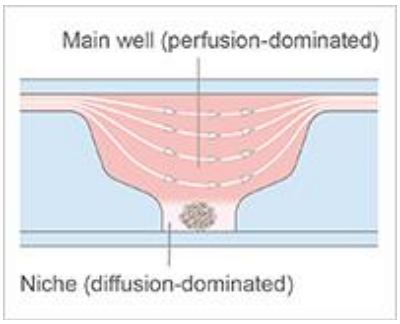

Figure 5. Continuous perfusion of medium in the main well and diffusion of nutrients to the niche without disturbing the sample. By ibidi ® (µ-Slide Spheroid Perfusion).

Although the designs of the LOC devices are based on the same principle, each one has its attributes as shown in Table 1. To acquire images, the Lionheart FX automated microscope must be configured each time a new labware is used. It needs the precise dimensions of the vessel (labware measurements), in this case, the LOC device, and of

each well (measurements and location). As the ibidi ® LOC device is commercially made, all the measurements are consistent. On the other hand, as the PDMS LOC device is fabricated manually, the dimensions and well locations vary slightly from device to device, so the microscope needs to be reconfigured each time a new PDMS LOC device is used (Agilent, 2022).

The commercial ibidi® LOC device has 21 wells distributed over three channels, while the custom-made PDMS LOC device has 39 wells within one channel that can be observed microscopically. Thus, three different conditions can be tested simultaneously in the commercially available one as opposed to just one in the custom-made device, but this design can be easily modified as per requirement to have more channels (Figure 6).

Figure 6. µ-Slide Spheroid Perfusion from ibidi ® (left) and PDMS LOC device (right).

The seeding processes are also different. The ibidi ® LOC device has a separate coverslip, which allows for individual seeding of each well before sealing the channels for flow application. The PDMS LOC device allows just one seeding process, as it has only one channel that distributes to all wells. In addition, cells can be easily recovered from the ibidi ® one at the end of the experiment, as the coverslip can be removed so the content of each well can be recovered separately. On the contrary, in the PDMS device, cells can be recovered only by applying pressure, so all cells will be recovered at once.

The seeding process also affects the distribution of cells inside the wells. Well by well seeding in the ibidi device ensures a known number of cells per well as opposed to the PDMS device where the number of cells per well varies. The shape of the well bottom also plays a role in how the cells settle in the well. In ibidi ® LOC device, the bottom is flat, so cells are distributed randomly, producing a heterogeneous distribution. In the case of the PDMS LOC device, as the wells are concave (U bottom), the cells tend to accumulate in the center, producing a homogeneous distribution. (Figure 7).

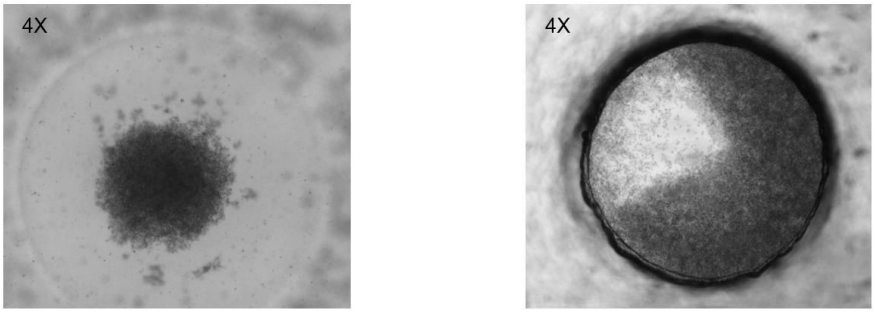

Figure 7. Distribution of cells in the PDMS LOC device (left) and in the ibidi ® LOC device (right).

Regarding the material, both LOC devices use biocompatible polymers. As stated in the name, the custom-made device uses PDMS, which makes it possible to reuse it by applying an adequate cleaning protocol. Many LOC devices are made of PDMS, as it shows fine biocompatibility and transparency for microscopy applications. It has been reported that PDMS can absorb small molecules, which can bias the results of experiments. As it has not yet been fully clarified whether the properties of PDMS influence cell culture in a negative way, it is so far the most commonly used substance thanks to its convenient prototyping suitability. Other types of biomaterials available are polystyrene or polylactic acid. In the case of the ibidi ® LOC device, it uses a proprietary bioinert synthetic polymer that allows one use only (Velvé Casquillas & Houssin, 2022).

Table 1. Comparison between LOC devices, custom made (PDMS) vs. commercial (ibidi ®).

CHIP / CHARACTERISTIC	PDMS	IBIDI
MICROSCOPE CONFIGURATION	Variable (Personalized for each chip)	Constant
WELLS	39	21
INLETS	1 (single channel) *	3 (separate rows)
COVERSLIP	non-removable / fixed	separate
WELL BOTTOM	U bottom	Flat bottom
RECOVERY OF CELLS	Difficult (Pressure-dependent)	Easy (Coverslip can be removed)
DISTRIBUTION OF CELLS PER WELL	Homogeneous (Accumulation in the center)	Heterogeneous
REUSABLE	Yes	No

*Modifiable

Considering all these characteristics and analyzing the pros and cons of each design, the next steps of the research were performed using the ibidi ® LOC device.

Adequate medium to cultivate Jurkat cells

To establish an adequate medium to cultivate Jurkat cells, experiments were performed using a 96-well plate. The culture medium with a range of serum concentrations and also the influence of poly-L-lysine coating was tested.

By comparing RPMI (Figure 8 A and B) and adv. RPMI (Figure 8 C and D), the second one was more efficient, as more area was covered by cells. Even without serum, the area covered by cells increase similarly compared to serum medium. Adv. RPMI contains ethanolamine, glutathione, ascorbic acid, insulin, transferrin, AlbuMAX™ I lipid-rich bovine serum albumin, trace elements of sodium selenite, ammonium metavanadate, cupric sulfate, and manganous chloride, which allow serum reduction with no change in growth rate or morphology of cells (Thermo Fischer Scientific, 2022). This reduction also helps to have more reliable results as FBS can interfere with or activate T-cells.

In the case of the poly-L-lysine coating, the treated wells (Figure 8 B and D) present different results. When cultivated with RPMI, cells in the coated wells cover more are that the cells in the untreated ones. On the contrary, with adv. RPMI, cells in the uncoated wells cover more are that the cells in the treated ones. This may be due to the fact that when using the Incucyte® it is not possible to obtain images of the entire well, so the cells in suspension move and change position, obtaining images of different cells at each time point. Considering these results, the medium selected for the next experiments was adv. RPMI with 4% FBS, which allows for the addition of growth factors if need arises. Since the entire well could be imaged, slight changes in the position of cells during imaging did not affect the analysis. Therefore, the wells of the LOC devices were not coated with poly-L-lysine for further experiments.

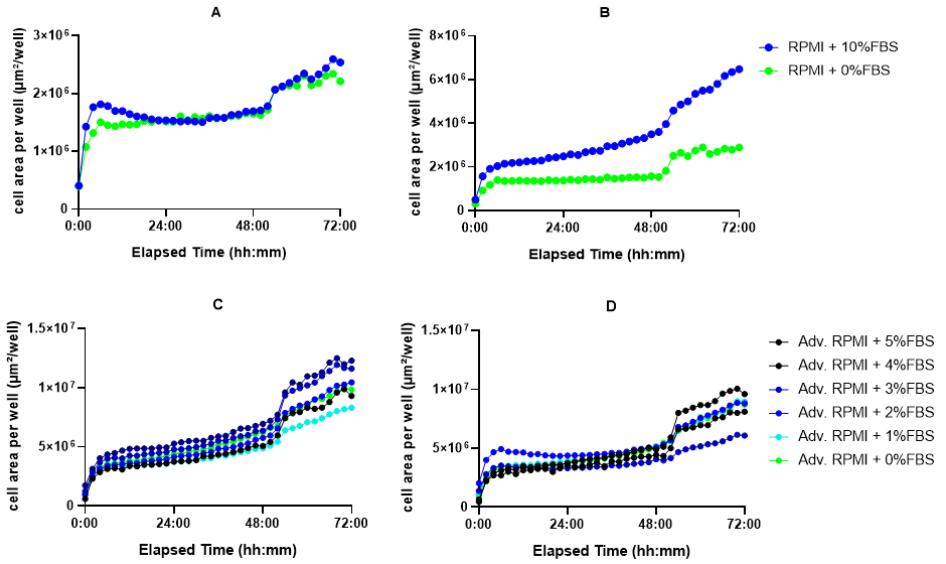

Figure 8. Comparison between RPMI and advanced RPMI with different FBS concentrations. A. RPMI uncoated, B. RPMI coated with poly-L-lysine, C. adv. RPMI uncoated, D adv. RPMI coated with poly-L-lysine.

Flow conditions

Several flow rates were tested in the ibidi ® LOC device. The capacity of the MFS flow sensor used in the microfluidic system goes from 2 µl/min to 80 µl/min. It was found that at the highest flow rate allowed, the cells were not removed from the well. This confirms the fact that the design of the LOC device allows high flow rates without disturbing the cells or influencing their behavior (ibidi GmbH, 2021).

To set the adequate flow conditions for the experiment, two flow conditions were tested, 50 µl/min for 2 min (channel A) and 10 µl/min for 5 min (channel B). The chosen flow parameters ensured that the total channel volume (45 µl) was completely exchanged. As seen in Figure 9, both flow rates influence the cell behavior similarly.

The faster flow rate (50 µl/min for 2 min) was chosen for the next experiments based on the fact that the medium exchange is faster with fewer fluctuations in the flow rate. Besides, as the cell density was too low (100.000 cells/ml = 200 cells/well) and was difficult to visualize cell proliferation, for the next experiments, the cell density was increased to 500.000 cells/ml (i.e., 1000 cells/well).

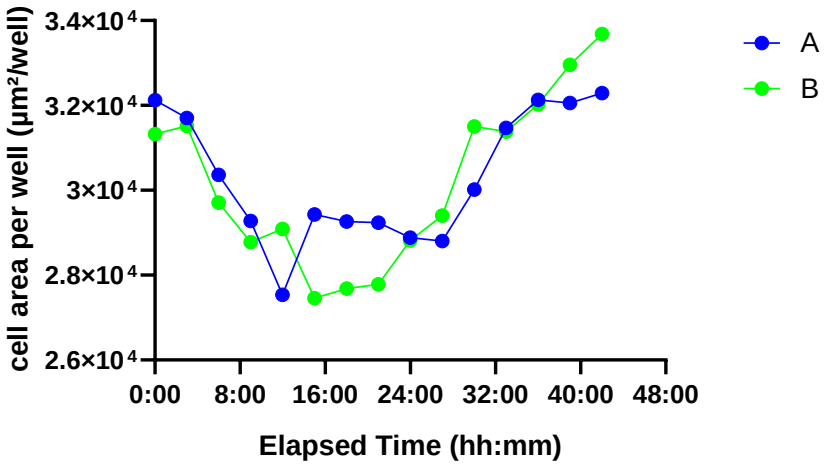

Figure 9. Comparison of flow rate influence in cell proliferation 50µl/min for 2min (channel A - blue) and 10 µl/min for 5min (channel B - green) performed in an ibidi ® LOC device.

Seeding Protocol

As mentioned in the 'LOC Devices' section, the ibidi ® LOC device allows two types of seeding. Figure 10 A shows the results of seeding by well, and Figure 10 B the result of seeding by channel. It can be seen how seeding by well gives a more stable starting cell number between wells and channels. On the contrary, when seeding by channel, the starting point diverges between wells.

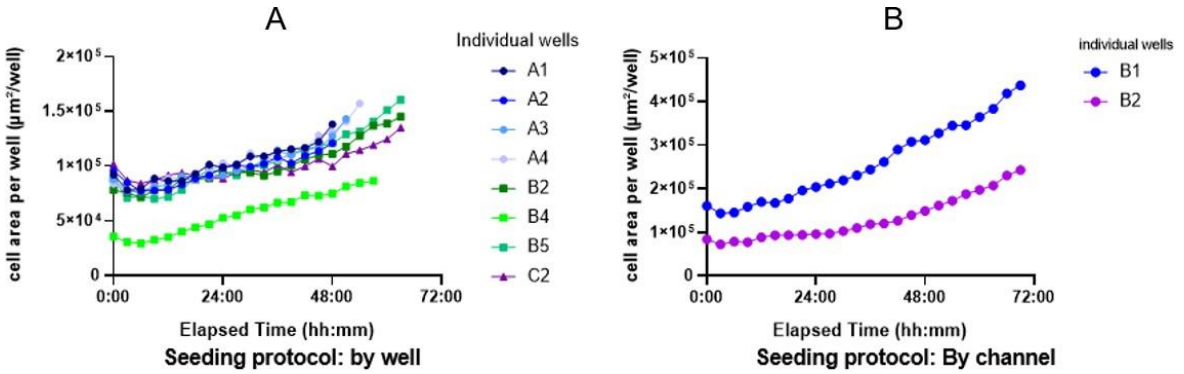

Figure 10. Comparison between seeding protocols A. Wells seeded individually, B. Seeding by channel

Furthermore, considering that the total well area is 5 x 10^5 µm^2 and using the values obtained from the image analysis (area covered by cells per well), the percentage of well area covered by cells was obtained. Although both experiments shown in Figure 10 were performed with the same initial cell density, Figure 10 A presents a coverage of 40% and Figure 10 B a coverage of almost 90% at the end of the assay. This confirms how cells react differently and have distinct proliferation rates although they are grown under the same conditions (Vormittag et al., 2018).

An assay was performed in an ibidi ® LOC device using different initial seeding densities in each channel, 1 x 10^5, 2.5 x 10^5 and 5 x 10^5 cells/ml. In the case of the first densities, the quantity of cells was too low, and no cells were visible. Due to this, the minimum initial seeding density established was 5 x 10^5 cells/ml.

Using the minimal initial seeding density (5 x 10^5 cells/ml), a 6-day experiment was performed. As seen in Figure 11, at the end of the experiment, the entire area of the well was covered with cells. The cell viability was determined by applying the trypan blue protocol using the Bio-Rad TC10TM (Bio-Rad, USA) automated cell counter, the starting value was 70%, but after 144 h it raised to 92%. As perfusion was applied every 3h, the continuous exchange of nutrients and gas allowed the possibility of long-term experiments **and a better environment for cells to grow.**

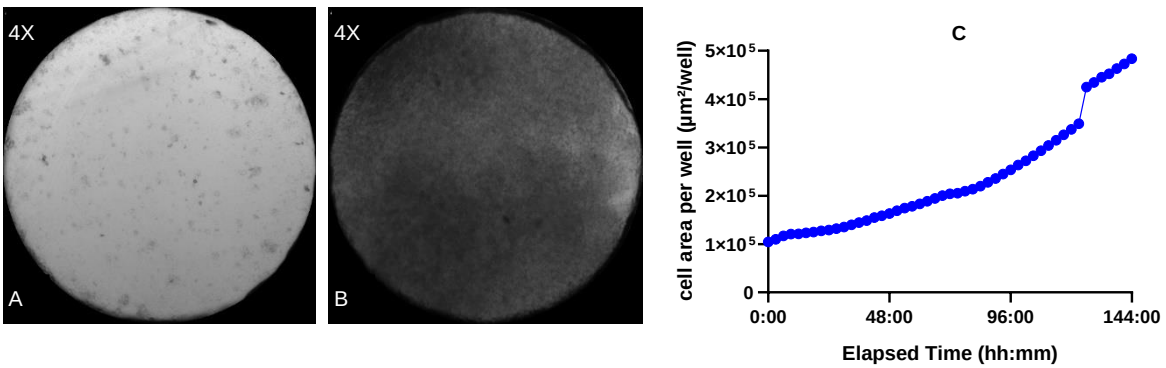

Figure 11. Long-term (6-day) experiment with Jurkat cells. A. Image at 0h, starting condition. B. Image at 144h, full area covered by cells. C. Average of the area covered by cells per well in one channel (7 wells) in an ibidi ® LOC device.

Validation of the system

To validate the system, the LOC device was compared to a 96-well plate and the expansion of Jurkat T cells was measured by the area covered by cells. The fold expansion was calculated by dividing the number of cells present at the end of the culture (72 h) by the number of cells at the beginning of the culture (0 h). In a 96-well plate, 200 µl of medium per well containing 20 000 cells was distributed, but in the ibidi ® LOC device, only 2 µl per well containing 1000 cells was used. In Figure 12 we ca see that the fold expansion seen in the 96-well plate is greater, but the difference was not significant. This confirms that the use of LOC devices in an experiment can be beneficial as the expansion of cells is similar to the commonly used labware, but with the advantage of using less medium and sample volumes.

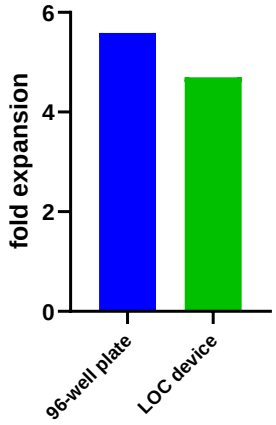

Figure 12. Comparison between the fold expansion of Jurkat cells in a 96-well plate and in the ibidi ® LOC device after a 72 h culture.

In Figure 13 we can see how the expansion of cells in both platforms is similar. By comparing Figure 13 A and B, the last one shows a more stable tendency line.

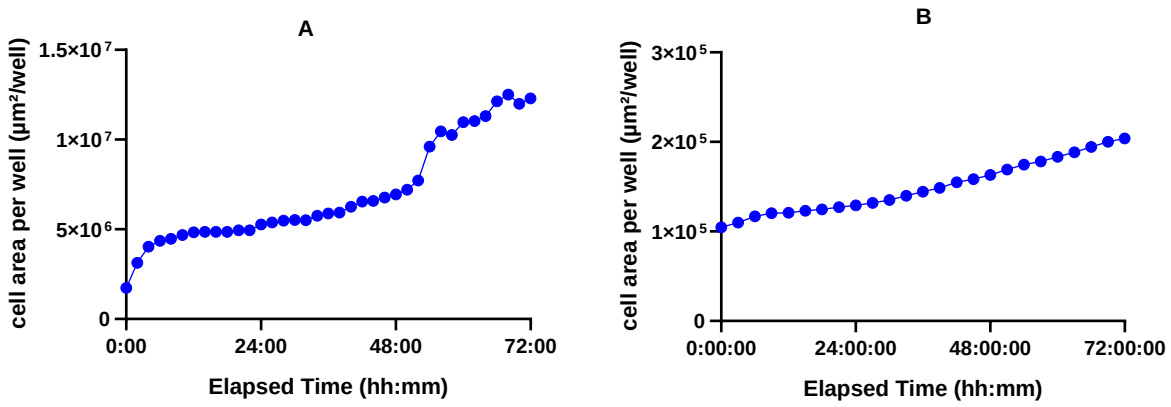

Figure 13. Area covered by cells during a 72-h experiment. A.96-well plate, each time point shows the average value of a row (6 wells). B. ibidi ® LOC device, each time point shows the average value of a channel (7 wells).

For the coating assay, Poly-L-lysine and Poly-D-lysine were tested in the LOC device. As seen in Figure 14, the coated channels have less area covered by cells. Poly-D (or L)-Lysine is a synthetic positively charged polymer that strongly captures cells and has been used for imaging suspension cells. New studies show that, although polylysine is an inert compound, it can alter the activity of membrane proteins such as the T-cell antigen receptor (TCR) and also by fixing the cells to the bottom, it prevents the cells from forming rosette like structures so, proliferation is slowed down (Santos et al., 2018). When comparing to the assay performed in a 96 well plate, the same tendency is observed in the experiment with adv. RPMI (Figure 8 D), but not with the one with RPMI (Figure 8 B).

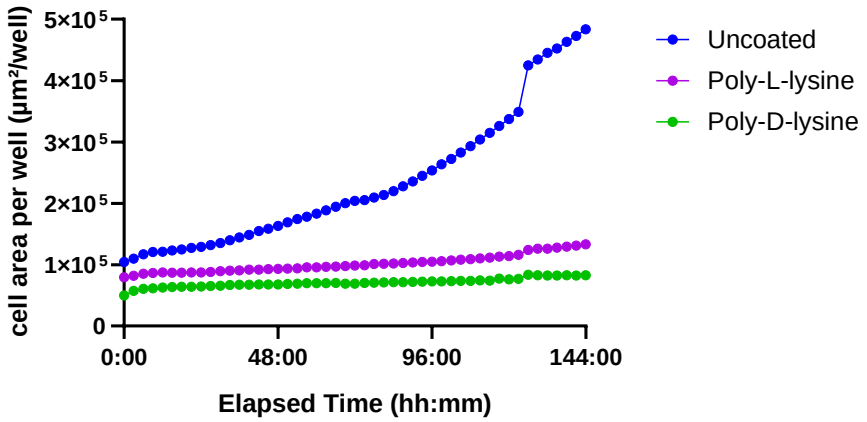

Figure 14. Area covered by cells during a 6-day assay using Poly-L-lysine and Poly-D-lysine coating.

In the case of the etoposide assay (Figure 15), we can confirm that this compound causes cell death in both platforms, proving that the LOC devices are as efficient as the 96-well plate.

Etoposide is a topoisomerase II inhibitor widely used in model studies for apoptosis. Although Jurkat T cells lack both p53 and Bax, essential proteins for apoptosis, it can bee seen how they are sensitive to the compound, confirming the results found by Karpinich et al. (2006).

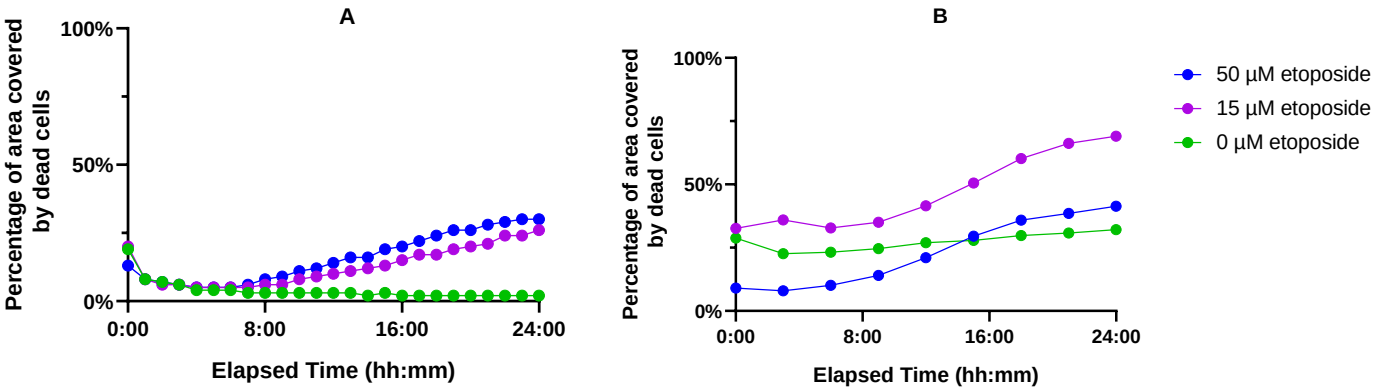

Figure 15. Percentage of dead cells during the etoposide assay in A. 96 well plate and B. ibidi ® LOC device.

The principal reason for fluctuation between results in both platforms as seen in the previous experiments is the characteristics of the wells. The Incucyte® Live-Cell Analysis System was used for imaging the 96-well plate, which takes 4 images per well at 20 x. Since for each image acquisition the system moves the stage to focus on each well, the cells in suspension will move from place to place, resulting in inaccurate coverage values. This also happens when imaging the ibidi ® LOC device with the Lionheart FX microscope, but as the wells are smaller, the entire well can be imaged at 4 x magnification so the analysis is more robust (Figure 16).

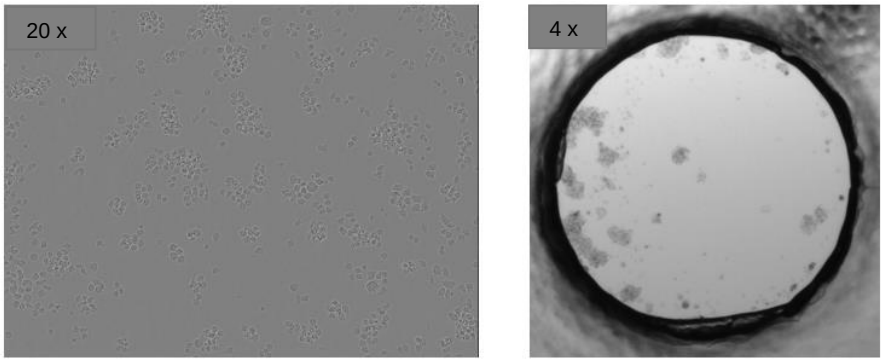

Figure 16. Comparison between the images taken from the 96-well plate (left) and the LOC device (right).

CONCLUSION

In this study, it was demonstrated that the proliferation of suspension cells, in this case, the leukemic Jurkat T cell line, can be achieved in the microfluidic system as proposed here, and it was validated by comparing it against the commonly used 96-well plate.

Regarding LOC devices, this labware is a closed cell culture system, which is preferred due to the assurance of sterility throughout the experimental processes. In addition to this, the one-way flow of fluids ensures that the culture process is free from contamination. LOC devices also work as a microfluidic bioreactor where different types of cells can be grown and maintained. Although both LOC device designs are similar, each one has its attributes. Depending on the objective of the assay, a different design can be used. The size of the LOC device also allows the use of a smaller initial seeding density than in a 96-well plate.

Although the expansion rate of cells is similar in both platforms, the microfluidic system has some improvements. As it uses pressure-driven flow control technology, the flow rate is pulseless and more precise, giving an advantage over syringe pumps or peristaltic pumps. Also, due to the precise flow control, it helps to reduce reagent wastage. Additionally, it is possible to connect multiple reservoirs and labware to the same platform, making it possible to parallelize experiments.

The system is also more robust than the conventional 96-well plate and also than other microfluidic systems. As almost all the assays can be automated, human intervention is reduced, decreasing the reproducibility issues. Likewise, as it is possible to image the whole well using an automated microscope, the results obtained using the microfluidic system are more reliable.

For future experiments, it is expected to work with primary cell lines to validate the system and test its uses in the protocols for CAR T-cells. The small/miniaturized setups presented here allow experiments to be conducted with a very small sample size, which, due to the small amount of patient material available, multiple growth conditions can be tested before scaling up.

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