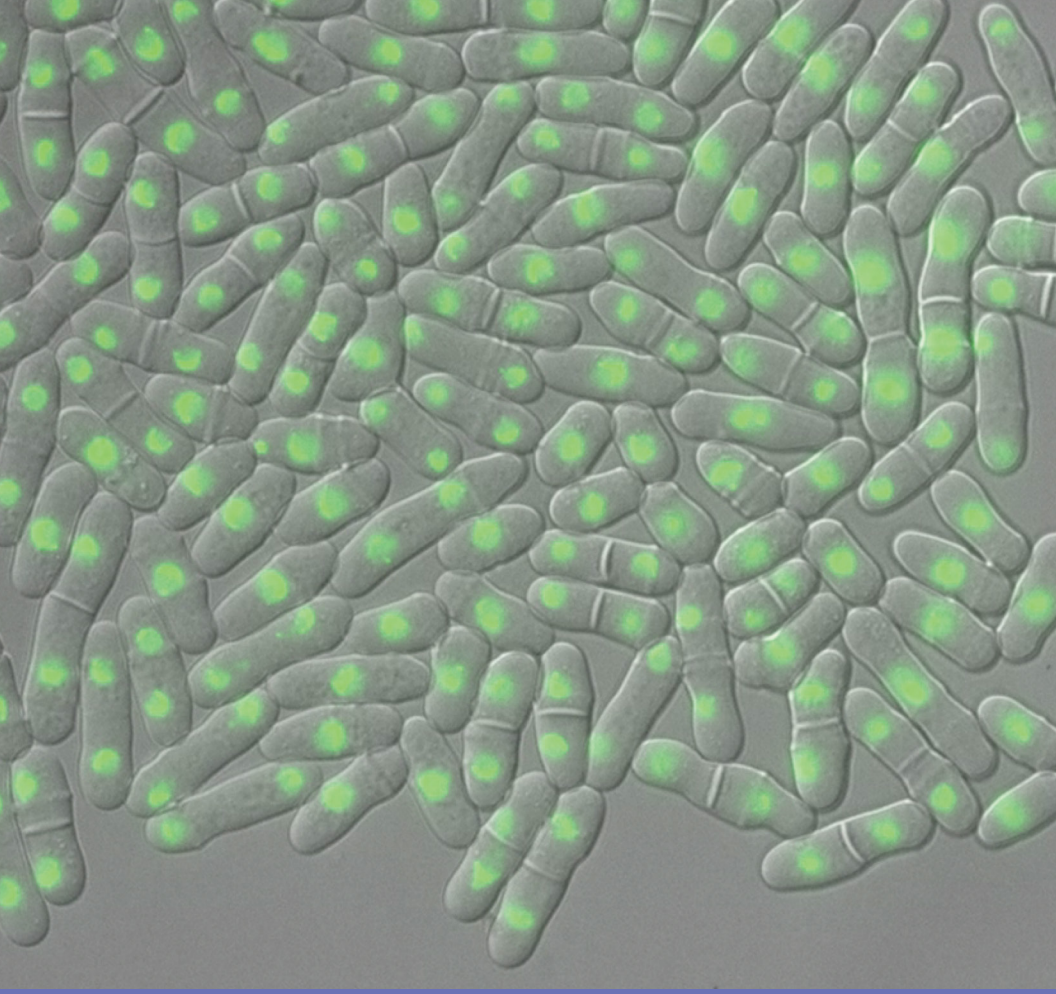

Microfluidics and Live-cell Imaging

Essential Knowledge Briefings

CURRENT PROTOCOLS
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- Intuitive software enables rapid setup of detailed protocols for truly automated, hands-free cell culture

Continuous culture for more physiologically relevant data

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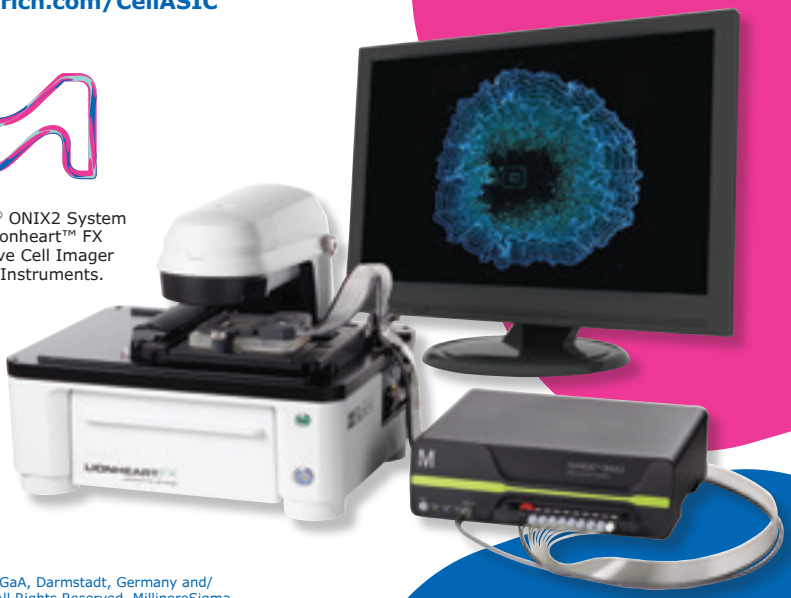

The CellASIC® ONIX2 System shown with Lionheart™ FX Automated Live Cell Imager from BioTek® Instruments.

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EKB Written by Gwen Taylor, Ph.D.

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INTRODUCTION

1.1. What is microfluidics in cell culture?

Researchers have been devising ways to grow cells in culture in controlled laboratory conditions since the late 1800s, and a staggering array of culture methods has been developed in the intervening years. Simple systems may consist of shaking bacterial cultures in a flask of growth media in an incubator, while more complex systems may involve growing eukaryotic cells in atmospheric environments that differ from the ambient laboratory air, e.g., hypoxic conditions (reduced oxygen, a common characteristic of cancer cells in the interior of solid tumors that is linked to chemo-resistance and poor clinical outcomes). Other culture variations include different physical environments, such as simple two-dimensional (2D) monolayer cultures grown in flasks, or three-dimensional (3D) cultures that more closely mimic the environment a cell would experience *in vivo*.

Attaining some of the more complex culture configurations can require specialized equipment and a healthy dose of investigator ingenuity to create a culture to mimic; for example, the microenvironment of a vascularized tumor. Regardless, cell culture had typically been designed on a scale convenient for manipulation by human hands, meaning that substantial amounts of reagents and laboratory space were involved. Such culture systems also are typically static and do not replicate the changing physiological parameters experienced by cells in their native environment.

Microfluidics encompasses systems that can precisely manipulate very small (microliter, nanoliter and smaller) volumes of fluids using channels, pumps and valves to control flow and mixing. Design and creation of microfluidic systems combines expertise from biology, biochemistry, engineering and physics as the behavior of fluids is very different at microscale compared to what is observed at the larger, bulk scale (see the section titled *Flow behavior of aqueous solutions at microscale* below).

Microfluidic cell culture involves devices and techniques for growing, maintaining and analyzing both adherent and non-adherent cells in microscale volumes. The channels in microfluidics devices are typically in the range of 100 nm to 500 µm in diameter, and systems have been created

to accommodate cells of all types, shapes and sizes. For comparison, eukaryotic cells typically have diameters between 7 and 100 µm, while prokaryotic (bacterial) cells range from 0.1 to 5.0 µm. Yeast cells, which are eukaryotes, can vary greatly in size, although most are 3 to 4 µm in diameter.

A microfluidic chip consists of a set of microchannels etched or molded into a material (glass, silicon or polymer). The microchannels are connected to create the desired features, for example, to mix, pump or modify the biochemical environment. This network of microchannels is connected to external features by very small holes pierced through the chip through which liquids or gases are injected and removed through tubing, syringe adapters or even simple holes in the chip. The flow of liquids and gases is controlled either with external active systems (e.g., pressure controllers or pumps) or passively (e.g., hydrostatic pressure). Figure 1 shows one type of commercially available polymer-based plate.

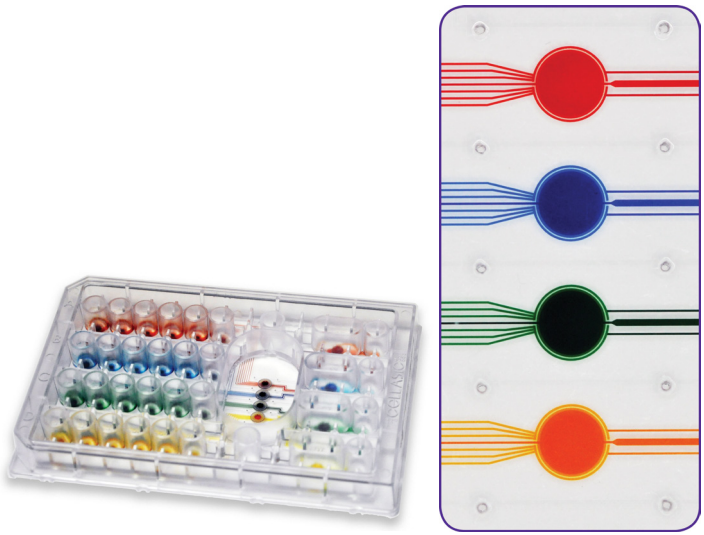
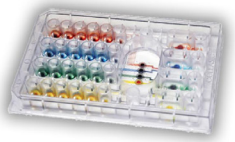


Figure 1: CellASIC® (MilliporeSigma) microfluidic plate and magnified view of four culture chambers and associated channels.

The number and shape of the channels and culture chambers in a microfluidic system are typically chosen based on the type of cells under study. For example, chambers and channels can be configured to grow single neurons such that the soma is isolated from axons, enabling spatially restricted studies of nerve injury. Another example is culture plates that have low ceilings that are used to grow monolayers of cells in a single focal plane. Also, if a perfusion system is to be used, channels will need to be incorporated in order to add or remove media and cells.

In microfluidic devices, the goal is to create more *in vivo*-like systems by controlling the microenvironment (e.g., cell matrix, atmosphere, flow rate, chemical gradients, pH, temperature). This control is more easily achieved and maintained in microfluidic devices compared to traditional instruments for several reasons discussed below. As a result, microfluidic technologies for cell-based assays have the potential to increase the biological relevance of cell models while maintaining or increasing the throughput of current methods.

Advantages of microfluidic systems over conventional-scale culture



Requirement	Conventional Cell Culture	Microfluidic Cell Culture
Temperature/Gas Control	Crude control due to large liquid volumes	Dynamic and precise control via small volumes
Nutrient and metabolite exchange	Infrequent manual exchange of large media volumes	Consistent and precise exchange of media
Monitoring of cellular responses through imaging.	Largely unfeasible	High capability
Parallelization of assays.	Largely unfeasible	High capability
Automation of cell culture maintenance	Expensive and requires large specialized equipment	High capacity using compact and inexpensive components
Single cell analysis and experimentation	Largely unfeasible	High-throughput capacity.

Table 1 presents some basic cell culture requirements and describes advantages microfluidic systems hold over conventional culture systems (e.g., Petri dishes, culture flasks or multiwell plates).

Apparatus design flexibility

One advantage of microfluidic devices over conventional culture systems is in the flexibility of equipment design, which can be tailored for individual cell types. Cell health can be affected by the configuration of the platform and operating conditions, and it is important to design the microfluidics system for cell culture in a manner that minimizes the exposure of cells to damaging stressors. For example, abrupt geometries should be avoided as they tend to favor bubble formation which can clog channels and even cause cell death at the gas-liquid interface, while channels that are deep and wide preclude the development of shear stress. Varma and Voldman (2018) give practical guidelines regarding device design and operation to minimize cell stress along with recommendations for standardizing assessments of cell health.

Perfusion and flexibility of liquid handling

In typical static cultures, the medium is applied to the cells in a batch-wise manner. While this approach is economical and simple, it presents problems for long-term cell culture, including a high risk of contamination due to repeated manual interventions and variation of the cell environment. Perfusion culture provides a more sterile, stable and quantifiable culture environment as a result of continuous nutrient supply and waste removal.

Microfluidic systems can be designed to deliver continuous perfusion to cells to introduce soluble factors or to create chemical gradients for motile cells. Perfusion culture provides the additional advantage of allowing long-term study under a microscope. For live-cell imaging with microfluidics, it is critical to be able to replace culture medium without opening the perfusion chamber to prevent evaporation and to maintain precise control over environmental variables, such as temperature and pH. Closed perfusion chambers are typically designed with ports to allow addition and removal of solutions during the experiment.

Reduced reagent consumption

Microfluidic cell culture simultaneously offers reduced consumption of reagents, reduced contamination risk and efficient high-throughput experimentation. In addition, the greatly reduced amount of fluids needed can dramatically reduce the time and cost of both culture and analysis, making these systems particularly useful for cell-based assays. Table 2 shows differences in several parameters among various cell culture methods.

	Surface Area (cm^2)	Seeding Density (cells per volume)	Growth Media Volume (mL)	Media Exchange (hr)
Dish (35mm)	9	0.3×10^6	2	48-96
12 well culture plate	4	0.1×10^6	1-2	48
T-25 Flask	25	0.7×10^6	3-5	48-96
CellASIC® ONIX M04L-03 plate	0.32	0.125 x 10^6	0.005	0.5-1*

Table 2: Comparison of Conventional and microfluidic cell culture critical parameters.

Automation

A goal in many experimental designs is to automate as much of the process as possible, both to allow the researcher to gather more data over a given period of time, and to minimize the potentially deleterious effects of manual intervention in procedures. Microfluidics is ideally suited for automation. For example, Zhang et al. (2019) created an ultra-multiplexed microfluidic system that combined multimode cell culture (single-cell, 2D and 3D), generation of dynamic chemical inputs, and 1500 individually addressable cell culture units on a single device. The system can perform programmed delivery of thousands of fluidic inputs to designated on-chip culture units while monitoring and analyzing cellular responses via live-cell microscopy and end-point biochemical analysis methods. In a typical 1-week-long experiment, this system tracks ~30,000 individual cells cultured under 1500 dynamic individual conditions by performing ~106

pipetting steps with nanoliter precision and creates millions of single-cell data points. That's some automation!

Current microfabrication techniques require a significant amount of engineering knowledge and facilities that put homemade microfluidics technology out of reach for many researchers.

Although investigators with significant engineering and physics expertise can create their own systems, there are commercially available, turn-key microfluidics culture systems available for researchers who have less specialized backgrounds. The most complete systems combine microfluidics plates for adherent and suspension cell phenotypes in sizes ranging from yeast to large mammalian cells, along with a manifold for controlling environmental parameters that is controlled by intuitive software. All-in-one systems such as these democratize the advantages of microfluidic cell culture for a wider swath of researchers by eliminating the need for engineering expertise to accommodate the goals and *in vitro* models of the individual lab.

3D culture

The more closely a cell culture system can replicate the cells' native, *in vivo* conditions, the more closely those cells will replicate the behaviors and responses of cells in the body. There are many significant differences between 2D (monolayer) and 3D culture, but one of the most prominent is the mechanical environment. The stiffness of glass or plastic is much higher than that of soft tissue, and this directly affects the cells' ability to adhere, spread, migrate, and differentiate. Microfluidic systems are very amenable to 3D culture to allow physiologically relevant cell growth and easy microscopic observation. Castieux et al. (2019) review microfluidic 3D culture techniques, as well as integration of detection schemes into these systems.

Control of gases in microculture

The gaseous microenvironment plays an important role *in vivo*, but it is challenging to precisely control gas levels *in vitro* for cell culture. However, technological advances in microfluidics have led to the development of new ways to manipulate and control cellular microenvironments for *in vitro* cell studies. Wu et al. (2018) review recent developments for the control of gaseous microenvironments in microfluidic cell culture devices and discuss the advantages and limitations of current devices.

There are also commercially available microfluidic systems that allow researchers with limited experience to set up gas-controlled microscale cell culture experiments and begin collecting data very rapidly (e.g., the CellASIC® ONIX2 system from MilliporeSigma). The CellASIC® system allows control of gas composition, flow rate and pressure, as well as dynamic switching of any of these parameters during an experiment.

Creation and maintenance of chemical gradients

Cell migration is stimulated and directed by the interaction of cells with the extracellular matrix, neighboring cells, or chemoattractants. This migration plays a fundamental role in a large array of processes including gastrulation, neural development, wound healing and tumor metastasis.

There are commercially available microfluidics devices that capitalize on the laminar behavior of fluids at microscale to produce stable gradients for prolonged periods of time. One such device, designed for use on the CellASIC® ONIX microfluidic platform, enables precision-controlled chemoattractant diffusion across perfusion barriers to create a gradient in the culture-viewing area (Figure 2). Perfusion inlets and outlets form a continuous-flow "infinite source/sink" that maintains a stable concentration gradient profile for days, all the while also allowing live-cell imaging. The flexible format of the plate enables changes in gradient directionality, turning gradients on and off, and toggling between gradient and single-solution exposure.

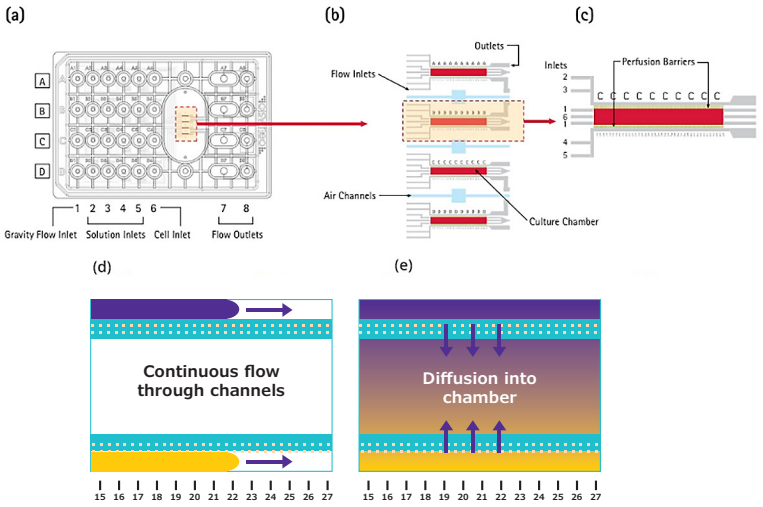


Figure 2: (a) The CellASIC® ONIX M04G Microfluidic Plate has four independent culture chambers (A-D). (b) All four culture chambers are located under a single viewing window. (c) The chamber is bound by perfusion barriers on the top and bottom edges to separate the chamber from flow channels. Gradients are established by simultaneously flowing media of different compositions through the upper and lower channels. (d) Due to continuous perfusion, a stable gradient can be maintained for extended periods (>2 days). Figure from MilliporeSigma.

In addition to the commercial options for microfluidic gradient-forming devices, there are numerous examples in the literature of custom-made devices (e.g., Ayuso et al., 2019; Schwarz et al., 2016; Chang et al., 2014).

1.2. Theory behind the combination of microfluidic cell culture with live-cell imaging

Most live-cell imaging studies involve the use of either intense illumination or fluorescent probes and reporters in conjunction with various types of microscopy. However, one of the most significant challenges in live-cell imaging is to overcome photobleaching and phototoxicity while maintaining cell health. **Photobleaching** (fading) is the process by which a fluorescence-emitting molecule is chemically altered by high-intensity or prolonged illumination such that it will be irreversibly unable to fluoresce. **Phototoxicity** is the process by which the imaged cells are damaged upon illumination with high laser power or for prolonged periods. Both phe-

nomena are a result of high-energy electrons from fluorescent excitation not being released as photons, but instead reacting with oxygen to produce reactive oxygen species, which cause toxic effects. Phototoxicity can cause cell death. Both photobleaching and phototoxicity can negatively influence cell survival and data quality.

Most cells and many cellular processes are negatively affected by excessive light. Therefore, the light used for imaging should be kept to a minimum and any unnecessary exposure reduced as much as possible. Using bright, photostable fluorescent proteins is beneficial to minimize the likelihood of affecting biological processes.

In addition to minimizing photobleaching and phototoxicity, there are several imaging-related parameters that need to be optimized to produce the highest quality data throughout a microfluidic cell-based experiment.

- Use microfluidic culture plates that have optical-quality glass bottoms to achieve high-quality microscopic imaging.
- The cell media and perfusion system must not only provide the physiological conditions necessary to keep the cells alive, but also have sufficient clarity to produce the required image quality and an optimized signal-to-noise ratio.
- The imaging system must be compatible with the microfluidic system to permit image acquisition, while maintaining incubation conditions to keep cells healthy.

1.3. How is live-cell imaging different from endpoint imaging?

Live-cell vs. endpoint imaging

Endpoint images of static cell culture experiments can be of live or fixed (dead) cells, whereas live-cell imaging is a non-destructive method that focuses on the observation of live cells in real time, and over time. Endpoint images are most useful for experiments that do not involve much cell movement. However, live-cell assays are particularly well suited for the assessment of fast signaling responses and experiments where multiple cell

types interact, which require both spatial and temporal data to interpret the interactions. For live-cell imaging, the researcher needs several tools: reagents to keep the cells alive over the observation period, dyes or other markers to label specific molecules or structures while maintaining viability, an incubator to provide the right environment, plus a microscope and a digital imaging system. The necessary hardware can be assembled from individual components, but there are also ready-to-use, comprehensive solutions commercially available.

Physiological relevance

Traditional endpoint measurement techniques typically use fixed cells and require staining or artificial labelling. Some cell-staining protocols require initial cell membrane permeabilization to allow larger molecules such as antibodies to access the interior of the cell. This process results in loss of the soluble contents of the cell. These artificial conditions mean that the resulting data do not reliably represent physiologically relevant cellular responses. Live-cell imaging allows cellular structures, processes, behavior and function to be studied in their native environments, making the resulting data less prone to experimental artefacts.

Efficiency/time

Live-cell imaging can also streamline experimental workflows. Controls and treated samples can be placed in the same plate and followed over an extended period of time. This approach can dramatically reduce the experimental set-up time and complexity, as well as the number of samples required compared to endpoint experiments. In addition, live-cell imaging typically does not require the calibration that endpoint assays need, which can also save time.

Data storage and processing power capabilities

Endpoint imaging typically results in one or a few static images acquired at the end of an experiment. Live-cell imaging generates large numbers of images over extended time periods. This means that the live-cell imaging system must have sufficient data storage capacity for potentially hundreds of thousands of large image files, as well as substantial processing power for image stitching and image analysis software.

Fixing and staining

Cells can be difficult to observe under a microscope without first being stained, and most stains kill cells. Endpoint assays often employ fixed and stained cells as the fixing process protects and stabilizes gross structures at a specific timepoint. Additionally, cells may need to be fixed if the sample is to be analyzed by more than one technique and it is important to ensure there is no cell movement or degradation between the different modes of measurement. However, fixing cells not only kills the cells, but can also introduce artefacts. For example, prolonged fixation can chemically mask epitopes and prevent antibody binding for immunohistochemistry.

Fortunately, there are new generations of dyes and stains that label live cells with little to no deleterious effects on cell health. There is a vast array of dyes that specifically label organelles and other cell structures (e.g., DNA, endoplasmic reticulum, Golgi, actin filaments, lysosomes, mitochondria and others). There are also cell-permeable live-cell dyes for applications such as apoptosis detection, cell viability and hypoxia. Several dye suppliers have excellent websites to help researchers select the best dyes for their needs.

Phototoxicity

Many cells and tissues are never exposed to light *in vivo*, and high doses of light can damage DNA, raise cellular temperatures, or cause phototoxicity. Therefore, the tools used for live-cell imaging must be as gentle as possible, especially over multiple or long exposures (also *see the section on Theory behind the combination of microfluidic cell culture with live-cell imaging above*). This translates into using a microscope and detection system that can render images from the least light possible. The signal-to-background (noise) ratio can be optimized by using reagents that reduce extracellular fluorescence and increase fluorophore photostability. It is also important to use media specifically formulated to maintain cell health during live-cell imaging while reducing or eliminating background fluorescence. The addition of a background suppressor can help reduce extracellular background fluorescence, and antifade mounting media for live cells can be employed to reduce photobleaching.

HISTORICAL BACKGROUND

2.1 Brief history of microfluidics, including challenges and limitations

Historical background

The field of microfluidics arose from four primary disciplines: molecular analysis, biodefense, molecular biology and microelectronics. The field really began with microelectronics, where the processes of photoengraving and photolithography were instrumental in the construction of transistors in the 1940s and 1950s. Inkjet technology built on these achievements and Richard Sweet's inkjet printer, built in 1965, was the first true microfluidic device. Stephen Terry and his colleagues at Stanford extended microfluidic technology into the world of molecular analysis and developed the first miniaturized gas chromatograph in 1979 that allowed the separation of chemical compounds by flowing small amounts of a sample through narrow tubes or capillaries. High-pressure liquid chromatography (HPLC) and capillary electrophoresis (CE) technologies followed in the coming decades, which revolutionized chemical analysis.

In the 1980s, the foundational research for 3D printing was being laid and substantial advances were made in the design and manufacture of valves, mixers and pumps for microfluidics systems.

In the 1990s the Human Genome Project was ramping up with the goal of mapping the entire human genome within 15 years. It rapidly became evident that the current technologies were not going to be sufficient to reach this goal. Researchers began investigating scaled-down solutions and materials other than silicon to facilitate DNA-sequencing technologies that required optical transparency for light-based detection methods. Capillary electrophoresis (CE) and CE arrays were developed in the early 1990s that allowed separation of DNA based on size, a crucial step in DNA sequencing.

Starting in 1994, the United States' Defense Advanced Research Projects Agency (DARPA) substantially contributed financially to the growth of microelectromechanical systems (MEMs) and the development

of miniaturized and portable "laboratories on a chip" with the main goal of detecting chemical and biological weapons. These programs were the main stimulus for the rapid growth of academic microfluidic technology.

Also in the 1990s, the first microfluidic chip-based polymerase chain reaction (PCR) system was developed, which allowed researchers to incorporate sample preparation as well as detection and analysis into a single microfluidic device. PCR is a DNA amplification technique in which an enzyme is used to rapidly amplify as little as a single molecule of DNA in a very small reaction volume by several orders of magnitude via temperature cycling.

In the early 2000s, investigators began creating microfluidic chips in which cells were seeded in 3D cultures to recreate the microenvironment and microarchitecture of a specific human tissue or organ (organ-on-a-chip). There are many published papers about linking these types of chips together to create "human/body-on-a-chip" systems. A goal of this field is to use these devices to replace animal testing in drug development.

A major advancement in microfluidic device construction was achieved in 2006 with the development of the elastomer poly(dimethylsiloxane) (PDMS) by George Whitesides and his group at Harvard. PDMS has many advantageous characteristics for fabrication and continues to be the most commonly used material for microfluidics devices.

Flow behavior of aqueous solutions at microscale

It is important to understand the physics of fluids at microscale. Fluids (which is water for most biological applications) behave quite differently at very small scales (microliters, nanoliters, even picoliters (10^{-12} liter)) compared to bulk fluids in many aspects, including surface tension, evaporation, flow and mixing. On larger (macro) scales, fluids mix convectively. An example of this type of mixing is what is observed when milk is added to coffee. In a microfluidic environment, water moves in laminar (smooth) flow. An example of laminar flow is that of honey through a pipe. When

two laminar fluid streams come together in a microchannel, they flow in parallel, without turbulence, and the only mixing that occurs is a result of diffusion of molecules across the interface between the fluids, which is a slow process.

Surface tension and capillary forces dominate gravity for fluid movement at microscale. At macroscale, pressures well above or below atmospheric pressure and gravity dominate fluid dynamics, while surface tension and capillary forces are essentially negligible. At microscale, this behavior is reversed. As volume decreases, the surface area-to-volume ratio increases, so surface tension and the capillary effect dominate the fluid mechanics. Controlling these forces is a primary goal of microfluidic systems.

2.2 Brief history of live-cell imaging techniques

One of the first examples of live-cell imaging was a time-lapse video made in 1907 by the Swiss biologist, Julius Ries. He created a 2-minute film of images acquired over 14 hours of observing the fertilization and development of a sea urchin egg. Optical (light) microscopes were the major tools available until the 1940s when the phase contrast microscope revolutionized live-cell imaging as it allowed researchers to view unstained, living cells in detail. Previously, cells had to be stained to be viewed through a light microscope and stains typically kill cells. The subsequent development of quantitative phase contrast microscopy, fluorescent microscopy and numerous other types of microscopy along with a vast array of dyes and fluorescent proteins has given researchers a vast array of tools with which to observe living cells. Holotomographic microscopy is a recently developed technology that uses a laser and refractive index measurement to produce label-free quantitative images. This is a major advancement in that phototoxicity and other staining-derived disadvantages are non-issues due to digital staining based on cells' refractive index. However, it is very new and not yet widely used.

The process of imaging a biological sample damages the sample to some extent, so live-cell microscopists face challenges not encountered

by those working with fixed cells. Illumination wavelength and photon dose are two of the most important imaging parameters to consider when performing live-cell imaging. There should be stringent control of both the wavelength(s) and the total photon dose delivered to the sample. The efficiency of the detection system is equally important, such that the lower the detection efficiency, the more illuminating photons will be required to produce an image. Experts recommend aiming for having healthy cells rather acquiring than the best images, because beautiful images often make for unhealthy cells.

Maintaining homeostatic temperatures (37°C for human cells) during imaging is also critically important. Many cells types will stall and even reverse the cell cycle at 20° to 22°C, and between 23° and 35°C, the timing of mitosis is altered, indicating stress on the cells. There are two ways to control the temperature of a cell culture for live-cell imaging. One employs a heated specimen holder that attaches to the stage of the microscope, and the other involves enclosing the entire microscope in a heated box. Both configurations require sometimes complex solutions to allow the investigator to monitor and maintain the other critical parameters of the culture, namely the composition of the gas environment (O2/CO2), humidity, pH and osmolality.

Individual cells display substantial heterogeneity, but this variation is eliminated by population-average measurements. However, with live-cell microscopy, data can be collected from a single cell, or from many cells in parallel, thereby providing direct information about cell heterogeneity. Live-cell imaging approaches also provide other advantages, including the ability to make measurements at the temporal frequency necessary to sample the dynamics of most biological processes. Such real-time, single-cell measurements eliminate artefacts that can arise from attempting to reconstruct time courses from snapshots of different cells taken at different times. Snapshot measurements cannot distinguish temporal from cell-to-cell variability.

CURRENT MICROFLUIDICS LIVE-CELL IMAGING TECHNOLOGIES

3.1 What is available?

For researchers who want to design their own microfluidic chips for specific applications, soft photolithography describes a collection of methods for fabricating custom chips/plates with channels and chambers. It is "soft" because it uses elastomeric (elastic) materials as opposed to rigid silicon or glass. Microscale features are created on the elastomeric polymers using stamps, molds and photomasks. Investigators can also design custom microfluidic chips that are then fabricated by commercial services.

Babic et al. (2018) describe an easy-to-build and re-usable microfluidic system for live-cell imaging using liquid silicone rubber for fabrication of the microfluidic chip. The system can be constructed without the need for extensive technical expertise or microfabrication equipment. Similarly, Chen et al. (2016) developed PDMS-free microdevices for live-cell imaging in an effort to avoid the biomolecule adsorption sometimes experienced with PDMS. Their platform also provides for maintenance of precise sample temperature both above and below ambient as well as for rapid temperature shifts. Finally, changes in medium composition and temperature can be efficiently achieved within the chips while recording cell behavior by microscopy.

There are also commercially available turn-key solutions for live imaging of cells grown in microfluidic culture. A major advantage of systems such as the CellASIC® ONIX microfluidic platform is the ability to proceed rapidly to cell-based experiments rather than spending large amounts of time creating the chips/plates and assembling the hardware to conduct the experiment. Such an approach can literally save years of development time.

The CellASIC® perfusion-based system integrates with an existing microscope to enable dynamic, time-lapse experiments. Experiments can be conducted with mammalian, bacterial or yeast cells in 2D or 3D over many days. The vacuum-sealed plate system allows hands-free changes of media perfusion, gasses and temperature. The system allows continuous

observation of cells over longer periods of time due to continuous perfusion capabilities that mimic *in vivo*-like conditions. It is feasible to rapidly introduce changes to the cell environment (insults such as antibiotics, other drugs, stresses) and observe resulting changes in cell shape and behavior.

Coluccio et al. (2019) provide a recent review of microfluidic platforms for cell culture along with their capacities for high throughput analysis, automation capability, and interface to sensors and integration (see Further Reading).

3.1.1 Materials that do not influence availability of biomolecules

Very early in the design of any cell-based experiment, it is crucial to test the biocompatibility of all the materials with which the cell will come into contact. This includes both hardware (potential for biomolecule or cell adsorption) and all reagents (potential for undesired reactions or cell damage). Most microfluidic chips and devices need surface treatment to adapt their surface properties to the application and to limit nonspecific adsorption. Shirtcliffe et al. (2013) review surface treatments for microfluidic biocompatibility.

Silicon

Silicon was one of the first materials used in microfluidics due to its use in microelectronics. Advantages of silicon in microfluidic applications lie in its thermal conductivity, surface stability, solvent compatibility and well-understood manufacturing protocols. The main drawback of silicon microfluidic chips is optical opacity that makes optical detection impossible.

Glass

Glass has the same above-mentioned advantages of silicon. Its well-known surface chemistries, optical transparency and high-pressure resistance make it an excellent choice for many applications. Glass is also biocompatible, chemically inert, hydrophilic and allows efficient coatings. The main shortcoming of glass microfluidic chips is its relatively high cost.

Polymers

Polymers offer an attractive alternative to glass and silicon as they are easy to access, inexpensive, robust and have faster fabrication processes. Many polymers can be used to fabricate microfluidic chips, including

- Polystyrene (PS)
- Polycarbonate (PC)
- Polyvinyl chloride (PVC)
- Cyclic Olefin Copolymer (COC)
- Polymethylmethacrylate (PMMA)
- Polydimethylsiloxane (PDMS)

In the late 1990s, George Whitesides and his group at Harvard developed the elastomeric material poly(dimethylsiloxane) (PDMS), which quickly became (and continues to be) the most popular material for the manufacture of microfluidic devices. The main advantages of PDMS microfluidic chips include:

- Oxygen and gas permeability, which is advantageous for long-term cell-based experiments
- Optical transparency
- Elastomeric properties
- Robustness
- Non-toxicity
- Biocompatibility
- Ease of creating complex microfluidic designs by stacking multiple layers
- Relatively low cost

One of the main drawbacks of PDMS chips is its hydrophobic nature. Consequently, introducing aqueous solutions into the microchannels is difficult and hydrophobic analytes can adsorb onto the PDMS surface, thus interfering with analysis. There are now PDMS surface modifications available to avoid issues due to hydrophobicity (e.g., Shirtcliffe et al., 2013). Another issue with PDMS chips is that they are not suitable for high-pressure operation as it can alter channel geometry, rendering the system

prone to leaking at elevated pressure. Bubble formation from passage of gas through PDMS can also be problematic. Another drawback is its poor chemical compatibility with many organic solvents, which makes PDMS suitable mainly for aqueous applications.

In sum, polymeric materials have become the material of choice in biological and medical applications. Ultimately, it is the application that must guide the researcher in the selection of the most appropriate material for their microfluidic application.

3.1.2 Required architecture for suspension cells

Cells can be classified as adherent or non-adherent based on whether they have to attach to a substrate to proliferate. Those that require a substrate are adherent (anchorage-dependent) and those that can grow in suspension are non-adherent. Mammalian cells that come from tissue are typically adherent, while yeast and bacteria are usually non-adherent and are grown in suspension culture.

Non-adherent cells are generally not expected to have any interaction with surfaces, but such cells need to be trapped for reliable imaging. As shown in Figure 3, CellASIC® ONIX Y-series microfluidic plates keep yeast cells in a single focal plane, allowing observation and induction of cell events during high-magnification analysis over many generations (16+ hours).

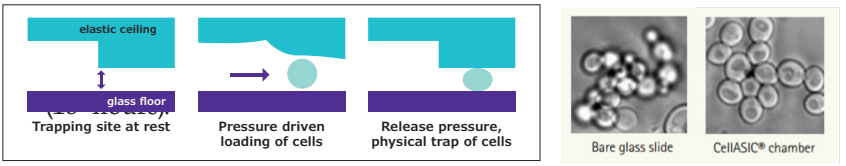


Figure 3: Side view of microfluidic plate with a ceiling height similar to yeast cells to restrict their growth in a single focal plane and maintain x,y position over time.

3.2 Cost and complexity challenges of microfluidic live-cell imaging systems

The technical demands for creating microfluidics cell culture systems (plate design and fabrication) are substantial and integrating live-cell imaging hardware and software adds significantly to the complexity of troubleshooting and successfully conducting experiments.

The availability of versatile, easy-to-use microfluidic live-imaging systems such as the CellASIC® ONIX provides an advantage for less technically sophisticated end-users who wish to conduct cell-based assays without first having to develop a microfluidic system. The assay development time saved by using such prefabricated devices with automated systems can amount to years.

3.2.1 Reduction in scale, reagents and cost

Microfluidic live-imaging systems occupy a much smaller footprint than the facilities needed for standard tissue culture and imaging. For example, the CellASIC® ONIX microfluidic platform comprising a computer, controller and microscope occupies approximately 4 feet of bench space while typical mammalian cell culture requires incubators and a copious amount of single-use plastic cultureware in addition to the computer and microscope required for gathering and analyzing images.

As shown in Table 2, microfluidic cell culture systems use dramatically smaller amounts of both cells and reagents. Compared to a 35-mm culture dish, a microfluidic chip will require more than 5000-fold less growth medium. Combining these savings with the greatly increased capacity of microfluidic systems to run multiplexed and parallel assays results in a potentially massive increase in research productivity with a substantial savings of reagent cost and time.

3.2.2 Controlling delivery of fluids and gases

Fluids

As explained above, aqueous fluids adopt laminar flow behavior at microscale, and microfluidics devices must use appropriate techniques to control laminar flow. This means exploiting surface tension and capillary

forces or using external devices such a syringe, peristaltic pumps or pressure controllers. (see Flow Control in Microfluidics in Article Links)

Gases

The importance of control of the gaseous microenvironment is discussed above. A common technique for regulating gas composition and delivery is through use of a controller unit. In addition, microfluidic plates should be constructed from gas-permeable materials with aeration channels to minimize diffusive effects.

Wu et al. (2018) review recent developments for the control of gaseous microenvironments in microfluidic cell culture devices and discuss the advantages and limitations of current devices.

3.3 Existing applications

The CellASIC® ONIX2 microfluidic live-imaging platform is highly adaptable and has been used for many published studies involving mammalian cells, yeast, bacteria and algae. The array of plates and expansive range of control of microenvironmental parameters gives the researcher virtually unlimited ability to conduct cell-based studies that require ongoing microscopic observation.

Studies using the ONIX2 system have been published on topics such as:

- co-culture of adherent and non-adherent cells
- 2D and 3D cultures
- single-cell studies
- cell signaling
- chemotaxis with motion tracking
- autophagy
- phagocytosis
- biofilms, cell growth and division

3.3.1 Engineering behind the plates and the different types that exist for different cell types

The family of CellASIC® ONIX2 microfluidic plates all have a 96-well plate footprint that is compatible with any standard inverted microscope, but each is specifically designed for different applications. The plates are made of PDMS, PMMA, glass and polycarbonate and there are different plates for mammalian, bacterial and yeast cells. There are also different plates for different purposes such as gradient formation or single-plane growth restriction.

The plates contain four independent culture chambers under a single analysis window as well as #1.5 (170 µm) thick glass bottom for microscopic analysis. Perfusion barriers allow continuous mass transport without shear stress and integrated channels allow gas diffusion. The plates are pre-primed, disposable and immediately ready for use out of the package.

The M-series microfluidic plates for mammalian cells enable high quality, long term live cell analysis with the ability to switch between five inlet solutions. Continuous perfusion experiments typically run for 4-72 hours on the microscope stage, enabling single-cell tracking during exposure, washout, and switching.

Both the B-Series (for bacterial cells) and the Y-series (for yeast cells) microfluidic plates keep the non-adherent cells in a single focal plane, allowing the investigator to follow and induce cell events during high magnification analysis over many generations (16+ hr), with the capability for laminar flow switching between five inlet solutions and complete solution change in under a minute.

3.3.2 Ability to create stable gradients

CellASIC® M04 gradient plates enable precision-controlled chemoattractant diffusion across perfusion barriers to create a gradient in the culture-viewing area for live-cell analysis. There are four culture chambers in the plate for parallel comparison of up to four different cell types or exposure conditions. Each chamber has four switchable upstream solution channels, and each chamber is bracketed by perfusion barriers. Stable

gradients are established by simultaneously flowing media of different compositions through upper and lower channels. The flexible format of the plate enables changes in gradient directionality, turning gradients on and off, and toggling between gradient and single-solution exposure. The glass coverslide bottom surface allows high-resolution optical viewing.

3.3.3 Ability to track cell behaviors affected by chemical gradients, i.e., migration, invasion, proliferation.

The movement of cells in response to chemical gradients can be analyzed using software that tracks individual cells through a series of time-lapse images. For example, Image J (NIH), Manual Tracking (NIH) and Chemotaxis tool (ibidi) software packages were used in a study of the effect of a serum gradient on metastatic breast cancer cell migration. There are many programs for analysis of microscopic time-lapse images.

CASE STUDIES

CASE STUDY 1: Primary neuron culture and analysis

When neurons are grown in culture they develop into a network and create a useful model for studying the central nervous system. Having the neurons in culture allows researchers to measure the responses of the cells to external stimuli, such as changes in temperature, chemical signals, electrical signals, cell-cell and cell-matrix contacts, in a much more controlled environment compared to studying intact neurons in a live organism. However, there are several particularly challenging problems associated with growing and maintaining clinically relevant neuronal networks in culture. One is their short lifespan. The long timelines associated with studying neuronal plasticity (often on the scale of months) makes extending the lifespan of neurons in vitro *a crucial goal.*

Another challenge is that primary neurons are very sensitive to microenvironmental parameters such as temperature, pH, osmolarity, oxygen availability, nutrient availability, cell-cell communication and extracellular matrix coating. However, little is known about how these microenvironment parameters and their dynamics affect the stabilization and health of primary neurons in culture.

Microfluidic technology has been used to culture neurons for various studies. In one study, a commercially available microfluidic platform (CellASIC® ONIX) was used to optimize the growth of rat primary neurons. The neurons were cultured on the microfluidic platform for 21 days and microscopic images of live cells were acquired every 3 days to evaluate the health of the colonies. The total length of neurites was measured and compared with that of colonies cultivated in standard dish culture. The cultured cells in the microfluidic platform were characterized by immunostaining (Figure 4).

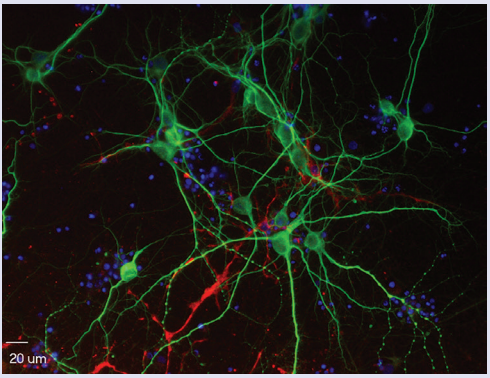


Figure 4: Immunocytochemistry of rat primary cortical neurons cultured, stained and analyzed on the microfluidic platform. The neurons were cultured on the device for 19 days, and the neuron marker (MAP2, green) and the astrocyte marker (GFAP, red) were identified by immunocytochemistry using anti-MAP2 and anti-GFAP antibodies. Nuclei were stained with Hoechst 33342 (blue). From EMD Millipore Application Note.

Microfluidics systems can also be used to isolate neuron soma from axons, enabling spatially restricted studies of injury. For example, Kim et al. (2009) inflicted laser-induced injury to central and peripheral

nervous system axons grown in a microfluidic apparatus and monitored the post-injury sequence of events from initial degeneration to subsequent regeneration.

In another application, Kane et al. (2019) constructed an automated cell culture platform for long-term maintenance and monitoring of different cells in three-dimensional microfluidic cell culture devices. The system, termed the Pelican, can be equipped for time-lapse imaging microscopy and electrophysiology monitoring to assess cellular activity. In an illustrative study, the authors used the system to perform automated cell culture of Parkinson's patient-derived neuroepithelial stem cells and monitor their differentiation into dopaminergic neurons. They assessed the health of the cells with an automated image-acquisition pipeline. After 24 days in culture, calcium imaging and immunofluorescence assays were performed to characterize the dopaminergic neurons. In addition, three-dimensional imaging revealed mature and interconnected neuronal populations within the microfluidic cell culture chips.

CASE STUDY 2: Dynamic live-cell imaging of bacterial biofilms

Biofilms are thin, slimy films of microorganisms that adhere to a surface that is either submerged in or exposed to an aqueous solution. Plaque on teeth is a common example, but there are many types of biofilms such as on the exterior of seagoing ships, on the shower floor, on plants to defend against pathogens, and even in the human large intestine. Biofilms initially form by attachment of free-floating microorganisms (e.g., bacteria, fungi, protozoa, algae) to a surface and may include a single species or a diverse array of microorganisms. The biofilm then grows by a combination of cell division and recruitment.

Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species due to the dense and protected environment of the film. For example, cells in a biofilm

may become increasingly resistant to antibiotics. Infections associated with biofilm growth usually are challenging to eradicate, mostly due to the fact that mature biofilms display tolerance towards antibiotics and the immune response.

Biofilms are therefore medically important in a wide variety of microbial infections, such as bacterial vaginosis, urinary tract infections, catheter infections, middle-ear infections and many other conditions. Bacterial biofilms may also impair skin wound healing and interfere with the efficiency of topical antibacterial treatments in healing or treating infected skin wounds.

Liu et al. (2015) used a microfluidic culture platform to study how biofilms can reconcile the opposing benefits of growth and protection during biofilm development. Cells at the outer edges of a biofilm protect cells in the interior from external attack but also deprive them of nutrients (Figure 5). A major challenge in studying such dynamic systems is in the measurement and control of the culture environment together with continuous monitoring of changes over time.

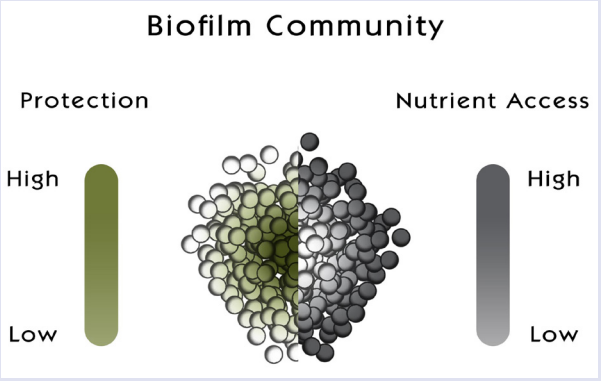


Figure 5: Biofilms must reconcile opposing demands for protection from external challenges (gradient indicated in green) and access to nutrients (gradient indicated in gray). Adapted from Liu et al., 2015.

In this study, single-strain biofilms of Bacillus subtilis *were grown using the CellASIC® ONIX2 microfluidics platform. The culture plates used had exceptionally large chambers and a silicone ceiling at a height that restricted cell growth to a single focal plane and maintained cell x,y position over time. This configuration allowed the formation of colonies (biofilms) containing millions of cells. Biofilm growth was monitored by phase contrast microscopy, with images acquired every 10 min. Images were then analyzed using ImageJ software to detect regions of biofilm expansion.*

Upon tracking physical movement within the biofilm, the authors observed oscillations in the growth patterns. The biofilms would grow for a period of time and then growth would halt in the peripheral cells. Following a series of medium supplementation experiments, they found that the periodic halting in biofilm growth resulted from metabolic co-dependence between cells in the biofilm periphery and interior that is driven by glutamate consumption and ammonium production, respectively. This co-dependence leads to periodic halting in biofilm growth, increasing nutrient availability for the sheltered interior cells and preventing them from starving to death. Their findings may inspire new strategies to control biofilm growth.

4.0 Cell migration across a chemoattractant gradient

Directed migration of cells is critical in biological processes ranging from development and morphogenesis to immune response, wound healing, and regeneration. Cell migration is directed by the interaction of cells with the extracellular matrix (ECM), neighboring cells, and biomolecules present in the milieu. However, there are few techniques to specifically direct, manipulate, and observe cell migration *in vitro* and *in vivo*. In an interesting effort to achieve control of cell migration to user-defined locations, independent of native chemotaxis receptors, Jason Park and coworkers (Park et al., 2015) at UC-San Francisco conceived of a strategy that involved genetically modifying motile cells with an engineered G protein-coupled receptor (GPCR) so the cells would bind to a bioinert drug-like small molecule, clozapine-N-oxide (CNO). This type of engineered GPCR is known as a designer receptor exclusively activated by a designer drug (DREADD), and it does not respond to endogenous ligands. Similarly, CNO does not bind to any naturally occurring receptors on native cells.

The authors demonstrated the directed control of cell movement using motile cells expressing the DREADD and a microfluidics-plus-imaging system employing a special gradient-generating plate (M04G; CellASIC®, MilliporeSigma) that enables continuous perfusion culture for live-cell analysis (Figure 6). Each of the four chambers in the plate has four switchable upstream solution channels, permitting formation of stable gradients. The chamber is bracketed by perfusion barriers, and stable gradients are established by simultaneously flowing media of different compositions through upper and lower channels. The laminar flow on the microfluidic plate provides rapid and uniform solution exchange and the glass coverslide bottom ensures high-resolution optical viewing through an inverted microscope.

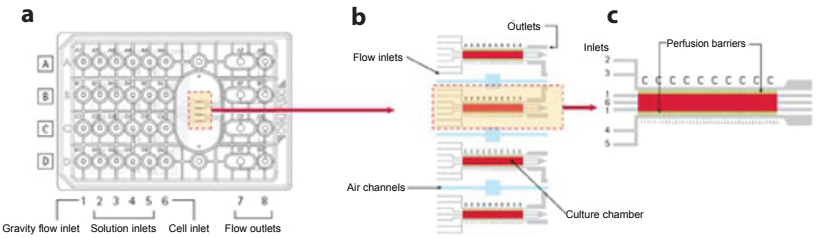


Figure 6: (a) The CellASIC® M04G Microfluidic Plate has four independent culture chambers (A-D), each with a gravity flow inlet (1), four solution inlets (2-5), a cell inlet (6), and two shared outlet wells (7 and 8). Each row of wells (A-D) addresses the corresponding culture chamber. (b) All four culture chambers are located under a single viewing window to minimize travel distance for high-magnification phase objectives. (c) The chamber is bracketed by perfusion barriers on the top and bottom edges to separate the chamber from flow channels. Inlet wells 2 and 3 flow media into the upper channel, while 4 and 5 flow media through the lower channel. Gradients are established by simultaneously flowing media of different compositions through the upper and lower channels. Figure from MilliporeSigma.

HL-60 neutrophils stably expressing the DREADD engineered to recognize CNO were flowed into the fibronectin-coated culture chambers of the microfluidic gradient plate and allowed to adhere. Migration buffer was then flowed through the chamber to wash away nonadherent and dead cells. A diffusive gradient of the chemoattractant, CNO, was applied (visualized by a fluorescent red tracer dye), and cells were tracked by time-lapse fluorescence microscopy (see Movie 5 and Figure 2B from Park et al., 2015).

The cells containing the DREADD migrated up the gradient of CNO. The authors also demonstrated similar cell behavior using other systems and other cell types including *in vivo*-directed movement of T lymphocytes modified with the engineered receptor to CNO-releasing beads implanted in a live mouse. This technology thus provides a generalizable tool to systematically control cell migration *in vitro* and *in vivo*.

4.1 Culture and analysis of cells in hypoxic conditions in a microfluidic system with real-time imaging

Hypoxia is defined as oxygen deficiency and, under normal circumstances, is toxic to cells. However, tumor cells often acquire the ability to adapt and grow in hypoxic conditions. Experimental and clinical evidence demonstrates a strong correlation between these adaptations and resist-

ance to chemotherapy and radiation therapies, as well as the progression of malignancy. Understanding the mechanisms of hypoxic responses is critical to the development of therapeutics targeting tumor progression. However, standard culture methods typically do not allow precise control of oxygen pressure.

The hypoxic responses of several types of human cancer cells were studied using a microfluidic system and assay-optimized plates, which enable precise control over micro-environmental conditions, including gas and media content (see ***An integrated platform for real-time dynamic culturing and analysis of hypoxia with single cell resolution*** application note in the Article Links section). Results indicated that hypoxia impacts numerous cell processes and functions including response to cytotoxic agents, mechanisms of cell death, and invasive capacity.

Several markers for the detection of hypoxia by imaging have been developed. BioTracker 520 Green Hypoxia Dye is a fluorescent imaging probe for the detection of hypoxia in living cells. This dye can be used in live-cell fluorescence imaging and flow cytometry applications. Reductive cleavage of the BioTracker dye occurs under hypoxic conditions, generating a compound that produces bright green fluorescence. Lower oxygen levels, such as occur in more severe hypoxic conditions, produce greater fluorescence intensities. [see BioTracker 520 ONIX video]

In another study, Germain and coworkers (2016) fabricated a microfluidic culture system capable of rapidly changing local oxygen concentrations to determine changes in drug resistance in prostate cancer cells. They found that the cells they utilized both rapidly and reversibly adapted to hypoxic conditions as evidenced by changes in resistance to the anti-cancer compound staurosporine.

WHAT'S NEXT

5.1 Anticipated Imaging Technology

As research into dynamic processes such as cell migration and development increases, techniques capable of capturing real-time, three-dimensional data will become indispensable for understanding biological systems. (see Article Links for more information)

- Super-resolution microscopy with resolution below the diffraction limit of light.
  - *Super Resolution Microscopy Use Burgeoning*
- New microscope cameras with greater sensitivity for use in very low light conditions.
  - *Capturing the Infinitesimal: Choosing the Right Camera for Your Microscope*
- Microscope technologies and software that enable better quantitative image analysis of label-free images.
  - *Look Alive with Live-Cell Imaging*
- Lattice light-sheet microscopy which is gentle on live samples and causes very low phototoxicity.
  - *Lattice Light Sheet Microscope*
- Multi-photon imaging to achieve greater depth of imaging in live tissues.
  - *Live-cell imaging: Deeper, faster, wider*
- 3D microscopy to image highly sensitive stem cells over days.
  - *Breakthrough 3D Live Cell Imaging Technology Changes the Future of Stem Cell Research*

5.2 Anticipated Fabrication Technology Advances

The future development in microfluidic devices will likely focus on new fabrication methods and biomaterials. For example, standard microfluidics imaging methods employ only two sides (top and bottom) of the sample for multi-angle imaging, but Hochstetter (2019) has devised a method to generate devices with four optically-clear sides for imaging (top, bottom, front, and back).

Pore plates for cell migration assays utilize parallel chambers (2, 3, or 4) separated by a physical perfusion barrier. Cells can be seeded in all three chambers. These plates are useful for researchers doing 3D culture (organoids, spheroids) using their homemade microchips that have etching for microfluidics. These new plates can facilitate 3D analysis with:

- Unique single-cell analysis compared to insert's population analysis
- Trapezoidal posts optimized for 3D culture in center chamber
- Solution switching capability in the two side chambers
- Gel compartment loaded directly from plate
- Gradient formation and interstitial flow dynamics.
- Compatible gels (i.e. Matrigel, Collagen, Fibrinogen, Alginate)

Engineers expanding the capabilities of the CellASIC® ONIX2 system are developing a universal manifold. This will enhance the system's versatility by enabling the use of any microfluidic slides with the ONIX2 system, which is currently designed to be compatible with system-specific microfluidic plates.

5.3 What are some new applications on the horizon for specific cell types?

In vitro Vascularization of Organoids at Microscale

One company, Mimetas, has developed a microfluidic system to study in vitro vascularization of human tissues such as organoids, spheroids and explant tissues. Two perfusion channels flank one larger extracellular matrix (ECM) channel. In the perfusion channels, human endothelial cells are introduced and a solution of angiogenic factors is then placed in the central ECM channel, forming a gradient of these factors. Angiogenic sprouts grow in the direction of the increasing gradient and functional microvessels are formed. A vascular network develops over several days and then 3D tissues (e.g., organoids) are placed in contact with the network and can become connected to the system of human blood vessels, thus achieving in vitro vascularization (Figure 7).

This system facilitates perfusion of the tissue and allows drug administration through the vasculature. Perfusion of the network and vasculature-tissue interactions can be studied using fluorescent dyes and the glass bottom of the plate allows capture of high-quality images on an inverted microscope. Tissues grafted in this system can be extracted, sectioned and stained to visualize the penetration of the tissue by the sprouted endothelial cells.

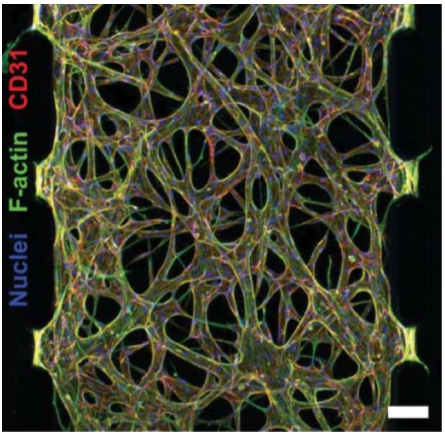


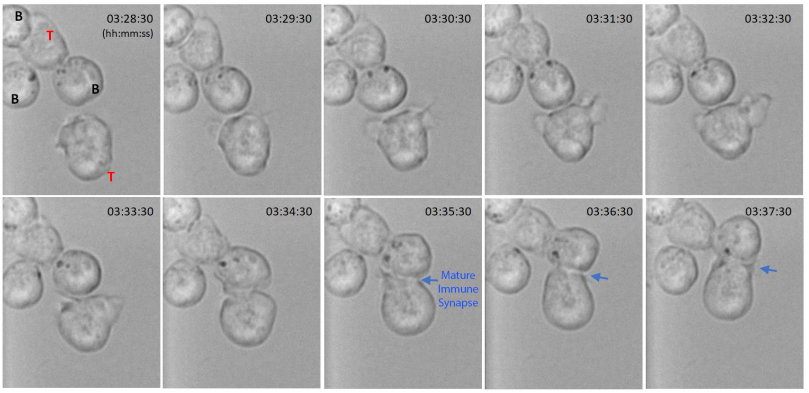
Figure 7: Perfusable capillary network generated in a microdevice. Vascular network formed by vasculogenesis on a chip. Scale bar is 100 µm. Figure 3c from Miura T, Yokokawa R. Tissue culture on a chip: Developmental biology applications of self-organized capillary networks in microfluidic devices. *Dev Growth Differ.* **2016;58(6):505–515. doi:10.1111/dgd.12292**

Co-culture enhancements

It is possible to grow co-cultures of different cell types on the same chip to study cell-cell or cell-environment interactions. Lee et al. (2018) report a microfluidic co-culture model that integrates tumor spheroids with pancreatic stellate cells in a 3D collagen matrix to mimic the *in vivo* tumor microenvironment and recapitulate epithelial-mesenchymal transition and to study chemoresistance.

There have also been successful microscale co-cultures of adherent and immobilized non-adherent cells to study the interaction of cancer and immune cells. (see Real-time imaging of adherent and non-adherent cell interactions: utility of an automated microfluidic trap platform to recapitulate in vivo cell culture microenvironment in Article Links)

Another application involves pad trap plates that utilize pillars that extend from the ceiling to trap suspension cells within a single focal plane for real-time imaging. Jurkat T cells and Raji B cells were trapped together and then loaded with superantigen to create immune synapses (Figure 8).



File: M04T_Immune Synapse Formation.jpg

Figure 8: Frame-by-frame analysis of Jurkat T cells and Raji B cells forming an immune synapse. Figure from MilliporeSigma.

Human/body/lab on a chip

With a goal of replacing animals in preclinical drug screening and toxin testing, scientists have linked microfluidic organs-on-a-chip (e.g., lung, kidney, heart, intestine, others) to simulate a "body-on-a-chip". Although the field is still in its infancy, Edington et al. (2018) report integrating 10 organs-on-a-chip.

CONCLUSION

The combination of microfluidic cell culture and microscopic imaging techniques has opened a new world of biologically relevant, live-cell research by allowing the investigator to control all aspects of the cell culture microenvironment to create highly *in vivo*-like systems on very small scales. Once available only to laboratories possessing high levels of technical expertise, the technology is now available as turn-key systems that allow investigators with little technical training to set up and conduct experiments extremely rapidly. Ongoing hardware and software improvements will provide greatly expanded opportunities to study cellular behavior, responses, and interactions. The possible applications are essentially limited only by the researcher's imagination.

ARTICLE LINKS

1. An integrated platform for real-time dynamic culturing and analysis of hypoxia with single cell resolution. https://www.emdmillipore.com/Web-US-Site/en_CA/-/USD/ShowDocument-Pronet?id=201709.056
2. Park et al (2005) – Movie: https://www.pnas.org/highwire/filestream/615730/field_highwire_adjunct_files/5/sm05.mov
3. BioTracker 520 ONIX – video: https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-culture/hypoxia-detection-assays.html
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10. Breakthrough 3D Live Cell Imaging Technology Changes the Future of Stem Cell Research: https://www.technologynetworks.com/cell-science/product-news/breakthrough-3d-live-cell-imaging-technology-changes-the-future-of-stem-cell-research-299824

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   Recent review of microfluidic devices used for culturing and monitoring cells. Describes design options with their various technological features.

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   Provides a historical perspective of microfluidic devices, tools and technologies. Describes advances in materials and processes and gives a perspective on future directions.

3. Zhong, Qifeng, Haibo Ding, Bingbing Gao, Zhenzhu He, and Zhongze Gu. "Advances of Microfluidics in Biomedical Engineering." *Advanced Materials Technologies* **4**, no. 6 (2019): 1800663. https://doi.org/10.1002/admt.201800663.

   Reviews recent advances in microfluidics for biomedical engineering, emphasizing basic concepts and research trends. Covers recent research in microfluidics for bioassays, biofabrication, and tissue engineering.

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