

Microfluidics with fluid walls

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*To my parents,
For their unconditional support.*

*Parintilor mei,
Pentru sprijinul neconditionat.*

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Abstract

A high variety of microfluidic platforms that aim to miniaturize and automate biomedical assays have been developed in the last decades, but these have failed to be integrated as common equipment in laboratories. This is due to concerns about biocompatibility, issues about the ease of use and the limited range of readouts that the system can accommodate.

This thesis describes a powerful and versatile alternative to conventional microfluidics, in which solid, obscuring walls are replaced by morphing fluid walls. Various methods are used to reshape cell media on Petri dishes into almost any imaginable pattern. The simplicity of the technique eliminates the need for expensive machinery and equipment. The system is shown to be biocompatible and to have potential for high-throughput cell-based analysis. This technology allows the user to fabricate a wide variety of microfluidic devices truly on demand (within minutes).

Beyond simple proof-of-concept applications, this technology produces several important results: miniaturized CRISPR-Cas9 assays in volumes smaller than 1 μl , a fluorescent method for measuring small volumes, a method to reconfigure cellular environments around living cells, a way to accelerate single-cell cloning, beating the Poisson distribution in single-cell cloning, designing and creating a perfusion device which can continuously feed cells over days, and a method for dislodging attached cells and creating wounds in vitro. In its entirety, this thesis puts forward a set of methods to model and reshape cellular environments using apparatus accessible to common biology laboratories: a 3-axis traverse, syringe pumps, petri dishes, cell media and FC40.

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Chapter 1: Introduction

More than a decade ago researchers recognised the potential of microfluidic devices to significantly impact the field of life sciences (1-2). An assessment of the contribution that microfluidics has had to biomedical research shows that the adoption of this promising technology has not matched the initial optimism. Nearly 90% of the microfluidic papers are published in engineering journals, with the rest in biomedical and multidisciplinary journals (3). This is because for the majority of microfluidic devices there is no progression beyond the proof-of-concept (PoC) experiments. Microfluidic platforms need to have a set of basic capabilities in order to make possible the adoption of the technology by the biomedical field: biocompatibility; a way of introducing samples and reagents in the system; means of moving the fluids around the system in order to create mixtures of reagents, and a method that allows the retrieval of the sample for subsequent analysis (4). Most microfluidic platforms address only some of these capabilities, making them highly specialised. The purpose of this thesis is to introduce a microfluidic technology that meets the main needs of biomedical research and that has a demonstrated wide range of applicability in this field.

The underlying principles of this technology rely on building micro-structures, where fluid walls replace solid ones. At the macroscale, objects are made out of solids, as gravity is the dominant force. In the microworld, however, interfacial forces dominate gravitational ones, so air:water or oil:water interfaces can act as robust walls. This paradigm enabled the emergence of open microfluidics. These are mainly 2.5D environments, where at least one of the containing walls is an interface between two fluids (5-7). These platforms are generally easier to integrate into biological assays, as they offer optical and physical access to the samples. However, most of them still require some form of surface treatment, affecting the biocompatibility of the device, as well as complicating the manufacturing process.

Recently, Walsh *et al.* (8) introduced a technology that requires no surface alteration, where the only building materials used are those in the biocompatible trio 1) cell media, 2)

polystyrene, 3) FC40 (a chemically inert fluorocarbon). The manufacturing process is simplified, such that fluid structures are printed on petri dishes with a needle brought close to the surface, infusing media constantly. The newly created traces remain pinned due to adhesion forces, and then the whole structure is covered with FC40 to prevent evaporation and contamination. This thesis represents a development of this initial concept presented by Walsh *et al.* New methods to reshape fluids stemming from the original idea are developed, and then used to demonstrate their applicability in biological workflows.

Objectives

This thesis aims to address the following list of objectives:

- Describe and understand the role of microfluidic technologies in biomedical research and identify the state-of-the-art technologies in the field.
- Identify and present the relevant theoretical aspects of fluidic chambers.
- Develop a method to create micro-environments for cells in culture that can accommodate the main workflows in cell biology.
- Assess the biocompatibility of the platform with the main cell-based assays.
- Apply the method to reconfigure micro-environments around living cells, during the experiment.
- Analyse the optical properties of the micro-environments and assess their suitability to accelerate single cell cloning.
- Develop a non-contact fluid-shaping method to improve precision and reliability of the technology.
- Design and implement a method to beat the Poisson distribution in single-cell cloning.
- Investigate a method to dislodge and detach cells as part of the passaging workflow or in wound-healing assays.

This thesis consists of 3 chapters introducing the relevant literature and theory, followed by 5 chapters containing published or submitted articles, and the relevant conclusions and proposed future work. The flow of the thesis is as follows:

Chapter 2 offers a brief literature review of the main uses of microfluidic devices in the biomedical field. The three applications explored here—drug screening, single-cell isolation, and whole-organism assays—were chosen to match the assays explored with this technology.

Chapter 3 explores the relevant theoretical concepts of microfluidics. The physics of fluid flow at both macro- and micro-scale are complex. Here, only aspects required for the understanding of the material contained in this thesis are presented. These include: the geometry of the interfaces for the main fluidic structures (droplets and conduits), the contact angle hysteresis, passive pumping and the Laplace pressure that drives it.

Chapter 4 represents a manuscript published in PNAS in June 2018, and introduces the main method used throughout this thesis. A petri dish is overlaid with a layer of cell media, followed by a layer of FC40 on top. A Teflon stylus is used to push the FC40 into contact with the polystyrene substrate, splitting the existing media. FC40 preferentially wets the polystyrene, so it stays pinned there, subdividing the bulk media into multiple reservoirs. Chambers newly formed are stable and robust, while offering excellent optical and physical access to the contents. The platform is demonstrated to be biocompatible and to accommodate most of the main workflows in cell biology: feeding, replating, cloning, cryopreservation, fixation plus immuno-labelling, lysis plus RT-PCR, and transfection plus genome-editing. Furthermore, the platform is shown to accommodate some whole-organism assays—development and drug treatment of *C. elegans*.

Chapter 5 explores the ability to reconfigure environments around living cells, a task impossible with the existing platforms, and has been published in July 2019 in Science Advances. Once built, a fluidic structure can be removed by adding sufficient volume to it, such that it breaches the advancing contact angle and bursts. Same kind of or new structure can then be built on the same footprint. The user has full flexibility of when and where to build dividing walls to customise the cellular macro-environment. Applications such as drug treatment and wound healing demonstrate its suitability for complex workflows.

Chapter 6, published in SLAS Technology, explores an important property of microfluidic environments with fluid walls—the optical clarity. The lack of solid, obstructing walls

removes edge effects (dark regions around the edges), making the contents of the chambers fully accessible to the observer. The theoretical aspects of this optical phenomenon are presented, and the platform is shown to facilitate cloning assays; it has the potential to shorten the length of cloning experiments from 1-2 months (typically required for one or two rounds of cloning in multi-well plates) to 7-8 days.

Chapter 7 is a paper accepted for publication in Advanced Science and presents an important milestone in this work. As **Chapter 4** describes it, the main method to print fluid walls was using a stylus. This was found to have a few disadvantages stemming from the effect of contact, such as protein build-up and play in the x-y plane, which reduced the accuracy and precision greatly. To circumvent these limitations, a contactless method was developed, where fluid walls are printed using a micro-jet. A needle jetting FC40 replaces the stylus, pushing the static layer of FC40 in contact with the polystyrene. This is shown to offer improved resolution and reproducibility, which made possible beating the Poisson distribution in cloning and creating a perfusion circuit that can feed cells continuously for more than a week. These, together with the other circuits built, demonstrate the ability to create microfluidic devices on demand (within minutes) with great flexibility. Furthermore, due to the disrupting shear rates it causes, jetting is proposed as a method to dislodge attached cells without using any reagents.

Chapter 8 is a paper submitted for publication and an extension to the ability to dislodge attached cells presented in **Chapter 7**. By tuning the parameters for flowrates and movement of the traverse, the same system is shown to accurately and reliably produce wounds with almost any 2D shape in confluent layers of cells.

Finally, **Chapter 9** represents a discussion of the presented work, where the main disadvantages are summarised and a path for future work is proposed.

In summary, this thesis attempts to do more than just to describe a method for creating microfluidic devices. In its entirety, it puts forward a set of methods to model and reshape cellular environments using apparatus accessible to the average biology laboratory: a 3-axis traverse, syringe pumps, petri dishes, cell media and FC40.

Chapter 2: Literature Review

Introduction

Microfluidics is a technology that makes possible the manipulation of small volumes of fluids constrained to a sub-millimetre scale. By means of creating micro-channels with precisely defined geometries or micro-droplets, microfluidics facilitates and enables experimental work in biological research. Complete laboratory protocols performed inside chambers of a few square millimetres allow researchers to use reduced volumes of samples and reagents resulting in a significant cost and waste reduction. Additionally, for small volumes the favourable surface-to-volume ratio of reagent (Fick's law) results in a decrease in the reaction time, facilitating high-throughput experiments.

Microfluidics was first used in the inkjet printing industry in the 1950s with small tubes as means of carrying ink and later found uses in molecular analysis, biodefence, molecular biology and microelectronics (4). Although numerous applications have been exploited using microfluidic devices, material choice has had a significant role in diminishing the impact of microfluidics in biological research. In an attempt to closely mimic responses observed in conventional macroscopic culture, biologists have been reluctant to add new variables to the system, such as unfamiliar materials. Yet, most microfluidic platforms rely on materials or chemicals outside the biological breadth as building blocks, scaffolds, or coatings for the devices.

This chapter will look at one of the main ways to divide the field of microfluidics (micro-chambers and micro-droplets), as the technology presented in this thesis lies at the intersection between the two. Three of the main applications in the biomedical field will then be explored - drug screening, single-cell isolation and whole-organism assays.

Micro-chambers vs micro-droplets

In the field of microfluidics devices, two approaches for cell-analysis assays can be distinguished: the isolation of cells in micro-chambers and the encapsulation of cells in micro-droplets. Microfluidic chips based on micro-chambers are the most commercialised devices, with well-known products such as diabetes or pregnancy testing. These devices use the principle of passive liquid transport via capillary forces (9-10). The majority of these devices are made from polydimethylsiloxane (PDMS) and require expensive manufacturing processes and extended timescales for the prototyping phase. For constrictions smaller than 1 mm with solid walls, air bubbles can pose difficulties in controlling the flow in the system. Also, specialised equipment and advanced training is often required to use a device that has a restricted applicability. Furthermore, PDMS has been found to have biocompatibility issues when uncoated, as it extracts toxins that interact with organic solvents (11-13).

Droplet-based microfluidics has proven to be a powerful tool used in cell-based assays mainly because it employs extremely low volumes (pico- to nanolitre). In this system aqueous flow is divided into droplets surrounded by a water-immiscible carrier fluid, which is often a fluorinated oil. The oil-water interface ensures cells and reagents are completely encapsulated preventing diffusion and cross-contamination between droplets. Accurate control of size, shape and frequency is required for developing a high-throughput system. For this purpose, hydrodynamic (14-16), electrohydrodynamic (17-18), thermocapillary (19-20) and acoustic-based (21-22) technologies have been used to control droplet formation making use of fluid flow characteristics, electrical and acoustic signal, respectively (23).

Drug screening

The development of new drugs is at an acute demand in the light of the ever-increasing drug resistance and emergence of new diseases. Finding a new drug is a lengthy process which can last more than a decade, resulting in colossal sums of money (approximately \$650 million per drug; (24)) spent by pharmaceutical companies. A staggering 97% of the drugs

undergoing clinical trials failed to receive FDA approval (25). One of the factors responsible for this is the questionable quality of preclinical data, a reason for which researchers have pleaded for raising standards of research at this phase (26). This results in an increased expenditure for drug companies. Microfluidic-based systems present numerous advantages compared to conventional drug screening methods: as volumes are minimised, so are waste, sample size, cost and time (23).

Cell arrays are one of the main deliverables of microfluidics in drug screenings. These offer the capability to run high-throughput drug screening in volumes of several orders of magnitude smaller to study the response of single cells (27), populations of cells (28), or cell-cell pairs (29) to different chemical stimuli.

Finding the optimal dose of drugs to be delivered is crucial for maximising the positive effects and minimising toxicity. This is typically achieved by generating biochemical concentration gradients and microfluidics proves to be an excellent platform for this, as the user has spatial and temporal control of the flow (30). Besides the classical live-dead readout, these prove to be suitable for detecting more advanced markers for changes in phenotype, such as bacterial chemotaxis, cancer cell migration, and axon growth (31-33). Typically, these technologies make use of droplet microfluidics, which generate discrete microreactors with fixed concentrations (34-35) or, continuous-flow based microfluidics, which use interconnected microchannels with a tree-like architecture to dilute chemicals. Concentration gradients can also be created by exploiting the laminar nature of flow in some microfluidic devices (40). More recently, Walsh *et al.* developed a method which allows the generation of sharp gradients of concentrations using three parallel streams of fluids (8).

Multi-well plates have been used for decades as enclosures for cell-based assays, but some studies report that micro-volumes are a better representation of the physiological environments experienced by cells at microscale levels. For instance, Doskey *et al.* showed that assays performed in small volumes present a significant difference in response to drug treatments when compared to 96-well plates (41).

Single-cell isolation

Conventionally, core research in biomedical sciences, such as cancer research, was based on experiments carried on populations of cells. However, this means that the measured response represents an average of the bulk of cells and valuable information is lost, causing a paradigm shift in the last decade (42). As a consequence, the field of single-cell analysis has emerged as a way of studying heterogeneity in cell populations (43-45) and microfluidics was the main technology used in this quest.

The traditional way of isolating single-cells by manually pipetting cell suspension into the reservoirs of standard multi-well plates (with 96, 384 and 1536 wells) is laborious, time-consuming and wasteful. Numerous microfluidic devices which accelerate and automate single-cell assays have been reported. Here, we distinguish between those based on passive encapsulation (making use of the so-called single-cell traps; (46-48)), and those using droplet-based encapsulation (49-52). In the case of the latter, the throughput is limited by Poisson statistics, which dictates the number of resulting droplets containing single cells (**Figure 1**). In practice, this translates into an encapsulation rate of less than 30% of the available droplets (or wells in multi-well plates).

Beating the Poisson distribution is a well-known challenge in microfluidics (53) and several methods have been proposed to circumvent this limitation, such as on-demand encapsulation (54), post-encapsulation sorting (55-57), and inertial cell ordering (58-59). However, despite the high throughput encapsulation, most of these microfluidic systems can only perform the encapsulation step and still require transfer to other platforms for cell growth, drug delivery and downstream analysis. Nevertheless, microfluidics is to be credited for the high throughput in single cell investigations (60) and is expected to play an important role in disentangling the heterogeneity of single cells.

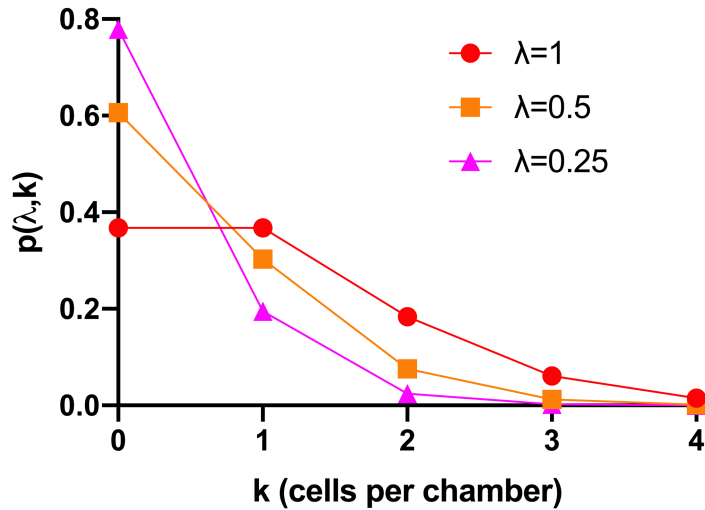


Figure 1: Poisson distribution. The proportion of drops $p(\lambda, k)$ containing a number of k cells is illustrated for different values of the cell density λ (cells per unit volume). The cell density to be used is then chosen based on the maximum accepted probability of having chambers with 2 or more cells.

Whole organisms

Cellular models are a critical milestone for developing a robust knowledge of basic processes inside cells. However, studies on more complex systems are required if we are to understand development, progression of diseases and how drugs react in the human body. Microfluidics has been used extensively to accommodate assays on small model organisms (61) and three of the main ones are highlighted here.

Caenorhabditis elegans (*C. elegans*), is a free-living nematode widely used in developmental biology. Due to its transparent nature, the entire cell lineage of the organism has been mapped (62). Microfluidic tools have been used to immobilize *C. elegans* in trapping channels (63), image them (64), and study the effects of mechanical (65) or chemical stimuli on the worms (66).

The zebrafish *Danio Rerio* is also transparent and well-characterised, with a fully sequenced genome (67). As it has a very rapid embryonic development, many transgenic models have been generated for studying human diseases such as diabetes (68), Parkinson's

disease (69), muscular dystrophies (70) and other genetic diseases (71). In order to accelerate the standard, laborious laboratory techniques where embryonic culture is done manually, popular microfluidic formats were adapted for zebrafish assays (72). Microfluidic array systems were used in drug screenings (73) and gradient generators were used to fine tune the concentration of successful drugs (74). Additionally, tasks like trapping (75), perfusion (76), and even transfection of embryos (77) were successfully brought to practice in such microfluidic environments.

The fruit fly *Drosophila Melanogaster* has been used in studies of genetics and developmental biology for over a century and the eight Nobel prizes awarded for research on this organism serve as a testimony for its immense contribution to science (78). As adults, *Drosophila* fly in the air, so microfluidic devices developed targeted the embryo stage. Notably, the main objective was to accurately control the position and orientation of the embryos to allow imaging and study of morphogenesis (79-82).

Microfluidic technologies have played an important part in advancing our knowledge in developmental biology based on whole-organism models. These are much less costly than mammalian models and remain one of the few viable options for large-scale culture. In combination with other advanced technologies (e.g. high-speed image acquisition), the power of microfluidics can be further enhanced, resulting in a robust, well-established research tool.

The problem of increasing adoption of microfluidic technologies in mainstream biomedical research is still unsolved. The history of science shows that adoption of new technologies complementing existing methods is often a gradual process. The advantage engineers now have is that they can draw on almost half-a-decade of microfluidic technologies to democratize microfluidics.

Biological terminology

Throughout this thesis reference is being made to various reagents and workflows in cell biology. The following section has the role to introduce the most common elements of cell culture practice, such that the reader is able to better understand the steps involved in the experiments described.

Dulbecco's Modified Eagle's medium (DMEM) is one of the most commonly used cell culture media. Phosphate-buffered saline (PBS) is a buffer solution used in cell culture. It is a water-based salt solution, and it is mainly used to rinse cells and to dilute substances. Fetal bovine serum (FBS) is the most widely used serum supplement for culturing cells *in vitro*. It comes from blood drawn from a bovine fetus and contains various growth factors which help cells survive, grow and divide.

The following describes the typical workflow for maintaining cells in culture. Mammalian cells are typically grown in flasks inside incubators at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS. Cells divide and grow in confluence, covering the available surface. When they reach 70-80% confluence, cells are typically ready to be used in experiments. If cells are adherent (attach to the substrate), they have to be detached in order to be transferred. The detachment is done through a PBS wash, followed by an exposure to Trypsin (an enzyme that digests the projections cells use for attachment). This allows cells to go in suspension and be transferred to well-plates, Petri dishes or other cell-culture dishes, process called 'plating cells'. Newly plated cells are exposed to a set of conditions/reagents of interest in order to trigger a response. This response is measured using downstream analysis methods such as microscopy, polymerase chain reaction (PCR), sequencing, etc. Common intermediate steps include fixing (preservation of samples from decay) and lysing (breaking down the membranes of cells) biological samples. Different cell lines require different conditions for each one of these steps. Precise culturing workflows should be available from commercial suppliers of individual cell lines, and these should be followed accordingly for an optimum viability of the samples.

Chapter 3: Theory

Chambers with fluid walls have interfaces that morph under the influence of hydrostatic and Laplace pressures acting upon them. In order to use these circuits as environments for cells in culture, one needs to know the key parameters of the fluidic architecture: height, volume, shear stress, and pressures. This requires the geometry of the interface to be fully defined. This proves to be a difficult task, as the chambers used throughout the thesis have a wide variety of footprints (circular, square, rectangular, triangular, polygonal), resulting in complex shapes of the interfaces. In this chapter, the simplest geometries will be analyzed, droplets and conduits, for which an analytical solution can be derived. Chambers with more complex footprints require extensive analysis, which is beyond the scope of this thesis.

Sessile droplets with circular footprints are analyzed first, followed by conduits (long channels which generally act as connections between two reservoirs). The concept of contact angle hysteresis is explained, and the associated contact angles explored. Finally, a passive pumping system is analyzed as an example that incorporates both droplets and conduits to accommodate flow in the system.

Sessile droplets

The balance between gravity and surface tension dictates the shape of a droplet. This is the reason why large droplets deposited on planar surfaces have flattened shapes (gravity dominates), whereas small droplets maintain a perfectly spherical shape (surface tension dominates). This transition in geometry happens when the ratio of the droplet's Laplace pressure to its hydrostatic pressure approaches 1 (5). Applied for a spherical drop on a perfectly hydrophobic surface (with a contact angle of 180°) this reads:

$$\frac{\Delta P_{Laplace}}{\Delta P_{Hydrostatic}} = \frac{2\gamma/R}{\Delta\rho g 2R} = 1$$

where γ is the surface tension, R is the radius of the droplet, g the gravitational constant, and $\Delta\rho$ the difference in density between the droplet and the surrounding fluid. So, the two pressures have the same magnitude when

$$R = \sqrt{\frac{\gamma}{\Delta\rho g}} = L_c$$

This value is called the “capillary length”, L_c . A dimensionless group, the Bond number, can be formed:

$$Bo = \frac{\Delta\rho g R^2}{\gamma}$$

where, a value $Bo \ll 1$ indicates that the droplet will have a spherical shape. For water droplets the capillary length is usually of the order of 2 mm, but this value can vary with surface tension and difference in density.

In this thesis, most of the droplets fall below the capillary length, having a spherical shape. Such a sessile droplet is characterized by any three of the four parameters: the contact radius a , the sphere radius R , the contact angle θ and the height of the droplet h (**Figure 1**).

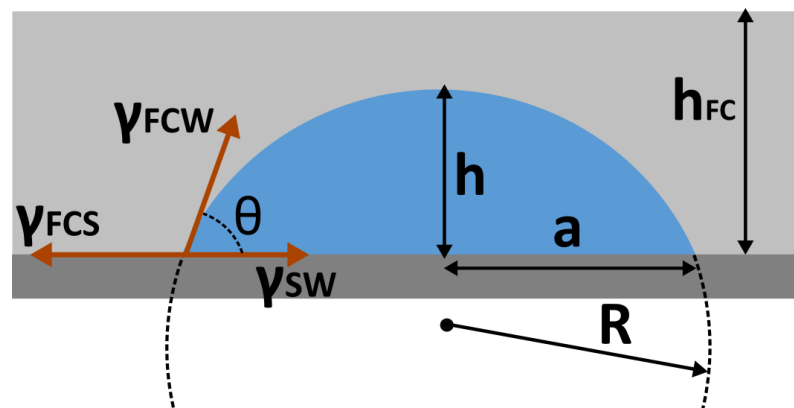


Figure 1. Shape of a sessile droplet. The parameters affecting the geometry of the interface: the half width (a), the radius of curvature (R), the contact angle (θ), and the height (h). The point at which the contact angle is measured shows the schematic of the surface tensions at the triple contact line: solid-gas (γ_{FCS}), liquid-gas (γ_{FCW}), and solid-liquid (γ_{SW}).

The relationship between these parameters is obtained using Pythagoras' theorem:

$$(R - h)^2 + a^2 = R^2 \Rightarrow R = \frac{h^2 + a^2}{2h}$$

$$h = R(1 - \cos\theta)$$

$$a = R\sin\theta$$

$$h = \frac{a}{\sin\theta} (1 - \cos\theta)$$

The volume of a sessile droplet, V_{cap} , is the same as the cap of a sphere:

$$V_{cap} = \frac{\pi h}{6} (3a^2 + h^2) \xrightarrow{a^2 = 2Rh - h^2} \frac{\pi h^2}{3} (3R - h)$$

V_{cap} can also be expressed in terms of contact angle (θ), drop height (h), and radius of wetted footprint (a):

$$V_{cap} = \frac{\pi(2 - 3\cos\theta + \cos^3\theta)}{3(1 - \cos\theta)^3} h^3 \xrightarrow{h = \frac{a}{\sin\theta} (1 - \cos\theta)} \frac{\pi(2 - 3\cos\theta + \cos^3\theta)}{3} \frac{a^3}{\sin^3\theta}$$

The Laplace pressure for a sphere or a cap of a sphere (5) is given by:

$$\Delta P_L = \frac{2\gamma}{R}.$$

Contact angle

In practice, a given combination of solid-liquid-gas will result in a continuous range of contact angle values, depending on the volume. Fluid can be added to a sessile droplet without increasing the footprint up to a limit determined by the advancing contact angle, θ_A ; however, once θ_A is breached, footprint area increases (**Figure 2 - left**). Similarly, the footprint shrinks when liquid is removed and the receding contact angle, θ_R , is breached (**Figure 2 - right**). This is known as the contact angle hysteresis.

Circular sessile droplets have a constant contact angle along the pinning lines. Throughout this thesis, however, droplets have polygonal bases, which produce variable contact angles along the edges the footprints. **Figure 3** illustrates this phenomenon using Surface Evolver simulation results as datapoints. Surface Evolver is a software that computes liquid interfaces by means of minimizing the surface area of a given body. Simulation results

show contact angles along the pinning lines for bodies of constant volume and base area, but different polygonal footprints and side lengths: triangle ($l_t = 2.2$), square ($l_s = 1.41$), pentagon ($l_p = 1.08$), hexagon ($l_h = 0.88$), octagon ($l_o = 0.64$), and circle (with radius $R = 0.8$). For all polygons, contact angles are at a minimum at the corners, and increase until they reach a maximum halfway between two adjacent vertices.

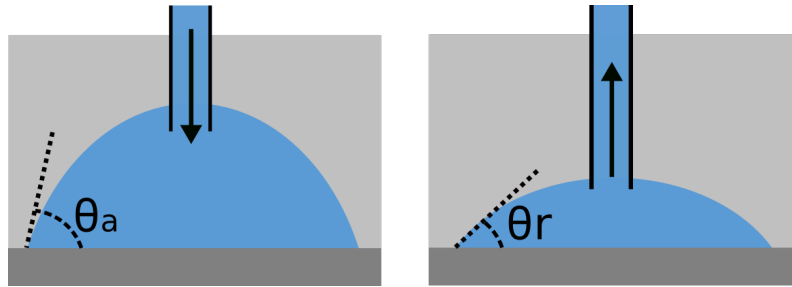


Figure 2. Contact angle hysteresis. Left – the maximum contact angle the droplet can sustain when adding volume to it, before the pinning line advances. Right – the minimum contact angle reached when withdrawing fluid from a droplet, before the pinning line recedes.

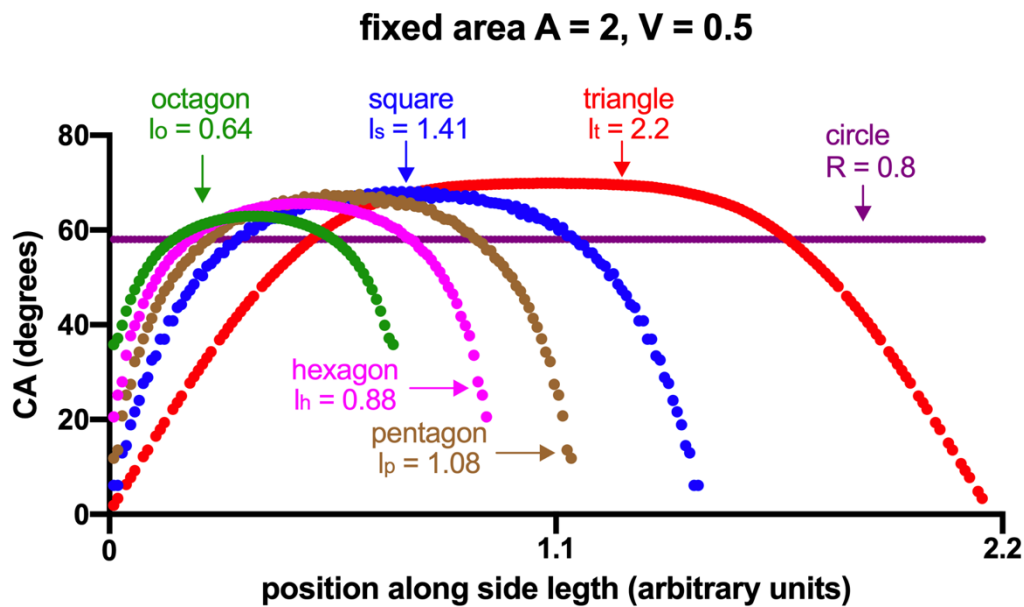


Figure 3. Variation of contact angles along the edges of droplets with various footprint. Datapoints represent results of simulations done with Surface Evolver, where all droplets have the same area of the base ($A = 2$) and the same volume ($V = 0.5$), but different polygons as footprints.

Conduits

Let us now consider a droplet which has as a footprint a rectangle with a high aspect ratio ($L_c \gg a_c$) and with a characteristic length smaller than the capillary length ($h_c < a_c < 2 \text{ mm}$). We call these structures “conduits” and they are used in our system as connections between reservoirs. **Figure 4** illustrates the shape of a conduit on a polystyrene surface completely covered by FC40.

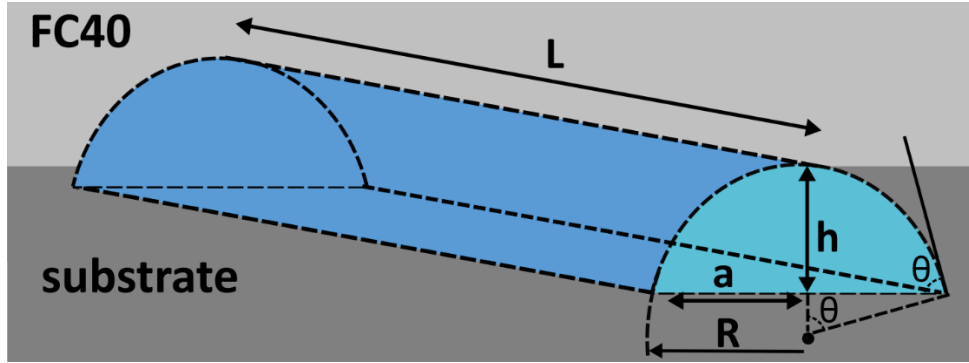


Figure 4. The geometry of a conduit under FC40. Its characteristic length scale is considered to be less than 2 mm, such that the cross-section of the conduit is a circular segment.

Having the contact angle, θ , expressed in degrees, the area of the cross section is:

$$A_{cross\ section} = \underbrace{\pi R^2 \left(\frac{2\theta}{360} \right)}_{\text{area of sector}} - \underbrace{a(R-h)}_{\text{area of triangle}} = \frac{1}{2} R^2 \left(\frac{\theta\pi}{90} - \sin 2\theta \right) = \frac{a^2}{2 \sin^2 \theta} \left(\frac{\theta\pi}{90} - \sin 2\theta \right)$$

The volume contained is:

$$V_{conduit} = L * A_{cross\ section}$$

$$V_{conduit} = \frac{a^2}{2 \sin^2 \theta} \left(\frac{\theta\pi}{90} - \sin 2\theta \right) * L$$

Using this relationship, the contact angle of a conduit can be computed provided its volume, length and contact radius are known. As one radius of curvature is negligible (along the conduit), the Laplace pressure across the interface is simply:

$$\Delta P_{conduit} = \frac{\gamma}{R_{conduit}}$$

Passive pumping

Apart from sessile droplets and conduits (which are isolated chambers), this thesis also includes more complex networks of chambers. The simplest network consists of two droplets connected by a conduit, submersed under FC40 (**Figure 5**). Together, they form a passive pumping system, where flow is driven by the difference in pressures between the two droplets (9). Passive flow in a system with morphing walls is complex, as changes in interfaces cause changes in both Laplace and hydrostatic pressures. Below, an expression for the pressure difference driving the flow, ΔP , is derived to be used in iterative calculations. In depth analysis of the system goes beyond the scope of this thesis.

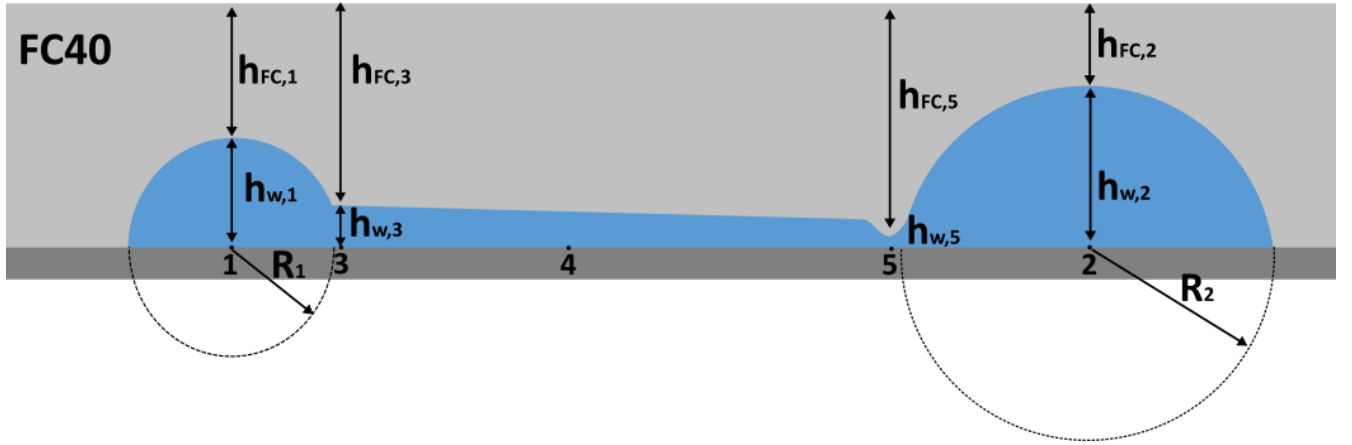


Figure 5. Passive pumping. Two sessile droplets completely submersed under FC40 are connected by a fluidic conduit. Given the difference in hydrostatic pressures is negligible compared to the difference in Laplace pressures, flow will occur from left to right (higher to lower Laplace pressure).

At each point in the system, the total pressure is the sum of the hydrostatic and Laplace pressures. Hence, inside the conduit total pressure are

$$P_3 = \rho_w g h_{w,3} + \rho_{FC} g h_{FC,3} + \frac{\gamma}{R_{conduit}}$$

$$P_5 = \rho_w g h_{w,5} + \rho_{FC} g h_{FC,5} + \frac{\gamma}{R_{conduit}}$$

and pressures inside droplets 1 and 2 are:

$$P_1 = \rho_w g h_{w,1} + \rho_{FC} g h_{FC,1} + \frac{2\gamma}{R_1}$$

$$P_2 = \rho_w g h_{w,2} + \rho_{FC} g h_{FC,2} + \frac{2\gamma}{R_2}$$

Therefore, the pressure difference driving the flow between the two droplets is:

$$\begin{aligned} \Delta P = P_2 - P_1 &= \rho_w g h_{w,2} - \rho_w g h_{w,1} + \rho_{FC} g h_{FC,2} - \rho_{FC} g h_{FC,1} + \frac{2\gamma}{R_2} - \frac{2\gamma}{R_1} \\ &= \rho_w g (h_{w,2} - h_{w,1}) + \rho_{FC} g (h_{FC,2} - h_{FC,1}) + 2\gamma \left(\frac{1}{R_2} - \frac{1}{R_1} \right) \end{aligned}$$

Noting that

$$\Delta h_{2,1} = h_{w,2} - h_{w,1}$$

$$\Delta \rho_{w,FC} = \rho_w - \rho_{FC}$$

ΔP becomes

$$\Delta P = \Delta \rho_{w,FC} g \Delta h_{2,1} + 2\gamma \left(\frac{1}{R_2} - \frac{1}{R_1} \right).$$

The theoretical concepts presented in this chapter should provide a sufficiently comprehensive foundation, such that the reader is able to follow the content of the thesis. The physics of structures with fluid walls represent a largely unexplored realm, and a path to explore these in more detail is discussed as part of the future work proposed in **Chapter 9**.

Chapter 4: Microfluidic chambers using fluid walls for cell biology

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Abstract

Many proofs of concept have demonstrated the potential of microfluidics in cell biology. However, the technology remains inaccessible to many biologists as it often requires complex manufacturing facilities such as soft lithography; and uses materials foreign to traditional cell biology such as polydimethylsiloxane. Here we present a novel method for creating microfluidic environments by simply reshaping fluids on a substrate. For cell biology applications, we use cell media on a virgin petri dish overlaid with an immiscible fluorocarbon. A hydrophobic/fluorophilic stylus then reshapes the media into any pattern through the creation of liquid walls of fluorocarbon. Microfluidic arrangements suitable for cell culture are made in minutes using materials familiar to biologists. The versatility of the method is demonstrated by creating analogs of a common platform in cell biology - the microtiter plate. Using this vehicle we then demonstrate many manipulations required for cell culture and downstream analysis including, feeding, replating, cloning, cryopreservation, fixation plus immuno-labeling, lysis plus RT-PCR, and transfection plus genome-editing. We also show that mammalian cells grow and respond to stimuli normally, and worm eggs develop into adults. Reconfiguration of the fluidic walls is also possible and utilised for fixation/immuno-labeling resulting in a rapid method to screen antibodies with minimal consumables. This simple approach provides biologists access to microfluidics, without the usual expertise or equipment requirements, while also providing the freedom to re-configure designs on demand.

Significance statement

Despite improvements in our ability to manipulate ever-smaller volumes, most workflows in cell biology still use volumes of many micro-liters. We describe a new method for creating microfluidic arrangements containing sub micro-liter volumes. It exploits the interfacial forces that are dominant at the microscale, and confines liquids with fluid (not solid) walls. We demonstrate many basic manipulations required for cell culture and some widely used downstream workflows. The method eliminates many of the problem associated with manufacture of conventional microfluidic devices, and enables reconfiguration during use, thereby providing a simple on-demand approach for fabrication of microfluidic devices using materials familiar to biology.

Introduction

Microfluidics addresses the manipulation of tiny volumes, typically less than one micro-liter. Despite many proofs of concept involving common protocols in cell biology, uptake of microfluidics by biologists is limited. Some well-known and documented reasons include the cost and complexity of manufacture of microfluidic devices (which might involve soft lithography and clean rooms), concerns regarding biocompatibility (materials used for fabrication like polydimethylsiloxane – PDMS – and the associated solvents are not traditionally applied in cell biology), and the inaccessibility of cells once introduced into enclosed spaces within devices. This prompts the development of many alternatives including “open” and “paper-based” microfluidics.

Microtiter plates are widely used during liquid handling; each is essentially an array of miniature test-tubes with 96, 384, or 1,536 wells in a uniform footprint where wells have working volumes of ~100-400, ~15-150 and ~3-10 micro-liters, respectively. Arrays with more wells and volumes down to a few femto-liters have been developed (1-3). Arrays of aqueous drops sitting on flat – usually patterned – surfaces, and overlaid with an immiscible liquid to prevent evaporation have also been fabricated (4-9); here, liquid walls/ceilings confine the

aqueous phase. The burgeoning field of droplet-based microfluidics also uses fluid walls to confine liquids (10-12). However, when compared to the widespread use of microtiter plates, few of these alternatives are incorporated into workflows in cell biology (13); consequently, most still involve volumes of many micro-liters.

A recent method, termed Freestyle Fluidics (FF), allows fabrication of microfluidic circuits by dispensing cell media in a desired pattern on a Petri dish and overlaying it with an immiscible liquid (14). The aqueous phase is bounded by fluid walls – the interface between water and immiscible liquid. One of the resultant circuits was used in a chemotaxis experiment with bacterial cells, and several benefits compared to traditional circuits were demonstrated. Here, we also create microfluidics patterns with fluid walls. However, instead of depositing the aqueous phase in the desired pattern on the substrate and then overlaying the immiscible liquid, we simply reshape the two fluids already on the substrate and allow interfacial forces to build fluid walls accurately, reproducibly, and immediately. At the microscale, these walls prove to be strong, pliant, and resilient; they morph above unchanging footprints when nano-liter volumes are added/removed. Although any 2D pattern can be made, we demonstrate the method and its versatility by creating analogs of a familiar experimental platform in cell biology, the microtiter plate. We show mammalian cells grow and respond to stimuli normally, and worm eggs develop into adults. We also demonstrate many basic manipulations involved in cell biology (i.e., cell feeding, replating, cloning, cryopreservation) plus some common downstream workflows (i.e., fixation/immuno-labeling, cell lysis/RT-PCR, transfection/genome-editing). Furthermore, we go beyond what is possible with existing microfluidics, and reconfigure the fluidic structures in real time. We suggest this method provides biologists with an easy entrée into microfluidics.

Results

Methodology

Figure 1A illustrates the fabrication of a 2 x 2 grid. The bottom of a standard polystyrene Petri dish is completely covered with cell media, excess media removed, and the residual thin film overlaid with an immiscible liquid. This overlay can be less dense than water, for example a hydrocarbon. Perhaps counter-intuitively, it can be denser like FC40 – a transparent fully-fluorinated liquid (density 1.855 g/ml) that is widely used in droplet-based microfluidics with applications in biology; at the microscale, effects due to gravity and buoyancy become negligible, and interfacial forces pin the aqueous phase to the plastic. A hydrophobic and fluorophilic stylus – with a conical tip made of polytetrafluoroethylene (Teflon) and held by a 3-axis traverse (a “printer”) – is now lowered through both liquids until it just touches the dish. As FC40 wets Teflon and polystyrene better than water, the tip (now coated with FC40) brings fluorocarbon down to wet the substrate. When the stylus moves laterally, the aqueous liquid is displaced from the surface to leave a track of FC40 pinned to plastic by interfacial forces. Drawing more lines creates a grid.

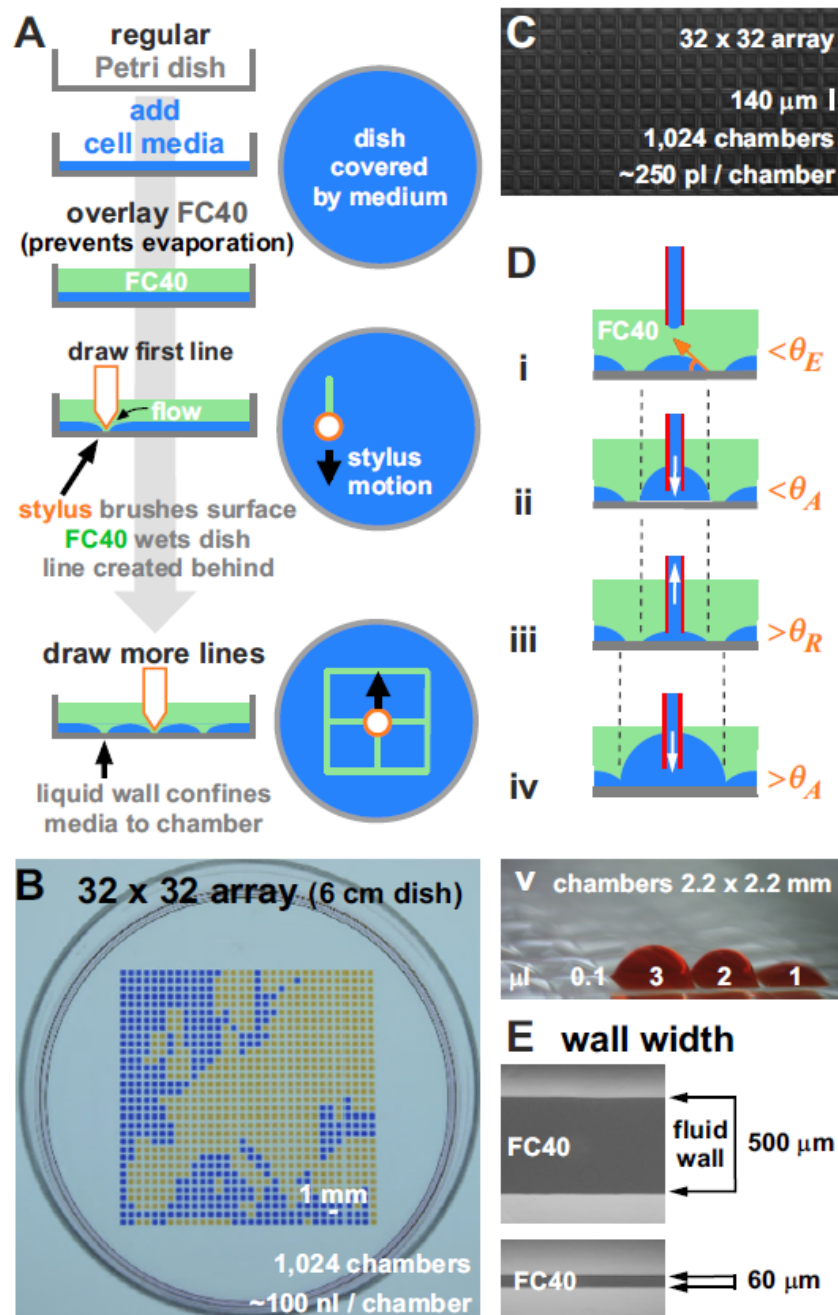


Figure 1. Building fluid walls.

A. Principle. A Petri dish is filled with DMEM + 10% FBS, most media removed, and the residual thin film overlaid with FC40 (shown green here). A hydrophobic stylus is now lowered to touch the dish, and bring FC40 to the surface. As the stylus moves horizontally, media is pushed aside, and FC40 takes its place. This creates a track of FC40 pinned to the substrate, and a liquid wall of FC40 divides the aqueous layer. Drawing more lines creates a grid.

B. A 32 x 32 grid made in ~4 min. After printing, 70 nl yellow or blue dye is added to each chamber.

C. A high-density grid (with square footprints) made with a thin stylus (73% surface covered by media).

D. Adding and subtracting media.

(i) The contact angle is $< \theta_E$ ($\sim 70^\circ$). (ii) Media can be added without altering the footprint until θ_A is reached ($\theta_A > \theta_E$). (iii) Media can be removed without altering the footprint until θ_R is reached ($< 3^\circ$). (iv) If θ_A is exceeded, the pinning line breaks and chambers merge. (v) Within limits imposed by θ_A and θ_E , grids are used like conventional plates; aqueous liquids are pipetted into (out of) chambers through FC40 instead of air. Here, 1-3 μl dye were added to chambers initially containing 0.1 μl media. Note the maximum contact angle in the square drop with 3 μl is $> 70^\circ$.

E. Stylus width determines wall width. Lines were made using styli with wider and narrower tips than in (B), and regions between chambers imaged.

Grids with few or many chambers can be made in minutes (**Fig. 1B,C; Fig. S1; Movie S1**); for example, chamber density in the grid in **Figure 1C** is analogous to that of a microplate with 393,216 wells. Here and subsequently, colored dyes are often pipetted into chambers to aid visualization; they play no role in stabilizing liquid structures. Individual chambers are used much like wells in conventional microplates; liquids are simply pipetted into (or removed from) them through FC40 instead of air (**Fig. 1D**). This can be achieved without altering the footprint on the dish. Here, consider a sessile water drop in air sitting on a standard polystyrene Petri dish. The drop is shaped like the cap of a sphere, and its footprint depends on the equilibrium contact angle (the angle, θ_E , at the air—water—substrate interface) (15,16). When tissue-culture medium without serum replaces water, θ_E is $\sim 50^\circ$, and θ_E increases to $\sim 70^\circ$ if FC40 replaces air (14). Slightly more medium can now be added without increasing the footprint up to a limit determined by the advancing contact angle, θ_A ($\theta_A > \theta_E$); once θ_A is breached, footprint area increases. Similarly, when medium is removed, the footprint shrinks once the receding contact angle, θ_R , is reached. However, θ_R is $< 3^\circ$, so at least 95% of a 5 μL drop of medium can be removed without altering the footprint (14). Hereafter, medium with serum will generally be used, and θ_A becomes $> 70^\circ$. The significant difference between θ_A and θ_R allows addition and removal of liquids above unchanging footprints (**Fig. 1Dv**). The spacing between chambers can also be varied by using styli with variable tips widths (**Fig. 1E**).

FC40 plays several additional roles. Fluorocarbons like FC40 were developed during the Manhattan Project as materials resisting attack by highly-reactive uranium hexafluoride; consequently, they are arguably the most inert liquids known, and are the carrier fluid of choice in droplet-based microfluidics. For example, FC40 carries the vital gases (O_2 , CO_2) so effectively it has been used as a blood substitute (17) and its close relatives for liquid ventilation of human preterm neonates (18,19). FC40 also prevents the underlying aqueous layer from evaporating (the solubility of water in FC40 is < 7 ppm by weight at room temperature) whilst isolating each chamber from others in a dish (and from the surroundings) – thereby preventing contamination and communication between adjacent chambers. For example, if bacteria are pipetted manually through FC40 into every second chamber in a grid,

bacteria grow only in inoculated chambers as the rest remain sterile (**Fig. 2A**). Moreover, it stabilizes pinning lines sufficiently that grids can be shaken without altering footprints (**Movie S2**). While we generally use FC40, the method works equally well with silicone and hydrocarbon oils; glass surfaces can also be used, as can polystyrene coated with more cell-friendly materials (**Fig. 2B-D**).

Varying initial chamber volume

The volume of liquid initially in a chamber can be controlled in various ways. In one, different amounts of media are added during the first step; however, the interface has curvature and hence the fluid is not an equal height across the film region. Therefore, it is convenient to use the approach in **Figure 2E**. Three 6 cm dishes are covered with thin films plus blue dye, FC40 added, 4 lines drawn in each to create 3 large central chambers (volumes $\sim 10\ \mu\text{l}$) to which more media plus dye are added (i.e., 0, 32, or 64 μl); finally, the now-different volumes are split into 3 grids. Each of the 64 chambers in the 3 dishes ends up with ~ 150 , ~ 650 , or $\sim 1,150\ \text{nl}$.

Essentially all grids illustrated here are made by passing the stylus down the center-line of a large square or rectangle to divide them symmetrically into two; then, repeated symmetrical divisions (**Fig. S2Ai**) yield chambers with equal footprints and volumes. This is demonstrated using fluorescein instead of blue dye; chambers in a grid end up with similar fluorescence intensities – and so volumes (chambers in **Fig. S2Aii** have a volume of $850\ \text{nl} \pm 4\%$ (SD); see **Fig. S2** for additional results, the calibration method, and reproducibility in a repeat experiment). However, if the stylus passes asymmetrically through a chamber offset from the center-line, resulting sub-chambers can end up with very different volumes (in **Fig. S2B**, they have 100 – 1,000 nl in an 8 x 8 grid).

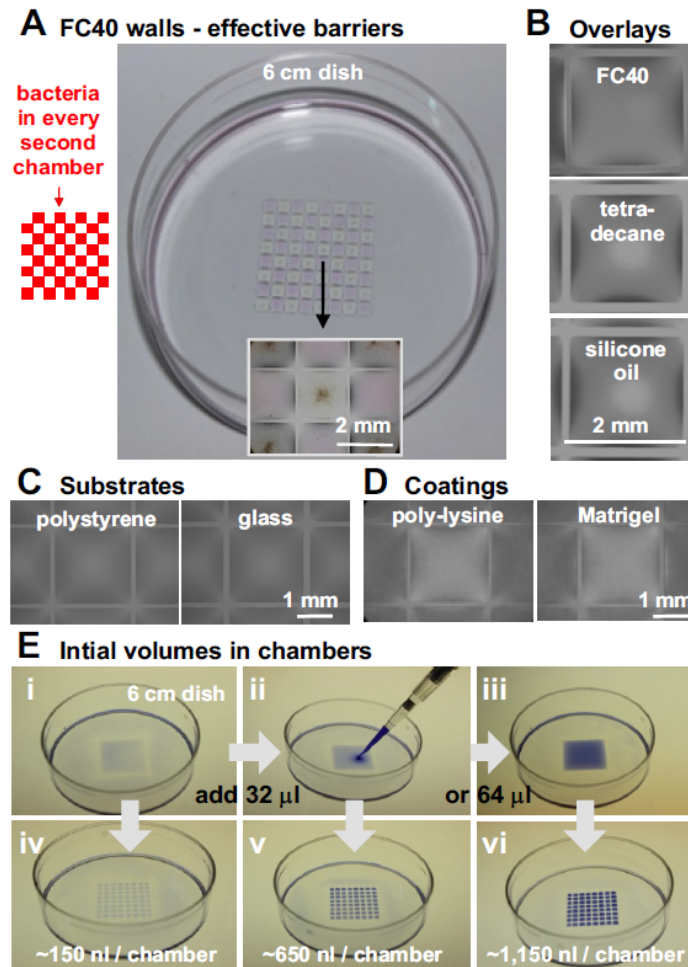


Figure 2. Some different ways of making grids.

A. FC40 isolates chambers effectively. 1.5 μ l medium $\pm$ *E. coli* was pipetted manually into every second chamber in an 8 x 8 grid (2 x 2 mm chambers; cartoon). After incubation (24 h; 37°C), a phase-contrast image is collected. Bacteria grow only in inoculated drops (seen as aggregates in chambers containing exhausted slightly-yellow media) as the rest remain sterile (slightly-pink chambers). Inset: aggregates and granularity indicate presence of bacteria.

B-D. Various overlays and substrates yield stable pinning lines. Films of media on polystyrene (uncoated except in D) or glass (C) were overlaid with 2 ml FC40 (or other oils in B), grids created, and phase-contrast images collected.

E. Preparing grids with different starting volumes (chambers 2.25 x 2.25 mm). **(i-iii)** Three dishes are covered with media + blue dye, FC40 added, four 20 mm lines drawn to create one central square in each dish (volume $\sim$ 10 μ l), and 0, 32, or 64 μ l pipetted into squares. **(iv-vi)** Subdivision yields grids.

Adding and removing liquids

Liquids can be delivered to conventional wells and our chambers in the same way – by immersing a pipette tip into a recipient well/chamber (**Fig. 1Dii**). However, when repeating this process with the same pipette, liquids can be carried over from one well/chamber to the next – and so pipets are usually washed or replaced between deliveries. Therefore, we investigated methods not requiring washing or replacement. In our printer, the pipette is a stainless steel tube mounted next to the stylus on a 3-axis traverse. In one method, the pipette tip is positioned in the FC40 above a recipient chamber, and liquid ejected into it through a transient “liquid bridge” (**Fig. 3A**). As the pump driving ejection starts and stops, and the tip moves down, up, and across, we call this “discontinuous” delivery. This method has been used to deliver one liquid to many chambers without carry-over (20). **Figure 3B** and **Movie S3** illustrate a liquid bridge without detectable upward transfer of red dye from recipient chamber to delivering pipette that could cause carry-over. We demonstrate lack of carry-over in another way. Bacteria are inoculated into every second chamber in a grid, and media delivered discontinuously into all chambers using the same tip (**Fig. 3Ci**); on incubation, bacteria grow only in inoculated chambers as others remain sterile (**Fig. 3Cii**). This confirms that bacteria are not carried over when media is delivered, and that FC40 provides a sterile barrier between chambers.

With grids, there are no solid walls, and so no need to raise or lower the pipette as it traverses, or to start and stop a pump; consequently, one liquid can be delivered to many chambers by a pipette as it moves at constant speed and height, ejecting liquid continuously (**Fig. 3D**). Such “scanning” can deliver ~70 nl to each of 1,024 chambers in 90 s (**Fig. 3E**, **Movie S4**). The variation in delivery of 280 nl to a 16 x 16 grid (measured as in **Fig. S2**) was <10% (**Supplementary Information**) mostly due to the transient motion. Such delivery occurs without detectable carry-over – exemplified by failure to transfer fluorescent beads between chambers (**Fig. 3F**). This illustrates another advantage of fluid walls: one liquid can be

delivered through one tip speedily to many chambers without carry-over because FC40 always remains in contact with the tip.

Our chambers have square footprints, and contact angles vary around the footprint (**Fig. S3**). Therefore, we estimate maximum volumes that can be added to chambers based on scaling relative to a sessile circular drop with θ_A of 70° and θ_R of 3° (values for media without serum) and diameter equal to chamber width. Then, a 1 mm square chamber in **Figure 1B** is limited to minimum and maximum volumes of 4 and 120 nl (**Table S1** gives working volumes for chambers with different footprints; all are conservative estimates as adding serum to medium increases θ_A).

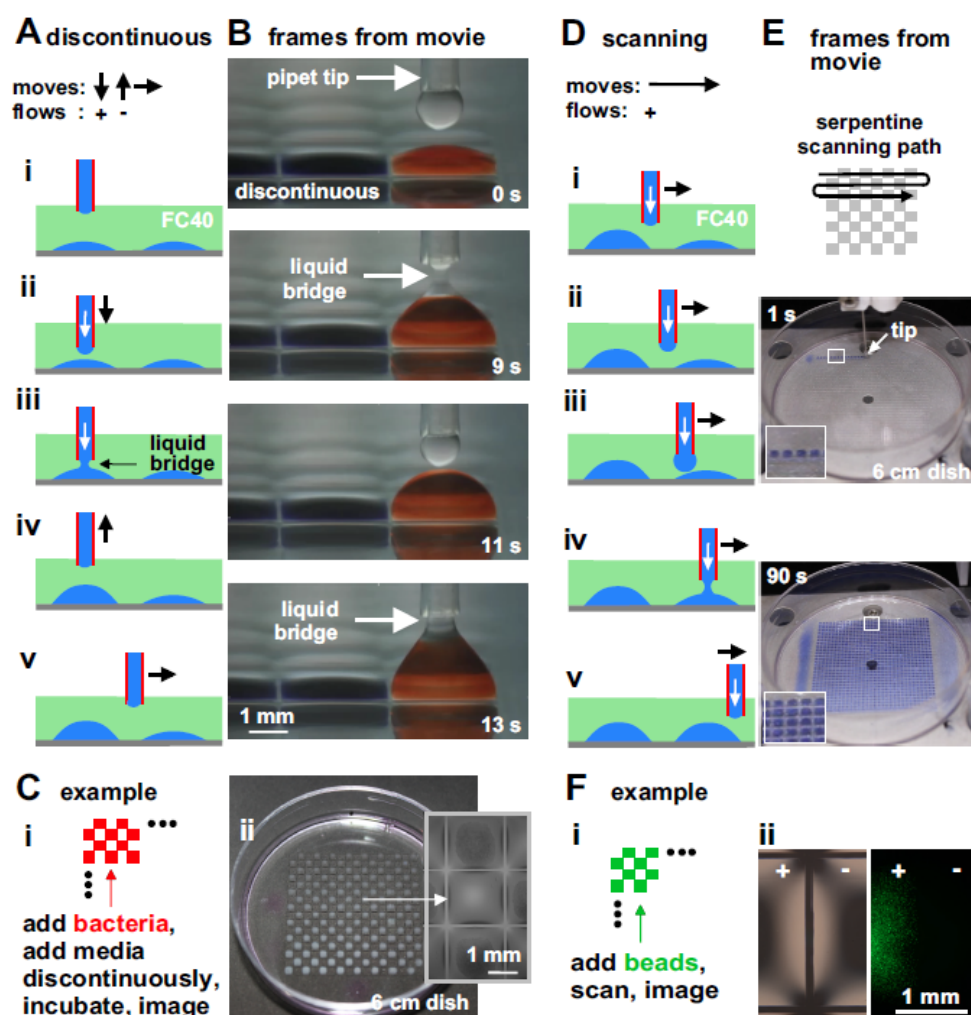


Figure 3. Adding liquids discontinuously and continuously.

A. Discontinuous. (i) The pipette is (ii) lowered (black arrow), and the pump ejects liquid (white arrow) that (iii) eventually merges with the chamber; (iv) after stopping the pump, the pipette is raised and (v) moves to the next chamber.

B. Frames from **Movie S3** illustrating no red dye moves up through a liquid bridge to the pipet. Note that contact angles at the centers of chamber edges are $\sim 90^\circ$.

C. Lack of carry-over of bacteria during discontinuous delivery (2 x 2 mm chamber, ~ 150 nl). (i) 300 nl LB $\pm$ $\sim 20,000$ *E. coli* is added manually to every second chamber, and then 500 nl LB to all chambers by discontinuous delivery. (ii) After 3 d at 20°C , imaging shows bacteria (white) grow only in inoculated chambers.

D. Continuous (“scanning”). (i-iii) The pipette maintains a constant height ($450\ \mu\text{m}$) above the substrate as it traverses (black arrow) continuously ejecting liquid (white arrow). (iv) Liquid is

delivered to the chamber. (v) Continuing traverse breaks the liquid bridge (maximum final chamber height 380 μm).

E. Frames from **Movie S4**. The tip scans (15 mm/s) along a serpentine path delivering ~70 nl medium + blue dye per chamber. Insets: 4x magnifications.

F. No carry-over of fluorescent beads between chambers during scanning (16 x 16 grid, 2 x 2 mm chambers, ~150 nl). (i) 300 nl media $\pm$ 9,000 fluorescent beads (1 μm diameter) are added manually to every second chamber in the grid. 500 nl medium is now added by scanning to each chamber. (ii) Phase-contrast and fluorescent images; no beads are carried over between chambers.

Biocompatibility – cells and organisms

Grids are made using bio-inert components – tissue-culture media and polystyrene dishes used by biologists, plus FC40. To confirm bio-compatibility, a grid was prepared, NM18 (a line of mouse mammary tumor cells) plated by scanning, and the dish placed in a conventional CO₂ incubator (O₂ and CO₂ exchange freely through FC40); cells grow normally (**Fig. 4A**) – as do all cells tested to date. Manipulations used in conventional tissue-culture are easily adapted to grids including feeding, trypsinization, replating (**Fig. 4A,B**), and cloning (below). Cells can be grown on polystyrene or glass, and on polystyrene dishes coated with poly-lysine, Matrigel, collagen, fibronectin, and laminin (**Fig. 2D, Fig. S4A,B**). Cells can be adherent or non-adherent (**Fig. S4B,C**). They can also be cryopreserved in grids (**Fig. 4C**), as in conventional microfluidic devices (21, 22); this shows that these pinning lines are stable, which is remarkable given the complex effects occurring when oil-water emulsions are frozen and thawed (23-25). Thus far, fluid walls have been made before adding cells; however, they can also be built around pre-plated cells (**Fig. 4D**). Consequently, fluid walls can be built around cells of particular interest already growing in a dish, so those with a characteristic morphology, or expressing a particular fluorescent marker, can be isolated. Note here that the microfluidic pattern is modified during use, which is impossible with most microfluidic chips. Note also that FC40 provides an additional line of defense against contamination, as it is impermeable to bacteria (**Fig. 3C**). These experiments show the standard procedures required for mammalian cell culture can be carried out in grids using sub micro-liter volumes.

We next demonstrate that whole organisms develop normally. *Caenorhabditis elegans* is a roundworm ~1 mm long that swims by undulatory locomotion; dorsal and ventral muscles contract alternately to generate bending waves along the worm's axis (26). Worms have been studied in conventional microfluidic devices (27) and droplet-based systems (28). We wished to see if pinning lines are strong enough to withstand swimming forces; they are (**Fig. 4Ei; Movie S5**). After pipetting individual eggs manually into chambers, followed by food (i.e., bacteria), eggs develop normally into adults (**Fig. 4Eii**). Worms also respond as expected to

stress. Individuals of a strain (29) that expresses the green fluorescent protein (GFP) in response to trauma were grown $\pm$ 300 mM NaCl; osmotic stress induces GFP expression (**Fig. 4Eiii**). These experiments confirm biocompatibility, plus the strength and excellent optical properties of fluid walls.

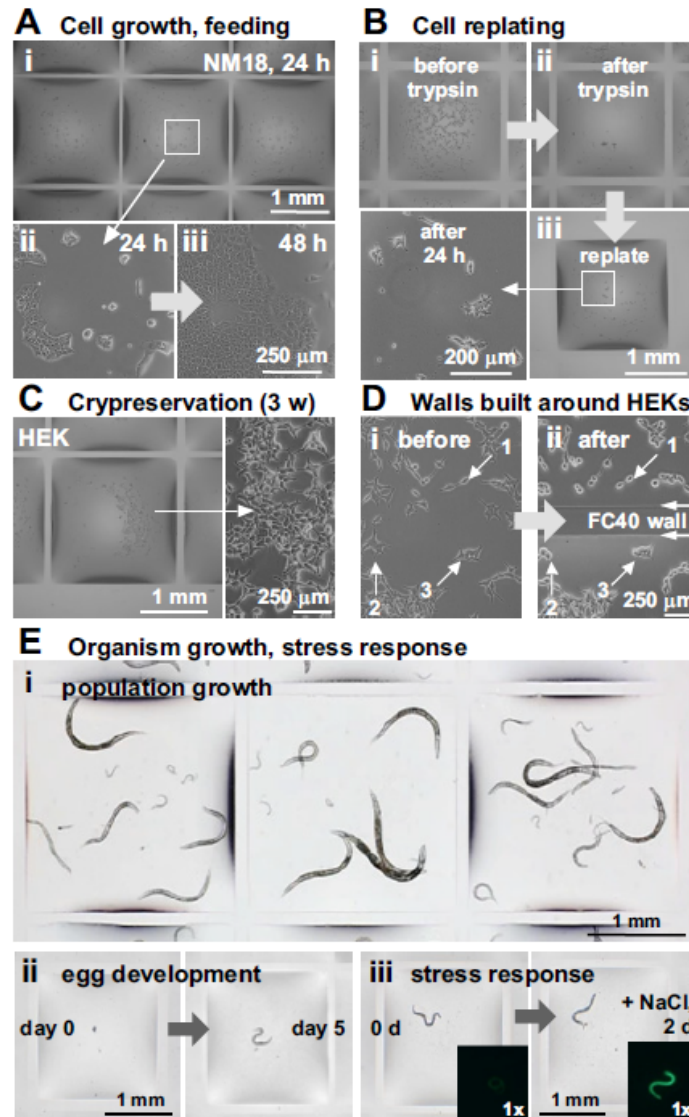


Figure 4. Biocompatibility (2 x 2 mm chambers, initial volume 150 nl).

A. Growth, feeding. (i) 250 NM18 in 800 nl are added to chambers by scanning, grown (24 h), fed (800 nl removed, 800 nl fresh media added), and imaged. (ii) High-power view of selected region. (iii) Cells are re-grown (24 h), and re-imaged; they grow normally.

B. Trypsinization, replating. (i) 500 HEKs in 500 nl are plated by scanning, grown (24 h), and imaged. (ii) After trypsinization, 1 μ l is retrieved, and the chamber re-imaged; most cells have been removed. (iii) Retrieved cells are deposited in a new chamber, and this chamber imaged. (iv) After growth (24 h), imaging confirms successful cell transfer.

C. Cryopreservation. HEKs are cryopreserved in grids for 3 w at -80°C, thawed, and cells re-grown (24 h). Pinning lines and cells survive freezing and thawing.

D. Creating fluid walls around growing cells. (i) HEKs (2×10^6) are plated, grown (24 h), and imaged. (ii) A grid is prepared around these adherent cells, and the same region of the dish re-imaged. A fluid wall divides the field; cells 1-3 remain in their original positions, as others in the path of the stylus are moved.

E. *C. elegans* develops and responds to osmotic stress normally. (i) Frame from **Movie S5** illustrating worms living in a grid. (ii) Single eggs in 500 nl S medium are deposited manually in chambers and fed daily; after 5 d, the resulting adult is imaged. (iii) Individual trauma-sensitive worms (strain CB7317) in 500 nl S medium are deposited manually in chambers, and 500 nl medium + bacteria $\pm$ 600 mM NaCl added; after 2 d, worms are imaged. This strain expresses GFP in response to osmotic stress, and worms exposed to 300 mM NaCl fluoresce green. Insets: fluorescence images.

Reconfiguration of fluid walls – antibody labelling

We next show fluid walls can be removed and rebuilt in the same place (**Fig. 5Ai**). After plating in grids, NM18 cells are fixed, the dish removed from the printer, emptied of FC40, and washed with PBS; this destroys wall integrity, although some FC40 can remain pinned along initial footprints. After mounting the dish back on the printer, fluid walls were re-built in their original– positions, although they can be built in different places if required.

As breaking/making fluid walls is so easy, we incorporated it into an immuno-labeling workflow. NM18 cells are induced by tumor growth factor $\beta 1$ (TGF $\beta 1$) to reorganize their cytoskeleton and undergo the epithelial-to-mesenchymal transition (EMT; 30). NM18 in some chambers are treated with TGF $\beta 1$, fixed, and then the workflow involves cycles of destruction of fluid walls (when cells in all chambers are batch-washed, and – in one case – permeabilized) and wall rebuilding (so different reagents can be added to selected chambers; **Fig. S5**). After finally remaking walls, cells are imaged. Cell morphology and fluorescence are as expected: TGF $\beta 1$ increases actin bundling (detected by phalloidin labeling) and vimentin expression (detected by immuno-labeling) – two markers of the EMT (**Fig. 5Aii**). This confirms the self-healing properties of walls (they were pierced and resealed 8 times) and demonstrates that immuno-labeling can be performed in grids. This approach can be scaled to screen hundreds of antibodies with considerable cost savings while also accelerating the workflow.

Pinning lines can withstand detergents – RT-PCR

A common cell-based workflow involves lysing cells with detergents followed by RT-PCR to measure mRNA levels. We expected this would be impossible as detergents reduce interfacial tension and destabilize pinning lines. However, it turns out that footprints in chambers made with medium plus serum are stable (**Fig. 5Bi,ii**). We exploit this using HEKs that encode a GFP reporter gene controlled by a promoter switched on by tumor necrosis factor alpha (TNF α); when exposed to the cytokine, cells fluoresce green ((14) **Fig. 5Biii**) and RT-PCR confirms levels of GFP mRNA increase (**Fig. 5B**, legend). This shows pinning lines

can withstand destabilizing effects of detergents, presumably due to denaturation/cross-linking of serum components.

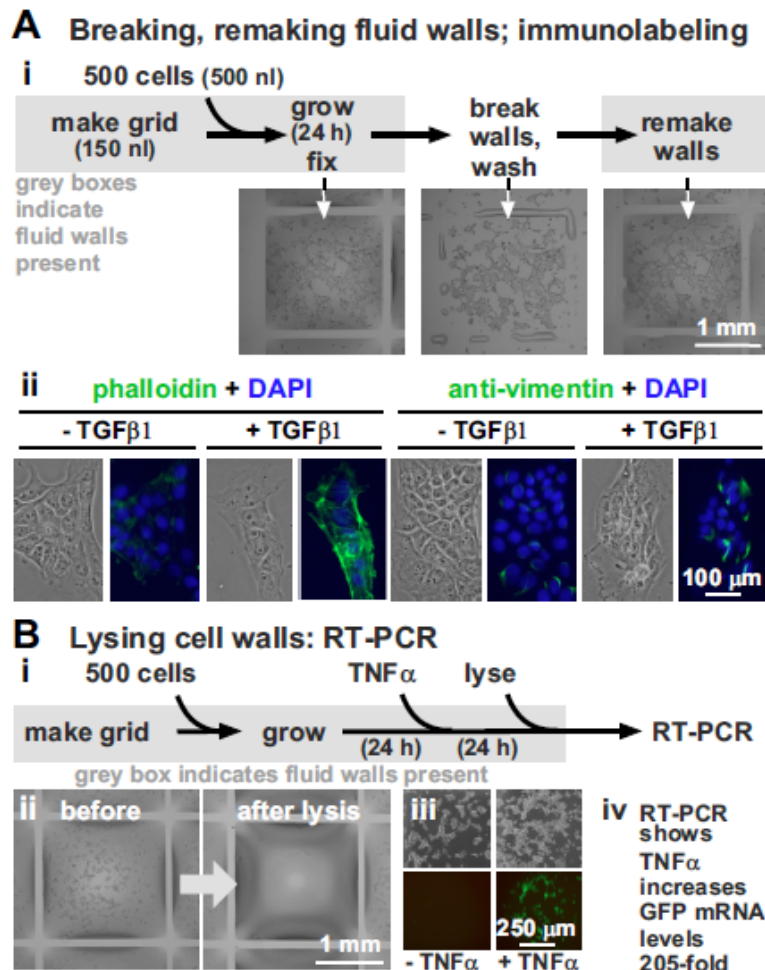


Figure 5. Rebuilding fluid walls, and destroying cell walls (2 x 2 mm chambers). 6 cm dishes were mounted in positioning rings – tightly-fitting circular sleeves enabling a dish plus ring to be removed from a printer and remounted so walls can be rebuilt in the same place.

A. Breaking and remaking fluid walls during immuno-labeling. (i) NM18 are plated by “scanning”, grown, fixed, fluid walls broken, all cells batch permeabilized with Triton X100, and walls remade. Fluid walls survive fixation, but are destroyed by emptying the dish of FC40 and washing with PBS (some FC40 remains pinned to the dish). After returning the dish to the printer and overlaying FC40, fluid walls are rebuilt. (ii) Cells respond to TGFβ1 as expected. The workflow involves cycles of making and destroying fluid walls (Fig. S5); when walls are

present individual chambers are treated differently, and when walls are absent all cells in all the different parts of the dish are treated similarly. Cells are seeded by scanning in chambers, grown $\pm$ TGF β 1, and fixed. Next, walls are destroyed, fixative washed away, cells permeabilized, walls rebuilt, phalloidin or anti-vimentin (both conjugated with Alexa 488) added to selected chambers, walls destroyed, cells washed, walls rebuilt, DAPI added, walls destroyed, cells washed, walls rebuilt, and phase and fluorescent images collected. TGF β 1 induces actin bundling and increases vimentin expression (detected by phalloidin- and immuno-labeling respectively).

B. Lysing cells and RT-PCR. (i) HEK-293 reporter cells are plated by scanning in chambers, grown (24 h), medium $\pm$ TNF α (10 ng/ml) added, cells regrown (24 h) to allow the cytokine to induce GFP expression before washing and lysis, and lysates transferred from chambers to micro-centrifuge tubes for assessment of levels of GFP mRNA by RT-PCR. (ii) Pinning lines survive lysis. (iii) Phase-contrast (top) and fluorescence images (bottom) show TNF α induces GFP expression. RT PCR also shows TNF α increases GFP mRNA levels 205-fold (lower and upper bounds: 174- and 241-fold) normalized relative to the control (lower and upper bounds: 0.63- and 1.6-fold; $n = 4$).

Example of a complex cell-based workflow – genome editing

We next performed a complex workflow – editing the *Casp6* gene using CRISPR-Cas9 (31,32) (**Fig. 6A**). Here, NM18 growing in grids are transfected with an empty plasmid, or plasmid 880 or 881 which encode Cas9 and a puromycin-resistance gene plus a guide RNA targeting a region of *Casp6*. After adding puromycin, cells are grown for 4 days; puromycin kills untransfected cells (which lack the resistance gene), whereas roughly half transfected ones remain alive (indicative of high transfection rates; **Fig. S6B**). Cells are now transferred from chambers to conventional plates, expanded, and single cells plated in new chambers. Some chambers receive one cell (**Fig. 6B**), others two, and some none (based on the Poisson distribution). Many single cells grow into colonies (cloning efficiency 71%; **Fig. 6B** legend). The printer then picks clones and transfers them to micro-centrifuge tubes. After expanding clones conventionally followed by DNA amplification and sequencing, clones are found to have deletions at appropriate target sites (**Fig. 6C**); they also no longer express Casp6 protein (**Fig. S6C**). Here, walls are pierced 16 times, and pinning lines survive treatment with transfection reagent. Significantly, fluid walls provide excellent optical clarity; there are no solid walls to obscure the view (contrast cloning in conventional flat microplates, where cells often sit against surrounding walls and “edge effects” prevent them from being seen clearly). Moreover, when cells are seeded centrally in grids, fluid dynamics and chamber geometry ensure few end up near pinning lines; pre-existing (cell-free) media is forced by incoming cell-containing media towards edges (**Fig. S7**). Consequently, users can have confidence in monoclonality. Since growing clones can be identified earlier, they can be picked sooner (in **Fig. 6C**, after 8 d – which contrasts with >14 d in conventional plates).

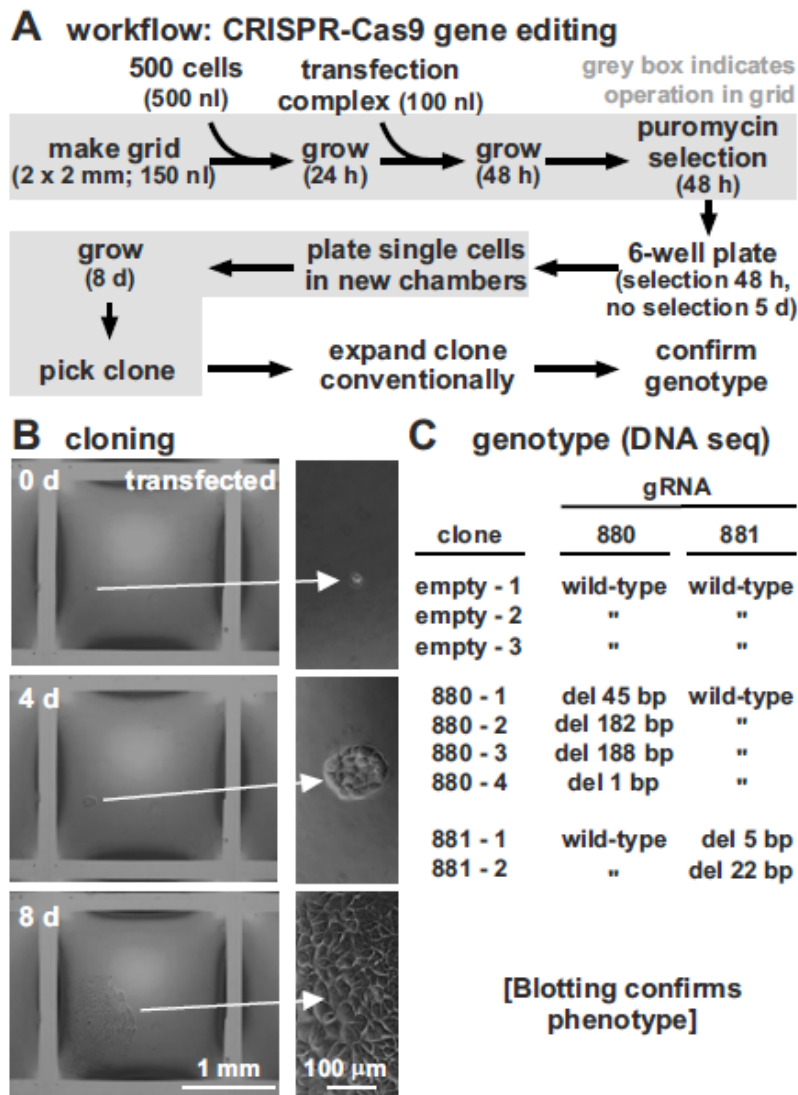


Figure 6. A complex workflow – deriving a NM18 clone with a mutated *Casp6* gene using CRISPR-Cas9.

A. Workflow. Cells are transfected with empty vector (encoding Cas9 + puromycin resistance), or plasmid 880 or 881 (which additionally encode a guide RNA – gRNA – targeting different parts of *Casp6*).

B. Cloning. After transfection, puromycin selection (Fig. S6), and expanding cells conventionally, the printer delivered ~0.2 cells (by scanning) to each chamber; images show the single cell divides over 8 d. Initially, 42 of 256 chambers (40 expected from Poisson distribution) contained 1 cell; after 8 d, 30 had colonies (71% cloning efficiency).

C. Genotyping. After picking colonies (as Fig. 4B), the printer delivers cells to a micro-centrifuge tube; cells were then expanded conventionally, DNA purified, the *Casp6* region

amplified by PCR and cloned in bacteria, and genotypes determined by sequencing. Clones derived from transfections with plasmids 880 and 881 have deletions (del) in expected regions, unlike those receiving empty vector. Immunoblotting confirms the phenotype (Fig. S6).

Conclusion

We describe a microfluidic platform for miniaturizing workflows in cell biology. Grids are made by covering the surface of a Petri dish with a thin layer of media, overlaying FC40, and using a Teflon stylus to reshape the aqueous phase into an array of individual chambers; each chamber is isolated from neighbours by liquid walls of FC40 (**Fig. 1A-C**). At the microscale, effects due to gravity and buoyancy are negligible, and the aqueous phase remains pinned to the plastic; consequently, such grids can be used much like conventional microplates – liquids are simply pipetted through FC40 instead of air (**Fig. 1D**).

During fabrication, this platform has many advantages compared to other methods. It does not require a dedicated facility or specialized equipment beyond a syringe pump and an automated positioning system to drive the stylus; then, fluid walls/ceilings are built accurately, reproducibly, and immediately by interfacial forces (e.g., our stylus generally traverses at 25 mm/s, and builds 256 and 1,024 chambers in a 6 cm dish in ~2 and ~4 min, respectively; **Movie S1**). Building is scalable; for example, the array in **Figure 1C** has a chamber density equivalent to a microplate with 393,216 wells. High-density grids are created efficiently (drawing 2-fold more lines yields 4-fold more chambers), and ~90% of the surface area is available for cell culture in contrast to ~40% in a conventional 1,536-well plate (**Fig. S1B** – calculated assuming fluid walls are 100 μm wide).

During use, there are additional advantages. First, pinning lines are stable, and fluid walls pliant and resilient. They withstand agitation (**Movie S2**), they morph above unchanging footprints when liquids are added/removed (**Fig. 1Dv**), and self-heal when pipets are inserted through them (in **Fig. 6**, walls were pierced 16 times). Second, uniform volumes can be added to many chambers quickly and easily; as there are no solid walls, there is no need to

raise/lower and/or wash the delivering pipet, or start/stop the pump. Instead, one pipette can scan through FC40 at constant speed and height, ejecting liquid continuously to feed chambers through liquid bridges that are formed transiently. Such scanning can deliver 70 nl to each of 1,024 chambers in 90 s without detectable carry-over (**Fig. 3E**, **Movie S4**). Third, all points in the aqueous phase are accessible, and the walls confining the aqueous phase can even be reconfigured during use (impossible with conventional microfluidic devices); for example, new walls can be added (**Fig. 4D**), and existing ones destroyed and then rebuilt in the same place (**Fig. 5A**).

The platform has another significant advantage: it is biocompatible if used with the culture media and dishes (including coated ones; **Fig. S4A,B**) familiar to biologists, plus what is arguably the most bio-inert immiscible liquid available – FC40 (17-19). Cells from bacteria to human cells can grow normally in grids (**Figs 2A, 3C, 4A,B**), worm eggs develop into adults (**Fig. 4Eii**), and cells/organisms respond as expected to stimuli (stress, puromycin, TGF β , TNF α ; **Figs 4Eiii, Fig. 5**). We also miniaturize the basic manipulations involved in mammalian cell culture (e.g., feeding, replating, cryopreservation, cloning; **Figs 4A-C; Fig. 6B**), and some widely-used downstream workflows (fixation and immuno-labeling, cell lysis and RT-PCR, transfection and genome-editing; **Figs 5,6**). As walls can be built around living cells of interest (**Fig. 4D**) – perhaps ones with characteristic morphologies or expressing particular fluorescent markers – selected cells can easily be isolated from others (for subsequent growth or analyses) without touching them. We also anticipate grids will prove especially useful for cloning. With conventional microplates, single cells often sit against surrounding walls and cannot be imaged clearly because of “edge effects”. In contrast, fluid walls lack obscuring walls and so yield excellent optical clarity, and – as cells are deposited centrally in chambers, and as fluid dynamics and geometry ensure few end up at the edges of footprints (**Fig. S7**) – users can be confident which chambers contain only one cell. Moreover, clones can be picked sooner (e.g., after 8 d in **Fig. 6B**). Additionally, FC40 provides an extra barrier to contamination, isolating each chamber from others in one dish, and from the external world (**Fig. 2A**).

As with any platform, ours has limitations. First, an immiscible liquid is required to limit evaporation (as is generally the case wherever sub micro-liter volumes are handled). Second, liquids and surfaces must be matched to ensure pinning lines are stable. We use the following approach to test new combinations rapidly. A drop of aqueous liquid is placed on the substrate and overlaid with FC40, and most aqueous phase removed. If pinning lines do not retract, the combination can probably be used. If pinning lines retract, we try making a grid with combinations known to work, and then replace liquids with ones desired for operation. Third, there are limits to upper and lower volumes that chambers can accommodate without change in footprint. These limits depend on advancing and receding contact angles; for example, with angles of 70° and 3°, a 2 x 2 mm chamber has working volumes of 35 - 1,100 nL, and a 140 x 140 µm chamber 9 – 270 pL (**Table SI**).

In summary, we have developed a versatile platform for manufacturing microfluidic patterns, exemplified by making arrays of square chambers with nano-liter volumes. These grids are used like conventional microplates, except liquids are pipetting through FC40 instead of air. We anticipate they will prove especially useful in cell biology as they are made with materials familiar to users, they can be incorporated into common workflows, and they provide considerable savings in consumables.

Materials and methods

General reagents and equipment

FC40 was purchased from Acota. It is bio-inert, not found in regulatory lists of dangerous organic chemicals (<http://www.acota.co.uk/assets/data-centre/msds/3m/3mfc40msds.pdf>), and it should not be confused with the volatile chlorofluorocarbons that release the chlorine radicals destroying the ozone layer. If grids are to be kept for days, extra FC40 should be added when needed (14). Evaporated FC40 has no untoward effects on any of many different cell types grown conventionally in the same incubator at the same time over 3 years. All other

fluids and materials were from Sigma Aldrich unless otherwise stated. Where indicated, aqueous drops contain water-soluble dyes (e.g., Allura Red, toluidine blue, resazurin).

Most grids were fabricated, and small volumes delivered to them, using an isoCell (iotaSciences Ltd, Oxford, UK). This is essentially a tool-head driven by a 3-axis traverse and appropriate software; the tool-head holds a stylus – a Teflon rod (3.8 mm diameter) with a conical tip (angle at tip $\sim 50^\circ$) – and a stainless-steel dispensing needle (width 0.5 mm outer diameter) connected to a syringe pump. This Teflon stylus was used to make all circuits except for ones shown in **Figures 1C, 1Dv, S1B, and C, and 3** where the rod was replaced by a Teflon tube (outer diameter $\sim 750\ \mu\text{m}$; Cole Parmer, Vernon Hills, Ill). As the dispensing needle is hydrophilic, liquid can run up the outside instead of into a chamber as wanted, and this both makes accurate delivery of small volumes unreliable (33) and increases carry-over contamination when delivering one liquid to many chambers in a grid from one dispensing tube. Therefore, a hydrophobic sleeve (a piece of Teflon tubing) is included around the tip of the dispensing tube to prevent runback (see **Movie S4**). The central work-place holds a 6 cm dish, plus micro-centrifuge tubes containing reagents (often tissue-culture media, 70% ethanol for sterilization; **Movie S4**). The 6 cm dish is placed in a positioning ring – a tightly-fitting circular sleeve bearing a protrusion that ensures the dish plus ring can be mounted, removed, and remounted in the IsoCell workplace in the same orientation. Where sterility is required, the printer is placed in a bio-safety cabinet, and sterile procedures used throughout (e.g., the stylus tip is sterilized with 70% ethanol, and the software includes cycles for aspirating 70% ethanol into the tube connected to the dispensing needle and ejecting the ethanol into a waste tube). When printing grids on rectangular flat (1-well) polystyrene microtiter plates (127.7 x 85.5 mm; Nunclon from Thermo Fisher Scientific) – the 3D-traverse system described by Walsh et al. (14) fitted with a stylus – a Teflon tube – was used.

Printing and operation of grids

Grids were generally fabricated using the isoCell and “6 cm” polystyrene tissue-culture dishes (Falcon – 60 mm TC-treated cell culture dish product #353002, and Corning – 60mm

TC-Treated Culture Dish, product #430166; these dishes have internal diameters of ~5 cm). 6 cm dishes were coated where stated with poly-lysine, Matrigel (Corning, Wiesbaden, Germany), fibronectin, laminin, or collagen (Cell Applications Inc, San Diego, CA) by covering the bottom of the dish with the liquid coating using the concentrations, times, and temperatures suggested by the manufacturer (except for collagen – below), removing most of the liquid coating to leave a thin film on the surface, and (without allowing the coating to dry) immediately creating grids. For collagen, a 0.5x dilution (2.5 $\mu\text{g}/\text{cm}^2$) was used, as the thicker coating prevented FC40 from wetting the surface. Glass substrates were either glass microscope slides/coverslips in 6 cm dishes, or 35 mm glass bottom dishes (No 0, MatTek, Ashland, MA). DMEM plus 10% FBS was used to make all grids described except those for use with worms; when the term “media” is used in the context of mammalian cell culture, it should be assumed that serum is present unless stated otherwise. Typically, 1 ml DMEM + 10% FBS is pipetted manually into a 6 cm dish, medium swirled around so the bottom is covered completely when the dish is horizontal, the dish tilted, and 0.9 ml medium removed and discarded. The bottom is now completely covered by a thin film of medium. 3 ml FC40 is manually pipetted into the dish so a layer of FC40 covers the media. Steps prior to overlaying FC40 are carried out quickly if the grid is to be used with cells, so the pH of the medium remains unchanged. After placing the dish in the work-area on the printer, the system uses in-built software to “home” the tool head, select the stylus, “draw” lines to create the grid (stylus speed typically 25 mm/s), deselect the stylus, and go “home”. Additionally, the software can select a pipette (the “pen”), deliver nano-liter volumes to selected chambers (by switching on and off an in-built syringe pump), deselect the stylus, and go “home”.

Detailed methods for individual Figures are described in SI.

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Supplementary materials

Methods used for individual figures

For the 32 x 32 grid in **Figure 1B**, 70 nl medium + yellow or blue dye were delivered to selected chambers to create the wanted pattern. For **Figure 1C**, the grid was made in a 6 cm dish using a stylus constructed from a Teflon tube. For **Figure 1E**, medium was added to chambers on each side of the FC40 wall to increase wall—chamber contrast.

For **Figures 2A** and **3C**, *Escherichia coli* (HB101) were inoculated directly from frozen stocks into Luria-Bertani broth (LB), and used as described. For **Figure 3C**, 300 nl LB medium $\pm$ ~20,000 *E. coli* was added to every second chamber, and 500 nl LB was added (ejection rate 8.3 μ l/s; as in **Fig. 3A**) to each chamber (minimum tip—dish-surface distance ~0.6 mm;

maximum chamber height increases from ~0.34 to ~0.63 mm). In **Figure 3E**, the dispensing tip scans (15 mm/s) along a serpentine scanning path as it delivers ~70 nl medium + blue dye per chamber (ejection rate 0.7 μ l/s; maximal chamber height increases from ~0.15 to ~0.34 mm). In **Figure 3F**, 300 nl media $\pm$ 9,000 fluorescent beads (1 μ m diameter) are added to each chamber as indicated, and then 500 nl media is now added by scanning (traverse rate 1.67 mm/s, ejection rate 0.4 μ l/s) to every chamber (tip—dish-surface distance ~0.65 mm; maximum chamber height increases from ~0.34 to ~0.63 mm). For a 16 x 16 grid, the standard deviation found after delivering 280 nl by scanning to each chamber (measured as in **Fig. S2**) was found to be $\pm$ 27 nl. When larger volumes are delivered (up to the maximum final volume given in **Table S1**), the uncertainty in volume delivered should remain the same as the main source of variation results from differences in initial chamber height. [**Figures 3-6 and S4** illustrate delivery of 70-1,000 nl by scanning to chambers of different size.]

For **Figure 4A**, NM18 cells (34) were used. For **Figure 4B**, 500 HEKs in 500 nl are plated by scanning, grown (24 h), and imaged. Next, 800 nl medium is removed, cells washed with PBS (1 μ l added, 1 μ l removed after 1 min), 1 μ l trypsin (TrypLE, Gibco #12563011, Gaithersburg, MD) added, the dish incubated (5 min; 37°C) and transferred to a printer, 1 μ l retrieved, and the chamber re-imaged. Finally, the retrieved 1 μ l (containing suspended cells) is mixed with 10 μ l fresh medium (to neutralize trypsin) in a micro-centrifuge tube, 1 μ l of the mixture deposited in a new chamber, this chamber imaged, and – after growth (24 h) – re-imaged. For **Figure 4C**, 500 HEK cells in 500 nl were seeded in grids in DMEM + 20% FBS + 10% DMSO, and immediately put in a -80°C freezer in an expanded polystyrene box surrounded by paper tissues, stored for 3 w, chambers thawed by placing the dish at room temperature, chambers washed (700 nl removed, 700 nl fresh medium added) 3 times (some cells were lost), and cells re-grown (24 h).

For experiments involving *C. elegans* (strain CB3717²⁹), worms were grown on agar plates using *E. coli* OP50 as food (35). The strain used has been genetically modified to express GFP in response to trauma; it also expresses RFP constitutively, but results using

this marker are not illustrated here. Grids were made with S medium + 0.03 mg/ml BSA (added to stabilized pinning lines), and worms in them grown at room temperature. Prior to collection of still images, dishes were placed at -20°C for 10 min to temporarily immobilize worms, and sometimes worms were washed with fresh medium to remove feces and bacteria. For **Figure 4Eii**, single eggs in 0.5 µl S medium were deposited manually in chambers, 0.5 µl medium + bacteria added, and 0.8 µl removed daily and replaced with an equal volume of fresh S medium + bacteria; after 5 d, the resulting adult was imaged. For **Figure 4Eiii**, individual worms in 0.5 µl S medium were deposited manually in chambers, and 0.5 µl S medium + bacteria ± 600 mM NaCl added; after 2 d, worms were imaged. Average fluorescence intensities/pixel over 5 worms treated ± NaCl for 2 d were 1 ± 0.1 and 4 ± 1.5 arbitrary units (± SD).

For **Figure 5Ai**, NM18 cells (500 in 500 nl) were plated by scanning in each chamber (16 x 16 grids, 2 x 2 mm chambers, 150 nl initial volume) in a 6 cm dish mounted in a positioning ring, grown (24 h), and the dish remounted on the isoCell. After removing 650 nl, cells were fixed by adding 500 nl 4% paraformaldehyde in PBS, and one chamber imaged. After fixation (15 min), the dish (still in the positioning ring) was removed from the isoCell, and fluid walls destroyed by manually emptying the dish of FC40 and washing with PBS. The same chamber was reimaged. The dish was then remounted on the isoCell, FC40 added, and the grid recreated in the same place as before. Finally, the same chamber was imaged. For **Figure 5Aii**, NM18 (500 in 500 nl) were plated by scanning in each chamber (16 x 16 grids, 2 x 2 mm chambers, 150 nl initial volume) in a dish mounted in a positioning ring, grown in a CO₂ incubator (24 h), the dish returned to the isoCell, 400 nl media ± TGFβ1 (Peprotech, London, UK) added (final concentration 5 ng/ml), cells regrown (24 h), fed on the isoCell (by removing 800 nl medium, and replacing 800 nl media ± TGFβ1), regrown, refed on the isoCell (by removing 800 nl and adding 800 nl media ± TGFβ1, and cells regrown (24 h). After fixation and destruction of fluid walls (as above), cells in all chambers were batch permeabilized manually by adding PBS plus 0.1% Triton X100 to the dish. After washing with PBS to remove detergent, the dish was remounted in the IsoCell, FC40 added, and fluid walls rebuilt in their

original places. Next, 500 nl PBS $\pm$ phalloidin-iFluor 488 (1/1000 dilution; Abcam; Cambridge, UK) or $\pm$ an anti-vimentin antibody conjugated with Alexa 488 (1/800 dilution; Cell Signaling Technology, Leiden, The Netherlands) were added to selected chambers, and the dish incubated (4°C, 15 h). Next, fluid walls were destroyed, the dish washed with PBS, fluid walls remade in the same place (all as before), 500 nl DAPI added (final concentration 1 μ g/ml) and the dish incubated (20°C, 30 min). Finally, fluid walls were destroyed (as above), the dish washed with PBS, walls remade to facilitate identification of particular cells, and individual chambers imaged.

For **Figure 5B**, HEK-293 reporter cells (NF- κ B/293/GFP-LucTM Transcriptional Reporter Cell Line; System Biosciences, catalogue number TR860A-I) were grown as recommended by the manufacturer in DMEM plus 10% FBS. They encode a GFP gene under the control of the minimal cytomegalovirus promoter downstream of four copies of the NF- κ B consensus transcriptional-response element, and GFP expression can be induced by TNF α . 500 cells in 500 nl were plated by scanning in chambers, grown (24 h), 400 nl medium $\pm$ TNF α (10 ng/ml; Peprotech, London, UK) added, cells regrown (24 h) and imaged, washed with PBS (by removing 1 μ l medium, adding 0.8 μ l PBS, and retrieving 0.8 μ l), and lysed by adding 0.5 μ l RNA lysis buffer (Zymo Research, Orange, CA); the same chamber as before was now re-imaged. Next, levels of GFP mRNA were assessed by real-time RT-PCR. 0.5 μ l cell lysate was transferred from a chamber to a micro-centrifuge tube containing 100 μ l RNA lysis buffer and levels of GFP mRNA assessed by RT-PCR. Total cell RNA was isolated using the Quick-RNA MicroPrep kit (Zymo Research), and cDNA synthesis and RT-PCR performed using the SuperScript[®] III Platinum[®] One-Step qRT-PCR kit (Thermo-Fisher Scientific) with DNase treatment, according to the manufacturer's instructions. Gene-specific primers for GFP were synthesized commercially (Sigma-Aldrich; forward primer GATGGGCTACGGCTTCTACC, reverse primer GTACTTCTCGATGCGGGTGT). Each mRNA value was normalized to levels of transcripts of the house-keeping gene, 5s rRNA (forward primer TACGGCCATACCACCCTGAA, reverse primer GCGGTCTCCCATCCAAGTAC). Cycling profiles (Eppendorf realplex² master cycler; melting-curve analysis) were: 50°C for 5', then

95°C for 5', followed by 40 cycles of 95°C for 15" and 60°C for 30", and finally 40°C for 1'. Results are reported as relative gene expression. The fold-change in gene expression relative to the control was calculated by the $2^{-\Delta\Delta CT}$ method (36).

For **Figure 6**, the *Casp6* gene in NM18 cells was edited using the CRISPR-Cas9 system and plasmids 880 and 881; transfections involving no plasmid, and plasmids lacking inserts, provided controls. The guide RNA in plasmid 880 targeted the sequence TCCATCTTGTA CTGCTCGGCTGG, and that in 881 targeted CGTTGGTGCCCCGCCTCTCTGGG. These plasmids were constructed by inserting oligonucleotides into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene, Cambridge, MA). After making a grid (16 x 16, chambers 2 x 2 mm, 150 nl), cells (500 in 500 nl) were delivered by scanning (**Fig. 3D**) to each chamber, and grown (24 h) in a CO₂ incubator. Transfection complexes were formed in a micro-centrifuge tube by manually pipetting 100 µl serum-free DMEM plus 1.5 µl TransIT-X2 (Mirus Bio LLC, Madison, WI) and ~2 µl plasmid DNA (1 µg), briefly vortexing, and then transferring 50 µl into a well on a glass slide (Nexterion Slide Glass B MPX 16N; Schott, Jena, Germany) contained in a rectangular container (custom made on a 3D printer) that fits into the station for micro-centrifuge tubes at the front of the isoCell. The slide in the container was now overlaid with FC40 and incubated (30 min, room temperature) to allow complexes to form. Next, the container plus slide was transferred to the isoCell. After placing the dish containing cells on the isoCell, the printer added 100 nl complexes to each chamber in the grid followed by 250 nl medium (so total chamber volume becomes 1 µl). Cells were regrown (48 h), fed with medium plus puromycin (the isoCell removed 800 nl media and added 800 nl fresh media ± 1 µg/ml puromycin to kill wild-type cells and select for ones expressing a transfected puromycin-resistance gene), grown in a CO₂ incubator (24 h), and refed ± puromycin (as above). After further growth (24 h; **Fig. S6B** provides images of cells growing in grids ± puromycin at this stage), the isoCell retrieved cells from chambers containing puromycin (as **Fig. 4B**), and transferred them to micro-centrifuge tubes. Cells were now manually transferred to conventional 6-well plates, grown (48 h) in medium + puromycin, and regrown (5 d) without selection. Cells were harvested, counted, and diluted to 400 cells/ml,

and the isoCell then transferred ~0.2 cells in 500 nl into each chamber in a new 16 x 16 grid (2 x 2 mm, 150 nl) followed by 200 nl medium containing serum (giving a total of 1 μ l). Chambers containing one cell were imaged and recorded, the grid transferred to a CO₂ incubator for 8 days, and chambers re-imaged. Cells derived from a single clone were then transferred from a chamber to a micro-centrifuge tube by the isoCell, expanded conventionally, and genomic DNA purified and amplified by PCR, followed by TOPO TA cloning (Thermo Fisher Scientific and Life Technologies, Carlsbad, CA) and sequencing. Phenotypes of edited clones were also monitored by immuno-blotting (**Fig. S6**). Cells grown conventionally were harvested in Pierce RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Fisher Scientific, Loughborough, UK) containing protease inhibitor cocktail (Fisher Scientific), protein concentrations determined using the Bradford method and kit (Sigma-Aldrich, Dorset, UK), and whole cell lysates resolved by 12% SDS-PAGE and transferred to ImmunoBlot PVDF membranes (Bio-Rad Laboratories, Herts, UK). Next, membranes were incubated overnight with primary antibody in 5% w/v non-fat dry milk, 1X TBS plus 0.1% Tween 20 at 4°C with gentle shaking. Antibodies used were rabbit polyclonal anti-Caspase-6 (Cat# 9762) and anti-rabbit IgG, HRP-linked antibody (Cat #7074; Cell Signaling Technology, Beverly, MA). After stripping membranes with Restore Plus Membrane Stripping Buffer (Thermo Scientific), membranes were re-probed with mouse monoclonal IgG GAPDH (G-9) (sc-365062) and goat anti-mouse IgG-HRP (sc-2005 F0115) from Santa Cruz Technology (San Diego, CA).

Imaging

Images of dishes were taken using a digital SLR camera (Canon 600D). Bright-field, phase-contrast, and fluorescence microscope images of grids with and without cells were collected using either a camera (AxioCam MRm) attached to a microscope equipped for live-cell imaging (Zeiss Axioskop 40; Olympus LWD A20 PL 20x lens) or a zoom lens and digital SLR camera (Nikon D7100 DSLR) connected to an epi-fluorescent microscope (Olympus IX53; 1.25X, 4X, 10X, 25X objectives) with translation stage and overhead illuminator

(Olympus IX3 with filters). For **Figure S2**, the objective used was an Olympus UPlanFI 4x (0.13 NA, 17 WD). Fluorescence intensities in **Figure 4Eiii** were quantified using ImageJ; for each image, background was subtracted, the area containing the specimen selected, and the average intensity per pixel determined and normalized relative to the control (non-treated specimen).

Supplementary tables

Table S1. Some characteristics of conventional microplates and grids.

Microplates (solid walls)				Grids (fluid walls)			
well number	inter-well spacing (mm)	working volume (μl)		inter-chamber spacing (mm)	working volume (μl)		example
		min	max		min	max	
96	9	~50	~360	9	3.6	113	
384	4.5	~15	~100	4.5	0.44	14	
1,536	2.25	~3	~10	2.25	0.05	1.6	Fig S1A
				2	0.035	1.1	Fig 2A
				1	0.004	0.12	Fig 1B
393,216	0.140			0.140	9×10^{-6}	270×10^{-4}	Fig 1C

Values for microplates are from manufacturers' data sheets. For grids, inter-chamber spacing includes the width of a 100 μm FC40 wall except for the highest-density one where the width was 20 μm; minimum (min) and maximum (max) working volumes – which are proportional to the cube of the characteristic length scale (the width of the chamber) – are conservatively estimated by scaling relative to a sessile drop with circular footprint with diameter equal to chamber width using θ_A of 70° (serum-free medium) and θ_R of 3° (both values from ref 14 for serum-free media). Maximum and minimum values for media plus serum are higher and lower than indicated, respectively.

Supplementary figures

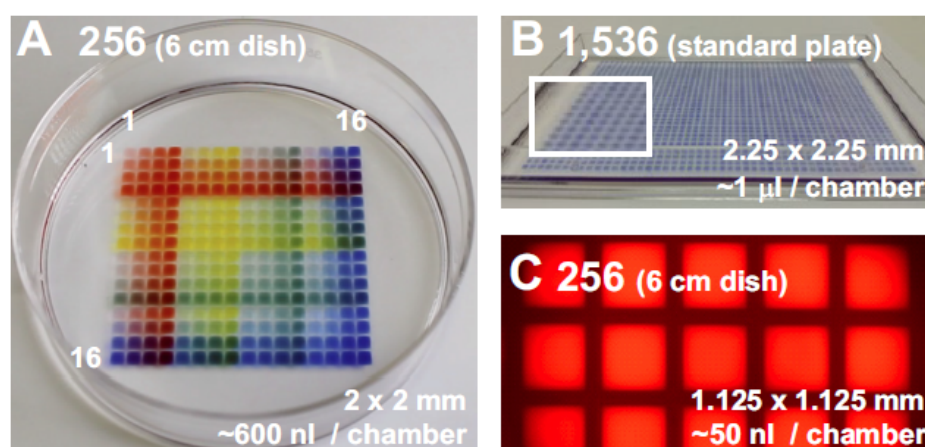


Figure S1. Grids of different sizes are made easily.

A. 16 x 16 grid which could be used for a combinatorial drug screen. 16 different dyes (representing 16 different drugs) were added in all pairwise combinations to 2 x 2 mm chambers (200 nl/chamber). Dye 1 (400 nl) was delivered to each chamber in row 1 and column 1, dye 2 to row 2 and column 2, and so on. Consequently, chambers on the diagonal (top-left to bottom-right) contain one dye, whilst others contain two in all pair-wise combinations (duplicates on each side of the diagonal). The stability of this grid is illustrated in **Movie S2**.

B. A 48 x 32 grid made in a 1-well microplate of standard area (i.e., a rectangular polystyrene Petri dish, 127.76 x 85.48 mm). Center-to-center spacings of chambers are 1/4 of those in a 96-well microplate, and like those in a 1,536-well microplate. After printing, blue dye was added to each chamber to aid visualization. Inset: 2x zoom.

C. Fluorescence micrograph of 16 x 16 grid with 256 chambers made in a 6 cm dish using medium + blue dye (which fluoresces red). Center-to-center spacings are 1/8 of those in a 96-well microplate.

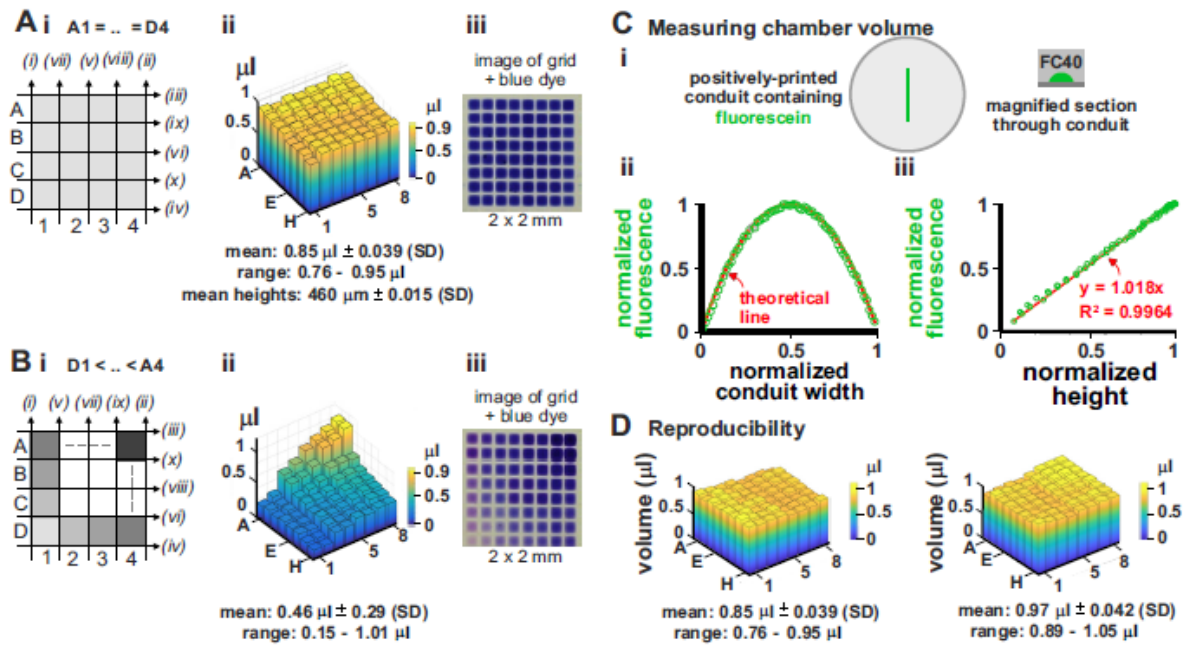


Figure S2. Creating chambers with the same or different starting volumes by altering stylus path (chambers 2 x 2 mm). Two dishes are covered with media + fluorescein, FC40 added, four 20 mm lines drawn to create one central square in each dish (volume ~10 μl), and 50 or 32 μl (in **A** and **B**, respectively) pipetted into each square (as **Fig. 2Eii**). The stylus tip is a cone that displaces equal amounts of liquid to each side as it moves across the substrate at 25 mm/s, and it creates grids by symmetrical or asymmetrical division. The fluorescence intensity/chamber (which reflects volume) is measured by microscopy.

A. Symmetrical division. (i) Stylus path (arrows – stylus direction; roman numerals – drawing sequence). Lines (i)-(iv) create a square, and then (vi)-(x) a 4 x 4 grid as successive lines pass through centers of pre-existing aqueous bodies. Additional lines (not shown) create an 8 x 8 grid in which chambers have similar volumes (volume A1 = B1 ... C4 = D4). (ii) Measurement of fluorescence intensity/chamber shows chambers contain similar volumes. (iii) Image of grid created using blue dye instead of fluorescein.

B. Asymmetrical division. Chambers with different volumes created as in (A) except the stylus follows a different path. (i) Lines (i)-(iv) create a square (as before), but (v)-(x) scan progressively from lower-left to upper-right to divide pre-existing aqueous bodies asymmetrically (except for last two); this progressively shifts the center of mass of the larger

body of media up and to the right, and chamber volume $D1 < C1 \dots B4 < A4$. Additional lines (not shown) create an 8 x 8 grid. (ii) Measurement of fluorescence intensity/chamber shows chambers contain different volumes. (iii) Image of grid created using blue dye instead of fluorescein; chambers at lower left clearly contain less dye.

C. Method used to measure chamber volume. (i) A straight 30 mm conduit containing media + 10% FBS supplemented with fluorescein is positively printed in air on a 6 cm dish (0.5 mm diameter pen) using a syringe pump connected to a hollow stainless-steel dispensing needle, and immediately overlaid with FC40 to prevent evaporation (14). As the cross-section of this aqueous conduit is shaped like the cap of a sphere, it has a known radius, maximum height, and width. (ii) Normalized fluorescence intensity across the conduit. Using a fluorescence image of a central section of the conduit, the fluorescence intensity (arbitrary units per 50 x 50 pixels across conduit) is measured (green circles). The red curve is the one expected if the conduit section is shaped like the cap of a sphere, and if intensity is proportional to cap height. All image processing steps were carried out in Matlab. (iii) Relating fluorescence to height. Data from (ii) is replotted by relating intensity to height.

D. Two grids were made by symmetrical division, and chamber volumes calculated (as in **C**). Chambers have similar fluorescence intensities – and so volumes. Left-hand plot prepared using data from (**Aii**).

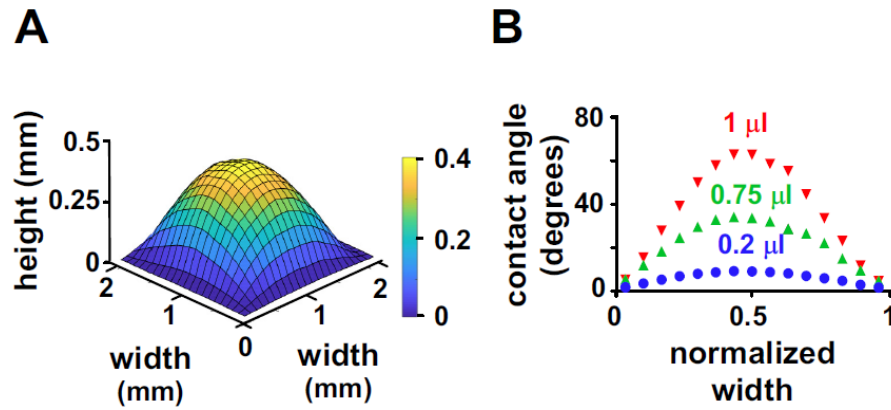


Figure S3. Chamber shape.

A. Overall shape. 2 x 2 mm chamber was made (as in **Fig. 2E**) using medium plus fluorescein; its volume was 850 nl (measured as in **Fig. S2C**). The (maximum) contact angle (measured as in **B**) at the midlines of edges is 52°. A droplet of the same volume with a spherical footprint of 2 mm diameter has a contact angle of 54°; consequently, calculations based on the use of droplets with spherical footprints in **Table SI** provide good estimates of working volumes.

B. Contact angles along a pinning line of a 2 x 2 mm square chamber holding 0.2, 0.75 and 1 μ l medium plus fluorescein. After preparing height profiles as in **(A)**, average heights of units of 150 x 150 pixels along a chamber edge were determined (2,250 pixels per 2 mm edge), and contact angles given by the middle of the edge of that unit (zero height) and the middle of each unit (average height) calculated (using Matlab).

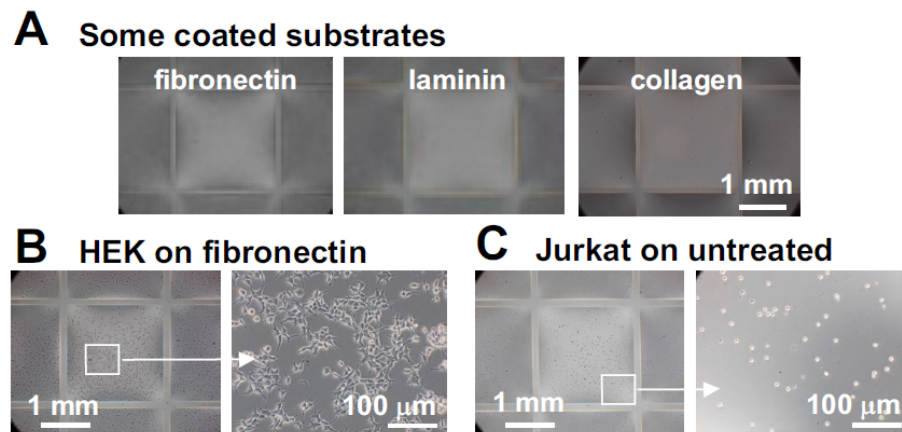


Figure S4. Phase-contrast images of grids prepared on dishes with different coatings, and using adherent and non-adherent cells (delivered by scanning).

A. Pinning lines are stable on dishes coated with various substrates (2.25 x 2.25 mm chambers; no cells added).

B. Adherent HEKs grow normally on fibronectin. 800 cells in 1 μl were delivered to each chamber, grown (24 h), and imaged (zoom shows region of chamber).

C. Non-adherent Jurkat cells (an immortalized line of human T lymphocyte cells) grow in suspension. 660 cells in 1 μl were delivered to each chamber, grown (24 h), and imaged (zoom shows region of chamber).

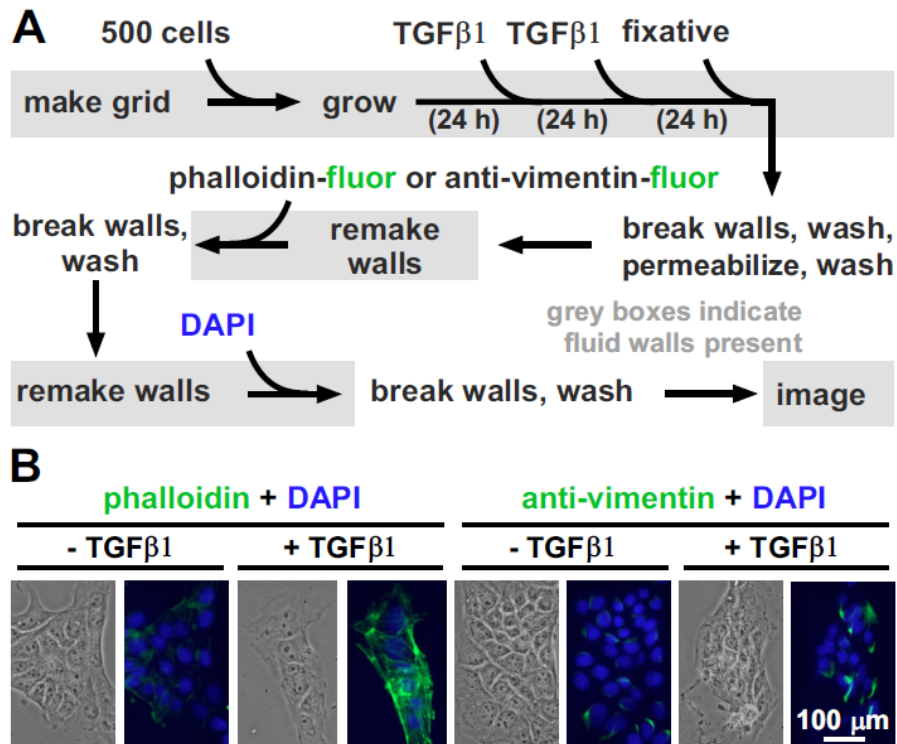


Figure S5. Breaking, remaking fluid walls during immuno-labeling. This Figure provides the workflow used in Figure 5Aii.

A. The workflow. NM18 are plated by “scanning”, grown, treated twice with $\pm$ TGF β 1, and fixed. Fluid walls were now broken (as in Fig. 5Ai), and all the fixed cells in the dish simultaneously batch-washed and permeabilized with Triton X100. Fluid walls were now remade, and phalloidin-Alexa488 or anti-vimentin-Alexa488 added to individual chambers. Next, fluid walls were re-broken, all cells in the dish simultaneously batch-washed. Walls were now remade, and DAPI added to chambers. After breaking walls, and batch washing, walls were remade, and central areas of different chambers imaged.

B. Phase and fluorescent images show TGF β 1 induces actin bundling and increases vimentin expression (detected by phalloidin- and immuno-labeling respectively). These images are reproduced from Figure 5Aii for convenience.

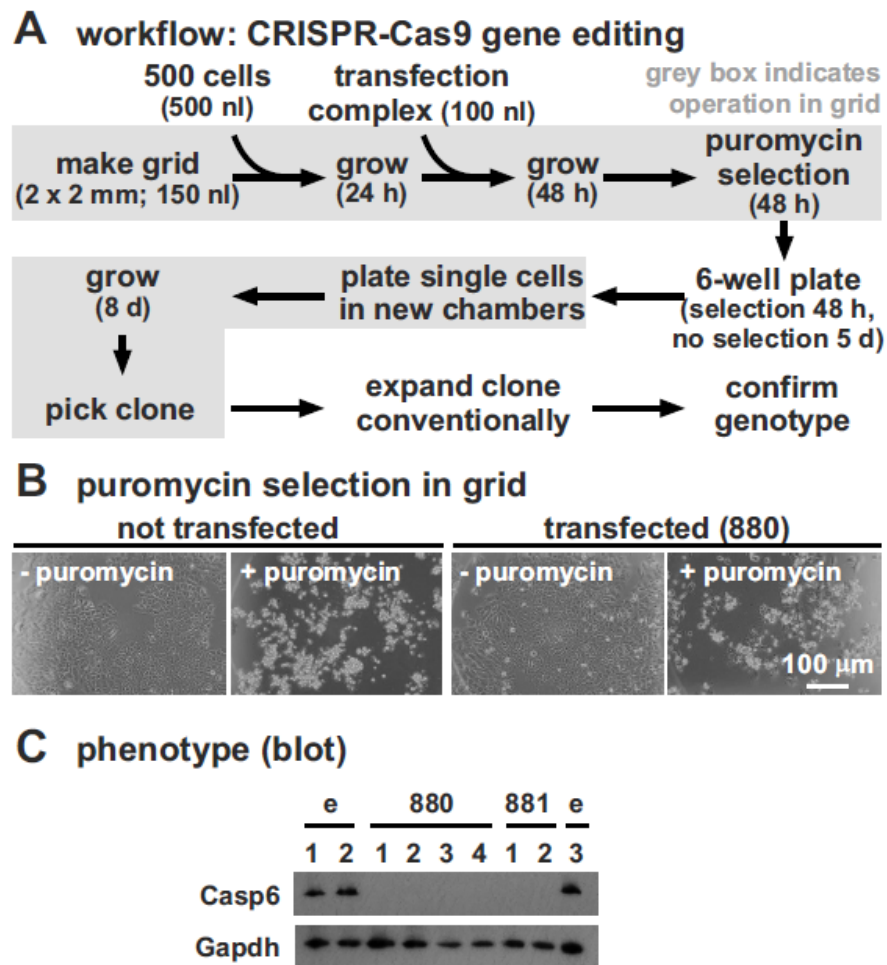


Figure S6. Data supporting the complex workflow illustrated in Figure 6 (deriving a NM18 clone with a mutated *Casp6* gene using CRISPR-Cas9).

A. Workflow (reproduced from Fig. 6A for convenience).

B. Puromycin selection. Views of chamber centers after puromycin selection (48 h). Untransfected cells are refractile and rounded (indicative of cell death); in contrast, ~50% transfected cells remain non-refractile and attached (and viable).

C. Phenotyping picked clones. After expanding the picked clones conventionally, immunoblotting confirms clones derived from transfections with 880 and 881 lose Casp6 (loading control – Gapdh), unlike those with empty vector (e).

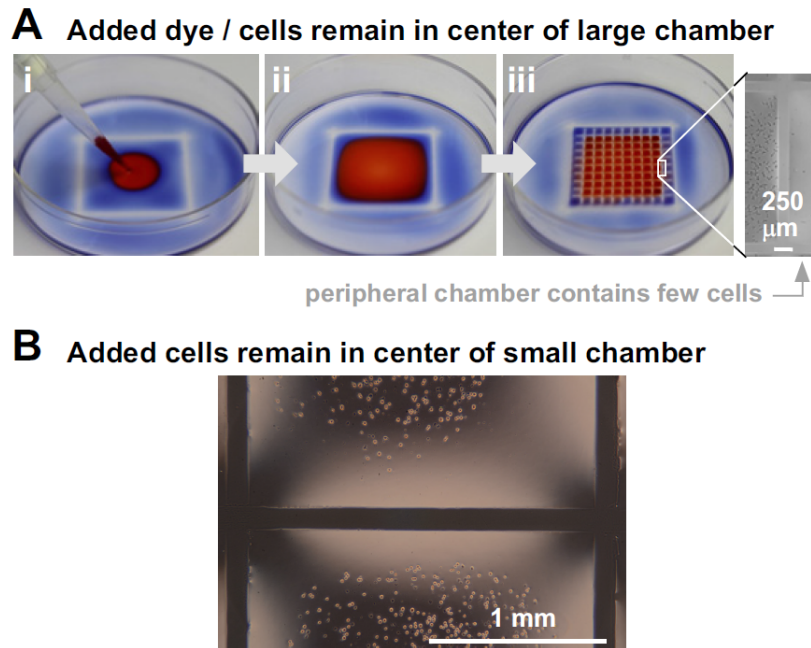


Figure S7. Cells tend to be found centrally in chambers (6 cm dishes).

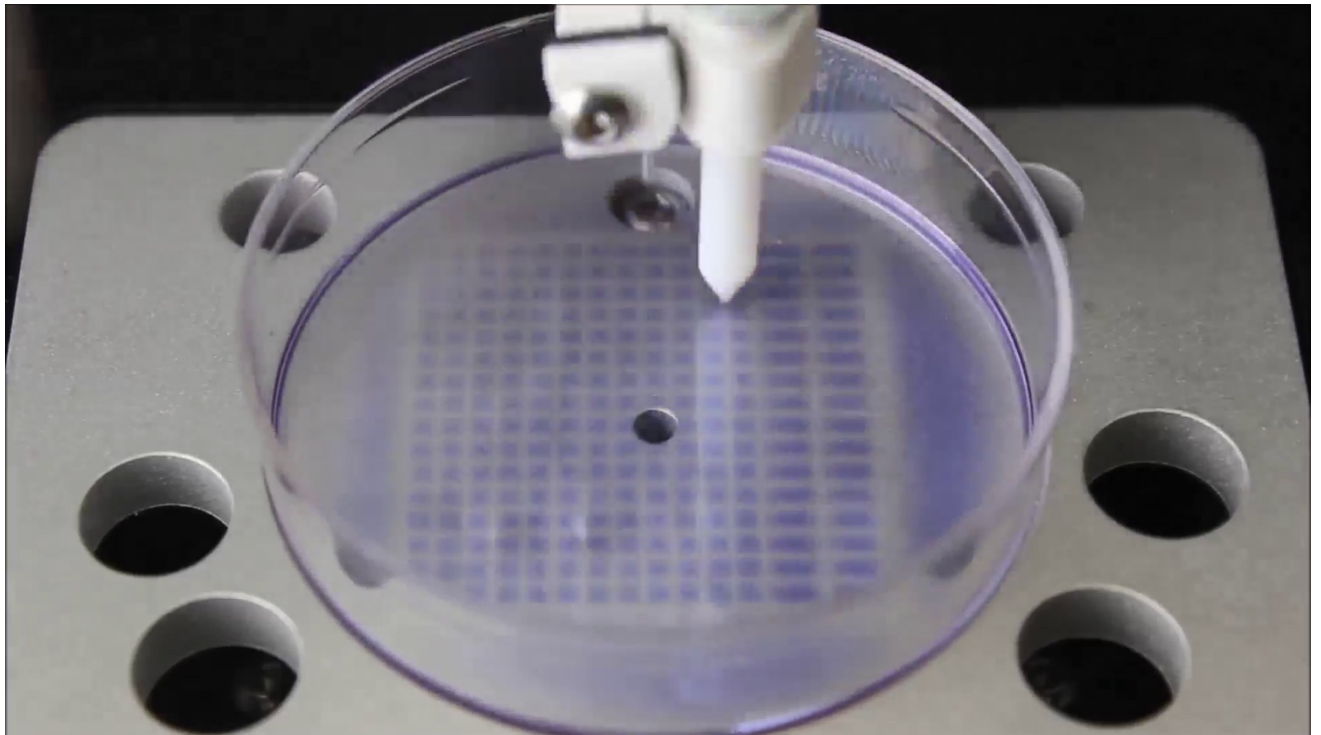
A. Large chamber. Medium plus blue dye is added to a dish, most medium removed to leave a thin film covering the bottom, and one 28 x 28 mm printed in the center of a dish (as **Fig. 2E**). (i) 30 μ l red dye is now pipetted into the middle of the square. (ii) Red dye spreads to push blue dye to the edges of the square. (iii) After splitting the central square into a 10 x 10 grid (2.25 x 2.25 mm chambers), peripheral chambers mainly contain blue dye, and those in the central 8 x 8 grid mainly red dye. Inset: when blue dye is omitted, and 20,000 HEKs replace red dye, few cells are found in a peripheral chamber.

B. Cell distributions in small chambers. NM18 were seeded in 2 x 2 mm chambers, and a bright-field image collected immediately. There are no cells at the edge of the chamber close to the pinning line.

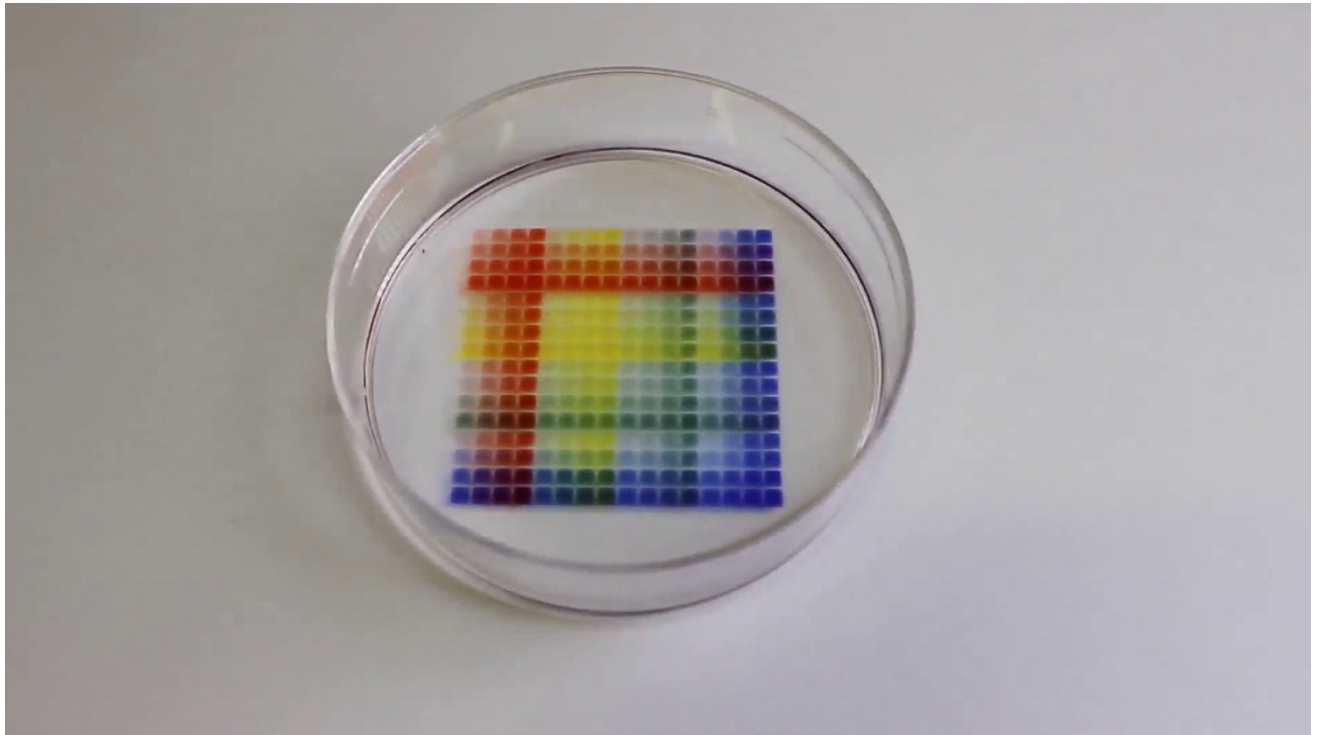
Supplementary movies

Movies can be found at <https://www.pnas.org/content/115/26/E5926/tab-figures-data> .

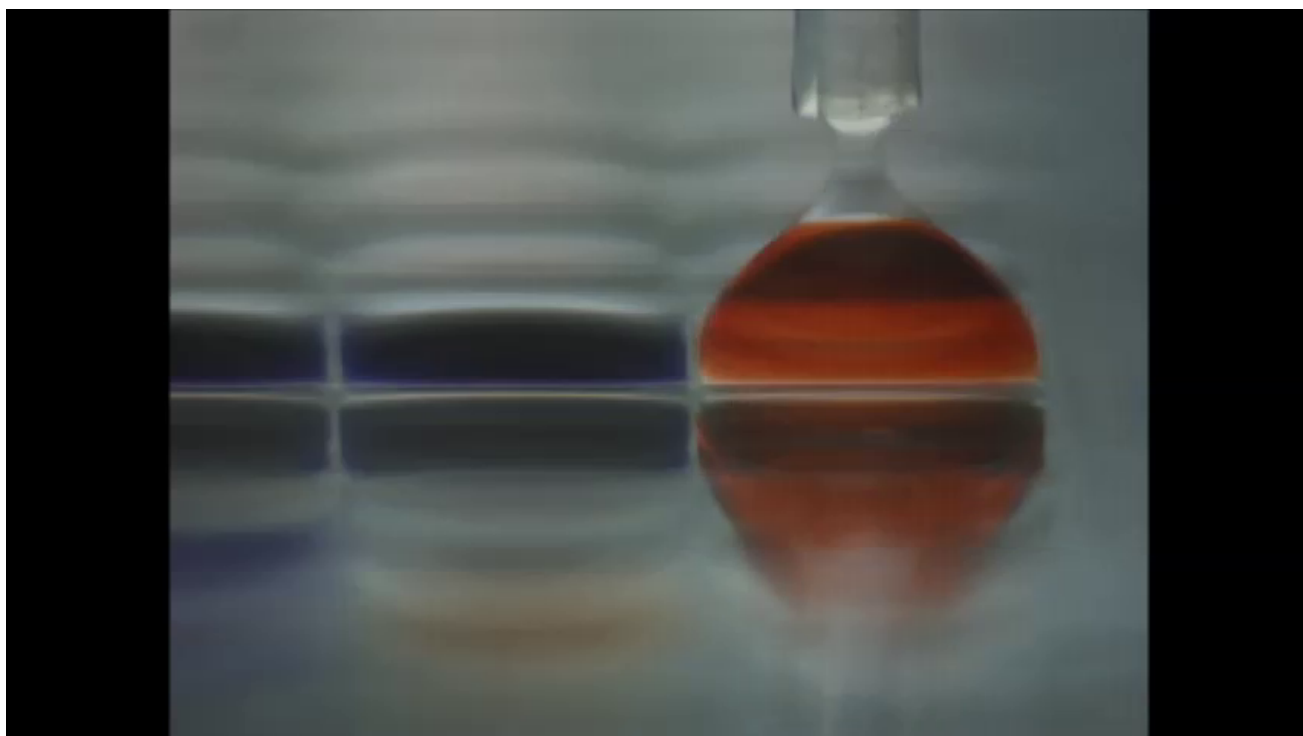
Movie S1. Printing a 16 x 16 grid in a 6 cm dish (the movie runs in real time for 4 s, and then is speeded up 10x for the remainder). The dish initially contains a thin layer of medium plus blue dye overlaid with FC40, and the white Teflon stylus draws a grid.



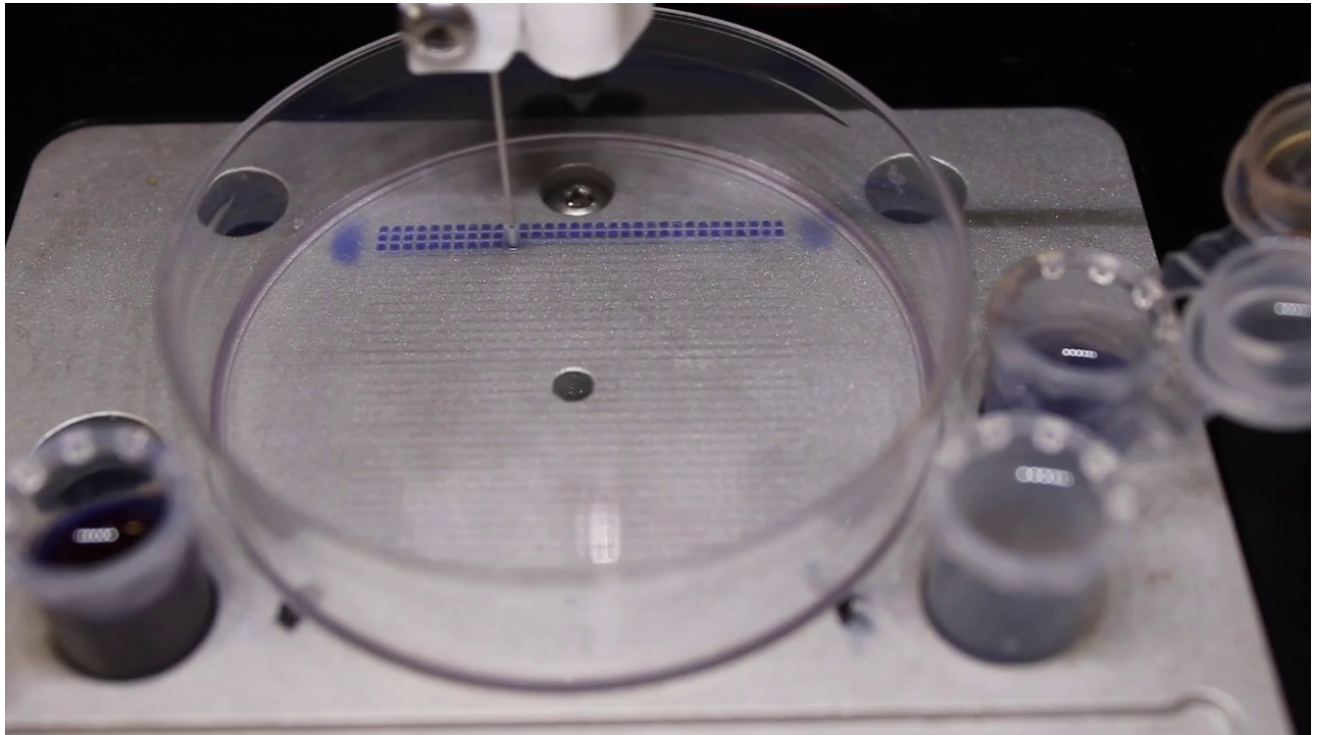
Movie S2. A grid overlaid with FC40 can survive violent agitation (the movie runs in real time). The grid was printed on a 6 cm dish, 16 different dyes delivered in all pairwise combinations to chambers (see **Fig. S1A**), the dish placed on a shaker, and the lid of the dish removed. The movie begins when the shaker was started; as the speed of shaking increases, waves form in the FC40 and increase in height. Footprints of chambers remain unchanged throughout. Of course, if chambers made with serum have contact angles of $>90^\circ$ and are closely packed and separated from others by thin fluid walls, shaking can cause neighbors to merge.



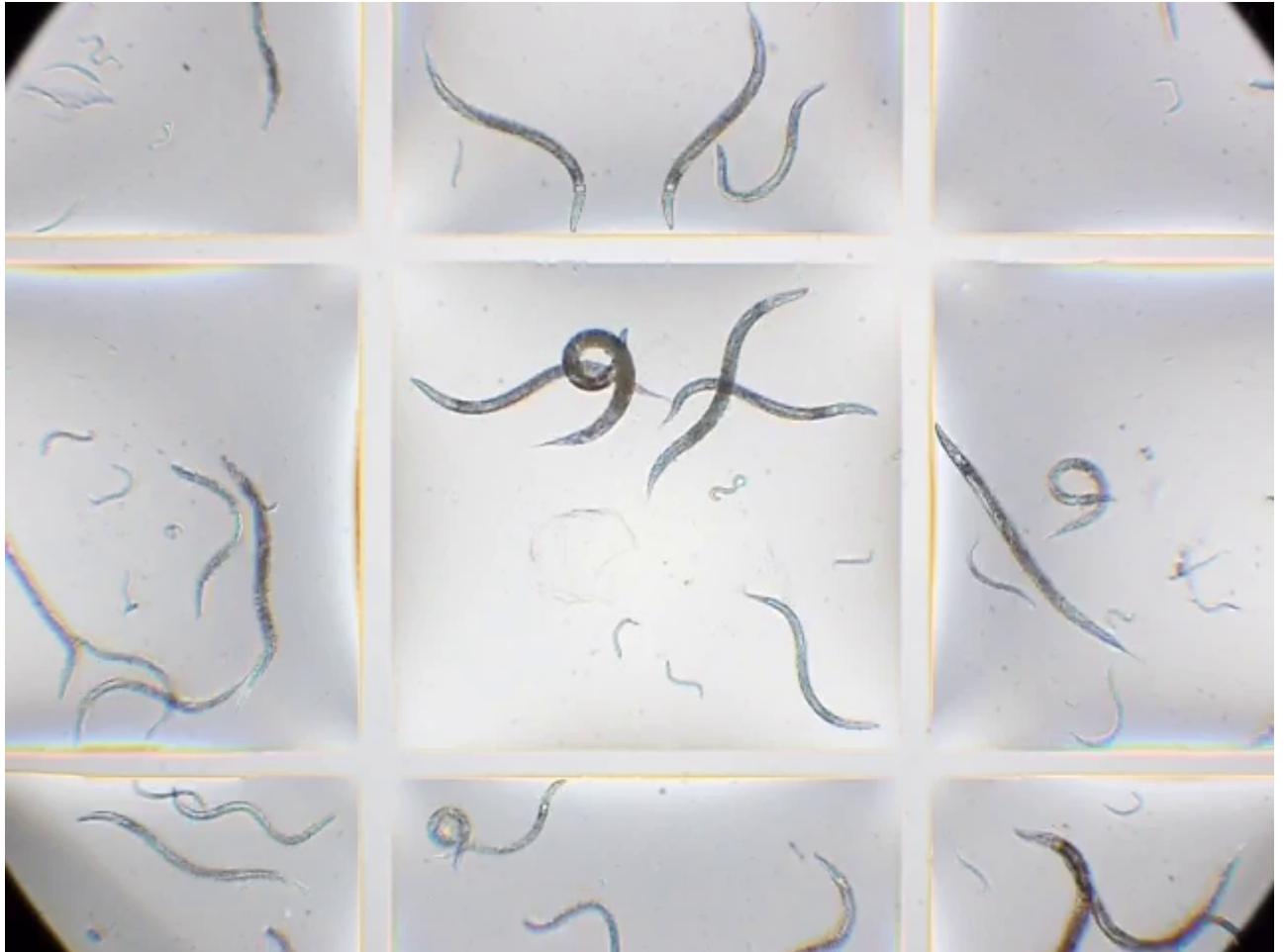
Movie S3. Adding fluid via a liquid bridge to a chamber (the movie runs in real time, and frames from it are reproduced in **Fig. 3B**). A 16 x 8 grid (2.25 x 2.25 mm chambers, rectangular dish) was made using medium, and red and blue dyes delivered to chambers. Between 0-13 s, medium is continuously ejected from the pipette tip, which is immersed in FC40 and positioned above the recipient chamber containing red dye. The pipette is a hydrophilic tube with a hydrophobic sleeve at the end. At 0 s, ejected medium begins to form a bubble at the end of the tip. By 9 s, the bubble has grown sufficiently to touch the surface of the chamber, and a liquid bridge forms. Note that there is no detectable transfer upwards of red dye through this bridge. By 11 s, the bridge has broken, and no red dye can be detected in the bubble on the tip. After 13 s, a new bridge has formed, and again no dye is being transferred upwards. The tip will now move to a new position above another chamber, and deliver medium to it. This movie indicates that essentially no red dye will be transferred between chambers during this process.



Movie S4. Delivering liquid to a grid by scanning along a serpentine path (the movie runs in real time, it is truncated after delivering to 3 rows, and frames from it are reproduced in **Fig. 3E**). A 32 x 32 grid (initially with ~40 nl per 1 x 1 mm chamber) was made in a 6 cm dish using medium. The movie begins as a hollow stainless-steel dispensing needle with a Teflon sleeve at its tip dispenses blue dye as it scans along a serpentine path delivering ~70 nl to each of 1,024 chambers in 90 s (some dye is added to the left and right of the grid).



Movie S5. Fluid walls withstand forces exerted by living worms at room temperature (a frame from this movie is illustrated in **Fig. 4Ei**). An 8 x 8 grid (chambers 2 x 2 mm) was made using S medium + 0.03 mg/ml BSA, bacteria added as food, dishes left at room temperature, worms washed in S medium and transferred to new chambers, and living worms imaged.



Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

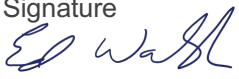
Title of Paper	Microfluidic chambers using fluid walls for cell biology
Publication Status	Published
Publication Details	C. Soitu, A. Feuerborn, A. N. Tan, H. Walker, P. A. Walsh, A. A. Castrejón-Pita, P. R. Cook, and E. J. Walsh PNAS June 26, 2018 115 (26) E5926-E5933

Student Confirmation

Student Name:	Cristian Soitu		
Contribution to the Paper	Lead author, performed experiments (apart from those in Fig. 5B, 6C and S6C), collected, contributed to data analysis and interpretation, contributed to manuscript writing and editing.		
Signature <i>Cristian Soitu</i>	Date	15.09.2020	

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Prof Edmond J. Walsh		
Supervisor comments		
Signature 	Date	9/10/2020

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 5: Raising fluid walls around living cells

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Abstract

An effective transformation of the cell-culture dishes that biologists use every day into microfluidic devices would open many avenues for miniaturizing cell-based workflows. In this article, we report a simple method for creating microfluidic arrangements around cells already growing on the surface of standard Petri dishes, using the interface between immiscible fluids as a ‘building material’. Conventional dishes are repurposed into sophisticated microfluidic devices by reshaping, on demand, the fluid structures around living cells. Moreover, these microfluidics arrangements can be further reconfigured during experiments, which is impossible with most existing microfluidic platforms. The method is demonstrated using workflows involving cell cloning, the selection of a particular clone from amongst others in a dish, drug treatments, and wound healing. The versatility of the approach and its biologically-friendly aspects may hasten uptake by biologists of microfluidics so the technology finally fulfills its potential.

Introduction

Microfluidic-based approaches have been applied to many workflows in biology; however, uptake by mainstream biology labs remains slow. Many reasons for this have been given, including the materials used are not congenial to cell growth, architectures are enclosed and so inaccessible, geometries are predetermined and cannot be reconfigured during experiments, costs of manufacture and operation are high, and workflows typically designed by engineers often do not align with the pre-existing ones that biologists have spent decades developing (1-3).

Open microfluidic approaches have sought to address some of these issues through their low barriers to fabrication and operation. However, such platforms often require established live-cell-based assays to be compatible with, or adapted to, materials and methods not traditionally used in biology – as is the case with suspended (4), in-air (5) and paper-based microfluidics (6). Another open approach uses inserts to form hydrogel walls within wells of standard microplates, so substrates familiar to biologists can be repurposed for microfluidics (7). However, these methods have been demonstrated by creating microfluidic arrangements prior to incorporating cells. A more biologically-relevant approach would be to form arrangements on the Petri dishes in which biologists traditionally grow their cells. In other words, begin with what biologists already use, and fabricate the required microfluidic devices on their pre-existing platform.

In the macro-world, solid materials are used as the building blocks for most objects, and the idea of creating structures with liquid walls does not work – gravity collapses them into puddles. Therefore, liquids are held in chambers with solid walls at the macro-scale and in most microfluidic devices. However, as length scales become smaller, interfacial forces dominate gravitational ones (i.e., small Bond numbers) and confinement of liquids by fluid interfaces, analogous to solid materials, becomes possible; therefore, liquid walls can replace solid ones. While the creation of 3D constructs with fluid walls has been previously demonstrated using nanoparticle surfactants, the biocompatibility of the system remains to be

assessed (8). Recently, we developed a method for making arrays of isolated microfluidic chambers on virgin Petri dishes to accommodate some major workflows used in cell biology (e.g., cell feeding and transfer, cloning, cryopreservation, fixation and immunolabeling, cell lysis and RT-PCR, CRISPR-Cas9 gene editing); in these cases, cells were added post fabrication (9). Here, we create various microfluidic arrangements on standard Petri dishes already containing adherent cells and go on to reconfigure them in real time. Moreover, we isolate and retrieve cell clones, and perform proof-of-concept drug tests and wound-healing assays. The ability to create and reconfigure microfluidic circuits on Petri dishes as cells grow and divide should find many applications in biology, and yield more relevant phenotypic and genotypic responses when compared to standard microfluidic assays.

Results

Methodology

Fig. 1A illustrates the approach; fluids sitting in a standard Petri dish are reshaped to create a wanted microfluidic arrangement. In summary, the bottom of a virgin Petri dish is covered with tissue-culture medium, and most removed to leave a thin film covering the polystyrene substrate. The thin film is overlaid with an immiscible fluorocarbon, FC40, to prevent evaporation; this additionally provides a physical barrier against external contaminants, and so maintains sterility of the medium. A hydrophobic and fluorophilic stylus (which has a conical tip made of polytetrafluoroethylene – PTFE, or “Teflon”) is lowered through the two liquid layers to bring it into contact with the bottom of the dish. As the fluorocarbon preferentially wets Teflon (compared to water), FC40 is brought down to the substrate and displaces the aqueous phase (as it also preferentially wets the substrate). The Teflon stylus is moved laterally over the surface to create a wanted microfluidic arrangement – in this case, a square. The apparatus that draws the pattern – a “printer” – consists of a tool-head that carries the stylus attached to a three-axis traverse controlled by appropriate software; the tool-head also carries a dispensing tube connected through a Teflon tube to a

syringe pump that can add/remove nanoliter volumes to/from any point in the microfluidic arrangement. This approach brings the advantages of open microfluidic platforms to standard cell-culture ware.

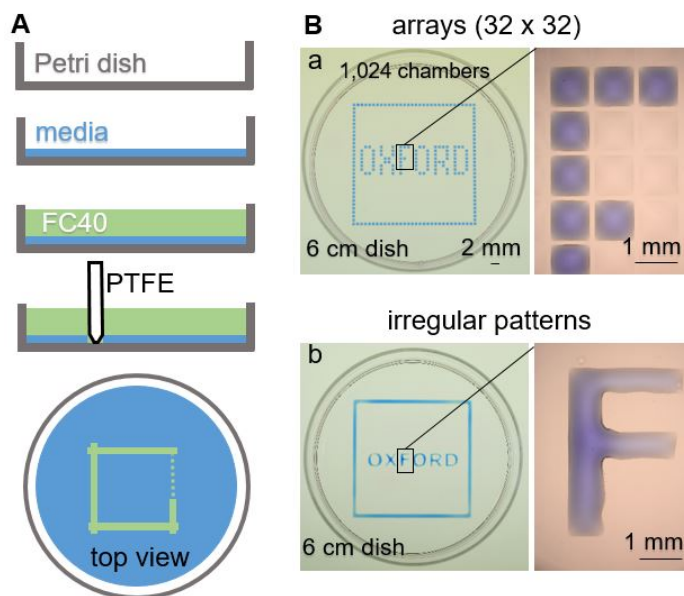


Figure 1. Chamber construction. **A.** Principle. DMEM + 10% FBS is added to a virgin Petri dish, and most medium removed to leave a thin film covering the bottom, which is overlaid with FC40. The stylus is moved across the bottom to create a microfluidic arrangement. When complete, the initial volume of DMEM + 10% FBS will be divided into two parts separated by a continuous liquid wall of FC40 pinned to the substrate.

B. Different patterns. **(a)** Forming equally-spaced vertical and horizontal lines creates an array (32 x 32; 1 mm spacing). Next, 60 nl blue dye is added by the printer to selected chambers; peripheral chambers receive blue dye to give the blue square, and internal ones give the word “OXFORD”. The magnification (right) shows individual chambers without and with dye. **(b)** A similar pattern created by forming two squares (one slightly larger than the other) with the stylus, and then adding dye manually to the space in between; each letter is made by forming its sides, and again manually filling the interior. The magnification shows the letter “F” is one continuous body of liquid.

The aqueous phase can be shaped in various ways, for example by forming horizontal and vertical lines of equal length to create a grid. For instance, Fig. 1B(a) illustrates the “printing” of a 32 x 32 array of 1 x 1 mm chambers using tissue-culture medium (which appears light pink at the macro-scale due to the phenol red added as a pH indicator) and FC40 (which is colorless). The volume of medium initially in each chamber in this array is so small (i.e., ~25 nl (9)) that individual chambers appear colorless and invisible at low magnification. Therefore, to aid visualization, 60 nl of blue dye is added to selected chambers; each peripheral one receives blue dye (giving the blue square), as do selected ones in the interior (giving the word “OXFORD”). The amount of fluid that can be added to (or removed from) a chamber without altering its footprint depends on the advancing and receding contact angles, θ_A and θ_R (1, 9). An analogous microfluidic arrangement made by forming both curved and straight lines of various lengths is illustrated in Fig. 1B(b). Here, the outer border is made by forming two squares (one slightly larger than the other), and then adding dye to the space in between; each letter is made by forming its sides, and again filling the interior.

Reconfiguring microfluidic arrangements

The method is extended to reconfigure a microfluidic arrangement during the assay; fluid walls are built, destroyed, and rebuilt in different shapes. A circle within a triangle within a square (9 mm sides) is “printed” (Fig. 2(1); this – and following frames – are taken from Movie 1), and microliter quantities of 3 dyes of different colors added to the three shapes; FC40 walls prevent dyes from mixing (Fig. 2(2-4)). The circular wall is now destroyed by pumping yellow dye through the dispensing tube into the centre (Fig. 2(5)). After adding 3 μ l, θ_A of the circular pinning line is exceeded, the circular wall ruptures, and contents spill out into the triangle (Fig. 2(6)). After adding 24 μ l, the triangular wall ruptures, and contents spill into the square (Fig. 2(7)). Now, the two internal walls are rebuilt, but this time the circle encloses a triangle. As printing through a thick aqueous film is difficult, most volume within the square is removed (Fig. 2(8)), new walls built (Fig. 2(9)), and different colored dyes added to the three

volumes (Fig. 2(10-12)). These results show that FC40 walls can be destroyed and rebuilt in any desired 2D shape, and that they confine liquids effectively.

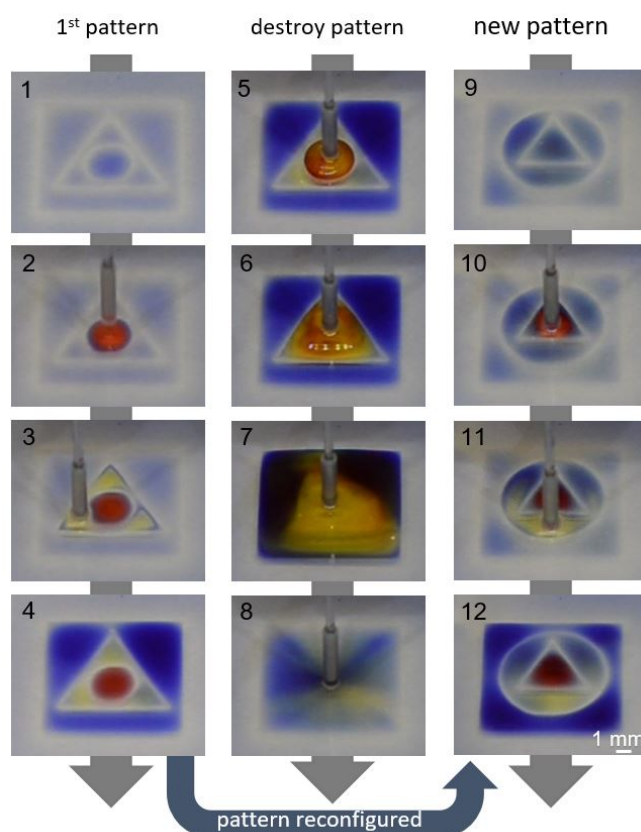


Figure 2. Reconfiguring microfluidic arrangements. Images show frames from Movie S1. (1) An initial pattern is printed: a circle (radius 1.5 mm) inside a triangle (side 7 mm) inside a square (side 9 mm). (2-4) Different dyes are added to each compartment (1.5 μ l red dye, 1.5 μ l yellow, 5 μ l blue); dyes are confined within FC40 walls. (5) More yellow dye is added to the circle. (6) After adding 3 μ l yellow dye, the circular pinning line ruptures and contents spill into the triangle. (7) After adding 24 μ l, the triangular pinning line ruptures and contents spill into the square. (8) 60 μ l is withdrawn from the square. (9) A new pattern is printed – a triangle (side 4.5 mm) in a circle (radius 3.3 mm) – in the initial square. (10-12) Colored dyes are added to the 3 different compartments as before.

Building fluid walls around single cells and clones

Arrays of chambers (“grids”) have been used in various workflows in cell biology; Fig. 3A recapitulates the cloning of mouse mammary tumor cells (NM18) and follows a previous workflow (9) where grids are initially created and cells added later. Using a 16 x 16 array (illustrated with red dye in Fig. 3A(a)), a single mouse tumor cell in 600 nl is dispensed into every fifth chamber in the grid (so Poisson statistics ensure few chambers contain two cells), and the dish placed in a conventional CO₂ incubator. As FC40 is freely permeable to O₂ and CO₂, cells grow like their counterparts lacking a fluorocarbon overlay (Fig. 3A(b)). A quantitative analysis, presented in Fig. S2, demonstrates that there is no significant difference between the two systems in the size of the colonies after 8 days. This confirms the biocompatibility of the platform. Moreover, when compared to imaging microplates (where solid walls around individual wells can obscure views), here fluid walls/ceilings provide excellent optical clarity across the complete footprint of a chamber – a critical feature required to give users confidence that the chamber contains only one cell.

It is often useful to isolate one clone from others in a set of many clones (e.g., because cells in that clone express a particular phenotype or protein). Therefore, we now reverse the process: single cells are allowed to grow into clones before surrounding them with fluid walls. Fig. 3B(a) illustrates the shape of the chambers used, and Fig. 3B(b) the experiment (see also Movie 2). Cells are plated sparsely, grown, and walls built around resulting colonies. Each of the three shapes illustrated has different advantages (e.g., circular chambers have the most stable pinning lines for a given volume and footprint area, while triangles and rectangles can tessellate and so use the maximum surface area). This shows that fluid walls with different 2D footprints can easily be built around living cells to provide assurance that colonies remain isolated from each other during subsequent treatment or retrieval. While there are many examples where cells are grown in confined spaces on pre-patterned surfaces (10-13), these often require surface treatments prior to adding cells.

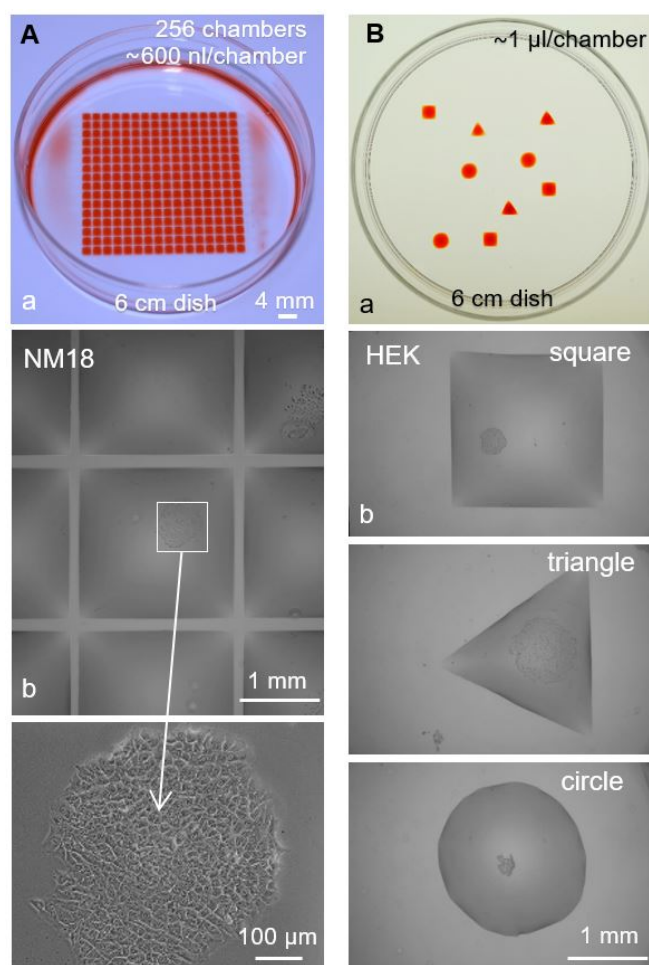


Figure 3. Building walls before or after cloning cells.

A. Building walls before plating single cells in individual chambers. After making grids (16 x 16, 2 mm spacing; 6 cm dishes), dye or cells (NM18) were added by the printer. **(a)** Red dye (600 nl) is added to each chamber to illustrate the grid used. **(b)** Medium (600 nl) containing 0.2 cells is added to each chamber (so only a few chambers receive 1 cell), dishes incubated (8 d), and chambers imaged; the central chamber contains one colony (see magnification).

B. Building walls around living clones. **(a)** Red dye (1 μ l) is added to various shapes printed in a dish; the dye is added to aid visualization (see also Movie S2). **(b)** Cells (HEKs) were plated in a dish (density ~ 1 cell/cm²), and allowed to grow for 8 d into colonies. Next, most medium is removed to leave a thin film on the surface, FC40 added, various shapes drawn around colonies, each chamber filled with 1 μ l medium using the printer, and images collected. The 3 images come from different dishes.

Clone picking

We now partially automate clone picking. Our approach relies on a “reference plate” – a glass support bearing a matrix of unique identifiers (i.e., A1, A2, ... etc.; Fig. 4A). After placing a dish containing colonies on this plate, clone locations are recorded using a microscope by focusing successively on the plate and clone (Fig. 4B(a,b)), and inputted into a script that controls wall printing at specific positions (Fig. 4B(c)). Once built, these walls effectively isolate wanted clones from others in the dish. A clone is now picked using a conventional – but miniaturized – workflow. The printer washes cells by adding/removing PBS, adds trypsin, and the dish is transferred to an incubator to enhance cell detachment; next, the printer retrieves the cell-rich suspension (emptying the chamber; Fig. 4C(a,b)). Finally, recovered cells are plated manually and grown conventionally; they multiply as expected (Fig. 4C(c)).

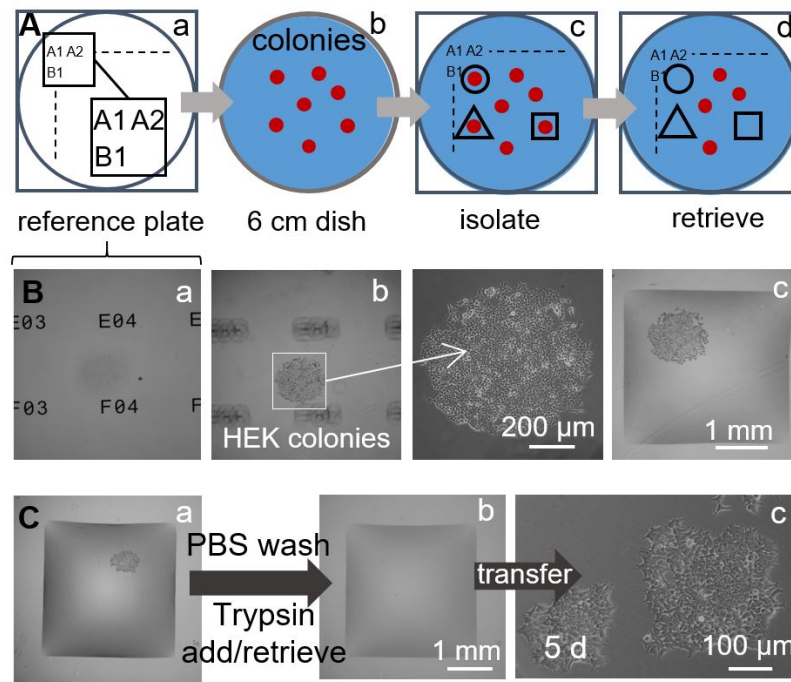


Figure 4. Semi-automating selective clone-picking (HEKs). The printer adds/removes a microliter to/from chambers at different stages.

A. Approach. (a) Locations on a glass “reference plate” are marked by unique identifiers (i.e., A1, A2, ...B1...). (b) A 6 cm dish with colonies (red) is placed on the reference plate. (c) After recording colony locations and inputting them into a script, fluid walls are printed around selected clones (black lines). (d) Clones are retrieved from these chambers.

B. Isolating a clone. HEKs were plated at low density ($\sim 1 \text{ cell/cm}^2$), grown (8 d) into clones, the dish placed on a reference plate, and walls built around selected clones. Three different z-axis views of one clone are shown. (a) Reference plate with unique identifiers in focus. (b) Colony in focus (identifiers out-of-focus) with magnification. (c) The colony after building surrounding walls.

C. Clone picking. (a) Square wall built around one living colony. The printer washes cells by adding/retrieving 1 μl PBS; it then adds 1 μl trypsin. (b) The dish is incubated (37°C ; 5 min) to detach cells from the surface, and the printer retrieves 1 μl containing the cell-rich suspension (and transfers it to a micro-centrifuge tube) to leave the now-empty chamber. (c) Retrieved cells are plated manually in a 12-well microtiter plate and grown conventionally for 5 d; cells attach and grow.

Fluid walls are effective barriers to drugs

We next demonstrated that fluid walls confine liquids effectively. Puromycin is a small-molecule translational inhibitor that kills mammalian cells, and is used here to demonstrate that an FC40 wall effectively prevents leakage of the poison out of square chambers (arranged as a 3 x 3 grid) drawn around cells already growing on a dish. The central chamber receives only growth medium, whereas the drug was delivered to the surrounding 8 chambers at a concentration ten-fold higher than the lethal one (i.e., 10 $\mu\text{g/ml}$). After 24 hours, only cells within outer chambers die (less than 40% viability), and cells in the central one remain alive (Fig. 5A). A second example exploits a particular property of this line of HEKs that had been genetically-modified to encode a GFP-reporter gene controlled by a promoter switched on by tumor necrosis factor alpha (TNF- α) – when exposed to the cytokine, cells fluoresce green (14). Pairs of chambers with various shapes were built around cells already growing in a dish; the larger one encompasses the smaller one. As before, the flexibility of the technique is demonstrated by drawing a square inside a circle, a triangle inside a square, and a circle inside a square (Fig. 5B). After adding the cytokine to one or other chamber (inner for Fig. 5B(a,b), and outer for Fig. 5B(c)), only cells in treated chambers fluoresce green. These results show that fluid walls form effective barriers to these drugs, and allow side-by-side comparison of treatments of cells growing in the same dish. It is easy to see how this approach might be extended to screen many drugs in different isolated regions of one dish.

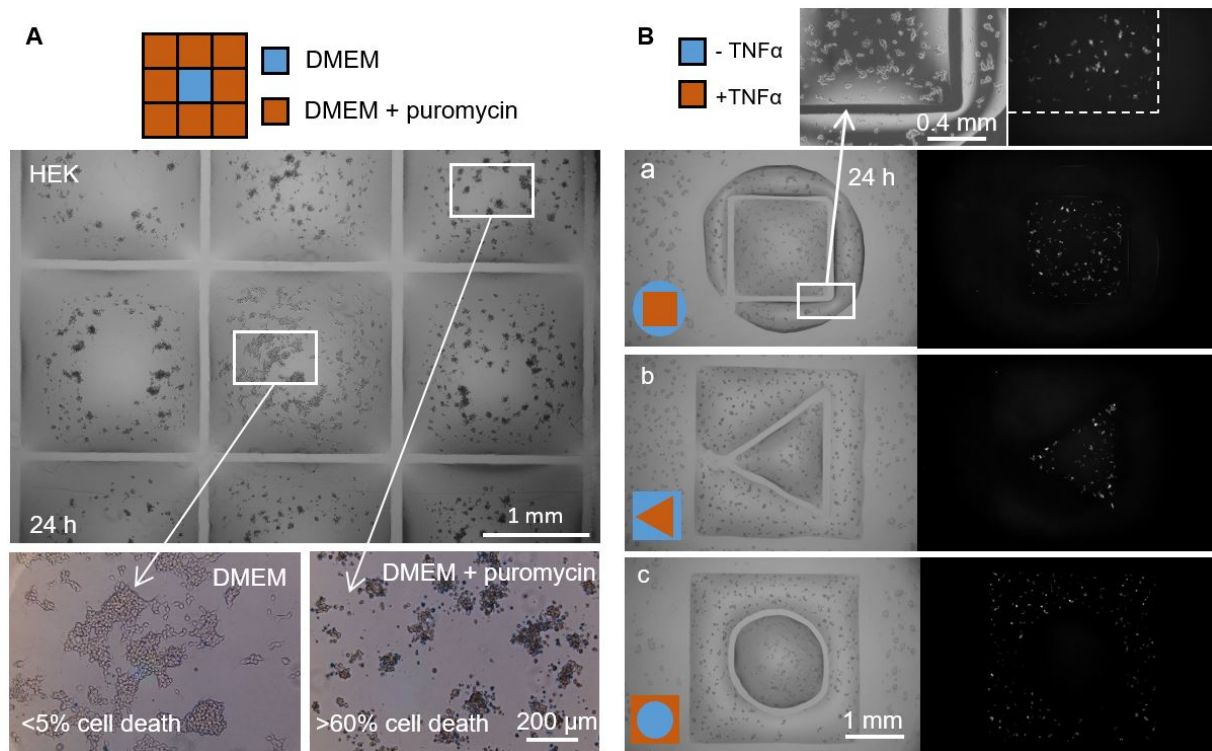


Figure 5. Two drug treatments side-by-side with untreated cells. Fluid walls were built around HEKs (300,000 cells; 6 cm dish) grown for 24 h.

A. Puromycin (3 x 3 grid; 2 x 2 mm chambers). The printer adds 1 μ l medium to the central chamber and 1 μ l medium + puromycin to peripheral ones (final concentration 10 μ g/ml) as indicated in the cartoon. Cell viability is assessed after incubation (37°; 24 h) using a Trypan blue exclusion assay. Cells in outer chambers are dead (more than 60% in each one) whereas those in the central one remain alive (less than 5% cell death). This assay has been replicated 3 times.

B. TNF- α . Pairs of chambers with distinct shapes are printed, one surrounding the other. The printer adds 0.5 μ l medium $\pm$ TNF- α (final concentration 10 ng/ml) to one or other volume (as in cartoons). As cells encode a GFP-reporter gene controlled by a promoter switched on by TNF- α , they fluoresce green on exposure to the cytokine. Fluorescence images show that only cells in the treated volume fluoresce green. Volume pairs had the following dimensions: (a) square (side 1.8 mm) in circle (radius 1.75 mm); (b) triangle (side 1 mm) in square (side 3.5 mm); (c) circle (radius 1 mm) in square (side 3.5 mm).

Wound healing

Wound-healing assays generally involve comparing cells growing in different conditions in different dishes (15-17), and various microfluidics devices have been developed for such assays (18- 19). As many chambers can be built in one dish, and as liquid walls are easily destroyed, we exploit these features to monitor two wounds healing in one dish in which different parts of the dish have been coated in different ways. Two coatings were used for a proof-of-concept experiment. Matrigel is a gelatinous protein mixture secreted by sarcoma cells that is often used in studies of cell invasion (20) and fibronectin is a glycoprotein of the extra-cellular matrix that enhances wound healing (21). The workflow (Fig. 6A) involves building two rectangular chambers, and filling them with Matrigel or fibronectin to coat different parts of one surface; fluid walls are now destroyed by thorough washing, and HEKs plated in the dish. Cells soon form a monolayer throughout the dish except in regions originally containing walls (Fig. 6B(a), Fig. 6C(a)); these are marked by residual FC40 droplets and their sparser cell content presumably results from some modification of surface. After wounding by dragging the Teflon stylus across the monolayer (Fig. 6B(b), Fig. 6C(b)), cells – now in a common liquid environment and on a common substrate that bears different coatings in different positions – migrate into the two wounds at slightly different rates (quantitative data shown in Fig. S3). This workflow involves coating the surface before plating cells and wounding; it can be modified so the coating is added later, at the time when cells begin migrating into newly-formed wounds (Fig. S4). It is easy to see how these workflows might be adapted to test the effects of different coatings applied at different times to arrays of wounds, each located in different regions of one Petri dish; then, healing can occur in a common environment.

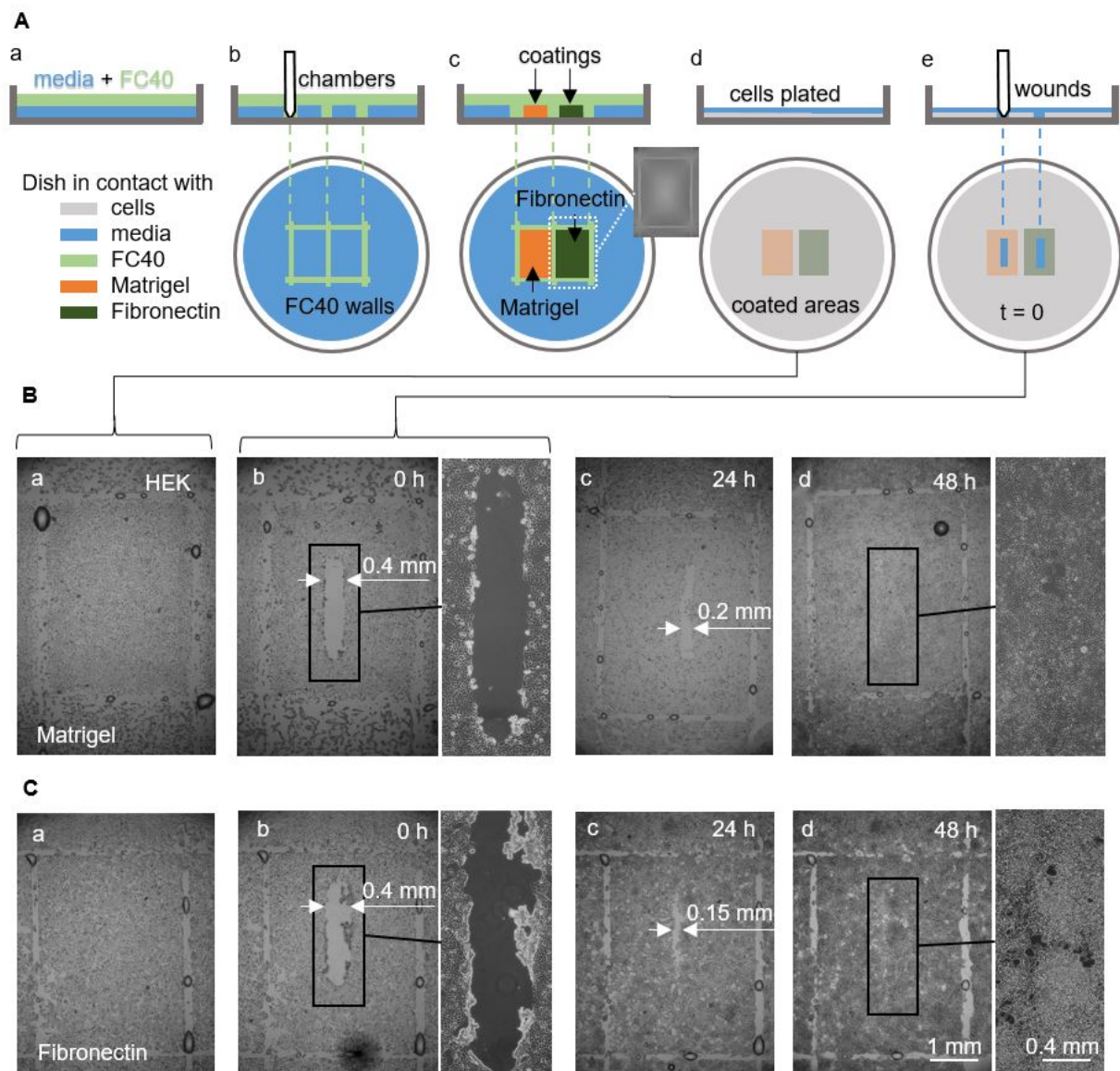


Figure 6. A proof-of-concept wound-healing assay using one dish pre-coated with Matrigel and fibronectin in different regions.

A. Cartoon illustrating workflow. **(a)** A thin layer of medium is overlaid with FC40. **(b)** Two chambers (3 x 4 mm each) are printed side-by-side. **(c)** Surfaces in chambers are coated with Matrigel or fibronectin (2 μ l; final concentration of 1 μ g/ cm^2 ; 1 h); the inset shows an image of one chamber. Fluid walls are now destroyed, and the dish washed with 3 ml medium to remove unattached coatings. **(d)** 600,000 HEKs are plated in the dish. **(e)** After 24 h cells have formed a monolayer, and a wound (0.4 x 2 mm) is created by scraping the stylus over the surface to remove cells in its path. Healing of the wound is now monitored microscopically.

B,C. Images of wounds in monolayers grown on Matrigel or fibronectin. **(a,b)** Immediately before and after wounding (some droplets of FC40 remain where walls originally stood). **(c)** As expected, after 24 h, cell growth reduces wound widths to <0.2 mm and <0.15 mm with Matrigel and fibronectin, respectively. **(d)** By 48 h, wounds have completely healed.

Conclusion

A microfluidic platform for miniaturizing workflows in cell biology was recently demonstrated (9); it is extended here to form microfluidic arrangements around pre-plated adherent cells that are then used for various proof-of-principle assays (e.g., cell cloning, drug screening, wound healing). This platform has many advantages. First and foremost, it can be used to repurpose the cell-culture ware familiar to biologists into complex microfluidic arrangements (**Fig. 5B**) that can support elaborate workflows (Figs. 4-6, S4). This contrasts with conventional approaches that usually require biological assays to be adapted to suit pre-engineered microfluidic devices. Second, the microfluidic arrangements made using this platform have fluid walls/ceilings that are built accurately and reproducibly by interfacial forces (Fig. 1B, Fig 2, Fig. 3A(a)). Third, the platform is highly flexible and customizable. Fourth, the replacement of solid walls with fluid ones brings all the benefits of open microfluidics without some of the limitations; for example, all points in the arrangement remain fully visible and accessible throughout an experiment because FC40 is both optically transparent and provides no barrier to a pipette tip. Fifth, the resultant pinning lines around chambers are stable (e.g., in Figs. 3-5, S4, fluid walls remain intact as dishes were carried between printer, CO₂ incubator, and microscope several times). Sixth and uniquely, these microfluidic arrangements can be reconfigured in real time during an experiment (Fig. 2, Fig. 5, Fig. 6, Fig. S4). Notable limitations include: arrangements are restricted to the two dimensions of the substrate, liquid walls are inevitably more fragile than solid ones (although robust enough to withstand agitation exceeding that occurring during normal use; Fig. 1-6; (9)), and the liquids and surfaces used must be matched to ensure that pinning lines are stable (e.g., some surfactants reduce volumes that may be held within chambers before pinning lines are compromised). Nevertheless, we hope this combination of features and advantages will provide a sufficiently low barrier to entry that many biologists will begin to exploit the power of microfluidics.

Materials and Methods

General reagents, equipment

FC40 was supplied by IotaSciences. All other fluids and materials were purchased from Sigma Aldrich unless otherwise stated. Water-soluble dyes (e.g., Allura Red, toluidine blue, resazurin) are used where indicated. Matrigel (Corning, #3542777) and fibronectin (Sigma Aldrich, #F1141) were used for the wounding experiment.

All microfluidic arrangements were made on 6 cm polystyrene tissue-culture dishes (Falcon – 60 mm TC-treated cell culture dish product #353002) using custom-written software and a printer (IotaSciences Ltd, Oxford, UK). The printer is essentially a tool-head driven by a 3-axis traverse and appropriate software; the tool-head holds a stylus – a Teflon rod (3.8 mm diameter) with a conical tip (angle at tip 50°) – and stainless-steel dispensing needle (width 0.5 mm outer diameter) connected to a syringe pump that is used to add/remove nano-liter volumes from chambers. A hydrophobic sleeve (a section of Teflon tubing) is added around the end of the dispensing needle to prevent liquids running up the outside instead of being delivered to the chamber. The stage of the printer can accommodate a 6 cm dish and micro-centrifuge tubes for storing needed reagents (e.g., cell-culture media, 70% ethanol for sterilization, tubes for waste). A tightly-fitting circular sleeve around each dish acts as a positioning ring to ensure the dish can be removed and added back on to the printer in the original orientation. The printer can be placed in a bio-safety cabinet if sterility is required; then, standard procedures for sterility are used (e.g., stylus and tubes are sterilized with 70% ethanol).

Cells

Adherent human embryonic kidney cells (HEKs) were grown in DMEM + 10% FBS (Gibco, Life Sciences), and sub-cultured using trypsin (TrypLE, Gibco #12563011, Gaithersburg, MD). These HEKs were genetically-modified reporter cells (NF- κ B/293/GFP-LucTM Transcriptional Reporter Cell Line; System Biosciences, catalogue number TR860A-I);

they encode a GFP gene under the control of the minimal cytomegalovirus promoter downstream of four copies of the NF- κ B consensus transcriptional-response element, and GFP expression can be induced by TNF α (used at a final concentration of 10 ng/ml, and obtained from Peprotech, London, UK).

For Fig. 3A and Fig. S2, a line of mouse mammary tumor cells (NM18) was used. To obtain clones, cells were plated at densities of 0.2 cells/well (96-well plate) and 0.2 cells/chamber (16x16 grid). Fluidic chambers in the grid stored 600 nl of medium, which was replenished every 3 days.

Printing and operation of grids

DMEM plus 10% FBS was used to make all grids; when the term “medium” is used, it should be assumed that serum is present. Typically, 1 ml medium is pipetted into the dish so the medium completely covers the bottom, the dish tilted, ~0.9 ml medium retrieved and discarded, the thin film of medium left behind on the bottom of the dish overlaid with 3 ml FC40, and a grid or individual chambers made using the printer essentially as described previously (9).

The reference plate used in Fig. 4 is a custom-made product supplied by IotaSciences; it consists of a glass support with a series of unique identifiers printed on it in a 16 x 16 matrix (i.e., A1-A16 on the first row, and P1-P16 on the last row; 1.9 mm spacing between identifiers).

Imaging

Images of dishes were taken using a digital SLR camera (Nikon D610). Bright-field, phase-contrast, and fluorescence microscope images of grids with and without cells were collected using a zoom lens and digital SLR camera (Nikon D7100 DSLR) connected to an epi-fluorescent microscope (Olympus IX53; 1.25X, 4X, 10X, 25X objectives) with translation stage and overhead illuminator (Olympus IX3 with filters). Images in Fig. 1B, 1C, and 3B had brightness increased by 20%. For the fluorescence images in Fig. 5B, contrast and brightness was enhanced using ImageJ, and for Fig. 6B, 6C, and S4B contrast was increased by 40%.

Images of wounds used for the graph in Fig. S3 have been collected daily and post-processing was performed using ImageJ. Areas free of cells (wounds) were manually selected and measured.

Images of colonies used for Fig. S2 were taken after 8 days from plating. The outline of each colony was selected manually and the area of its footprint measured using ImageJ.

Cost

FC40 is the only reagent required by the technique additional to those already used in conventional cell culture. Based on the price for FC40 (Acota, bottle of 2.7 l; product no #51142-49-5), and each dish receiving 2 ml, the FC40 cost/dish is £0.4. Fluidic chambers use volumes up to 100-1000 fold smaller than 96-well plates, resulting in linear reduction in the cost of reagents required. Furthermore, the excellent optics of the chambers have positive effects on media costs during cloning. Thus, users can reliably detect chambers containing only one cell immediately after plating; this is impossible in a microplate well as the solid walls obscure views of any cells abutting walls (this is why many perform a second round of cloning to increase the chances that a 'clone' is truly derived from only one cell). Moreover, clones can be picked sooner (e.g., after 8 days in the examples shown in Fig. 3(b)); this compares with the ~14 days or more usually taken using a microplate well (again, the excellent optics enable users to detect the whole clone). In terms of equipment, the printer (a 3-axis traverse plus syringe pump) can vary in cost depending on the specifications. In our case, this was provided by iotaSciences as an early prototype research tool for development purposes.

Acknowledgments

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Author contributions: C.S., A.F., A.A.C.-P., P.R.C., and E.J.W. designed research, C.S., C.D., and E.J.W. performed experiments, and all authors wrote the paper.

Data availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Competing interests: Oxford University Innovation – the technology transfer company of The University of Oxford – has filed provisional patent applications on behalf of C.S., A.F., P.R.C., and E.J.W. partly based on this study. A.F., P.R.C., and E.J.W. each hold equity, and were directors in iotaSciences Ltd, a company that is exploiting this technology. iotaSciences Ltd. provides a scholarship for C.S. and C.D, and a salary for A.F.. E.J.W. and P.R.C. are consultants for iotaSciences.

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Supplementary materials

Supplementary figures

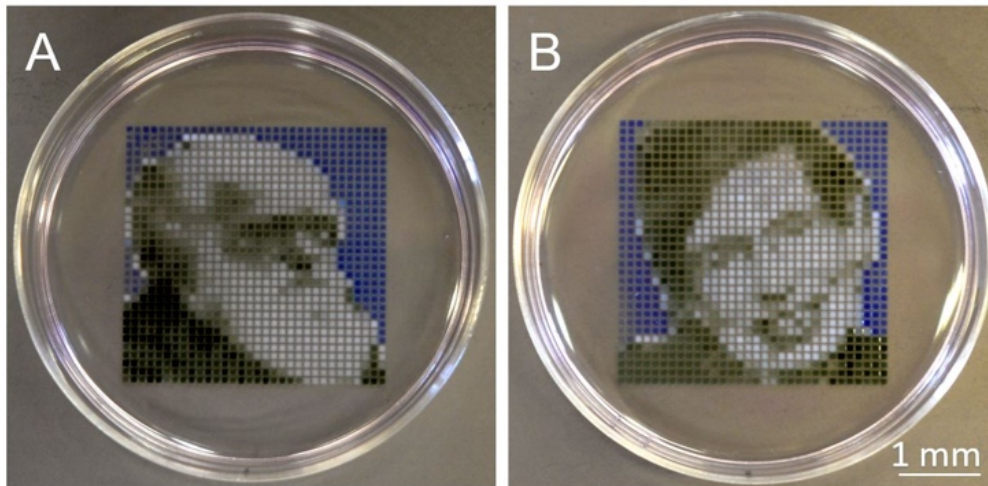


Figure S1. Portraits made on 6 cm dishes. Grids (32 x 32 arrays; 1 mm spacing) were made, and a palette of 16 colors (blue and 15 shades of grey) prepared on a glass slide designed to hold 16 droplets. In Matlab, initial portraits have been converted to grayscale images, and their resolution was changed to match the size of the grids (i.e., 32 x 32 pixels). Intensities of individual pixels in the image were distributed into 15 divisions, each corresponding to a shade of grey. The background around heads was cropped and allocated the color blue. Volumes of 60 nl of blue dye, or one of the 15 shades of grey dye, were then added to corresponding chambers to reproduce portraits of **(A)** Charles Darwin and **(B)** Stephen Hawking.

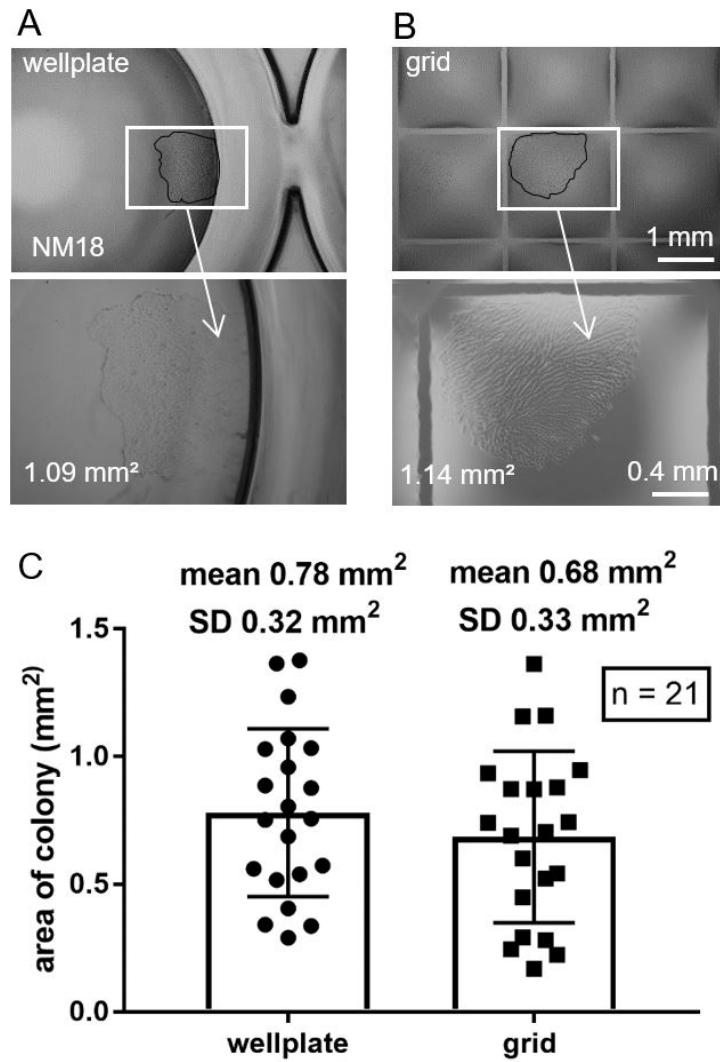


Figure S2. Growth of clones in chambers in grids compared to wells in microplates. Single NM18 cells were plated either in chambers in 96-well plates, or in chambers in 16x16 grids; after growth for 8 days, images of clones were collected, and areas of clones (a measure of growth) determined using ImageJ. **(A,B)** Typical images. Top: images of parts of wells/chambers (black lines mark colony edges). Bottom: areas indicated above are shown at higher magnification (numbers at bottom left give clone areas). **(C)** Areas of colonies grown in wells and chambers are similar (p value = 0.358, unpaired t -test). One would expect a clone in a chamber (which has $1/8^{\text{th}}$ the area of a well) to be slightly smaller due to the effects of contact inhibition (as cells stop growing when they reach the edge of the available area).

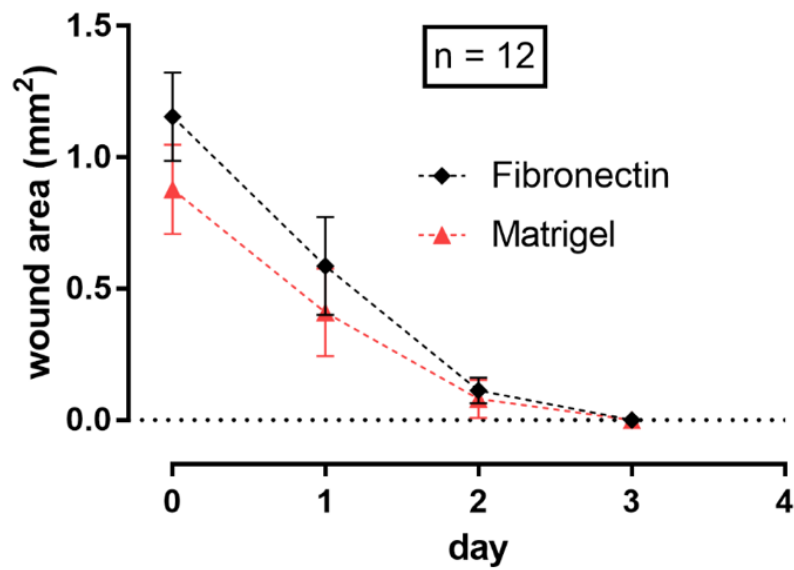


Figure S3. Wound healing rates (HEK cells). Using images collected as in Fig. 6, the area of unfilled wound at different times was measured using ImageJ. Although wounds were created using the same Teflon rod, wound area on fibronectin-coated dishes is initially larger than on Matrigel-coated dishes because cells on fibronectin have stronger cell:cell interactions and more tend to be removed from the surface as the rod passes. Both wounds heal completely after 3 days. The difference in healing rates between wounds on fibronectin (mean 0.384 mm²/day, SD 0.056 mm²/day) and those on Matrigel (mean 0.292 mm²/day, SD 0.056 mm²/day) is significant (p value = 0.0006, unpaired t-test).

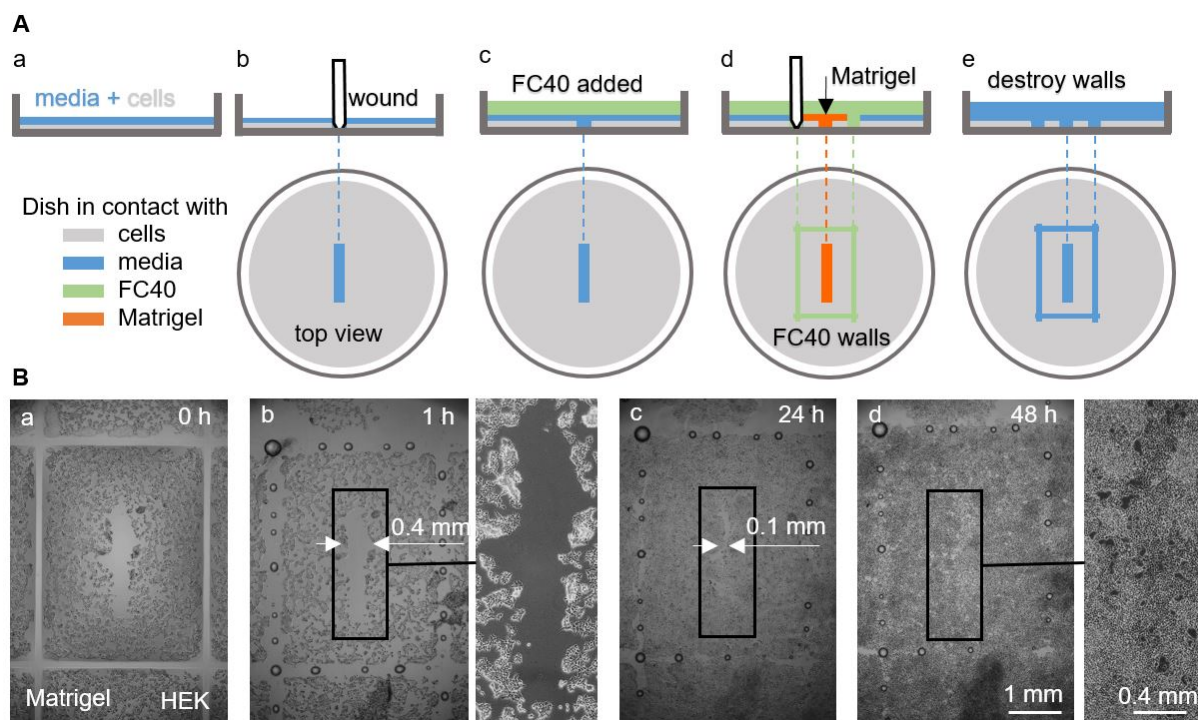


Figure S4. A wound-healing assay where Matrigel is added after wounding. The workflow in Fig. 6 involved coating the surface before plating cells and wounding; here, the coating is added after plating and wounding (and cells begin their migration into the wound in the presence of Matrigel in the medium).

A. Cartoon illustrating the workflow. (a) 600,000 HEKs were plated on a dish and grown for 24 h to confluency. (b) The stylus is scraped across the surface to remove cells in its path and create a wound (0.4 x 2 mm). (c) Most medium is now removed manually to leave a thin film in the dish, and FC40 (3 ml) added (again manually). (d) A rectangular chamber (3 x 4 mm) is built around the wound to isolate it from the rest of the dish, and Matrigel added to this chamber (2 μ l; final concentration of 1 μ g/cm²; cells are incubated for 1 h). In an experiment, a number of wounds would be created in one dish, chambers built around them, and individual chambers filled with different agents so that their effects can be compared. (e) Fluid walls are now destroyed (FC40 is removed manually, and the dish washed thoroughly with 3 ml medium to remove unattached Matrigel); wound healing is monitored by microscopy.

B. Images of wounds at different times. (a) The wounded region immediately after building the surrounding wall (before adding Matrigel). (b) The same region after incubation (1 h) in

Matrigel, wall destruction, and removal of unattached Matrigel (FC40 is removed manually, and the dish washed thoroughly with 3 ml medium); traces of FC40 remain. (c) After further incubation (24 h), cells have grown into the wound (wound width is <0.1 mm). Healing occurs slightly faster than in Fig. 6. (d) After 48 h, wounds heal completely.

Supplementary movies

Movies can be found at <https://advances.sciencemag.org/content/5/6/eaav8002/tab-figures-data> .

Movie S1. Reconfiguring patterns. An initial pattern (a circle within a triangle within a square with 9 mm sides) is printed, and different dyes added to each enclosure. The three dyes do not mix, being separated by FC40 walls. Walls around the two inner compartments are destroyed successively by pumping a large volume (55 μ l) of yellow dye into the centre. Most of the fluid (60 μ l) is now retrieved from the square, and a new pattern created (a triangle within a circle within the surviving square). Dyes are now added to the different compartments as before. The movie plays at 3x speed.

Movie S2. Creating chambers for the isolation of clones. Sets of three chambers of various shapes – circle, square (sides 2 mm), and triangle – are created, and red dye (1 μ l) is added to each chamber. The movie plays in real time as the first two patterns are printed, and then at 3x the speed.

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

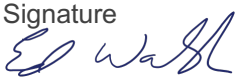
Title of Paper	Raising fluid walls around living cells
Publication Status	Published
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Student Confirmation

Student Name:	Cristian Soitu		
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Signature <i>Cristian Soitu</i>	Date	15.09.2020	

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Prof Edmond J. Walsh		
Supervisor comments		
Signature 	Date	9/10/2020

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Chapter 6: Using fluid walls for single-cell cloning provides assurance in monoclonality

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Abstract

Single-cell isolation and cloning are essential steps in many applications ranging from the production of bio-therapeutics to stem-cell therapy. Having confidence in monoclonality in such applications is essential from both research and commercial perspectives, for example to ensure data is of high quality, and regulatory requirements are met. Consequently, several approaches have been developed to improve confidence in monoclonality. However, ensuring monoclonality using standard well-plate formats remains challenging, primarily due to edge-effects; the solid wall around a well can prevent a clear view of how many cells might be in a well. We describe a method that eliminates such edge effects: solid confining walls are replaced by transparent fluid ones, and standard low-cost optics can confirm monoclonality.

Introduction

Single-cell isolation and cloning are critical steps in many high-value biological workflows, including the production of monoclonal antibodies (1-3), stem-cell therapy (4), and gene editing (5). For these applications, it is of paramount importance that a selected colony derives from a single progenitor. The method of limiting dilution is commonly used to isolate single cells (6-10); a cell suspension is dispensed into wells of a microtiter plate using a distribution of less than one cell per well. Cell numbers per well follow Poisson's distribution. However, assurance that there is one, and only one, cell per well is challenging, so users often take cells from the resulting colony and perform a second round of cloning to increase confidence in monoclonality—adding significant time and cost to the workflow.

The challenge of assuring monoclonality has been well documented by regulatory authorities (11), who note it is insufficient to show a single colony in a well during a cloning round. Instead, it should be demonstrated that only a single cell was originally plated in a well, ideally by imaging the entire well with sufficient resolution to identify the single progenitor. Identification of single cells in flat-bottomed well-plates becomes complicated in the region where the well base meets the vertical wall. This junction produces an “edge-effect”, which usually manifests itself as dark/blurred regions due to artefacts resulting from the effects of refractive index and curvature; this makes single-cell identification both challenging and time-consuming. Fluorescent labelling is commonly used to determine if just one cell is present, but this brings additional cost and may adversely affect cell viability—and so may not be suitable for some workflows. Another difficulty when utilizing well plates is cell-settling time; this can be reduced using a centrifuge, but this may also be harmful and laborious.

Many methods, more advanced and costly than limiting dilution, are routinely used or currently in development, to facilitate, automate, and accelerate cloning in standard microplate formats. The most established is Fluorescence-Activated Cell Sorting (FACS) (12-13), which allows fluorescently-labelled cells to be sorted rapidly and efficiently. However, cell labelling plus the high pressure and shear rates involved may adversely affect viability (14,18),

especially of sensitive cells. Other methods include inkjet printing (15-17), manual cell-picking, and the use of optical tweezers or laser microdissection (18); some use label-free optical methods, and deposition into wells to give a higher fraction containing single cells than is achievable using limiting dilution—allowing one to “beat” Poisson’s limit. However, most provide confirmation that a single cell is present prior to deposition into a well, rather than after deposition. A wide range of microfluidic devices have also been developed for single-cell cloning (18-20), but have disadvantages associated with cell-proliferation rate, colony retrieval, and non-standard formats that are not easily integrated into existing workflows.

We now describe a simple microfluidic method that can provide confidence in monoclonality, and is microplate compatible. Critically, rather than working around difficulties associated with edge effects, cell labelling, and spin-down requirements, we eliminate them by confining liquids within fluid walls rather than solid ones. In its simplest form, a sessile drop of media (perhaps containing a single cell) is placed on a standard polystyrene dish used for tissue culture, and some of the media is withdrawn by an automated aspirator. This leaves a relatively flat drop pinned to the dish by interfacial forces. The drop is bounded on the lower side by polystyrene, and on the upper side by an air/media interface. This interface is optically transparent, and—after collecting an image using a standard microscope—one can obtain a clear view of whether the drop contains a single cell. Optical clarity can further be enhanced by overlaying the drop with an immiscible liquid that has a refractive index closer to that of medium; we use the bio-compatible fluorocarbon, FC40. Arrays of square droplets—which we call ‘GRIDs’—are easily made with chambers arranged in any chosen format, and which can hold volumes from a micro-liter down to a few nano-liters (22-24). For proof-of-concept, we study GRIDs with 256 individual chambers sitting in a standard 60 mm tissue-culture dish. In principle, the method can be incorporated into high-throughput workflows by increasing chamber density in a 60 mm dish (by 256-fold (23)) or surface area (e.g. using a standard one-well microplate (23)).

Materials and Methods

Theory

The maximum angle, μ_m , for which light rays are accepted by a microscope objective in air can be calculated with knowledge of the numerical aperture (NA)

$$\mu_m = \sin^{-1} NA$$

Rays with angles exceeding μ_m do not reach the image plane, so result in dark regions (21). As rays pass through the curved liquid surface of a sessile drop, the change in refractive index results in refraction according to Snell's law. Consider a drop of water (refractive index $n = 1.33$) sitting on the bottom of a well in a polystyrene microplate ($n = 1.58$) in air ($n = 1$). If drop radius is less than the capillary length ($\lambda_c < \sqrt{\frac{\gamma}{\Delta\rho g}}$, where λ_c corresponds to capillary length, γ is surface tension, g is gravitational acceleration, and $\Delta\rho$ is the mass density difference of the fluids), then gravity has a negligible effect and the drop has the shape of a spherical cap. For a spherical cap (**Figure 1A**, blue area), the maximum height, h , radius of curvature, R , volume, V_{cap} , footprint radius, a , and contact angle, θ , are related through²²

$$V_{cap} = \frac{\pi(2 - 3\cos\theta + \cos^3\theta)}{3} \frac{a^3}{\sin^3\theta}$$

$$h = \frac{a}{\sin\theta} (1 - \cos\theta)$$

$$R = \frac{h^2 + a^2}{2h}$$

For a known volume and footprint area, the entire drop geometry can be evaluated. Then, the angle between a tangent at any point on the surface and the horizontal, α , (**Figure 1A,B**) is given by

$$\alpha = \sin^{-1} \frac{R_a}{R}$$

Using this angle, the trajectory of a light ray through a sessile drop can be determined using Snell's law. **Figure 1B** illustrates paths of parallel rays through a drop on polystyrene as they pass through air/water interfaces (continuous lines) or FC40/water interfaces (dashed lines). If the rays are refracted such that their exit angle, μ , exceeds μ_m , then the image will appear dark in the regions shown in **Figure 1C**, where R_a coincides with $\mu = \mu_m$. In white regions $\mu < \mu_m$, and in dark regions $\mu > \mu_m$. When the drop is overlaid with an immiscible fluid such as the fluorocarbon, FC40, with $n = 1.29$, at a given radius μ is lower than in air due to the higher refractive index (green paths in **Figure 1B**).

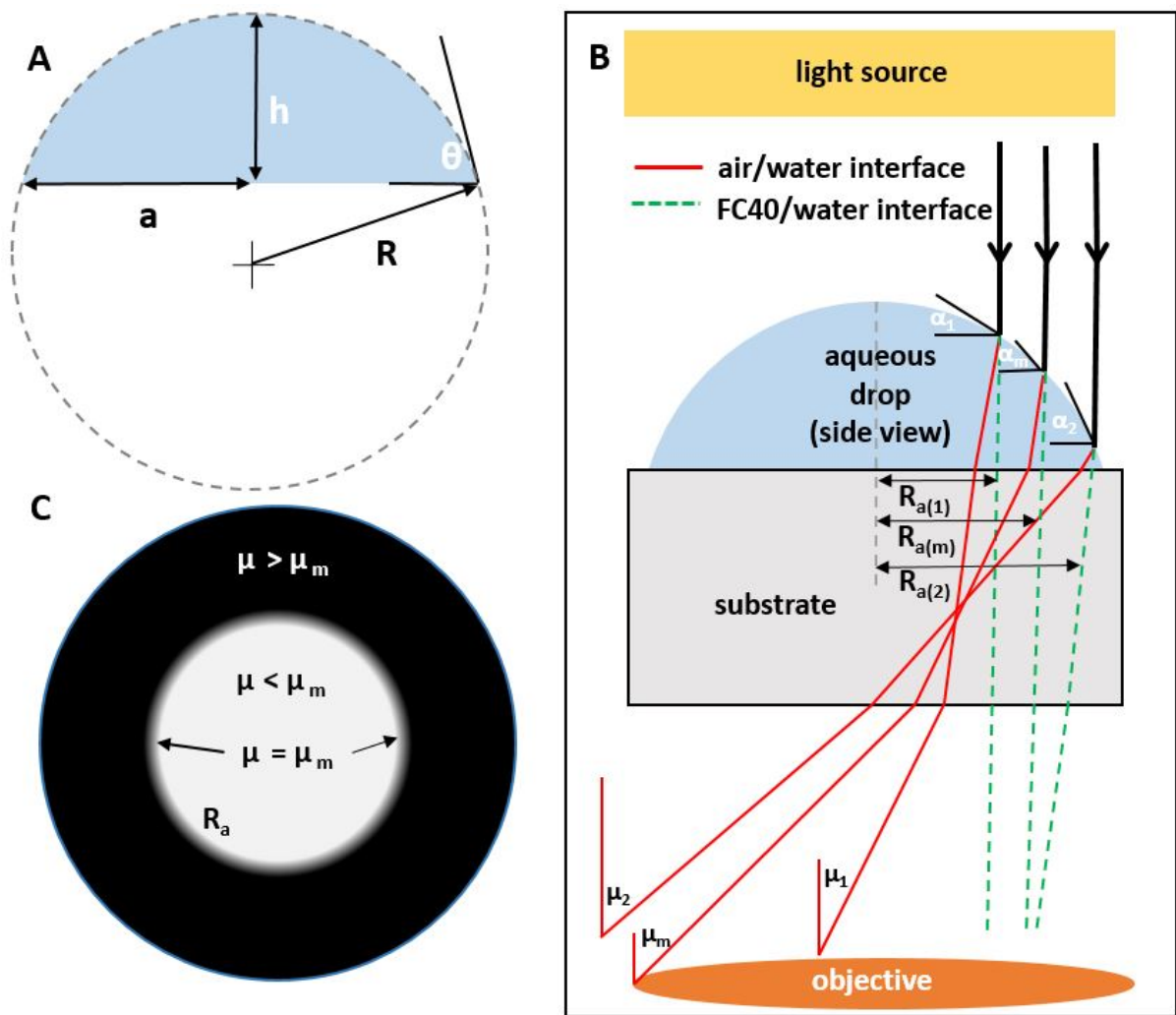


Figure 1. Schematics illustrating drop parameters **(A)** Geometry of a sessile drop. **(B)** Light paths through a sessile drop on a polystyrene substrate (for illustrative purposes only). Different incident angles at the drop surface (α_1 , α_m , α_2) result in different exit angles (μ_1 , μ_m , μ_2). **(C)** Refracted light enters the objective when $\mu < \mu_m$; when $\mu > \mu_m$, dark regions obstruct visibility of drop contents.

Reagents and equipment

All reagents and materials were purchased from Sigma-Aldrich, unless otherwise specified. Matrigel (Corning, #3542777) was used for **Figure 5A,iii**. FC40 was supplied by IotaSciences Ltd (Oxford, UK). All GRIDs and sessile droplets were made on 60 mm TC-treated cell-culture dishes (Falcon, #353002). Where indicated, droplets contain Allura Red dye. All fluidic arrangements were created and manipulated using a custom mini-printer (IotaSciences) and custom software. This printer consists of a three-axis traverse, which holds a Teflon rod (used to create fluid walls of FC40), and a dispensing needle connected to a syringe pump that is used to add/remove liquids to/from chambers in GRIDs and droplets.

Cell culture

All cell lines were grown routinely in cell culture flasks at 5% CO₂ at 37 °C. Preliminary tests to demonstrate reliable single-cell identification in droplets (**Figures 2 and 3**) used human embryonic kidney (HEK) cells cultured in Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS; Gibco). Subsequent plating and cloning in GRIDs were performed using both HEKs and mouse embryonic stem (ES) cells (EK.CCE cell line, derived from a single XY blastocyst-stage embryo of the 129/Sv//Ev strain (25)). The latter were cultured in DMEM (Invitrogen) supplemented with 15% FBS, 1% penicillin/streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol, 1% glutamine (Invitrogen), 1% minimum essential media (MEM) non-essential amino acids (Gibco), 1mM sodium pyruvate, and 1000 U/ml ESGRO leukemia inhibitory factor (LIF) on gelatin-coated plates.

Cells were plated by "scanning" (23) in 1.9 x 1.9 mm chambers in GRIDs that contained 400 nL of medium, at densities of 0.1 and 1 cell/chamber. In assays assessing the effects of different feeding regimes (**Suppl. Figures S2B and S3**), replenishment was done by removing 350 nL of old medium and adding the same volume of fresh medium, using the custom-built mini-printer.

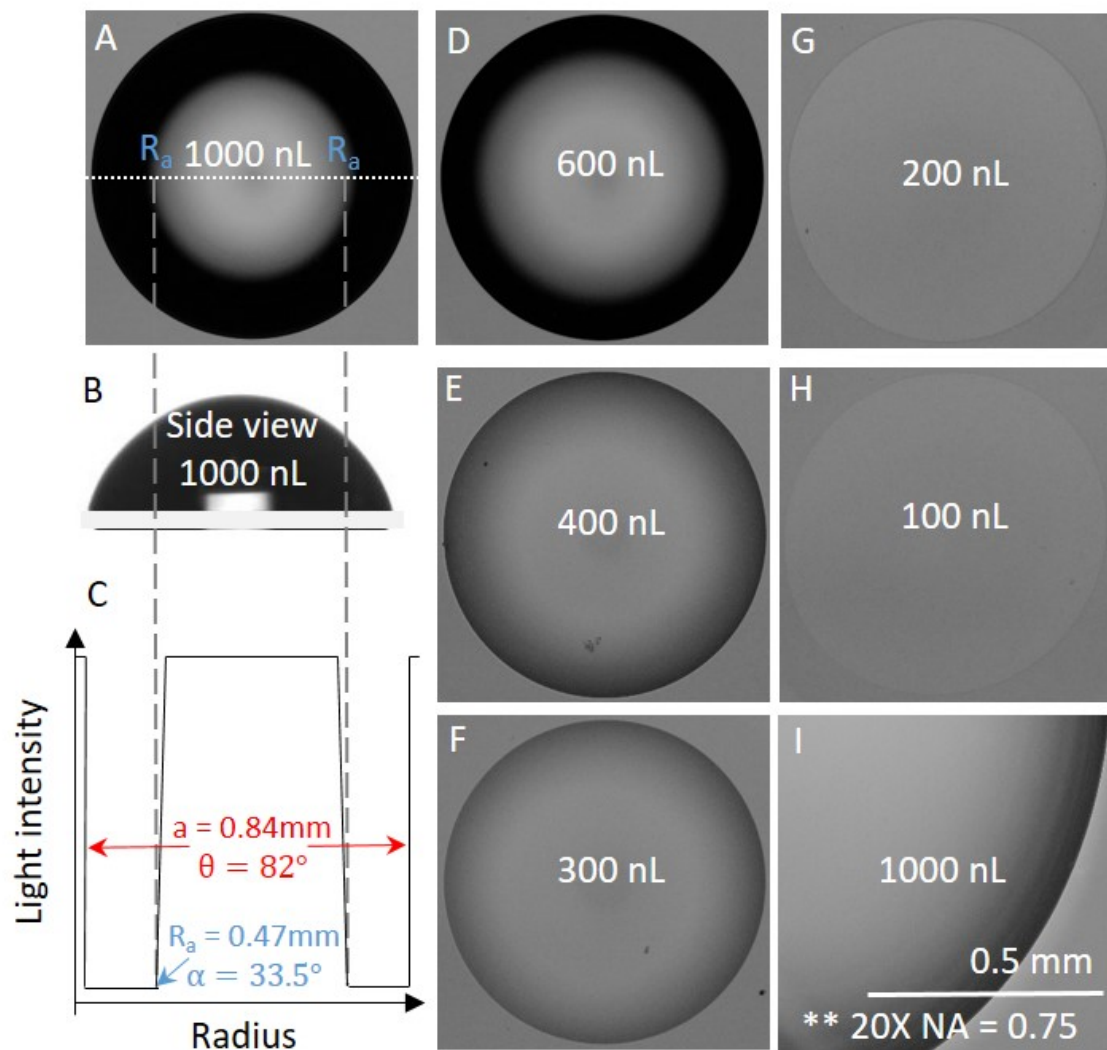


Figure 2. Images of drops of media with different volumes but identical footprints sitting in air on a polystyrene dish. Drops of 1,000 nL were deposited, and 0-900 nL removed. Footprints remain identical (diameters 1.68 mm) because media is firmly pinned to the dish as the receding contact angle is never reached (as that angle is sufficiently low). **(A-C)** Top (10X objective, NA 0.25) and side views (taken with FTA instrument) of 1,000 nL drop, plus a cartoon illustrating the intensity along the dotted line across the diameter in (A). **(D-H)** Top views of drops with smaller volumes (10X objective, NA 0.25); reducing volume reduces curvature, so less of the chamber is obscured by the dark ring. **(I)** Top view of 1,000 nL drop taken with a different objective (20X; NA 0.75).

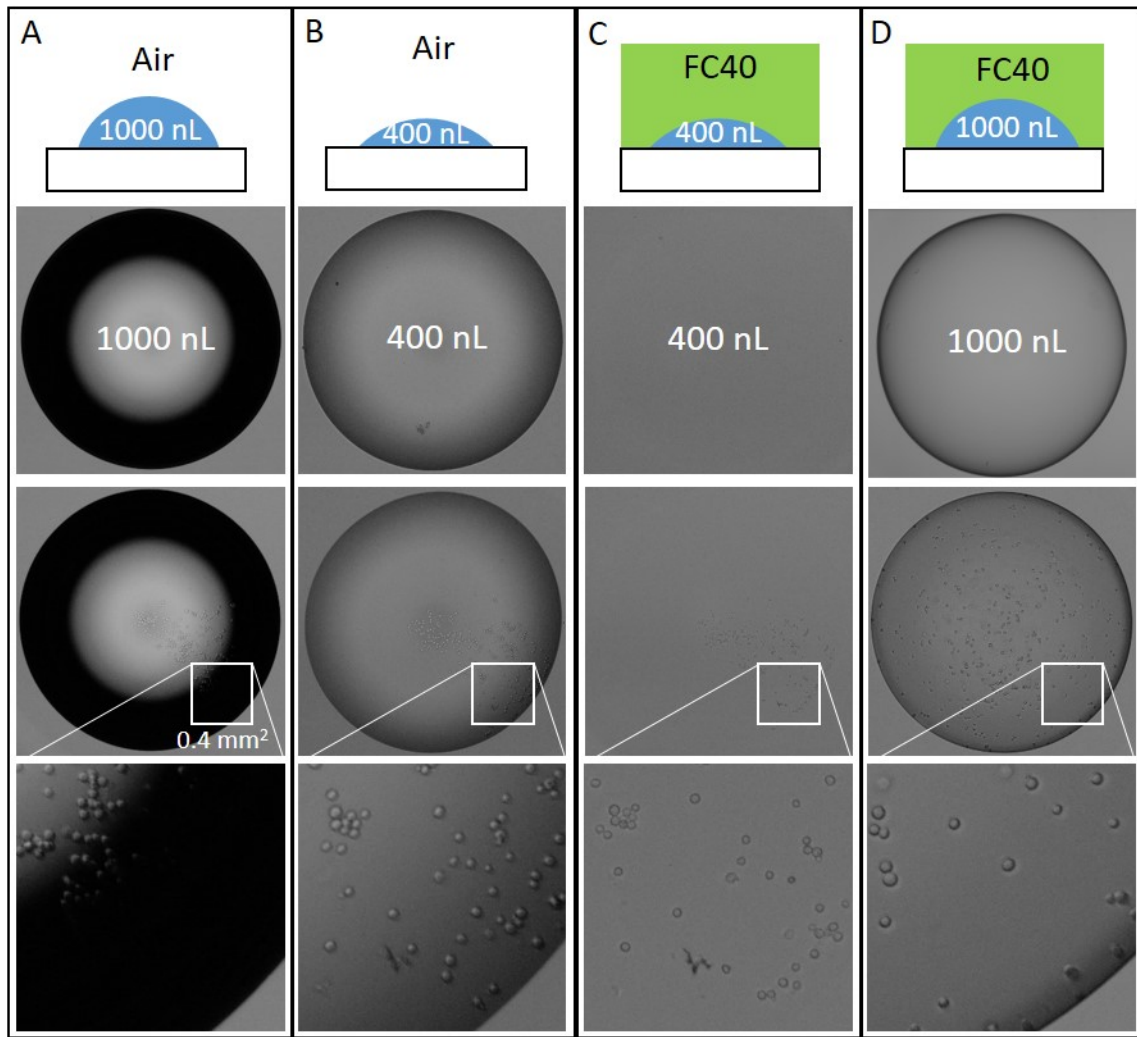


Figure 3. Identifying cells in sessile drops. All drops have the same footprint area, but contain 1,000 or 400 nL $\pm$ HEKs. Images were collected immediately after plating, so cells had not yet adhered to the surface. Images were collected with a 10X objective (NA 0.25). (A – D). Sessile drops were prepared in 4 ways. (i) Illustrations of the experimental setup in each column. (ii) Images of drops of DMEM + 10% FBS under air or FC40. (iii) Similar drop to the one above but containing HEK cells. (iv) Digital magnification (0.4 x 0.4 mm) of the area shown above.

Imaging droplets

To identify single cells in standard 96-well plates it is important that the entire region in the well where cells might have been deposited can be imaged clearly; in general, enhanced optical clarity lowers microscopy and labor costs. We will show that replacing the solid walls that confine cells with liquid ones improves optical clarity obtained using standard low-cost microscope objectives. This method was validated by placing eight 1 μ L sessile drops of cell media (DMEM + 10% FBS) on a standard polystyrene Petri dish used for tissue culture. Fluid was extracted from seven using a syringe pump, to leave drops with 100 – 1,000 nL (**Figure 2**). All drops have the same footprint area, as FBS prevents the pinning line from receding²⁶, and drops with the lowest volumes have the smallest contact angles. Drops were imaged less than ten seconds after formation to minimize evaporation using a Nikon D610 DSLR mounted on an IX53 inverted microscope (Olympus), operating in bright-field mode, and fitted with a 10X objective (Olympus A10 PL; NA = 0.25). The method works in both bright-field and phase-contrast modes, although the former was exclusively used here. Contact angles, θ , were calculated using equations from the theory section above from the measured footprint area (obtained using the calibration ruler in the microscope) and volume. They were also measured directly by the sessile-drop method using First Ten Angstroms' (FTA) instrument and software. For the latter method, drops were formed by ejecting a 1 μ L drop using a needle (33G blunt NanoFil needle, World Precision Instruments) connected to a syringe pump (Harvard Ultra) through a Teflon tube. Drops were gently transferred to the surface of a square cut from the base of a Corning 60 mm suspension-culture dish made from polystyrene, and then imaged from the side. The resultant equilibrium contact angles in air were $\sim 82^\circ$ and $\sim 80^\circ$ using the analytical and sessile-drop methods, respectively.

Imaging GRIDs

Supplementary **Figure S1A** illustrates how GRIDs in standard tissue-culture dishes are created, using a dispensing needle mounted on a three-axis traverse system. Cells are plated in individual chambers in a GRID using a swift scanning motion. Due to the low volumes

contained within these chambers (i.e. 400 nL) and the FC40 overlay, the rectangular footprints yield similar optical clarity to droplets in **Figure 3Cii**. For **Figures 4A, 4B, 5Aiii, and 5B** the same inverted microscope and objective was used to image each individual chamber.

Images in **Figures 4C, 5Ai, and 5Aii** were taken using an IncuCyte ZOOM, a commercial live-cell imaging system (Sartorius, Gottingen, Germany). Data illustrated in supplementary **Figures S2 and S3** was collected using the IncuCyte and a second commercial imaging system, cytoSMART Omni (cytoSMART, Eindhoven, Netherlands). For these experiments, GRIDs were imaged immediately after plating single-cell suspensions. Images were analysed to locate chambers containing single cells and cells in chambers fed using the regimes indicated (**Suppl. Figures S2B and S3**); colonies were imaged after 8 days.

Results

Consider the image in **Figure 2A** of a 1,000 nL drop of tissue-culture medium sitting on a polystyrene Petri dish in air. A black ring surrounds the grey central area. As drop radius is less than the capillary length, the drop's shape is that of a spherical cap (**Figure 2B**). The radius, R_a , at the transition point between bright to dark regions was measured (**Figure 2C**), and then light-ray paths and diffraction angles were calculated (**Figure 1B**; Methods); some results are shown in the first row of **Table 1**.

Drops with smaller volumes but similar footprints were made by depositing 1,000 nL, and then removing up to 900 nL (**Figures 2D-H**). Footprints remain unchanged on removal because the receding contact angle is sufficiently low it is never reached; consequently, media does not recede and remains firmly pinned to the dish (23), as illustrated in **Movie 1**. For all drops in **Figure 2**, a ray of light entering a drop vertically at R_a results in an average $\mu_m = 12.8^\circ$ (SD of 0.6°). The value of μ_m can be calculated using the NA of the objective, and is $\mu_m = 14.5^\circ$. Considering the simplified assumption that parallel rays enter the drop, this agreement is satisfactory; moreover, the approximately constant value of μ_m for a range of drop volumes further supports the validity of this analysis. The maximum refracted angle, μ_{max} , for all drops

considered, occurs at the pinning line of the 1,000 nL drops, and is 48° at a value of $\alpha = \theta = 82^\circ$. Imaging a drop with an objective with a higher numerical aperture (i.e., $NA = 0.75$, compared to 0.25) gave a dark ring with reduced width (**Figure 2I**) and $\mu_m = 48.6^\circ$ (**Table 1**), as expected. **Table 1** also shows that drops in panels G and H result in $\mu < 10^\circ$ everywhere, and dark regions disappear. The value of μ can be reduced further by overlaying drops with an immiscible fluid of refractive index closer to the one of cell medium, like FC40 ($n = 1.29$); μ_m falls from an average of 12.8° in air to 1.3° in FC40 (**Table 1**). These results show that all regions within a drop can be imaged, providing drop volume is sufficiently small.

Drop	Volume (μL)	R_a (μm)	θ°	h (μm)	$\alpha^\circ@R_a$	$\mu_m^\circ@ \alpha$ (air)	$\mu_m^\circ@ \alpha(\text{FC40})$
A	1	466	82	726	34	12.0	1.5
B	0.8	485	73	618	34	12.1	1.1
C	0.7	531	68	558	36	13.0	1.2
D	0.6	581	61	492	37	13.7	1.3
E	0.4	696	45	346	36	13.1	1.2
F	0.3	835	35	263	35	12.6	1.2
G*	0.2	835	24	177	24	8.2	0.8
H*	0.1	835	13	91	13	4.2	0.4
I*	1	835	82	726	82	48	10.9

*Calculation based on $\alpha = \theta$ at the pinning line.

Table 1. Geometric parameters calculated for drops like those shown in Figure 2 assuming their shape is equivalent to the cap of a sphere.

Imaging cells in sessile drops

To evaluate whether individual cells can be seen clearly in such drops, drops (now containing HEK cells) with constant footprints but varying volumes were prepared on tissue-culture-treated dishes and imaged immediately using a standard low-cost 10X objective

(**Figure 3**). As before, a reduction in volume and adding an FC40 overlay reduces the width of the dark ring, and the combination eliminates it completely; consequently, cells throughout the volume can be seen clearly. Of course, use of higher magnifications and numerical apertures further increase resolution. As 1,000 nL drops have a substantial height (**Table 1**), some cells may not have settled on to the bottom when images are collected; however, they can still be detected, even though they may be out of focus (e.g., arrowed cells in **Figure 3Div**). Of course, using drops with smaller volumes (and so heights) brings essentially all cells in the volume into sharp focus (**Figure 3Civ**). These results show how the slight edge effects associated with the use of fluid walls, and problems associated with the failure of cells to settle rapidly, are easily mitigated.

Comparing optics in conventional wells with their equivalents made with fluid walls

Although arrays of sessile drops under FC40 that have circular footprints are made easily (22), we now prefer to use alternatives with square footprints (23). This geometry is useful for creating a high number of chambers efficiently, while maximizing the surface area used. We call such arrays 'GRIDs'. GRIDs are made by reshaping media and FC40 already sitting in a dish (**Figure S1** (23)). First, the bottom of a standard Petri dish is covered with a thin film of media (approximately 50 μ L), a 1 mL FC40 overlay added to prevent evaporation, and a Teflon stylus (approximately 100 μ m wide) lowered on to the bottom of the dish. As FC40 wets the stylus and polystyrene better than media, it runs down to the substrate. Moving the stylus sideways then "prints" a fluid wall of FC40 that is tightly attached to that substrate, held by interfacial forces. Drawing more lines then creates a GRID, in which neighboring chambers containing media are separated by liquid walls of FC40. GRIDs with hundreds to thousands of chambers containing volumes down to a few nano-liters can be made, using the custom mini-printer. Here, we use GRIDs with 256 chambers made on 6 cm dishes in under 90 seconds (23). Liquids can be added to, and removed from, individual chambers simply by pipetting through the liquid FC40 walls; then, within wide limits determined by advancing and

receding contact angles, the fluid walls morph above an unchanging footprint. The walls also prove robust during handling (23); for example, a dish containing a GRID can be inverted (so essentially all FC40 drains out of the dish, leaving only a thin layer covering the wells and fluid walls—approximately a few microns high), but all chambers (and their aqueous contents) remain tightly attached to the dish (**Figure S1B**). Cells are deposited into chambers using a high-speed scanning motion (23) performed by the mini-printer, in which a dispensing needle moves at constant speed and height above the chamber as it infuses a cell suspension. Each chamber receives the same volume (**Movie 2**) and—as heights are so small—cells settle quickly. Cells in GRIDs are then grown in conventional CO₂ incubators just like their counterparts in microplates, as FC40 is freely permeable to the vital gases, O₂ and CO₂. Previous work has shown that liquid FC40 walls effectively isolate reagents and cells in one chamber from others in the same GRID; this is largely due to the very low solubility of water in the fluorocarbon (i.e., <7 ppm by weight at room temperature (22-24)). Consequently, after inoculating bacteria into every second chamber in a GRID, bacteria grow only in inoculated chambers as the rest remain sterile (23). While solid walls in a microplate provide an effective barrier between chambers, FC40 provides an additional sterile barrier over chambers. We now compare edge effects given by solid walls found in a conventional microplate with those given by the transparent and liquid ones in such GRIDs.

Figure 4A illustrates images of two colonies of adherent HEK cells, one growing near the solid wall of a microplate well, the other at a fluid wall in a GRID. The solid wall gives a significant edge effect (the dark annulus), and individual cells in the clone cannot be resolved even in the digital zoom. In contrast, there are few edge effects near the fluid walls (which appear as light-grey lines in the top panel), and individual cells throughout the colony are easily resolved in the zoom. It is even possible to distinguish between a single cell and two sitting side-by-side close to a fluid wall (**Figure 4B**). Moreover, chamber dimensions are easily shrunk to user's needs (by printing fluid walls closer together), without affecting cell visualization (**Figure 4C**).

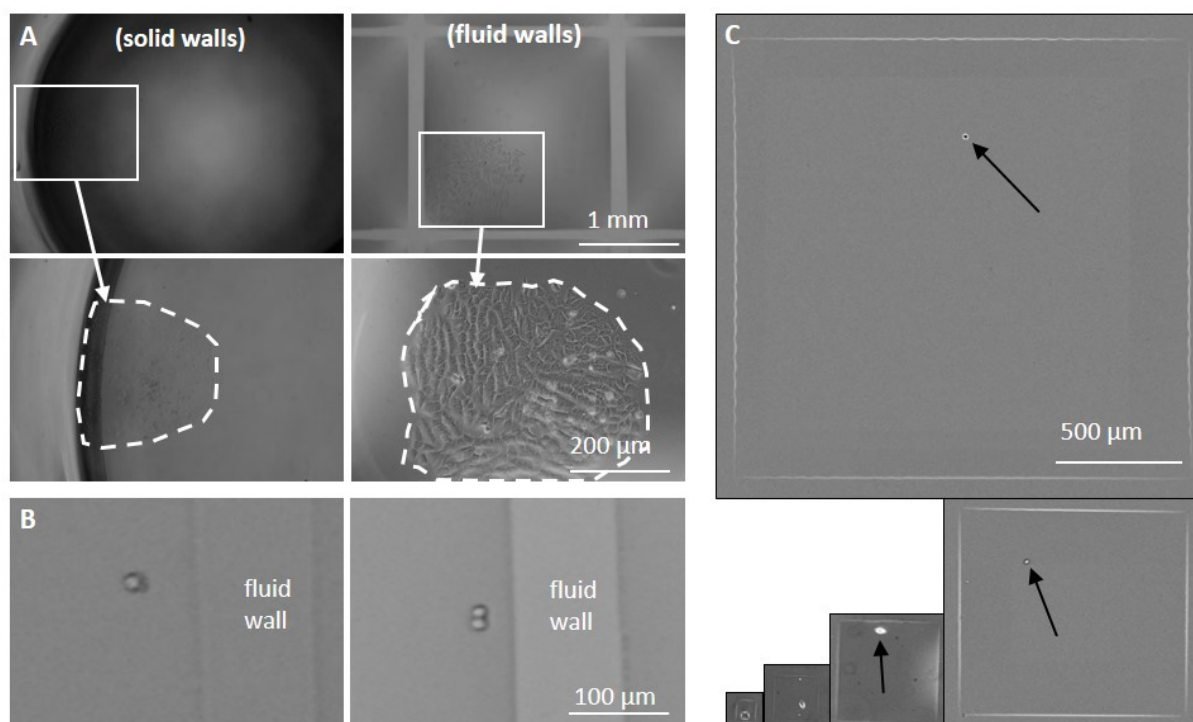


Figure 4. Comparing optics in chambers with solid and fluid walls. **(A)** Images of colonies of HEK cells growing close to walls; magnifications of areas indicated are shown below, where dotted lines mark clone limits. Left: the solid wall of a 96-well plate introduces an edge effect that partially obscures the view of the clone. Right: fluid walls present no equivalent edge effect, and individual cells in the clone are seen clearly. **(B)** A single cell (left) and doublet (right) are easily identified immediately after plating, even though they lie close to fluid walls. **(C)** A gallery of 5 square chambers where the lengths of edges are reduced by half from 1.9 mm at top, down to 110 μm , each containing one cell.

Cloning

As well as providing excellent optics for single-cell identification, GRIDs offer a suitable environment for cloning. After plating HEKs in a GRID, an image scan across the GRID allows chambers containing single progenitors to be identified rapidly, and subsequently their growth into colonies can be tracked similarly (**Figure 5Ai**). In addition to ensuring monoclonality, cloning efficiencies can be determined by identifying formed colonies soon after plating. For example, we used an arbitrary threshold of ≥ 16 cells derived from a single progenitor to call a clone the group of cells on the left in **Figure 5Aii** (but not the group on the right). This call was made after 8 days, which contrasts with the 21 days we generally use with these cells in a well in a conventional microplate. The versatility of the platform also enables chambers to be made with different coatings, prior to seeding single cells (**Figure 5Aiii**; the different coatings have complex effects on cell morphology, growth rate, and colony compaction (27)). Additionally, the excellent optics and small chamber size even allow multiple colonies to be identified in a single image obtained with a standard 4X objective (**Figure 5B**). Here, note the dark areas next to the fluid walls that vary in thickness (and are non-existent at the corners). Chamber shape approaches the cap of a perfect sphere at the center, but this cap sits on a square footprint. Consequently, the contact angle along the pinning line varies from a minimum at each corner to a maximum halfway between each pair of adjacent corners; this underlies the variations in thickness of the dark areas (**Figure 1**). As in the case of drops with circular footprints (**Figure 3**), thickness at any specified point can be reduced by reducing the contact angle at that point (by withdrawing medium from the chamber). Notwithstanding these dark areas, it still generally remains possible to identify single cells and clones at any point in a chamber (as in the case of the clone in the chamber at top-right in **Figure 5B**).

The process of dispensing cell suspensions into GRIDs is rapid. For example, it takes less than 90 seconds to plate cells into the 256 chambers in a GRID used here²³, and resulting cell distributions follow Poissonian statistics (**Figure S2A**). The same printer used to make GRIDs and plate cells can also replace spent media, and so feeding protocols are easily

varied. For example, in **Figure S2B** we show that frequent feeding has little impact on cloning efficiency, suggesting that the 400 nL media initially present in a chamber is sufficient to support colony growth for 8 days. However, different feeding regimes can have slight effects on colony size (**Figure S3**). In addition, retrieval of colonies (after adding trypsin) and transfer into other GRIDs or conventional microtiter plates (by aspirating the detached cells) is also done using the printer. Retrieval efficiency was assessed by transferring colonies from fluid chambers to 96-wellplates and 56 out of 57 samples reattached and grew.

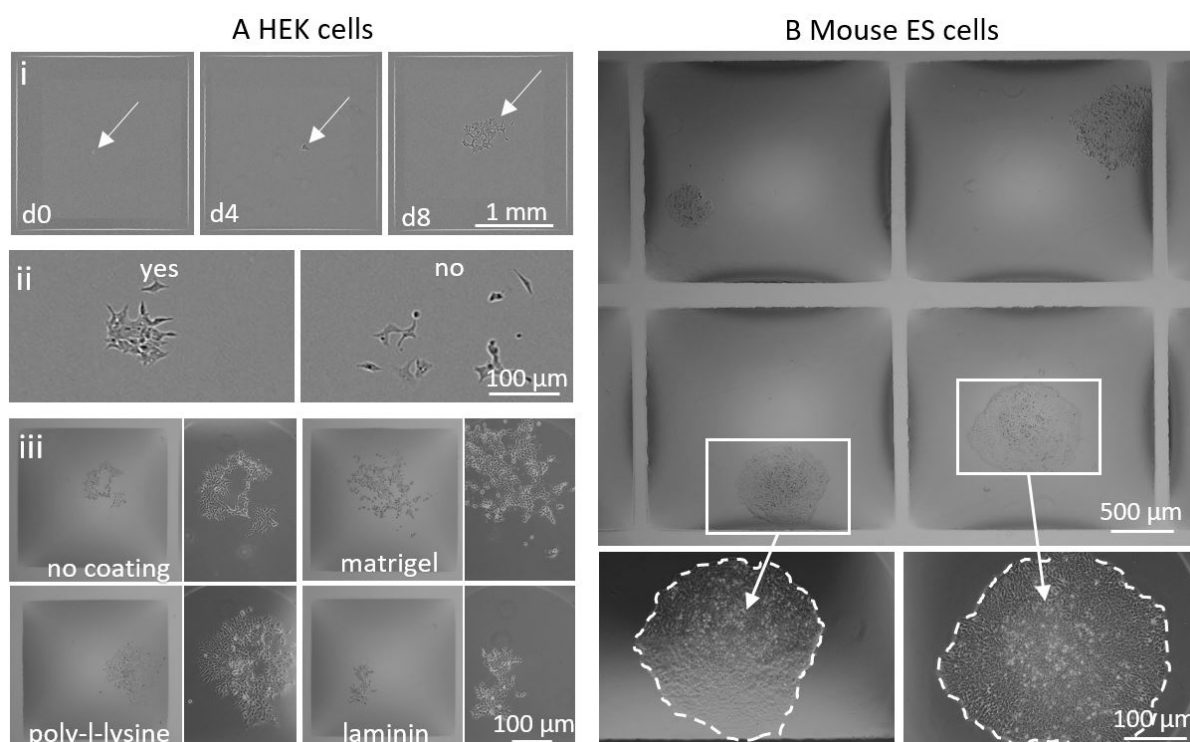


Figure 5. Cloning in chambers (1.9 x 1.9 mm) with fluid walls. **(A)** HEK cells. (i) Images of a whole chamber containing a single cell that develops into a colony over 8 days. The single cell seen at d0 moves slightly to the right in d4, prior to forming the colony. (ii) An arbitrary threshold (i.e., ≥ 16 cells derived from one progenitor) is used to define a colony; only the colony on the left meets this criterion. (iii) Fluidic chambers can be created on substrates $\pm$ different coatings (each pair of images shows a view of the complete chamber on the left, and zoom on the right). Coatings visibly affect the morphology of colonies, which ranges from compact (poly-L-lysine) to spread (Matrigel). **(B)** Mouse ES cells grow as more compact colonies than HEKs, imaged using a 4X objective. The upper image shows a view of 4 chambers, each containing an 8-day old colony derived from a single cell; zooms are shown below (dotted lines indicate colony limits). Edge effects are more pronounced here due to the addition of media for feeding, thereby increasing the GRIDs' contact angles (see Theory) resulting in the shaded regions around the edges. Single cells were identified prior to the addition of media.

Conclusion

Single-cell isolation and cloning is a core part of many workflows, and there is a growing need to ensure monoclonality. Unfortunately, this proves to be difficult and time-consuming when imaging wells in micro-plates, as solid walls obscure regions at the periphery, making it difficult—if not impossible—to ensure the well contains only one cell. As a result, methods for in-well identification of single cells usually require plates to be spun to settle cells, fluorescent tagging, or expensive optics. Here, we present a simple method to eliminate issues associated with edge effects, cell-labelling, and settling time that uses standard bright-field or phase-contrast optics. Importantly, obscuring solid walls used conventionally to confine liquids are replaced by transparent fluid ones. Then, contact angles of the resulting drops are reduced so the obscured region shrinks until contents of the whole drop become visible. Adding an overlay of the fluorocarbon, FC40, both prevents evaporation of small volumes, and improves optical clarity, as its refractive index more closely matches that of the aqueous phase than air. Arrays of drops/chambers with fluid walls were created in which single cells and their resulting clones are identified easily. The method provides an in-well method for confirming monoclonality that is user-friendly, versatile, biocompatible, and easily adapted to high-throughput.

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Competing interests. Oxford University Innovation—the technology transfer company of The University of Oxford—has filed provisional patent applications on behalf of C.S., P.R.C., and E.J.W. partly based on this study. P.R.C., and E.J.W. each hold equity in, and obtain remuneration from, iotaSciences Ltd, a company that is exploiting this technology. iotaSciences Ltd. provides scholarships for C.S. and C.D.

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Supplementary materials

Supplementary figures

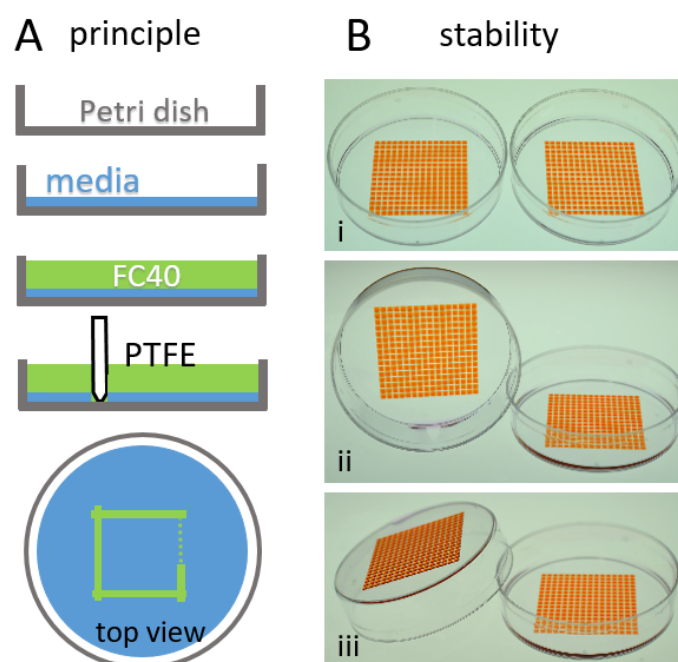


Figure S1. Making GRIDs, and some demonstrations of their stability. **(A)** Steps in making a GRID. 1 mL of cell-culture media (DMEM) containing 10% FBS is added to a virgin Petri dish, and subsequently most (~0.95 mL) removed by pipetting. This leaves a very thin film of media that has wetted the surface of the dish (approximately 50 μ L). 1 mL of FC40 is then added on top of the media to limit evaporation. A stylus made of polytetrafluoroethylene (PTFE, Teflon) is lowered on to the surface of the dish. As FC40 wets Teflon and polystyrene better than water, the fluorocarbon becomes pinned to the substrate around the tip of the stylus. The stylus is then dragged along the surface in the pattern shown; it pushes media aside, to leave a fluid wall of FC40 pinned to the polystyrene substrate. In the case shown, one central chamber isolated by fluid FC40 walls from surrounding media is created. “Printing” more lines (not shown), can then subdivide the original body of media into the wanted number of chambers. These chambers are used like the wells of a microplate, except that liquids are pipetted into them, and removed from them, through FC40 instead of air. **(B)** Stability of two GRIDs each containing 256 square chambers (1.9 x 1.9 mm) in a 6 cm dish. GRIDs are made

as in **(A)** using tissue-culture media. Each chamber initially contains ~200 nL, and—as media volumes are too small to be seen—200 nL red dye is added to each chamber to aid visualization. **(i)** Two GRIDs after manufacture. FC40 is transparent, and so invisible. **(ii)** The left-hand dish is tilted by 90°; most FC40 has spilt out, but a small residue can be seen at the bottom corner of the dish—chambers remain firmly pinned by interfacial forces to the dish. **(iii)** The left-hand dish has now been almost completely flipped over; chambers still remain pinned to the dish, and FC40 drains out almost entirely. This illustrates the dominance of interfacial forces at the microscale over gravitational ones, and how firmly chambers are attached to the dish.

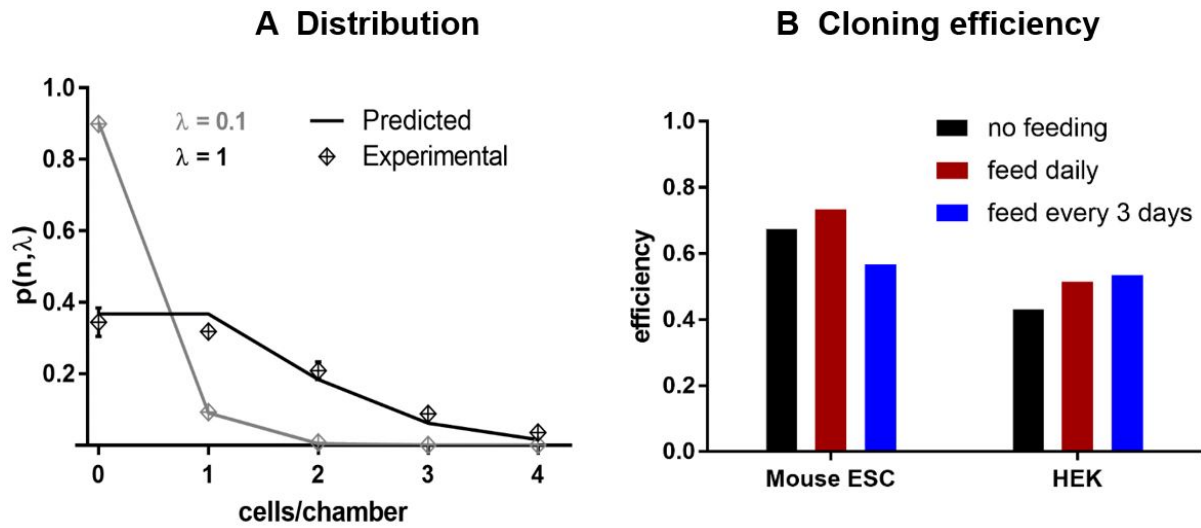


Figure S2. Cloning cells by limited dilution yields the expected Poissonian statistics, and efficiencies. A suspension of HEK cells, or mouse ESCs, are deposited by scanning²³ (~400 nL in each chamber in a GRID with 256 chambers of 1.9 x 1.9 mm in a 6 cm dish), and the number of cells per chamber immediately determined by microscopy using a 4X objective (as in Fig. 4B,C). **(A)** The process of dispensing a single-cell suspension of HEK cells in GRIDs does not introduce any bias in the distribution. Cells were seeded at densities, λ , of 0.1 and 1 cell/chamber. Distributions of numbers of cells in each chamber follow Poisson's distribution. **(B)** Cloning efficiencies of the two cell types assessed using three different feeding regimes. Cells are plated and imaged as in **(A)**, grown, and some fed by removing 350 nL spent medium and replacing it with 350 nL fresh medium. Chambers are reimaged after 8 days of growth, and the number of colonies with ≥ 16 cells derived from a single progenitor are recorded. The resulting cloning efficiencies are in the expected ranges.

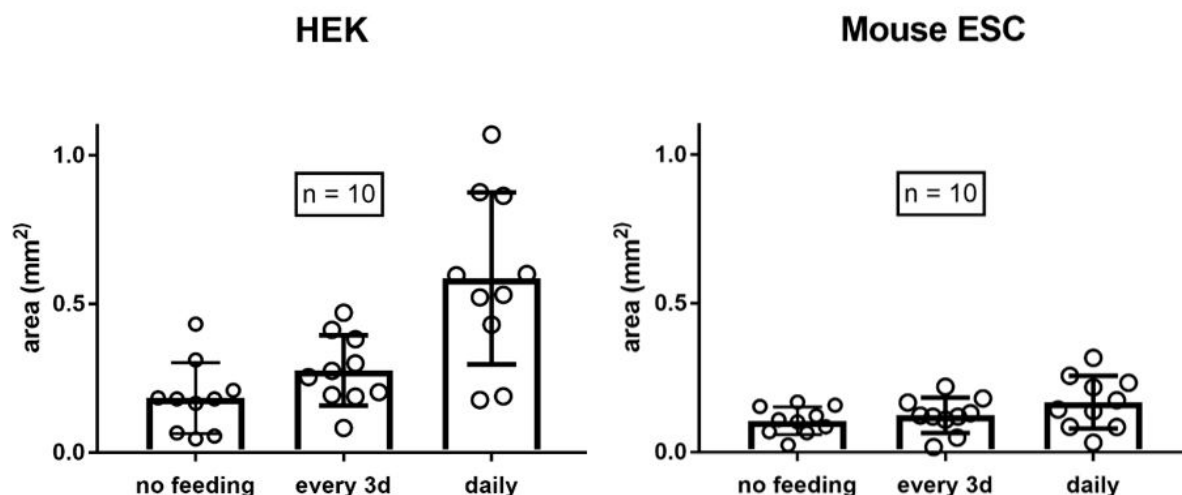


Figure S3. Size of colonies. Cells were cloned and fed as described in **Figure S2**, and areas occupied by clones scored in the images collected after 8 days. Areas occupied by the clones are as expected, and the different feeding regimes have marginal effects on colony size. This indicates that the 400 nL media in a chamber is nevertheless sufficient to support early clonal growth.

Supplementary movies

Movies can be found at <https://journals.sagepub.com/doi/suppl/10.1177/2472630319891135>.

Movie 1. Withdrawing fluid from a droplet. A withdrawing needle is withdrawing fluid from a sessile drop deposited under FC40. As the fluid is extracted, the pinning line remains fixed and does not recede.

Movie 2. Plating cells using a scanning motion. A needle infusing cell suspension at constant flowrate traverses at constant height above equally-spaced fluidic chambers, such that each one receives the same volume.

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

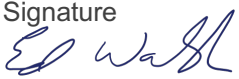
Title of Paper	Using fluid walls for single-cell cloning provides assurance in monoclonality
Publication Status	Published
Publication Details	C. Soitu, C. Deroy, A. A. Castrejón-Pita, P. R. Cook, and E. J. Walsh SLAS Technology, June 1, 2020, 25(3), 267-275

Student Confirmation

Student Name:	Cristian Soitu		
Contribution to the Paper	Lead author, performed cloning experiments, contributed to data collection and analysis for cloning experiments, prepared figures 4, 5, S1, S2, S3, contributed to writing and editing of the corresponding part of the manuscript.		
Signature <i>Cristian Soitu</i>	Date	15.09.2020	

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By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Prof Edmond J. Walsh		
Supervisor comments		
Signature 	Date	9/10/2020

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Chapter 7: Jet-printing microfluidic devices on demand

Authors: Cristian Soitu, Nicholas Stovall-Kurtz, Cyril Deroy, Alfonso A. Castrejón-Pita, Peter R. Cook, and Edmond J. Walsh

Accepted for publication: Advanced Science, September 2020.

Abstract

There is an unmet demand for microfluidics in biomedicine. This paper describes contactless fabrication of microfluidic circuits on standard Petri dishes using just a dispensing needle, syringe pump, 3-way traverse, cell-culture media, and an immiscible fluorocarbon (FC40). A submerged micro-jet of FC40 is projected through FC40 and media on to the bottom of a dish, where it washes media away to leave liquid fluorocarbon walls pinned to the substrate by interfacial forces. Such fluid walls can be built into almost any imaginable 2D circuit in minutes, which is exploited to clone cells in a way that beats the Poisson limit, sub-culture adherent cells, and feed arrays of cells continuously for a week. This general method should have wide application in biomedicine.

Introduction

As sensitivities of methods for detecting biomolecules improve, demand for handling ever-smaller volumes increases – and this drives development of microfluidic approaches (1-3). However, few of these are found in biomedical workflows, with the exception of those involving microplates. Why? Reasons given include: devices are expensive and take days/weeks to make, they are complicated to operate, their contents are inaccessible, and they are not made with cell-friendly materials (1).

In the everyday world, gravity is such a dominant force that most objects are made with solids, and one cannot contemplate building them out of liquids, which just collapse into puddles. Consequently, liquids are always contained by solid walls, otherwise they drain away. In the microworld, gravity becomes irrelevant, and interfacial forces dominate (think of water-striders skimming over ponds, and dew-drops sticking to blades of grass). Consequently, an interface between two fluids can act as a robust wall separating the two. This paradigm enabled the emergence of open microfluidics (4), where solid walls are replaced by air:water (5) or oil:water interfaces (6-8).

Open microfluidic devices are generally easier to integrate into biomedical workflows as they offer better optical and physical access to samples, reduced adhesion of reagents to solid surfaces and are resistance to blocking by air bubbles. However, despite the ever-increasing efforts to simplify manufacturing workflows for such devices, most of them still require etching of the substrate (9), surface treatment (10) or contact (7), or some combination of these (8) to confine fluidic structures. Such complex manufacturing processes deter many biologists who favor fast flexible prototyping without having to compromise biocompatibility (11). Recently, microfluidic arrangements were created simply by dragging a Teflon rod/stylus resting on the surface of a dish to reshape cell-culture media and an immiscible overlay into the wanted pattern (7). However, motion of the stylus relative to its holder introduced play in the x-y plane, reducing accuracy and precision. Moreover, proteins in media aggregate on the stylus to

reduce reproducibility and increase risks of cross contamination. Additionally, the stylus needs frequent change due to wear. All these drawbacks stem from the effects of contact.

Here, we describe a contactless method to fabricate microfluidic devices on demand, where the only 'building' materials used are those in the biocompatible trio – cell media, the immiscible fluorocarbon FC40, and a polystyrene Petri dish. FC40 is 'jetted' from a dispensing needle through bulk FC40 and media on to the untreated bottom of a dish. Complex microfluidic structures with features $<50\text{ }\mu\text{m}$ in size can be produced reproducibly with high accuracy in minutes. The aqueous phase is confined by fluid walls – media:FC40 interfaces – which are robust yet easily pierced (so liquids can be added/removed through them at any preselected point) whilst being transparent. The physics underlying flow during such jetting is complex (12-15); therefore, we establish appropriate conditions. We then exploit jetting to 'beat' the Poisson limit to clone single mammalian cells by limited dilution, sub-culture them (again using a contactless method), and perfuse them steadily with fresh media for a week.

Results

Approach

Figure 1a illustrates the approach. The bottom of a standard tissue-culture dish is covered with a film of cell-growth media, and an FC40 overlay added to prevent evaporation. A dispensing needle filled with FC40, connected to a syringe pump, and held by a 3-way traverse is now lowered below the surface of the fluorocarbon; starting the pump jets FC40 on to the dish to push media aside. As FC40 has a low equilibrium contact angle (CA) on polystyrene ($< 10^\circ$), it wets it better than media (equilibrium CA $\sim 50^\circ$ (6)), so it adheres to the bottom. Moving the micro-jet sideways then creates a line of FC40 on the dish, and drawing more lines creates a grid with 256 chambers in <2 min (Figure 1b; Movie 1). Each chamber is isolated from others by liquid walls of FC40 pinned to polystyrene. Interfacial forces dictate chamber geometry – a spherical cap sitting on a square footprint (height $\sim 75\text{ }\mu\text{m}$; volume $\sim 100\text{ nl}$). Up to $\sim 900\text{ nl}$ more media can be pipetted into chambers as fluid walls morph above unchanging

footprints. Chambers are then used like wells in microplates: liquids are added/removed to/from them by pipetting through FC40 instead of air. The maximum and minimum volumes that can be held in chambers without altering footprints are determined by advancing and receding contact angles; addition of too much media inevitably merges adjacent chambers. Even so, chambers accept a manyfold wider range of volume than equally-spaced wells in a microplate, whilst containing $\sim 1,000^{\text{th}}$ the volume (7). Consequently, if chambers contain cells, the volume ratio of intra- to extra-cellular fluid more closely resembles that *in vivo*. Importantly, this method is contactless: the nozzle touches neither dish nor media. Moreover, one pipette tip can add/extract reagents to/from many chambers without detectable cross-contamination (shown – for example – by seeding bacteria in every other chamber, adding media to all through one tip, and finding that bacteria grow only in inoculated chambers as others remain sterile (7)). In other words, a tip is washed effectively by passage through FC40 between chambers, and – when using cells – we make doubly sure by additionally washing in 70% ethanol.

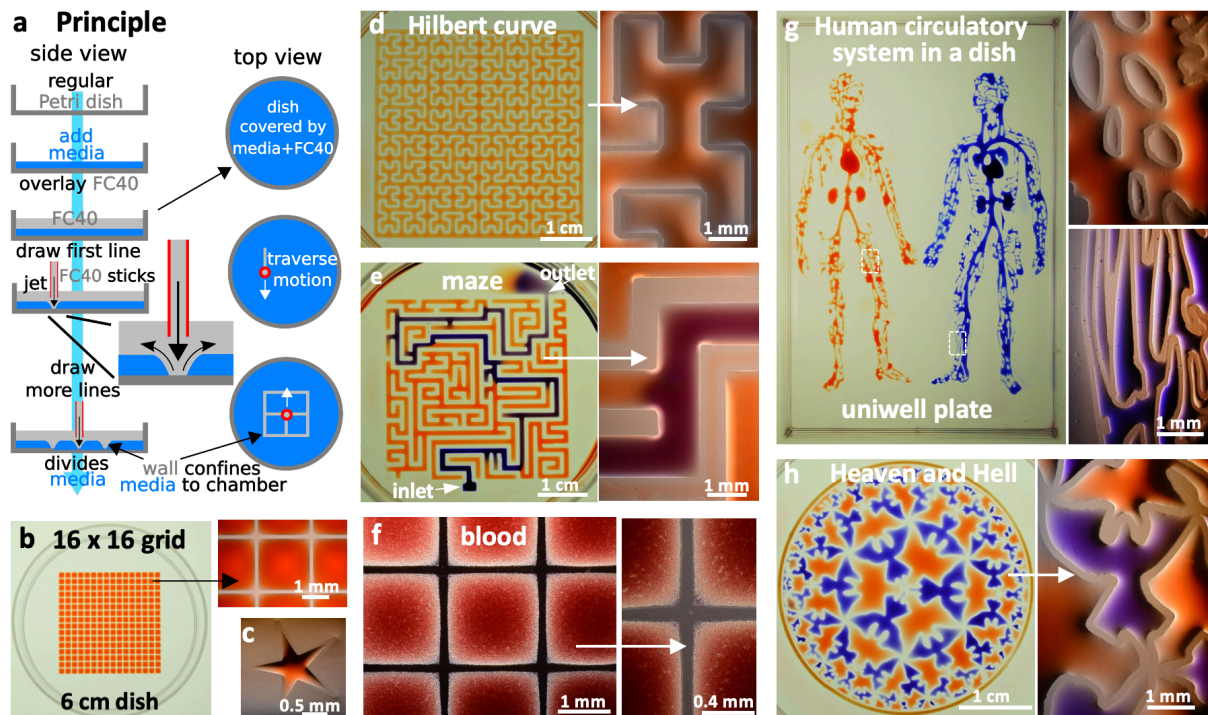


Figure 1. Principle. **a.** A ‘micro-jet’ of an immiscible fluorocarbon (FC40) is projected through FC40 and media on to the bottom of a dish; media is pushed aside, and FC40 sticks to the bottom (it wets polystyrene better than media). Moving the jet now draws lines to form a grid with FC40 walls. **b.** A 16 x 16 grid (inter-chamber spacing 1.9 mm; 600 nl red dye added to each chamber; zoom shows fluid walls). **c.** Star-shaped walls (red dye added after jetting). **d.** A square with one internal wall shaped like a Hilbert curve (a continuous line with many 90° and 45° turns). Red dye was added at many points; it diffused around the wall throughout the square. Zoom shows some turns. **e.** Path through a maze with a single inlet and outlet. The circuit was built by jetting FC40 through media plus red dye; when blue dye is pumped into the inlet, it takes the path of least resistance through the maze. Zoom shows some walls. **f.** Part of 16 x 16 grid where FC40 is jetted through blood instead of media. Zoom shows the jet leaves no cells in what are now FC40 walls. **g.** Human circulatory system in a uniwell plate with dimensions of a 96-well plate. Red and blue dyes were infused into major ‘arteries’ and ‘veins’ which then flow/diffuse throughout each system. Zooms show regions in white rectangles. **h.** Walls were built into pattern inspired by “Circle Limit IV (Heaven and Hell)” by M.C. Escher - WikiArt. Patterns for Figure 1d,e,g were obtained from the following

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Jetting allows walls to be built with a precision only limited by that of the traverse – illustrated by building a star-shaped chamber (Figure 1c), a square enclosing a single continuous wall shaped like a Hilbert curve (Figure 1d; fabrication time ~5 min), and a microfluidic maze where blue dye infused into the inlet solves the problem of finding the path to the sole exit (Figure 1e; conduits have maximum widths of 1 mm, and heights of ~350 μm). Reproducibility is demonstrated by constructing a fractal circuit in which a central input is connected through conduits (footprint width 450 μm , maximum height ~160 μm) that each make 5 right-angled turns to one of 64 outlets; dye infused into the input reaches all outlets simultaneously, showing that conduit cross-sections and lengths are similar throughout (**Figure S1**). The method is not restricted to media, which can be replaced by whole human blood (Figure 1f). Nor is it restricted to regular patterns, illustrated by the ‘human circulatory system’ (Figure 1g; jetting time ~90 min) and M.C. Escher’s ‘Heaven and Hell’ design inspired by “Circle Limit IV (Heaven and Hell)” by M.C. Escher, WikiArt, (Figure 1h; jetting time ~5 min). These circuits emphasize the ability to reproduce almost any pre-existing pattern with features of ever-decreasing size (‘veins’ or ‘angels’). In these and subsequent examples, dyes are added solely to aid visualization; they play no role in stabilizing liquid structures. We also use ‘media’ to describe DMEM plus 10% FBS unless stated otherwise. In addition, fluid walls are sufficiently stable to be carried up/downstairs between labs, incubators, and microscopes – just like any dish filled with liquid (see Movie 2). These examples illustrate the versatility of jetting and show that circuits with almost any imaginable 2D shape can be built quickly and accurately using cell-friendly materials.

Building walls and conduits with different widths

In theory, many parameters including interfacial properties, jet momentum, densities, and kinematic viscosities influence wall stability and shape. FC40 has a density of 1.855 g/ml, and intuition suggests that buoyancy should force media above the fluorocarbon; however, media remains pinned to a pre-wetted dish as interfacial forces are stronger than buoyancy forces. Other parameters were varied to assess their impact on wall construction (**Figure 2a**;

Figure S2a). When the nozzle is high above the substrate (i.e., H is large), no walls form; the momentum of the jet falls as it travels through FC40 (which has a kinematic viscosity of 2.2 cSt compared to 1 for water), making it insufficient to displace media (Figure 2bi, red symbol). Lowering the nozzle decreases the loss to the point when the final jet momentum that hits the substrate is large enough to push media aside. FC40 then remains pinned to the substrate to give a wall. Examination of streamlines (Movie 3) shows that jet width increases with distance from the nozzle, so further lowering yields progressively narrower walls as the jet impinges on progressively-smaller substrate areas. Even so, wall width proves to be relatively tolerant to changes in H (Figure 2bi), so walls can be constructed using a nozzle positioned at the same height above dishes from different manufacturers that turn out to be slightly bowed to different degrees. At a low volumetric flow rate ($\dot{Q}$), momentum is insufficient and no wall forms; increasing $\dot{Q}$ yields walls with increasing width (Figure 2bii). Nozzle diameter (D_{nozzle}), traverse velocity ($V_{traverse}$) and interfacial properties (especially presence of FBS) all affect wall width (Figure 2bii,iii; see Figure S2b for data on E8+, mTesR, and StemFlex media). Wider walls can be made by overlapping jetting lines (Figure 2c). Conduit width is easily adjusted down to at least 35 μm by jetting walls closer together (Figure 2d). We have therefore identified a range of conditions allowing wall formation even on commercially-available dishes that are not completely flat.

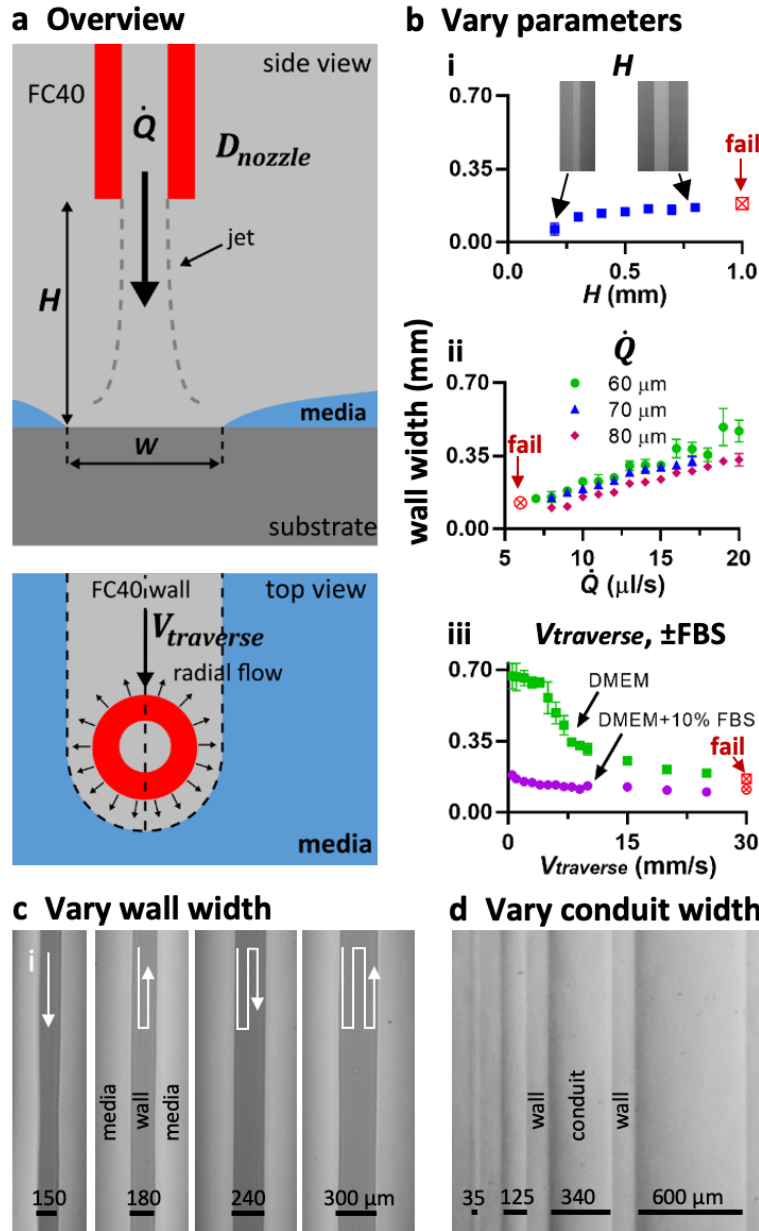


Figure 2. Varying jetting parameters. Unless stated otherwise throughout the paper, $\dot{Q} = 8 \mu\text{l/s}$, H (height of nozzle above substrate) = 0.4 mm, D_{nozzle} (diameter of jetting nozzle) = 60 μm , $V_{traverse}$ (lateral traverse speed) = 10 mm/s, and media was DMEM + 10% FBS.

a. Overview. A submerged FC40 micro-jet drives media off the substrate to create an FC40 wall, and some parameters varied are indicated.

b. Controlling wall width (red symbols: continuous walls fail to form) by varying: (i) jet height (inset: images of walls when $H = 0.2$ and 0.8 mm). The jet width increases with distance from the nozzle, so lowering the nozzle yields progressively narrower wall. (ii) Flow rate and nozzle

diameter. Wall width increases as jet momentum increases (by increasing flowrate or reducing nozzle diameter) (iii) Traverse velocity and serum content. Proteins in the serum causes an increased adhesion force at the pinning lines, so the 'sweeping' action of the jet momentum is diminished, resulting in smaller wall widths.

c. Varying wall width by overlapping walls. Wall width can be increased by setting the separation between jetting paths to be smaller than the wall width generated by one pass.

d. Making conduits with different widths (walls all have equal widths) by varying the distance between consecutive paths. $H = 0.3 \text{ mm}$, $\dot{Q} = 5 \text{ } \mu\text{l/s}$, $D_{\text{nozzle}} = 60 \text{ } \mu\text{m}$, $V_{\text{traverse}} = 1.8 \text{ mm/s}$.

Controlling flow through circuits

Flow through these circuits can easily be driven by external pumps; fluid walls/ceilings spontaneously seal around connecting hydrophilic tubes on insertion, and then morph above unchanging footprints during flow. However, adding external pumps adds complexity and cost. As intrinsic differences in Laplace pressure can also drive flow through microfluidic circuits (6,16), we demonstrated this in these circuits (**Figure S3**).

Thus far, we have jetted FC40. We now create a microfluidic ‘valve’ by jetting media (**Figure S4**, Movie 4). We first build a conduit with a dead-end (using an FC40 jet), and make a hole through the end wall (using a media jet traversing down the conduit axis) to allow flow through the hole. We next rebuild the original end wall (using an FC40 jet that traverses across the conduit) to stop flow. Such valves can be opened and closed as many times as needed.

‘Beating’ the Poisson limit during cell cloning

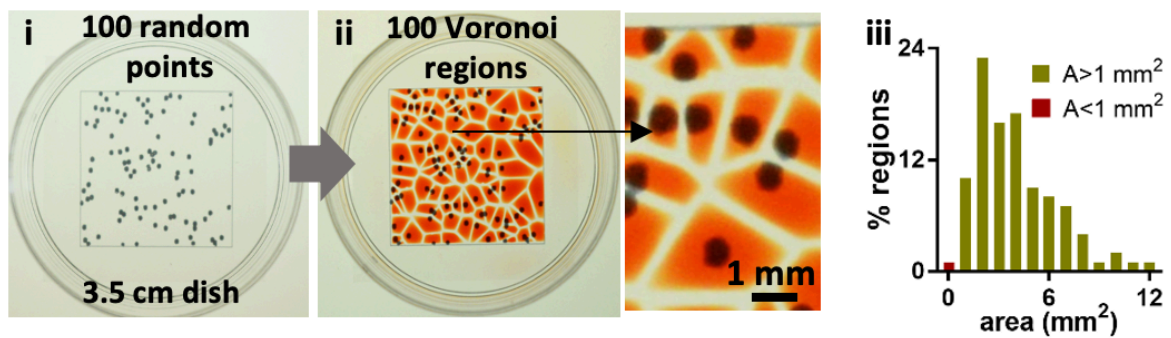
Mammalian cells are often cloned by splitting a dilute cell suspension amongst wells in a microplate so most wells get no cells, a few get one, and fewer still get two or more – before singletons are selected, grown, and resulting colonies picked. This approach is wasteful, as Poisson statistics ensure that wells with singletons are in a minority. Although many approaches (including fluorescence activated cell sorting, and combinations of printing plus imaging (17)) can increase the number of wells with singletons and so ‘beat’ the Poisson limit, cost and complexity prevent widespread adoption (18-19). Moreover, solid plastic walls around each well in a microplate yield ‘edge effects’ that obscure cells close to walls (20). Such effects are so severe that many users never check to see whether a well contains just one cell immediately after plating, and go through a 2nd cloning round to increase the chances of achieving monoclonality. This prompted development of an approach that allies jetting with use of Voronoi diagrams.

The approach is illustrated using 100 randomly-distributed dots as cell surrogates printed on a transparency stuck on the underside of a dish (**Figure 3ai**). After imaging and mapping dot positions, we build an analogue of a ‘cloning ring’ around each dot by jetting

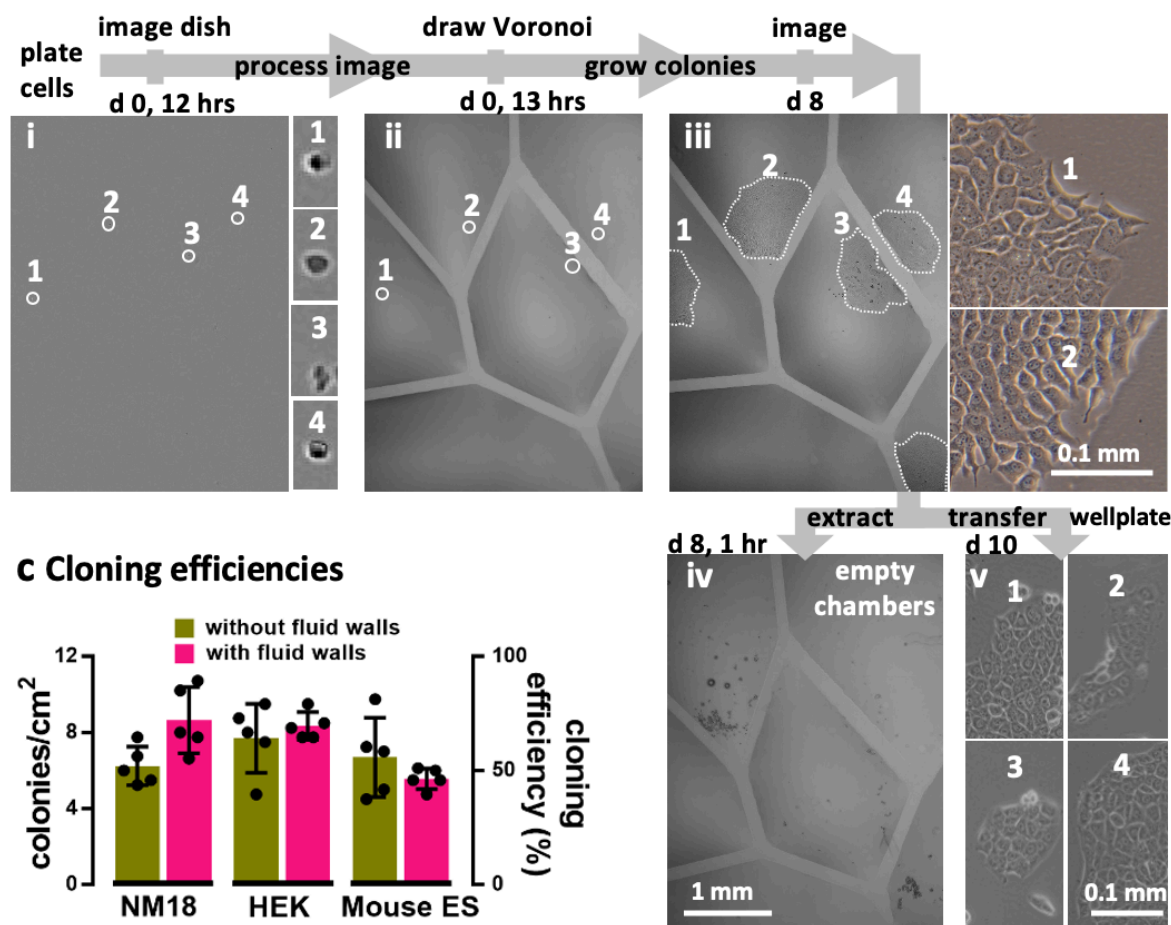
surrounding FC40 walls. Instead of jetting circles, we compute the relevant Voronoi diagram – a set of polygons where each contains one dot – and jet polygonal walls around dots (Figure 3a_{ii}; **Figure S5a**). More fluid can be added to/retrieved from such polygons, within the bounds of advancing and receding contact angles (Movie 5). Then, the Poisson limit is ‘beaten’ in the sense that every polygon contains one dot. In practice, some polygons prove unusable – FC40 walls have a thickness (W) and two dots may lie $<W$ apart (which is too small to accommodate a new wall), and nozzle width may be greater than polygon width (so polygons merge when media is added subsequently). The fraction of such lost polygons inevitably increases as dot density increases; fortunately, densities used for cloning ensure few are lost (e.g., only 2% with 100 dots/cells per 2 cm square; Figure 3a_{iii}). Importantly, the user has the freedom to set any lower and/or upper limits on the acceptable areas. An alternative approach also involves jetting a circular or square wall around each cell to give chambers with similar volumes, but this yields many fewer isolated clones per dish than the numbers achieved here.

Figure 3b illustrates an experiment. Mouse NM18 cells are plated, imaged (Figure 3b_i), positions of viable cells identified, the Voronoi diagram computed, and polygons jetted around each living cell (Figure 3b_{ii}). Once colonies grow (Figure 3b_{iii}), trypsin is added to polygons, cells removed (Figure 3b_{iv}) and transferred to standard 12-well plates, and clones expanded (Figure 3b_v). Cloning efficiencies with 3 mammalian cells (NM18, mouse ES, human embryonic kidney – HEK) are as high as those obtained conventionally (Figure 3c, Figure S5b,c); moreover, >90% cells identified as viable adherent ones in original images are isolated successfully in polygons, and >95% picked colonies are successfully transferred to (and expanded in) wells in conventional plates. These results show the Poisson limit can be ‘beaten’, with clones picked after 8 days in this case (instead of ≥ 2 weeks) due to the excellent optical clarity afforded by fluid walls. This approach also gives users confidence that a polygon contains only one cell/colony, so they can forego a 2nd cloning round. More generally, any cell of interest in a complex population can be isolated (e.g., one with a particular morphology or expressing a fluorescent reporter).

a Principle



b Workflow



c Cloning efficiencies

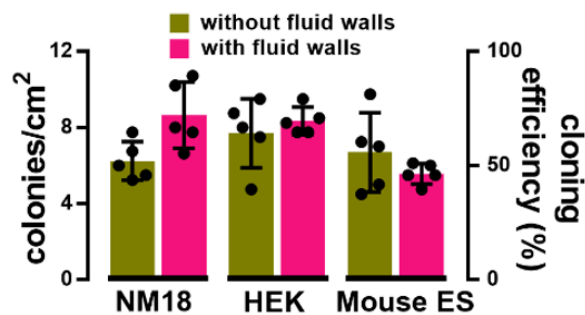


Figure 3. Beating the Poisson limit during cell cloning.

a. Principle illustrated using 100 randomly-distributed dots in a 2 cm square printed on clear film placed under a 35 mm dish. (i) Dish + dots. (ii) After recording dot positions, the Voronoi diagram is determined, polygonal walls built around each dot, and each polygonal chamber filled with dye (zoom shows some chambers). (iii) Distribution of areas in this Voronoi diagram. Two polygons had area (A) < 1 mm², a threshold determined by the minimum area (hence volume) the infusing needle can access.

b. Workflow illustrated using NM18 cells (12 cells/cm² or ~48 in 2 x 2 cm square) plated in a 35 mm dish. Phase-contrast images are of the same region, and numbered zooms show magnifications of different founder cells and their colonies. (i) Cells 1-4 12 h after plating. (ii) The same cells after jetting surrounding polygons. (iii) By day 8, cells develop into colonies (outlined by dotted lines). (iv) Emptied polygons (achieved by adding/removing PBS, adding trypsin, incubation, removing most cells). Over-emptying some chambers leaves FC40 (dark blobs) attached to the bottom. (v) After removal, each clone was re-seeded in a well in a 12-well plate, and imaged 2 days later.

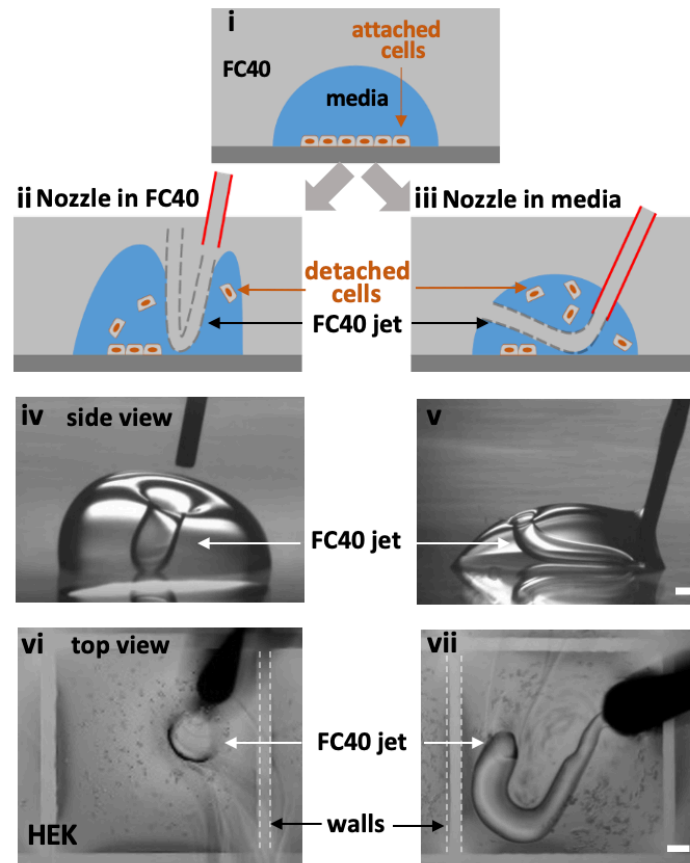
c. Cloning efficiencies. Each cell type was seeded (12 cells/cm²) in 10 x 35 mm dishes, colonies counted after 8 days, and cloning efficiencies calculated. 5 dishes were used to assess cloning efficiencies conventionally ('without fluid walls'), and 5 using polygons ('with fluid walls'). There was no or little significant difference between the two approaches (unpaired t test for HEK, ES, and NM18 gave p = 0.488, 0.3, and 0.033, respectively).

Sub-culturing cells using contactless jetting

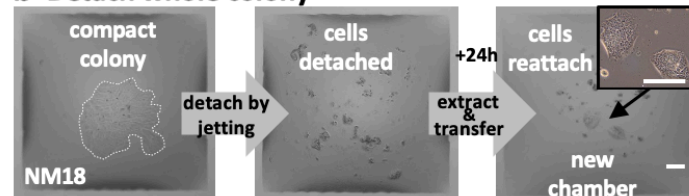
The momentum of an FC40 jet can be insufficient to form a wall but sufficient to dislodge adherent cells from a dish without using trypsin or EDTA (think of a jet-hose cleaning the bottom of a pool). There are two regimes. In one, the nozzle is placed in FC40 above media so jet momentum depresses part of the FC40–media interface to force it down so it plays on attached cells and detaches them (**Figure 4ai**). The jet minimizes its area of contact with the aqueous phase when rebounding back to rejoin the overlay (Movie 6, 7). Whole colonies can be dislodged, extracted, and replated (Figure 4b). Half a colony can be retrieved, as the other half is kept as back-up (Figure 4c, Movie 8). Importantly, this allows a colony to be sampled several times as it grows to allow developmental and differentiation markers to be checked, without introducing the growth setbacks of completely removing and then replating the cells. Naturally, different cell types require different amounts of shear for detachment, with consequential effects on viability. For example, loosely-attached HEKs and NM18 are easily detached as >95% remain viable, whereas tightly-attached ES cells are more difficult to dislodge and ~5% reattach and regrow (**Figure S6a**). The jet also produces vortices in chambers that can be used to mix small volumes (e.g., 200 nl human blood; Movie 9).

The second regime applies if the hydrophilic nozzle is lowered into media so it is wetted around its circumference. Now, jetted FC40 forms a squirming ‘worm’ surrounded on all sides by media as it makes its way back to the overlay, usually through a ‘hole’ in the chamber ceiling (Figure 4a, right; Movie 10). Both regimes allow adherent cells to be sub-cultured without adding enzymes or chemicals, and mix micro-volumes efficiently.

a Jetting regimes



b Detach whole colony



c Detach half a colony

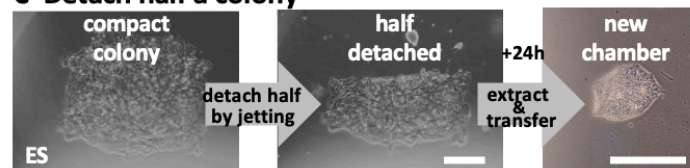


Figure 4. Dislodging attached living cells by jetting. Scale bars: 200 μm .

a. Two jetting regimes (illustrated using a drop of media and a hand-held jet viewed through a microscope; white dashes outline wall edges). (i-iii) With the nozzle in FC40 or in media, a jet can depress the FC40:media interface so it plays on, and detaches, cells. (iv-v). Side views of the two regimes operating on drops (volumes 3 and 1 μl) with circular footprints (without cells). FC40 jets are invisible. (vi,vii) HEKs are plated in a 16 x 16 grid (as Figure 1b), grown

for 24 h, and detached using the two regimes and a hand-held nozzle. Remarkably, fluid walls survive.

b,c. Detaching a whole or half a colony. A colony of NM18 (outlined by dotted white line) or ES cells growing in a chamber (as Figure 1b) are detached using a nozzle in FC40, detached cells removed and replated in a new chamber, where they grow.

A complex perfusion circuit with constant flow

We now illustrate the design, fabrication, and operation of a complex circuit that provides steady flows of fresh media for 7 d as cell grow in an array of chambers. Many conventional circuits doing this have been made (21-23), but they are custom-built and difficult to integrate into workflows in bio-labs. We designed one where every chamber receives fresh media. An external pump drives media through an input conduit (IC), on to one cell chamber (in a 4 x 12 array), and thence to an output conduit (OC), a choke (C), and finally into the sink (the rest of the dish). The choke acts to minimize pressure differences between input and sink, so equalizing flows through each cell chamber; **Figure 5ai**). Heights in chambers were quantified by measuring the intensities given by a red dye or fluorescein; all cell chambers yielded similar intensities (**Figure 5aii**; **Figure S7**). As fluid walls/ceilings morph during flow to reflect internal pressures, the demonstration that all chambers (which have identical footprints) have similar heights (and so similar volumes), confirms that all experience similar pressures, shear stresses, and flows (**Figure 5aiii**; **Figure S7**). This illustrates another advantage of fluid walls: chamber/conduit height serves as an inbuilt pressure and flow indicator.

Circuit operation is demonstrated using mouse C2C12 myoblasts that can differentiate over 7 d into mature myotubes; they fuse to form syncytia and express components of the neuromuscular junction at the expected rate – including the adaptor protein, DOK7 – a differentiation marker which in this case is tagged with EGFP (24). The undifferentiated (non-fluorescent) myoblasts can be added to each chamber immediately after fabrication; then, as chambers have Laplace pressures lower than flanking conduits, deposited cells remain in chambers. While this method is quick, it has the drawback that cells are exposed to equilibrating flows (and shear stresses) as they settle, and this can adversely affect some cell types (not shown). Therefore, cells are deposited as drops (in air) on a virgin dish at positions corresponding to those where the 48 chambers will eventually be built; on incubation, cells attach under shear-free conditions (**Figure 5bi**). Next, media is added to the whole dish to merge drops, FC40 overlaid, and the circuit built around the 48 groups of living cells (**Figure**

5bii, iii). The dish is now placed in a CO₂ incubator, and fresh media slowly pumped at 0.55 nl/s into the circuit over 7 d; then, media in each chamber is exchanged at a rate of 1 µl/day. Subsequently, cells grow and differentiate into myotubes, form syncytia, and become fluorescent (Figure 5c; Figure S7d). This shows that flows through a complex circuit can be equalized by judicious design, and that cells flourish and differentiate at the same rate and to the same extent as those grown conventionally. Although growth increases numbers and some cells migrate towards inlet and outlet channels to eventually meet those from neighboring chambers, microscopic analysis is easily restricted to those cells that remain in any particular chamber. If required, any chamber can be disconnected from the rest of the circuit by building new FC40 walls across input and output conduits, and its contents extracted for downstream analysis.

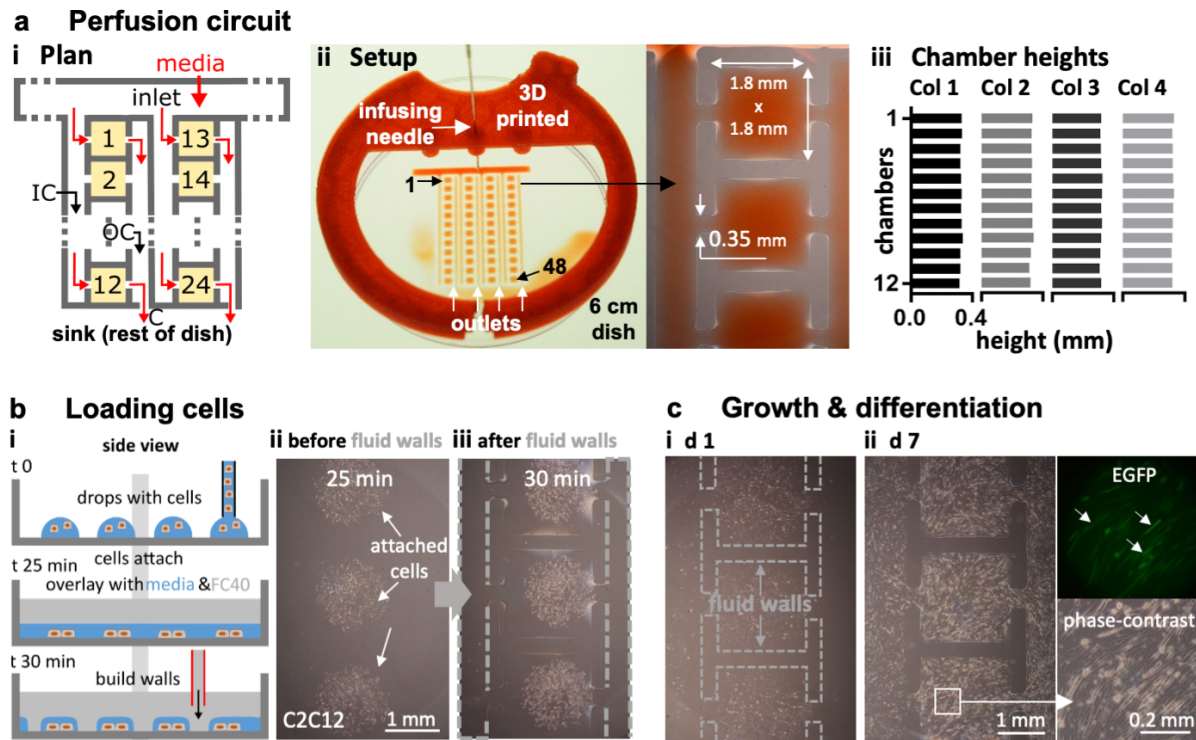


Figure 5. Perfusion circuit for continuous feeding 48 sets of differentiating myoblasts.

a. (i). Plan of the first two columns in the 4-column circuit. Media ($\pm$ dye) driven by an external pump into the inlet flows (red arrows) through an input conduit (IC), one cell chamber (chambers 1,2,...24 are orange), an output conduit (OC), a choke (C), and out into the dish (the sink). Grey lines indicate FC40 walls. (ii) Setup. Red dye is pumped into the inlet through a 3D-printed adapter (red) that fits on a 6 cm dish, through 48 chambers, and out into the dish (zoom shows some chambers). (iii) All chambers have similar maximum heights (average $\pm$ SD = $325 \pm 6.3 \mu\text{m}$; assessed using fluorescent dyes as in Figure S7) showing that all experience similar pressures, shear stresses, and flows.

b. Growth and differentiation of mouse C2C12 myoblasts. (i) Workflow. Myoblasts are deposited in drops, the dish filled with media and FC40 added, and finally the circuit is jetted around them. (ii,iii) Cells before/after building fluid walls. Dashed lines: edges of some walls.

c. Images of chambers as media replenished ($1 \mu\text{l/day/chamber}$); myoblasts grow to fill chambers, form syncytia, and express EGFP-DOK7 (day 7). Fluorescence and phase-contrast zooms: arrows mark fluorescing syncytia each containing >20 nuclei with length $>200 \mu\text{m}$ –

indicative of differentiation into myotubes. The speed and extent of growth and differentiation is like that of cells grown conventionally.

Conclusion

We describe a general and rapid method to fabricate microfluidic circuits on a standard polystyrene Petri dish. A micro-jet of FC40 is projected through FC40 and media on to the bottom of a dish; as FC40 hits the dish, it washes away media to leave a liquid wall of FC40 tightly 'pinned' to the substrate by interfacial forces (Figure 1a). Circuits with almost any imaginable 2D shape can be built in seconds to minutes (Figure 1b-h). We establish conditions required to build stable fluid walls (Figure 2), drive flow through conduits without using external pumps (Figure S3), and create 'valves' that can be opened and closed at will (Figure S4). We develop an approach for cloning dilute suspensions of mammalian cells that can beat the Poisson limit in the sense that almost every cell in the initial population is confined within its own fluid wall (Figure 3). We extend the approach to sub-culture attached cells without adding trypsin or EDTA (Figure 4), and to feed arrays of mammalian cells over long periods (Figure 5). Unfortunately, evaporation from static volumes of less than ~1 nl inevitably limits construction of ever-smaller devices (even though the solubility of water in FC40 is <7 ppm by weight).

Materials and methods

General reagents

All reagents and materials were purchased from Sigma Aldrich (St. Louis, Missouri), unless otherwise stated. FC40^{STAR} (iotaSciences Ltd, Oxfordshire, UK) is FC40 treated using a proprietary method that improves wall formation by jetting, and throughout we use the term 'FC40' to refer to it. Water-soluble dyes (e.g., Allura Red, toluidine blue, resazurin) are used where indicated.

Cells, cell culture

Whole human blood (anti-coagulated with EDTA) was obtained from anonymous blood donors through the National Health Service (NHS) Blood and Transplant Service (Oxford, UK); ethical approval was provided by The Interdivisional Research Ethics Committee of Oxford University (R63966/RE001).

Adherent human embryonic kidney cells (HEKs) were grown in DMEM (Gibco, Gaithersburg, MD) + 10% FBS; these HEKs were genetically-modified reporter cells (NF- κ B/293/GFP-LucTM Transcriptional Reporter Cell Line; System Biosciences, catalogue number TR860A-I), but reporter activity is not relevant here.

Mouse embryonic stem (ES) cells (EK.CCE line, derived from a single XY blastocyst-stage embryo of strain 129/Sv/Ec (25)) were routinely cultured in DMEM supplemented with 15% FBS, 1% penicillin/streptomycin (Gibco, #15140122), 0.1 mM 2-mercaptoethanol, 1% glutamine (Invitrogen, Loughborough, UK), 1% minimum essential media nonessential amino acids (Gibco), 1 mM sodium pyruvate, and 1000 U/mL leukemia inhibitory factor (LIF, ESGRO; Merck, Darmstadt, Germany; #L5158) on gelatin-coated plates (Merck, #ES-006-B). Such plates were used for Figures 3c and S5b.

Mouse mammary tumor cells (NM18, a derivative of the NMuMG line (26)) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% insulin (Sigma Aldrich, #10516).

C2C12, an immortalized mouse myoblast (24), were cultured in DMEM + 15% FBS. For the perfusion circuit in Figure 5, cells were seeded in DMEM + 15% FBS, and the FBS was reduced to 6% after 4 d, and to 2% after 5 d.

Printing and operating circuits

All circuits were made on tissue-culture-treated surfaces – 60 or 35 mm circular dishes (Corning, Merck product 430166 or #430165) or rectangular uniwell plates with overall dimensions of 96-well plates (Thermo Fisher Scientific, Waltham, MA) using custom-written software (see also below) and modified ‘Freestyle’ or ‘Pro’ printers (iotaSciences Ltd,

Oxfordshire, UK). Each printer consists of a 3-axis traverse, which controls movement of stainless steel needles (Adhesive Dispensing Ltd, Milton Keynes, UK) – usually a 60 μm inner-diameter nozzle jetting FC40 (outer diameter 0.5 mm), and a 0.5 mm outer-diameter needle (inner diameter 0.25 mm) used to add/remove media to/from chambers. The two needles are each connected via Teflon tubing to a programmable syringe pump. The Freestyle accepts 60 and 35 mm dishes, while the Pro has a larger working area and less mechanical backlash, so it was used to print the circuits in Figures 1d, 1e, 1g, S1 and 5a. Flows through circuits were driven by programmable syringe pumps (Harvard PhD Ultra I/W) connected via Teflon tubing to blunt stainless-steel dispensing needles (outer diameter 0.5 mm); needles were lowered through FC40 into the circuit at the appropriate position, when fluid walls spontaneously self-seal around inserted needles. We anticipate that fluidic arrangements with even finer features can be jetted using 3-way traverses with greater accuracy and precision.

DMEM plus 10% FBS was used to make all grids and circuits; when the term ‘medium’ is used, it should be assumed that 10% FBS is also present. Routinely, to prepare a grid or circuit, 1 ml medium is pipetted into a 60 mm dish to wet the entire bottom surface and ~0.9 ml retrieved to leave a thin film. This is then overlaid with 2-3 ml FC40 and the dish placed on the printer. Standard conditions used for jetting FC40 when making most circuits were: $D_{\text{nozzle}} = 60 \mu\text{m}$, $H = 0.4 \text{ mm}$, $\dot{Q} = 8 \mu\text{l/s}$, $V_{\text{traverse}} = 10 \text{ mm/s}$. A tightly-fitting 3D-printed circular sleeve around each dish acts as a positioning ring to ensure the dish can be removed and added back on to the printer in the original orientation. The adapter in Figure 5a_{ii} used to hold the needle feeding the perfusion circuit in a 60 mm dish was 3-D printed with polylactic acid (rigid.ink, Wetherby, UK).

Patterns in Figures 1c, 1d, 1e, 1g, 1h, S1, and 5a were constructed after reproducing the desired circuit in Inkscape (inkscape.org) and conversion to G-code, the programming language used by the printers. Patterns for Figures 1d, e, g and 1h were obtained from Wikipedia, CanStockPhoto, <http://www.udel.edu> and WikiArt, respectively.

For Figures 3 and S5, ImageJ (27) was used to detect and highlight attached single cells. Images were then processed in MATLAB (MathWorks, Cambridge, UK), where coordinates of single cells were recorded and Voronoi diagrams subsequently generated. The code used is available in Supplementary Materials. Finally, Inkscape was used to convert the diagrams into the G-code used to print polygons. Areas and centroids of each polygon were also computed, and the volumes to be added to each polygon calculated using the formula $V_{\text{cap}} = (A_{\text{wetted}} / 3.7)^{3/2}$ – where V_{cap} is the maximum volume that can be added to a polygon assuming an advancing contact angle of 50° (6), and A_{wetted} is the area of the polygon. The same volumes were removed from, and added to, each polygon when refreshing media, and during colony retrieval (which involved emptying chambers, PBS addition and removal, trypsin addition, and removal of the resulting cell suspension). Retrieved cells were plated in 12-well plates (Greiner Bio-One, Kremsmünster, Austria; #665180) and allowed to reattach and grow.

Imaging

Images of dishes in Figures 1b, 1d, 1e, 1g, 1h, S1, S2c, 3a, S3c, 4a, S4c, 5a, and S7a were taken using a digital SLR camera (Nikon D610). All insets in these figures, and fluorescence images in Figures 5c and S7d were collected using a zoom lens and digital SLR camera (Nikon D7100 DSLR) connected to an epi-fluorescent microscope (Olympus IX53; 1.25X, 4X, 10X, 25X objectives) with translation stage and overhead illuminator (Olympus IX3 with filters).

For Figure 3, 35 mm dishes were imaged using a phase-contrast live-cell imaging system and a 4x objective (IncuCyte Zoom, Sartorius, Gottingen, Germany).

For fluorescent images of cells in Figure 5c and Fig S7d an exposure time of 1 s was used. Quantification of EGFP intensity was done in ImageJ by subtracting the background and averaging the intensity across the entire image. Brightness was then increased by 40% for these images for a better visualisation of cells expressing EGFP.

Movies 3, 6, 7, and 10 were taken using a pendant-drop instrument (First Ten Angstroms 1000, Cambridge, UK).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (San Diego, CA). For Figure 2b each data point represents an average of $n = 9$ tests performed across 3 different dishes. For Figure 3c, 5 dishes were used for each cell line and each of the two conditions (with and without fluid walls). To assess whether there is a significant difference between cloning efficiencies for the two conditions, unpaired t tests were performed. For Figure S7bii, a second order polynomial was fitted to the experimental data. This polynomial was then used to infer heights of chambers from fluorescence intensity.

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Competing financial interests. Oxford University Innovation – the technology transfer company of The University of Oxford – has filed provisional patent applications on behalf of C.S., P.R.C., and E.J.W. partly based on this study. P.R.C. and E.J.W. each hold equity in, and receive fees from, iotaSciences Ltd, a company exploiting this technology; iotaSciences Ltd also provided printers, FC40^{STAR}, and scholarships for C.S. and C.D.

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Supplementary materials

Supplementary figures

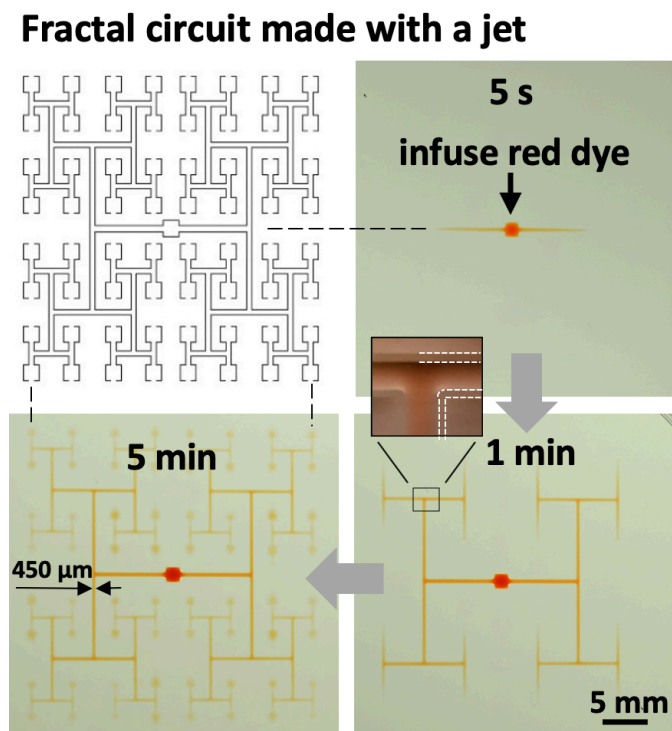
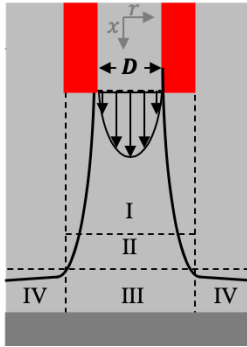
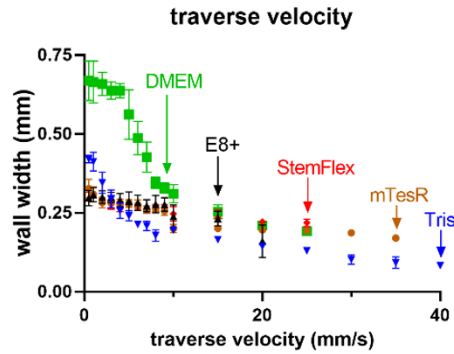
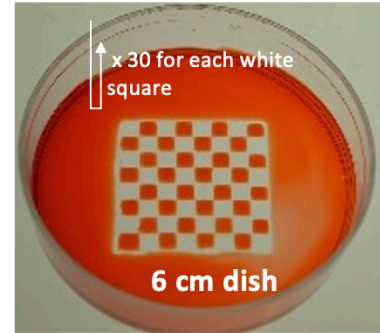


Figure S1. Making a fractal circuit using a jet. The jet provides great accuracy and precision, as the jetting needle is held by the traverse at a defined x,y,z position (so there is no play in the system), and it contacts neither media nor dish. This circuit is constructed with high accuracy in ~6 min from T-shaped segments (plan shown at top left; conduits have footprint widths of 450 μm) connecting a central input to 64 outlets and the rest of the dish (the sink). Three images show the circuit in operation; red dye was infused into the input and it reaches each outlet simultaneously, showing that all branches have similar dimensions. Inset: one junction with some wall edges highlighted white.

a Submerged jet**b Vary traverse velocity, media****c Overlapping walls****Figure S2.**

- Flow profiles in/around a submerged jet impinging on a flat substrate (Deshpande and Vaishnav, 1982). Five arrows illustrate the flow profile on emergence from the nozzle, and then there are 4 regions of jet flow assuming a fully-developed exit-flow velocity-profile. I: free jet region where jet diameter progressively increases. II: transition-to-wall zone. III: stagnation zone. IV: wall-jet region. In our case, the substrate is initially covered with media and removed by jet momentum and the shearing force in the wall-jet zone as the jet traverses.
- Effect of traverse velocity on wall width using different media ($H = 0.5$ mm, $\dot{Q} = 8 \mu\text{l/s}$; 4 mg/ml resazurin dye added to 20 mM TrisHCl). No media tested gave stable walls with $V_{traverse} > 35$ mm/s (as dwell time over any one area in the dish is too short for the jet to remove media from the surface).
- Building wide overlapping walls (3 x 3 mm) by jetting through media + dye.

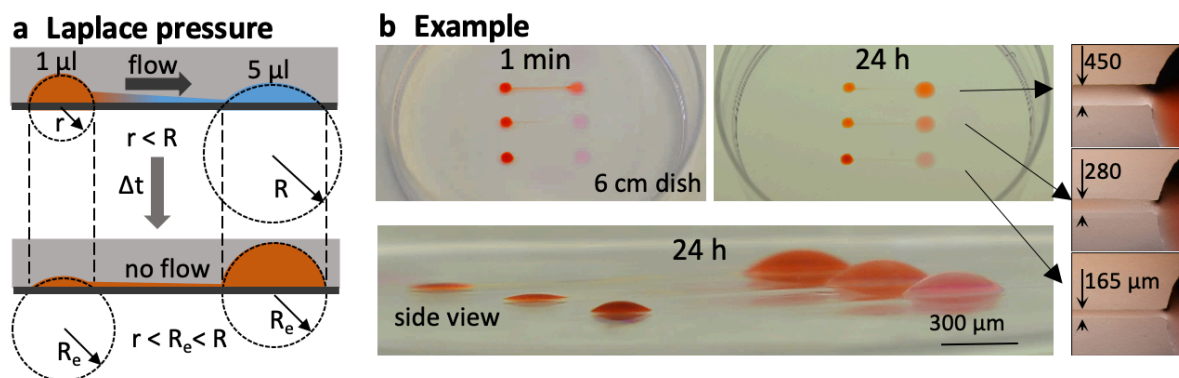


Figure S3. Controlling flow through conduits with different widths using differences in Laplace pressure.

a. Laplace pressure – principle. Top: a difference in Laplace pressure spontaneously drives flow as drops have different radii of curvature ($r < R$). Bottom; flow ceases when both drops have the same curvature and radius at equilibrium, R_e .

b. Example. Each of the 3 circuits was made by jetting one continuous wall; all 3 are initially identical (left- and right-hand chambers have diameters of 2.5 and 5 mm respectively) except for conduit width which varies from 450-165 μm . Red dye (5 μl) is added to left-hand drops to increase Laplace pressure to drive flow to the right. After 1 min, some dye in the upper circuit (but not others) reaches the right-hand drop. After 24 h, the left-hand upper drop is nearly flat (and so empty) as essentially all dye has been discharged through the widest conduit to the right-hand one. In contrast, the left-hand lower drop remains rounded and continues to drive flow through the narrowest conduit. Insets: zooms of conduits with widths indicated.

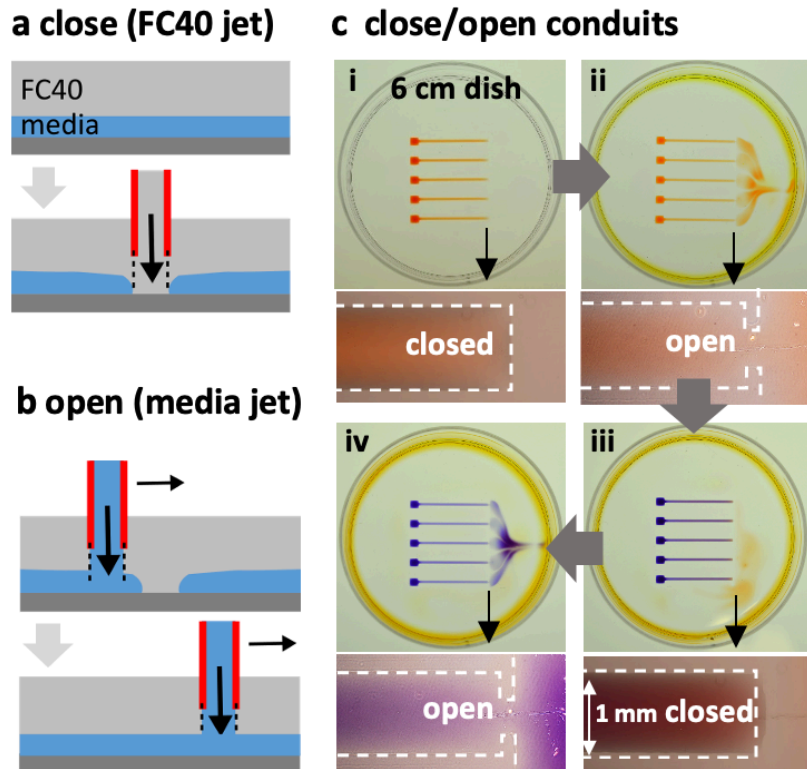


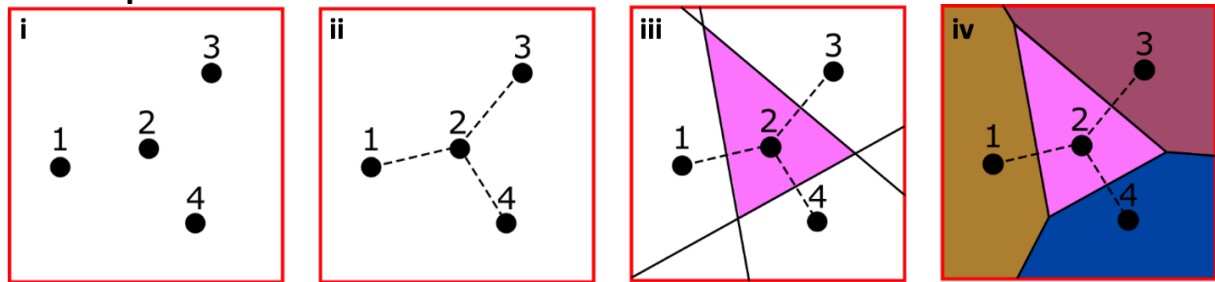
Figure S4. A microfluidic valve.

a. Closing an open conduit. A new wall across a conduit is built by jetting FC40 (nozzle moves into page).

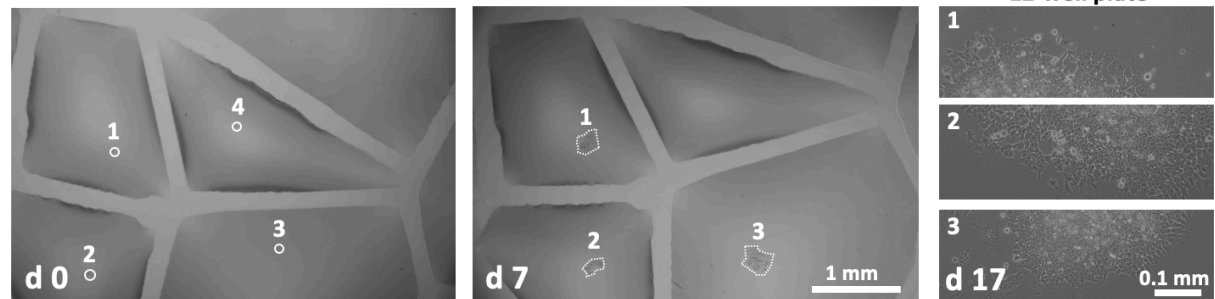
b. Opening a closed conduit. A wall across a conduit is removed by jetting media through the wall (nozzle moves to right).

c. Example. Insets show conduit ends, with dashed lines marking FC40 walls. (i). Five continuous FC40 walls were printed to create 5 identical circuits (each a reservoir with a 2 x 2 mm footprint connected to a conduit 1 mm wide), and 2 μ l red dye added to each reservoir. Red dye is confined to each circuit as the surrounding FC40 wall is continuous. (ii) Media was jetted ($H = 0.4$ mm, $\dot{Q} = 8$ μ l/s, $V_{traverse} = 15$ mm/s) to open end walls. Red dye spills out into the dish (which acts as a sink, as it has a low Laplace pressure). (iii) Ends of conduits were closed by jetting FC40 (conditions as for media) to rebuild walls, and 2 μ l blue dye added to reservoirs to test for leaks. No blue dye escapes, showing new walls provide perfect seals. (iv) Reopening end walls allows escape of blue dye. The pattern indicates flow through each end is similar, so openings have equal widths.

a Principle



b Mouse ES cells



c HEK cells

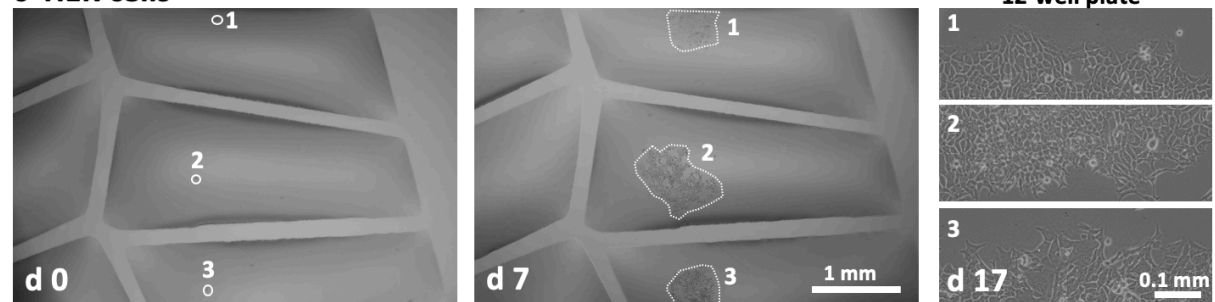


Figure S5. Drawing Voronoi diagrams, and examples of their use.

a. Principle. (i) In this example (4 points randomly-distributed in a square), (ii) we draw straight lines between each point (here shown for point 2) and its nearest neighbors. (iii) The corresponding Voronoi polygon is formed using half-plane intersections for all lines. (iv) The same procedure is applied for other points to produce the Voronoi diagram.

b, c. Example Voronoi diagrams used during cloning of ES and HEK cells. Details are as Figure 3, and each pair of images on the left show the same region of the dish immediately after plating cells (d 0; circles surround single cells), and prior to picking colonies (d 7; dotted lines outline colonies). Right-hand panels show views of picked colonies growing in conventional 12-well plates at d 17. Note that, in principle, the local concentration of autocrine factors secreted by cells might be different in polygons with different volumes, and as such a

concentration might be many-fold higher than that in a well in a 96-well plate (as volumes are about 1,000 times smaller), and as a higher concentration of autocrine factors might increase cloning efficiency, one might expect improved efficiencies in Voronoi polygons compared to well plates. However, in practice, cloning efficiencies of 3 cell types tested in polygons and conventional plates proved to be similar (Fig. 3c).

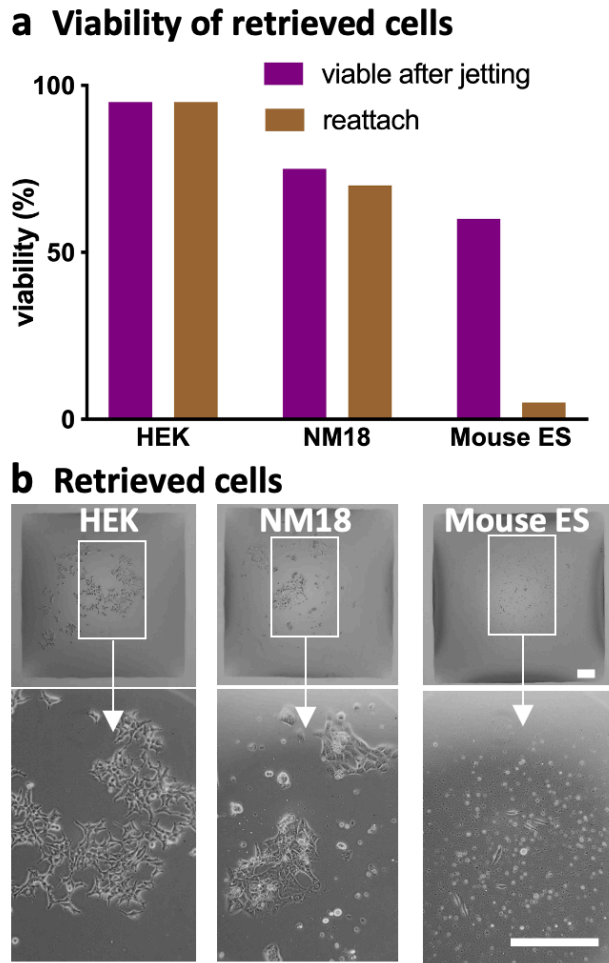
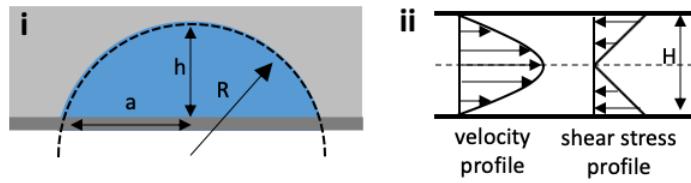


Figure S6. Cell viability after detachment by FC40 jetting. Cells (HEK, NM18, ES) in single colonies in a chamber (as in Figure 1b) were dislodged by FC40 jetting with the nozzle in FC40 and detached cells retrieved from the chamber; after assessing cell viability by trypan-blue staining, cells were transferred to new chambers and the percentage reattaching assessed.

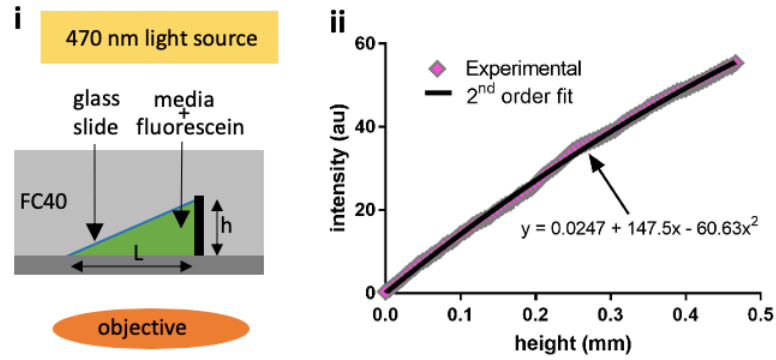
a. Viability of retrieved cells. As might be expected, viability and reattachment rates depend on jet momentum required for detachment. Thus, loosely-attached HEKs are easily detached by jetting to remain viable and reattach, whereas tightly-attached ES are detached with difficulty and have poorer viabilities and reattachment rates.

b. Images of retrieved cells 24 h after replating in new chambers. Bars: 200 μ m. Zooms show magnifications.

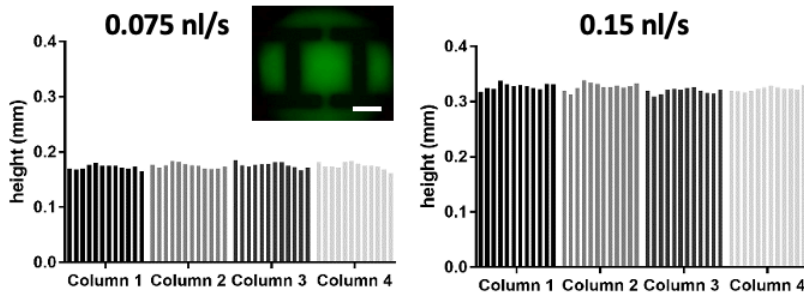
a Assumptions



b Measuring height using fluorescence microscopy



c Maximum chamber heights



d Differentiation of myoblasts into myotubes

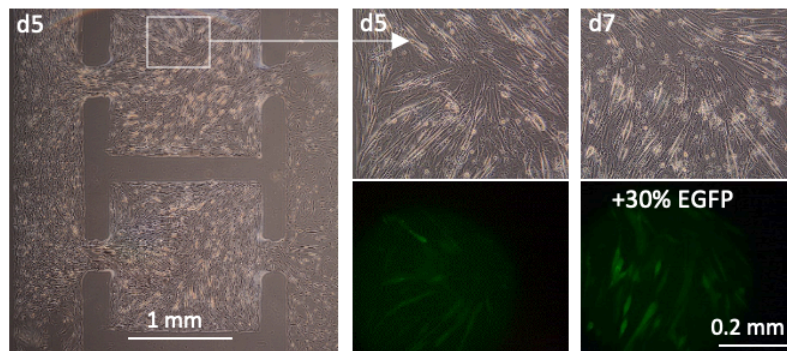


Figure S7. Confirming that cells in different chambers in the perfusion circuit in Figure 5 experience the same pressures and velocity profiles (and so rates of media replenishment and shear stresses).

a. Assumptions. (i) The 3D geometry of a cell chamber is complex, resembling that of a spherical cap sitting on a rectangular base,^[7] for simplicity, we assume it is a perfect cap of a

sphere ($2a$ = chamber width, R = sphere radius). (ii) Then, the velocity and shear-stress profiles between bottom and top of any section through a chamber are functions of height, H , at that point in the section. Identical heights in all chambers indicate that cells in each chamber experience similar pressures, shear stresses, and flows.

b. Measuring maximum chamber height using fluorescence microscopy. (i) Construction of a calibration curve relating fluorescence intensity to height of fluorescein. A mixture of cell media and fluorescein is added between a glass slide propped against a pillar (height, h) and the bottom of a Petri dish, FC40 overlaid, the construct imaged, and the intensity at each point along the slide recorded. (ii). Relation between fluorescence intensity (arbitrary units, au) and height (calculated knowing dimensions of the triangle in (i)). Black line: best fit of a second-order polynomial.

c. Maximum chamber heights in circuits perfused at different rates. Media plus fluorescein was perfused through chambers in circuits ($\dot{Q}$ = 0.075 and 0.15 nl/s; data for 0.15 nl/s is reproduced from Figure 5a_{iii}), fluorescence images collected (inset: fluorescence image of part of a circuit, bar = 1 mm), and chamber heights calculated using the calibration curve. Average heights were $175 \mu\text{m} \pm 5 \mu\text{m}$ and $325 \mu\text{m} \pm 6.3 \mu\text{m}$ SD at 0.075 and 0.15 nl/s, respectively. This indicates that for a given circuit and flow rate, heights of different chambers are similar, and that chamber height changes with flow rate (as fluid walls morph above unchanging footprints to accommodate the pressure difference). As chamber heights reflect both $\dot{Q}$ and velocity profiles, each chamber in a circuit experiences the same rate of media replenishment and shear stress.

d. Control test to show the increase of EGFP signal with differentiation. C2C12 cells were cultured for 7 d in the perfusion circuit. Insets show phase-contrast and fluorescence images of the same location inside a chamber at d 5 and d 7. For an exposure time of 1 s, the average intensity of signal per pixel increases by 30% between the two time points, indicating increased expression of the tagged DOK7 (and so differentiation into a myotube).

Supplementary Movies

Movies have been attached.

Movie 1. Jetting a grid. The movie runs at 4x speed. A Petri dish (6 cm) – filled with a skim of media mixed with blue dye plus an FC40 overlay – sits on a printer, and the nozzle of a hollow needle ($D_{nozzle} = 60\ \mu\text{m}$) is held by a 3-way traverse in FC40 above media in the dish ($H = 0.4\ \text{mm}$). The movie begins as the pump infuses FC40 ($\dot{Q} = 8\ \mu\text{l/s}$) and the traverse traces the path of a grid ($V_{traverse} = 10\ \text{mm/s}$). The invisible jet of FC40 pierces the layer of media, pushing it aside to contact the substrate. As FC40 wets polystyrene preferentially, it remains firmly pinned to it. A grid with 256 aqueous chambers, each surrounded by transparent FC40 walls, is created in $<2\ \text{min}$.

Movie 2. A 16×16 grid withstands violent agitation. The movie runs in real time. A grid like the one in Figure 1b was constructed, each chamber filled with 600 nl red dye, and the dish placed on a shaker.

Movie 3. Jetting Novec 649 from a nozzle submerged in FC40 confirms that jet width increases with distance from the nozzle. The movie runs in real time (10 frames/sec). This side view depicts a nozzle of a 34G needle ($D_{nozzle} = 80\ \mu\text{m}$, outer diameter $220\ \mu\text{m}$) submerged in FC40. Then, another fluorocarbon (i.e., Novec 649) – chosen because it is miscible with FC40 but has a different refractive index so interfaces between the two transparent liquids can be seen – is jetted from the nozzle ($D_{nozzle} = 60\ \mu\text{m}$) through FC40. Jet width increases with distance from the nozzle (see also Deshpande and Vaishnav, 1982). Turbulence at the end of the movie results from Novec 649 rebounding from the substrate.

Movie 4. A microfluidic valve. The movie plays at 1x or 4x real time as indicated. It illustrates the experiment shown in Figure S4. The 6 cm dish initially contains 5 identical circuits, each a reservoir connected to a conduit 1 mm wide with a dead-end, and each bounded by one continuous wall. Then, the printer adds $2\ \mu\text{l}$ red dye to each reservoir (walls are intact and dye stays within each circuit), jets media to open end walls (red dye spills out

into the dish), jets FC40 to rebuild end walls, adds 2 μl blue dye to reservoirs to test for leaks (no blue dye escapes), and jets media to reopen end walls (so now blue dye does escape).

Movie 5. Visualizing a Voronoi diagram. The movie is speeded up 4x, and illustrates the kind of experiment shown in Figure 3a where an area of 20 x 20 mm in a 35 mm dish was subdivided into 64 Voronoi polygons, each containing one dot. Polygons are initially invisible due to the low volume each contains. The movie shows addition of media + black dye by a printer through FC40 to each polygon (added volume is proportional to polygon area, and ranges from 0.25–2.8 μl).

Movie 6. FC40 from a nozzle held above drops of media deforms FC40–media interfaces so a depressed section plays on the bottom. The movie runs in real time (10 frames/sec). It depicts a side view of a nozzle (a 34G needle, $D_{\text{nozzle}} = 80 \mu\text{m}$, outer diameter 220 μm) held below the surface of FC40 but above drops of media (shaped like caps of spheres with volumes of 1–3 μl) sitting in a dish. The movie begins as the nozzle jets FC40, and traverses above drops. Although the jet is invisible, it deforms sections of the FC40–media interface in each drop as it passes. These sections are forced down to the substrate, and can be used to detach adherent cells.

Movie 7. Jetting Novec 649 from a nozzle held above a drop of media deforms the FC40–media interface so a depressed section plays on the bottom. The movie runs in real time (10 frames/sec). In Movie S3, FC40 is invisible, and there is no indication of any turbulent flow after the jet leaves the drop. Here, the jet contains another fluorocarbon (Novec 649) that is miscible with FC40 but has a different refractive index so interfaces between all 3 liquids can be seen. Initially the nozzle (34G needle, $D_{\text{nozzle}} = 80 \mu\text{m}$, outer diameter 220 μm) is held below the surface of FC40 but above a 1 μl drop of media. Novec 649 is then jetted on to the drop, the nozzle traverses back and forth above the drop, and a depressed section of the FC40–media interface plays on the bottom.

Movie 8. Detaching half a stem-cell colony growing in a chamber in a grid. The movie runs in real time, and images were collected on a microscope from above. The movie depicts removing part of a living colony of mouse ES cells from a chamber (as in Figure 1b) using a

hand-held FC40 jet ($D_{nozzle} = 60 \mu\text{m}$; $\dot{Q} = 9 \mu\text{l/s}$). The nozzle is positioned in FC40 and above the media–FC40 interface throughout, and the jet depresses a section of the interface so it plays on the bottom of the chamber to detach some cells in the colony. Figure 4c illustrates accurate removal of half a colony using the printer, but then imaging through a microscope from above (as in this movie) is impossible.

Movie 9. Mixing blood using the regime described in Figure 4a where the nozzle is held below an FC40:aqueous interface. The movie runs in real time. A grid like the one in Figure 1b was made, 0.2 μl whole human blood added to two chambers, and a manually-held jet used to create vortices in chambers, and so mix the blood.

Movie 10. Jetting FC40 from a hydrophilic nozzle submerged in a drop of media yields a ‘squirming worm’ (which can be used to detach cells). The movie runs at 5x speed (10 frames/s). This side view depicts a 1 μl drop of media (initially shaped like the cap of a sphere) under FC40, and the hydrophilic nozzle ($D_{nozzle} = 80 \mu\text{m}$) is below the surface of FC40 but above the drop. After 1s, the pump jets FC40 ($\dot{Q} = 10 \mu\text{l/s}$) on to the drop to deform the FC40–media interface. After 5 s, the hydrophilic nozzle is lowered towards the drop. By 12 s, the nozzle enters the drop and is wetted around its circumference; consequently, the FC40 jet is surrounded on all sides by media as it leaves the drop to rejoin the overlay (which it does at the back, and so out of sight). After ~24 s, flow rate is increased ($\dot{Q} = 15 \mu\text{l/s}$), and the jet rearranges and extends from the nozzle to the right to appear as a ‘squirming worm’. By 35 s, the ‘exit hole’ of the jet from the drop is clearly visible. At ~85 s, the flow rate is reduced ($\dot{Q} = 10 \mu\text{l/s}$), and at ~125 s increased again ($\dot{Q} = 15 \mu\text{l/s}$). The worm continues to squirm in the drop until the end of the movie.

Supplementary code

```
% used to create Voronoi diagrams

image = imread('single_cells.tif'); % read image with single cells to be analysed.

%get the size of the image
[rows, columns, numberOfColorChannels] = size(image);

%show the image
imshow(secondlevelTIFF);

%project a grid on top of the image to help with the identification of single cells
grid_lines_every = 1000;

for row = 1 : grid_lines_every : rows
    line([1, columns], [row, row], 'Color', 'r');
end

for col = 1 : grid_lines_every : columns
    line([col, col], [1, rows], 'Color', 'r');
end %record position of each cell

X = []; Y = []; points = []; i = 0; datacursormode on;

while (i < 1000)
    dcm_obj = datacursormode(figure(1));
    pause
    cursor_info = getCursorInfo(dcm_obj); position = cursor_info.Position;
    X = [X, position(1)]; Y = [Y, rows - position(2)]; i = i + 1;
end

datacursormode off %store positions in txt files
dlmwrite('Xpos.txt', X); dlmwrite('Ypos.txt', Y);

%apply Voronoi diagram to the set of points
voronoi(X,Y);
```

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

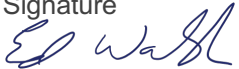
Title of Paper	Jet-printing microfluidic devices on demand
Publication Status	Accepted for Publication
Publication Details	C. Soitu, N. Stovall-Kurtz, C. Deroy, A. A. Castrejón-Pita, P. R. Cook, E. J. Walsh. Advanced Science, 2020

Student Confirmation

Student Name:	Cristian Soitu		
Contribution to the Paper	Lead author, led experimental work, led data collection and analysis, coordinated and contributed to manuscript writing and editing.		
Signature <i>Cristian Soitu</i>	Date	15.09.2020	

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Prof Edmond J. Walsh		
Supervisor comments		
Signature 	Date	9/10/2020

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 8: Creating wounds in vitro using micro-jets

Authors: Cristian Soitu, Mirela Panea, Alfonso A. Castrejón-Pita, David Beeson, Peter R. Cook, and Edmond J. Walsh

Submitted for publication

Abstract

Many wound-healing assays are used in cell biology and biomedicine; they are often labor intensive and/or require specialized and costly equipment. We describe a contactless method to create wounds in vitro using micro-jets of either media or an immiscible fluorocarbon (i.e., FC40). We combine this with another method that allows rapid fabrication on standard Petri dishes of arrays of micro-chambers; after filling a dish with a skim of media overlaid with FC40, the two liquids are reshaped into a grid. This combination allows automation and multiplexing of wound-healing assays.

Introduction

Wound-healing assays are widely used to investigate, for example, skin repair, angiogenesis, morphogenesis, and metastasis (1-5). Cell movements in and around wounds are complex, and governed by four major interlinked parameters: cell autonomous behaviours, cell-cell interactions, matrix compositions, and concentrations of critical solutes (6). These are often analysed *in vitro* by creating a wound—a cell-free area—in a confluent monolayer of cells (either by excluding cells from initially entering it, or removing cells from it), and then tracking cells using well-established pipelines (7).

For cell exclusion, a barrier (in the form of a solid, liquid, or gel) is used, but this can leave residues on the substrate which may alter cell behaviour (6). The ‘scratch assay’ is commonly applied to remove cells, and its versatility, simplicity, and low cost make it popular (8). However, it is performed by hand, and so often lacks reproducibility (e.g., because application of too much force damages the substrate or underlying matrix and – if automated – the associated machinery can be costly. Wounds can also be created, for example, by electrical pulses (9), laser ablation (10), or laminar flows (11), but these approaches have not been widely adopted, mainly due to the requirement for specialised equipment.

We previously developed methods to create microfluidic micro-chambers and more complex microfluidic circuits on standard Petri dishes in seconds (12-14). Liquids are usually confined within chambers and inter-connecting pipes by solid walls; in our devices, they are confined by fluid ones. This is possible because interfacial forces become powerful at the microscale, whilst gravitational ones become irrelevant. Consequently, interfaces between cell-culture media and an immiscible fluorocarbon like FC40 can act as robust walls separating the two liquids; interfacial forces tightly pin these walls to dishes. FC40 is biocompatible, chemically-inert, and freely permeable to O₂ and CO₂ (15-16). We also applied a variant of the traditional ‘scratch assay’ to create wounds in cell monolayers growing in chambers surrounded by fluid walls; a rod made of polytetrafluoroethylene (PTFE) was dragged through a monolayer under its own weight (13). As arrays of hundreds of chambers can be constructed

in minutes on dishes (12-13), this gives obvious potential to screen for drugs that might affect healing. However, our variant suffers the same general disadvantages of traditional scratch assays (e.g., if scratching tools are used once the assay becomes expensive, and if used repeatedly they may wear and so alter wound geometry).

Here, we introduce a contactless method to create wounds in 2D monolayers using micro-jets. Stationary jets of media have been used previously to detach cells, and so quantify the strength of cell adhesion (17-18). We attach a micro-jet to a 3-axis traverse, and so create wounds with almost any 2D geometry in seconds by jetting media or FC40 onto the monolayer. As arrays of chambers can be made using the media and Petri dishes familiar to biologists, and as dishes already fit so seamlessly into their incubators, microscopes, and other workflows, we anticipate this assay will find wide application in biomedicine.

Results

Principle of wounding

Figure 1 illustrates two ways of creating wounds with micro-jets in confluent monolayers of human C2C12 myoblasts. In the first (**Fig 1A**), the nozzle of a steel needle (held by a 3-way traverse, connected to a syringe pump, and filled with media) is lowered until 0.4 mm above cells. When it starts jetting media, streamlines resulting from impact with the substrate (18) produce a shear stress that dislodges attached cells (**Fig S1A**). Moving the nozzle along a linear path then creates a linear wound filled with media.

In the second (**Fig. 1B**), the monolayer and media are covered by a layer of FC40, the nozzle of a steel needle filled with FC40 is lowered until just above the surface of the media, and the nozzle jets FC40 instead of media (**Fig 1B**). Now, FC40 streamlines push media aside and detaches cells, and – once the nozzle has passed – media refills the wound (**Fig S1B**). The width of wounds created by both methods is easily varied by overlapping jetting lines (**Fig. 1**).

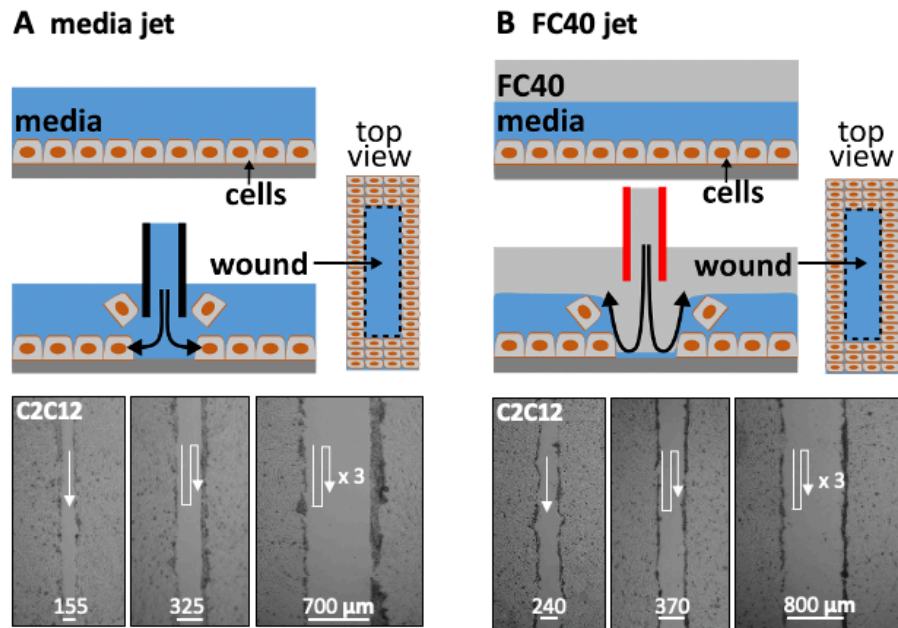


Figure 1. Creating wounds in a monolayer of mouse C2C12 myoblasts. White arrows: jetting paths.

A. With media jet. Top: the nozzle of a steel needle filled with media is lowered until just above the monolayer, and starts jetting media; jet momentum detaches some cells from the surface, and moving the nozzle along a predefined path (here into the page) creates a wound. Below: images of wounds of different widths produced by jetting overlapping lines.

B. With FC40 jet. Top: the nozzle of a steel needle filled with FC40 is lowered into an FC40 overlay until just above the underlying media, and starts jetting FC40; jet momentum forces the FC40:media interface down so it plays on the monolayer to dislodge cells, and moving the nozzle again creates a wound. Below: images of wounds with different widths created by jetting overlapping lines.

Parameters affecting wound dimensions

Experimentalists often study a number of linear wounds of the same length and width. As wound length is easily controlled either by starting or stopping the pump at the appropriate time or place, we analyzed various parameters affecting wound width in detail, including nozzle diameter (D_{nozzle}), height of the nozzle above the substrate (H), volumetric flowrate ($\dot{Q}$), and speed of the traverse ($V_{traverse}$) when making wounds (length 2.5 mm) in C2C12 myoblasts. At a low media flowrate of 7.5 $\mu\text{l/s}$, jet momentum is insufficient to clear all cells off the substrate in the jetting line; we call this ‘failure’ (**Fig. 2Ai**, left). Increasing flow then produces progressively wider wounds (**Fig. 2Ai**, right). With an FC40 jet, failure occurs at a lower flowrate of $\sim 5.4 \mu\text{l/s}$, and increasing flows again produce wider wounds (**Fig 2Aii**). Quantitative analysis confirms these general trends: failure occurs at low flows, increasing flows give wider wounds, and FC40 has a lower failure point and gives narrower wounds (**Fig. 2Aiii**).

Flow profiles resulting as a jet emerges from a submerged and stationary nozzle to impinge on a flat substrate are complex (19). In our case, the substrate is (initially) covered with cells that are removed by jet momentum and shearing forces in the last region by the passing jet. The trends described above (**Fig. 2**) are consistent with jet momentum playing a critical role. For example, the longer a jet spends above a specific point, the more persistent are the shearing forces so that more cells are dislodged (resulting in wider wounds), and when jet momentum falls below a critical value, wound production fails. Moreover, FC40 is ~ 1.8 times denser than media so it gives narrower wounds, with failure occurring at a lower flowrate and higher traverse rate.

Note that passage of an FC40 jet not only clears cells from the target area, but often leaves a column of FC40 droplets down the centerline of the wound (**Fig. 2Aii**). This phenomenon is studied in detail elsewhere (20), and results from the complete displacement of all media from the substrate coupled to preferential wetting of polystyrene by FC40 compared to media; then, increasing the flowrate yields FC40 drops spaced progressively

closer together, until they coalesce to give a line of FC40 pinned to the dish. These results show that linear wounds with chosen lengths and widths can easily be made by jetting.

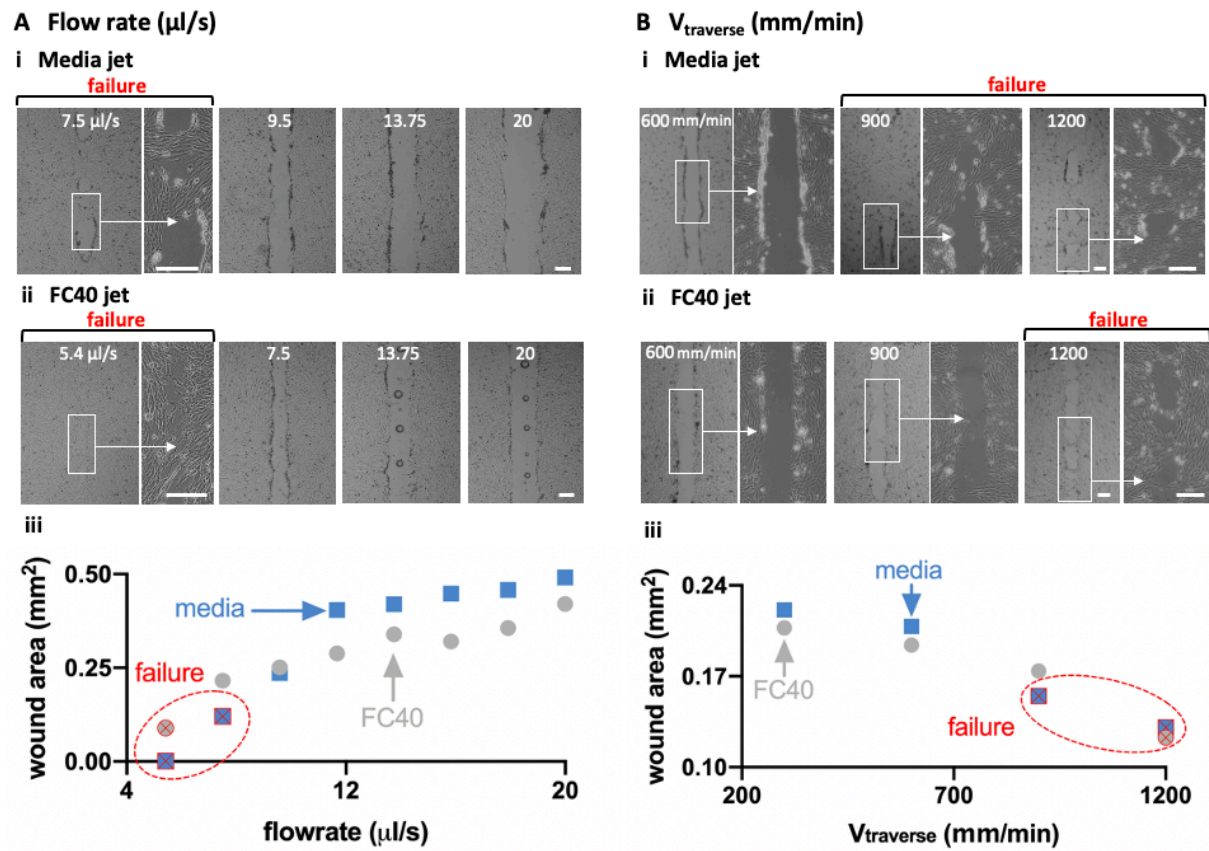


Figure 2. Effects of varying flowrate ($\dot{Q}$) and traverse speed (V_{traverse}) on wound width and area ($D_{\text{nozzle}} = 60 \mu\text{m}$, $H = 0.4 \mu\text{m}$, wound length = 2.5 mm). Scale bars are 200 μm wide.

A. Varying $\dot{Q}$ as V_{traverse} is held constant at 300 mm/min. (i) For media jets, wound failure occurs at 7.5 $\mu\text{l/s}$, and increasing flow produces wider wounds. (ii) For FC40 jets, wound failure occurs at 5.4 $\mu\text{l/s}$, and increasing flows again yield wider wounds (often with a central column of FC40 drops that adhere to the dish). (iii) Relationship between the cell-free wound area and flowrate.

B. Varying V_{traverse} as $\dot{Q}$ is held constant at 8 $\mu\text{l/s}$. (i) For a media jet, increasing the traverse rate leads to wound failure as the dwell time over a given area falls. (ii) For an FC40 jet, failure occurs at a higher traverse rate. (iii) Relationship between the cell-free wound area and traverse rate.

Complex wound patterns

Assay geometry is another important parameter affecting migration into a wound (21). Wounds with almost any imaginable 2D pattern are easily generated by altering the path followed by the micro-jet – for example, from one straight or circular line (**Fig. 3A,B**), through many straight lines in a grid (**Fig. 3C**), to two curved lines plus some straight ones to represent the two backbone strands and base-pairs in a double helix of DNA (**Fig. 3D**, upper panel).

Wound healing

We illustrate wound healing using a wound shaped like a double helix, in which one helical 'strand' is thicker than the other. On incubation at 37°C, cells repopulate cleared areas – a process termed 'wound healing'. In this case, the thin strand heals quicker than the thick one, to leave a 'single RNA strand' (**Fig. 3D**). The other wounds illustrated in **Figure 3A-C** also heal in <24 h (**Fig. S2**). These results show that wounds produced by jetting can heal as usual.

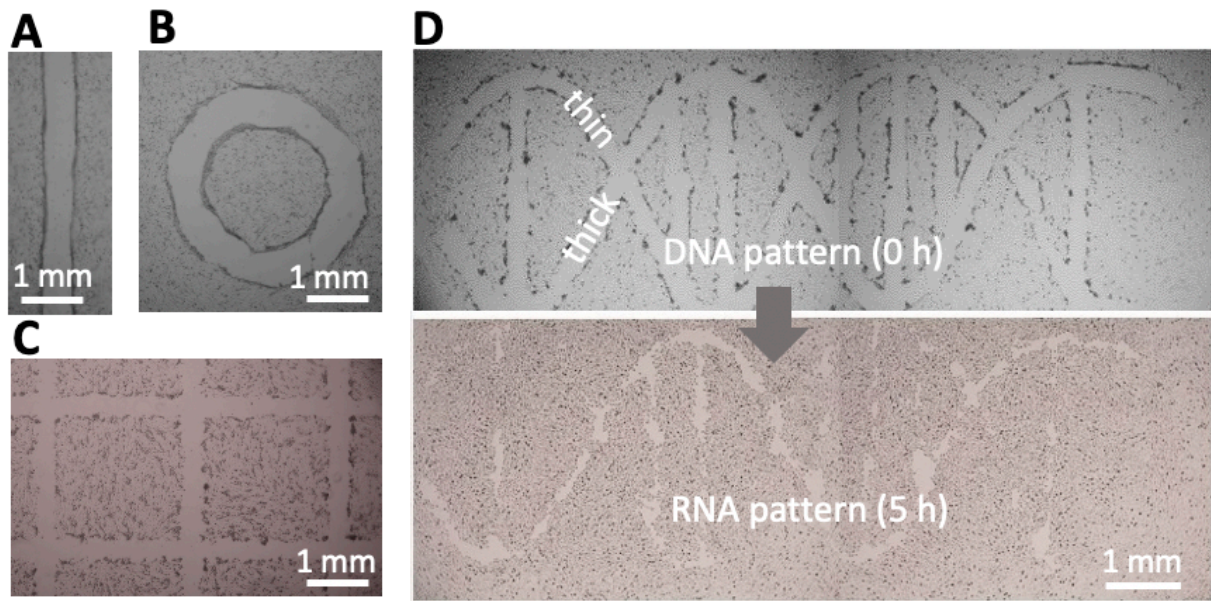


Figure 3. Wounding patterns. All shapes were created in 6 cm Petri dishes using media jets. **A.** Line. **B.** Circle. **C.** A complex wound (part of 16x16 grid). **D.** A wound shaped like a double helix of DNA (with one thick backbone strand + attached base, and one thin strand + base). After incubation (5 h), the thin strand has almost completely healed, so the structure looks like a single strand of RNA.

Multiplexed wound production

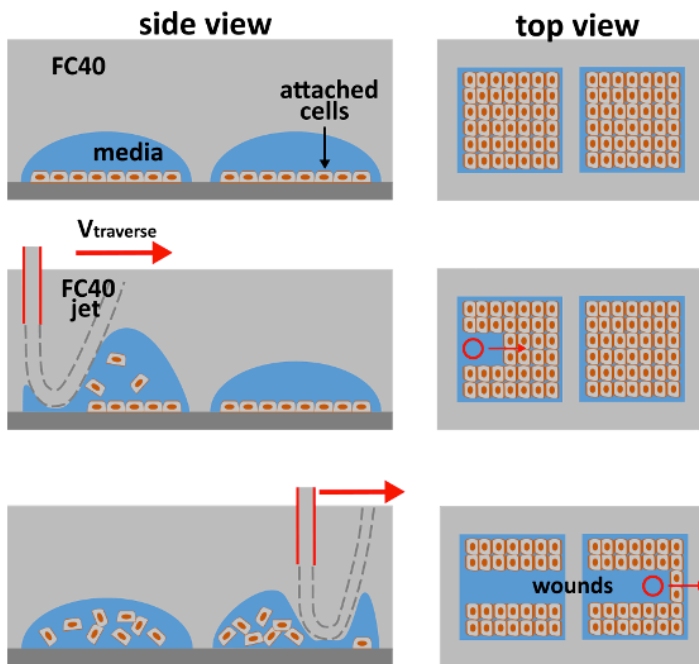
This wound-healing assay is readily multiplexed. We illustrate this first using 'GRIDs' – arrays of 256 microfluidic chambers in a 6 cm dish (**Fig. 4A**; (14)). These are designed to hold one-hundredth of the volume of a well in a 96-well plate. Thus, each chamber in such a GRID has a square footprint of 1.9 x 1.9 mm, each contains <1 μ l, and each is isolated from the others by fluid walls and ceilings of FC40. However, such chambers are used much like wells in microtiter plates; liquids are added or removed simply by pipetting through FC40 instead of air. Then, fluid walls morph above an unchanging footprint within limits imposed by the advancing and receding contact angles around the footprint. When too much volume is added, the advancing contact angle is exceeded, the footprint expands, and neighboring chambers merge. Unfortunately, the maximum volume of media each chamber can hold is less than the extra volume added when creating long linear wounds (2-6 μ l) by jetting media. Therefore, we use an FC40 jet here.

Figure 4Ai illustrates the principle. C2C12 myoblasts are seeded in each chamber, grown until confluent, and then the FC40 jet traverses across the middle of a chamber; the jet transiently forces the FC40:media interface down so it plays on the monolayer to dislodge cells, before the added FC40 returns to join the bulk FC40 in the overlay. Imaging then reveals the resulting wounds in the middle of each chamber (**Fig. 4Aii**).

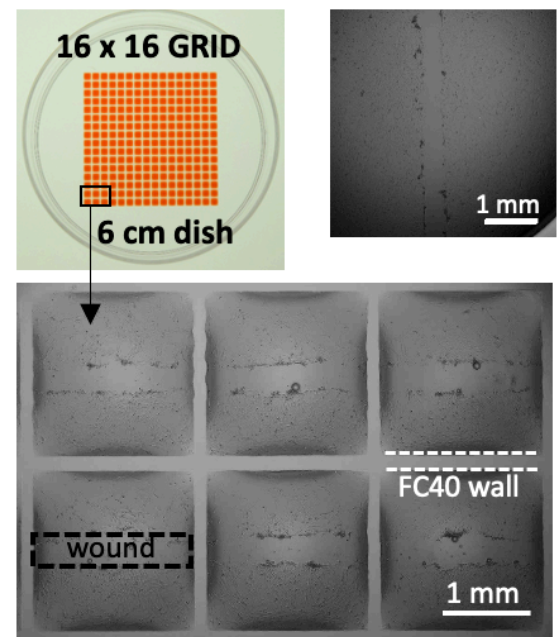
Wounding can also be multiplexed using conventional 96-well plates and a media jet – as these can hold larger volumes (**Fig 4B**). Note the large black ring close to the well wall (**Fig. 4B**). This 'edge effect' prevents a clear view of much of the bottom of the well, and is due to the solid surrounding wall obscuring the view. In contrast, edge effects in chambers with fluid walls are much less (**Fig 4Aii**), and can be essentially eliminated using smaller volumes (22). These results show that wound-healing assays using FC40 or media can easily be multiplexed. This opens up the possibility of using jetting for drug screening.

A In GRIDs

i Principle



ii Proof-of-principle



B In well-plates

media jet

Figure 4. Multiplexed wound production.

A. In GRIDs, with FC40 jet. (i) Principle. Cells are seeded in GRID chambers, grown to confluency, and the jet traverses above the centerline of each row; as it passes, the jet forces FC40;media interfaces down so they play on monolayers to detach cells. (ii) Image of GRID. Chambers initially held 100 nl media, and 400 nl red dye was added to aid visualization. Zoom in: images of wounds in 6 chambers.

B. In well of a conventional 96-well plate, with media jet.

Discussion

We describe a general contactless method to create wounds in a monolayer of a human cell; micro-jets of media or an immiscible liquid (FC40) are projected on to the monolayer to detach cells (**Fig. 1, Fig. S1**). We establish working conditions and failure parameters for one cell type (C2C12s myoblasts) and describe ways to alter wound dimensions (**Fig 2**). Wounds can have almost any imaginable 2D pattern (**Fig 3**), they heal as expected (**Fig. 3D, Fig S2**), and the approach can be multiplexed (e.g., using either an FC40 jet and a 6 cm dish with 256 chambers, or a media jet and a multi-well plate; **Fig. 4**).

Notable limitations of the technology include the following. (i) A 3-axis traverse and syringe pump are essential (to produce and control the required jet). (ii) Whilst the method is a general one, conditions have to be tuned to produce wounds with specified widths in monolayers of different cell density and type, as both degree of confluency and cell type affect wound production (Wang *et al.*, 2015). (iii) When using an FC40 jet, occasional FC40 drops are left behind in the wound, and these can potentially act as a physical barrier that prevents migration of cells into all areas of the wound (**Fig. 2A**).

In summary, we have developed a contactless method to produce wounds *in vitro*, using only biocompatible fluids. Due to its versatility and reproducibility compared to other methods, we anticipate it will find wide application in biomedicine.

Materials and methods

General reagents

All reagents and materials were purchased from Sigma Aldrich (St. Louis, Missouri), unless otherwise stated. FC40 was provided by IotaSciences Ltd (Oxford, UK). In **Figure 4A**, allura Red – a water-soluble dye – was added to each chamber to aid visualisation.

Cells

C2C12, an immortalized mouse myoblast cell line (23), was cultured in DMEM + 15% FBS. In preparation for wounding experiments, cells were plated in 60 mm tissue-culture-treated dishes (Corning, Merck product 430166) at a density of 10^6 cells/dish. Wounds were created ~48 h after plating. For **Figure 4B**, cells were plated on 96-well plates (Corning, product 3596) at a density of 15,000 cells/well, and used for wounding ~48 h later.

Wounding and printing of grids

All wounds were made using custom-written software and modified 'Freestyle' (used with 6 cm dishes) or 'Pro' printers (used with 96-well plates; **Fig. 4B**) provided by iotaSciences Ltd. Each printer consists of a 3-axis traverse, which moves two stainless-steel dispensing needles (Adhesive Dispensing Ltd, Milton Keynes, UK), one with a nozzle used for jetting media or FC40 (60 μ m ID, 0.5 mm OD mm), and the other to add/remove media to/from chambers (250 μ m ID, 0.5 mm OD). The two needles are connected to syringe pumps, which control the jetting/dispensing of media/FC40.

GRIDs (**Fig. 4A**) were made with MEM plus 10% FBS. To make a grid, 1 ml media is added to a 6 cm dish to cover the entire surface, and ~0.9 ml removed to leave a thin film. This is then covered with 2 ml FC40, and the grid created as described by Soitu *et al*, 2018. Unless otherwise stated, wounds were created using $D_{nozzle} = 60 \mu\text{m}$, $H = 0.4 \text{ mm}$, $\dot{Q} = 8 \mu\text{l/s}$, and $V_{traverse} = 300 \text{ mm/min}$.

The DNA-shaped wound in **Figure 3D** was constructed after drawing the pattern in Inkscape (inkscape.org) followed by conversion to G-code, the programming language used by the printer.

Imaging

All images of wounds were collected using a digital SLR camera (Nikon D7100 DSLR) connected to an epi-fluorescent microscope (Olympus IX53; 1.25X, 4X, 10X, 25X objectives) with translation stage and overhead illuminator (Olympus IX3 with filters). Brightness and

contrast was enhanced by 20% for these images, for better visualisation of wounds. The image of the grid in **Figure 4B** was taken using a digital SLR camera (Nikon D610). Images of wounds in **Figure 2** were analysed using ImageJ (Rasband, 1997-2018) and areas of wounds extracted.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (San Diego, CA).

Acknowledgements

Author contributions. C.S., P.R.C., and E.J.W. designed research, C.S., M.P., and E.J.W. performed experiments, and all authors wrote the paper.

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Competing financial interests. Oxford University Innovation – the technology transfer company of The University of Oxford – has filed provisional patent applications on behalf of C.S., P.R.C., and E.J.W. partly based on this study. P.R.C., and E.J.W. each hold equity in iotaSciences Ltd, a company that is exploiting this technology. iotaSciences Ltd. provides a scholarship for C.S..

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Supplementary materials

Supplementary figures

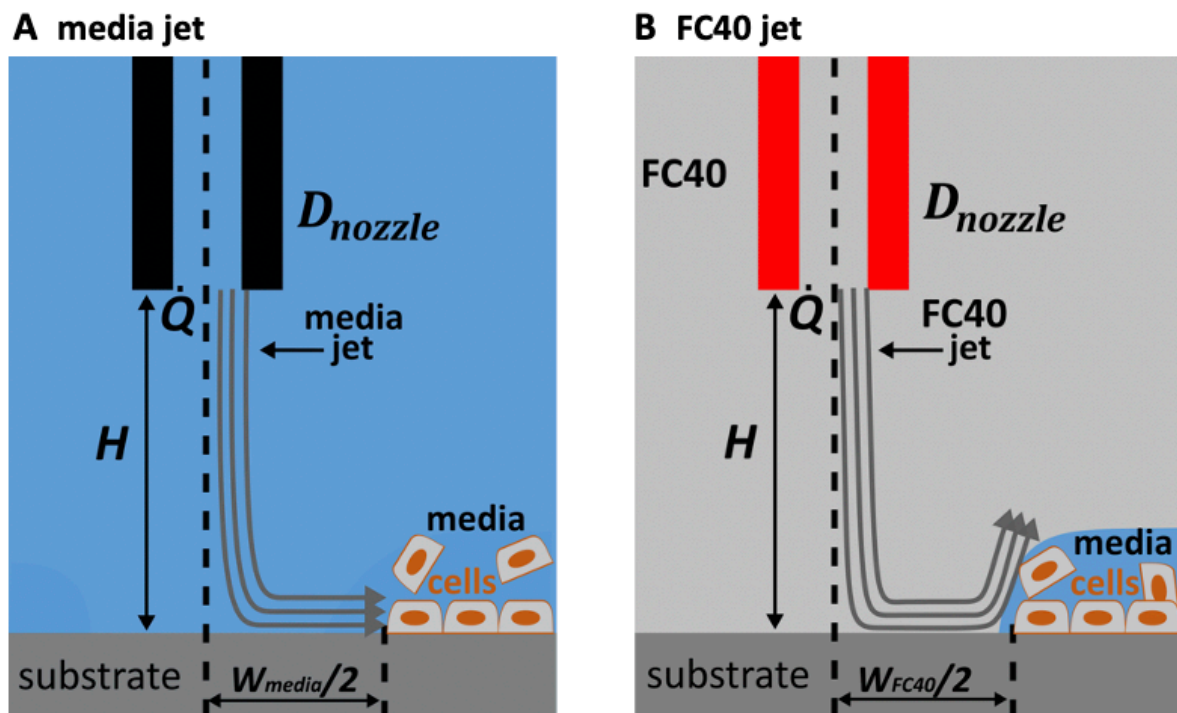


Figure S1. Parameters affecting wound width. Cells are detached by shear produced by the media or FC jet to create a wound of width W_{media} . Unless otherwise stated, all experiments were performed using D_{nozzle} (nozzle diameter) = 60 μm , $\dot{Q}$ (volumetric flowrate) = 8 $\mu\text{l/s}$, H (height of nozzle above substrate) = 0.4 μm , $V_{traverse}$ (traverse speed) = 300 mm/min. Streamlines of jets (grey) are for illustrative purposes only.

A. Nozzle in media.

B. Nozzle in FC40. Here, the FC40 jet displaces media (in addition to cells) from the substrate, and – as the jet moves on – media flows back into the ‘wound’ area.

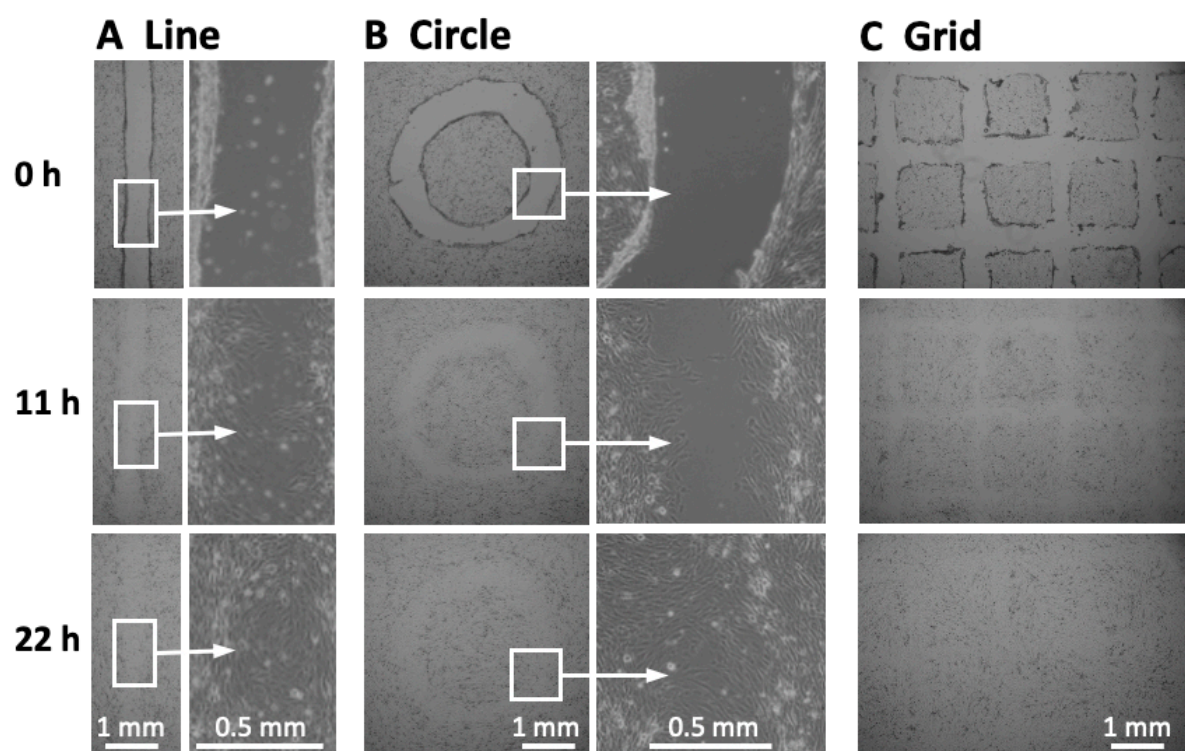


Figure S2. Healing of wounds similar to those shown in **Figure 3**. Images were collected at the times indicated. In all cases wounded areas are entirely repopulated with cells manage within 22 h, although the different wounds yield subtle differences in the structure of the cell sheet at the final time. **A.** Line. **B.** Circle. **C.** Grid.

Statement of Authorship for joint/multi-authored papers for PGR thesis

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The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

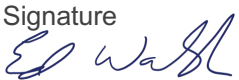
Title of Paper	Creating wounds <i>in vitro</i> using micro-jets
Publication Status	Submitted for Publication
Publication Details	C. Soitu, M. Panea, A. A. Castrejón-Pita, D. Beeson, P. R. Cook, and E. J. Walsh

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Student Name:	Cristian Soitu		
Contribution to the Paper	Performed experiments, contributed to data collection and analysis, contributed to manuscript writing and editing.		
Signature <i>Cristian Soitu</i>	Date	15.09.2020	

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Chapter 9: Conclusions and future work

Conclusions

This thesis represents a comprehensive introduction into the field of microfluidics with fluid walls. In its entirety, it describes a collection of methods and equipment harnessed to fabricate microfluidic devices on demand. The literature review in **Chapter 2** depicted the state-of-the-art microfluidic technologies used in biomedical research, as well as the main applications of those. **Chapter 3** presented the relevant theoretical aspects (geometry, contact angle, Laplace pressure) of structures with fluid walls. **Chapter 4** described the underlying principle behind printing fluid walls, together with a demonstration of how it can accommodate the main workflows in cell biology. The platform was found to be biocompatible with both cell- and organism-based assays. In **Chapter 5**, the ability to reconfigure cellular environments was explored, and applied to drug-treatment and wounding assays. The optical properties of fluidic chambers were investigated in **Chapter 6** and were found to facilitate and significantly accelerate single-cell cloning workflows. **Chapter 7** reported an important milestone in this work – a contactless method for printing fluid walls. Jetting was shown to improve precision and reproducibility, which made possible the use of Voronoi regions to beat the Poisson distribution in single-cell cloning. The same method was adapted to dislodge attached cells, and this was extended further to create wounds in confluent sheets of cells (**Chapter 8**).

Beyond simple proof-of-concept applications, this thesis provided along the way several important results: miniaturized CRISPR-Cas9 assays in volumes smaller than 1 μl , a fluorescent method for measuring small volumes (**Chapter 4**), a method to reconfigure cellular environments around living cells (**Chapter 5**), a way to accelerate single-cell cloning (**Chapter 6**), a method to beat the Poisson distribution in single cell cloning, a perfusion device which can continuously feed cells over days (**Chapter 7**), and a method for dislodging attached cells and creating wounds (**Chapter 8**).

The wide variety of applications mentioned illustrates perhaps the most important advantage of this technology - its versatility. The same setup used to accelerate single cell cloning was used to create perfusion devices, or wounds in monolayers of cells. Existing standard platforms specialize on one application only and are very costly. For instance, commercial devices designed to isolate single cells in wells of microtiter plates cost upwards of £100,000. Together with the greatly reduced costs required for the very small volumes of reagents used, this technology can generate savings of the order of hundreds of thousands of pounds. At a minimum, freestyle fluidics can serve as a rapid prototyping technology before committing to more durable, permanent, established platforms.

Importantly, this technology addresses one of the tacit problems of biomedical research. While the scientific community undoubtedly contributes to the advancement of our society, this comes at a large environmental cost. It is estimated that each of the millions of laboratories around the world uses five to ten times more energy per square meter than office buildings (83). The millions of metric tons of laboratory plastic waste produced each year is a price too high to pay. By minimizing workflows, freestyle fluidics drastically reduces the amount of plastic waste. One single Petri dish can accommodate more than 256 different experiments, for which three 96-well plates would be normally required. Furthermore, the washing steps that the printer is capable of performing eliminates the need to use countless pipette tips along the way. Finally, the 1000-fold reduction in volumes used by microfluidic platforms produces a similar reduction in amounts of harmful chemical substances released to the environment. All of the arguments above seem to suggest that, when it comes to sustainability, smaller is better.

Like any emergent technology, this one also has disadvantages. Several limitations have been presented at the end of each chapter and here, two of the most important ones are reiterated in the light of the future work proposed. First, and perhaps the most important, chambers created here are 2.5D at best and, so far, it has been difficult to create 3D structures. As the cell culture models in both academia and industry experience a shift towards 3D (spheroids, organoids, etc.), it becomes important to accommodate similar assays in

microfluidic environments with fluid walls. Second, the physical parameters inside these circuits (such as pressure and shear stresses) are not trivial to derive. Simple geometries can be analytically resolved, but even these required a good understanding of fluid mechanics principles. Consequently, circuit-designing tasks are largely inaccessible for biologists and require specialized expertise. Fluidic chambers have walls that morph as pressure inside them changes, which makes this a complex problem. Nevertheless, these shortcomings should find resolution through future work.

The complex capabilities of microfluidics with fluid walls should open many avenues for biologists wanting to miniaturize workflows, beyond the applications explore here. There is hope that the collection of features and advantages presented throughout the thesis will lead to widespread adoption of this technology, such that microfluidics finally fulfils its predicted potential in life sciences.

Future work

The emerging technology of microfluidics with fluid walls has the potential to contribute significantly to research in neuroscience. As major directions for future work, I propose the following sub-aims: 1) to develop a framework that recapitulates human brain development at the micro-scale and 2) to develop a platform that allows the construction of human neuronal networks, which would be the equivalent of a biological computer.

Finally, significant work is required to build a solid theoretical framework for the emergent field of microfluidics with fluid walls and several steps towards this goal are enumerated below.

Brain organoid formation

Brain development in humans is a remarkable self-organization process, which continues after birth, but this process is mostly inaccessible for mechanistic investigation. The importance of having accurate models for the human brain cannot be overstated – it would allow us to investigate the mechanisms involved in neurodegenerative diseases (e.g. Parkinson's, Alzheimer's, amyotrophic lateral sclerosis), with hope to inform and develop treatments.

Organoids research has emerged in recent years to address this need. Due to their ability to differentiate and self-organize into organ-like structures, organoids became one of the most physiologically relevant *in vitro* models we have. The typical workflow for generating brain organoids involves forming 3D cellular aggregates from stem cells, called embryoid bodies (EBs), growing those in a Matrigel matrix, followed by culture in suspension into spinning bioreactors for maturation (84). Organoids corresponding to different regions of the brain are then fused forming “assembloids” (85). However, despite the rapid advances in the field, there are caveats limiting the full potential of the technology. First, the entire workflow is usually done by manual pipetting all the samples and reagents, which results in a costly, wasteful and time-consuming process. Second, due to using unpatterned protocols, there is significant variability in the internal morphological features of the organoids, which often also have a necrotic core once they grow in size (86). Finally, connectivity between organoids happens in a random manner as functional separation of different brain regions is difficult to achieve, and yet critical in reproducing the complex set of chemical cues in human brains.

Conventional microfluidics arguably provides the best way to address these shortcomings, but neuroscientists rarely use microfluidic devices for reasons that include the long lead times required for prototyping, the complexity of operation, combined with poor optics, sample access. This section brings forward a path to overcome the aforementioned challenges. Specifically, I propose addressing the following goals:

1. Develop methods that streamline and standardize EB formation and minimize organoid variability.
2. Create devices designed to functionally separate individual neurons, populations of neurons or cerebral organoids with the purpose of controlling the formation of, probing, and manipulating neuronal connections.

1. EB formation and variability

EB formation is usually done in the conventional manner using AggreWell plates, which can be laborious and expensive. GRIDs offer a way to automate this workflow and minimize waste, cost and volumes of reagents (each chamber contains less than 1 μ l). This can be achieved by encapsulating inside chambers either single iPSCs (which grow into organoids; **Fig. 1**) or populations of cells which aggregate (not shown here). This would then become an ideal system for running screening assays with middle-to-high throughput and excellent optics.

As the next step, I propose repurposing the perfusion circuit in **Chapter 7** to accommodate EBs under precisely controlled flow conditions to address two current limitations of the 3D culture – the intra-organoid variability and necrosis at the core of the spheroid. The main factors affecting brain development are extracellular matrix, growth factors, and fluid flow. Although it is believed that a precise control of the media flow on brain organoid could minimise organoid variability and necrosis (87), static systems are currently preferred in practice.

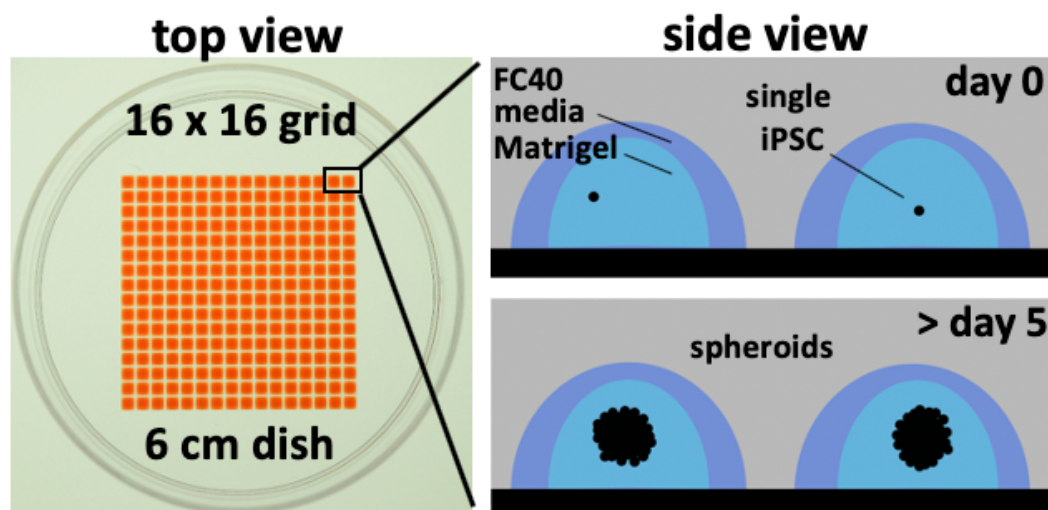


Figure 1. Spheroid formation. Single cells embedded in a Matrigel matrix, encapsulated in turn in media droplets grow into spheroids.

2. Connecting cerebral organoids

The ultimate aim of this sub-section is to design a system that functionally connects organoids mimicking different brain regions (e.g. dorsal and ventral). Such cerebral organoids models have already been used to study neurological disorders such as microcephaly and autism (88-89). The proposed system traps each organoid in its own fluidic compartment with its own set of chemical cues (**Figure 2**). Each pair of enclosures is connected through a channel which allows bundles of axons to squeeze through. Such fluidic circuits offer both physical and optical access to samples making it ideal for integrating electrophysiology and optogenetics tools in the workflow. For instance, one would like to observe how organoid C responds when A and B are stimulated, and then gauge the same response in disease models. The underlying physical principles of systems with fluid walls change when the characteristic size of the compartments (height in this case) is greater than 2-3 mm (which is the capillary length scale), whereas organoids can reach diameters of up to 5 mm. For this reason, this element of the proposed work receives a high-risk score.

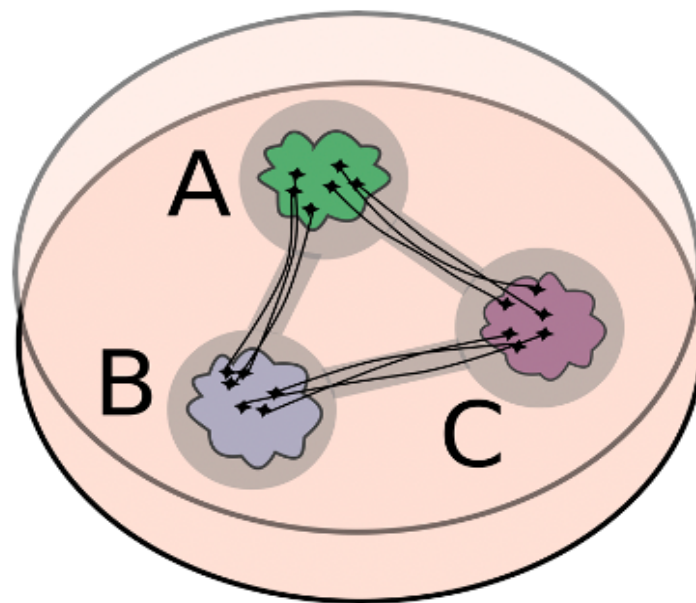


Figure 2. Connected brain organoids

Neuronal networks in a dish

Artificial intelligence (AI) is altering contemporary life, and natural intelligence is its muse. However, modelling neural networks underpinning AI and the analysis of connectivity in an animal brain are evolving as largely separate fields acting at different scales (e.g., AI usually works with millions of computer nodes, but neuroscientists record signals from hundreds of neurons). Existing *in vitro* approaches do not yet permit construction of human neuronal networks with a simple pre-specified pattern, and – once made – recording any connectivity remains difficult.

Microfluidics with fluid walls has the potential to provide a platform to study neuronal activity *in vitro* and reproduce (at least partially) the spatial complexity of a brain. It should allow neuronal stimulation and recording with existing approaches (e.g., optogenetics, calcium imaging, patch-clamping, using multi-electrode arrays – MEAs). Repeated, patterned stimulation of neurons in the input layer should produce neuroplasticity across the network, making this the first biological computer to use neurons *in vitro*. The initial aim to create a simple *in vitro* network in which particular neurons make pre-determined connections with other specified neurons. This would create a model ‘artificial brain’ in 2D that can be used to study the ways neurons make both physical and electrical connections. Preliminary studies have already been performed using neural stem cells derived from human induced pluripotent cells (iPSCs), which differentiate in circuits with fluid walls. Differentiated neurons will form a network in the system by extending axons through conduits (**Figure 3**). Channel height is adjusted to allow just axons to enter (but not somas), and – once axons grow through conduits – they connect with other axons. Neurons in such circuits are initially naïve; they have never fired before (unlike ones from animal brains or spinal cords that carry an unknown pre-history that complicates analysis of subsequent electrical activity). As fluid walls are transparent and self-heal around inserted probes, development of axonal connectivity throughout the network can be monitored microscopically (e.g., by phase-contrast, calcium imaging). Any point in the network can also be stimulated after inserting electrodes/patch-clamps, as alterations in

outputs are recorded at any other chosen point. As circuits can be constructed on glass, it is even feasible to use commercially available multi-electrode arrays to stimulate and record from multiple points simultaneously. Uniquely, users have complete flexibility over when/where to build/alter fluid walls, and when/where to stimulate/record. Once this workflow is optimized and functional, the project can progress towards the next step – building 3D networks.

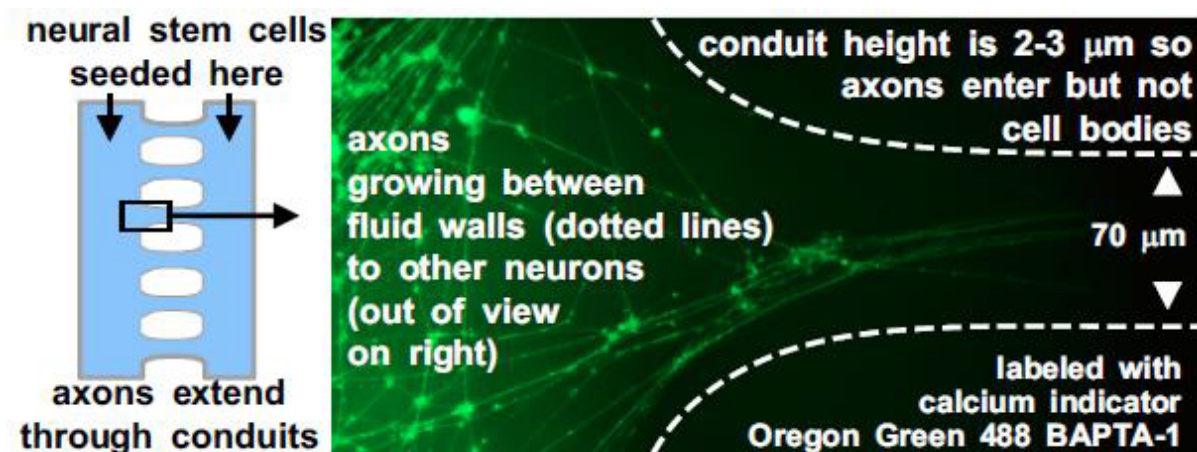


Figure 3. Creating a neural network in a Petri dish. Circuit plan (the rectangular region is shown on the right). Cells differentiate into cortical neurons and extend axons through a channel (dotted lines) towards others out-of-view on the right. All points in any cell in the network are accessible through fluid walls from above.

Theoretical framework

The second major direction proposed for future work is to continue the development of the theoretical framework of microfluidics with fluid walls. In this system chambers have fixed footprints, but their interfaces morph under the action of hydrostatic and Laplace pressure. This results in idiosyncratic geometries, hence unknown heights, contact angles, curvatures and inner pressures.

I propose using Surface Evolver, a software for simulating liquid interfaces, to build a collection of physical relationships that can be used to derive the heights, contact angles and

inner pressures for unconventional footprints (n-sided regular and irregular polygons). Surface Evolver has full flexibility in setting the initial conditions and constraints, which make it very useful, but also very problematic. This is why, simulations should be validated through experiments. For instance, the fluorescence method for measuring small volumes described in **Chapter 4** could be now used to extract the physical parameters of chambers with polygonal footprint. Upon successful completion of this aim, one can now start analyzing flow between chambers. Full knowledge of flow parameters would allow the user to fully define the shear stresses cells experience inside the enclosures.

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Dissemination of research

Often, academic research has little or no impact if it is not communicated effectively. In order to increase the visibility of my research, throughout my DPhil I engaged in a host of dissemination activities, as detailed below.

Published works

- ❖ C. Soitu, N. Stovall-Kurtz, C. Deroy, A.A. Castrejon-Pita, P.R. Cook, E.J. Walsh. Jet-printing microfluidic devices on demand. *Advanced Science* 2001854 (2020).
- ❖ C. Soitu, C. Deroy, A.A. Castrejón-Pita, P.R. Cook, E.J. Walsh, Using fluid walls for single-cell cloning provides assurance in monoclonality. *SLAS Technology* 25(3), 267-275 (2020).
- ❖ C. Soitu, A. Feuerborn, C. Deroy A.A. Castrejon-Pita, P.R. Cook, E.J. Walsh, Raising Fluid Walls Around Living Cells. *Science Advances*. 2019, 5, eaav8002.
- ❖ C. Soitu, A. Feuerborn, A.N. Tann, H. Walker, P.A. Walsh, A.A. Castrejon-Pita, P.R. Cook, E.J. Walsh, Microfluidic chambers using fluid walls for cell biology. *PNAS* 115, E5926-E5933 (2018).

Conference presentations

- ❖ SLAS Europe 2019, Barcelona (2019) - Podium presentation.
- ❖ International Conference on Lab-on-a-Chip Technologies, Rome (2019) - Podium presentation.
- ❖ SLAS2019, Washington (2019) - Podium presentation.
- ❖ BioMedEng18, London (2018) - Podium presentation.
- ❖ SLAS2018 - San Diego (2018) Poster presentation.
- ❖ Microfluidics Congress UK, London (2017) - Poster presentation.

Awards

- ❖ SLAS Innovation Award for my work on 'Microfluidic chambers with fluid walls' (2019).
- ❖ Best podium presentation - International Conference on Lab-on-a-Chip Technologies, Rome (2019).
- ❖ People's Choice Award - Sir William Dunn School of Pathology Art Competition (2018).
- ❖ Best poster presentation - 4Bio Summit - 3rd Microfluidics Congress, London, (2017).

Outreach activities

- ❖ Mentor Romanian Science Festival (2019).
- ❖ Mentor Oxbridge China Lecture Circuit (2018).

Teaching

- ❖ College tutor for Mathematics, University of Oxford, (2019-2020).
- ❖ College tutor for Fluid Mechanics, University of Oxford, (2018-2019).
- ❖ Demonstrator for Next Generation Sequencing Workshop, University of Oxford, (2017).