Drop-based microfluidic devices for encapsulation of single cells

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We use microfluidic devices to encapsulate, incubate, and manipulate individual cells in picoliter aqueous drops in a carrier fluid at rates of up to several hundred Hz. We use a modular approach with individual devices for each function, thereby significantly increasing the robustness of our system and making it highly flexible and adaptable to a variety of cell-based assays. The small volumes of the drops enables the concentrations of secreted molecules to rapidly attain detectable levels. We show that single hybridoma cells in 33 pL drops secrete detectable concentrations of antibodies in only 6 h and remain fully viable. These devices hold the promise of developing microfluidic cell cytometers and cell sorters with much greater functionality, allowing assays to be performed on individual cells in their own microenvironment prior to analysis and sorting.

Introduction

The ability to gather statistical information over large populations of cells has revolutionized the study of cellular function and is finding increasing utility in diagnostics. This is typically accomplished using flow cytometry of cells labeled with fluorescent probes, which measures the response of individual cells at a sufficiently high rate to collect statistical information. Cells with the desired characteristics can also be purified, if desired, using a fluorescence-activated cell sorter (FACS), again at high rates. However, in a flow cytometer or FACS, cells are interrogated by flowing them past the detector in a continuous stream of buffer. As there is no diffusional barrier between the cells, it is impossible to assay or sort cells based on properties of molecules they secrete; assays are restricted to molecules either confined within the cells or bound to the cell surface. In addition, it is difficult to perform assays which entail mixing discrete reagents with each cell shortly before measurement.

Considerably enhanced functionality could be achieved by encapsulating each cell in a small, isolated volume, which would confine the cell, any reagents, and any secreted molecules, while attaining high concentrations of solutes even with small absolute numbers. One means to accomplish this is through the use of picoliter to nanoliter microwells lithographically fabricated on a plate;[1] however, this limits any ultimate sorting of the cells. An alternate method is to encapsulate the cells in monodisperse picoliter aqueous drops in an inert carrier fluid using microfluidic devices.[2–9] Encapsulation of single mammalian cells has been demonstrated using optical trapping and microfluidic drop generation[6,9] and *E. coli* cells have been encapsulated in drops.[7] However, full functionality requires a device to encapsulate single cells, incubate, and analyze the cells, while maintaining cell viability and healthy metabolism. Achieving this functionality, while producing a robust device capable of operating in flexible and varied environments, is very challenging. Such a device, however, would represent an essential first step towards a new class of microfluidic FACS machines.

Here we describe the use of drop-based microfluidic devices to encapsulate single mammalian cells in distinct pL-sized drops to isolate them in their own microenvironment. Because the volume of each drop is restricted, molecules secreted by an individual cell can rapidly attain detectable concentrations. We use distinct microfluidic devices for encapsulation, incubation, manipulation, and analysis, significantly enhancing robustness and flexibility. We demonstrate the power of these devices by encapsulating individual mouse hybridoma cells in drops, where they remain viable for several hours while secreting antibodies at a rate similar to cells in bulk. Moreover the cells can be recovered from the drops and cultured.

Experimental

Microfluidic device fabrication

Microfluidic flow chambers are fabricated by soft lithography.[10] Negative photo resist SU-8 2025 or SU-8 2100 (MicroChem, Newton, MA) is deposited onto clean silicon wafers to a thickness of 25 μm, 40 μm, or 100 μm, and patterned by exposure to UV light through a transparency photomask (CAD/Art Services, Bandon, OR). The microstructure is developed and Sylgard 184 poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI) is mixed with crosslinker (ratio 10 : 1), degassed thoroughly, poured onto the photoresist patterns, and cured for at least 1 h at 65 °C. The PDMS replicas are peeled off the wafer and bonded to glass slides after oxygen-plasma activation

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of both surfaces. The microfluidic channels are treated with Aquapel (PPG Industries, Pittsburgh, PA) by filling the channels with the solution as received and subsequently flushing them with air prior to the experiments; this improves the wetting of the channels with fluorinated oil. Polyethylene tubing with an inner diameter of 0.38 mm and an outer diameter of 1.09 mm (Becton Dickinson, Franklin Lakes, NJ) connects the channels to the syringes. Glass syringes are used to load the fluids into the devices. Flow rates are controlled by syringe pumps.

Distinct devices are fabricated for encapsulation, incubation, and analysis. Optimum devices for drop formation and cell encapsulation are 40 μm high with a 35 μm-wide nozzle. To vary the drop size, we also use a channel height of 25 μm and different nozzle widths. Devices for cell incubation are 100 μm high, the channel width is 500 μm, and the length is 2.88 m. Devices for analysis can include various on-chip functionalities, but in all cases require an interface between the incubation and analysis chips. This is accomplished with a nozzle to reinject the drops into the channels; it is similar in geometry to the drop-formation nozzle, but is larger, with a 40 μm height and at least a 40 μm width, to facilitate the flow of drops into the devices. All inlet channels are equipped with patterned filters which prevent dust particles from clogging the channels downstream.

Cell culture

We grow 2C6 hybridoma cells, producing an anti-ovalbumin IgE (gift from Lester Kobzik), in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g L^{-1} glucose, L-glutamine, and sodium pyruvate (Mediatec, Inc. Herndon, VA) supplemented with 10% (v/v) fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 1% Penicillin/Streptomycin. The cells are split every 3 days under sterile conditions and incubated at 37 °C and 5% CO_2.

Cell encapsulation and incubation

Cells are grown on culture dishes to a density of 1.2 to 2.5 × 10^6 cells mL^{-1}. Prior to the experiments, cells are washed at least once and resuspended in fresh media. The cell density is adjusted to the desired value, which depends on the average density per drop and the drop size. Hybridoma cells are ~10 μm in diameter and the total volume of medium available to each cell is many times its own volume. Fluorinert FC40 fluorocarbon oil (3 M, St. Paul, MN) is used to suspend the drops. To stabilize the drops a PFPE–PEG block-copolymer surfactant[11] is added to the suspending oil at a concentration of 1.8% (w/w). This surfactant provides excellent drop stability against coalescence while ensuring good biocompatibility of the inner drop interface. For drop formation, the outer, carrier-oil flow rate is 300 μL h^{-1} and the inner, aqueous flow rate is 30 μL h^{-1} leading to a drop production rate of 250 Hz. At this rate we fill the incubation device in 40 min. The cells are incubated by placing the whole device in a cell incubator at 37 °C and 5% CO_2.

Drop formation is imaged with a high-speed Phantom V5 camera (Vision Research, Inc., Wayne, NJ) and individual frames are analyzed to determine the statistics of the number of cells per drop. For each dilution, we collected images of 350 drops at each of three different points in time during the course of the experiment.

Cell recovery and viability

Cells are recovered from collected emulsions by diluting the emulsion with 10× its fluid volume of fresh media and adding 15% its volume of drop release reagent (RainDance Technologies, Inc., Lexington, MA). The mixture is incubated for 2 min to allow the oil and release agent to settle. The supernatant containing the cells is transferred to a fresh vial. In separate tests of this procedure we observe no effect on cell viability. To optimize our experimental conditions we test for the cell viability in each case using a live-dead assay. We use 1 μM calcein-AM (Invitrogen, Carlsbad, CA, green fluorescence, live stain) and 1 μM ethidium-homodimer-1 (Invitrogen, red fluorescence, dead stain) in phosphate buffered saline (PBS). We incubate the cells with the stains for 45 min at room temperature (RT) in the dark, and analyze representative images of the sample using fluorescence micrographs. We determine the viability from the fraction of live cells. This assay provides a means to compare viability under different experimental conditions.

Recultivation of the cells

The supernatant with the recovered cells is transferred into 96 well plates and incubated at 37 °C and 5% CO_2.

ELISA for antibody production

Expression of anti-ovalbumin antibodies in bulk and in drops is determined by a kinetic enzyme-linked immunosorbent assay (ELISA). Cells are placed on ice prior to encapsulation for 30 min and maintained at 4 °C while being washed 2 times to remove any remaining antibodies from the suspension and to prevent premature antibody production. The supernatant from each wash is tested for antibody content. For comparison, one reference culture treated in an identical manner as the cells used for encapsulation is placed into a culture dish at the same high density (10 × 10^6 cells mL^{-1}) and incubated in bulk for 6 h at 37 °C and 5% CO_2. Cells in drops are maintained at 37 °C and 5% CO_2 on the incubation chip for 6 h. Emulsions are broken and ELISAs are performed on culture supernatants after centrifugation to remove any remaining hybridomas. Briefly, 50 μL ovalbumin (Sigma, St. Louis, MO) (1 mg mL^{-1}) in PBS is added to separate wells of a 96-well plate (control wells contained only PBS) and incubated for at least 5 h at RT. The antigen solution is removed, and the wells are washed 3 times with 1× Tris-buffered saline (TBS) containing 0.2% Tween-20 (TBST) for 5 min each. The wells are blocked with 200 μL 3% bovine serum albumin (BSA) in PBS for at least 2 h at RT. The wells are then washed 3 times with TBST, incubating each step for 5 min. Culture supernatant dilutions are prepared in 3% BSA in PBS, and 50 μL of the dilutions are added to each well and incubated for 1 h. The wells are washed 3 times with TBST for 5 min each. The secondary rat anti-mouse antibody horse radish peroxidase (HRP) conjugate (clone 23G3, Southern Biotech, Birmingham, AL) is prepared in 3% BSA in PBS at 1 : 1000 dilution, added to the wells and incubated for 1 h at RT. The wells are washed 3 times with TBST for 5 min each, and 100 μL of fresh substrate (*o*-phenylenediamine dihydrochloride, Pierce, Rockford, IL) in buffer solution is added to each well. The absorbance at 450 nm is read every 10 s for 10 min using the kinetic measurement mode

of a plate reader. We plot the measured signal as a function of time and determine the initial slope which provides a measure of the relative antibody concentration. The control signal obtained from wells with no protein is subtracted from the measured values.

Results and discussion

We use several distinct components for our all-microfluidic approach to single cell experiments: encapsulation, incubation, and manipulation devices, as indicated by the boxes in Fig. 1.

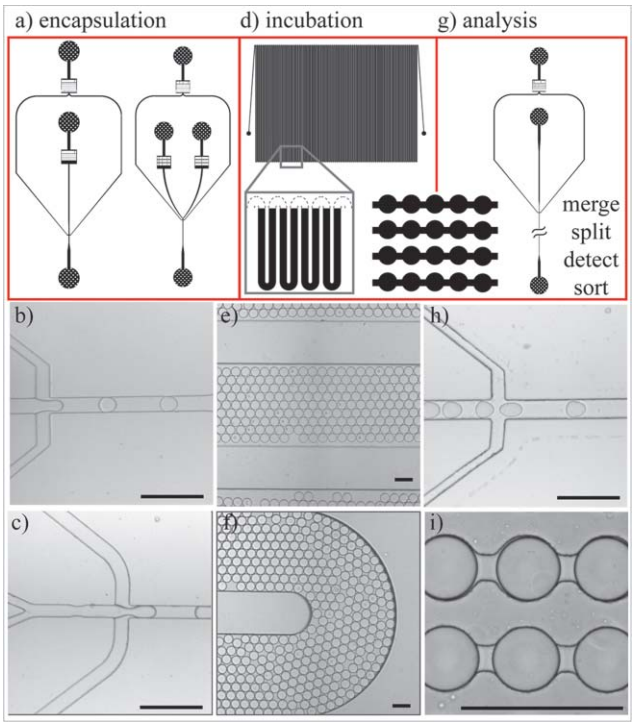

Fig. 1 Modular design of the microfluidic components. The individual components can be flexibly combined to meet specific experimental requirements and are connected by means of external tubing. Schematics and micrographs showing design of (a–c) encapsulation chips with single or double aqueous inlet, (d–f) incubation chips, and (g–h) interface to analysis (merging, splitting, detection, sorting) chips. Schematics: (a) single-inlet (left) and double-inlet (right) encapsulation, (d) top: serpentine incubation channel, bottom left: close-up of the serpentine incubation channel, bottom right: incubation channel for time-resolved studies, (g) reinjection for further drop handling. Micrographs: (b) single-inlet encapsulation, (c) double-inlet encapsulation, (e, f) serpentine incubation channel, (h) reinjection for further drop handling, (i) incubation channel for time-resolved studies from schematic shown in lower right part of d. All scale bars are 100 μm.

To illustrate the utility of our modular approach to drop based cell handling, we use a line of hybridoma cells which secrete anti-ovalbumin IgE antibodies. These hybridomas are suspension cells simplifying their handling in drops.

The basic cell encapsulation device uses a flow focussing geometry[5] to produce drops, as shown schematically on the left of Fig. 1a. Additional inlets can be incorporated on chip to mix reagents with the cells just before they are encapsulated, as shown schematically on the right of Fig. 1a. Three inlet channels, coming from the left, convert to form a nozzle as shown in the optical micrographs in Fig. 1b and 1c. In both cases, the center stream contains the cell suspension while the side streams contain the oil phase. The drop volume can easily be varied between ~0.5 pL and ~1.8 nL, corresponding to spherical drops of diameter 10 μm to 150 μm. This is accomplished by matching the size of the nozzle orifice to the drop diameter and operating the device in the dripping regime.[12] Fine tuning of the drop's size for a given nozzle can be accomplished by varying the inner, aqueous flow rate or the overall flow rate; this also leads to variation in the drop production frequency. The modular nature of the device enables the nozzle dimension and hence the drop size to be readily changed without affecting any other components.

Individual syringe pumps are used to control the flow of the oil and the cell suspension. Here we focus exclusively on suspension cells; however, adherent cells can also be studied by first growing the cells on small beads and then encapsulating the beads. To prevent settling of the cells and maintain the desired density we constantly stir the suspension. We typically use a 5 mL syringe containing 1 mL of cell suspension, ensuring that the depth of the volume is comparable to its height, thus enabling it to be easily mixed using a small magnetic stir bar. A convenient means of stirring the sample, while preventing clogging of the syringe, is to maintain it at a 45° upward angle and to place a stir plate on top of it. Using this scheme the encapsulation efficiency is typically approximately 70%. If we account for this factor we can reliably and reproducibly obtain the desired cell distribution in the drops.

Single cell studies require that virtually all drops contain at most one cell, so that the majority of drops contain no cell at all since the encapsulation process follows Poisson statistics. Production of drops encapsulating individual cells is shown in Fig. 2a, where black arrows highlight the cell-bearing drops. The Poisson distribution for cells is given by

$$f(\lambda;n)=\frac{\lambda^n e^{-\lambda}}{n!}, \qquad (1)$$

where n is the number of cells in the drop and λ is the average number of cells per drop; we adjust λ by controlling the cell density. We illustrate the distribution of cells in drops for $\lambda =$ 0.1, 0.3, and 0.5; these are typical values of interest for single cell experiments as they ensure that very few drops contain multiple cells. In each case, the results are in good agreement with those calculated from Poisson statistics for the values of λ used as shown in Fig. 2b. By using $\lambda = 0.3$ we obtain single cells in roughly 22% of the drops while ensuring that fewer than 4% have two or more cells. Although the number of single-cell-bearing drops is rather low, for these experiments this is not severe, given the high production and screening rate that can be achieved with microfluidic devices.

The incubation device consists of a long serpentine channel with a volume of 144 μL enabling it to hold a large quantity of drops, as shown schematically in the top of Fig. 1d. Cell-bearing drops produced in the encapsulation device can be redirected into the incubation device by means of external tubing. Inside the device the flow rate of the carrier oil is faster than that of the drops, thereby concentrating the emulsion.

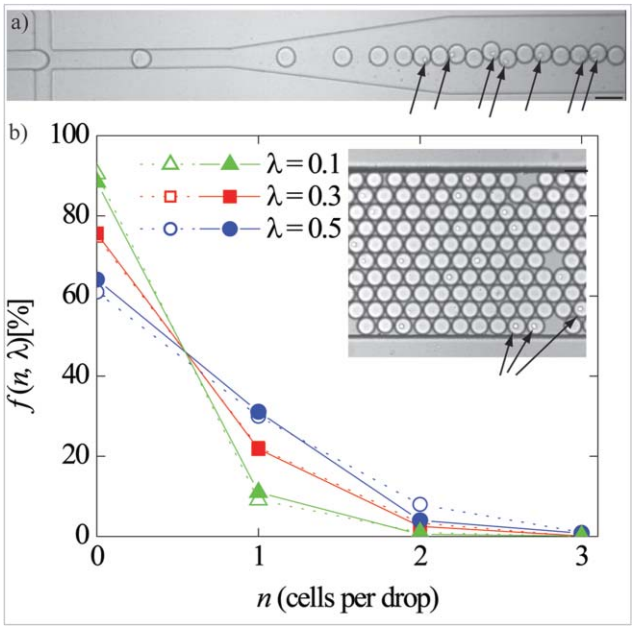

Fig. 2 Encapsulation of individual cells. (a) Single cells encapsulated in 33 pL drops. The cell-bearing drops are highlighted by the arrows. (b) Poisson distribution for 3 different cell densities. Open symbols: predicted values from Poisson statistics; solid symbols: experimental results. Lines are a guide to the eye. Inset: micrograph showing cells in drops in incubation device.

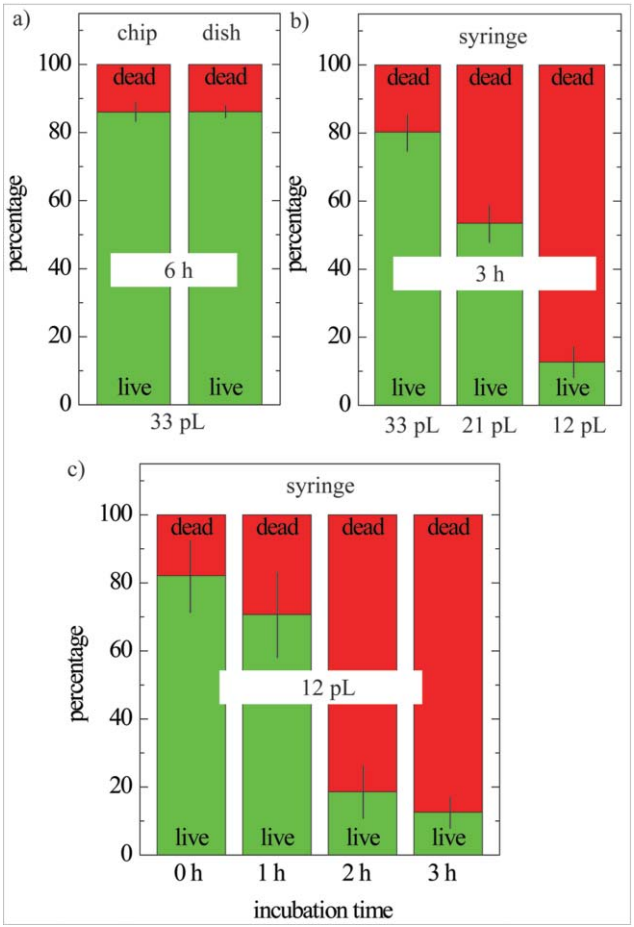

Fig. 3 Cell survival during incubation in drops. (a) Comparison for survival on chip (6 h, 33 pL drops, $n = 1167$ cells) compared to survival in a culture dish (6 h, $n = 3681$). (b) Survival in a syringe for different drop sizes (3 h, 33 pL: $n = 319$, 21 pL: $n = 301$, 12 pL: $n = 426$). In larger drops survival is increased. On chip we obtain survival rates similar to bulk incubation. (c) Time dependence of cell survival in small drops (12 pL volume, in syringe, 0 h: $n = 84$, 1 h: $n = 63$, 2 h: $n = 161$, 3 h: $n = 426$). Survival decreases dramatically even after short times.

Interestingly, because of their buoyancy the drops collect at the top of the channel where they form a well-packed single layer, as shown in Fig. 1e and f. Despite the high packing of the drops the surfactant ensures stability and we observe virtually no uncontrolled coalescence.[11]

The incubation device can be detached from the encapsulation device and placed in a cell incubator or other storage container to maintain the desired temperature and gas atmosphere. By carefully maintaining the channels filled with oil, any deleterious effects of air in the channels can be avoided. The permeability of both the PDMS[13] and the fluorocarbon carrier oil[14] to gas enables sufficient exchange to keep the cells at the level set by the environment; this is facilitated by their monolayer packing. The water saturated atmosphere prevents evaporation of water from the drops ensuring they retain the desired size and concentration. Independent studies over long periods of time confirmed that the drop diameter shrinks by less than 3.5% after 72 h; thus, for the much shorter incubation times used in these experiments the shrinkage is completely negligible.

To ascertain cell viability we break the emulsion after incubation, recover the cells, and perform live-dead assays. After incubation for a period of 6 h we find a survival rate of approximately 85%; by comparison, an identical survival rate is found for cells incubated on culture dishes as shown in Fig. 3a. Maintaining the cells in drops and on chip for all functions greatly increases both the convenience and usefulness of these devices, and these results confirm that this is feasible.

For comparison, we also sometimes collect drops directly into a syringe where the piston has been removed to allow gas exchange. In this case the monolayer packing of the drops is no longer maintained even when the syringe is placed almost horizontally to increase the surface area of the fluid; as a result cell viability is degraded, after only 3 h the survival rate is already only 80% as shown in Fig. 3b. These results confirm the importance of the monolayer packing in our microfluidic incubation device for these hybridoma cells.

A confined cell-culture volume without perfusion leads to a decrease in nutrient levels and an increase in waste levels, compromising cell proliferation and growth.[15,16] We therefore also test the survival rate as the drop size is decreased and find poorer results for drops of volumes 21 pL and 12 pL as shown in Fig. 3b. This is clearly a function of incubation time with the survival rate decreasing dramatically with increasing time as shown in Fig. 3c. We use drops of roughly 33 pL volume in our microfluidic incubation device ensuring good cell survival for at least 6 h.

This inverse relationship between drop size and survival time is consistent with studies using other mammalian cell lines (Jurkat and HEK293T), in which microfluidic systems were used to compartmentalized single cells in larger (660 pL) drops

in Fluorinert FC40 fluorocarbon oil stabilized with a PFPE-dimorpholinophosphate surfactant. In these larger drops the cells survive and proliferate for several days before viability starts to decrease.[8]

In addition to live-dead tests for cell viability we perform more rigorous experiments to ensure that cell metabolism is not harmed by their encapsulation We accomplish this by breaking the emulsion, recovering the cells, and recultivating them on microplates. Normal growth is observed; cells split directly from bulk are indistinguishable from those recultivated from the broken emulsion, as shown by the images, taken after 2 days growth, in Fig. 4a and b. This is a convincing demonstration of the viability of cells encapsulated in drops, and confirms that new cell lines could, in principle, be established from encapsulated cells.

We also ascertain that the production of antibodies is not hindered by the confinement of the hybridomas in the small volume of the drops. To prepare the hybridomas for this test, we start at a density of $\sim 2 \times 10^6$ cells mL^{-1} and grow the cells for 3 days, at which time the density has increased to $\sim 8 \times 10^6$ cells mL^{-1}. We measure the concentration of antibody in the supernatant with an ELISA, Fig. 4c (grey). We wash the cells with fresh media twice, checking to ensure that the antibody concentration in the supernatant has decreased to negligible value, as shown in Fig. 4c (green). We adjust the density to 10×10^6 cells mL^{-1}, and encapsulate the cells. We break a portion of the emulsion immediately and ensure that there is very little antibody production during the encapsulation process, as shown in Fig. 4c (orange). We incubate the remaining drops for 6 h on our incubation device, and then break this emulsion. The antibody concentration has increased significantly as shown in Fig. 4c (red). As a control, we compare the measured results with those obtained from cells cultured on a dish for 6 h at the same initial density, 10×10^6 cells mL^{-1}; virtually the same concentration is measured, as shown in Fig. 4c (blue). Assuming a typical rate of immunoglobulin secretion by hybridomas of 5000 molecules s^{-1},[1,17] we estimate the antibody concentration in the supernatant to be about 10^{15} molecules mL^{-1} after 6 h. We ensure that all of the ELISA measurements are performed in a regime where the signal is not saturated by performing additional experiments at ten-fold and one-hundred-fold dilutions; the measured relative concentrations all decrease proportionately, verifying the consistency of the results, as shown in Fig. 4d. This confirms that the cells are viable and that the metabolism of the encapsulated hybridoma cells is not degraded by their confinement. It also highlights a unique feature of these drop-based microfluidic devices: the ability to rapidly attain high concentrations of secreted molecules in the confined volumes of the drops.

After on-chip incubation, further analysis of the cells and the drop contents can be performed with the analysis device. This requires transferring the emulsion from the incubation device to the analysis device. A syringe pump is connected by external tubing to the inlet of the incubation device and carrier fluid is used to drive the emulsion through additional external tubing connecting it to the analysis chip. We use a flow-focussing geometry at the inlet of the analysis chip, with the auxiliary oil channels adjusting the spacing between the drops as shown in Fig. 1g and 1h. This leads to a uniform

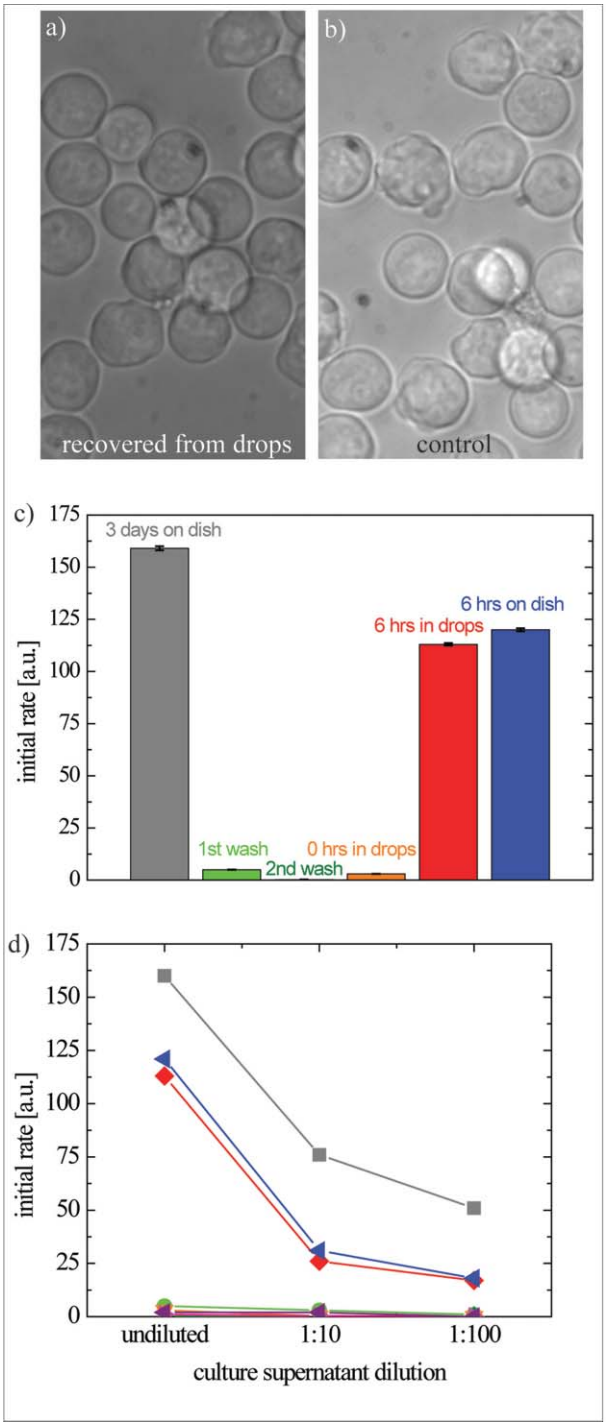

Fig. 4 Metabolic activity during and after encapsulation. (a) Cells were encapsulated, incubated for 6 h on chip, recovered from the emulsion and plated. Image was taken after 2 days. (b) Control, cells grown directly on culture dish. Both images show healthy, growing cells. (c) Antibody production in drops. Gray: after three days on culture dish; light green: after first wash; dark green after second wash; orange: encapsulated cells with no incubation time; red: encapsulated cells with 6 h incubation time; blue: cells incubated for 6 h on a culture dish; error bars correspond to the uncertanty in the linear fit to the initial enzyme reaction rate in the kinetic ELISA. (d) Initial rates of the ELISA for different dilutions of culture supernatant. Color code as in (c). Additional controls (purple, pink): empty emulsion drops, 0 and 6 h incubation time.

flow of drops, which can then be run into other modules fabricated on the analysis device. Potential examples include drop merging,[18] splitting,[19] detecting[7,20] or sorting,[21] depending on the assay desired. Alternatively, we can load the drops onto a microfluidic device designed to store ordered arrays of drops,[22] shown schematically in the bottom of Fig. 1d. This allows individual drops to be monitored, as shown in Fig. 1i, enabling time-resolved single-cell analysis.

Conclusions

We present a modular, and therefore highly flexible, drop-based microfluidic system which combines distinct devices to encapsulate, incubate, and manipulate single cells in small drops ($\leq$33 pL), enabling the concentrations of secreted molecules to rapidly attain detectable levels. The advantage of the modular concept is its flexibility, allowing adjustment to specific experimental requirements. All components are placed on physically separate chips which are connected by means of external tubing. Thus components can easily be exchanged to address the different experimental demands encountered when varying assays. Moreover, dysfunctional chips can easily be replaced, mitigating the inevitable experimental problems caused by clogging or leakage.

We show that antibody production, cell survival, and proliferation upon recovery are ensured despite the encapsulation in the confined geometry of the drops. These represent important preconditions for single cell experiments, such as screening for monoclonal antibodies, using drop-based microfluidics. Indeed, the small volume of the drops means that a single hybridoma cell in a drop secretes detectable concentrations of antibodies in only 6 h. The modular design of the devices also allows for adjustment to many other functional single cell assays where statistical information from large populations of individual cells is to be collected while each cell is isolated in its own microenvironment. This will separate the encapsulation, incubation, analysis, and sorting steps of assays, enabling greatly increased functionality to be incorporated. For example, drops containing other reagents or elements of a library could be merged with the cell-bearing drops prior to incubation or to sorting. The successful encapsulation of cells which retain viability in the drops is an essential step that will enable the development of a new class of flow cytometry and FACS instrumentation using drop-based microfluidics.

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