Chapter 15

Microfluidic Platform for Parallel Single Cell Analysis for Diagnostic Applications

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Abstract

Cell populations are heterogeneous: they can comprise different cell types or even cells at different stages of the cell cycle and/or of biological processes. Furthermore, molecular processes taking place in cells are stochastic in nature. Therefore, cellular analysis must be brought down to the single cell level to get useful insight into biological processes, and to access essential molecular information that would be lost when using a cell population analysis approach. Furthermore, to fully characterize a cell population, ideally, information both at the single cell level and on the whole cell population is required, which calls for analyzing each individual cell in a population in a parallel manner. This single cell level analysis approach is particularly important for diagnostic applications to unravel molecular perturbations at the onset of a disease, to identify biomarkers, and for personalized medicine, not only because of the heterogeneity of the cell sample, but also due to the availability of a reduced amount of cells, or even unique cells. This chapter presents a versatile platform meant for the parallel analysis of individual cells, with a particular focus on diagnostic applications and the analysis of cancer cells. We first describe one essential step of this parallel single cell analysis protocol, which is the trapping of individual cells in dedicated structures. Following this, we report different steps of a whole analytical process, including on-chip cell staining and imaging, cell membrane permeabilization and/or lysis using either chemical or physical means, and retrieval of the cell molecular content in dedicated channels for further analysis. This series of experiments illustrates the versatility of the herein-presented platform and its suitability for various analysis schemes and different analytical purposes.

Key words Single cell, Parallelization, Microfluidics, Circulating tumor cells, Cell trapping, Cell permeabilization

1 Introduction

Cell analysis is conventionally conducted at the scale of a population, consisting of a million to a billion of cells, yielding average data at the population level. It is however now well acknowledged that cell populations and tissues are heterogeneous [1]. Tissues, for instance, comprise different cells types. Furthermore, in any cell population, cells are asynchronous for cell cycles and biological processes such as, e.g., apoptosis and cell differentiation.

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Finally, rare cells or stochastic events occurring in individual cells often bear essential information for the understanding of fundamental processes. Consequently, in general, cellular analysis must be brought down to the single cell level to get useful insight into biological processes, and not to miss the information, which would be lost when using an analytical approach at the level of the whole cell population.

Analyzing cells at the single cell level is also highly relevant for diagnostic applications, not only when heterogeneous populations are encountered but also when only a handful of cells or even individual cells are available for analysis. First, as for any tissue, tumors comprise various cell types including cancer cells and cancer stem cells (CSCs) as well as stromal cells such as fibroblasts [2]. Only a small portion of the cells (<20 %) does bear the hallmarks of the disease [2], which become masked using a population analysis approach. Therefore, all cells must be analyzed separately to unravel molecular perturbations at the origin of the disease, to identify biomarkers and for the development of personalized medicine [3]. Similarly, circulating tumor cells (CTCs), found in liquid biopsies, are highly diluted among a large amount of blood cells (1–10 CTCs/mL blood *vs.* $>10^9$ blood cells), and they exhibit extremely different phenotypes [4], which calls out for a single cell analysis approach for their molecular characterization, after their selective isolation. Ideally, in these cases, information at both the single cell and population levels is required to actually fully characterize heterogeneous samples. Therefore, an optimal analytical strategy consists of examining each individual cell present in a complex population, would that be a solid or a liquid biopsy, in a parallel manner. We refer to this strategy here as parallel single cell analysis or PaSCAl [5]. For other diagnostic applications, only a few cells are available. For preimplantation genetic diagnostics (PGS), for instance, typically one or two blastomeres are isolated from the developing embryos for genetic diagnosis and screening [6]. Similarly, the analysis of fetal cells in maternal blood is conducted on a limited amount of cells [7], and in case of bacteremia, only a small number of bacteria must be detected and characterized from whole blood samples.

Microfluidics is a mature technology for the manipulation, processing, and high-sensitive analysis of minute amounts of sample [8], and, as such, it has opened new avenues in the field of single cell analysis [9, 10]. Specifically, microfluidic technology has enabled the development of a toolbox for experimentation at the single cell level [9]; for single cell manipulation and isolation; for cell characterization and/or analysis at the single cell level [11, 12]; for single cell engineering [13, 14]; for stimulation of individual cells, by taking advantage for instance of the unique flow properties at the microscale [15, 16]; or for single cell culture [17]. While different formats have been reported for the realization of microfluidic platforms for (parallel) single cell analysis, depending on the targeted

analysis scheme and the used readout technique, these platforms share the same common features. One particular essential function common to all platforms is the ability to manipulate, trap, and isolate individual cells, and different approaches have reported to that end [18]: using integrated microstructures [13, 19] for mechanical trapping of cells; electrical trapping of cells using either electrophoresis [20] or dielectrophoresis [21]; through the encapsulation of individual cells in nL-droplets [22]; using chemical patterns [23]; or using optical tweezers [16] (*see* **Note 1** for detailed examples of alternative platforms for parallel single cell analysis).

Here, we describe one specific microfluidic platform for parallel single cell analysis relying on mechanical trapping of cells using lateral microstructures. This platform includes a series of trapping sites for the capture of a series of individual cells (Fig. 1), and each trapping site is directly coupled to a separate side-channel for the in situ analysis of the cell molecular content. While the herein-reported platform, which comprises different layers of structures, is fabricated from an elastomeric material (PDMS or polydimethylsiloxane), similar structures can easily be produced from silicon, glass, or another polymer material. Interestingly, this platform is highly versatile since it is compatible with a great variety of analysis schemes, it lends itself well to the implementation of multistep analytical processes, and it is easily scalable for the analysis of 100's to 1000's of cells. For instance, after trapping, isolated cells can be stained in situ and subsequently imaged using bright-field or fluorescence microscopy, as well as high-resolution imaging. Similarly, cells can be exposed to soluble stimuli and their response followed in real time using imaging techniques. Alternatively, the cell membrane can be ruptured, and the intracellular content recovered in the individual channels located behind each trapping site for molecular analysis. Finally, several of these steps can be combined

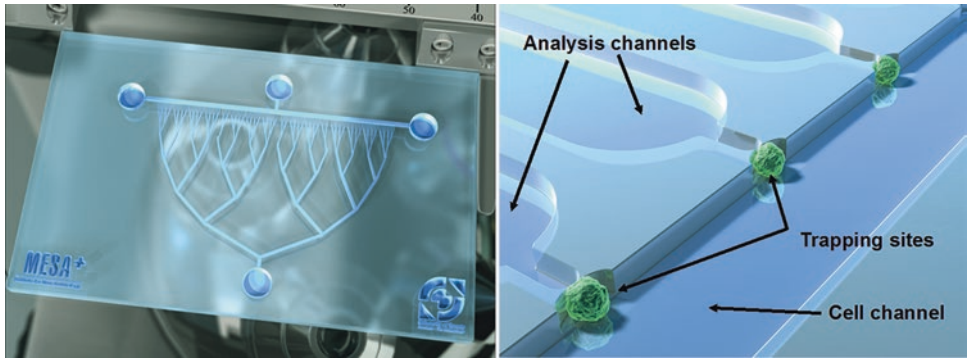

Fig. 1 Parallel single cell analysis—the concept. Artistic impressions of the microfluidic device comprising a main channel structure in which a cell suspension is introduced, and cells trapped in dedicated pockets present on one side of the channel. Each trapping site is connected to a separate side channel for eventual analysis of the cell molecular content. Artistic impressions by Nymus 3D

to access complementary information using different analysis strategies, or to identify changes at the molecular level after chemical stimulation of the cells. In this chapter, after a thorough discussion on parallel single cell trapping, different possible and independent steps of a complex analysis process are presented, and the utilization of various analysis protocols using both invasive and noninvasive approaches is demonstrated.

2 Materials

2.1 Cell Culture

1. Cells are grown in standard and commercially available culture medium supplemented with additives, as detailed below. The nature of the medium depends on the cell type.
2. P3X63Ag8 mouse myeloma cells are cultured in RPMI medium supplemented with 10 % v/v fetal bovine serum (FBS), 2 % v/v penicillin and streptomycin, 0.4 mg/mL fungizone, and 1 % v/v L-glutamine.
3. MCF-7 human breast cancer cells are cultured in DMEM medium supplemented with 10 % v/v fetal bovine serum (FBS), 2 % v/v penicillin and streptomycin, 0.4 mg/mL fungizone, and 1 % v/v L-glutamine.
4. A 1x trypsin solution in PBS buffer is employed to harvest MCF-7 cells for medium refreshment during culture or for cell sample preparation before experimentation.
5. Medium and serum are purchased from Invitrogen, and other supplements from Sigma-Aldrich.

2.2 Experimentation Buffer

All experiments are conducted in HEPES buffer (10 mM HEPES, pH = 7.4 composed of 10 mM HEPES, 140 mM NaCl, 2.68 mM KCl, 1.7 mM $MgCl_2$, 25 mM Glucose). All solutions to be inserted in the microfluidic device should be filtered (*see* **Note 2**). In some experiments, the experimentation buffer is supplemented with BSA (*see* **Note 3**).

2.3 Cell Staining (Off-Chip/On-Chip)

1. Various stains are employed in the experiments, for different purposes, as detailed below.
2. Calcein AM is first employed to visualize cells in the microfluidic devices. Next to this, this dye allows monitoring the rupture of the cell membrane, which is accompanied by leakage of Calcein out of the cells. Cells (typically 10^6 cells/mL) are stained using a Calcein AM working solution at 1 μg/mL (Invitrogen). This working solution is prepared by diluting 1000 times a Calcein AM stock solution (1 mg/mL in anhydrous DMSO) in culture medium.
3. Propidium Iodide (PI) is a cell-impermeable DNA-intercalating dye, which is widely used to detect damages in the cell membrane,

and therefore here cell membrane poration. PI is used at a concentration of 10 μg/mL. A PI working solution is prepared by diluting an aqueous stock solution (in DI water) in HEPES buffer (10 mM, pH 7.4).

4. The cell nucleus is stained in some experiments with Hoechst 33342 (Invitrogen). Hoechst is used at a concentration of 1 μg/mL for staining of a cell suspension at ca. 10^6 cells/mL, and a working solution is prepared by diluting 1000 times in culture medium a stock solution (1 mg/mL in anhydrous DMSO).
5. EpCAM is a receptor specifically expressed by cancer epithelial cells, such as MCF-7 cells, which can therefore be identified using a fluorescently labeled anti-EpCAM probe. A 1 μg/mL PE-anti-EpCAM solution in HEPES (10 mM, pH 7.4) is used to stain and identify cancer cells in a mixed MCF-7:P3X63Ag8 cell population.
6. In one series of experiments, the cell RNA content is stained with a selective probe, SYTO RNA Select. An intermediate SYTO RNA Select solution is prepared by diluting 1 μL of a stock solution (5 mM in anhydrous DMSO), in 1 mL of medium, which has been pre-warmed at 37 °C. Following this, the working staining solution is obtained by diluting the intermediate solution five or ten times with cell culture medium pre-warmed at 37 °C to yield a final SYTO RNA Select concentration of 0.5 or 1 μM.

2.4 Cell Fixation

One series of experiments is performed on fixed cells (*see* **Note 4**). Cell fixation is achieved here using an ice-cold methanol (−20 °C) solution (*see* **Note 5**), consisting of a 1:9 fresh PBS:methanol mixture, and which has been kept for at least 30 min at −20 °C.

2.5 Permeabilization/Lysis Experiments

1. Different approaches—relying on either physical or chemical means—are evaluated here for cell membrane permeabilization (*see* **Note 6**), for either permanent cell rupture or reversible membrane poration, as detailed in the following.
2. Cell chemical lysis is performed using a detergent solution based on 1 wt% LiDS (Sigma-Aldrich) in HEPES buffer (10 mM, pH 7.4).
3. Chemical (reversible) membrane poration is carried out using a 10 μg/mL digitonin solution in Ca^{2+}-free buffer (*see* **Note 7**), which is here HEPES buffer (10 mM, pH 7.4). The buffer is also supplemented with PI (10 μg/mL) for visualization of the cell membrane permeabilization.
4. Physical cell lysis relies here on the application of a high electric field across the cells. These experiments are conducted using a cell suspension in plain experimental buffer (HEPES 10 mM, pH 7.4)

2.6 Flow Visualization

A fluorescent solution of fluorescein sodium salt (Sigma-Aldrich) at a concentration of 1 mM in HEPES buffer (10 mM, pH 7.4) is utilized for visualization of the flows in the channels.

2.7 Microfluidic Device

1. All experiments are conducted using microfluidic devices fabricated from an elastomer material (PDMS or polydimethylsiloxane) which are bonded to a glass substrate.
2. All devices share the same main features (Fig. 2), which are: (1) a main channel, (2) a series of lateral cell trapping structures placed on one side of the main channel, and (3) a series of analysis or side-channels, each of them being connected to an individual cell trapping site.
3. In all devices, the main channel has a height of 30–50 μm and a width of 100 μm. This main channel is connected to one single outlet and to one or multiple inlets for the introduction of the cell suspension and chemicals.
4. Each device comprises 16 or 32 independent trapping structures and associated analysis channels. All trapping sites consist of a half-circular shaped pocket (radius of ca. 10 μm) having the same height as the main channel, and equipped with a shallow and narrow structure to prevent the cells from entering the analysis channel. All analysis channels are connected to one single reservoir in which a negative pressure is applied for cell trapping.
5. Two main designs (**Design 1** and **Design 2**) have been developed for different series of experiments and different purposes (Fig. 2); they share the same main features while exhibiting

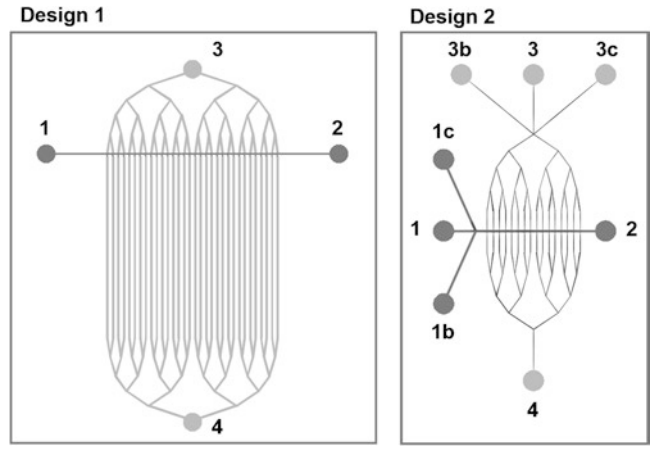

Fig. 2 Microfluidic device for parallel single cell analysis. Designs of the two devices utilized for the experiments presented here: Design 1 (*left*) and Design 2 (*right*) (see text for more explanations). Reservoirs are numbered, and these numbers are referred to in the text

small alterations regarding the design of the trapping sites and associated analysis channels, as discussed in the following.

6. **Design 1** (Fig. 2, left) uses a 2-layer fluidic structure [24].The height of the analysis channels is the same as that of the constrictions behind the traps, which is <5 μm (*see* **Note 8**). The analysis channels are made relatively wide (e.g., 100-μm width) to avoid high pressure drops in the analysis channels (*see* **Note 9**). In this design, the constricted section has a length of 30–40 μm and a width of 4–7 μm.
7. **Design 2** (Fig. 2, right) relies on a 3-layer fluidic structure [5]. The constriction behind the trap presents a height of 2 μm only to ensure cells cannot squeeze themselves into the analysis channel upon trapping. The analysis channel has a height of 10 μm and a width of 10 μm. In design 2, the length of the constricted section ranges from 0 to 30 μm.
8. All microdevices include, next to the array of analysis channels, a mirrored array of side-channels on the other side of the main channel, and these mirrored channels are also connected to one common reservoir. For cell electrical lysis, external electrodes are inserted in the common reservoirs of these two arrays of side-channels, and the electrical path between the electrodes for each cell is equivalent, which allows applying the same voltage on each cell.
9. Fig. 2 presents the two main designs used for the experiments presented in this chapter, and reservoirs have been numbered for both designs.

2.8 Experimental Setup

1. All experiments are conducted on an inverted microscope (Olympus IX51) equipped with an epi-fluorescence unit, suitable filter cubes, a camera (ColorView II, Olympus Soft Imaging Solutions), and connected to a computer for monitoring of the experiments and image acquisition. Dedicated software (AnalySIS docu 5.0) is used for image acquisition.
2. Fluids are actuated in the microfluidic system using different pieces of equipment, as detailed in the following.
3. Cell trapping in the microfluidic device is monitored using a pressure-controlled system (Maesflo, Fluigent) operated in the negative pressure range and controlled via dedicated software (Maesflo 4c v0.6.1).
4. Chemicals are introduced in the microfluidic device at a given flow-rate in the main channel, using a syringe-pump (Harvard PHD 22/2000, Hugo Sachs Elektronik-Harvard Apparatus GmbH), which is used in the suction mode.
5. A HV source (IBI 411, IBIS Technologies BV) is employed for cell electrical permeabilization and establishment of an

electro-osmotic flow in the analysis channel for cell content retrieval. The HV is applied using Pt electrodes, which are inserted in the reservoirs 3 and 4 of the microfluidic device.

3 Methods

3.1 Device Fabrication

The microfluidic devices are fabricated using soft-lithography techniques from an elastomer material (PDMS or polydimethylsiloxane). First, a silicon/SU-8 mold is fabricated in the cleanrooms of the MESA+ Institute for Nanotechnology and treated with a hydrophobic FDTS (1H,1H,2H,2H-perfluorodecyltrichlorosilane) coating to facilitate the release of the PDMS structures. Next, a 10:1 PDMS pre-polymer:curing agent mixture is prepared, thoroughly degassed and poured on the mold. PDMS is cured overnight in an oven at 60 °C. After curing, the PDMS structures are released from the mold, and reservoirs are punched with a sharp blunt needle. The PDMS layer is subsequently cleaned with isopropanol, carefully dried and exposed to oxygen plasma for its activation before bonding to a clean glass substrate. Figure 3 presents the picture of a microfluidic device (Design 2), as well as an enlarged view of one trapping structure (Design 2).

3.2 Device Preparation

Before experimentation, the microfluidic channels are incubated with a filtered BSA solution (5 % in HEPES buffer, 10 mM, pH 7.4) for 2 h to prevent undesired cell adsorption on the channel walls, followed by thorough rinsing with HEPES buffer.

3.3 Cell Culture and Cell Sample Preparation

1. Cells are cultured in their respective medium (RPMI for P3X63Ag8 and DMEM for MCF-7 cells), and the medium is typically refreshed twice a week. Specifically, for P3X63Ag8 cells, the cell suspension is collected from a standard T25 culture flask, centrifuged to remove dead cells and debris and ca.

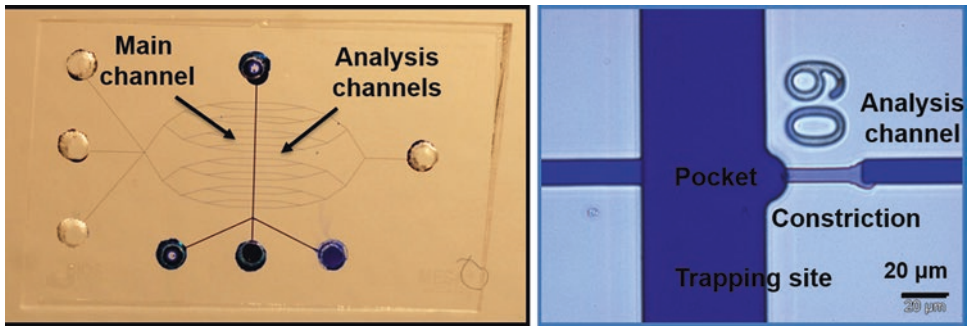

Fig. 3 Microfluidic device for parallel single cell analysis. Pictures of an actual device (Design 2) (*left*) whose microfluidic structures have been filled with *blue ink* for visualization purpose and of one trapping site (*right*), showing the three-layer structure

one sixth of the cells is resuspended in 5 mL of fresh medium. MCF-7 cells, which are adherent cells, are first washed with PBS, and detached from the culture flask surface using 1 mL of 1x trypsin for 2 min at 37 °C. Following this, the resulting cell suspension in PBS is treated in the same way as the P3X63Ag8 cells.

2. Depending on the experiments and the specific targeted goal, cells are exposed to different preparation steps, as discussed in the following for three specific experiments.
3. First, experiments are conducted both on live cells and fixed cells (*see* **Note 4**), fixation being performed prior to the introduction of the cells in the microfluidic device. To that end, cells are slowly and carefully resuspended in an ice-cold 1:9 PBS:methanol solution while gently pipetting to avoid cell aggregation. After ca. 10 min incubation at 4 °C, the cells are washed with cold PBS at least twice, and resuspended in experimentation buffer.
4. For cell trapping experiments, cells are stained with Calcein and/or Hoechst, these dyes being used to easily visualize the cells in the channels and trapping structures. In one series of experiments, these two dyes allow distinguishing two cell populations. Specifically, when a mixed MCF-7:P3X63Ag8 cell sample is used, one cell type is stained with one dye (Hoechst only, here) while the other cell type is stained with both probes (Hoechst & Calcein). For both dyes, the staining procedure is the same: the cell sample is resuspended in the staining solution, and incubated for 30 min at 37 °C. Thereafter, the cells are washed at least twice in HEPES buffer (10 mM, pH 7.4) to remove the excess of dye.
5. For cell lysis and cell content recovery experiments, cells are only loaded with Calcein AM using the same protocol as described above. Calcein is utilized here to probe pore formation in the membrane, which gives rise to Calcein leakage out of the cells, and allows monitoring cell content retrieval in the analysis channels.
6. One specific series of experiments aims at recovering the nucleic acid content of the cells. For these experiments, cells are exposed to a dual staining procedure using Hoechst 3342 and SYTO RNA Select, to separately label their DNA and RNA contents, respectively. Typically, 1 mL of cell solution is resuspended in the staining solution, which consists of 1 mL of warm medium containing the SYTO RNA Select probe and to which 1 μL of Hoechst stock solution is added. After 30–45 min incubation, the cell sample is washed 1–2× with buffer. This dual staining protocol can be applied to both living and fixed cells.

3.4 Cell Loading

1. Cells are loaded in the device using the passive pumping technique [25] (*see* **Note 10**). Specifically, a 15 μL droplet of HEPES buffer is introduced in reservoir 2, and a 1.5 μL droplet of a cell suspension in HEPES buffer in reservoir 1. Thereby, a flow is created from reservoir 1 to reservoir 2, and cells are transported with the flow in the main channel with a mild velocity of 100–200 μm/s.
2. Alternatively, the cell suspension can be deposited in one inlet reservoir, and suction applied using a syringe pump connected to reservoir 2 to create a constant and well-defined flow in the main channel.

3.5 Cell Trapping

While loading the cell suspension (10^6 cells/mL) in the microfluidic device and the main channel, a mild negative pressure (−30 mBar) is applied from the common back reservoir (#4) to attract cells in the different trapping sites, using a Maesflo pressure controller. The applied pressure is controlled via dedicated software with a great precision (as low as 0.345 mBar) and can easily be fine-tuned, depending on the exact design of the microfluidic structures. Once a cell is captured in a trap, the trap is blocked provided there is a good sealing between the cell and the trap, so that no other cell can be attracted in the trapping site. Once all traps have been filled with cells (ideally with one single cell), the pressure is reduced and maintained to −10 mBar to keep the cells in the traps, while flushing other solutions in the main channel.

Typically, using these conditions, parallel cell trapping is achieved within 2–5 min in a device equipped with 16–32 traps, and both proposed designs (**Design 1** and **Design 2**) yield similar trapping results. Figure 4 shows an array of individual cells trapped in a microfluidic device (Design 2). The trapping efficiency is characterized here with respect to the number of cells isolated per trapping site (0, 1 or more cells) (*see* **Note 11**). For both designs, the single cell trapping yield is high, and typically amounts to 70 % (as tested for several devices and different experimenters).

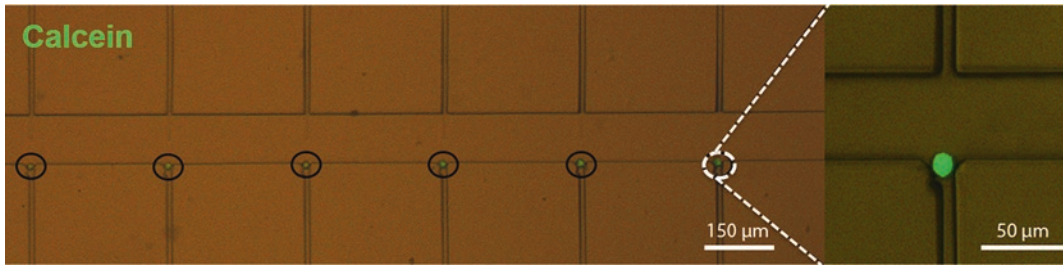

Fig. 4 Parallel single cell trapping. Picture of a portion of the main channel (Design 2) showing the trapping of a series of seven individual cells in separate trapping sites (*left*) and enlarged view of one captured cell. Cells are stained with Calcein for visualization purposes

Interestingly, the exact design of the trapping structure is found to have an effect on the single cell trapping yield: the shortest constriction structure gives the best single cell trapping yield (85 %), and other constrictions lengths result in similar trapping yields. Next, in typically 10–20 % of the trapping sites, multiple cells are found [5].

For in vitro diagnostic applications, or more in general, for medical analysis, the cell sample to be analyzed is in nature heterogeneous, and comprises cells of different sizes. To simulate a heterogeneous sample consisting of cells with different sizes, and to demonstrate the ability to work with mixed cell populations, a 1:1 MCF-7:P3X63Ag8 cell sample is prepared off-chip. Prior to this, MCF-7 cells are stained with both Hoechst and Calcein, while P3X63Ag8 cells are stained with Hoechst only before mixing the cells in a 1:1 ratio. The cell trapping efficiency and single cell trapping yield is not altered when working with mixed samples comprising two types of cells with slightly different sizes. Furthermore, this experiment confirms the suitability of the proposed device to handle mixed cell populations, which is particularly attractive in the future for the analysis of CTC samples that always contain residual WBCs.

3.6 On-Chip Cell Staining

After trapping in the herein presented microfluidic device, cells can be exposed to various soluble factors in a highly controlled manner by taking advantage of the main microfluidic channel, for their chemical stimulation or their staining. The latter approach allows detecting or identifying targeted cells in a mixed cell population, using specific (fluorescent) staining agents. For instance, cancer cells can be identified in a heterogeneous population using EpCAM (Epithelial Cell Adhesion Molecule), a marker expressed by epithelial cancer cells only, and this approach has proven to be powerful to identify CTCs after their purification from blood, or to detect CTCs in complex samples [26].

Here, to demonstrate the possibility to identify specific cells in a mixed population in our microfluidic device, a heterogeneous cell sample combining EpCAM-positive MCF-7 cancer cells and EpCAM-negative P3X63Ag8 cells in a 1:1 ratio is prepared and trapped as described above. Following this, and while maintaining a mild negative pressure (−10 mBar) from the back reservoir (#4), a staining solution containing Hoechst and PE-Anti-EpCAM is injected in the main channel for on-chip cell staining. After 30–60 min incubation with the staining solution followed by on-chip cell washing with HEPES buffer, cells are imaged in the device using fluorescence microscopy. As shown in Fig. 5, after on-chip staining, MCF-7 cells exhibit both blue and red labels, for Hoechst and PE, respectively, while P3X63Ag8 are only labeled with Hoechst (blue label).

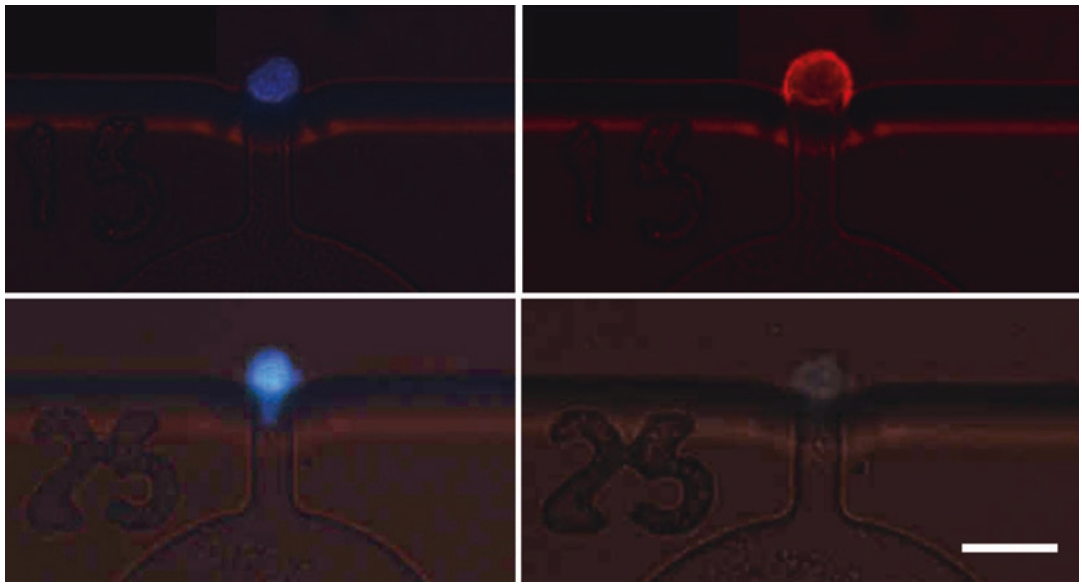

Fig. 5 In situ cell staining in the microfluidic device to identify cells in a mixed cell population. Enlarged view of two cells (P3x63Ag8 (*Bottom*) and MCF-7 (*Top*)) Differential staining of P3x63Ag8 and MCF-7 cells in a device, using Hoechst (*Left*) that label all cells, and an PE-conjugated antibody aiming at the membrane marker EpCAM (*right*) which is solely expressed by epithelial cancer cells (MCF-7 cells, here) [5]. Figure reprinted from [5] John Wiley and Sons with authorization

3.7 Cell Permeabilization/Lysis

1. In a second possible and more invasive analytical scheme, the cell membrane is ruptured to access the intracellular content. Different approaches are conceivable for cell membrane permeabilization, e.g., for reversible/temporary pore formation and sampling from the cell or irreversible cell poration, and using either chemical or physical means (*see* **Note 6**). Importantly, all these different approaches are compatible with the herein-proposed microfluidic platform, as detailed in the following.
2. For reversible permeabilization of live cells, the non-ionic detergent digitonin is utilized (*see* **Note 7**). Digitonin forms a complex with cholesterol present in the plasma membrane, and upon aggregation of several digitonin-cholesterol complexes, a membrane-spanning pore is formed [27] with a diameter around 8–10 nm. Here, the digitonin solution is applied in reservoir 1 (inlet), and pumped at a constant flow-rate using a syringe-pump connected to reservoir 2, while maintaining a mild negative pressure (−10 mBar) from the common back reservoir (#4) using the Maesflo system to prevent cells from escaping the trapping structures. The digitonin solution is supplemented with the cell-impermeable fluorescent probe, PI, to study the process of cell membrane poration. Upon direct exposure to digitonin, the cell opens up, becomes positive to PI while slowly swelling, which reflects the differences in osmolarity

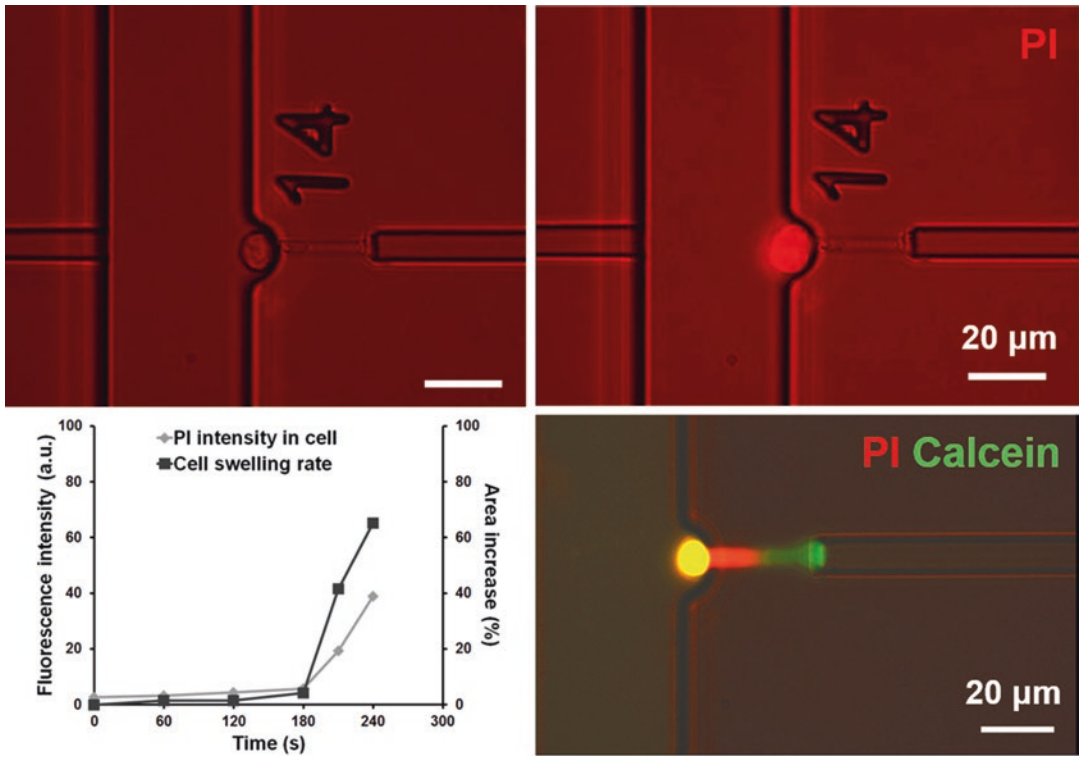

Fig. 6 Chemical (reversible) poration using digitonin of P3x63Ag8 cells in a microfluidic device (Design 2). Pictures of an individual cell in a trapping site during the permeabilization process using 10 µg/mL of digitonin in HEPES buffer (10 mM, pH 7.4). The buffer is supplemented with PI to monitor the process of cell membrane poration. The pictures taken at 0 s (*top left*) and 240 s (*top right*) indicate swelling of the cell and entry of PI in the cell after exposure to digitonin, which is also quantified over time for this particular cell (*bottom left*). Image of a cell previously loaded with Calcein, which is tightly secured in a trapping site, and simultaneously exposed to digitonin and PI as before. Interestingly, no release of Calcein is detected and PI slowly diffuses into the cell from its main channel side. Figure adapted from John Wiley and Sons with authorization [5]

in the buffer and intracellular medium (Fig. 6). Furthermore, after typically 3 min exposure, cell swelling and PI uptake dramatically increases (Fig. 6, bottom left) PI being still mostly found on the side of the cell facing the main channel, where digitonin is introduced. Surprisingly, no significant leakage of Calcein, which was loaded into the cells prior to experiments, is observed.

3. In a second series of experiments, cells are chemically lyzed via their exposure to a detergent solution, which induces dissolution of their cytoplasmic membrane. As a detergent, LiDS is used here: LiDS is milder than SDS, so that the process of cell lysis is slower, which allows progressive recovery of the cell content in the side channels. Furthermore, LiDS has been reported to be selective to the cell cytoplasmic membrane and to not induce lysis of the intracellular membranes, which is

particularly interesting for the separate isolation and analysis of the cell cytoplasm and its organelles. The LiDS solution (concentration of 1 wt%) is flushed in the main channel while maintaining a negative pressure from the back reservoir, not only to keep the cells in the trap, but also to possibly isolate the cell content in the analysis channels. In this experiment, P3X63Ag8 cells, which have previously been fixed and stained with Hoechst and RNA SYTO Select, are used. First, as in the presence of digitonin, the cell is swelling upon exposure to LiDS. Furthermore, and more interestingly, the nucleus seems to remain intact after exposure to LiDS 1 wt%, while the cytoplasm content is found back in the analysis channel, which is evidenced, respectively, by the remaining blue stain in the cells, and the presence of green dye in the channel in the vicinity of the trapping site (Fig. 7).

4. In a last experiment, the cell membrane permeabilization is demonstrated using an electric field (*see* **Note 12**) and the so-called technique of electroporation [28]. External electrodes are inserted in reservoirs 3 and 4, for the application of a HV across the cells captured in the trapping sites for their permeabilization, as well as in the analysis channels for cell content extraction (*see* **Note 13**). Typically, voltages of 200–300 V are applied (*see* **Note 14**). Such voltages give rise to an electro-osmotic flow velocity in the side channels of 10's μm/s (measured velocity of 78 μm/s upon application of 300 V), and to a theoretical voltage drop across the trapping sites higher than 1 kV/cm, as required for the creation of pores in the cell membrane. Within less than one second, cell membrane poration is observed, as evidenced by the release of Calcein, which has previously been loaded in the cells. More importantly, under these conditions, the intracellular content is selectively extracted in the analysis channels (Fig. 8).

3.8 Cell Content Retrieval

1. Two approaches have been explored to retrieve the cell content in the analysis channels during lysis for further cell content analysis (*see* **Note 15**).
2. In a first approach, demonstrated here in combination with the chemical lysis of cells using LiDS (see Subheading 3.7 **step 3** above and Fig. 7), the cell content is selectively extracted in the analysis channels by applying a mild negative pressure from the common suction port (#4) while exposing the cell to the detergents.
3. In a second approach where lysis occurs upon application of an electric field between reservoirs 3 and 4 and across the cell (see Subheading 3.7 **step 4** above), the same electric field allows creating an electro-osmotic flow in the side-channels, which is used here for the controlled transport of the cell content in the analysis channels (*see* **Note 14e** and Fig. 8).

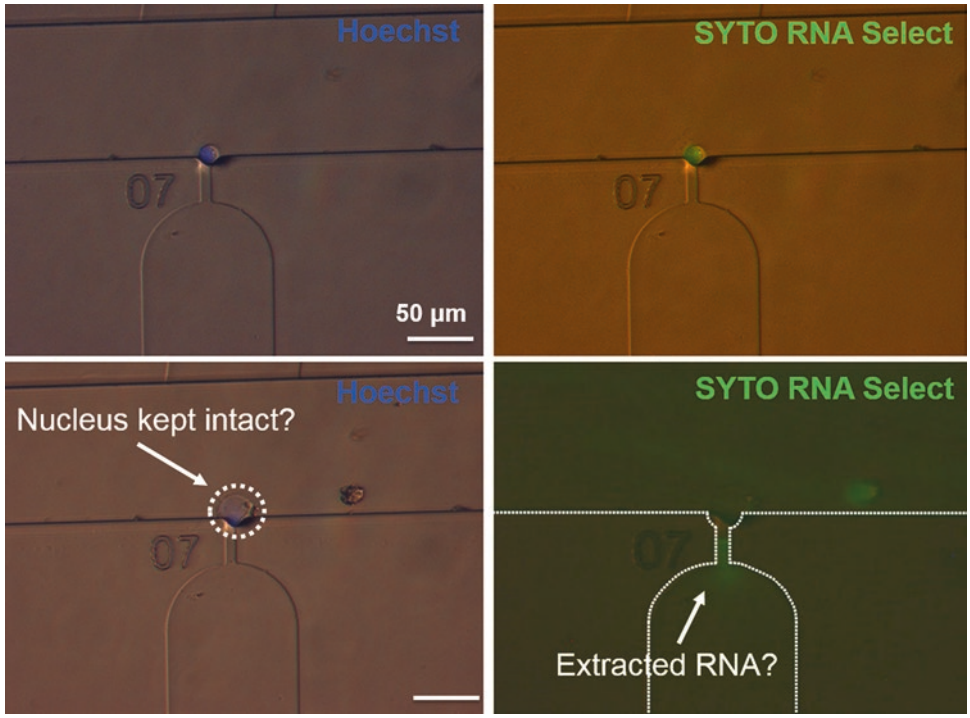

Fig. 7 Chemical permeabilization sing LiDS of P3x63Ag8 cells in a microfluidic device (Design 1) combined with pressure-based extraction of the cell content in the analysis channels. Pictures of an individual cell in a trapping site before (*top*) and after (*bottom*) the permeabilization process using 1 wt% LiDS, extracted from a time-lapse movie. Cells were stained with both Hoechst (*left*) and RNA SYTO Select (*right*) prior to their injection in the microfluidic device and their trapping, to monitor both processes of cell lysis and cell content recovery. The pictures after lysis (*bottom*) show swelling of the cells upon exposure to LiDS for ca. 60 s, together with extraction of the RNA in the analysis channel, and seem to indicate that the nucleus has been kept intact

4 Notes

1. Other platforms and approaches have been reported for (parallel) single cell analysis that rely on different strategies for single cell trapping. A first platform aiming at parallel single cell analysis consisted of an array of microfabricated wells [19, 46] in which individual cells could be trapped, provided optimization of the structure sizes and of the seeding protocol. In an alternative approach, a 2D array of lateral trapping structures was placed in a microfluidic chamber [29, 47]; this array was demonstrated for live cell imaging and monitoring of specific biological processes.
2. All solutions to be injected in the microfluidic devices must be carefully filtered beforehand to remove all debris that could easily clog the small features as found here in the trapping structures. In our experiments, we did observe clogging of

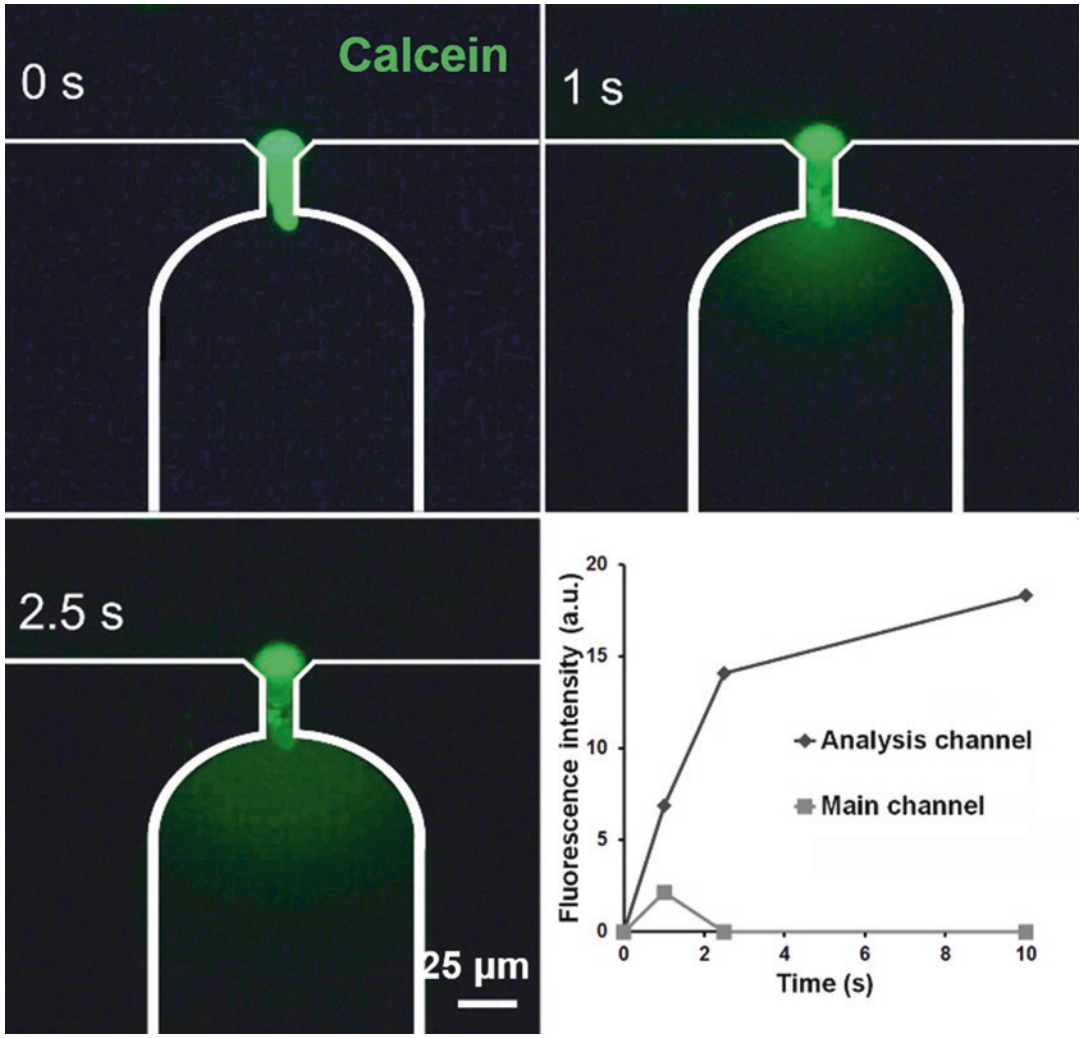

Fig. 8 Electrical permeabilization of P3x63Ag8 cells in a microfluidic device (Design 1) combined with EOF-based extraction of the cell content in the analysis channels. Pictures extracted from a time-lapse movie, illustrating the cell membrane poration and simultaneous recovery of the cell content in the analysis channel (Top *right* and *left*, and *bottom left*). Cells were stained with Calcein prior to their injection and trapping in the device to monitor both processes of membrane poration and content extraction. Variations in the green fluorescence intensity as a function of time in the analysis channel and main channel for this particular cell, on which a HV of 300 V has been applied (*Bottom right*). Figure reprinted from [5] John Wiley and Sons with authorization

some traps, even after careful filtration, which contributed to a decrease in the trapping yield.

3. If no special care is taken, cells in suspension tend to aggregate to form clusters. Cell aggregation results in trapping of multiple cells and not of individual cells, as targeted. To avoid this, cells in the solution must be resuspended through careful pipetting. Alternatively, the cell experimentation buffer can be

supplemented with a few % of BSA or accutase to prevent cell aggregation.

4. For most applications, live cells are preferred, especially for imaging-based monitoring of cellular processes [29]. However, fixed cells can be more advantageous than live cells: they are easier to trap since their membrane has been made more rigid.
5. For cell fixation, ice-cold methanol is preferred here since it does not affect intracellular biomolecules, as formaldehyde would do.
6. Two lysis schemes have been tested and validated here, using detergents (chemical lysis) and an electric field (physical lysis). Other schemes are also possible, such as the use of a laser pulse which induces the formation of a cavitation bubble [32, 33], surface acoustic waves [34], or mechanical lysis using sharp nanostructures [35] or beads. In general, physical means (e.g., electric field, sonolysis, etc.) are preferred since they can be made cell-specific. Lysis also occurs on a faster time scale than when using chemicals, which is particularly interesting to study fast processes in cells such as signaling pathways [36]. Furthermore, loss of biological materials through diffusion is limited. Finally, chemicals represent a source of contamination, and their use results in an overall dilution of the cell content sample.
7. The process of pore formation using digitonin is sensitive to the presence of calcium ions: pore formation induced by digitonin only occurs when no Ca^{2+} is present in solution [31], so that the buffer should not comprise any calcium ions. Interestingly, digitonin-induced pore formation is reversible and the addition of a calcium salt in solution assists resealing of the pore [31], a capability that has not been explored here, but would allow sampling out of cells in a seamless manner.
8. Different types of trapping structures have been tested here, with several alterations in their design (e.g., number of layers of structures, exact dimensions of the constricted section, etc.). In general, a compromise must be found between the ease of fabrication and cell trapping efficiency: larger structures are easier to produce, but they let more cells go through. In contrast, small structures are advantageous as discussed below (*see* **Notes 16** and **17**) since they offer better sealing of the cells in the trapping sites. Working with fixed cells allows the use of capture sites with larger dimensions, while experimentation using live cells calls out for smaller dimensions.
9. All devices reported here have been fabricated using PDMS. This elastomeric material is particularly attractive for prototyping, and the production of single-use devices, especially in an academic environment. However, PDMS suffers from a number of limitations. For instance, in Design 1 (Fig. 2, left), the analysis channels have a high aspect ratio (100-μm width and

2-μm height), and their dimensions were chosen to avoid cell squeezing in the analysis channels while limiting the pressure drop in these channels. However, when no particular care was taken, channel collapse was observed during bonding of the devices, and the analysis channels were "lost." Similarly, the realization of small structures can be challenging due to the thermal sensitivity of the PDMS material. This thermal sensitivity becomes a significant issue, if the PDMS fluidic layer must be aligned to other structures on the glass substrates. For instance, for cell electrolysis, integrated electrodes would be preferred (*see* **Note 13**), which is not conceivable when using PDMS. Last but not least, extraction of the cell content in the analysis channels relies in one series of experiments on the establishment of an electro-osmotic flow (EOF). However, the surface charges on the PDMS walls are ill-defined, so that the resulting EOF flow was not well controlled and subsequently not reproducible.

10. Cells have been introduced in the system using two main approaches, both involving the deposition of the cell suspension in the main inlet reservoir of the device (#1) and creation of a flow from the main outlet reservoir (#2): either using the passive pumping technique [25] or using a syringe-pump that allows the establishment of a steady flow in the main channel. Alternatively, cells could be inserted using a pumping system connected to the inlet reservoir, such as a syringe-pump or a pressure-controlled system (e.g., Maesflo system operated in the positive pressure mode). However, the latter approaches suffer from one major issue: cells tend to sediment in syringes or vials, so that active shaking is required to limit this. Therefore, here, we preferred using strategies involving aspiration of the cells from the inlet reservoir (#1).
11. Here, the trapping yield was defined with respect to the number of trapping sites present in the device and using the number of traps in which one or multiple cells were captured. Typically, for each experiment, 100's to 1000's of cells were injected in the device. However, for in vitro diagnostic applications, when a reduced number of cells is available, e.g., a handful of cells for CTC analysis, all cells injected in the device must be captured. Therefore, the trapping yield should be corrected for the number of cells injected in the device, and the trapping protocol must be altered to ensure every single cell inserted in the device is captured.
12. Electrical lysis has been demonstrated here. Compared to other physical lysis techniques, the process can be made reversible or irreversible by tuning the electric field parameters (amplitude of the signal, or time duration of the treatment). Furthermore, while applying an electric field for rupturing the cell membrane, an electro-osmotic flow is created in the side channels, which means that the cell membrane permeabilization

can directly be combined with the recovery of the cell content for further analysis.

13. Here, to create an electric field, external electrodes are inserted in the reservoirs 3 and 4 of the device and all trapping sites are exposed simultaneously to the same electric field. Using integrated and individually addressable electrodes in the future would allow making the process cell-specific, while using much lower voltage to reach the kV/cm threshold required for cell membrane poration [13]. It should be however noted that integrating the electrodes in the device would most probably imply changing materials to fabricate the devices, since the alignment of electrode structures with a series of micrometer-sized trapping structures using PDMS would be highly challenging or even not possible (*see* **Note 9**).
14. A DC voltage a few 100's V has been applied in these experiments for both cell electrical lysis and creation of an electro-osmotic flow. Under these conditions, the cells were porated but not entirely lyzed. A better strategy would rely on the use of a series of short pulses of a higher voltage for lyzing the cells, followed by a much lower DC signal for the EOF.
15. While experiments presented here are limited to cell trapping followed by cell staining and exploration of different permeabilization approaches, the herein-proposed platform is ideally suited for a great variety of analytical schemes. Cell imaging was demonstrated here to recognize cancer cells in a mixed population, but it can easily be extended to in situ FISH analysis, for instance, to determine gene copy numbers in individual cancer cells [37] or to examine possible aneuploidy or detect chromosome imbalance in blastomeres [38]. Imaging could also be performed using label-free and less invasive techniques such as Raman spectroscopy [39], provided the bottom substrate is replaced for CaF_2. After trapping, cells could alternatively be exposed in a controlled way to various drugs or chemical stimuli, and their response recorded in situ in the device, e.g., using time-lapse imaging, or after cell lysis to study specific changes in their intracellular content [40]. After cell permeabilization and retrieval of the content of individual cells in separate side channel, molecular analysis can be performed, e.g., to examine gene expression profiles using RT-PCR [41] and/or integrated mRNA microarrays, to unravel mutation patterns in the genome of individual cells using whole genome amplification [42], to examine the activity or expression level of specific proteins using enzymatic assays [43], capillary electrophoresis (CE) [44] or even integrated antibody arrays [45].
16. In these experiments, two trapping modes were observed, while using the same conditions for trapping and the same trapping structures. Specifically, cells could be either sitting in

front of the traps, or partially sucked in the traps. While no specific parameters could be identified that would influence the way cells would be positioned in the capture site, this trapping mode has an impact on the overall analytical process: for instance, on the risks to capture multiple cells in one site, or to release cells when applying a flow in the main channel; on the efficient recovery of the cell content in the analysis channels without any risk of loss or contamination; or on the efficiency of the electrical lysis.

17. Cells are trapped here by applying a negative pressure from the common back reservoir [4] to attract cells in the trapping sites. While this approach is preferable to have good sealing of the cells in the trapping structure that is important for multiple reasons (*see* **Note 16**, above), it also requires the use of smaller structures to limit cell loss through their passage as a whole in the analysis channels (*see* **Note 8**). Specifically, our experiments revealed that the constricted section should be no higher than 2–3 μm when working with live cells. In another approach, inspired by the work of Seki's group [30], a focusing flow could be used in the main channel to assist cell capture by pushing individual cells in the trapping sites. This milder approach is preferred to maximize the trapping yield, with respect to the number of cells available for the analysis and injected in the device, and to limit the stress applied on the cells. However, using this approach, there exists a risk that cells are not tightly trapped, which raises other issues, as discussed above (*see* **Notes 8** and **16**).

While these bottom and lateral trapping approaches are easily scalable for the capture of thousands of cells, they are exclusively limited to imaging of cells. These two platforms have therefore been upgraded to become compatible with intracellular molecular analysis, through the confinement of each individual cell in a closed compartment. For the former platform, a glass microscope was placed on the microwell array, after cell trapping to create a series of closed microreactors. This strategy has, for instance, been reported by Gong et al. for targeted gene expression analysis in 125 pL microchambers [48] using RT-PCR. Compartmentalization can also be achieved by using microchambers and/or valves realized by soft lithography. Individual cells have been brought into nL-chambers with the help of an optical tweezer [49], and lyzed in situ. In this device, the capture chamber was decorated with an antibody array for analysis of the number of protein copy number (here, for p53) in the lysate of the individual cells. Entirely closed chambers with a well-defined volume can easily be created using PDMS valves, which allow enclosing individual cells. Marcus et al. have, for instance, pioneered this strategy for mRNA analysis from individual cells [41].

More recently, Yang et al. reported whole genome amplification from individual cells in such microchambers for eventual genome sequencing and mapping of mutation patterns, at the single cell level [42]. In an alternative approach, the actuation layer comprised a more complex doughnut-like structure, which was pushed down to yield a closed or partially closed chamber [50]. Cells trapped in this chamber were lyzed chemically and their NADPH content analyzed in situ.

Individual cells can also easily be isolated in (sub)-nL droplets produced in a continuous immiscible oil phase [22] in a very high-throughput manner (up to 1000 droplets per second). In a seminal paper, Brouzes et al. demonstrated the potential of this droplet microfluidic platform for screening of a library of drugs on individual cells [51]. More recently, the same strategy has been applied to monitor drug uptake and cell viability in cells either sensitive or resistant to a specific treatment [52].

Finally, in a radically different approach, high-throughput single cell analysis was achieved in a flow-through manner, using optical lysis of individual cells at a channel intersection followed by online CE separation of their content and detection of targeted compounds using fluorescence microscopy [53], to eventually elucidate alterations in kinase activation and intracellular signalization pathways [36].

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