A new design of a macro-to-micro interface that can be used for simple and reliable control of comprehensive microfluidic cell culture processes is introduced making microfluidic procedures easily accessible to biological laboratories. The novel macro-to-micro interface is evaluated by adapting a workflow for single-cell, cell pair, and cell cluster encapsulation into hydrogel beads acting as 3D microenvironment with subsequent long-term cultivation. For the first time, the coupling of single-cell time-lapse microscopy data with phenotypic (immunofluorescence) and genotypic (single-molecule RNA fluorescence in situ hybridization) endpoint measurements as well as downstream compatibility in a single chip is shown. The presented platform will be a valuable tool for performing studies of dynamic biological processes coupled to a multiparametric endpoint characterization at the single-cell level as well as for gaining more detailed mechanistic insights into gene function relationships and the behavior of biological systems.

1. Introduction

Microfluidic systems have been used in several eukaryotic cell culture applications such as analyzing tumor organoids,[1,2] cell–cell interactions,[1,4] single-cell immune dynamics,[3] and the analysis of stem cell behavior including differentiation studies[6,7] but also for the single-cell culture of prokaryotes.[8–10] Although these studies show the potential of microfluidics for cell culture applications, microfluidic procedures are still difficult to implement in biological laboratories without intensive investments into equipment and personal resources. In particular, microfluidic devices for performing long-term cultivation of cells are often error-prone due to technical issues such as bubble formation, leakage problems occurring during the cultivation period, the possibility to work under fully sterile conditions, complicated handling due to a large number of tubing connections, and the lack of compatibility with established laboratory infrastructure and bioassays. Performing experiments in desired cell culture microenvironments (2D or 3D model) possesses another additional challenge. The physiological relevance of a 3D microenvironment has been extensively studied elsewhere.[11,12] Recent studies show that, especially for clinically relevant processes, 2D cell culture systems have limitations as they result in abnormal phenotypes.[13]

In addition to the biological issues, major technical hurdles exist with 2D cultivation in available microfluidic devices. These hurdles include immobilization of non-adherent cells such as cells derived from the hematopoietic system to prevent cell loss during medium exchange and detaching adherent cells from the chip for downstream analysis. Both procedures can substantially alter the inherent cell phenotype.[14,15] In comparison, 3D cell culture models gained significant relevance in the past years due to their biocompatibility, tissue like water content, high porosity, permeability, and in mimicking mechanical properties of the extracellular matrix resulting in a higher physiological relevance.[16] Despite its biological advantages, 3D cell culture hampers the combination of time-lapse data with endpoint measurements such as immunostainings as the hydrogel itself acts as a diffusion barrier. This diffusion barrier impedes supply with fresh nutrients, removal of waste products, and the implementation of efficient washing processes for endpoint staining protocols. In addition, embedding cells into macro 3D matrices complicates cell retrieval after cell cultivation as the hydrogel has to be removed enzymatically or chemically to release embedded cells.[17]

In this work, we evaluate a new design of a macro-to-micro interface that can be used for simple and reliable control of microfluidic processes including comprehensive cell culture processes. The presented macro-to-micro interface overcomes significant technical challenges thereby making microfluidic cell culture procedures accessible for biological laboratories. By integrating a workflow that has been described previously,[6] we overcome mentioned limitations and are providing a valuable tool for cultivating single cells, cell–cell pairs, and low cell numbers in spherical hydrogel beads acting as a 3D microenvironment. At the same time, we combine time-lapse (fluorescence) microscopy data with endpoint measurements that provide...
additional phenotypic (immunofluorescence, IF) and genotypic (single-molecule RNA fluorescence in situ hybridization, smRNA FISH) information. Additionally, we evaluate cell retrieval efficiencies for subsequent downstream analyses after performing time-lapse microscopy and endpoint stainings. We show the potential of our system by using it for the encapsulation of breast cancer cells (human mammary gland cells; MDA-MB-468, MDA-MB-231, and MCF7) and primary cells (human umbilical vein endothelial cells, HUVEC) into spherical hydrogel matrices, their cultivation, time-lapse fluorescence analysis, and endpoint analysis using IF staining and smRNA FISH as well as endpoint retrieval.

The presented macro-to-micro interface and its combination with a new microfluidic cell culture format overcomes significant limitations of conventional 2D and 3D cell culture systems and provides an important tool for in-depth analysis of biological systems and for deciphering gene function relationships at a single-cell level.

2. Results

2.1. Basic Design of Macro-to-Micro Interface and Cartridge Holder

The macro-to-micro interface is represented by a microfluidic cartridge that is controlled using a cartridge holder containing an electric and pneumatic interface for precise process control. The cartridge holder is an incubation chamber that acts as a closed cell incubator without the need for an additional environmental chamber.

The microfluidic cartridge which acts as a macro-to-micro interface consists of a computerized numerical control (CNC)-milled three-layer poly(methylmethacrylate) (PMMA) manifold, a microstructured polydimethylsiloxane (PDMS) membrane with punched holes and a glass substrate acting as a solid support for generating a planar focus plane (Figure 1). Moreover, the glass substrate acts as an optical medium with a defined thickness and defined optical characteristics which are required to be compatible with established imaging set-ups. The CNC-milled PMMA part has in total eight inlet reservoirs and four outlet reservoirs which can be used for supplying various types of liquids such as aqueous fluids, oily fluids, or cell suspensions to microfluidic structures. Fluid flows can be controlled by applying a controlled pressure to the headspace of the reservoirs (Figure 1A).

The reservoirs have a conical shape (Figure 1E) thereby enabling the work with very low sample volumes (<10 μL) which is especially critical for the work with limited sample material such as clinical samples. The PMMA manifold has openings in a vertical direction that contain sealing rings (Figure 1C). Thus, the headspace of the reservoirs can be closed from the top. A...
pressure can be applied to the closed reservoir by using a channel addressable from the bottom of the cartridge (Figure 1E).

For performing microfluidic cell culture experiments, the cartridge is placed into an incubation chamber which consists of a cartridge holder and a lid (Figure 2). The cartridge holder has a footprint of a standard microtiter plate (127.76 mm × 85.48 mm) and a maximum thickness of 22 ± 0.7 mm. This footprint makes the whole set-up compatible with established inverted microscope set-ups (Figure 2A). The incubation chamber is acting as a pneumatic interface that supplies defined pressures (0–600 mbar) and gas atmosphere (such as 5–7% CO₂) to the different headspaces of the inlet and outlet reservoirs (Figure 2B). Additionally, it provides electric interfaces for the cartridge and the lid, for example, for temperature control. The cartridge can be placed into the cartridge holder and is pushed within the cartridge holder to a reference point using an integrated spring mechanism (Figure 2C). Afterward, the cartridge is fixed in its position with a clamping mechanism which in turn fixes the positions of the microstructures relative to the outer walls of the cartridge holder. This is especially critical when the incubation chamber has to be moved during experiments, for example, to exchange medium or to perform different staining protocols as it ensures that the programmed imaging positions are not changing. The lid of the incubation chamber seals the cartridge from the top. The cartridge holder, as well as the lid, have in-built heating elements that enable fast heating of the incubation chamber as well as heating of the inserted cartridge (Figure 2D). In addition, the heated lid reduces evaporation of medium or liquids located within the reservoirs.

2.2. Microfluidic Design

In order to validate the previously described microfluidic cartridge for interfacing with microfluidic structures we integrated a workflow consisting of single-cell encapsulation into hydrogel beads, cell-laden hydrogel bead trapping, cultivation, time-lapse analysis, endpoint staining, and retrieval. To this end, we adjusted previously described microfluidic geometries to make them compatible with the macro-to-micro interface (Figure S1, Supporting Information). The microfluidic design consists of a conventional flow-focusing geometry[19] for the Poisson-distributed encapsulation of cells into highly monodisperse hydrogel beads.[20] (Figure S1C, Supporting Information). Separated from the encapsulation geometry, four individual microfluidic channels are connected to the second column of inlets as well as to the outlets (Figure S1B,C, Supporting Information). The microfluidic channels contain sequentially arranged hydrodynamic traps for the trapping of cell-laden hydrogel beads.[6] Each channel contains 665 trapping geometries with a grid of 7 rows and 95 columns. Thus, the geometry has a total capacity of 2660 trapping positions.

In the following sections, we will characterize the incubation chamber as well as the macro-to-micro interface connected to the previously described microfluidic geometry in terms of response times, heating times, generation of a defined gas atmosphere, priming and washing procedures, and its use for performing comprehensive microfluidic cell culture applications.

2.3. Characterization of System Parameters

To be compatible with established microscope set-ups, the incubation chamber is connected via tubing to the control unit which supplies defined pressures to the headspaces of the reservoirs of the macro-to-micro interface. We characterized the pneumatic connection between the control unit and the incubation chamber (tubing length: 80 cm, outer diameter: 1.27 mm, inner...
diameter: 0.86 mm) in terms of response times (Figure S2, Supporting Information). We define here the response time as the time required to achieve a pressure within the headspace of a reservoir which is at least 99% of the target pressure. Typical response times for the reservoirs P1 to P4 are 2.3 ± 0.11 s (target pressure: 500 mbar) (Figure S2, Supporting Information) with a response time of the control unit for pressure built up of 0.96 ± 0.07 s. Thus, the selected tubing length results in a time delay of 1.34 ± 0.18 s.

The macro-to-micro interface is equipped with a temperature sensor that is located directly above the glass substrate (position at which cells are cultivated). The incubation chamber has heating elements made of a printed circuit board (PCB) (total wattage: 78.6 W) that enable fast heating of the incubation chamber as well as of the lid (Figure S3, Supporting Information). The incubation chamber can be reliably heated within 30 min from RT to 37 °C (Figure S3A, Supporting Information). The macro-to-micro interface has a mass of 50.5 g which increases the heat capacity thereby increasing temperature stability which is especially important to decrease effects of temperature fluctuations on cell behavior. For the exchange of liquids within the reservoirs, the opening of the incubation system is required and we evaluated the impact of the removal of the heated lid on the temperature stability (Figure S3B,C, Supporting Information). Upon removal of the lid, the temperature of the macro-to-micro interface was maintained at 37.18 ± 0.04 °C for up to 10 min which is sufficient for exchanging liquids. In terms of its long-term temperature stability, we maintained a temperature of 37.11 ± 0.02 °C with a minimum temperature of 37.04 °C and a maximum temperature of 37.16 °C over 24 h (Figure S4G, Supporting Information). Most cell culture applications require a defined gas atmosphere such as 5% CO₂ which is necessary for pH buffering when bicarbonate-based buffer system is used. To generate a defined gas atmosphere within the headspace of the reservoirs, we implemented a gas washing procedure that enables the exchange of gas located within the headspaces of the reservoirs. By using the described gas exchange procedure as well as pre-equilibrated medium we maintained a stable pH over 24 h (Figure S4F, Supporting Information).

Air bubbles within microdevices can affect their function and may lead to failure as air bubbles change not only the hydrodynamic resistance of microchannels but also affect cells through the high surface tension at the air–liquid interface. Thus, the complete removal of any air located within microchannels is a critical aspect for error-free operation and a prerequisite for performing long-term cell culture. To implement a reliable priming procedure, PDMS was selected as material as it is cell compatible and known to be gas permeable which allows the removal of any air within microchannels by pressurizing the whole microdevice. For efficient priming, we first perfused the channels using a pressure gradient (Figure S5, Supporting Information) with subsequent pressurization at a higher pressure (300 mbar) resulting in the stable removal of any air bubbles within 10 min.

2.4. Cell Encapsulation

In terms of the presented macro-to-micro interface, volume flows within microchannels are generated by defined pressure gradients. The encapsulation process of cells into hydrogel matrices driven by a pressure-controlled system is highly dependent on the viscosity of the used hydrogel which is mostly affected by the temperature of the system. In this test, a low-melting agarose with a melting point of 65 °C that remains fluid at 37 °C and starts to solidify at temperatures between 26 and 30 °C was used as biopolymer for cell encapsulation. Thus, the whole system has to be kept at a constant temperature of 37 °C to enable reproducible cell encapsulation into monodisperse matrices. We characterized the encapsulation process in terms of the minimum required volume for performing cell encapsulations, monodispersity, reproducibility, roundness of generated hydrogel beads, and cell viability (Figure 3).

The minimum required volume for cell encapsulation was 8 µL with a cell concentration of 3500 cells µL⁻¹. The macro-to-micro interface contains conical reservoirs with an opening that is connected to a 1 mm CNC-milled PMMA channel which is directly connected to the corresponding microfluidic structures (Figure 1E). This geometry enables the precise positioning of very low volumes as well as direct transfer of the sample to the droplet generator. In addition, due to the low sample volume, droplet formation takes less than 2 min (Figure S5C, Supporting Information) and results in the formation of ~7700 droplets. This number is sufficient for filling the whole trapping geometry.

We generated hydrogel matrices in different batches as well as with macro-to-micro interfaces fabricated in different batches and evaluated hydrogel matrix size (Figure 3G) and roundness (Figure S6A, Supporting Information). The encapsulation process was reproducible between different macro-to-micro interfaces and resulted in hydrogel matrices with a size of 75.45 ± 6.21 µm in diameter. Also, repeating the encapsulation process with the same macro-to-micro interface resulted in a narrow size distribution with a mean bead size of 85.53 ± 5.29 µm in diameter.

In addition, we evaluated the influence of the encapsulation process on the viability of MDA-MB-468 breast cancer cells. To this end, we encapsulated cells into hydrogel matrices and performed a calcine and propidium iodide staining. The encapsulation process using the macro-to-micro interface did not affect cell viability as shown in Figure 3E,F.

2.5. Trapping, Stimulation, Perfusion, and Cultivation Processes

One aim of this study was to couple endpoint measurements such as immunostainings or smRNA FISH data to time-lapse functional phenotypes thereby deciphering gene function relationships. To establish protocols for implementing cell cultivation, stimulation, and washing processes we characterized the macro-to-micro interface in terms of its retention time and diffusion time. In addition, we evaluated possibilities for performing (periodic) perfusion as well as batch culture (Figure 4).

We define the retention time as the time a soluble compound needs to pass through a microfluidic channel (Figure 4C). To determine the retention time for the trapping geometry we added fluorescein isothiocyanate (FITC) solution to the inlet reservoir and applied a pressure difference (100 mbar). We then measured the fluorescence intensity over time at different locations.
After 3.75 min, 80% of the original fluorescence intensity was achieved. An additional exchange of medium resulted in a further increase of fluorescence intensity up to 99% of the original fluorescence intensity within 14 min in total (Figure 4C). Thus, efficient washing can be performed within less than 15 min.

For certain research applications a controlled stimulation and trigger is necessary. We evaluated the time until a compound diffuses from the inlet reservoir to the first cell position after exchanging the fluid in an inlet reservoir (diffusion time) (Figure 4D). To this end, we filled the macro-to-micro interface with a FITC solution and measured the fluorescence intensity within the channel over several hours. After 2 h, we measured an increase in fluorescence intensity indicating a diffusion of FITC from the inlet reservoir to the first trapping position (Figure 4D). Thus, for studies that require stimulation of cells, a time frame of 2 h is available to program the imaging set-up and to supply a stimulus in a controlled manner by applying a pressure gradient.

For cultivation of cells, the supply of fresh nutrients and removal of waste products is essential. The cell-laden hydrogel beads are positioned sequentially within the microfluidic channel as shown in Figures 1A, B and 4. Thus, for the establishment of a cell cultivation method we tested the time required to exchange the medium within the whole channel at a low pressure difference which results in a low volume flow thereby reducing shear stresses. To not affect cell behavior, we generated a low flow condition by applying a pressure difference of 5 mbar and replaced a FITC solution within \( \approx 40 \) min (Figure 4H). For cell culture, we then used a periodic perfusion protocol (Figure S4A–E, Supporting Information). Periodic perfusion has been reported to result in more homogeneous cell culture conditions.\[^{23}\]

We then tested the influence of the hydrostatic pressure which is a consequence of different height levels of liquids within the inlet and outlet reservoirs on perfusion rates. We used a suspension of tracer particles to monitor particle movement within the microfluidic channel (Figure 4E,I). We observed a directed movement with an average velocity of 13.7 ± 6.38 \( \mu \text{m s}^{-1} \) for a height difference of 5 mm. The channel cross-section is 0.008 mm\(^2\) resulting in an average volume flow of

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**Figure 3.** Generation of cell-laden hydrogel matrices using the macro-to-micro interface. A) Flow-focusing geometry used for cell encapsulation into monodisperse droplets. B) Individual frames from a high-speed recording of a droplet formation at the flow-focusing geometry, time in ms. C) Droplets located within CNC-milled channel of the cartridge after droplet formation. D) Intra- and G) inter-chip reproducibility. For determination of the reproducibility of droplet formation using the same cartridge, ten runs of cell encapsulation were performed and hydrogel bead sizes were measured (1396 beads were measured in total with at least 28 beads per run). The average size across all runs was 85.53 ± 5.29 \( \mu \text{m} \). G) Inter-cartridge reproducibility was determined using three separately fabricated cartridges. Mean: 75.45 ± 6.21 \( \mu \text{m} \) (in total 408 beads were analyzed). E) Representative microscopy images of cell viability assay. Green: viable cells stained with calcein. Red: dead cells stained with propidium iodide. F) Cell viability. MDA-MB-468 breast cancer cells were encapsulated into hydrogel matrices. Viability of cells was assessed directly after the encapsulation process (0 h), after 24 h and after 48 h by calcein and propidium iodide staining. Untreated cells and cells treated with ethanol were used as positive and negative control, respectively. Shown values are mean values ± standard deviation of three independent runs. At least 400 cells were analyzed for each condition. (n.s. = not significant; **** \( p < 0.0001 \)).
6.6 ± 3.1 nL min⁻¹. In comparison, the average particle velocity was 4.8 ± 2.74 μm s⁻¹ for particles in the channel with an equal height level corresponding to a volume flow of 2.3 ± 1.3 nL min⁻¹. Thus, by adjusting the liquid levels we significantly reduced the volume flow which can be necessary for the accumulation of soluble factors required for cell survival. This makes the system compatible to different cell culture protocols such as batch culture, perfusion, and periodic perfusion culture.

2.6. Combination of Dynamic Cell History with Corresponding Endpoint Phenotypes

Establishment of long-term 3D single-cell culture systems for correlating functional cell phenotypes and underlying molecular mechanisms is of particular interest for various research applications in the fields of immunology, developmental biology, oncology, and microbiology. To validate the macro-to-micro interface’s capability to analyze asynchronous and complex biological processes, we performed four assays: cell division, stimulation and live-cell fluorescence staining, endpoint combined staining for smRNA FISH and IF, and lastly, bead retrieval for further downstream analysis such as qPCR. The combination of these assays allows the linkage of dynamic cell history with the cell phenotype and genotype at a single-cell resolution within complex cell populations in 3D microenvironments.

Cell division plays a vital role in all living organisms, as it is essential for growth, repair of damaged tissues, healing, regeneration, and reproduction. To analyze the cell division process with the presented macro- to-micro interface, single MDA-MB-468 breast cancer cells were encapsulated in hydrogel beads, and the cell growth was monitored over 45 h (Figure 5). Out of 530 single cells analyzed, we detected the first cell division within 45 h for ~80% of cells (Figure 5B). In contrast, the second and third division per position started after 30 h with a maximum of 25.7% and 17.5% after 45 h, respectively. During the 45 h of cell cultivation, we detected 13.2% dead cells. To be able to analyze the cell cycle duration between the first and second cell division (second cycle) and the third and fourth cell division (third cycle), the cell cultivation period was extended to 80 h. In 40% of the analyzed cells, the second cell cycle duration was 22 h. In contrast, we observed a very heterogeneous cell cycle duration for daughter cells in the third division 20–70 h (Figure 5C). Resolving lineage relationships between cells can enhance our understanding of the pathology of cancer cells. To perform lineage tracing of MDA-MB-468 breast cancer cells, we tracked the cell divisions of five different single-cells in a time-lapse image series (Figure 5D and Figure S7, Supporting Information). Our results show the heterogeneity in the time...
interval between cell division in different cells and the daughter cells originated from the same mother cells.

To determine whether the system is suitable for analyzing time-resolved responses from primary cells upon cytokine stimulation, we encapsulated primary HUVEC in hydrogel beads and cultured them under normal cell conditions (Ctrl) or stimulated them with 50 ng mL\(^{-1}\) tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) for 27 h (Figure 6). In 2D culture, treatment of HUVEC with TNF\(\alpha\) has been reported to induce oxidative stress, inflammation, and apoptosis in a dose-dependent manner.\[24\] After 27 h, the cells which were treated with TNF\(\alpha\) showed a significantly higher death rate. To distinguish between apoptotic and necrotic cells in real time over 27 h, live-cell fluorescence staining reagents, NucView (caspase-3) and NucBlue (for detecting the dead cells) were added to Ctrl and TNF\(\alpha\) treated cells. Our results in a 3D microenvironment validated the previous observations shown in 2D and showed that TNF\(\alpha\) treatment induces cell apoptosis and cell death in HUVEC. Only a few dead cells were

Figure 6. The impact of tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) on the cell viability investigated on HUVEC encapsulated in hydrogel beads. A) HUVEC encapsulated inside the hydrogel beads were treated either with standard culture medium or 50 ng mL\(^{-1}\) TNF\(\alpha\). During 27 h of treatment, cells were stained first with NucBlue Fixed Cell ReadyProbes (DAPI) and then with NucBlue Live ReadyProbes (Hoechst 33342) reagents to determine the number of the dead and the total number of cells, respectively. Images were analyzed using ImageJ. To calculate the percentage of the dead cells, we evaluated \(n=31\) Ctrl cells and \(n=36\) TNF\(\alpha\) cells at 27 h. Means ± SD are shown. t-test yields \(* * * * \) \(p < 0.0001\). B) Representative time-lapse bright field and fluorescent images of HUVEC encapsulated in hydrogel beads treated with standard culture medium (upper panel, B) or 50 ng mL\(^{-1}\) TNF\(\alpha\) (lower panel, C). To distinguish between apoptotic and necrotic cell death NucView caspase-3 (red) and NucBlue Live Reagents (blue) were added to each channel.
observed in the cells cultured with standard culture medium after 12 h, whereas the cells treated with TNFα showed 100% double staining for NucView and NucBlue after 9 h of culture (Figure 6). These results proved the capability of the system to analyze time-resolved responses for both cell stimulation assays and live-cell staining.

Also, we tested the system for endpoint IF staining of cells cultivated for live-cell time-lapse analysis (Figure 7 and Video S1, Supporting Information). For this reason, MDA-MB-468 breast cancer cells were cultivated at a single-cell level inside the hydrogel beads, and a time-lapse recording was performed to analyze cell divisions for 41 h. At the endpoint of the experiment, to combine the functional phenotype data with protein expression, we fixed the cells and stained them for aldehyde dehydrogenases (ALDH1/2, cytoplasm) and NOTCH1 (intracellular membrane) which are known to be involved in the cell fate decision in cancer and stem cells. First, we tested the efficiency of the IF staining method on-chip and determined the required time for staining of encapsulated cells. AF488 NOTCH1 antibody was added to different reservoirs, and IF intensity was measured inside the channels, beads, and cells during the antibody incubation time (3 h) and subsequent washing steps. During antibody incubation, we observed only an increase of IF intensity inside the cells, and the fluorescent intensity remained constant inside the channels and beads. After the second washing step (210 min), the fluorescence intensity was reduced by almost 100% inside the channels and beads by 40% inside the cells (Figure 7A,B). The IF staining of MDA-MB-468 cells with AF488 NOTCH1 (green) and AF 647 ALDH1/2 (red) or isotype control antibodies showed double-positive signals in both daughter cells (Figure 7C and Figure S8, Supporting Information). These results showed the specificity of the method to effectively stain the cells within the hydrogel beads.

In addition, we tested the capability of the system for deciphering gene function relationships at a single-cell level (Figure 8). To do so, we combined the detection of mRNAs by smRNA FISH staining and protein expression by IF staining.

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Figure 7. Immunofluorescence analysis of MDA-MB-468 cells encapsulated in hydrogel beads. A) After adding AF488 conjugated NOTCH1 antibody to each channel, the mean fluorescence intensity of the GFP signal was measured every 1.75 min to assess the efficiency and sensitivity of IF staining. For the analysis, the mean fluorescence intensity was measured inside the chip’s microfluidic channels (n=8), beads (n=6), and encapsulated cells (n=4). The arrow indicates the first washing step. B) Representative epifluorescence images after adding AF488 conjugated NOTCH1 antibody (20 min), before first washing step (180 min) and after second washing step (210 min), the dashed lines represent the micro trap, the dotted circle represents the position of the hydrogel bead, the arrow indicates the cell. C) After 41 h culturing, MDA-MB-468 cells were fixed and stained with AF488 conjugated NOTCH1 (green) and conjugated AF647 ALDH1/2 (red) antibodies.

Figure 8. Endpoint IF-combined smRNA FISH analysis of encapsulated cells and direct correlation to time-lapse microscopy data. A) Representative fluorescent images of combined smRNA FISH and IF staining on-chip are shown for CHO, MCF7, and MDA-MB-231 cells. Cells were encapsulated into hydrogel beads and stained with 0.68 μmol L⁻¹ E-cadherin probe-pool conjugated with Cy3 (orange) and anti-E-cadherin antibody (red). B) After 24 h culturing, MCF7 and MDA-MB-231 cells were fixed and stained with 0.68 μmol L⁻¹ E-cadherin probe-pool conjugated with Cy3 (orange) and anti-E-cadherin antibody (red). For smRNA FISH analysis, cells were imaged using a LSM800 confocal microscope (Zeiss).
Chinese hamster ovary (CHO) cells (negative control), MCF7, and MDA-MB-231 cells were encapsulated into hydrogel beads and stained for E-cadherin mRNA (probe pool) and E-cadherin protein.

MCF7 cells are considered as a non-aggressive breast cancer cell line and positive for E-cadherin expression. In contrast, MDA-MB-231 cells are aggressive and poorly differentiated cells and do not express E-cadherin. The E-cadherin IF staining was detected in MCF7 cells at cell–cell contacts and slightly in the cytoplasm. In contrast, smRNA FISH staining could be detected in the MCF7 and some of the MDA-MB-231 cells (Figure 8A). The reduced E-cadherin mRNA level in MDA-MB-231 cells compared to MCF7 cells was also proved by qPCR (Figure S9, Supporting Information). Finally, the time-lapse microscopy of encapsulated MCF7 and MDA-MB-231 cells was coupled with smRNA and IF staining to combine functional phenotype with protein and RNA analysis (Figure 8B). During time-lapse bright field microscopy, MCF7 cells showed a tendency toward cluster formation and direct attachment to colocated cells. In comparison, MDA-MB-231 showed reduced cluster formation. This behavior can be explained by the corresponding E-cadherin expression (combined smRNA FISH and IF data) affecting cell–cell adhesion and thereby showing a direct gene function relationship.

2.7. Cell Recovery

To enable further downstream analysis such as qPCR of cells after on-chip cultivation, the ability to recover beads from the macro-to-micro interface was assessed. To this end, immobilized beads were removed from the microfluidic structures by applying a reverse volume flow that pushes hydrogel beads out of the trapping structures (Figure S6D, Supporting Information). Retrieved hydrogel beads were then collected in the inlet reservoirs. Here we define the recovery rate as the number of beads that were collected in the inlet reservoirs in relation to the number of beads that were immobilized before. Recovery rates of 90.9–95.4% were achieved (Figure S6D, Supporting Information). Purity of the recovered bead population was tested by performing two trapping processes with differently colored bead populations with washing steps to remove untrapped beads. Here we define the purity of the bead population as the number of beads of the trapped bead population in relation to the amount of all collected beads which shows the efficiency to remove untrapped beads from the macro-to-micro interface. A purity of the collected bead population of more than 99% was detected (Figure S6D, Supporting Information) which indicates a high efficiency to remove untrapped beads using the established washing steps.

3. Discussion

Microfluidics has become an important and powerful tool for biology but access to microfluidic procedures is still limited by the availability of suitable systems. Easy to use and reliable instruments are thus necessary to bring microfluidics into standard biological laboratories thereby enabling to exploit the full potential of microfluidics for the analysis of biological systems. For microfluidic prototyping, tubings are usually connected directly to the microfluidic chips and placed into an environmental chamber for cell culture and live-cell imaging. On the one hand, this offers huge advantages as fast evaluation of these microfluidic geometries can be performed. On the other hand, this results in an error-prone and time-consuming set-up and the implementation of a fully sterile workflow is significantly hampered. Especially the work with very small sample volumes (<10 μL) and limited sample material is challenging. Meanwhile, some commercial microfluidic cell culture cartridges are available having a 96-well plate footprint but rely on an external environmental chamber for live-cell imaging. In contrast, other commercial systems that include an environmental system are still limited to 2D cell culture and bulk experiments even though they offer other advantages such as fully integrated cell culture handling, imaging, data storage, and analysis. In addition, the work with small sample volumes as well as the integration of highly temperature-sensitive processes is hampered.

In this work, the combination of the microfluidic cartridge with an incubation chamber having a 96-well plate footprint offers significant advantages. First, we were able to remove any tubings directly connected to the microfluidic cartridge. Second, the incubation chamber enables the heating of the macro-to-micro interface when placed on a conventional microscope as well as when the system is opened under a flow hood for sterile handling. Thus it is possible to work with very low volumes (8 μL) of temperature-sensitive samples (low-melting agarose) and therefore with limited sample material such as primary cells. This allowed the integration of a single-cell cultivation and analysis workflow with cell-laden hydrogel matrices as powerful cell culture format.

Cell-laden hydrogel beads have been described previously and have been used for the encapsulation of single cells, cell pairs, and small cell clusters. Cell-laden hydrogel beads with a size of 70 to 150 μm in diameter offer a significant advantage in comparison to conventional 3D hydrogel formats. They act as a protective vehicle for transportation of cells as the hydrogel surrounding a cell protects it from shear forces. The small size of the hydrogel beads allows their transport and handling within microfluidic devices. In addition, encapsulation of cells into hydrogel beads is insensitive toward cell size thereby making this format compatible with prokaryotes and eukaryotes. Moreover, the hydrogel is acting as a 3D microenvironment which can give essential stimuli to cultivated cells. An additional advantage has been shown in the field of studying cell–cell interactions as the co-encapsulation of cell types results in a close co-localization for studying their isolated interaction. Cell-laden hydrogel beads represent a porous and diffusible system that is ideal for implementing staining procedures. In addition, the combination with microfluidic traps as shown in this study enables to preserve time-lapse information and to combine this information with endpoint data. The spherical hydrogel format also offers a reliable way for the analysis of non-adherent and suspension cells. The cultivation and time-lapse analysis of these cells is hampered by their floating characteristics making a time-lapse optical analysis difficult. This issue has been addressed in previous studies.
by seeding of non-adherent cells into microfluidic chambers whereas medium exchange has been performed using diffusion processes. In comparison, cell-laden hydrogel beads can be efficiently washed by perfusion without affecting and removing encapsulated cells thereby generating more homogeneous culture conditions. Finally, due to its small size and spherical shape, the hydrogel bead format provides a vehicle for simple cell retrieval and subsequent downstream analysis without the need for dissolving the hydrogel which has been a limitation in previous studies.[39]

With the novel established workflow, we showed the possibility of stimulating and inducing cell death in primary HUVEC with TNFα[24] and distinguished between necrosis and apoptosis events within individual cells. Moreover, by combining smRNA FISH and IF staining, we observed that MDA-MB-231 cells (aggressive breast cancer) express a low E-cadherin mRNA level, whereas, at the protein level, E-cadherin expression was entirely missing. In contrast, in non-invasive MCF7 cells, expression of E-cadherin mRNA and protein were detected equally good. These results were in line with a previous study showing that up-regulation of miRNA miR-221 in MDA-MB-231 suppresses E-cadherin protein expression and thereby increases tumorigenesis.[39]

4. Conclusions and Outlook

In this work, we evaluated a new macro-to-micro interface for interfacing with microfluidic structures and its use for performing comprehensive cell culture applications by integrating a single-cell cultivation and analysis workflow. We showed that the macro-to-micro interface is suitable for implementing microfluidic operations such as the encapsulation of cells into spherical hydrogel matrices as well as the long-term cultivation of cells located within immobilized hydrogel matrices. Importantly, the presented macro-to-micro interface is not limited to the microfluidic structures described herein but can also be combined with other microfluidic geometries. The presented system allowed the work under fully sterile conditions without the need to connect and disconnect any tubings to the microfluidic cartridge. Cell culture conditions were shown to be highly stable in terms of temperature stability and buffering capabilities using a defined gas atmosphere. In addition, the presented macro-to-micro interface was used for simple liquid exchange without significantly affecting microfluidic volume flows. This enabled the implementation of effective washing, staining, and stimulation procedures. In addition, the presented system, volume flows were highly controllable enabling the use of continuous and periodic perfusion as well as batch culture. The 96-well footprint, the small height and the use of a #1.5 cover glass makes the system compatible with established inverted microscopes as well as with confocal microscope setups without the need for an additional environmental chamber. Finally, effective priming procedures were implemented and a bubble-free state for reliable cell cultivation was maintained for several days.

By combining the presented macro-to-micro interface with a single-cell cultivation workflow we performed the cultivation of single breast cancer cells (MDA-MB-468, MDA-MB-231, and MCF7) and primary (HUVEC) cells. In addition, we coupled time-lapse (fluorescence) microscopy data with endpoint measurements such as immunostaining and smRNA FISH thereby deciphering direct gene function relationships on a single-cell level. Finally, we showed the effective endpoint retrieval of cell-laden hydrogel beads for further downstream analysis or cell expansion.

We envision to use the proposed macro-to-micro interface as a tool to make new microfluidic geometries quickly available to biological laboratories without the need for providing adjusted control systems as well as for performing fast prototyping of microfluidic geometries in particular for cell culture applications. The overall single-cell cultivation and analysis workflow described herein is a valuable tool for multiparametric single-cell analysis as well as for in-depth analysis of gene function relationships.

5. Experimental Section

Cell Culture: MDA-MB-231, MDA-MB-468, and MCF7 human breast adenocarcinoma cell lines were kindly provided by the group of Prof. Moerschbacher (University of Münster). MDA-MB-231 and MDA-MB-468 cells were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM, Merck, Darmstadt, Germany). MCF7 cells were cultured in RPMI-1640 medium (Merck). CHO cells were kindly provided by AG Grobe (University of Münster) and were cultured in DMEM:F12 medium (PAN-Biotec, Aidenbach, Germany). All media were supplemented with 10% fetal calf serum (FCS; Thermo Fisher, Waltham, MA, USA). Cell cultivation was carried out in a cell incubator at 37°C and 5% CO2 using T-25 Cell Culture Flasks (CELLSTAR, Greiner Bio-One, Kremsmünster, Austria).

Splitting of cells was performed at 80% confluency. Cells were cultured up to passage 30 at maximum. Primary HUVEC were kindly provided by Vlaams Instituut voor Biotechnologie (VIB, Flandern, Belgium). They were cultured up to passage six in ECGM-2 medium supplemented with SupplementPack (PromoCell, Heidelberg, Germany).

Characterization of Cartridge Holder and Macro-to-Micro Interface: Macro-to-micro interfaces (CellCity Array Chip), the incubation chamber (CellCity Incubator) as well as the control unit (CellCity Control Unit) were provided by evorion biotechnologies GmbH (Münster, Germany). For a characterization of the cartridge holder pressure sensors were connected to the headspace of each reservoir (P1–P5) and pressure values in the headspace of the reservoirs and in the control unit were measured. Response times to build up a target pressure (500 mbar) in the headspace of the reservoirs were determined. Temperature curves during preheating process and during handling of the open chamber were recorded using the temperature sensors in the cartridge, lid, and the bottom of the cartridge holder. Additionally, infrared images were taken using an infrared camera FLIR C3 (FLIR Systems Inc., Wilsonville, USA).

Encapsulation of Cells into Hydrogel Matrices: The reagents for cell encapsulation were provided by evorion biotechnologies (Münster, Germany) as part of the CellCity Array Kit. The cell encapsulation was done according to the manufacturers protocol.

Initially, hydrogel A was heated to 65°C for 30 min and then cooled down to 37°C and kept at 37°C for at least 15 min before further use. The cartridge and incubation chamber were preheated to 39°C for 1 h and all other solutions were preheated to 37°C. Handling was done under sterile conditions in a cell culture bench. For continuous heating, the incubation chamber was connected to the evorion clean bench box. Cells were prepared as a single-cell suspension. To this end, adherent cells were detached with a solution containing 0.05% trypsin and 0.53 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA) (Merck) following standard protocol. Cells were washed once with buffer A.
and the concentration was adjusted to 8000–10 000 cells μL\(^{-1}\) using buffer A. The cell suspension was then preheated to 37 °C for 15 min and mixed in a 1:1 ratio with the hydrogel solution. After preheating of cartridge and incubation chamber, the cartridge was primed. To this end, 150 and 50 μL of buffer B were added to the second column of channel inlet and outlet reservoirs, respectively. The detailed pressure protocol for priming is shown in Figure S5A, Supporting Information.

Droplets were formed at a flow-focusing geometry shown in Figure 3A. To this end, inlet reservoirs were refilled with 150 μL buffer B. Then, 150 μL bead formation solution and 8 μL hydrogel-cell suspension were added to the first and second inlets of the first column, respectively. The detailed droplet formation protocol can be seen in Figure SSC. Supporting Information. After the bead formation, droplets were collected at the outlet reservoir. To this end, the entire liquid in the reservoir was taken up. The reservoir has to be washed by pipetting up and down several times without releasing the collected droplets back into the reservoir. When releasing the droplets into a tube, the pipette tip should again be washed several times to avoid droplets sticking to the inside surface of the pipette tip. Droplets were incubated at 4 °C for 15 min to ensure hydrogel formation.

Finally, the bead suspension was demulsified. To this end, the volume of the lower phase was reduced to 100 μL. Then, 200 μL of trapping solution were added on top of the solution. Then, 100 μL of demulsification solution were added to the lower phase and the tube was inverted twice. After the separation of the water and the oil phase the beads were in the upper phase. Then, 200 μL of trapping solution were again added to the upper phase. Without inverting, the upper phase was removed and subsequently filtered with a 100 μm mesh filter (Sysmex, Kobe, Japan).

**Cartridge Reproducibility:** To determine intra-chip reproducibility of the droplet formation, ten runs of droplet formation were performed using one chip. To this end, reservoirs were refilled after droplet collection with fresh hydrogel and bead formation solution and the process of droplet formation was repeated. To test inter-chip reproducibility of the process, three runs of droplet formation were performed using three different chips. Bead size and roundness analysis was performed using a Neubauer improved cell counting chamber. Images were taken with an EVOS Auto FL 1 Imaging system (Thermo Fisher). Bead size and roundness were determined by manual measurement using ImageJ. The roundness is a dimensionless number defined as the area of the minimum enclosing circle divided by the detected area and varies in the range 0–1 where 1 is a perfect circle.

**Cell Viability:** To test the cell viability, encapsulated cells were cultivated in standard medium in a 96-well plate. Staining was performed using 0.5 μM mL\(^{-1}\) calcein AM (Thermo Fisher) and 2 μM mL\(^{-1}\) propidium iodide (Merck). Untreated cells were used as positive control. Cells treated with ethanol for 10 min prior to viability staining were used as negative control. Staining was performed directly after the encapsulation process, 24 h and 48 h. Viability was assessed by fluorescence imaging using the EVOS FL auto imaging system (GFP, RFP light cube; Thermo Fisher). Live and dead cells were counted manually. Significant differences of viability between encapsulated cells and controls were done with an unpaired, parametric T-test. Differences with \(p < 0.05\) were considered significant.

**Hydrodynamic and Hydrostatic Characterization:** For the hydrodynamic characterization, the microfluidic channels were initially primed with a 7 μg mL\(^{-1}\) FITC (Thermo Fisher) or phosphate buffered saline (PBS). Then, FITC or PBS were removed by washing with 50 μL PBS or FITC and 100 mbar at ports 1–4 for 5 min. The washing step was repeated two more times. Between each step, the inlets were emptied and refilled with 50 μL PBS or FITC solution. Images were taken every 1.75 min.

To test diffusion of soluble compounds into the channels, the microfluidic channels were primed with PBS. Then, 200 and 50 μL FITC were filled in inlets and outlets, respectively. No pressure was applied at all ports and images were taken every 5 min for 16 h. Finally, the medium exchange during on-chip cultivation was tested with microfluidic channels primed with PBS. Then, 200 and 50 μL FITC were filled in inlets and outlets, respectively. Additionally, pressure of 25 and 20 mbar were applied to ports 1–4 and port 5, respectively. Images were taken every 1.75 min for 1 h. All images were taken at the first, middle, and last position of each microfluidic channel with an EVOS FL Auto Imaging System. Images were analyzed with ImageJ (Version 1.51). Specifically, a region of interest within the channel was selected manually and mean fluorescence intensity for each position and time point was calculated automatically.

For characterization of hydrostatic parameters, polystyrene particles (Red4; Polyan, Berlin, Germany) were diluted in ddH\(_2\)O containing 70% v/v glycerol and 1% v/v Tween-20 to avoid sedimentation and aggregation of particles. The concentration was adjusted to 13 000 particles μL\(^{-1}\). Before the experiment start, channels were primed using PBS and then particle solution was washed twice into the microfluidic channels by applying a pressure of 100 mbar for 5 min. To determine the hydrostatic pressure that is generated by a volume difference between inlet and outlet reservoirs, two different settings were tested. For the first setup a high volume difference was tested. 150 and 0 μL particle solution were filled in the inlets and outlets, respectively. To determine the particle movement at a low volume difference, inlets and outlets were both filled with 75 μL of particle solution. Recording of particle movement was performed using a screen recorder (Movavi Software Limited, Limassoul, Cyprus), with a frame rate of 60 fps, while imaging in the RFP fluorescence channel using the EVOS FL Auto 1 imaging system. For each condition, 10 s of particle movement were recorded. Detection and tracking of single particles were performed using a particle tracking plugin in ImageJ.[40] Parameters for particle tracking were set as follows: radius 3, cutoff 0.001, displacement 5, and link range 4. For each condition ten particles were tracked in the first row of each microfluidic channel. These measurements were repeated with the same pressure applied at the inlet and outlet pressure ports (20 and 100 mbar).

**On-Chip Cultivation:** Cells encapsulated in hydrogel beads were generated as described above. Mean bead size was supposed to be around 75–88 μm to allow successful immobilization of the hydrogel beads inside the trapping channels. Bead concentration was adjusted to 10 beads μL\(^{-1}\) using the trapping solution. A volume of 150 μL bead suspension was added to each channel inlet reservoir. Trapping was performed by applying a pressure of 300 mbar at ports 1–4 for 4 min. Afterward, reservoirs were emptied and two washing steps were performed using complete cell culture medium (150 μL) for 5 min applying 200 and 100 mbar to ports 1–4, respectively, to remove untrapped beads. For cell cultivation with bicarbonate buffer systems, equilibrated medium was used which was incubated at 5% CO\(_2\) for at least 2 h before experiment begins. After trapping, cultivation was started by adding 200 μL equilibrated cell culture medium to the inlet reservoirs and 50 μL to the outlet reservoirs. The pH was kept constant during the experiment by connecting a 7% CO\(_2\) gas bottle to the control unit. A cultivation sequence was run for 24 h by applying a constant pressure of 20 mbar to all pressure ports. Every 1.5 h the pressure at the inlet reservoirs (PI–PM) was increased to 25 mbar for 30 min to allow exchange of medium inside the channel. After 24 h, the medium in inlet and outlet reservoirs was exchanged with fresh medium and cultivation was continued. A constant temperature of 37 °C was maintained throughout the experiment.

Time-lapse imaging was performed using an EVOS FL Auto 1 Imaging System (Thermo Fisher). Imaging positions were chosen manually using the EVOS software. Bright field images were acquired with a 40× objective and a monochrome, high-sensitivity interline CCD camera. Cell survival and cell events were assessed manually. Time intervals between cell divisions and lineage relationships were determined by manually tracking of division events.

**Immunofluorescence Staining:** The following steps were carried out within the microfluidic chip of the evorion CellCity system by using the characterized washing method (100 mbar, 5 min, see Section 5). The washing method was applied twice to flush the channels with the respective reagents and buffers. After each step, the residual reagent was removed from the outlet and inlet reservoirs before applying 50 μL fresh reagent to the inlet reservoirs. On-chip immunostaining was performed...
with the standard protocol from evorion. Encapsulated cells were fixed with pre-warmed 40 g L\(^{-1}\) PFA (Thermo Fisher) in PBS for 20 min at RT. Next, encapsulated cells were washed twice with PBS and permeabilized with cold 0.1% Triton X-100 for 10 min at RT. After washing twice with blocking buffer (1% bovine serum albumin, 3% goat serum in PBS), cells were stained with AF647 ALDH1/2 (1:20; H-8, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and AF 488 NOTCH1 (1:20; A-8, Santa Cruz Biotechnology) conjugated antibodies for 3 h at RT. Nuclei were stained by adding two drops of NuclBlue Fixed Cells (DAP); Thermo Fisher) to the blocking buffer. The cells were finally washed twice with blocking buffer and once with PBS. Isotype controls were performed in the same way as described above with mouse IgM Alexa Fluor 488 and mouse IgG2b Alexa Fluor 647 conjugated antibodies.

smRNA FISH-Combined Immunofluorescence Analysis: On-chip molecule smRNA FISH was performed with the microfluidic chip of the evorion CellCity system by using the characterized washing method (100 mbar, 5 min, see Section 5). Encapsulated cells were fixed with pre-warmed 40 g L\(^{-1}\) PFA (Thermo Fisher) and incubated for 20 min at RT. Next, cells were permeabilized with 0.1% v/v Triton X-100 in RNase-free PBS for 10 min at RT and washed twice with wash buffer (10% formamide and 0.02% RNase-free BSA in 2\(\times\) SSC). The cells were then incubated with 0.68 \(\mu\)mol L\(^{-1}\) E-cadherin probe-pool diluted in hybridization buffer (10% formamide, 0.02% RNase-free BSA, 5 mmol L\(^{-1}\) EDTA, 1 \(\mu\)L L\(^{-1}\) RNase inhibitor in 4\(\times\) SSC) overnight at 25 °C in the dark. The next day, the channels were washed thrice for 30 min at RT with wash buffer. For E-cadherin IF staining, cells were incubated with primary mouse anti-E-cadherin antibody (1:10; BD Biosciences, San Jose, CA, USA) in the blocking buffer (0.02% RNase-free BSA in RNase-free PBS) at RT for 1 h. Subsequently, cells were stained with secondary donkey anti-mouse antibody conjugated to Alexa Fluor 488 (1:200; Invitrogen, Karlsruhe, Germany) in blocking buffer for 1 h at RT. E-cadherin probe design and generation of the probe pool are shown in Table S1, Supporting Information.

RT-qPCR: PCR analysis for encapsulated MCF7 and MDA-MB-231 cells in hydrogel beads was performed as described previously.[4] Briefly, selected and isolated beads were transferred to a round bottom 96-well plate with a disposable glass micropipette. cDNA synthesis was performed using SuperScript IV CellsDirect cDNA synthesis kit following the manufacturer’s instructions (Invitrogen). Real-time PCR reactions were performed with a RotorGene SYBR Green PCR Kit in the RotorGene Cycler (both Qiagen). The following primers were used: E-cadherin-fw, 5\(\prime\)-GACCGGTGCAATCTTCAAA-3\(\prime\) and E-cadherin-rev, 5\(\prime\)-CCAGACGGAAGCTCGGAAAC-3\(\prime\); and TOP1-fw, 5\(\prime\)-GACCGGTGCAATCTTCAAA-3\(\prime\) and TOP1-rev, 5\(\prime\)-GACCGGTGCAATCTTCAAA-3\(\prime\). Cycle threshold (\(\text{CT}\)) values were normalized by the \(\Delta\text{ΔCT}\) method,[41] and TOP1 was used as a reference gene.

Cell Recovery: In order to assess recovery rates of encapsulated cells after immobilization and on-chip cultivation, the trapping process was performed two times. First, traps were filled with beads containing RFP labeled cells. Then, beads containing GFP labeled cells were added to the channel to fill any unoccupied trap positions. After each trapping process, three washing steps were performed to remove untrapped beads. To recover beads from the microfluidic channels, a backflow was used by applying a pressure of 500 mbar at the outlet port P5 for 1 min using 100 \(\mu\)L evorion’s bead recovery solution. This process was repeated two more times with exchanged solutions in between runs. In the last run pressure was continued until all channels were filled with air to ensure complete bead recovery by air pushing beads back into the inlet reservoir. Beads were collected in a 96-well plate and counted manually to assess the recovery rate.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
H.K.-B., R.W., F.S.B., C.E., V.F., M.R., S.B. are employees of evorion biotechnologies GmbH. evorion biotechnologies GmbH is patent applicant for the patent application WO2020229650 (A1) (name of inventors: H.K.-B., R.W., and S.B.) which covers the basic design and usage of the described macro-to-micro interface.

Author Contributions
H.K.-B. and R.W. contributed equally to this work. F.S.B performed experiments on system characterization and biological experiments. M.R. and J.S. performed biological experiments. V.F. and C.E. assisted with programming and data analysis. H.K.-B., R.W., F.S.B., M.R., and C.E. contributed to the writing of the manuscript.

Data Availability Statement
The data that support the findings of this study are available in the supplementary material of this article.

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hydrogel, immunostaining, live-cell imaging, single-cell analysis, single-cell lineage tracing, smRNA FISH

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