

Nano-Volume Well Array Chip for Large-Scale Propagation and High-Resolution Analysis of Individual Cancer Stem Cells

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Abstract

Cellular heterogeneity represents an increasingly appreciated aspect for research in life science. To address this issue, we have developed a nano-volume well array chip that allows larger-scale isolation and propagation of single cells. Notably, the chip enables single-cell analysis of freshly isolated primary cells at a high-resolution. With an average height of $130 \pm 10 \mu\text{m}$ and an average diameter of $80 \pm 10 \mu\text{m}$, each nano-volume well can hold up to 0.4 nL of volume, and is compatible with both adherent as well as 3D suspension cultures. Simultaneous time-lapse imaging of thousands of nano-volume wells allows to monitor cell division, as well as tracking of cell fate, and/or alterations in the microscopic cellular morphology and/or markers expression. To demonstrate its application, we employed the system for propagating and tracking of Cancer Stem Cells (CSCs). CSCs could be monitored over three consecutive days by time-lapse high-resolution imaging at the single-cell level. We could demonstrate that non-CSCs do not dedifferentiate into CSCs, while CSCs were able to give rise to both CSCs and non-CSCs by undergoing symmetric and asymmetric division, respectively. Altogether, we have developed a novel nano-volume well array chip that significantly ameliorates clonal propagation and high-resolution image analysis of rare cells.

Keywords: Pancreatic cancer; Cancer stem cells; Niche

Introduction

A major challenge in life science is to understand how individual cells process information and respond to alterations of the surrounding environment [1]. Much of our knowledge is based on the analysis of heterogeneous cell populations. However, cell-to-cell differences have been reported even for supposedly homogenous cell populations [2-4], and the ensemble behavior of a population may not represent the behavior of individual cells bearing distinct features. Therefore, it has become increasingly obvious that the challenge is no longer to demonstrate that populations of 'seemingly identical' cells are heterogeneous, but rather the daunting challenge has become to determine which components of the observed cellular heterogeneity serve a biological function or contain meaningful information.

For example, convincing evidence has been provided that the majority of tumors contain a distinct sub-population of cancer cells referred to as tumor-initiating cells or Cancer Stem Cells (CSCs) [5-7]. Apparently, these cells are the driving force behind the cancers' key feature including tumorigenicity, metastatic spread, and relapse of disease following treatment [8]. These CSCs regularly represent a rather small subpopulation of cancer cells, which can be enriched by means of sorting for surface markers, e.g. CD133, CD44, CD24 [9], or by cultivating them in 3D-suspension cultures [10]. However, even these enriched subpopulations of CSCs still represent heterogeneous populations of cells with distinct biological features including the ability to metastasize and require further characterization at the single cell level [11].

Based on our current understanding of CSCs and their dynamic behavior it appears rather unlikely that any marker combination could be suitable for detecting homogeneous populations of CSCs across large patient populations. Therefore, current investigations in the CSC field rely on studies using rather heterogeneous populations merely enriched for CSCs [12]. Assay readouts from these experiments mostly represent average responses from bulk cancer cells instead of individual

cells, which represent a major confounding factor due to cellular heterogeneity. Therefore, highly miniaturized *in vitro* culture platforms for propagation and analysis of CSCs at the single-cell level are urgently needed [9,13].

Microfluidic chips in poly(dimethylsiloxane) (PDMS) using soft lithography can be rapidly fabricated [14]. Specifically, micro well-shaped structures of 10-20 μm in diameter have been fabricated by using PDMS stamping of Poly(Ethylene Glycol) (PEG) onto silicon substrates and allowed for culturing of single mouse fibroblasts [15]. Another microwell system has been fabricated by surface engineering PEG on glass for culturing hepatocytes [16]. Larger wells were utilized in a cell-retaining PDMS structure for high-content analysis [17] and for studying stem cell fates [18]. Other authors have reported the development of microwells for cultivating adult stem cells, overcoming the limitation that these cells could not be propagated in conventional cell-culture conditions [19], or for investigating new features of self-renewal of hematopoietic stem cells at the single-cell level [20]. Finally, leukemic cancer cells have also been studied using a microwell system in order to analyze their cell heterogeneity and their response to different drugs [21], but no chip has been developed for the analysis and propagation of cancer stem cells to date and therefore represented the objective of the present study.

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Materials and Methods

Chip design and fabrication

The microchip pattern was designed with AutoCAD (Autodesk) and contained 14,000 wells distributed over a surface area of 30 cm². Each of the wells was designed to have a diameter of 80 µm with a center-to-center distance to the neighboring wells of 135 µm. While chips of any size can be used, for our experiments, we used 1 cm² of the chip, which contained ~4,600 wells and was bonded to a glass bottom well.

PDMS elastomer devices were fabricated using standard photolithography and molding technology [22]. Negative molds were produced at the Stanford Microfluidics Foundry (Stanford University, CA), which we then used to cast the PDMS chips. For this purpose, 5 gr of curing agent and base (1:10 ratio; Sylgard 184 Silicone Elastomer) were poured onto the mold and the mold was then spin-coated in two steps, first at 500 rpm for 12 sec and then at 1500 rpm for 9 sec, thus resulting in PDMS chip of approximately 130 µm in height. The thickness of the chip allowed for monitoring of the cells at high-resolution within the formed wells. After polymerization for 24 h at 65°C, the PDMS layer was peeled off the mold and cut into 1 cm² pieces. After treatment with oxygen plasma, the PDMS piece was transferred immediately to a glass bottom well in order to maintain chip sterility and decrease evaporation during the actual experiments. Bonding of the chip to the glass bottom ensured stable conditions during imaging.

Cell seeding

PBS was added to the well to cover the chip surface and subsequent brief transfer to vacuum chamber ensured removal of any air bubbles trapped in the nano-volume wells. Then, the chip was sterilized by UV exposure for 1h followed by manual loading of appropriate numbers of singularized cells onto the nano-volume well array chip. Cells were randomly positioned inside the wells by gravitation. 20 min after seeding the cells, loading of the chip was verified. Only occasionally some cells could be found on the surface of the chip, which varies between cell types. However, this could be addressed by additional washes before cells start adhering to the surface. Thus, to ensure that most nano-volume wells contain single cells, we loaded 8·10³ cells in 500 µL of media on 1 cm² chips placed when in 24-well plates; and 2·10³ cells in 150 µL on 1 cm² chips when they were placed in 8-well plates. Here, we have kept constant the dimensions of the chip and titrated the cell sample concentration (data not shown) as a factor for improving the occupancy of the nano-volume wells based on the utilized cell type (primary human pancreatic cancer cells). However, if further adaptations become necessary (e.g. when using other types of cells varying in size, shape, and/or deformability) factors such as well dimensions, sample volume applied to the chip, number of wells in the array, and the fractional area (area of the well opening multiplied by the number of wells and divided by the area of the chip) of the well openings could be modified to adapt capture efficiency and the distribution statistics [23].

Ultra-low adhesion coating

To prevent cell adhesion, a solution containing 1.2 % (w/v) pHEMA in 95% ethanol (12 mg/mL pHEMA) was prepared and filtered through a 0.2 µm pore filter to discard any dust particle. Chip bonded to the glass bottom well plate were then covered with the pHEMA solution and left to dry at 37°C overnight. Chips were covered with PBS and vacuum was applied to remove putative bubbles trapped in the nano-volume wells. Before usage, chips were sterilized by UV exposure for 1h before use.

Primary pancreatic cancer cells

Pancreatic tumors obtained from patient-derived xenografts were dissected into small pieces, then transferred into a homogenization tube with 2 mL FBS-free medium, and minced mechanically (gentleMACS Dissociator; Miltenyi). Subsequently, the homogenate was transferred to a tube with 2 mL of 0.25% collagenase, incubated under constant rotation for 1 h, at 37°C and 5% CO₂ water-saturated. The collagenase digestion was stopped by the addition of 5 mL of FBS-containing medium. The homogenate was passed through a 100 µm cell strainer to remove remaining clumps, centrifuged for 5 min at 1500 rpm, and then resuspended in medium. This step was repeated using a 70 µm and 40 µm cell strainer, respectively; and the cell pellet was finally resuspended in 3 mL of medium prior to seeding on the chip.

Sphere culture

Pancreatic cancer spheres were generated and expanded in DMEM-F12 (Invitrogen, Karlsruhe, Germany) supplemented with B-27 (Gibco, Karlsruhe, Germany), bFGF (PeproTech EC, London, UK), and 1% penicillin/streptomycin. A total of 5×10³ cells/mL were seeded in conventional ultra-low attachment tissue culture plates (Corning B.V., Schiphol-Rijk, The Netherlands) or pHEMA pre-treated nano-volume well arrays. For sphere formation with multicolor reporter systems, equal numbers of PDAC cells carrying SFFV-GFP, SFFV-Katushka, SFFV-CAE, or SFFV-CIT were used.

Adherent culture

Pancreatic cancer cells were expanded in RPMI (Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS and 1% penicillin/streptomycin.

Fluorescent labeling of the chip

A solution of 1.25 mg/mL Rhodamine B isothiocyanate-dextran (70.000wt, Sigma) in PBS was prepared. After bonding the chip to the glass bottom well, the chip surface was activated by oxygen plasma with the same protocol used for binding. Subsequently, the solution of Rhodamine was poured onto the chip, degassed using vacuum, and placed at 4°C overnight. The chip was washed with PBS before seeding the cells.

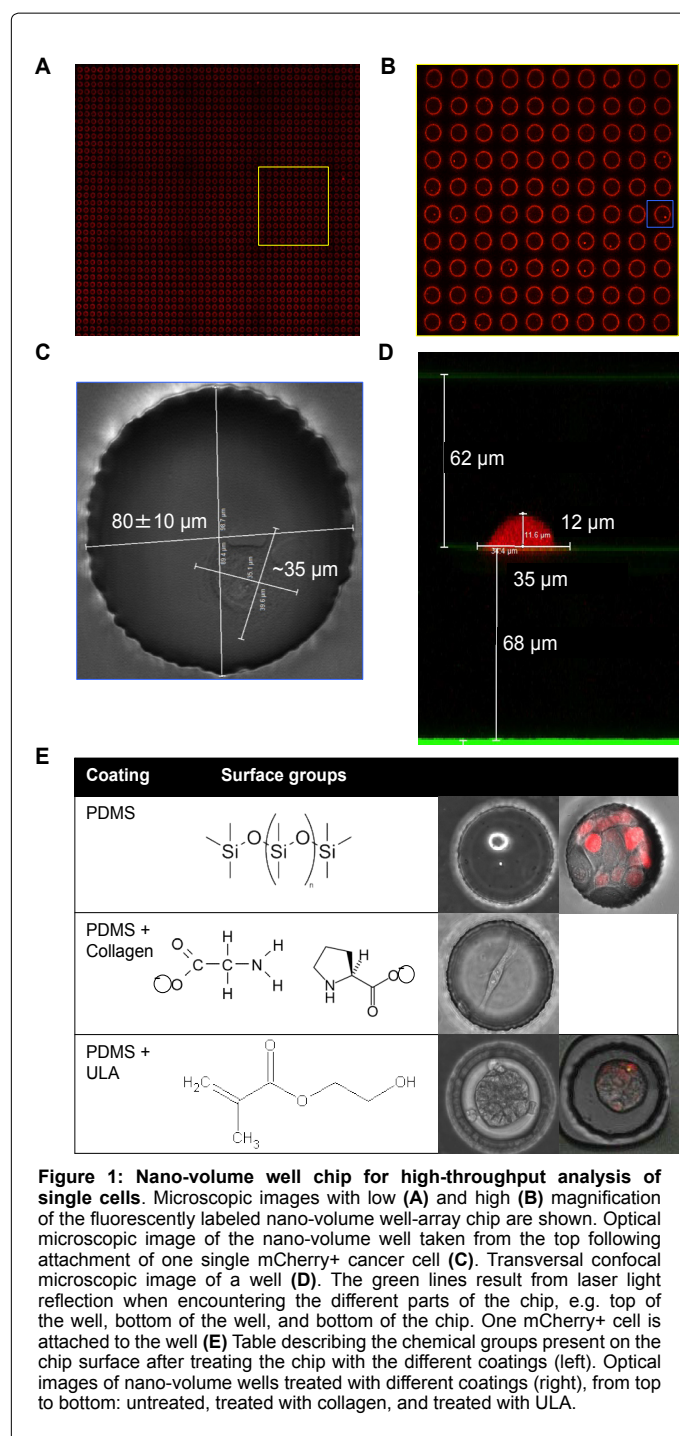
On-chip cell staining

First, the media was removed from the well by tilting the chip and sucking gently the media from the corner of the well. Afterwards, the chip was washed with PBS, and cells were fixed with 4% paraformaldehyde (PFA) for 15 min. No PDMS destruction was observed after PFA treatment. Subsequently, the cells were washed three times with PBST before adding the blocking buffer for 2h at RT. The primary antibody (EpCAM-Alexa488; Biolegend, San Diego, CA) was added overnight at 4°C, and after PBST washing, the secondary antibody was added for 2 h at RT. Finally, 1 µg/mL DAPI (4,6-diamidino-2-phenylindole) was added just prior to imaging.

Results

Chip design and characterization

The microchip was manufactured using standard soft-lithography techniques [22]. The post-array was made of polydimethylsiloxane (PDMS) and designed as a 1 cm² chip containing ~4,600 wells of 80 µm diameter (Figure 1A-1C). Prior to cell seeding, the chip was bound either to 8 or 24-well glass bottom plates using an oxygen plasma oven. Suspended cells were seeded in the nano-volume wells manually, and settled by gravitation. Cells



required between 15-60 min for attaching in the well, but they required up to 24 h when sorted by FACS as assessed by microscopy (data not shown). The structures of the chip as well as single cells trapped inside of the chip were imaged by confocal microscopy (Figure 1D). For imaging the chip, transversal microscopic images were recorded in order to define its final dimensions after the manufacturing process. The actual chip height was $130 \pm 10 \mu\text{m}$, and bearing nano-volume wells with an average depth of $60 \pm 10 \mu\text{m}$ and an average height of $80 \pm 10 \mu\text{m}$. This small height of the chip was of crucial importance for allowing high-resolution time-lapse imaging of single cells.

Adherent and anchorage-free culture conditions

Although CSCs regularly represent a smaller subpopulation of the cancer cells, they can be enriched by means of sorting for specific markers and by cultivating them in 3D-suspension cultures. Therefore, to enable the use of our chip for expanding cells in both adherent and suspension culture, we modified the chip surface. PDMS is a hydrophobic polymer (Figure 1E, upper row) and cells were expected to bind poorly as to polystyrene plates. Intriguingly, pancreatic cancer cells displayed a normal adherence behavior by gross appearance. Nevertheless, to ensure optimal surface contact we treated the surface of PDMS with $10 \mu\text{g}/\text{cm}^2$ rat tail collagen type I (Figure 1E, middle row). However, due to the high migratory capacity of cancer cells, they were freely moving from well to well during the experiment when the chips were covered with collagen, thus rendering the obtained data inaccurate for the utilized cancer cells, but the coating may still be applicable to other, less motile cells. Therefore, we reverted to plating the cells on untreated PDMS for our experiments.

In order to perform 3D-suspension cultures in the nano-volume well array, we next treated the wells with poly(2-hydroxyethyl methacrylate) (pHEMA), which was non-specifically adsorbed at the surface of PDMS. pHEMA is a neutrally charged hydrophilic hydrogel (Figure 1E, lower row) widely used to inhibit cell adhesion to surfaces in conventional culture vessels. Spheres could successfully be formed in the nano-volume well chip coated with pHEMA. This coating procedure is very advantageous since it is fast, very simple, and does not require any chemical reaction as would be needed for treatments using covalent linking [19,24,25]. Briefly, $150 \mu\text{L}$ of 1.2% of pHEMA was dispensed on the chip bound into a well of an 8-well plate. After overnight drying at 37°C , the chips were sterilized by UV exposure for 1h prior to use.

Clonal expansion of single cells

A key feature of CSCs represents their ability to self-renew, which is regularly tested in ultralow adhesion cultures. However, a major limitation of the readout of these assays is the varying degree of cell aggregation, which would lead to polyclonal spheres confounding the readout of the assay. To compare the ability of conventional non-adherent cell culture versus the nano-volume well chip for tracking clonal expansion, we performed experiments using primary pancreatic cancer cells derived from the same original tumor, but each of them stably expressing one of the following four fluorescent reporter genes: GFP (green), Katushka (red), CAE (blue), and CIT (yellow). Equal numbers of cells (2,000 cells per 1 mL of medium) from each group were seeded in the nano-volume well chip and in conventional 24-well plates, respectively, using both adherent and in 3D-suspension conditions. Images were taken on day 1 and 7 after seeding by a confocal microscope demonstrating that a large fraction of spheres forming in the 24-well plates were actually polyclonal, formed by more than one group of cells. In fact, 76% of them were formed by cells of all 4 different colors (Figures 2A-2C and S1). In contrast, all spheres formed in each of the nano-volume wells were monoclonal if indeed initiated from a single cell, which was the case in 75% of the wells (Figures 2B,2C and S1). Multiple cells per well were observed in 25% of the cases because cell-seeding distributions in multiwells follow the Poisson statistics [23].

In order to demonstrate that our chip is compatible with other single-cell assays, we also studied single-cell expansion in MatrigelTM and methylcellulose, two well-established matrices for performing single-cell colony-forming assays. For this purpose, we first seeded the

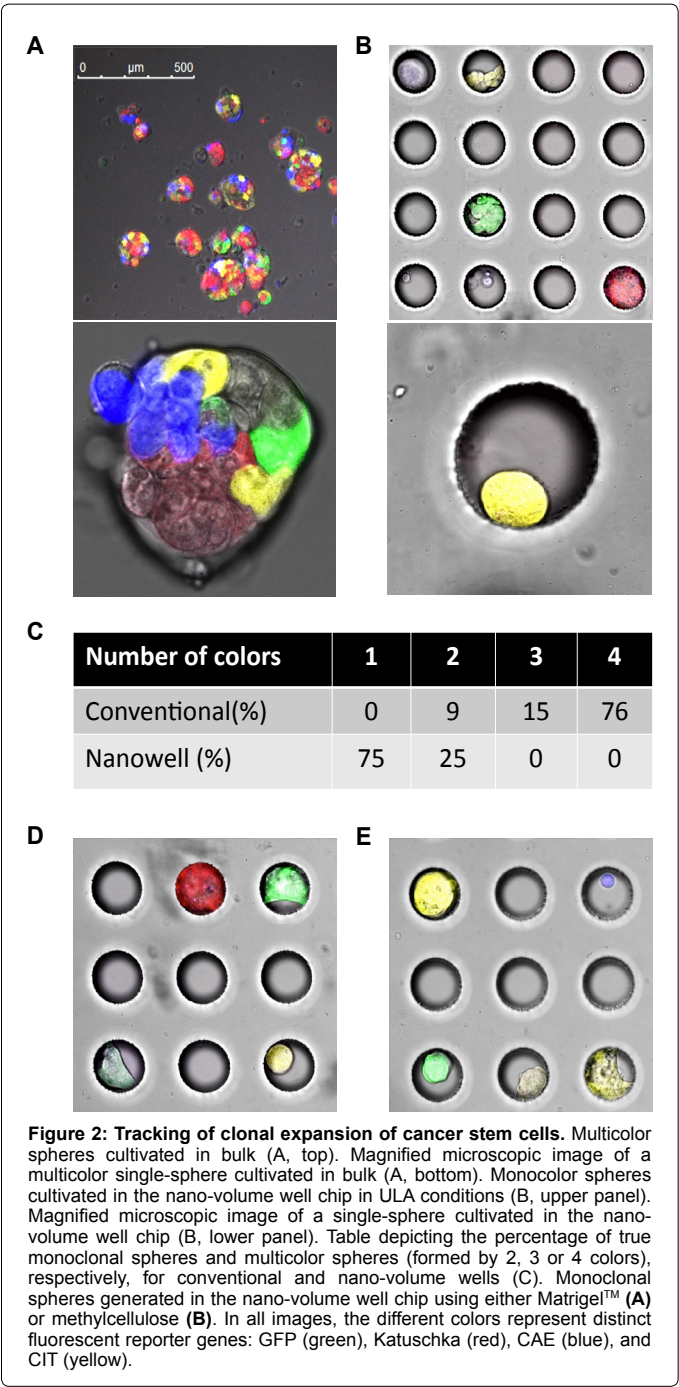


Figure 2: Tracking of clonal expansion of cancer stem cells. Multicolor spheres cultivated in bulk (A, top). Magnified microscopic image of a multicolor single-sphere cultivated in bulk (A, bottom). Monocolor spheres cultivated in the nano-volume well chip in ULA conditions (B, upper panel). Magnified microscopic image of a single-sphere cultivated in the nano-volume well chip (B, lower panel). Table depicting the percentage of true monoclonal spheres and multicolor spheres (formed by 2, 3 or 4 colors), respectively, for conventional and nano-volume wells (C). Monoclonal spheres generated in the nano-volume well chip using either Matrigel™ (A) or methylcellulose (B). In all images, the different colors represent distinct fluorescent reporter genes: GFP (green), Katuschka (red), CAE (blue), and CIT (yellow).

cells in the nano-volume wells (either PHEMA coated or uncoated), and after 20 min the medium was removed by tilting the chip and gently aspirating the media from the corner of the well. Subsequently, the respective matrix was added (a volume required to cover the chip surface) and the chip was kept at 37°C. Again, nano-volume wells seeded with single cells showed formation of monoclonal 3D-structures after 7 days of cultivation in Matrigel™ (Figure 2D) and methylcellulose (Figure 2E), respectively.

Picking of expanded cells from single nano-volume well

Isolation of such monoclonal spheres is critical for further studies at both molecular and functional levels. Therefore, we developed

an isolation method in order to retrieve spheres lacking any cross-contamination with neighboring clones while maintaining the viability of the clone. Needles with 100 μm gauges were installed on a microscope equipped with an XYZ-axis arm manipulator and allowed selective and reproducible picking of any desired clone. Figure 3A shows the nano-volume well chip before (upper panel) and after (lower panel) the picking process. The utilized needle was able to enter the nano-volume well and capture the sphere by mere capillary forces. Each of the isolated spheres could then be re-seeded on 96-well plates and expanded over an extended period demonstrating that the isolation process did not affect cellular viability. A representative example of an isolated sphere at baseline and after 2 and 4 weeks in culture is shown in Figure 3B.

On-chip phenotyping of cells

In order to further investigate and confirm the formation of spheres, we performed nuclear (DAPI) and cell surface staining (EpCAM conjugated to AF-488) in spheres and cell-monolayers after cultivation in nano-volume wells. For this purpose, we seeded single primary cancer cells on two different nano-volume well chips, either PHEMA-treated (for ULA conditions) or un-treated (for adherent expansion). After 7 days of cultivation, cells were stained as described in the methods section. The nano-volume well chips were then imaged using confocal microscopy, and Z-stacks were recorded to allow 3D reconstruction of the wells. Analyses of the microscopic images revealed a clear round shape of the spheres in PHEMA-treated nano-volume wells (Figure

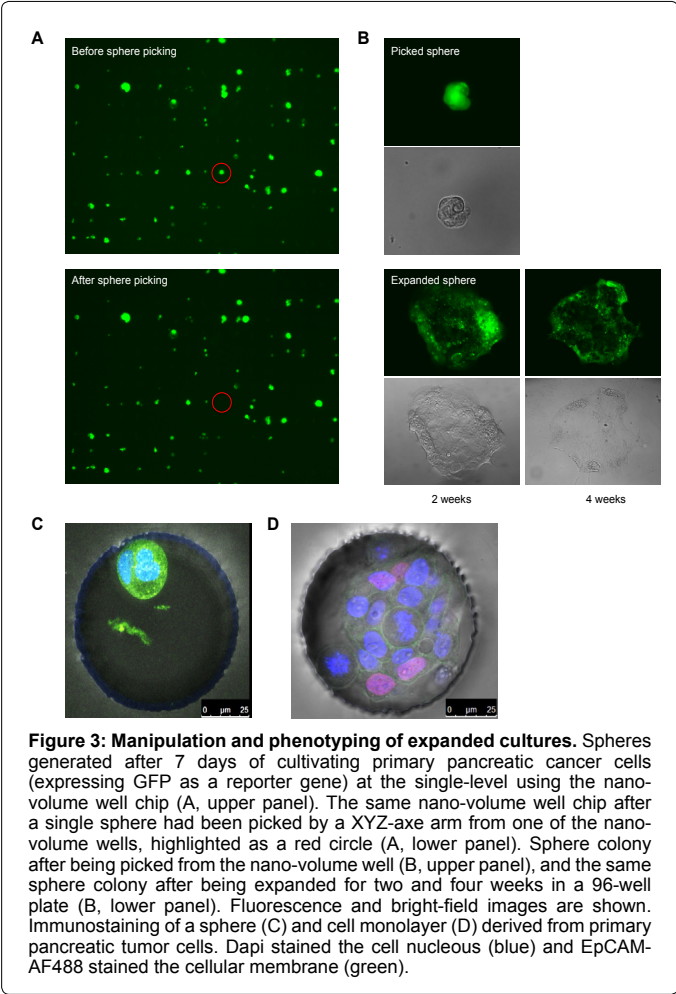
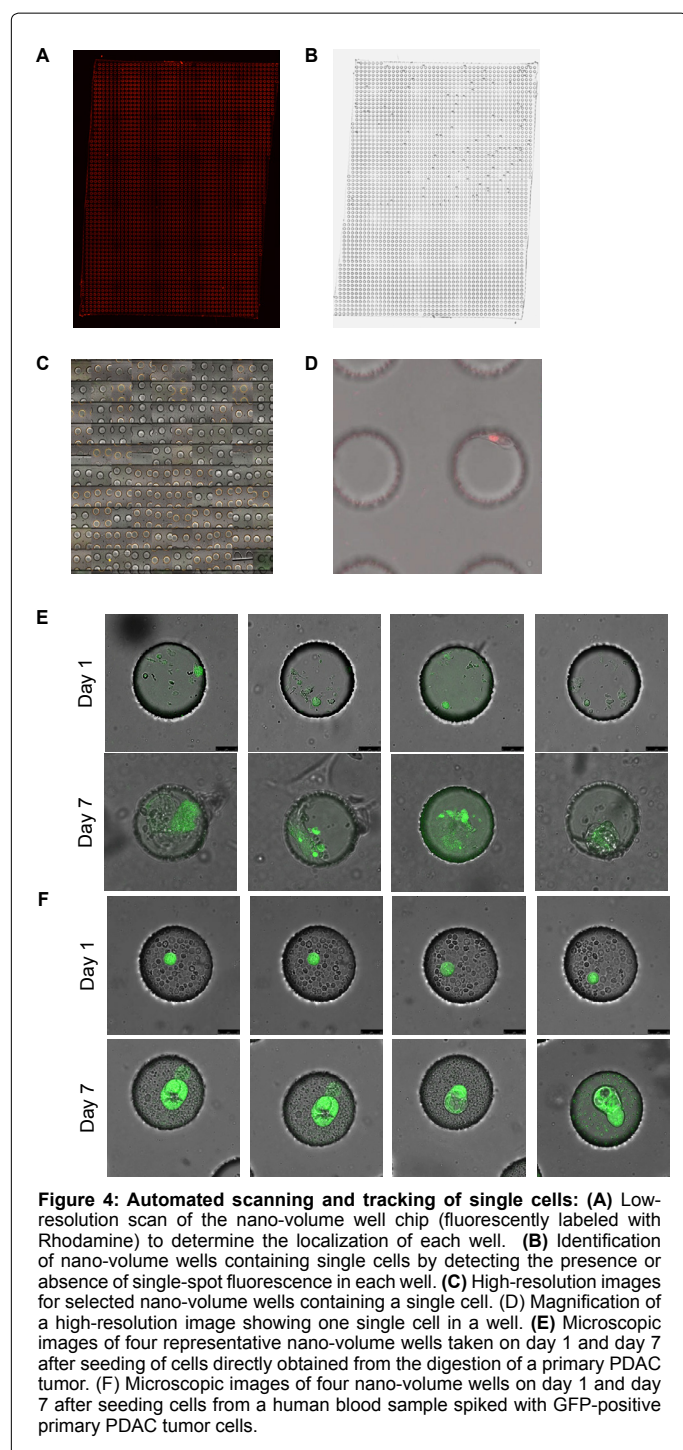


Figure 3: Manipulation and phenotyping of expanded cultures. Spheres generated after 7 days of cultivating primary pancreatic cancer cells (expressing GFP as a reporter gene) at the single-level using the nano-volume well chip (A, upper panel). The same nano-volume well chip after a single sphere had been picked by a XYZ-axis arm from one of the nano-volume wells, highlighted as a red circle (A, lower panel). Sphere colony after being picked from the nano-volume well (B, upper panel), and the same sphere colony after being expanded for two and four weeks in a 96-well plate (B, lower panel). Fluorescence and bright-field images are shown. Immunostaining of a sphere (C) and cell monolayer (D) derived from primary pancreatic tumor cells. Dapi stained the cell nucleus (blue) and EpCAM-AF488 stained the cellular membrane (green).



3C), however when using un-treated nano-volume wells, we observed both cell monolayers (Figure 3D,) and spheres, but this time bound slightly to the nano-volume well bottom. The fact that some cells show both, sphere development as well as partial attachment to non-pHEMA coated nano-volume wells, suggests cell heterogeneity, which reflects cellular heterogeneity, but if desired can be avoided by modifying the coating protocol data not shown.

Acquisition software

The comprehensive utilization of the nano-volume well array

systems for the use in high-resolution and -throughput screening of rare cells requires efficient analysis of the entire chip within a reasonable time. Importantly, the main factors determining cell seeding and distribution statistics are cell sample concentration, sample volume applied to the chip, number of wells on the array, and the surface of the well opening relative to the total chip area. Depending on these factors, a varying fraction of wells will remain empty or will contain more than one cell and needs to be prospectively excluded from the subsequent analysis. For that purpose, we used in house developed software (iMSRC) capable of self-operating the SP5 confocal microscope (Leica). First, the software acquires a low-resolution scan of the entire chip fluorescently labeled with Rhodamine (Figure 4A). The software then automatically localizes nano-volume wells containing single cells by quantifying the number and the presence or absence of fluorescence cells and saves the respective coordinates of the wells (Figure 4B). The software then records high-resolution images for each of the selected nano-volume wells containing a single cell (Figure 4C), e.g. every 15 min for several days. These images are afterwards merged to build time-lapse movies illustrating the process of cell division for hundreds of cells in a single experiment (Figure 4D).

Culturing single cells from fresh samples

Next, we aimed to evaluate two potential applications for the nano-volume well chip: i) culture of cells from fresh tumors on the single-level and ii) detection and culture of tumor cells isolated from a blood sample. For the first approach, a primary PDAC tumor was digested into single cell suspension; cells were counted and then seeded onto the nano-volume well chip (0.5 cm × 0.5 cm) at a concentration of 5,000 cells per chip. The cells were cultured in the presence of 30 μM Riboflavin over-night, a vitamin enriched in cancer stem cells allowing the prospective tracking of these rare cells (unpublished data, I.M.-L.) [26]. Confocal images were recorded on day 1 and day 7. Analysis of the images on day 1 revealed that a large fraction of the wells contained single auto fluorescent tumor cells (Figure 4E, upper panel). Images on day 7 verified that cells had not only survived but also had proliferated (Figure 4E, lower panel). Thus, demonstrating the possibility of cultivating cells directly from a tumor, and even more importantly, doing so at the single-level. Therefore, the nano-volume well chips providing the unique opportunity to study tumor heterogeneity and monitor the fate of the cells over time.

For evaluating the second putative application, 10³ GFP-positive primary PDAC cells were spiked into 5 mL of whole human blood. The blood sample was then processed using a protocol established in our lab for the isolation of viable circulating tumor cells. Briefly, Red Blood Cells (RBC) were lysed using the BD Pharmalyse buffer. Then, the sample was depleted of CD45+ cells, using automated Magnetic Cell Sorting (Auto MACS) [27]. Subsequently, the remaining cells were counted and seeded onto four nano-volume well chips (0.5 cm × 0.5 cm). Finally, confocal images were recorded on day 1 and day 7. Analysis showed that cells spiked in the blood sample could be detected in the chip directly after seeding (Figure 4F, upper panel), and further proliferated during 7 days within the nano-volume wells (Figure 4F, lower panel). These data demonstrate that the chip could be also used for detecting, expanding and monitoring tumor cells harvested from a blood sample, e.g. Circulating Tumor Cells (CTCs) [28].

Cell fate tracking

Finally, we aimed to demonstrate the potential of the chip to track cell fate in large numbers of heterogeneous cancer cells. First, cancer cells obtained from a freshly digested pancreatic tumor were enriched

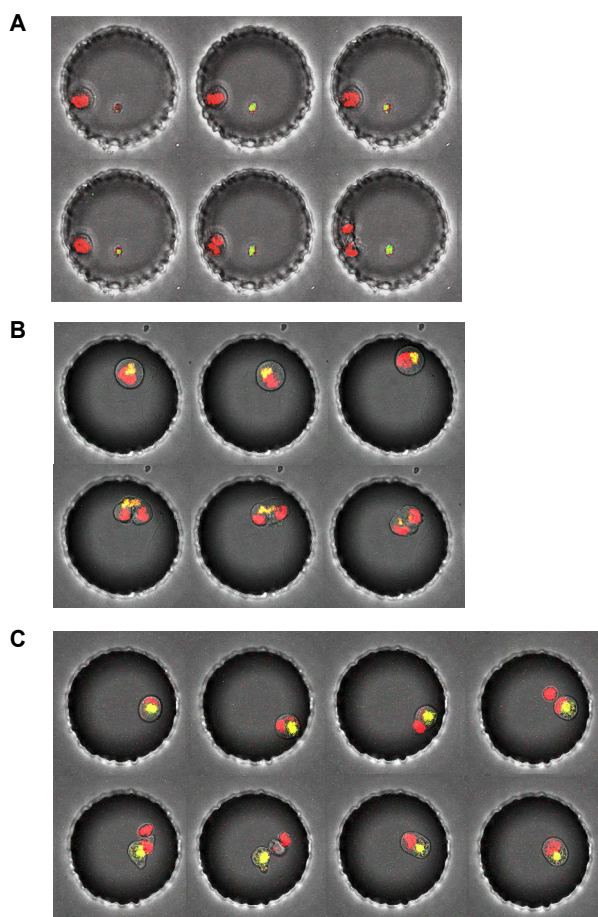


Figure 5: Automated scanning and tracking of single cells: (A) Sequence of images of a single fluorescence-negative cell giving rise to two fluorescence-negative cells. (B) Sequence of images of a single fluorescence-positive cell dividing symmetrically into two fluorescence-positive cells. (C) Sequence of images of a single fluorescence-positive cell dividing asymmetrically into one fluorescence-negative cell and one fluorescence-positive cell.

for cancer stem cells by low-purity FACS sorting for their native autofluorescence as described above (unpublished data, I.M.-L.) in order to enhance the frequency of cancer stem cells seeded onto the nano-volume well chip. A total of 10^3 sorted cells resuspended in 2 mL were added to a fluorescently-labeled nano-volume well array. Then, a fast low-resolution scan of the chip was performed to localize nano-volume wells containing single cells; thus discarding wells containing none or more than one cell. Next, the newly developed software was used to record high-resolution images for each of the selected nano-volume wells (bright-field and fluorescence), which was repeated every 15 min over an observation period of 72 h. Based on the high-yield, low purity sorting setup, analysis of the images demonstrated that about 50% of the sorted cells were actually non-autofluorescent when the imaging process started. Most importantly, the analysis of the images revealed that autofluorescence negative non-CSCs did not give rise to autofluorescence-positive CSCs (Figure 5A). In contrast, autofluorescent CSCs could give rise to both CSCs and non-CSCs respectively, by undergoing symmetric (Figure 5B), and asymmetric divisions (Figure 5C). The first cell division occurred within 48 h for 60% of the cells with no difference between autofluorescence positive and negative cells. The remaining 40% of the cells were either dead or

died during the imaging process. From the 60%, 10% of the videos were not conclusive, 35% underwent cell division and 15% didn't. From the 35%, 26% underwent symmetric cell divisions and 9% asymmetric cell divisions. Expectedly, cell death rate markedly decreased from 40% down to 20% when non-sorted cells were used and imaged only twice each 24 h.

Discussion

Here we report the successful development of a microfluidic device for studying the heterogeneity of cancer stem cells harbored within pancreatic cancer, but these rare cells have also been reported for other cancers and therefore our chip is broadly applicable. We demonstrate the successful expansion of primary pancreatic cancer cells derived from freshly digested tumors at the single-level. The chip also enabled for the extensive analysis of each individual clone, but at the same time in a highly parallelized manner allowed for the simultaneous study of clonal heterogeneity and cell propagation for several thousands of cells in a single experiment.

Although several different chips have been published to date, as outlined in the introduction, no development has been reported that would be suitable for the analysis and propagation of rare and heterogeneous cancer stem cells. Our chip specifically developed for this purpose is a nano-volume well-based array hosting several thousands of ~ 80 μm diameter wells, each of which holds up to 0.4 nL of volume. The untreated chip can be used for expanding cells in regular adherent conditions, which favors differentiation and therefore expansion of bulk cancer cells. However, we also developed a specific surface treatment of the chip to propagate cells in anchorage-independent 3D cultures for the generation of CSC-enriched spheres. Thus, for the purpose of studying cancer stem cells our nano-volume well chip provides several advantageous and unique features compared to conventional techniques.

First, cells do not necessarily require vigorous and therefore harsh sorting since several thousands of cells can be studied simultaneously, thus maintaining high cell viability. Intriguingly, we were able to expand tumor cells directly from freshly resected tumors at the single-cell level in the nano-volume wells. Due to the small size of the nano-volume wells as opposed to regular microtiter plates, it is reasonable to hypothesize that the cells are more capable to generate their own microenvironment, and paracrine factors (e.g. Nodal and Activin) are not rapidly diluted in the large volume of media used for standard cell culture [29,30]. Indeed, based on our experience single cells do not regularly proliferate in standard microtiter plates, but for the first time could be efficiently propagated in the herein presented nano-volume well chip. Thus, specific tumor cells can be expanded at the single level despite the unavoidable presence of contaminating tumor fibroblasts, thus allowing the possibility of studying tumor heterogeneity and cells properties over time. Indeed, increasing evidence demonstrates that even CSCs represent a heterogeneous population of cells with distinct features including metastatic activity and drug resistance that require investigation at the single cell level.

Secondly, the single cell propagation maintains monoclonality throughout the experiment, as the seeded cells are kept physically separated from each other. Subsequently, single cells formed true monoclonal colonies in the nano-volume wells under different conditions including the presence of matrices including Matrigel™ and methylcellulose. In contrast, in standard large-scale culture dishes as well as microtiter plates cells tend to aggregate and form multicolored and therefore polyclonal spheres as shown in our experiments. This was

still observed for optimized conditions at low cell density. Therefore, the physical separation of single cells is an important feature of the chip allowing true cell fate tracking experiments. Forth, cells cannot only be cultured and monitored, but can also be recovered from the nano-volume wells at the single-cell or after propagation at the single-sphere level. Single spheres can be selectively picked from a nano-volume well with a XYZ-axis arm micromanipulator, and subsequently can be further expanded as individual clones in a microtiter plates.

Thirdly, we show that even tumor cells spiked into blood samples can be detected and clonally expanded in the nano-volume wells chip. Here, after sample processing, all remaining cells could be plated on the chip and monitored over 7 days. Despite the efficient depletion of red and white blood cells, still thousands of contaminating blood cells remain in these samples. However, since tumor cells were captured in single wells, they could proliferate at the single-level demonstrating the enormous potential of our chip for detecting and propagating CTCs in blood samples of cancer patients for further molecular and cell biology studies. Future studies should address if personalized drug response profiles can be obtained with CTC-derived CSC cultures and whether this can predict drug response in individual patients.

Finally, we demonstrate the feasibility of cell fate tracking by monitoring the expression of a novel cancer stem cell marker, namely autofluorescence, during cell division using time-lapse imaging. Images could be recorded at high magnification and high-resolution as the nano-volume well chip is not only transparent, but also manufactured at a height of only 130 μm . Our experiments revealed that non-autofluorescent bulk cancer cells do not give rise to autofluorescent CSCs and therefore these data address an intensively discussed controversy as to whether CSCs can be replenished by non-CSCs. Moreover, we demonstrate that autofluorescent cancer cells can give rise to both autofluorescent and non-autofluorescent cells and thereby are capable of replenishing the entire spectrum of cancer cells range in the tumor. Therefore, the nano-volume well chips offer a plethora of new opportunities and should arise as a powerful tool for further advancing our understanding of cancer stem cell biology.

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