Mimicking Intravasation–Extravasation with a 3D Glass Nanofluidic Model for the Chemotaxis-Free Migration of Cancer Cells in Confined Spaces

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A new 3D nanofluidic biochip for the study of cancer cell migration and invasion is proposed. In this design, femtosecond laser-assisted etching is applied to create embedded microfluidic channels, with a base thickness of less than 100 µm for high-resolution imaging using inverted microscopes. The glass deformation is thermally controlled during fabrication to create pillar-like formations separated by narrow constricted channels with widths of less than 1 µm spanning lengths of more than tens of microns, mimicking the 3D intravasation–extravasation configuration. Time-lapse microscopy is used to observe the behavior of prostate cancer (PC3) cells in chemotaxant-free media over long time intervals as the cells invade the narrow spaces. The PC3 cells are observed to be capable of breaching the fabricated submicrometric intravasation-like barriers while retaining their viability and proliferation activity. The cells are further able to penetrate the extravasation-like confining spaces, confirming their dynamic adaptability as they pass through constricted channels with volumes much less than that of the cell nucleus.

1. Introduction

Although early diagnosis and cancer growth inhibition have led to clinical progress in cancer survival rate, cancer metastasis is still the main cause of morbidity and mortality for about 90% of cancer deaths.1 Cancer metastasis is the process by which cancer cells that developed in the primary tumor start to spread through the body via the bloodstream or lymphatic system and reach secondary sites where they may generate new tumors.2 Metastatic cancer cells are defined by several specific characteristics, such as poor adhesion, increased motility and invasiveness, survival during circulation in the bloodstream, and relocating to other tissues to colonize new sites.2 For the development of appropriate and effective treatments for metastatic cancer patients, it is highly desirable to understand the behavior and mechanisms of metastatic cancer cells during their journey. This process starts with cell detachment from the primary tumor, with the detached cancer cell invading the narrow confinements of the surrounding extracellular matrix at the beginning of the metastatic process and continuing to travel through connective tissues.3 It has been found that in tissues, an individual cancer cell can follow linear confinement of 2 µm in diameter for lengths of up to 750 µm or channels of a few micrometers in diameter and an interstitial space length of 150 µm.5 The cells can then penetrate even nanometric spaces to reach blood vessels during intravasation and travel as circulating tumor cells to eventually leave the vessels by extravasation and enter another tissue or organ (Figure 1).

Though somewhat different from each other, the processes of cell invasion, intravasation, and extravasation have much in common, including similar supporting signaling pathways, drastic morphological deformation, and rupture followed by repair of the cell nucleus.6 It is thus a challenge to create systems that mimic the in vivo environment, which has pushed many researchers to focus in recent years on the development of microfluidic biochips capable of recapitulating the relevant in vivo characteristics, along with imaging techniques for subcellular visualization, in order to better understand the mechanisms of cancer cell migration, in micro- and submicrometric environments in particular.7

In comparison with conventional in vitro culture platforms, microfluidic biochips offer well-defined 3D pathways for fluids with controlled geometries from submillimeter to submicrometer dimensions.8 In addition, they are cost effective because they require smaller volumes of reagents and allow reactions with high sensitivity and high spatiotemporal resolution.9
Therefore, microfluidic biochips offer great potential to study cell migration. Particularly, they provide platforms enabling both observation of collective cancer cell migration over long periods in vivo-like environments and more detailed visualization at unicellular and subcellular levels for a target cell, together at the desired spatial (micro- to nanometric) and temporal (minutes to seconds) scales by using advanced microscopic instruments. The best material for the platform is glass, similar to the glass-bottom Petri dishes most commonly used for live cell imaging, because of its excellent optical transparency and very low auto-fluorescence. Ideally, the base thickness (thickness between the bottom of the microfluidic channel and the rear surface of glass chip) should be less than 100 µm to allow high-resolution imaging because the inverted optical microscope typically used for this type of application has an optical lens with a very high numerical aperture (NA = 60× or 100×), necessitating a very short working distance (sometimes ≈200 µm).

Femtosecond laser assisted etching (FLAE), which is a subtractive processing method, can be used to create 3D microfluidic structures directly embedded in a glass microchip, tailored at the micrometric level, without any stacking or bonding procedure for the substrates.[10] FLAE of Foturan glass has been employed to fabricate biochips such as nano-aquariums for 3D observation of the rapid flagellar motion of Euglena gracilis[11] and elucidation of the gliding mechanism of Phormidium in soil for accelerating vegetable growth.[12] Furthermore, the integration of microelectronic components in the fabricated microfluidic channels by the subsequent femtosecond laser-induced selective metallization has been applied for the electrical manipulation of biological samples in nano-aquariums.[13] Through the combination of FLAE with successive two-photon polymerization (TPP), which is an additive processing technology, complex 3D shapes of polymeric structures with submicrometer dimensions have been successfully integrated inside glass microfluidic devices. This novel hybrid FLAE-TPP technique has been applied to build a multi-functional filter-mixer for the substrates.[10] FLAE of Foturan glass has been employed to fabricate biochips such as nano-aquariums for 3D observation of the rapid flagellar motion of Euglena gracilis.[11]

In the present study, we improved the fabrication resolution of FLAE by inducing glass deformation via post-thermal treatment (FLAE followed by nanoscale glass deformation: FLAE/NGD) to create submicrometer glass channels in ultrathin glass microchips. This not only eliminates the necessity of using a second laser step for polymerization to downsize the channel dimensions, but also enables the use of biochips made from a single material (glass). As described above, the fabricated glass microfluidic devices provide two important benefits: they create an in vivo-like environment with architectures down to the nanoscale, providing narrow constrictive spaces for cancer cell migration, and they enable unicellular and subcellular observation by high-resolution confocal microscopy for sensitive live cell brightfield and immunofluorescence imaging because of their ultrathin glass bases. Using this biochip, we observed the capability of PC3 cells to penetrate two consecutive narrow constrictions mimicking intravasation–extravasation events known to occur in the in vivo environment.

This study presents two significant contributions: the first is a biochip fabrication technology, and the second, a proof-of-concept cancer cell invasion study. With regard to the former, the laser fabrication of microfluidic channels with an ultrathin base thickness is very challenging because the classic FLAE process involves high-temperature thermal treatment, which can often lead to the deformation of the thin glass chips. Glass submicrometer constrictions inside the microfluidic channels were obtained for the first time using the proposed novel FLAE/NGD approach. Regarding the cancer cell invasion portion of the study, the chemotaxis-free migration and invasion of cancer cells in the submicrometer channels were revealed, providing new findings to elucidate the mechanisms of cancer cell migration, intravasation, and extravasation. Specifically, it was observed that PC3 cancer cells have low penetration thresholds to enter channels, breaching barriers through narrow channels of submicrometric widths to travel in an intravasation–extravasation configuration while retaining their viability and capacity for proliferation.

2. Results

2.1. Biochip Concept

The glass biochip proposed herein consists of three open microreservoirs at the front surface connected with L-shaped embedded microchannels (Figure 2a). Two of the microreservoirs (µR1, µR3) are used as cell inlets, and the third microreservoir (µR2) is necessary to produce two rows of symmetric channel arrays of submicrometer width as well as to eventually collect cells after their migration (from µR1 to µR2). The
channel Ch4 connecting μR1 to μR3 was necessary as a permanent experimental control to confirm cell migration in the absence of narrow constructions. The channel connecting μR1 to μR2 is composed of three chambers (Ch1, Ch2, and Ch3), which are delimited by two rows of glass elliptical pillars to mimic endothelial cells covering blood vessels (Figure 1). The pillars connect the ceiling to the base of the microfluidic channel and thus have the same height of ≈60 μm as the channel (Figure 2b,c), and their length in the longitudinal direction of the tunnel is ≈150 μm. These dimensions are slightly variable depending on parameters in the fabrication protocol, particularly the etching time. The lengths of Ch1 and Ch3, i.e., the distances between μR1 and the first row of pillars and between μR2 and the second row of pillars, are 500 μm, and the length of the central chamber, Ch2, bounded by the two rows of pillars is 200 μm. The width of the gaps between the pillars can be tailored by adjusting the process parameters of the etching and second thermal treatment. In particular, the second thermal treatment produces constriction channels with submicrometric widths as a result of the melting of the shallow glass surface (Figure 2d).

2.1.1. Biochip Fabrication: Reducing the Channel Dimensions to the Submicrometer Range

The L-shaped glass microfluidic platform was fabricated by femtosecond laser-assisted etching (FLAE), which is a subtractive 3D process with high controllability. It is necessary to optimize the experimental parameters (e.g., laser power, scanning mode and speed, scanning pitch, concentration of etchant, etching time, thermal treatment temperature and time) to achieve the desired geometry (Figure 2a).

A computer-automated design (CAD) program was developed for the laser scanning of the entire structure with special attention paid to the writing of the pillars, which had a rectangular shape in the initial design. To make the gap between each pillar as narrow as possible, a single straight line was scanned between them by laser, producing the narrowest modification. This laser irradiation produced latent images in the glass due to the photoreduction of Ag ions to Ag atoms induced by free electrons generated by the multiphoton absorption of the femtosecond laser beam. After the laser irradiation, the sample was exposed to an initial thermal treatment, by which precipitated Ag atoms were clustered to act as nuclei for the growth of the crystalline phase of lithium metasilicate at the laser-exposed regions. As a result, the modified patterns were clearly observable as dark brown regions inside the photosensitive glass (Figure 3a). These regions can be preferentially etched in a diluted HF acid solution at a rate ≈50 times higher than the non-laser exposed regions. The sample after the first annealing is shown in Figure 3a, with the red dotted squares labeled 1 and 2 highlighting two rectangular pillars, each of which appears transparent because they did not undergo laser modification. They are delineated by narrow dark brown lines produced by a single straight line scanned by a laser beam.

The etching time is a critical parameter in the development of uniform channels with similar widths over extended lengths. In the present experiment, an optimal etching time of 55 min in an 8% HF solution was applied to produce pillars after the second annealing with the narrowest channels spanning the length of the gaps between the pillars (Figure 3b). As shown in Figure 3b, the channels became tapered as a result of the isotropic etching during channel development from Ch1 and Ch3 toward Ch2. The minimum width of the channels at the optimal etching time was found to be ≈4 μm at the Ch2 junction. Thus, the originally rectangular pillars became trapezoidal, as shown by the dotted red trapezoids in Figure 3b.

Finally, a second thermal treatment at a temperature slightly below the melting temperature was carried out. In
this treatment, because the surface energy is lower than the bulk energy, shallow surface layers could be melted. The surface melting reformed the trapezoidal pillars to an elliptical shape, narrowing the gaps between pillars as a result of the reflow associated with the surface tension of the melted zones (Figure 3c). The volume of each pillar appeared to have increased after the reforming, which is probably responsible for the supply of additional glass from the melted surface at the floor and ceiling of the microchannels. Additionally, the second thermal treatment greatly smoothed the etched glass surfaces and offered high transparency. Such a smooth surface with an average roughness of less than 20 nm is essential to induce efficient cell migration and to allow the clear observation of the cells in the micro/nanochannels by optical microscopy.

2.1.2. Evaluation of Submicrometer Channels formed in Glass Biochips

Prior to performing experiments with the chips, the dimensions of the nanofluidic channels within the microfluidic channels were evaluated. To this end, the entire chip was filled with Rhodamine Red, and the chip was observed by confocal microscopy. The reconstructed 3D structure is shown in Figure 4 along with

Figure 3. Optical microscope images of the sample after each process step. a) Sample after laser direct writing followed by the first thermal treatment. The modified regions take on a dark brown color. The regions surrounded by small black or red dotted rectangles show the locations of the pillars. b) Sample after chemical etching in an 8% HF solution for 55 min. The pillars designed with rectangular shapes become trapezoidal columns (trapezoidal pillars 1 and 2 respectively correspond to rectangular pillars 1 and 2 in a). c) Sample after the second thermal treatment at 645 °C. The trapezoid pillars are reshaped into ovals. d) Fabricated ultrathin glass micro/nanofluidic biochip.

Figure 4. Imaging of submicrometer channels inside the microfluidic biochip using Rhodamine Red. a) Rhodamine fluorescence image showing one row of elliptical pillars. b) Top-down view magnified from the dotted square area in (a). c) Further magnified image in the xy plane from the uppermost region in (b). d) Side view of (c). e) 3D image reconstructed from a. f) 3D image reconstructed from b. g) Optical confocal image of submicrometer channels. The red arrow in the image shows a cell nucleus and the migration direction (Correlated with Movie 4.1 and Movie S2, Supporting Information).
the obtained 2D images. For better evaluation, one row of pillars was observed in detail (Figure 4a). Figure 4b shows a top-down view of two nanochannels magnified from the dotted square area in Figure 4a and Figure 4c shows detailed view further magnified from the uppermost area in Figure 4b. Figure 4e,f shows 3D-reconstructed images of the pillar-delimited nanochannels, and Figure 4g shows an optical confocal image confirming the formation of submicrometer channels of over 50 µm in length, with the channel width gradually enlarging to reach widths of more than 5 µm toward either end (Figure 4c). A live view of cross sections at different locations with the channel length can be seen in Movie 4.1 (front view) and Movie S2 (Supporting Information) (side view) during the migration of a cell through the narrow space. These movies demonstrate the change in the shape of the cell nucleus following the geometry of space. From the side view images and 3D reconstructions of the internal structure of the biochip, a slightly asymmetric 3D configuration similar to a rectangular enclosure with gabled rounded roof was identified. The asymmetry may be attributable to the originally trapezoidal shape of the pillars. The wider and higher entrance was designed to give a larger surface area for more efficient guiding of cells into the nanochannels, whereas the more constrictive zone at the center is the space dedicated for the study of cell migration. Indeed, a tapered shape was observed with a maximum height of $27 \pm 2.7$ µm at the entrance and exit, which was the same as the height of the microchannel ceiling, and a minimum height of 6.75 ± 0.67 µm at the center of the channel over a length of 110 µm ± 11 µm (Figure 4d). A more general view of the same structure is also shown in Figure S1a,b (Supporting Information), along with images of structures exhibiting different constriction dimensions, which can be controlled by simply changing the etching time (Figure S1c–e, Supporting Information). Indeed, by adding one or two supplementary minutes to the previously mentioned etching time (55 min), the gap between the pillars could be widened to create samples with wider constriction channel widths of 2 µm ± 0.2 µm or 5 µm ± 0.5 µm, respectively, after the thermal treatment (Figure S1, Supporting Information). By appropriately tuning the laser scanning process, thermal treatments, etching dilutions, and exposure intervals, the process can be well controlled, and samples with similar geometric characteristics (length, width, height) were successfully obtained with a mean standard deviation of ±10%. Small sample-to-sample variations appeared because of challenges in strictly controlling HF concentration and precise etching time. Nevertheless, in the present experiments, the same devices with unchanged geometric characteristics were used for repeated testing to eliminate any variability caused by the fabrication process. One of advantages of our glass nanofluidic devices is that they can be reused many times.

2.2. Observation of Cancer Cell Behavior in Micro/Nanofluidic Channels

The surfaces of all biochips were coated with collagen before testing the migration and invasion of PC3 cancer cells stably expressing mRFP2-histone within the micro/nanofluidic channels. After the cells were loaded into the reservoir µR1, conditions were provided for them to adhere, spread, migrate, and proliferate in a complete cell medium, but no other chemotaxants were supplied to induce chemotaxis during testing. Figure 5 shows the behaviors of the cells in a micro/nanofluidic channel at different times after the cells were loaded. The upper, middle, and lower figures correspond to optical images, fluorescence images of the stained cell nuclei, and detailed images merging the optical and fluorescence images, respectively. At first, the cells migrated not individually in the microchannels but rather collectively inside the channels (Figure 5a). They were able to spread on the ceiling (6 cells at 8 h, Figure 5a; 12 cells after 28 h, Figure 5b, middle row) and the walls but more preferentially existed on the floor (36 cells at 8 h, Figure 5a; 40 cells after 28 h, Figure 5b, middle row). Typically, 24–28 h after loading, the cells approached the first row of pillars (Figure 5b, left). Approximately 40–45 h after cell loading, the first cells detached from the population and entered the nanochannels formed by the gaps between the pillars (Figure 5c). A few hours later (49 h after loading), many cells were invading and migrating through the nanochannels. Eventually, 65 h after loading, cells were exiting from the nanochannels to start populating the second chamber, Ch2 (Figure 5d, from right to left).

The cell invasion experiments were continued under observation by confocal time-lapse microscopy. The main aim of these experiments was to observe cells penetrating the first row of pillars (intravasation row, hereafter referred to as the I-row), their behavior after passing through the constrictive spaces in the I-row (reflecting a potential loss in viability), and their eventual continuation toward the second extravasation row (E-row). A selection of frames from the recorded time lapses are shown in Figure 6. After the cell intravasation, the cells survived without any indication of loss in viability (i.e., all of the ~20 identified cells were viable during the observation period for a few hours after migration; Figure 6b). Furthermore, they were seen to actively migrate and even proliferate without any significant damage. Notably, invasive activity remained unchanged, as demonstrated by cells further penetrating the E-row (red arrows in Figure 6a,b). A viability test was carried out by calcein staining after several cells were found penetrating the E-row, confirming that cells in all chambers were viable (Figure S3, Supporting Information). Meanwhile, many cells were returning to Ch1 and again back to Ch2. Eventually, as a result of maintained proliferative activity, the cells occupied all chambers, numbering more than 100 cells in Ch2, far more than the starting number of 20–40 cells from loading at start. Interestingly, all cells passing through the nanochannels migrated toward the channel ceiling despite starting their migration from the floor (Figure 6c,d). This could be related with the larger surface area formed on the upper part of the exit of the nanochannels due to the asymmetric architecture. A more detailed image showing their migration toward the channel ceiling is provided in Figure S2 (Supporting Information). Movies corresponding to Figure 6 and Figure S2 (Supporting Information) are provided in the Supporting Information (Movies 6.1, 6.2, 6.3 and Movies S2.1 and S2.2 in the Supporting Information).

The speed of cells migrating in the narrow constrictions was then considered. The cells that migrated without stopping (with a nearly constant speed) were first monitored to evaluate the average migration speed with high precision. Then, because of
the asymmetric shape of the channels and the tapered architecture at both ends, the speeds of the cells within the narrower space inside the nanochannels were compared to those at the wider exits. Time-lapse confocal images provided data demonstrating the speed at which a PC3 cell could migrate over a distance of 50 µm within the submicrometer channels. It was observed that the fastest cells migrated with relatively constant speeds of 1.5 µm min⁻¹ ± 0.07 µm min⁻¹ in the nanochannels (Figure 7a,b and Movies 7.1, 7.2, and 7.3). In contrast, the speed decreased to 0.33 µm min⁻¹ ± 0.015 µm min⁻¹ during migration in channels with larger widths of 2 µm ± 0.2 µm (Figure 7c). The mean standard deviation for the migration speed was ±5% for ten counted cells migrating in similar narrow spaces. Another interesting aspect observed here was that cells maintained

Figure 5. Cell migration a) 8 h, b) 28 h, c) 49 h, and d) 65 h after cell loading in µR1. a,b) Ch1, and c,d) Ch2 and I-row nanochannels. The upper, middle, and lower images are optical images, fluorescence images of mRFP2-histone expressing cell nuclei, and detailed images merging the optical and fluorescence images, respectively. The target cells are indicated with short white arrows in the middle panels. Long white arrows show the migration direction. Scale bars are identical on each row. Correlated with Movies 5.1, 5.2, 5.3, and 5.4.

Figure 6. Cell migration and invasion in nanochannels: time-lapse confocal fluorescence of mRFP2-histone-expressing cell nuclei merged with optical images. a) Top-down view and b) reconstructed bird’s-eye view of the first cell (indicated by a red arrow) invading the second row of pillars (E-row). c,d) Side views showing cells migrating toward and remaining on the ceiling. a–c) Fluorescence images merged with optical images, and d) fluorescence image only. Correlated with Movies 6.1, 6.2, and 6.3. Yellow I and E indicate intravasation and extravasation rows.
their speed when reaching the tapered zone of ≈20–30 µm in width at the exit, where cell nuclei expanded to occupy the free volume. This can be seen in Figure 7b, which shows the red fluorescence from the stained cell nucleus becoming progressively larger as the PC3 cells migrate out from the submicrometric space. It is important to note that the cell stretches along the narrow constriction and the nucleus covers the entire volume while being deformed into the 3D sheet-like shape of the nanochannel. As shown in Movies 7.2 and 7.3, this results in transformation of the cell into a disk shape that glides from one side to the other.

Because very large nucleus stretch was observed (Figure 8a,b and Movie 8.1), the volume of the deformed cell nucleus during migration in the nanochannels was estimated. This was expected to provide insights regarding the degree to which the cells can deform without altering their viability. If the cell nucleus is assumed to be spherical, its volume can be estimated with the equation $V_{\text{sphere}} = \frac{4}{3} \pi r^3$, where $r$ is the radius of the nucleus. The average radius of the PC3 nucleus used in this study is ≈5 µm ± 1 µm, and thus the average volume of the nucleus is ≈524 µm$^3$. The nanochannel has a roughly rectangular cuboid shape with a length $l$ of 50 µm, a height $h$ of ≈6.75 µm, and a width $w$ of ≈1 µm, giving a volume of $V_{\text{nanochannel}} = lhw = 337.5$ µm$^3$. The actual volume of the nanochannel is expected to be smaller than this, because the cross section of the channel may be elliptical rather than rectangular. Thus, the estimated volume of the nucleus was much larger than that of the nanochannel. However, the cell nucleus was entirely confined in the nanochannel, as shown in Figure 8a,b and Movie 8.1, indicating that the nucleus became severely

**Figure 7.** Cell migration and invasion in a narrow submicrometer channel at different time intervals. a) Optical microscope image of cells migrating in nanochannels merged with fluorescence images from the mRFP2-histone-expressing cell nuclei. b) Time-lapse confocal fluorescence image from the stained nuclei of cells invading the narrow constriction (rectangular region indicated in a). Each frame corresponds to a 5 min interval. c) Time-lapse fluorescence images of a cell migrating within a 2-µm-wide channel. Correlated with Movies 7.1, 7.2 and 7.3.

**Figure 8.** Cell stretching inside the nanochannels. a,b) Grayscale fluorescence microscopy images of mRFP2-histone-expressing cell nuclei invading the narrow regions of the nanochannels. a) Obtained by merging fluorescence and optical microscope images of cells. The white arrow in (b) shows an elongated cell inside the nanochannel. c) 2D and d) 3D confocal fluorescence microscopy images of mRFP2-histone-expressing cells additionally stained for a membrane marker (green). In all images, the green arrows show the cell movement direction. Correlated with Movies 8.1, 8.2, and 8.3.
compressed inside the nanochannel. Apart from the nucleus, by staining the body, we observed that the cell body was much more elongated than the nucleus. The top of cell body could then reach the exit from the nanochannel within the tapered structure, in which the cell nucleus was located at the rear of the cell (Figure 8c,d and Movies 8.2 and 8.3). From the 3D reconstructed images, it appears that the compressed cell body has a thin sheet-like shape and occupies the entire narrow constricted region in the nanochannel from the entrance to the exit, whereas the cell nucleus deforms to a disk-like shape that is pulled into the constricted region of the channel later in the migration process.

From a general view of cells penetrating the I-row with both the cell nuclei and membranes stained, the nucleus positioning relative to the membrane could be observed during migration in narrow spaces (Figure 9 and Movie 9.1). The following three types of cell behavior were observed: 1) the cell membrane translated from one side to the other when the cell changed its migration direction (white arrow in Figure 9a–d, red arrow in Figure 9a–h), 2) the cell membrane extended into the nanochannel first and the nucleus trailed behind (orange arrow in Figure 9a–e), and 3) the position of the membrane and nucleus changed during migration despite the migration direction remaining constant (Figure 9e–g). For cells maintaining the migration direction, the corkscrew-like propulsion of the cells (better observed in Movie 9.1) at the channel exits was visualized (orange arrow in Figure 9e and yellow arrow in Figure 9g); this behavior could be explained by speed preservation in the tapered zones and may recapitulate the intravasation modality of tumor cells into the circulatory system. Quantitative data on the number of cells penetrating the intravasation barrier and further cell proliferation are summarized in Table 1.

In addition to being able to flexibly stretch even in narrow submicrometric spaces, the PC3 cells were capable of dividing after or during the migration (Figure 10a,b, better observed in Movie 10.1). This suggests that cancer cells maintain their original function of division after migrating through constrictive spaces. Importantly, the likelihood of division and proliferation was the same before and after the cells passed through these barriers. They were then able to continue migrating forward.

Table 1. Quantitative data on migrating PC3 cells collected from Movies 9.1, 9.2, and 9.3.

| Experiment | PC3 cells in Ch2 chamber at staring counting time (0 h) | PC3 cells in the middle chamber after 14 h | PC3 cells migrating forward keeping direction after 14 h | PC3 cells migrating forward−backward after 14 h | PC3 cells dividing following migration after 14 h |
|------------|------------------------------------------------------|-----------------------------------------|-----------------------------------------------------|-----------------------------------|---------------------------------------------|
| Experiment 1 (Video 9.1) | 30 | 42 | 4 | 3 | 4
| Experiment 1 (Video 9.2) | 16 | 28 (2 extravazating in Ch3) | 12 | 3 | 0
| Experiment 1 (Video 9.3) | 7 | 11 | 4 | 1 | 0

a)Time lapse confocal fluorescence microscopy; b)Time lapse fluorescence microscopy; For PC3 division after migration, we have counted the fluorescent nuclei of the dividing cells in the Video 9.1 processed after time lapse fluorescence microscopy.
or backward and sometimes cross paths with other cells in the same nanochannel (Figure 10c,d and Movie 10.2). The fact that two cells traveling in opposing directions were observed to pass each other in the narrow constricted region of the nanochannel suggests that PC3 cells are very flexible and could pass through even narrower spaces.

3. Advantages of Micro/Nanofluidic Glass Biochips

In vitro tests that allow fast quantitative evaluation of cancer cell invasiveness and migration were first proposed using Boyden chambers that can mimic the hierarchical organization of tissues.[17] In such assays, also known as transwell assays, the cells are placed on a membrane with a defined pore diameter of 3–8 µm, and they travel by chemotaxis into a lower chamber containing a chemoattractant solution that stimulates active cell transmigration.[18] However, in such top-to-bottom configurations, direct visualization of the cells as they travel through the micrometric spaces is not possible. New Boyden-like configurations able to develop a concentration gradient orthogonal to the z-plane were thus proposed for the direct visualization of cell migration.[19] Very recently, it was shown that such transwell assays using a microfluidic tumor–vessel co-culture system are able to demonstrate various phases of cancer metastasis in vitro (e.g., proliferation, migration, intravasation, and adherence).[20] With the recent development of lab-on-a-chip technology, biochips have been applied to cancer research and, in particular, to cancer cell chemotaxis through confined spaces.[6,21] Ideally, long, narrow spaces with a hierarchical organization would be used in such studies to offer models with a more in vivo-like environment.[5] In addition, microfluidic platforms containing architectures that can be scaled down to a single-cell level within a 3D geometry are preferable.[7,22] Such platforms have made it possible to demonstrate essential differences in cell migration dependent on the dimensionality of the microenvironment; for example, the migration speeds have been found to be different in 2D and 3D spaces, suggesting differences between their underlying mechanisms.[23] Further, in a 3D microfluidic model of tumor–endothelial cell interactions, tumor cell intravasation was successfully observed by high-resolution live imaging with endothelial barriers and in the presence of macrophages, and the interplay between endothelial permeability and tumor–endothelial signaling was demonstrated.[24] Later, a metastasis biochip model was used to create a cancer–endothelial cell interface and quantify the angiogenic response as well as tumor cell trans-endothelial migration by imaging.[25] Recently, a novel 3D microfluidic platform of concentric hydrogel layers was able to demonstrate that spontaneously formed vasculature enhances cancer cell invasion and reduction of vessel diameter while increasing permeability.[26]

In this study, we developed a 3D hierarchical biochip composed entirely of glass that can be scaled up or down by adjusting process parameters. Narrow submicrometer spaces were realized by implementing a newly developed processing method called FLAE/NGD. With this method, we were able to tailor the dimensions of both the widths and lengths of the channels in a true 3D microfluidic configuration. Devices containing narrow constricted channels with widths of 5 µm, 2 µm, and less than 1 µm were successfully prepared for the study of the migration and invasion processes of PC3 cancer
cells in a chemotaxis-free environment. The absence of chemoattractants can allow better sealing of the device, because there is no need for additional tubing or pipetting, and it makes it possible to obtain high-resolution images of living cells over long time intervals. Such an environment may be relevant for applications in which the interference of a supplementary chemoattractant in biomolecule migration processes should be avoided. However, few studies have been devoted to cancer cell invasion and migration through long, narrow spaces by means other than chemotaxis. In particular, cancer cell migration in a chemoattractant-free environment was observed for the first time a decade ago in a study that showed that cancer cells of different types can move unidirectionally, in a rapid and persistent manner, for hours when constricted in a 3D channel.[76] Polydimethylsiloxane (PDMS) microfluidic channels with a cross-sectional area comparable to the size of a single cell were designed in that study to mechanically constrain the migrating cancer cells. Their motility was spontaneous, occurring in the absence of external stimuli, suggesting that intrinsic mechanisms are involved in cancer cell motility induced by mechanical confinement. Indeed, it is expected that the physical characteristics of the tumor environment, such as the confinement of the growing tumor by the surrounding tissue, could be a trigger causing more rapid invasive cell migration.[77]

In contrast to cell migration on 2D surfaces, where locomotion is driven by integrin-mediated adhesion, in a 3D space, it has been demonstrated that confinement suppresses adhesion-based motility.[28] A biophysical mechanism could explain the migration mechanism inducing the amoeboid movement of certain cells, characterized by a crawling-like motion achieved by the formation of pseudopodia or blebs, that has been observed in cells traveling through narrow confined spaces within complex 3D environments.[7e,f,7] Very recently, it was proven that migrating leukocytes, which show amoeboid movement, lose spatial coherence when their microtubules are disrupted.[29] This revealed a link between the central organization of cellular polarity and the locomotion strategy of leukocytes. Such natural migration processes were tested in PANC-1 pancreatic cancer cells migrating through fibronectin-coated microchannels in a PDMS device with channel structures mimicking a 3D confined environment.[30] Cells migrated easily and without deformation through well-defined structures with relatively large sizes (W × H × L: 15 µm × 11 µm × 50 µm), whereas for narrower channels of ≈7 µm in width, only 7% of the PANC-1 cells that migrated through the channels suffered from severe deformations. Importantly, the cell speed increased as the channel narrowed, as was also observed in the present study. Ultimately, PANC-1 cells were not able to migrate through a 3 µm wide channel. In a very recent study, narrow PDMS microchannels were used to investigate the mesenchymal-to-amoeboid transition (MAT) migration mechanism, which is a spontaneous switch from focal adhesion-dependent movement to contractility-dependent amoeboid locomotion during invasion of cancer cells in confined spaces.[31] In addition to confirming this MAT behavior,[28] the authors demonstrated that the cell migration speed was faster in 3 µm wide narrow channels than in wider 10 µm channels. Experiments were carried out in serum-free media to avoid serum protein adsorption by the PDMS surface, which could sequester extracellular matrix (ECM) proteins. In another study, MDA-MB-231 breast cancer cell invasion through confined microchannels was shown to induce a change in migratory phenotype.[29] A microfluidics-based migration chamber using engineered PDMS consisting of four-walled channels of fixed height (10 µm) and variable widths (50, 20, 10, 6, and 3 µm) was used. The cell migration was somewhat induced by serum-based medium.

In the present experiments, a cell medium with fetal bovine serum (FBS) was used, and cells were allowed to migrate freely without any stimuli or chemoattractants. To reach the narrow constrictions, cells could travel to any side of the microchannels, including their floors, ceilings, or walls; however, faster movement was always observed in cells on the channel walls, in a process known as “contact guidance.”[31] Our observation indicates that there is a threshold cell density at which they stay together and individual cells do not travel alone inside the larger channels. Once they collectively approach the narrow channels, individual cells detach from the population and migrate through the narrow constriction. The geometry of the narrow constricted regions is different from that used in previous studies; specifically, the present 3D structure has a y–z asymmetric shape with tapered structures at the entrance and exit. Additionally, as the platform is composed of glass, it is much stiffer than PDMS devices used in previous studies. Indeed, the stiffness and tapered space could trigger the cancer cells to migrate to and invade narrow spaces. It was observed in this study that cells were able to penetrate and migrate through 5 µm, 2 µm, or even submicrometer spaces with volumes smaller than the volume of their nuclei. It was determined that PC3 cells could migrate rapidly, over a length of 50 µm in ≈1 h, in 3D confined spaces with dimensions of 0.9 µm × 6.75 µm. The narrower the confined spaces, the faster the cells migrated. Cell migration in confined spaces is characterized more by amoeboid invasion than integrin-mediated matrix attachment, the MAT transition also being correlated with an increase in cell speed.[71] The velocity mechanism in such an environment could be related to water permeation during the migration of cells.[76] Specifically, permeation induces a polarized distribution of ion pumps and aquaporins in the cell membrane with inflow at the leading edge of the cell and outflow at the trailing edge. Thus, tumor cell volume was found to be inversely correlated with cell velocity. In addition, to clearly observe individual cells in the narrow constrictions, a new method was used to fabricate an ultrathin glass biochip, with a thickness of less than 100 µm. This provides a similar environment to observation using glass-bottom Petri dishes with thin glass bottoms specially designed for inverted microscope imaging that allows the visualization of the shape of nuclei from different angles when the cells move through confined spaces.

The length and width of channels prepared in this study are similar to actual dimensions and shapes in an in vivo transendothelial environment, which is of great importance for the further study of both collective and individual migration of cancer cells. Additionally, the devices produced here contain the narrowest spaces ever investigated in a migration study and are expected to enable the elucidation of new cell characteristics. Because the micro/nanofluidic channels in these devices are embedded in the glass substrate, the evaporation of the
A novel micro/nanofluidic glass biochip was developed by implementing a new processing technique, FLAE/NGD. The fabricated biochip provides 3D hierarchical architectures with nanoscale characteristics and an ultrathin (<100 µm) chip base for high-resolution live cell imaging. Nanochannels narrower than 1 µm with a height of 6.75 µm and a length of over 50 µm were developed inside the glass itself. Prostate cancer (PC3) cells were cultivated and grown inside the glass biochip, and migration and invasion of the cultivated cells in narrow spaces were observed by high-resolution live cell imaging using confocal time-lapse microscopy. The following findings were obtained in this study.

1) PC3 cells can migrate through narrow submicrometer channels while retaining viability. The geometries of the channels, with narrow submicrometer asymmetric constricted regions and tapered extremities, are markedly different from those used in previous studies and can be considered an effective tool for obtaining new findings in cancer research.

2) The PC3 cells were observed to attain speeds of up to 1.5 µm min⁻¹ with migration speed varying inversely with the channel width. This is the highest migration speed reported for cancer cells during migration in narrow constrictions in chemottractant-free environments. Time-lapse microscopy studies can further allow for a better understanding of cancer cell invasiveness.

3) The volume of the cell nucleus is compressed during passage through nanochannels, and the cell nuclei are able to stretch along the 50 µm long nanochannel with no significant damage. This is an unprecedented level of cellular compression and stretching in submicrometric constrictions and chemottractant-free environments.

4) The cells are able to divide and proliferate after or even during the migration in the nanochannel. The probability of proliferation remained unchanged after migration. In addition, cells traveling in opposite directions can pass by each other in the nanochannels during the simultaneous penetration of multiple cells into the same nanochannel. Such behavior has not previously been reported, and this observation is coupled with further findings of unprecedented cellular compression and stretching in submicrometer channels.

The obtained results newly demonstrate the dynamic adaptability of cancer cells when exposed to asymmetric mechanical confinement.

5. Experimental Section

Design and Fabrication of Ultrathin Micro/Nanofluidic Biochips: Figure 11 shows the sequential procedural framework of biochip fabrication and observation of cancer cell migration including the design of the chip layout (Figure 11a); femtosecond laser direct writing (Figure 11b); formation of micro/nanofluidic channels in an ultrathin glass chip after chemical wet etching and post-thermal treatment (Figure 11c); loading and culturing of cells in the microreservoirs (Figure 11d); observation of cancer cell migration (Figure 11e); and recording, reconstruction, and analysis of the observation results (Figure 11f).

The 2D design of the laser writing scheme for biochip fabrication is shown in Figure 11a. The solid lines correspond to the scanning trajectories of the focused laser beam. The design consists of three circular reservoirs connected with two rectangular channels in an L-shape. One of the rectangles is divided into three subregions, connected by arrays of straight lines. The design was optimized by fabricating biochips several times with different writing schemes. In particular, the orthogonal writing scheme was employed for the rectangular regions on the left and right sides of the horizontal channel. This helped in reducing the etching time, which is necessary to ensure the uniformity of the biochip. Two layers were scanned with the 2D design for the rectangles by moving the laser focal point along the laser beam axis with a step of 10 µm to create microfluidic channels of an appropriate thickness, whereas 10 layers were used for the circles to form the open reservoirs.

A schematic of the laser direct writing system and fabrication procedure of the 3D glass microfluidic structures can be found in a previous study.[8] The second harmonic of an Yb-fiber laser beam (332 nm; 360 fs, 200 kHz) was employed for the direct writing of the glass (Figure 11b) at a power of 5 mW. Two lenses, L1 (f = 200 cm) and L2 (f = 50 cm), were used to reduce the diameter of the fundamental beam for efficient coupling with the second-harmonic generation (SHG) crystal. The laser power was adjusted with an attenuator. Two additional lenses, L3 (f = 30 cm) and L4 (f = 300 cm), were used for the generated green laser beam to expand the diameter in order to match the numerical aperture (NA) of the appropriate objective lens. A dielectric mirror with ultrahigh reflectivity at 522 nm allowed the green laser beam to be reflected toward the objective lens while allowing white light to pass through for process monitoring. The glass sample was placed on a 3D stage with two stepping motor controllers to move according to the 2D model (Figure 11a). The 3D stage controller, shutter, and charge-coupled device (CCD) camera were controlled by personal computer (PC) software.

Foturan glass (Mikroglas, Germany) was used for the biochip, as it provides suitable properties for the application, such as a high Young’s modulus, little absorption in the visible range, high chemical stability,
advances from 500 µm to 1000 µm into the sample. The stage controller moved the sample at a speed of 1000 µm s⁻¹ during irradiation, according to the pre-programmed patterns (Figure 11a,b). Next, the sample was annealed with a two-step treatment at 500 °C for 1 h followed by 605 °C for 1 h.

A subsequent chemical etching procedure in 8% HF solution was used to selectively remove the laser-modified regions, resulting in the formation of 3D microchannels embedded in the glass chip; this etching is made possible by the laser exposure increasing the etching rate by up to 50 times in a diluted HF solution in comparison with the rate in unexposed regions. These unexposed parts are also etched to a lesser degree with a rate of ≈1 µm min⁻¹ so that the final thickness of the chip decreases from 500 µm to ≈360–380 µm after etching for about 1 h. For the laser scanning at the bottom layer, the laser beam was focused 200 µm above the bottom surface of the glass substrate, so that the final base could reach a thickness of less than 100 µm. Such an ultrathin base is necessary for high-resolution imaging with inverted time-lapse microscopes.

After the etching, a second thermal treatment at 500 °C for 1 h, followed by temperatures as high as 645 °C for 18 h. The frame images were analyzed and compiled into movies.

**In Vitro Experiments: Cell Cultures:** On-chip in vitro experiments were designed to observe the behavior of metastatic prostate cells in submicrometer channels filled with a complete culture medium.

The microfluidic devices were sterilized with ethanol, coated with collagen, and then mounted in a glass-bottom Petri dish. The PC3 human cell line, derived from the bone metastasis of a 62-year-old male patient with prostate cancer, was purchased from RIKEN BRC Cell Bank. Replication-defective, self-inactivating lentivirus vectors were used to stain their nuclei. Complimentary deoxyribonucleic acid (cDNA)-encoding mRFP2-histone was cloned into a CSII-EF-MCS vector. The plasmid was transfected with a packaging plasmid (pCAG-HIVgp) and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) into 293T cells. High-titer viral solutions for mRFP2-histone were prepared and used for transduction into the PC3 cell line. The cells were loaded inside the microreservoir in the chip (Figure 11d), grown in RPMI 1640 supplemented with 10% FBS, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin, and maintained under a 5% CO₂ atmosphere at 37 °C.

**Cell Imaging:** Approximately 20–40 cells were resuspended in the growth medium and seeded in the left microreservoir (Figure 11d,e).

Live cell migration was recorded using both time-lapse fluorescence and laser scanning microscopy (LSM) (Figure 11e). The microscopes were equipped with a mini-incubator with a CO₂ supply, and the temperature was set to 37 °C. Images of the cells migrating inside the channels were captured at objective magnifications of 20 ×, 40 ×, and 60 × with NAs of 0.75, 0.95, and 1.20, respectively, to show their relative location along the microchannels at 20-s frame intervals for up to 5 days with time-lapse fluorescence microscopy, confirming that they maintained viability even after 5 days.

In the case of LSM, 25 stacked images with a 2.5 µm pitch were captured either every 5 min for 100 min or every 60 min for up to 18 h. The frame images were analyzed and compiled into movies. Reconstruction and analysis for LSM images were achieved by Olympus software (Figure 11f).
Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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