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Vertical Oxide Nanotubes Connected by Subsurface Microchannels

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ABSTRACT

We describe the fabrication of arrays of oxide nanotubes using a combination of bottom up and top down nanofabrication. The nanotubes are made from epitaxially grown semiconductor nanowires that are covered with an oxide layer using atomic layer deposition. The tips of the oxide-covered nanowires are removed by argon sputtering and the exposed semiconductor core is then selectively etched, leaving a hollow oxide tube. We show that it is possible to create fluidic connections to the nanotubes by a combination of electron beam lithography to precisely define the nanotube positions and controlled wet under-etching. DNA transport is demonstrated in the microchannel. Cells were successfully cultured on the nanotube arrays, demonstrating compatibility with cell-biological applications. Our device opens up the possibility of injecting molecules into cells with both spatial and temporal control.

KEYWORDS

Nanotube, cell injection, nanowire, gallium phosphide, reactive ion etching, wet etching, cell

1. Introduction

In the past few years, nanowires have attracted increased attention in biology with diverse applications ranging from electrical recordings and detection of biomarkers to cellular force measurements and axonal guidance [1–5]. Studies have shown nanowires to be biocompatible, both *in vitro* and *in vivo* [6–8]. One important application being explored is the injection of molecules into cells using semiconductor nanowires and carbon nanotubes as cell-penetrating delivery systems. This implementation of nanotube and nanowire technology holds great promise as a powerful tool for cell biologists [9–16].

In this paper we demonstrate a method to fabricate arrays of oxide nanotubes that are connected via a subsurface microchannel capable of transporting liquid. By connecting the channel to a reservoir it will be possible to inject molecules into cells cultured on the nanotube array. We also demonstrate that cells survive on these substrates.

In cell biology, foreign molecules, such as drug candidates or genetic material, are often introduced into cells to probe cellular functions. Several non-specific

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techniques, such as electroporation, viral vectors and liposome transfection are used extensively to introduce material into cells [17, 18].

Mechanical injection, such as microinjection and nanowire-assisted injection, offers a complementary method, which can yield additional experimental control, such as the ability to directly address individual cells, and enables other types of experiments not possible with the above-mentioned methods.

One commonly used mechanical injection method is microinjection in its various forms [17, 19]. The drawback of standard microinjection is that it is a serial technique, where each cell is penetrated in turn by a microneedle, severely limiting throughput [17, 19, 20]. Many attempts to improve throughput have been made, by for instance using arrays of microneedles to allow parallel injections [21] or increasing the single-cell injection rate using microfluidics or robotics [22, 23]. However, using micro capillaries (in the same size range as the cells) for cell injection can be detrimental to cell health, especially for inexperienced users [17, 19]. The solution to the problem of needle size, which is being pursued by several groups, is to fabricate the needles on the nanoscale.

The most obvious way to approach the nanoregime is to simply make increasingly sharper micropipettes with the advantage that these are often compatible with current microinjection setups [24–26].

Another approach is to use an atomic force microscope (AFM) to control the injection tip. Meister et al. used a technique similar to microinjection where an AFM with a hollow cantilever was used to penetrate cells [13]. Thanks to the hollow tip Meister et al. could use it to inject liquid into cells and they successfully injected several dyes into different cell types and even localized injections to subcellular compartments.

Han et al. used a focused ion beam to sharpen an AFM tip, which they successfully used to transfect human kidney cells and stem cells with a gene for green fluorescent protein [27]. In this application the plasmid was bound to the exterior of a 6 μm long solid Si needle with a diameter of 200 nm which was then inserted into a cell where the plasmid desorbed.

Another avenue being explored is the use of carbon nanotubes (CNTs) as delivery vehicles for cellular injections. The small size of the CNTs, in some cases with a diameter of a single nanometer, ensures low invasiveness. The most direct approach is to mount the CNT on either a glass capillary or an AFM tip and coat the CNT with molecules that are released once the CNT enters a cell [16, 28].

In a recent paper Singhal et al. used a large (outer diameter ~100 nm) multi-walled CNT mounted on a glass capillary to inject liquid into cells [15]. They also demonstrated that their system was capable of extruding liquid from the cytoplasm, conducting electro-chemical measurements and with a gold-particle coating it could be used for surface enhanced Raman spectroscopy of cellular domains.

The drawback of the above techniques [13–16, 23–25, 28] is that they all operate on single cells one at a time and thus inherit the throughput-limiting serial approach of the microinjection technique.

Using arrays of self-assembled semiconductor nanowires can massively increase the injection throughput by enabling injections to occur in parallel. The basic approach adopted by McKnight et al. [10], Shalek et al. [11], and Kim et al. [12] is to add the molecule to be injected on the surface of plain nanowires and then culture cells on top of the nanowires. They have all shown the successful transport of molecules into the cell interior. This approach depends on the binding of molecules to the nanowires when in the culture medium and the molecules then being released from the nanowires when these enter the cells, which places qualitative and especially quantitative limitations on the molecules that can be injected.

Park et al. [9] adopted a slightly different approach based instead on oxide nanotubes. The molecules to be injected was placed inside the tube. This approach is essentially the same as the solid nanowire arrays, as the molecules have to attach to the interior of the tubes and be released into the cytoplasm.

A more serious limitation to these arrays of coated nanowires is their one-shot nature. The cells are transfected when they are penetrated which occurs at a fixed time after seeding. There is no flexibility as to when the cells are injected, and multiple injections cannot be made.

Our group has recently begun investigating random arrays of semiconductor nanotubes for cellular injections

[29]. We have created random arrays of semiconductor nanotubes on a membrane. As a test of molecular transport, DNA was pulled through the nanotubes using electrophoresis [29]. This approach combines the parallelism of nanowire arrays with temporal control on the single-cell level without relying on binding–unbinding as in the work described above. Using several membranes connected to different reservoirs on a single sample it would be possible to gain spatial control of injections and to inject several molecules in parallel to different subpopulations of cells.

As an important step towards that goal, in this paper we show a method to fabricate oxide nanotubes connected to a microfluidic system to give us the ability to inject molecules into cells with both temporal and spatial control.

2. Method

2.1 Fabrication of hollow nanowires

The fabrication process used to create our oxide nanotubes, or hollow nanowires, is outlined below and summarized in Fig. 1.

Gold aerosol particles with a diameter of 80 nm were deposited with a surface density of 1 μm^{-2} on (111)B GaP substrates (Girmet Ltd, Moscow, Russia) as described in Ref. [30]. GaP nanowires were then grown from the gold seeds using metal organic vapour phase epitaxy (MOVPE) (Aix 200/4, Aixtron, Herzogenrath, Germany) as previously described [31]. In short, the nanowires were grown from the precursors phosphine (PH_3) and trimethylgallium (TMGa) at the locations specified by growth-catalysing gold seeds. The length of the nanowires was determined by the duration of the growth and were adjusted to between 2.5 and 4 μm with diameters determined by the size of the gold seeds (typically 40–80 nm). The nanowires were subsequently covered with aluminum oxide (Al_2O_3) using atomic layer deposition (ALD) (Savannah 100, Cambridge Nanotech Inc., Cambridge, Massachusetts, USA).

The samples were heated to 250 °C before the precursors H_2O and trimethylaluminium (TMAl) were alternately introduced into the chamber in short bursts and allowed to react on the surface, covering it with Al_2O_3 in a uniform way. A total of 500 cycles were used to create a ~50–55 nm layer on the samples.

Following this deposition, the coated nanowires were partially embedded in a resist layer. S1818

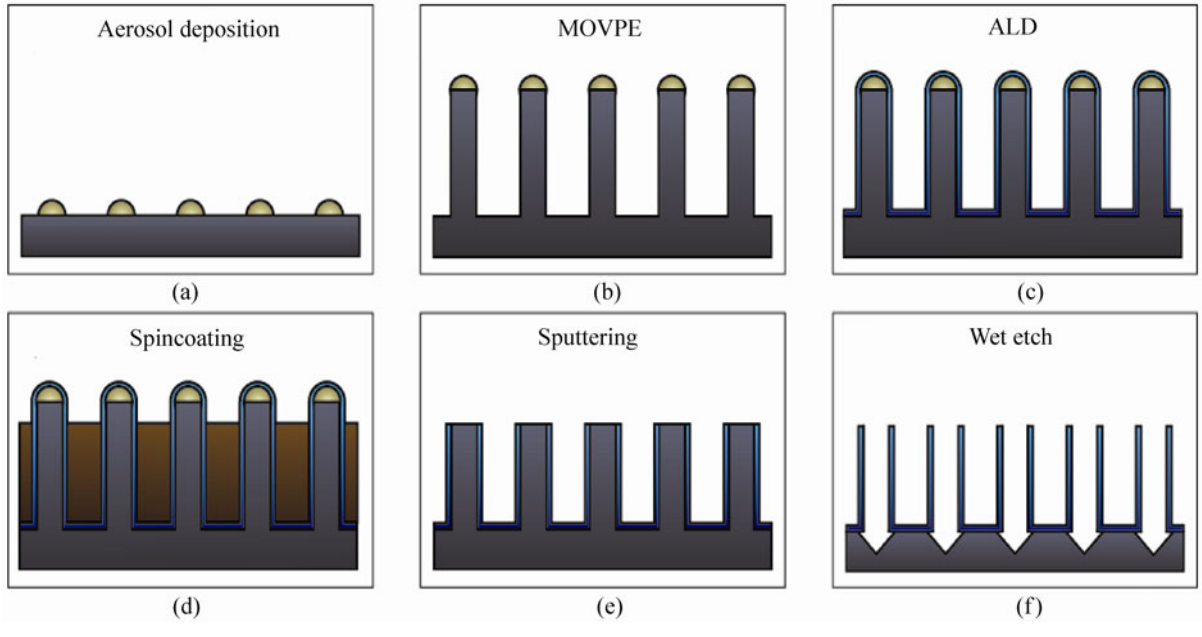

Figure 1 Outline of the hollow nanowire fabrication. (a) Gold aerosol nanoparticles were deposited on the GaP substrate. (b) GaP nanowires were grown from the gold catalyst particles using MOVPE. (c) The nanowires were covered with Al_2O_3 using ALD. (d) The nanowires were then covered with a resist layer (e) Argon sputtering was used to remove the tips protruding from the resist. The resist was then removed in a subsequent oxygen plasma step. (f) In the final step the GaP cores were selectively removed by wet etching, leaving intact oxide nanotubes

(Microchem Corp., Newton, Massachusetts, USA) was spun onto the samples at 4000 rpm for 45 s and then baked for 90 s on a hotplate at 115 ˚C. The tips of the nanowires, protruding from the resist, were then removed using argon sputtering (Plasmalab 100 System, Oxford Instruments, Oxfordshire, UK). A chamber pressure of 10 mTorr was used together with an Ar flow of 130 sccm, RF power of 100 W, ICP power of 1000 W, a sample temperature of 25 ˚C and a total runtime of 15 min. The resist applied in the previous step determines the final length of the hollow nanowires and ensures that they are not completely removed.

The resist was subsequently removed using oxygen plasma. The chamber pressure was set to 15 mTorr, the oxygen flow to 40 sccm, the RF power to 15 W, the ICP power to 1500 W and the sample temperature to 25 ˚C. The duration of the plasma was varied between 20 and 40 min, depending on batch to batch variations of resist thickness.

The exposed GaP cores of the nanowires were etched selectively using aqua regia (HCl 37%: HNO_3 65%, mixed to 3:1) for 2–3 min leaving intact Al_2O_3 tubes. As the etch rate depends on the solvent age [32], fresh aqua regia was prepared each time and allowed to age for 5 min before etching the samples. Note that a GaP nanowire etchant also etches the GaP substrate on which they are standing, creating a cavity under the Al_2O_3 layer covering the substrate. These cavities form the basis for the subsurface microfluidic channels outlined in the next section.

2.2 Fabrication of the subsurface channel

When the oxide nanotubes are hollowed out by the GaP selective wet etching process, the GaP substrate is also attacked and partially removed, creating cavities under the Al_2O_3 layer. By carefully controlling the distance between the nanowires and controlling the size of this under-etch (by controlling the etch time), it is possible to link several cavities in a long continuous tunnel as illustrated in Fig. 2.

To this end, electron beam lithography (EBL) was used to control the positions of the gold particles responsible for nanowire nucleation in MOVPE [31]. Polymethylmethacrylate, PMMA950 A5 (Microchem Corp., Newton, Massachusetts, USA), was spin-coated onto the GaP (111)B substrates at 5000 rpm for 30 s.

Figure 2 By carefully arranging the locations of the nanowires and tailoring the wet-etching time, it is possible to link neighbouring under-etched areas to create a continuous channel

The samples were then baked for 15 min on a hotplate at 160 ˚C. The resist was exposed using EBL (Raith150, Raith GmbH, Dortmund, Germany) at a single dot dose of 22 pAs at 20 kV. The nanowires were placed 1 μm apart in a square array, with each channel 5 μm wide and 1 cm long. Five similar channels were placed in parallel on each sample. The channels were terminated in a grid to increase fluid flow at the entrance of the channels.

Following EBL exposure, the samples were developed for 60 s in a 1:3 solution of methyl isobutyl ketone and iso-2-propanol (MIBK:IPA) at room temperature and the resist was then covered by a 20 nm Au layer using thermal evaporation. Lift-off was carried out by soaking the samples in boiling acetone for 25–35 min and then in fresh boiling acetone for another 5 min. This was followed by 1 min sonication with a subsequent 5 min soak in fresh boiling acetone. Finally the samples were washed in IPA and dried with N_2.

Following the creation of the gold dot pattern, nanowires were grown using MOVPE and coated in Al_2O_3 as described above. The tips of the coated nanowires, were also removed using argon sputtering, exposing the GaP core. The wet etching was subsequently carried out with an identical solution as that above but with an extended etching time of 20–25 min to ensure a continuous under-etched cavity.

To investigate the success of the fabrication of the hollow nanowires, scanning electron microscopy (SEM) was used to capture images of the different samples (FEI Nova™ Nanolab 600, FEI Company, Hillsboro, Oregon, USA and LEO-1560, Carl Zeiss SMT GmbH, Oberkochen, Germany).

2.3 Wetting experiment

To test the functionality of the fluidic connections, a

physical barrier was defined on the subsurface channel as shown in Fig. 3(f). λ-DNA (Fermentas, Burlington, Canada) was stained with YOYO-1 (Invitrogen, Carlsbad, California, USA) at a ratio of 1 dye molecule per 10 base pairs and suspended in a buffer consisting of 0.5xTBE (Tris/Borate/EDTA) with 4% (*w*/*v*) polyvinylpyrrolidone (PVP) and 3% (*v*/*v*) β-mercaptoethanol. The final DNA concentration was 0.5 μg/mL.

The DNA solution was then added to one side of the barrier and pure buffer was added to the other side which was then observed using fluorescence microscopy.

2.4 Cell cultures

To test whether cells can survive on our nanotube arrays, fibroblasts were cultured on the devices for 24 h before being fixed and stained for confocal microscopy. The cells were subsequently dehydrated and sputtered with metal for SEM.

A confluent culture of mouse fibroblasts, L929 (DSMZ, Braunschweig, Germany), was detached by trypsinization and resuspended in growth medium (RPMI 1640 supplemented with 8% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin). The cells were then seeded with a density of 1000 cells/mm^2 on the nanotube arrays placed in a Petri dish. The cells were allowed to adhere to the nanotube arrays for 2.5 h at 37 ˚C in a humidified CO_2 incubator with 5% CO_2 in air before the Petri dish was filled with growth medium and the cells

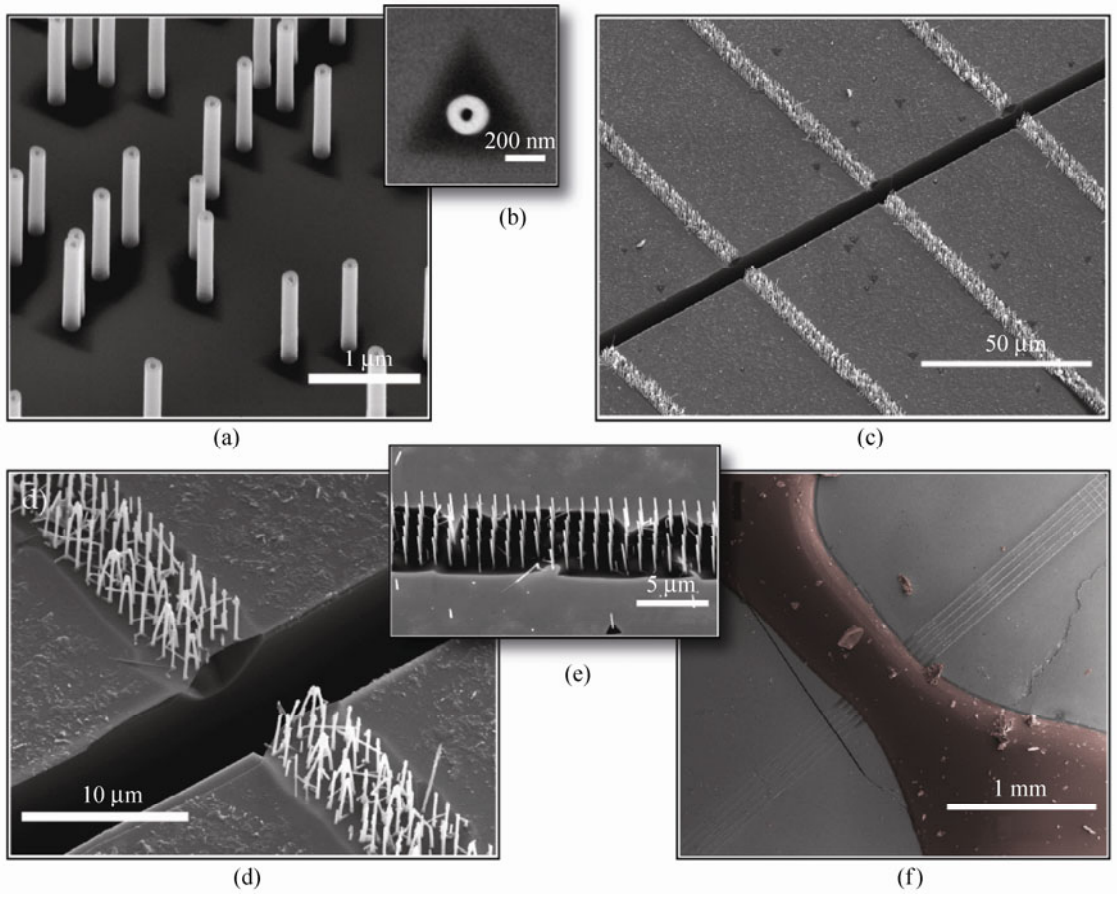

Figure 3 SEM micrographs showing hollow nanowires in a random pattern as well as EBL defined arrays. (a) Random array of hollow nanowires (tilt 30˚). (b) Single hollow nanowire seen from above. Note the dark triangular etched cavity. (c) Overview of several channels (tilt 30˚). A crack in the sample perpendicular to the nanotube arrays reveals the subsurface channel. (d) Close-up of one of the channels seen in c (tilt 30˚). (e) Close-up of the array of nanotubes and their subsurface channel (dark area surrounding the nanotubes) (tilt 30˚). (f) The device seen at a low magnification (tilt 0˚). The artificially coloured red glue creates a physical barrier separating the two halves of a channel used in the capillary wetting experiment outlined in Section 3.3. The five parallel channels can be seen as faint lines running from the top right corner to the bottom left

were incubated for another 24 h. Growth medium components were purchased from Biochrome (Berlin, Germany) and tissue culture plastics from Nunc (Roskilde, Denmark).

The cells were then fixed for 1 h at room temperature using Stefanini's fixative (2% paraformaldehyde and 15% picric acid in phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, pH 7.3)).

The cells were stained with Alexa Fluor® 488 conjugated to phalloidin (Invitrogen™, Carlsbad, California, USA) at a concentration of 33 nmol/L in PBS with 0.25% *v*/*v* Triton X-100 and 0.25% *w*/*v* bovine serum albumin. The cells were incubated for 2 h at room temperature and then rinsed with PBS. The cell nuclei were stained with bisbenzimide (Hoechst 33258) for 1 min at a concentration of 1 μg/mL in PBS. The cells were then studied in a confocal microscope (LSM 510, Carl Zeiss SMT GmbH, Oberkochen, Germany).

After confocal microscopy, the PBS covering the cells was replaced by ethanol in increasing concentration steps (20%, 50%, 70%, 90%, and 99.99%). The sample was then placed in a critical point dryer (CPD030, Leica Microsystems GmbH, Wetzlar, Germany) where the ethanol was replaced by liquid CO_2 which was then allowed to evaporate, drying the cells while preserving their structure. Finally the cells were sputter-coated with gold/palladium (Polaron SC7640, VG Microtech, Sussex, UK) and investigated using SEM (FEI Nova™ Nanolab 600, FEI Company, Hillsboro, Oregon, USA).

3. Results

3.1 Hollow nanowires

Figure 3(a) shows a random array of hollow nanowires where aerosol-deposited gold particles were used to seed the nanowire growth. The resulting nanowires have an outer diameter of ~180 nm and an inner diameter of ~80 nm. Note that these dimensions can be tailored for each specific application. In the figure the under-etched areas can clearly be seen as dark triangles. The triangular shape indicates that the wet etching is anisotropic, preferentially etching the {100} crystal planes.

3.2 Subsurface channel

The SEM images in Figs. 3(c)–3(e) show the ordered array of hollow nanowires connected by the subsurface microfluidic channel.

3.3 Wetting experiment

During the wetting experiment outlined in Section 2.3, a movie was acquired and a few frames are shown in Fig. 4.

In the three frames shown here, one horizontal channel can be seen. DNA has been added to the far left in the images and evaporation of the buffer is driving the flow to the right. An arrow indicates a single DNA molecule as it travels through the channel. The full movie can be found in the supplementary online material.

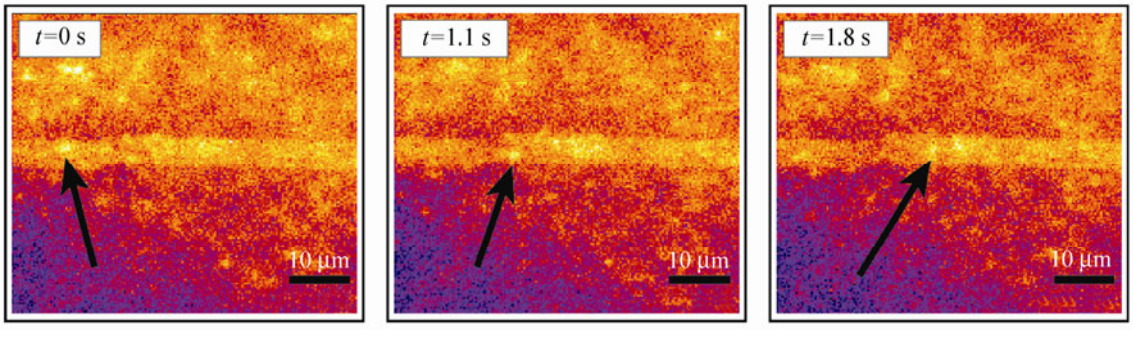

Figure 4 Fluorescence micrographs illustrating the wetting experiment. The channels of one device have been divided into two halves using a physical barrier as shown in Fig. 3(f). Before the images were captured DNA was added to one side of the barrier and buffer to the other side. The evaporation of buffer solution causes a flow which results in the movement of DNA localized in the microchannel. The arrows trace the path of a single DNA molecule as it moves through the channel

3.4 Cell cultures

After 24 h of culture, the fibroblasts seem to adhere well to the nanotubes, as shown in Fig. 5. In Fig. 6 it also seems as though the cell nuclei are pierced (dark spots in nucleus) and the cells' cytoskeleton interacts with the nanotubes (actin dots). Figure 7 shows two SEM images of fibroblasts on the devices.

A long-term cell viability study on nanotubes substrates using human breast epithelial cells (MCF-10A) and aerosol particle seeded arrays is underway in our laboratory. Preliminary results show reduced but intact mitochondrial respiration and DNA synthesis but lower cell densities compared to cells cultured on cover glass for up to 96 h (results not shown).

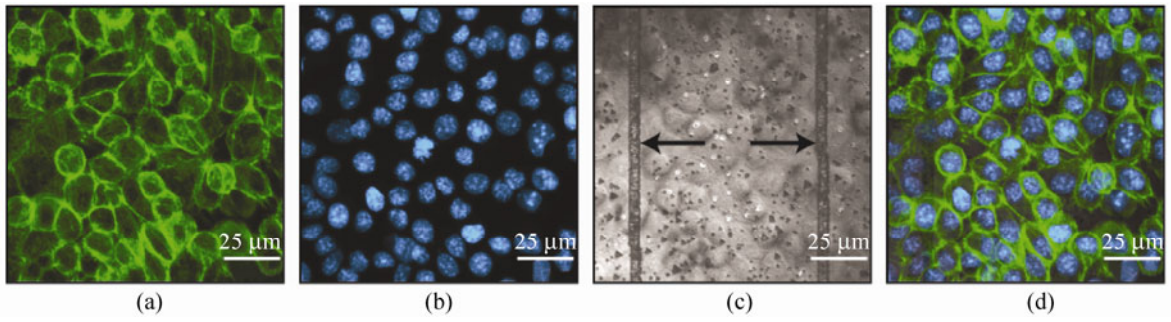

Figure 5 Flattened confocal stack showing fibroblasts cultured on the arrays of nanotubes. (a) Phalloidin labelled with Alexa Fluor® 488 bound to the actin filaments. (b) Bisbenzimide-stained cell nuclei DNA. (c) Laser scattered by the non-fluorescing substrate. Arrows indicate subsurface channels. (d) Composite image

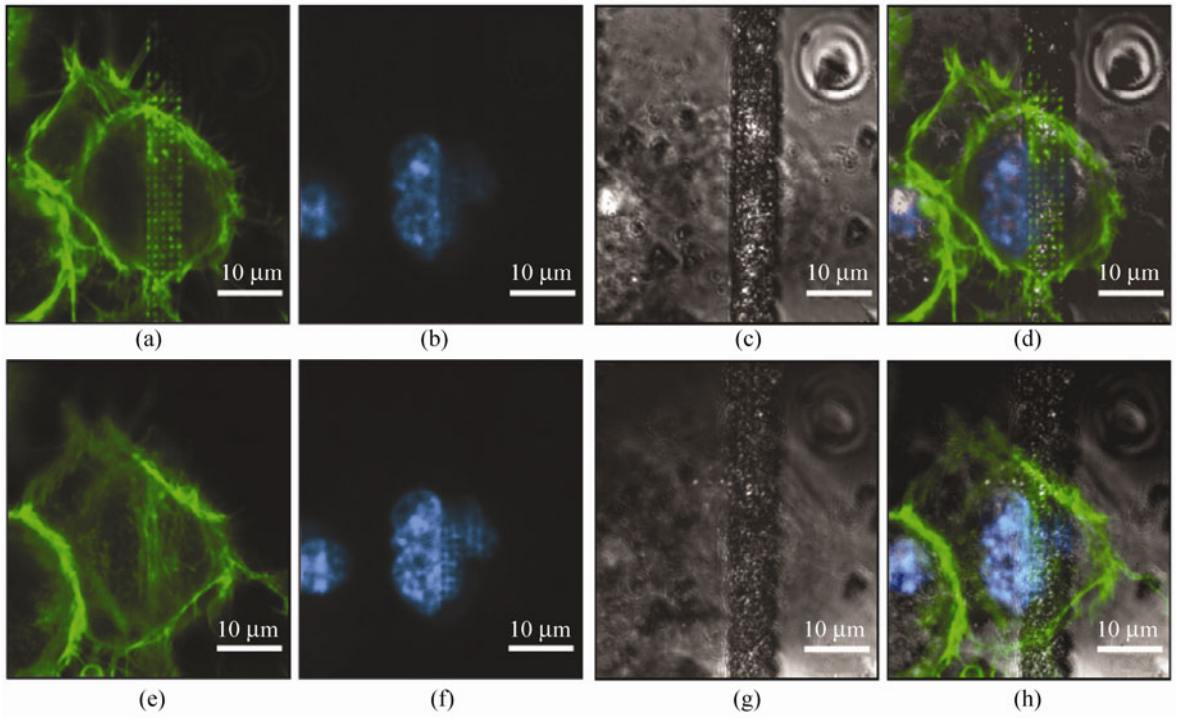

Figure 6 Confocal images (single height planes) showing a fibroblast interacting with the nanotubes. The cells were stained with phalloidin labelled with Alexa Fluor® 488 (green) and bisbenzimide (blue). Laser light scattered by the non-fluorescing substrate was shown as white and reveals the narrow channel (~7 μm wide vertical line in the centre of the images). Images (a)–(d) were acquired close to the surface of the substrate and it was clearly seen that the actin cytoskeleton was interacting with the nanotubes (green dots in a rectangular pattern). Images (e)–(h) were acquired further away from the substrate. Dark holes coinciding with the nanotubes were visible in the nucleus

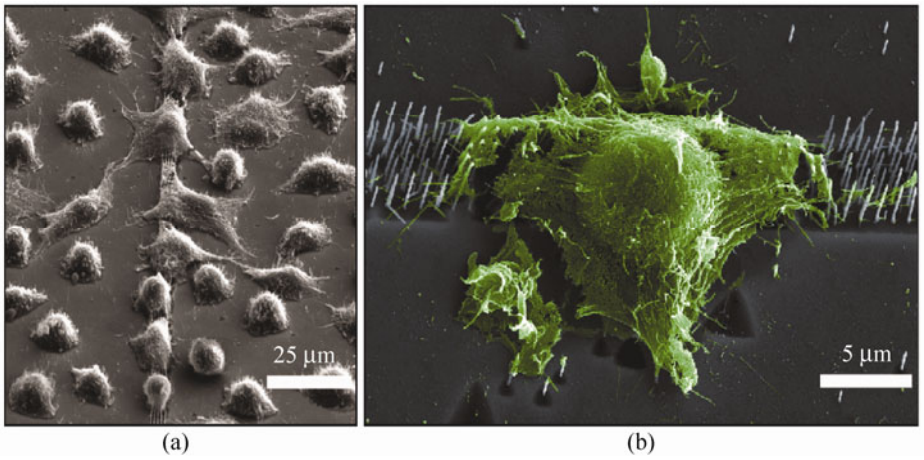

Figure 7 SEM images showing fibroblasts cultured on the arrays of nanotubes. (a) overview and (b) close up with artificial colours

4. Conclusions and outlook

We have successfully made oxide nanotubes connected to a subsurface microfluidic channel and demonstrated fluidic functionality by adding DNA to the subsurface channel. Cells have been cultured on the nanotube arrays maintaining full viability.

The next step of this project is to overcome the practical challenges related to interfacing the subsurface microchannel with a macroscopic fluid control system. When this connection has been established, molecules in the reservoir could be driven into the channel using electrophoresis or pressure driven flow, and subsequently diffuse into the cells that are pierced by the nanotubes as illustrated in Fig. 8.

In conclusion a versatile device is the result that opens up for highly parallel studies of single cells.

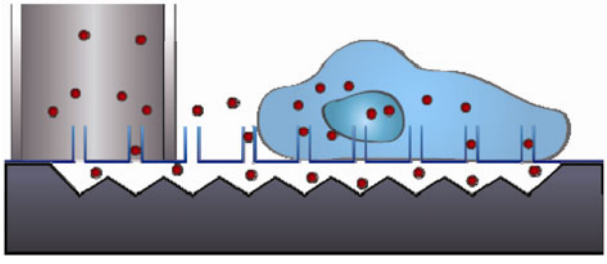

Figure 8 The nanotube system outlined in this paper will be used to inject molecules into cells cultured on the arrays of nanotubes

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Electronic Supplementary Material: A video of the wetting experiment summarized in Fig. 4 is available in the online version of this article at http://dx.doi.org/10.1007/s12274-012-0199-0.

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