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RESEARCH PAPER

Combining fluidic reservoirs and optical tweezers to control beads/living cells contacts

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Abstract We have developed a complete system based on holographic optical tweezers to realize multiple-point interactions between beads and cells with control of the stimulation places, timing and durations. We introduce microstereolithography as a 3D micromanufacturing approach to the rapid prototyping of three-dimensional fluidic microchambers of complex shapes, comprising wells, channels and walls, that are afterwards placed inside the sample and used to inject beads locally and keep them separated from cells in our assays. A custom reservoir designed to keep beads and cells separated in liquid samples has been realized and successfully tested. This allows us to deposit beads locally on the microscope cover glass placed under the reservoir outlet. Limited dispersion of beads under the outlet has been confirmed, and the ability of the polymeric structures to confine beads in a restricted area has been demonstrated. Examples of manipulations consisting at first in extracting several beads from the reservoir, making them travel to the target cell, and finally depositing on its outer membrane with respect to the shape of the target cell, are finally given.

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1 Introduction

In the past 10 years there has been an increasing interest in biochemical analysis of living single cells, showing that a lot of crucial biological events depend on specific molecular recognition at the cell plasma membrane. Moreover, recent studies in cell biology have shown that the cell is mechanically differentiated both spatially and temporally, leading to a regional approach in cell behaviour assays (Heidemann and Writz 2004). Hence, membrane dynamics must also be strongly dependant on both the temporal and spatial characteristics of the stimulus. As a consequence, it would probably be very interesting to be able to excite individual cells, by controlling the stimulation location, the stimulation timing and the stimulation duration.

In order to determine the local and/or temporal response to stimuli, and therefore to draw a map of cells sensitivity, one convenient way is to use optical tweezers to bind opsonized beads on the plasma membrane (Raucher and Sheetz 1999, 2000; Galbraith and Sheetz 1999). However, to study biological events in the 1 h timescale, it is necessary to keep beads and cells separated, in order to prevent unwanted beads to move freely in the sample and interact with the target cell during the experiment. Moreover, sequential deposition of several beads on a target cell makes rinsing not feasible (Monneret et al. 2006). Thus, microfluidics could provide a solution to create separate reservoirs containing beads or cells, and channels to connect them.

Several approaches to the micromachining of microfluidics devices are known (Andersson and Van den Berg 2003; Ziaie et al. 2004). Most of them are based on mainly planar designs because in-plane devices are the most convenient to fabricate with state-of-the-art planar technologies, comprising surface micromachining and etching. However, to facilitate measurements on adhesive cells, it is convenient to make cell culture inside a commercially available chambered coverglass, and then to experiment beads contacts inside the same chamber.

Some previous works combining optical tweezers and microfluidics have already been presented. Optical tweezers have been used as a tool to ensure continuous isolation of single bacteria cells in 5 μm high microchambers by removing out excess cells in order to realize on-chip single-cell microcultivation (Wakamoto et al. 2003; Umehara et al. 2003). In another experiment, microspheres have been transferred through the input port and directed to one of the two output ports of a T-channel, by using the trapping force of an optical tweezers to drag and release the objects within the laminar flow stream (Ozkan et al. 2003). Bacteria cells have also been moved from one reservoir to another without the media being dragged along by means of dual optical tweezers on a timescale of few seconds (Enger et al. 2004). Recently, multiple bacteria have been manipulated inside a 200 μm thick simple microchannel thanks to holographic optical tweezers (HOT) (Ferrari et al. 2005).

In this paper, we combine holographic multiple optical tweezers with a microfluidic system to create a versatile microlaboratory, with a spatial control of beads in cm^3-volume liquid samples containing living cells. More precisely, to keep target cells and handles separated, we defined custom microchambers which comprise fluidic reservoirs that are typically 100–1,000 μm big in diameter, in order to facilitate their manual filling, and 10 mm of height to be used inside macroscopic chambers. We also introduced microstereolithography (microSL) as a 3D micromanufacturing approach for the rapid prototyping of such custom microstructures. This technique reduces to only a few hours the time necessary to design, fabricate and test our microfluidic structures.

Organization of the paper is as follows. We first present the HOT setup we have developed, which is used to locally apply several beads on target cells, with specific beads patterns according to the target cell shape. We then describe our home-made microSL apparatus, and some examples of fabricated reservoirs. We demonstrate the possibility to use such reservoirs to confine silica or latex beads inside a macroscopic liquid sample. Finally, we demonstrate the combination of HOT with microSL to apply multiple beads patterns on target cells, the excess beads being kept enclosed inside a three-dimensional polymeric structure including a funnel and a confinement chamber.

2 Holographic optical tweezers

Optical tweezers (Ashkin et al. 1986) are one of the current methods commonly used in biology laboratories for manipulation of bioparticles like cells or bacteria (Neuman and Block 2004). As already mentioned in introduction, they are well adapted to trap latex or silica beads and drive them onto individual cells in order to probe some of the local properties of the membrane.

Holographic optical tweezers allow the trapping beam to be split up into more than one trap at different positions, by means of a diffractive optical element (DOE), which is generally a hologram. Most holographic setups place the traps in the Fourier-plane of the DOE implying that a linear phase at the DOE results in a lateral displacement of the optical trap. Originally demonstrated with a commercial diffractive 4×4 square array generator (Dufresne and Grier 1998), HOT have since then been implemented with a computer-addressed spatial light modulator as the DOE, allowing diffractive pattern to be quickly replaced in a cartoon fashion, and dynamically reconfigure the resulting spatial distribution of traps (Reicherter et al. 1999; Mogensen and Gluckstad 2000). HOT use holograms that are almost always phase holograms, also called kinoforms, as intensity holograms would decrease the power in the trapping beam. Moreover, diffractive elements which solely rely on a phase modulation of the incident beam lead, in ideal conditions, to a single reconstruction order without the annoying and power disturbing presence of the zero order (Lesem et al. 1969).

Figure 1 shows our version of HOT. It has been built by modifying an inverted microscope so that a laser beam can be introduced into the optical path right before the objective. This is achieved by means of a dichroic mirror, which does not compromise the original conventional and fluorescence imaging capabilities of the microscope.

The holographic optical bench is built around an optically addressed Hamamatsu X8267 programmable phase modulator (PPM). It has a 768×768 pixels resolution without sharp pixelation, suppressing the loss of power due to additional, widely spaced diffraction orders (Liesener et al. 2000). As a result,

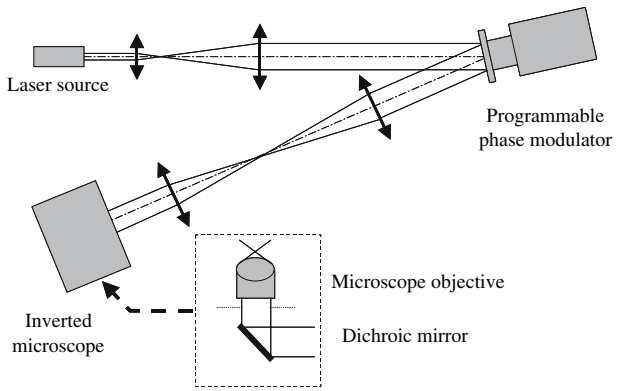

Fig. 1 Scheme of the HOT setup (*top view*)

typically over 80% of the incident light is diffracted into the optical traps. The linearly polarized laser beam comes from a near infrared 2 W fibre laser source (IPG Laser, Breunen, Germany); it is collimated by a first ×4 telescope to fit the PPM active surface, and finally resized from a second ×0.83 telescope to fill up the entrance pupil of a 40×, NA = 1.3 oil immersion objective (5 mm focal length). We use the PPM at an angle of incidence of 4.5°.

The incorporation of two 25 mm travel range, 2 μm resolution, crossed-roller bearing linear translation stages (Physik Instrumente, Karlsruhe, Germany) affords traps to make programmed travels between millimetric reservoirs and target cells.

The creation of separate holographic traps is achieved by diffracting a single collimated laser beam into a desired distribution of beams, from a specific complicated phase pattern DOE. Thus, the definition of the DOE, with respect to the diffraction efficiency, zero and high orders diffraction residuals, and computation time, is the key to holographic trapping. In our case, we used a modified Fourier-transform-based Gerchberg–Saxton algorithm (Gerchberg and Saxton 1972) in order to control the trap pattern interactively.

3 Microstereolithography

Stereolithography (SL, Jacobs 1992) corresponds to a layered manufacturing methodology in which objects are built as a stack of horizontal cross sections, each one being formed individually by a light-induced photopolymerization chemical reaction. Thin layers of liquid material are thus sequentially solidified and stacked from bottom to top to create complicated three-dimensional structures. Stereolithography has been improved to manufacture microparts, and the so-called microSL (Ikuta and Hirowatari 1993) is now one of the most promising manufacturing processes for rapid prototyping of microparts (Bertsch et al. 2000, 2003; Monneret et al. 2001). Moreover, it has been used to manufacture packaged sensors or microfluidic devices (Tse et al. 2003; Provin et al. 2001) and even ceramic microcomponents (Provin et al. 2003). Another advantage is that it may be used without the need for a cleanroom facility.

Figure 2 shows the system we developed, which integrates as major sub-systems an ultra-violet (UV) lamp coupled with a flexible light guide, a dynamic mask, a projection/reduction lens, a motorized translation stage, a motorized scraper and a personal computer.

The UV light beam comes from a 200 W high-pressure mercury-xenon lamp (LC5 system, Hamamatsu, Hamamatsu, Japan). Since the uniformity of the light illumination is a critical factor that determines the process reliability, a specific lens (fly-eye type, Hamamatsu) has to be introduced at the lamp flexible light guide output, providing a uniform illumination on the mask.

The dynamic binary mask is composed of a digital micromirror device (DMD, Texas Instruments, Dallas, TX, USA), designed for optical use in the UV range. The spatial light modulator consists of a 1,024 × 768 array of aluminium micromechanical mirrors with a 13.7 μm pitch. Each mirror can be individually deflected with two stable resting states tilted ±12° to the surface normal, about a hinged diagonal axis. When the mirrors are set at +12°, light originating from the uniform illumination lens is directed towards the photographic lens. On the other hand, when they are set at

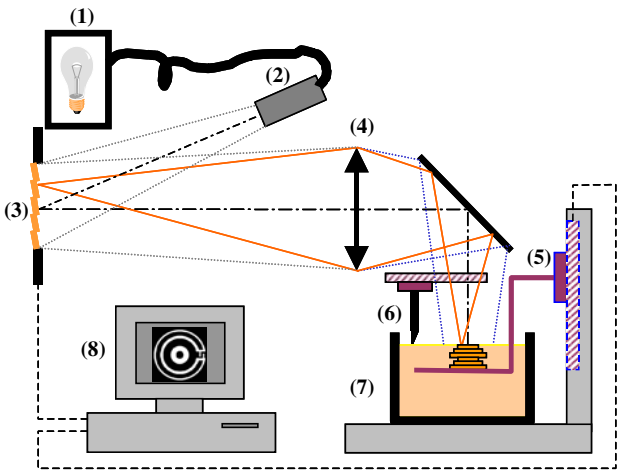

Fig. 2 Scheme of the μSL setup. (1) High-pressure Hg–Xe light source; (2) uniform illumination lens; (3) dynamic mask; (4) projection/reduction lens; (5) vertical moving stage; (6) scraper; (7) bath of liquid resin; (8) personal computer

–12°, light is deflected out of the imaging system. Moreover, the contrast ratio of the dynamic mask is sufficient to use it also as a shutter.

Photographic lenses (50 mm focal length, F/1.2) allow to image the dynamic binary mask onto the surface of the photocurable resin, with a variable magnification in order to control the size of the object. The vertically motorized translation stage moving the support gives the nominal value of the layer thickness. The scraper, a metallic blade, performs regular back and forth motions at the free surface level. The personal computer is used to control the support and scraper motions, and provides the input for the dynamic mask.

The principle of layer stacking used during the microfabrication process is as follows (Figs. 2, 3): a metallic plate supporting the object to be manufactured is positioned at a small distance below the liquid surface. The modulated light beam coming from the dynamic mask is transferred through the reduction lens, and an image is focused at the free surface of the liquid resin with a reduced feature size. This image defines a 2D binary pattern, which induces a polymerization reaction solidifying the resin in the illuminated areas, while leaving it liquid in the dark regions (step 1, Fig. 3). After the first layer is polymerized, the support sinks down one layer thickness in the resin. A scraper then applies new fresh material on top of the existing structure and makes the free surface uniform (step 2, Fig. 3). Because of the weak layer thickness (20–100 μm depending on the application), supplementary relaxation time is necessary to ensure the surface to be uniform (step 3, Fig. 3). Finally, a new pattern is displayed on the mask, and so on, following the shape of the object, which thus sinks stepwise in the resin bath as new solid layers are formed.

Once fabricated, the object is moved out of the bath and washed with a solvent. The adhesion on the metallic support is weak enough to prevent any damage when the solid micropart is lifted off.

The curable system used in this paper consists of 1% photoinitiator (Irgacure 819, Ciba Specialty Chemicals, Lambertheim, Germany) dissolved in a photocurable mixing of monomers, composed of 70% with pentaerythritol triacrylate (PETIA, UCB Chemicals,

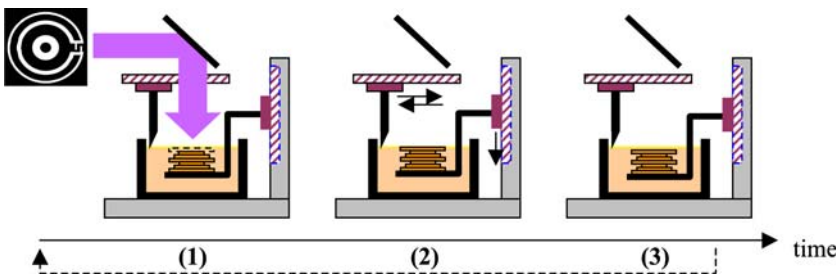

Fig. 3 Main steps of the fabrication process. (1) Pattern-shaped photocuring; (2) deposition of a new layer of liquid material; (3) relaxing the new layer of fresh resin

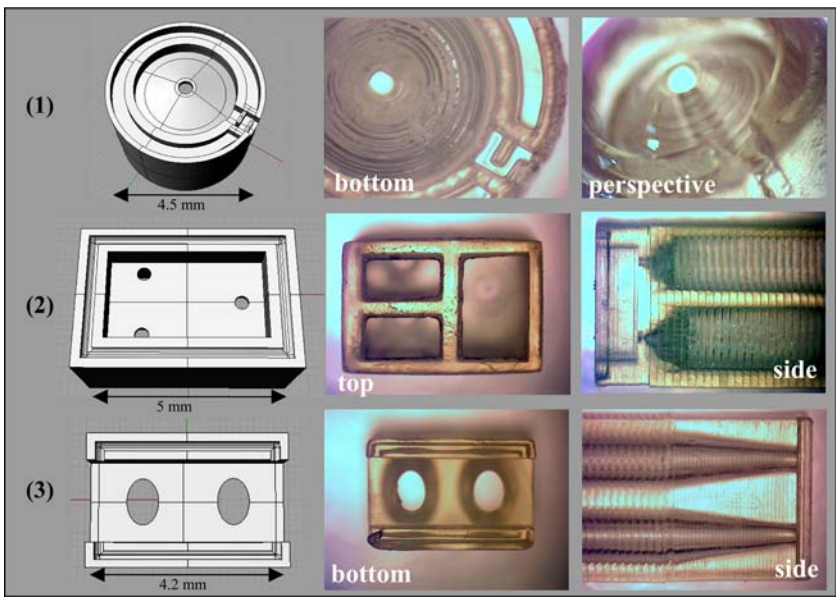

Fig. 4 Examples of 3D structures containing reservoirs. (1) Single-funnel structure with a zigzag-type chamber exit. (2) Triple-funnel with the three outflows directed towards a single closed chamber. (3) Double-funnel structure with an open chamber

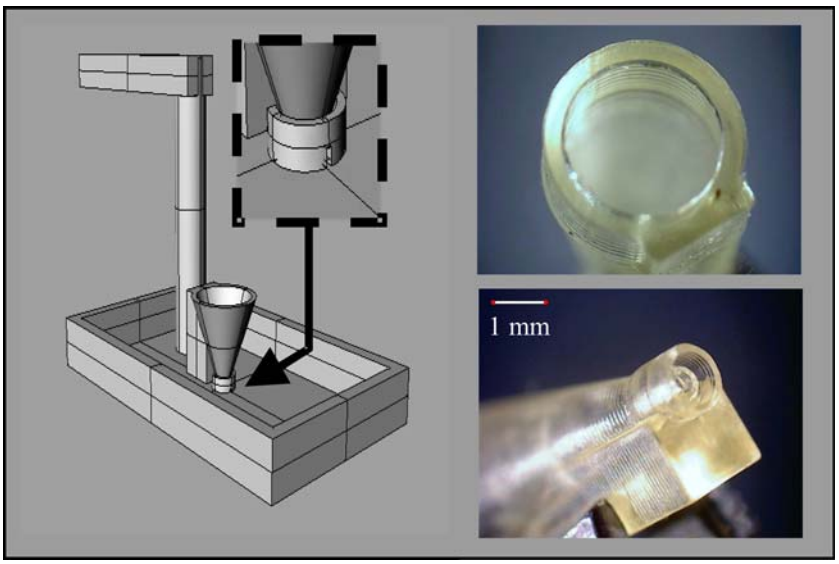

Fig. 5 A specific reservoir structure to keep beads and cells separated in a liquid sample composed of 75 layers, each one of thickness 100 μm

Brussels, Belgium) and 30% with 1,6-hexanediol diacrylate (HDDA, UCB Chemicals). With such a material, the fabrication rate is of two layers per minute, with a 800 ms exposure time of each frame for layers 50 μm thick.

It has also to be noted that the fabricated microstructures are not fully chemically inert and can lead to contamination of a biological sample, by dissolving insufficiently hardened resin remainders in the medium. This prevents us to use the structure close to living cells for times longer than 4 h. As a consequence, it is not possible to cultivate cells inside such structures with the actual material.

As for the conventional SL technique, the microfabricator can just be considered as a sophisticated output device for computers. The object to be built is generally designed with Computer Aided Design (CAD) tools like solid 3D modellers; then data transfer is performed using the binary STL encoding, which is the standard data transfer format for additive fabricators like SL devices. Because of the photocuring step, which is used to manufacture microobjects, the global resolution of the process obviously derives from the quality of the imaging optical system, but also from the chemical and physical properties of the photocurable mixture (Monneret et al. 1999).

Three-dimensional structures containing funnels, outlets and walls with different shapes have been fabricated with the actual apparatus. As examples, Fig. 4 shows three fabricated microstructures containing from one to three integrated funnels to inject from one to three particles types (cells, beads with given diameters or given coatings) into a sample, through an intermediate chamber providing a slit opening as an exit. Varying the layer thickness during the fabrication is possible and advised as it allows to optimize the fabrication time by increasing the thickness when simple and regular structures are being built, and decreasing it when details are needed (this can be easily seen in Fig. 4, structures 2, 3). Because of the relative big size of the reservoirs, the layer thickness was ranging between 50 and 100 μm, allowing them to be made in about 1 h.

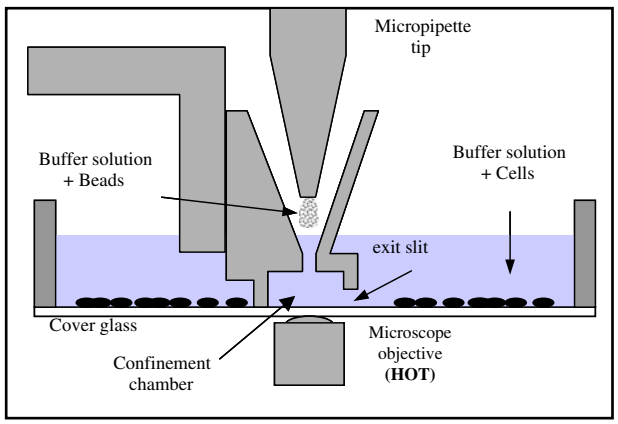

Fig. 6 Once fabricated, the reservoir is applied on a commercially available chambered coverglass. Once the beads are deposited on the coverglass, we use HOT to trap beads inside the confinement chamber. Then the sample is moved thanks to programmable stages, in order for the beads to pass through the exit slit and get onto the target cell. The reservoir is permanently placed inside the sample and never removed during the experiment

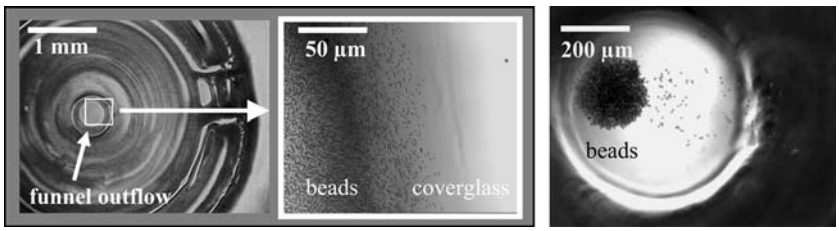

Fig. 7 Beads dispersion on the microscope cover glass placed under the funnel outflow. One can confirm the limited diffusion of beads on the glass, and hence demonstrate the ability of the polymeric structure to confine them in a restricted area inside the confinement chamber (*left*: silica beads, diameter 2.3 µm, *right*: latex beads, 5 µm diameter)

4 Results: controlling beads/living cells contacts

A custom reservoir structure has been realized in order to keep beads and cells separated in a liquid sample, and hence to allow the control of beads/cell interaction. This reservoir mainly consists of one funnel to inject the beads and one confinement chamber to keep them separated from the cells outside. It is composed of 75 layers, each one of 100 µm thickness (Fig. 5).

A typical experimental procedure is as follows: first, the reservoir is applied on a commercially available chambered coverglass (Fig. 5). Then a buffer solution is introduced into the entire microfluidic system to prevent air bubbles formation. Finally we inject inside the funnel a few µl of a new buffer-based solution containing the beads, by means of a manual micropipette. Shortly after, beads sink straight to the bottom with gravity (Fig. 6). Once the beads are deposited on the coverglass, we use HOT to trap beads inside the confinement chamber. Then the sample is moved thanks to programmable stages, in order for the beads to pass through the exit slit and get onto the target cell. The reservoir is permanently placed inside the sample and never removed during the experiment.

We first demonstrated the possibility to effectively control the deposition of microbeads on the coverglass, inside the confinement chamber. Figure 7 shows a relatively well-defined position of the beads (2.3 µm diameter silica beads, and 5 µm diameter latex ones) under the funnel outflow, showing that polymeric structures allow us to confine and maintain beads in a restricted area in a liquid macroscopic sample.

Our following results concerned application of beads patterns on a target cell. In such a case, the reservoir was applied on a chambered coverglass, which contained cultivated cells, as shown in Fig. 6. A cell was chosen to be the target, and its position was recorded as a reference. Then beads were injected inside the structure. In Fig. 8, three of them were trapped while sinking by means of HOT, and were moved outside the confinement chamber, up to the referenced target cell. Relative adjustments on the global positioning of the beads pattern above the target, but also on its scale were made in real time, by using the sample positioning system and by modifying the zoom factor of the displayed kinoform on the PPM. Afterwards, the beads were put into contact with the target cell, by moving the traps in the axial direction.

Another assay is presented in Fig. 9 that uses one of the most useful property of HOT, which allows to dynamically manipulate particles in order to adapt traps positions to the cell shape. Procedure is the same

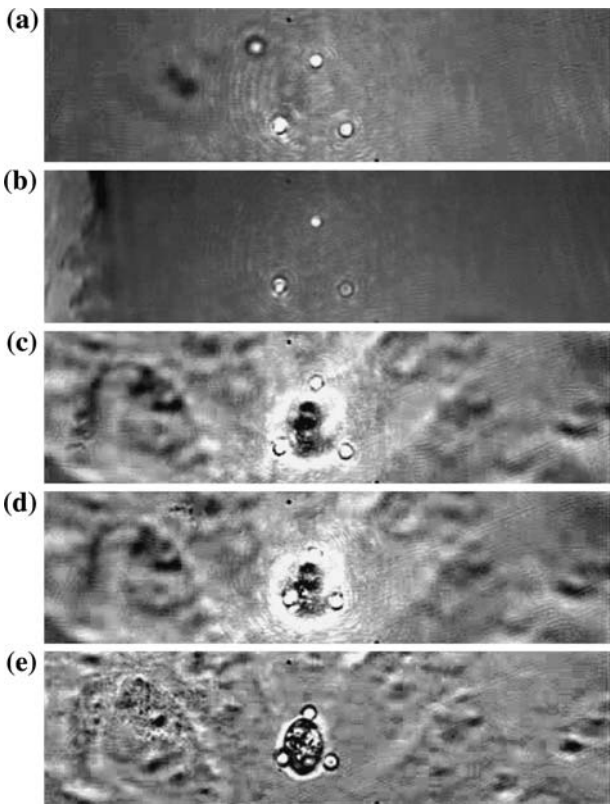

Fig. 8 Three silica beads are trapped inside the confinement chamber of the polymeric structure (**a**), and then moved out trough the slit opening (**b**), up to the target cell (**c**). After the size of the beads pattern is adjusted in real time (**d**), beads are put into contact with the cell, and the laser is turned off (**e**)

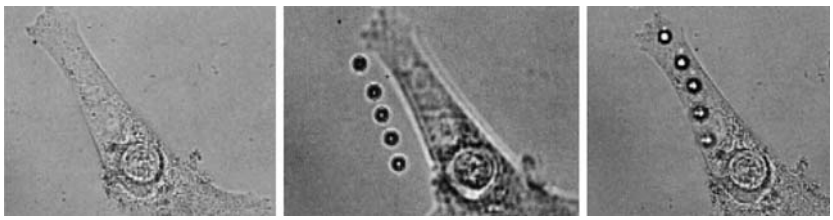

Fig. 9 A set of five latex beads of diameter 5 μm are extracted from the confinement chamber and put into contact with a selected COS-7 cell (*left*). The traps pattern was defined via the computer mouse in the proximity of the cell (*middle*), before the beads were bound on the cell membrane (*right*). Little changes in the pattern occurred during the bounding step (*laser turned off*)

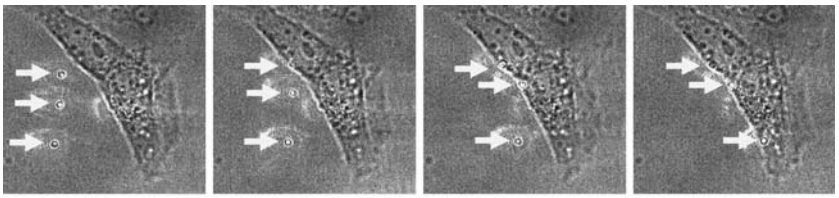

Fig. 10 A set of three silica beads are put into contact with the selected COS-7 cell via a sequence of kinoforms evaluated in real-time. The traps positions are input via the computer mouse

as for the previous experiment, but the beads pattern is interactively modified just before the beads are put into contact with the target cell.

A last example of assay is given on Fig. 10. Three beads were trapped and aligned inside the confinement chamber and moved outside up to a COS-7 cell chosen in a random way out of the cell culture. Then a sequence of kinoforms has been created to adapt to the target cell morphology and evaluated in real-time by a home-made calculation module (Belloni et al. 2006), in order to define and automatically control trajectories of the three selected beads. Movements are performed in steps of 1 pixel size at the SLM, which with the current magnification corresponds approximately to half a micron in the specimen. The assay shows how it is possible to stimulate the cell in several user-chosen sites of the plasma membrane. Moreover, adjusting the length of each trajectory also allows to control the timing of the successive sequential triggering events.

5 Conclusion

In this paper, we demonstrated that we are able to manufacture complex polymeric structures including custom chambers and microfluidics, in order to control the confinement of beads inside a liquid macroscopic sample. Beads injection into such chambers is easily accomplished because the confinement zone is placed under three-dimensional funnels allowing a manual filling with conventional micropipettes. We also described a HOT apparatus to present multiple beads to well-defined regions of individual target cells. The combination of optical tweezers and beads confinement allows us to fully control the temporal and spatial occurrence of multiple contacts with the target cell.

We are now developing a complete system (silica or latex beads covered with ligands and specifically labelled cells) to study the dynamics of activation of cell membranes when some of their specific receptors are stimulated, by presenting appropriate ligands-coated beads at varying surface densities to well-defined regions of individual target cells.

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