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Microfabricated glass devices for rapid single cell immobilization in mouse zygote microinjection

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Abstract This paper presents the design and microfabrication of a vacuum-based cell holding device for single-cell immobilization and the use of the device in mouse zygote microinjection. The device contains many through-holes, constructed via two-sided glass wet etching and polydimethylsiloxane (PDMS)-glass bonding. Experimental results of mouse zygote immobilization and microinjection demonstrate that the device is effective for rapid cell immobilization and does not produce negative effect on embryonic development.

Keywords Single cell immobilization · Cell holding device · Microfabrication · Cell microinjection

1 Introduction

Trapping/immobilization of single biological cells into a regular pattern has applications in many scenarios, such as molecule/drug screening (Castel et al. 2006) and fate/function studies (Chen and Davis 2006) at single cell levels. Existing cell trapping techniques utilize surface chemistry (Chen et al. 1997), dielectrophoresis (Voldman et al. 2002), optical tweezers (Jordan et al. 2005), ultrasonic trapping (Haake et al. 2005), magnetic trapping (Ino et al. 2008), and mechanical confinements (Rettig and Folch 2005; Carlo et al. 2006; Tan and Takeuchi 2007; Carlborg et al. 2007; Suzuki et al 2007).

Microinjection is a physical approach for introducing materials into cells for molecule screening, genetics, and reproductive studies, where target cells also need to be fixed for micropipette penetration. Conventionally, a holding micropipette is used to randomly locate dispersed cells and fix one cell at a time. Although robotics promises automated microinjection at a high speed with high reproducibility, the use of a holding micropipette for cell immobilization is a hurdle. The development of a microdevice that is capable of rapidly immobilizing many cells into a regular pattern can facilitate both manual and robotic microinjection of cells. Since differential interference contrast (DIC) microscopy is most commonly used in microinjection for cell imaging, glass should be chosen as the material for the construction of cell immobilization devices (Murphy 2001).

Among existing techniques, only mechanical confinements are capable of providing sufficient immobilization forces required by microinjection. These mechanical confinement structures include microwells (Rettig and Folch 2005), hydrodynamic traps (Carlo et al. 2006; Tan and Takeuchi 2007), and vacuum-based confinements (Carlborg et al. 2007; Suzuki et al. 2007). Cells trapped in microwells (Rettig and Folch 2005) can slightly move inside the microwells that do not provide secured immobilization during cell penetration. The hydrodynamic traps (Carlo et al. 2006; Tan and

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Takeuchi 2007) are built in closed microchannels, preventing an injection micropipette from accessing cells. The vacuum-based confinements employ an array of micrometer-sized through-holes connected to a vacuum chamber for immobilizing individual cells. In vacuum-based confinements, the construction of through-holes (e.g., 2–50 μm) on the immobilization devices is a critical procedure in the microfabrication process. Such through-holes have been formed on different materials (e.g., silicon, PDMS, and photoresist) (Carlborg et al. 2007; Suzuki et al. 2007; Matthews and Judy 2006). However, forming through-holes with a diameter $\leq$50 μm on glass substrates remains a challenge. Although laser micromachining can be used to drill high-aspect-ratio through-holes on glass substrates (Gattass and Mazur 2008), laser micromachined through-holes have rough surfaces along vertical walls, and minute amount of debris can cause shadows around through-holes in imaging.

This paper presents the design and microfabrication of a glass cell holding device with $\leq$40 μm through-holes, formed on a standard cover slip ($\leq$180 μm thick) for rapid immobilization of mouse zygotes in microinjection. Two-sided glass wet etching was used to form the devices. PDMS-glass bonding was used to form a vacuum chamber. Many trials of rapid immobilization and automated microinjection of mouse zygotes were conducted to determine possible negative effects of cell immobilization on post-injection embryonic development.

2 Device design and microfabrication

2.1 Device design

As illustrated in Fig. 1, the cell holding device consists of a top glass layer with an array of through-holes, a bottom glass layer, and a PDMS spacer for forming a vacuum chamber. Considering the size of mouse oocytes/zygotes (~100 μm), the size of the through-holes is designed to be 35 μm–40 μm. The use of glass in the microscopy light path meets the requirement of DIC imaging. Standard cover slips (size: 22 mm$\times$ 60 mm, thickness: ~180 μm, Fisher Scientific) are used as the top glass layer. Microscope slides (size: 76 mm$\times$ 26 mm, thickness: 1 mm, Fisher Scientific) are used as the bottom glass layer.

2.2 Microfabrication

The fabrication process is summarized in Fig. 2. In order to fabricate 35 μm–40 μm through-holes on a

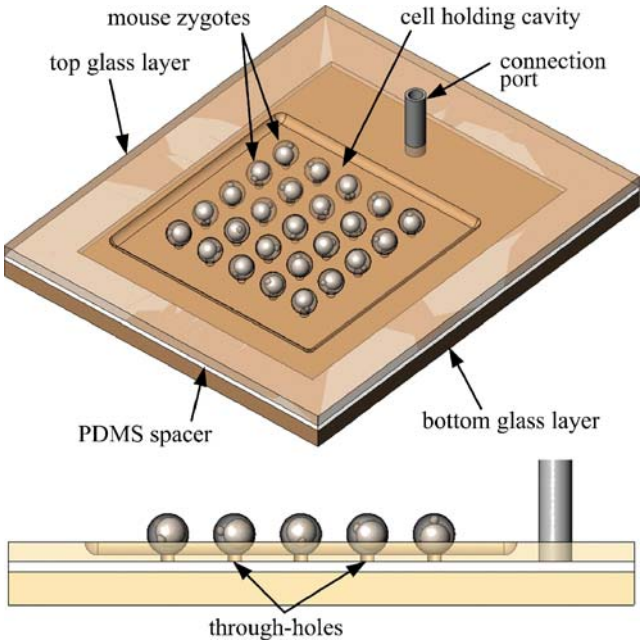

Fig. 1 Schematic of the vacuum-based cell holding device

cover slip, a ~155 μm deep cell holding cavity is first etched into the one side of the cover slip using hydrofluoric acid (HF) wet etching, leaving a ~25 μm thin layer of glass (Fig. 2(a)). Evaporated metal layers of Cr/Au (30 nm/800 nm) plus hard-baked positive photoresist (S1818, Shipley) are used as etch masks (Iliescu et al. 2007). The S1818 layers are capable of preventing penetration of HF solution through the Cr/Au layers.

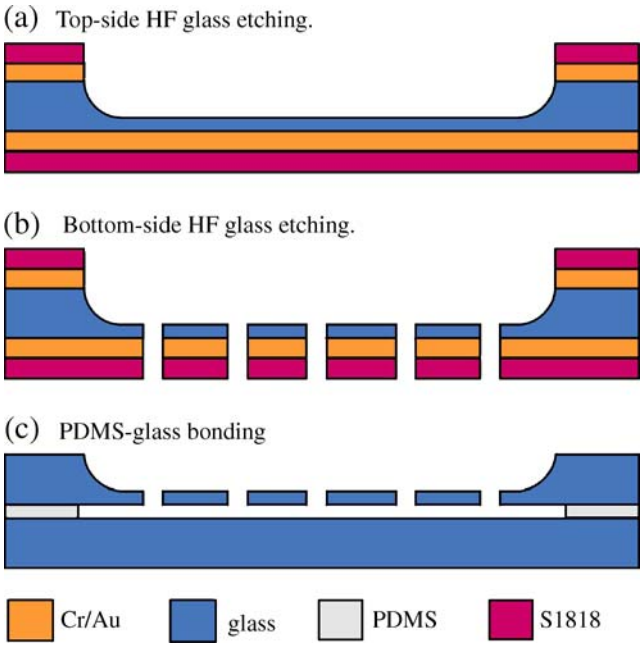

Fig. 2 Schematic of the vacuum-based cell holding device (**a**–**c**)

Etching rates of cover slips as a function of HF concentrations were experimentally determined, as shown in Fig. 3. No agitation was used during glass wet etching. High concentrations of HF cause rough bottom surfaces (inlet pictures in Fig. 3) of the cell holding cavities. Resulting poor optical clarity is also not acceptable for cell imaging. It was found that concentrations below 15% produced smooth surfaces under a 400× microscope objective (surface roughness $R_a \leq 100$ nm). A concentration of 14.3% was used in the final device fabrication, and the corresponding etching rate is 0.9 μm/min (Fig. 3). Bottom flatness of the cell holding cavity was measured to be ≤ 0.8 μm using a Wyko optical profilometer (Veeco Instruments).

After the formation of the cell holding cavity, S1818 on both sides is removed with acetone. The Cr/Au layers on the bottom side of the cover slip are then patterned using photolithography. A second time wet etching from both the top and bottom sides is conducted to form through-holes (Fig. 2(b)). In the meanwhile, a 1.7 mm through hole is also formed on the cover slip to construct a connection port (Fig. 4). Diameters of the etched micrometer-sized through-holes on each device are highly uniform with a standard deviation less than 0.5 μm. The thickness of the thin portion of the cover slip after through-hole formation is 10 μm–12 μm. Finally, S1818 and Cr/Au layers are removed, and the cover slip is cleaned in piranha solution.

The last step of fabrication is PDMS-glass bonding to form a vacuum chamber. PDMS prepolymer (mixing weight ratio of 10:1, Dow Corning) is spin-coated on a 1 mm thick glass slide at 500 rpm, and is then completely cured to form a spacer layer of ~100 μm in thickness. The PDMS spacer layer is carefully cut with a scalpel, oxygen plasma treated, and bonded with the pattered cover slip. Figure 4 shows a ready-to-use cell holding device with the inlet showing the zoomed-in view of through-holes.

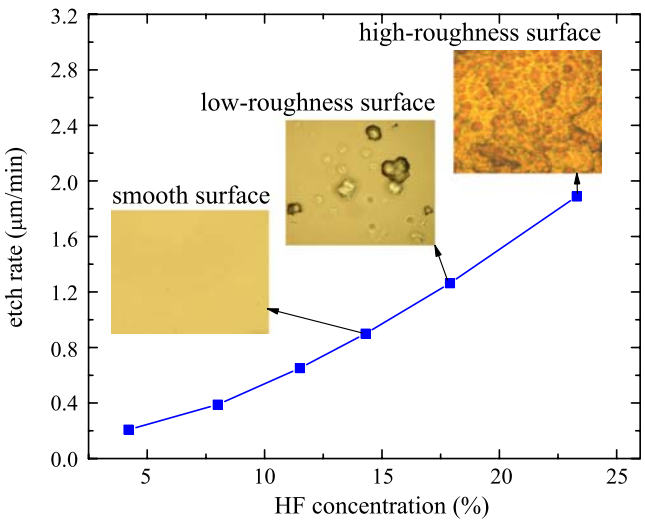

Fig. 3 Cover slip etching rates as a function of HF concentrations

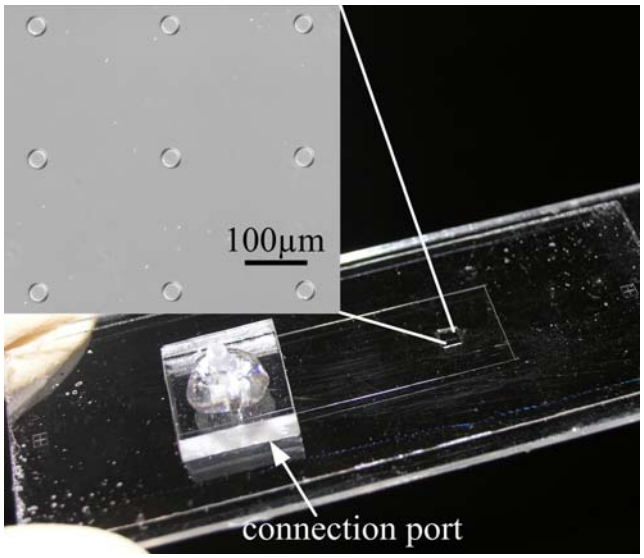

Fig. 4 A completed device. *Inlet picture* shows zoomed-in through holes

3 Experimental results and discussion

3.1 Mouse zygote immobilization

Devices with arrays of 3×3 and 5×5 through-holes were used for immobilizing mouse zygotes (98 ± 2 μm). The average diameter of the through holes is 37 ± 0.5 μm. Low pressures of 1.6 kPa–2.2 kPa were experimentally determined to be effective for holding the cells in place with sufficient forces during micropipette penetration.

For devices with 5×5 through-holes, a batch of mouse zygotes more than the number of through-holes (e.g., 30–35) are transferred to the cell holding cavity (Fig. 5(a)). With the application of a negative pressure, each through-hole traps a single cell. The immobilization process costs approximately 10 seconds. Extra untrapped cells are removed using a transfer pipette (Fig. 5(c)). The complete process including the removal of extra cells typically takes 31 s for devices with an array of 5×5 through-holes.

When a small number of zygotes need to be injected, devices with a lower number of through-holes (e.g., 3×3) are used. In this case, exactly the same number of cells are transferred to the cell holding cavity. In Fig. 6(a), only 4 cells of the 9 delivered cells are within

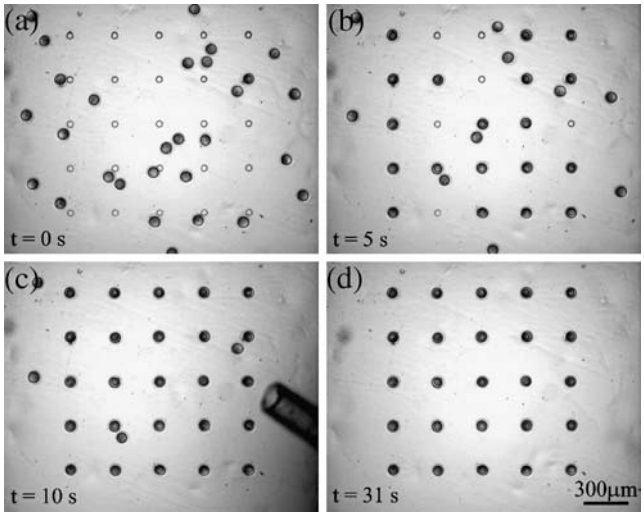

Fig. 5 Immobilization on a 5×5 array of mouse zygotes. (**a**) 30 mouse zygotes are transferred to the cell holding cavity. (**b**) With the application of a low sucking pressure (1.8 kPa), through-holes trap individual cells. 19 cells are immobilized within 5 s. (**c**) 25 cells are immobilized within 10 seconds. A transfer pipette is used to remove extra untrapped zygotes. (**d**) The immobilized 5×5 array of mouse zygotes. The complete process including removal of extra untrapped cells takes 31 s

the field of view. Due to the small number of through-holes and the smaller cell holding cavity, the 9 through-holes are capable of rapidly immobilizing 9 cells without requiring the delivery of extra cells. Therefore, the step of removing extra untrapped zygotes is not needed. The complete process for immobilizing 9 cells into a 3×3 array takes approximately 12 seconds.

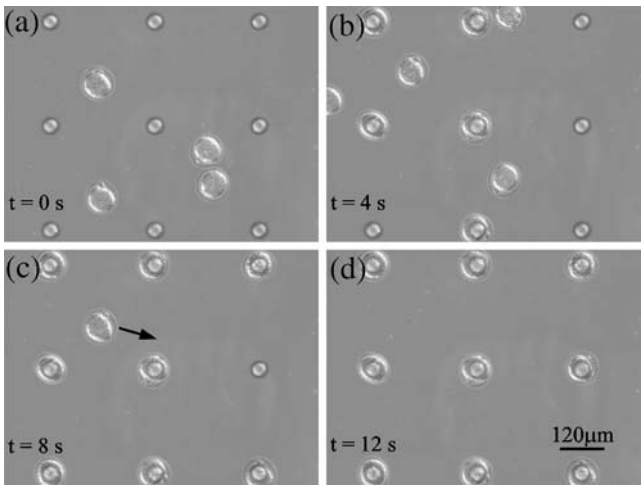

Fig. 6 Immobilization on a 3×3 array of mouse zygotes. (**a**) 9 zygotes are delivered into the cell holding cavity. 5 of them are out of field-of-view. (**b**) A 2 kPa sucking pressure initiates cell immobilization. (**c**) Last untrapped zygote is moving toward the final open through-hole. (**d**) The immobilized 3×3 array of mouse zygotes. The complete process takes 12 s

3.2 Robotic mouse zygote microinjection

Immobilizing mouse zygotes into a regular pattern makes cell search and immobilization easier and is an enabling factor for reliable automated microinjection. In order to quantify the possible negative effect of the cell holding device on post-injection embryonic development, the immobilized zygotes were injected by a robotic injection system (Liu and Sun 2009). The system (Fig. 7) integrates multiple micropositioning devices as well as motion control and image processing algorithms. An injection micropipette is controlled to diagonally penetrate the immobilized zygote and deliver materials into the cytoplasm center, as schematically shown in Fig. 8. A supplemental video clip demonstrates the injection process.

In experiments, PBS buffer was used as the injection material. Figure 9(a) shows a zygote penetrated with the micropipette tip at the cytoplasm center. An injection speed of 200 μm/sec and a retraction speed of 500 μm/sec were used in the experiments, which have proven to be optimal in terms of minimizing injection-induced cell lysis. The robotic system injected a total of 200 mouse zygotes (ICR strain) at a speed of 9 cells/min. The injected zygotes were thereafter cultured in KSOM medium (Specialty Media) for 72 hr (37 °C, 5% CO_2) to allow the zygotes to develop into blastocysts. Figure 9(b) shows robotically injected zygotes that successfully developed to the blastocyst stage.

The survival rate was defined as the ratio of the number of injected zygotes developing into the blastocyst stage to the total number of injected zygotes,

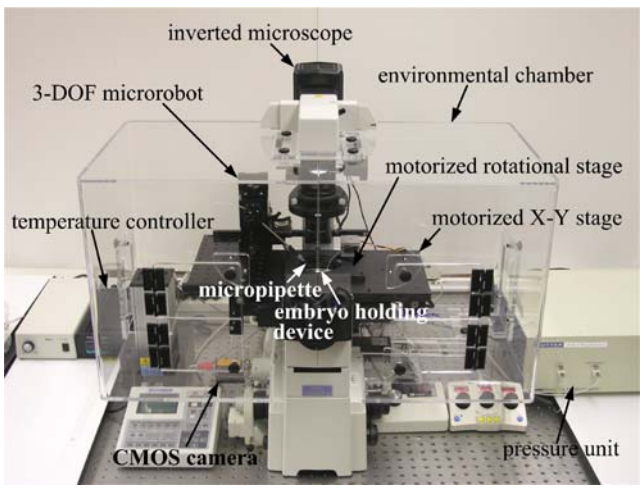

Fig. 7 A robotic system for automated mouse zygote microinjection. A cell holding device is used to immobilize an array of mouse zygotes

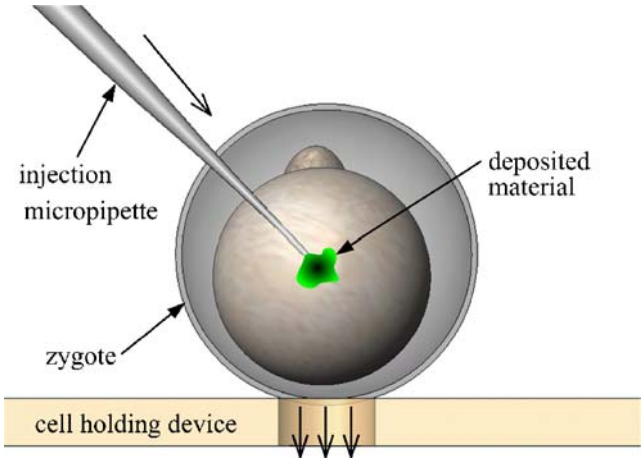

Fig. 8 Schematic of mouse zygote microinjection

quantitating the combined impact of vacuum-based cell immobilization and robotic microinjection. Control groups of non-injected zygotes were cultured under the same conditions (KSOM, 37 °C with 5% CO_2) to eliminate zygote quality differences across cell batches. Based on the 200 injected mouse zygotes, the cell holding device and the robotic injection system produced a survival rate of 89.8%, higher than the best survival rate (~80%) achieved by proficient injection technicians (over 12 years experience) using a holding micropipette and an injection micropipette. The result demonstrates that compared to conventional manual microinjection, the cell holding devices do not produce additional negative impact on embryonic development.

3.3 Discussion

In this study, cell immobilization was demonstrated using devices with 3×3 and 5×5 through-holes. The microfabrication process allows one to fabricate a single through-hole or hundreds of through-holes (e.g., 25 × 25 = 625) for large-scale injection of mouse zygotes. Tuning the microfabrication parameters, one can possibly build through-holes with diameters down to 5 μm on a standard cover slip, for example, for immobilizing smaller suspended cells (15–20 μm) for other single-cell studies that require the use of DIC optics and thus, glass microdevices.

Our present research uses these glass-based cell holding devices and the automated cell injection system for large-scale, high-throughput screening of molecules (proteins and drug compounds) and for clinical intracytoplasmic sperm injection (ICSI).

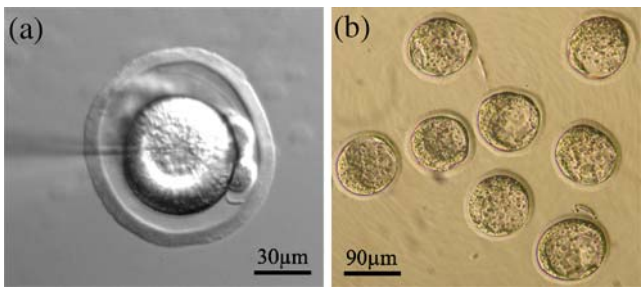

Fig. 9 (**a**) A mouse zygote penetrated by a micropipette before material deposition. The micropipette injects a cell in an diagonal direction. (**b**) Robotically injected mouse zygotes developing into blastocysts

4 Conclusion

This paper presented a glass-based, microfabricated cell holding device for single cell immobilization via the application of a low vacuum. A cell holding cavity and an array of through-holes were formed on a cover slip using isotropic two-sided glass wet etching. To form a device assembly, a microscope slide and a patterned thin layer of PDMS were used to bond to the etched cover slip. The device assembly is compatible with DIC imaging. Rapid immobilization of mouse zygotes was demonstrated using the cell holding devices. Automated injection of the immobilized mouse zygotes was conducted using a robotic injection system. The post-injection embryo culturing results demonstrated that the vacuum-based cell immobilization devices do not produce additional negative effects on embryonic development.

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