Mater. Res. Soc. Symp. Proc. Vol. 1346 © 2011 Materials Research Society
DOI: 10.1557/opl.2011.1076

Fabrication of Silicon on Borosilicate Glass Microarrays for Quantitative Live Cell Imaging

David T. Martin[1], Sergio Sandoval[2], Andy Carter[3], Mark Rodwell[3], Stefan Smith[4], Andrew C. Kummel[5], and Davorka Messmer[6]

Departments of [1]Electrical & Computer Engineering, [2]Bioengineering, [4]Mechanical & Aerospace Engineering, [5]Chemistry & Biochemistry, Nanoengineering, [6]Moores Cancer Center, University of California San Diego, 9500 Gilman Dr., La Jolla CA 92093.

Department of [3]Electrical & Computer Engineering, University of California, Santa Barbara, CA 93106

ABSTRACT

Planar arrays of microwells were fabricated in Silicon on borosilicate glass (pyrex) substrates in order to facilitate live cell fluorescence imaging experiments for cells sequestered inside their own individual microenvironments for incubation and quantification of single cell seceretions. Two methods of deep silicon etching were compared: cryogenic deep reactive ion etching (DRIE) and time multiplexed DIRE (Bosch Process). A 200um Si wafer was bonded to a 500um pyrex substrate. Cryogenic DRIE allowed for the reliable fabrication of 75-100um deep microwells with 60x60um openings across a 10x10mm substrate while the Bosh Process allowed for etching entirely through the Si layer, producing 200um deep microwells with transparent bottoms and steep sidewalls while maintaining the target 60x60um opening geometry.

INTRODUCTION

Investigating cancer from a systems biology perspective has been hindered by the lack of tools for a dynamic multiplexed analysis of surface and secreted proteins from small numbers of tumor cells. Several existing techniques such as flow cytometry, ELISA, or ELISPOT have been well established to measure to quantify secretants from a population of cells or detect surface protein expression. However, none of these techniques are suitable for quantifying secretants from individual cells or performing repeated measurements on live cells [1-5]. With this aim in mind, a planar microarray platform was designed to sequester cells in their own microenvironment to perform live cell imaging experiments to quantify secretant levels and detect surface protein expression in response to stimuli (Figure 1).

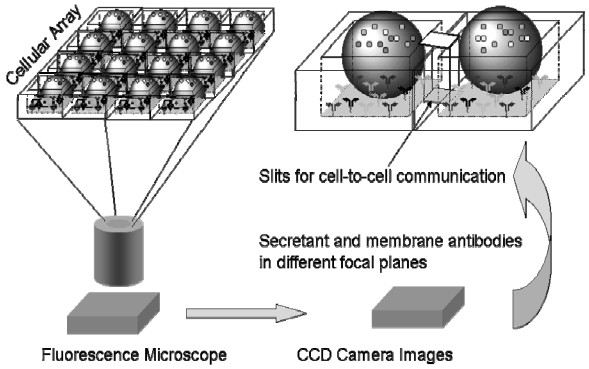

Figure 1. A planar array of microwells can contain individual cells in their own microenvironment for automated fluorescence based quantification of secretants. Cell-cell communication experiments can be conducted by opening diffusion slits between adjacent microwells.

An array of 60x60um square openings separated by 20um across a 10x10mm substrate consists of 15,625 different microwells on a single chip. The grid separation allows for easy image segmentation of each microwell with standard image processing techniques, and allows for quantitative measurements across multiple fluorescent channels. While plating cells in solution would lead to a random distribution across the microarray, the number of cells in each microwell can be easily counted through surface membrane staining; any microwell that did not contain only a single cell can be automatically filtered out from analysis. On chip cell staining and multiday survival have been demonstrated on microarrays 100um deep and a well opening to depth ratio of 10:1.

EXPERIMENTAL DETAILS

A 200um thick Si wafer was bonded to a 500um thick pyrex wafer, and subsequently diced into 12.5x12.5mm chips for individual sample preparation. A chromium hard mask was selected for cryogenic etching and microwell patterns consisting of 50x50um square openings separated by a 10um grid, and 60x60um square openings separated by a 20um grid were transferred to the chromium hard mask pattern through standard photolithographic techniques. The samples were mounted to a carrier wafer with vacuum oil and cryogenically etched with an Oxford P100 ICP Tool (Oxfordshire, UK). Etching parameters were established through the black silicon method [6], with a 1250W ICP power, 10W forward biasing power, 50 sccm SF6 flow rate, 6 sccm O2 flow rate, chamber pressure of 12mTorr, and cryogenic carrier wafer cooling at -115° C. Total etching time was varied from 45 to 120 minutes to achieve differing final depths.

Samples for Bosch process etching were prepared with a SiO_2 hard mask. The silicon surfaces of the samples were coated with a 1.5 um thick plasma enhanced chemical vapor deposition (PECVD) SiO_2 layer. The SiO_2 was patterned by optical lithography by 60x60um squares separated by a 20um grid. The SiO_2 was etched using an inductively coupled plasma (ICP) dry etching tool with a CHF_3-based etch recipe with a Panasonic ICP Tool (Osaka, Japan). After photoresist removal, the samples were etched with a Plasmatherm SLR Series DRIE (St. Petersburg, Florida) with a Bosch process recipe. An initial passivation polymer deposition step was employed in which 70 sccm of C_4F_8, 0.5 sccm SF_6, and 40 sccm Ar were flowed into the chamber under 825W of ICP power for 5 seconds. The C_4F_8 flow rate was reduced to 0.5 sccm, and the SF_6 flow rate was cycled between 50 sccm for 2 seconds (polymer re-deposition step), to 100 sccm for 6 seconds (etching step) for a total time of 2 hours. ICP power was held at 825W, forward bias was held at 9W, and the sample chuck was held at 20°C. Process parameters were first optimized on plain silicon substrates to minimize material costs.

After etching with the cryogenic or Bosch processes, samples were examined under optical microscopy and subsequently cleaved for sidewall examination with an electron microscope.

To test cell surface marker staining and multiday survival, 100um deep microwells fabricated in silicon using the Bosch process. THP-1 cells were incubated with Alexa488 conjugated MHC-class I antibody for 30 minutes as a positive staining control or with a non-specific Ig antibody as a negative control on separate microwell arrays. Both arrays were imaged under a fluorescent microscope without washing of any unbound antibody to test the ability to stain without removal of any cellular secretants. Cells were also plated onto a microarray and incubated for 5 days at 37°C inside a CO_2 incubator in culture media. A 50uL drop of propidium

iodine (PI), a viability stain, was dispersed onto the microarray and imaged 5 minutes later under a fluorescent microscope. This viability test was repeated with the addition of dead cells at the end of 5 days to verify proper PI staining.

DISCUSSION

Smooth, straight, and intact sidewalls that closely corresponded to the target microwell dimensions were critical to determine the total incubation volume of the microwells. Additionally, because accurate quantification of secretants was the ultimate goal, the microwell platform needs to be compatible with the highest resolution inverted confocal fluorescence microscope systems. Confocal microscopy not only has advantages in spatial resolution over conventional microscopy, but is also less susceptible to picking up out of focus background signal while allowing for increased scanning speed in automated systems [7]. For these reasons, a microwell architecture of silicon/pyrex substrates was proposed to allow for optimal imaging quality through transparent bottoms of the microwells. Through microfluidic dynamic simulations, it was determined that a microwell depth to opening length ratio of 10:1 was required to keep a majority of cellular secretants at the bottom of microwells for at least a minute before diffusion could significantly lower secretant concentration at the bottom of microwells. The secretant diffusion was modeled with the following equation:

$$c_t = \nabla^2 c = \frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial c}{\partial r}\right) + \frac{\partial^2 c}{\partial z^2} \quad (1)$$

where ct = concentration at time t, r = dimensionless radius, z = microwell height (z=0 at the top of the well). Solving numerically for a microwell with 10:1 height to opening ratio at 55 seconds shows 90% of a 15kD secreted protein remains at the bottom of a well. At 448 seconds only 10 of the secreted protein remains at the microwell bottom. In general, the diffusion time scales as (height/opening)^2.

Cryogenically Etched Samples vs Bosch Process Samples

The cryogenic etching process was found to be reliable in fabricating microwells with a depth from 75-100um with an average etching rate of 1.4um/min with both 50x50um and 60x60um square openings and a microwell depth to opening length to ratio between 1:1 and 2:1. However, the cryogenic process proved to be inconsistent when etching microwells with high aspect ratios (depth to opening length). Under identical process parameters (gas flow rates, plasma powers, and cooling temperature), a variety of defects were observed for etches lasting longer than 90 minutes (Figure 2).

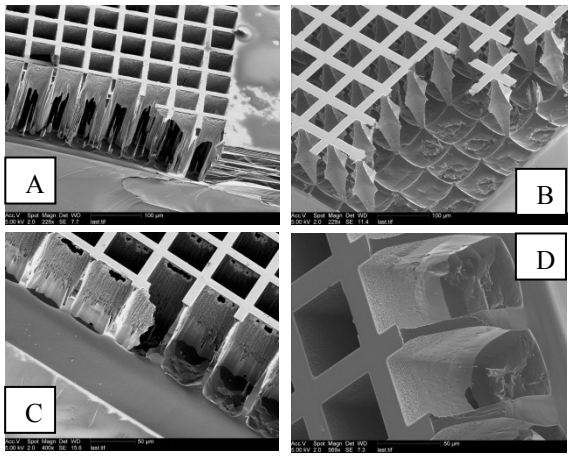

Figure 2. Cryogenically etched samples etched under identical process parameters. (A) Bottom sidewall punch through. (B) Pillaring and total wall punch through. (C) Mask undercutting and top of wall punch through. (D) Intact microwells with slight mask undercutting.

The major source of these defects and inconsistencies was likely mounting individual samples onto a carrier wafer rather than having the samples in direct contact the with cryogenic cooling stage. Stable temperature control is critical for cryogenic DRIE of silicon and any deviation from the optimal temperature for a given etching geometry can introduce undercutting and other defects [8]. The Oxford P100 is designed for 4" wafers, and individual samples were mounted to a carrier wafer with vacuum oil which formed an imperfect seal between the samples and carrier wafer. It was noticed that vacuum oil was consumed by the etching plasma which could have changed the thermal cooling performance throughout longer etching times. Additionally the carrier wafer itself would begin to etch and introduce variation into the process.

The Bosch process can be carried out at room temperature and therefore can avoid thermal management issues of small samples on carrier wafers while etching deep, straight openings in silicon [9]. Several test patterns of microwells were fabricated in plain silicon samples, including 20x20um square microwells of 100um depth separated by 10um sidewalls. A 60x60um square microwell separated by 20um sidewalls was chosen to test on the silicon/pyrex bonded substrates for a direct comparison to samples etched with the cryogenic process. All Bosch process samples exhibited nearly straight sidewalls for the entire depth of the microwells with no undercutting. Samples on the silicon/pyrex substrate stopped etching when the pyrex layer was reached, producing optically transparent microwell arrays (Figure 3).

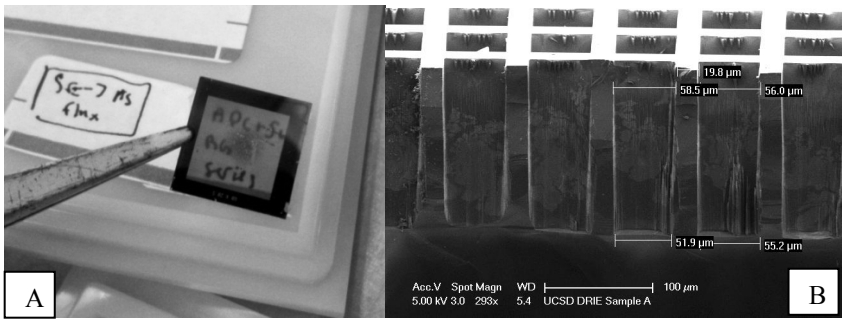

Figure 3. (A) Optical transparency of microwell array after etching through to pyrex substrate. (B) Electron microscopy cross section of cleaved sample.

A representative measurement of the dimensions of a microwell was taken near the center of the array; a 58.5 um opening at the top tapered down to 51.9 um at the bottom of the microwell. This 1.89° angle of the sidewall can likely be reduced further with process optimizations. The etching rate was not entirely uniform across the sample surface, leaving approximately 20% of microwells near the center of the chip with a small silicon ring at their

bottoms. Slightly extending the etching process time should fully etch the remaining amount of silicon in microwells at the substrate center without affecting the dimensions of microwells around the periphery. The success of fabricating 60x60um square opening microwells of 200um depth on silicon/pyrex substrates along with the previous success of fabricating 20x20um square microwells on plain silicon substrates indicates that 20x20um square microwells of 200um depth should be readily achievable and others have found great success in tuning the Bosch process to wide variety of etching geometries [10]. This would produce microwell arrays of a sufficient depth to opening ratio to limit secretant diffusion on transparent substrates for bottom-up fluorescent imaging.

Live Cell Experiments

To verify that surface markers of cells could be easily stained on-chip without any washing steps which would remove secretants, $5x10^4$ THP-1 cells were plated onto a microarray and allowed to settle for 10 minutes. The cells were incubated with an Alexa488 conjugated MHC-class I antibody for 30 minutes as a positive staining control or with a non-specific Ig antibody as a negative control on separate microwell arrays. The positively stained cells were readily visible without any interfering background signal. Negative cells were optically transparent and could only be seen on the top walls of microwells were light was reflected (Figure 4a, 4b). To verify that cells could be kept alive over several days to perform time course measurements, cells in culture media were plated onto a microarray and incubated for 5 days at 37°C inside a CO_2 incubator. PI was then introduced to test the viability of cells held in the same culture media for several days. No nonviable cells were found, and the viability test was repeated with the addition of dead cells at the end of 5 days to verify proper PI staining. Both nonviable cells and viable cells are clearly visible (Figure 4c, 4d).

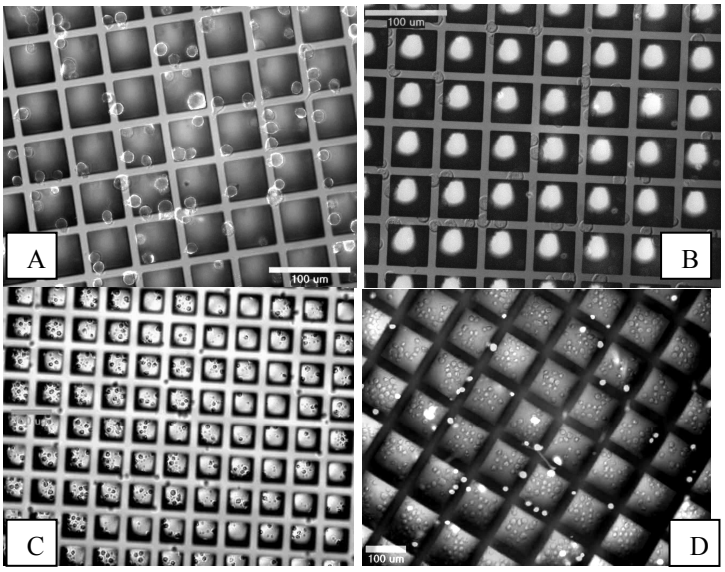

Figure 4. (A) Positively stained THP-1 cells, note membrane highlighting (B) Negatively stained THP-1 cells are transparent on top of walls, bright spot is reflected light from bottom of microwell. (C) Cells incubated for 5 days in media and then incubated with PI. No positively stained cells indicate viability. (D) Cells incubated for 5 days in media, introduced with dead cells, and again stained with PI. Both viable (transparent) and non-viable cells (solid white) are detected.

CONCLUSIONS

Bosch process DRIE is a preferable method for deep silicon etching over cryogenic etching when working with small substrates. Silicon microwell fabrication on a transparent

substrate shows promise for performing novel live cell experiments by isolating individual cells within their own microenvironment and isolating secretants for automated quantification.

ACKNOWLEDGMENTS

NIH-NCI is gratefully acknowledged for support of this work

REFERENCES

1. C. Czerkinsky, G. Andersson, H.P. Ekre, L.A. Nilsson, L. Klareskog, O. Ouchterlony, "Reverse ELISPOT assay for clonal analysis of cytokine production I. Enumeration of gamma-interferon-secreting cells", *J. of Immunological Methods* **110**, 1 (1988).
2. T. Jung, U. Schauer, C. Heusser, C. Neumann, C. Rieger, "Detection of intracellular cytokines by flow cytometry", *J. of Immunological Methods* **159**, 1-2 (1993).
3. R. Manz, M. Assenmacher, E. Pfluger, S. Miltenyi, A. Radbruch, "Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix" *Proc Natl Acad Sci* **92**,6 (1995).
4. C. Czerkinsky, Z. Moldoveanu, J. Mestecky, L. A. Nilsson, O. Ouchterlony, "A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells", *J. Immunol. Methods* **115**,1 (1988).
5. A. Rodriguez-Caballero, A. C. Garcia-Montero, C. Bueno, J. Almeida, R. Varro, R. Chen, A. Pandiella, A. Orfao, "A new simple whole blood flow cytometry-based method for simultaneous identification of activated cells and quantitative evaluation of cytokines released during activation", *Lab Invest* **84**, 10 (2004).
6. H. Jansen, M. de Boer, H. Wensink, B. Kloeck, M. Elwenspoek, "The black silicon method. VIII. A study of the performance of etching silicon using SF6/O2-based chemistry with cryogenical wafer cooling and a high density ICP source", *Microelectronics Journal* **32**, 9 (2001).
7. White, J.G., W.B. Amos, and M. Fordham, "An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy." *The Journal of Cell Biology* **105**,1 (1987).
8. M.J. de Boer, J. G. E. Gardeniers, H. V. Jansen, E. Smulders, M.-J. Glide, G. Roelofs, J. N. Sasserath, M. Elwenspoek, "Guidelines for etching silicon MEMS structures using fluorine high-density plasmas at cryogenic temperatures" *Journal of Microelectromechanical Systems* **11**, 4 (2002).
9. F. Laerme,., et al. in *Bosch deep silicon etching: improving uniformity and etch rate for advanced MEMS applications*, (Twelfth IEEE International Conference on MEMS '99, Orlando, Florida, 1999).
10. M. J. Walker, "Comparison of Bosch and cryogenic processes for patterning high aspect ratio features in silicon", Proc. SPIE 4407 (2001).