Available online at www.sciencedirect.com
SCIENCE DIRECT®
Sensors and Actuators A 114 (2004) 191–196
SENSORS AND ACTUATORS A PHYSICAL
www.elsevier.com/locate/sna

Characterization of fluidic microassembly for immobilization and positioning of *Drosophila* embryos in 2-D arrays

Ralph W. Bernstein [a,*], Xiaojing Zhang [b], Stefan Zappe [b], Matt Fish [c], Matthew Scott [c], Olav Solgaard [b]

[a] *Department of Microsystems, SINTEF Electronics and Cybernetics, P.O. Box 124, Blindern, 0314 Oslo, Norway*
[b] *Department of Electrical Engineering, Stanford University, Stanford, CA, USA*
[c] *Department of Developmental Biology, Stanford University, Stanford, CA, USA*

Received 2 July 2003; received in revised form 10 November 2003; accepted 21 November 2003

Available online 3 February 2004

Abstract

A technique for the positioning and immobilization of *Drosophila* embryos in 2-D arrays for use in high throughput microinjection experiments has been characterized. The method is based on fluidic microassembly, and immobilization yield, the number of misplaced embryos, alignment properties, and adhesion force of the embryos have been measured for samples with four different pad geometries. For samples with 250 μm × 400 μm sized rectangular pads, an immobilization yield of 85% was achieved. The adhesion force of the embryos was estimated at 36 ± 22 μN. A substantial amount of clustering was, however, observed. By reducing the pad size to 200 μm × 200 μm and changing the pad pitch and shape, the number of misplaced embryos was reduced to less than 5%. The adhesion force of embryos immobilized at the smaller pads was estimated at 14 ± 5.5 μN, resulting in lower immobilization yield for these samples. The self-assembly process facilitates rotational alignment to some degree, and an average of 40% of the immobilized embryos align within ±9° of the symmetry axis of the immobilization sites.

Keywords: Biosystems; Embryo; Fruit-fly; Fluidic assembly; Microinjection; Array

1. Introduction

The results from the genome projects open new opportunities for determination of gene functions important for development and disease. The *Drosophila* (fruit-fly) genome sequence has been particularly valuable, since 90 years of studies of this organism can be combined with the new molecular data to determine gene functions. To investigate the functions of the genes or the effect of drugs a rapid screening method is required. One powerful method is to inject embryos with double-stranded RNA, DNA or other biologically active materials [1,2]. Today such microinjection is carried out at single embryos; a very tedious process. The main goal of the presented work is to develop tools that enable reliable and precise injection of biological material into a large number of embryos within a short time-frame. In order to achieve this, new technology for handling, positioning, and microinjection of embryos has to be developed.

A critical part of the injection procedure is to position the embryos in well-ordered arrays so that they can be injected in parallel by matching linear or 2-D array of micromachined injectors [3–5]. A technique for immobilization and positioning of *Drosophila* embryos in 2-D arrays has recently been demonstrated [3]. Here we show that this method is capable of filling arrays with high yield and relatively few misplaced embryos. In this paper we focus on characterization of the immobilized embryos and report measurements of parameters important in injection experiments. These include immobilization yield, number of misplaced embryos, degree of self-alignment, and adhesion force.

2. Experimental

The applied immobilization scheme is based on a fluidic microassembly technique adopted from [6]. Cr/Au layers were deposited onto oxidized silicon wafers and arrays of Au pads with different geometries were patterned using photolithography and wet etching. Hydrophobic sites were established by formation of self-assembled monolayers

* Corresponding author. Tel.: +47-22067828; fax: +47-22067350.
E-mail address: ralph.bernstein@sintef.no (R.W. Bernstein).

0924-4247/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.sna.2003.11.021

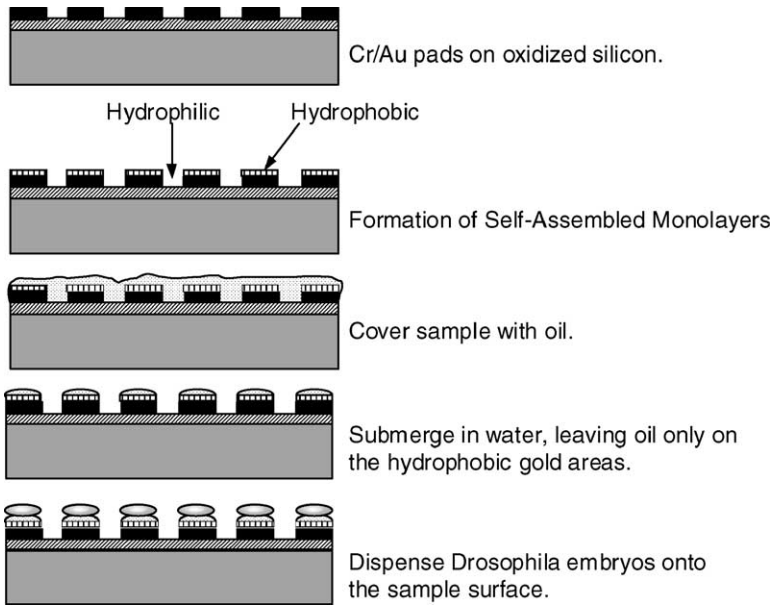

Fig. 1. Schematic process flow for the described fluidic microassembly immobilization technique.

selectively at the gold pads. Samples were immersed in a solution of 1 mM octadecanethiol in ethanol. This treatment renders the gold surfaces hydrophobic, while the oxidized silicon is kept hydrophilic [3,6]. After a rinse in ethanol the samples were covered with a film of polychlorotrifluoro-ethylene based oil. This oil is known to have low toxicity and it is regularly used to protect *Drosophila* embryos from dehydration during injection experiments and hatching.

The *Drosophila* embryo array assembly took place as described in [3]: Samples with hydrophobic sites, covered with oil were immersed in water, leaving oil only at the sites. The oil film acts as a lubricant and adhesive in the immobilization process. *Drosophila* embryos were dispensed onto the sample surface keeping the sample submerged in water. As a result embryos were immobilized only at the oil-covered pads. Excessive embryos were removed by a gentle rinse. The process flow is shown schematically in Fig. 1. Immobilization yield and alignment properties for the different array designs were evaluated by inspection of micrographs acquired from the assembly experiments.

The feasibility of using fluidic assembly to prepare arrays of embryos for use in injection applications strongly relies on the adhesion between the embryos and oil-covered pads. In order to assess the adhesion force, a water flow was directed at immobilized embryos by a micropipette (Fig. 2). By measuring the flow rate at which the embryo detaches from the pad, an estimate of the adhesion force was obtained. The flow rate was calibrated and measured by a SOTP60D pressure transducer from SensorOne.

3. Results and discussion

We report results from immobilization experiments carried out on samples with four different pad geometries. In the initial experiments rectangular pads measuring 250 μm × 400 μm were chosen to roughly correspond to the size of *Drosophila* embryos. In order to investigate the effect of pad geometry on rotational alignment in the self-assembly process, arrays of cross-shaped, truncated cross-shaped (boat), and circular-shaped adhesion sites were evaluated.

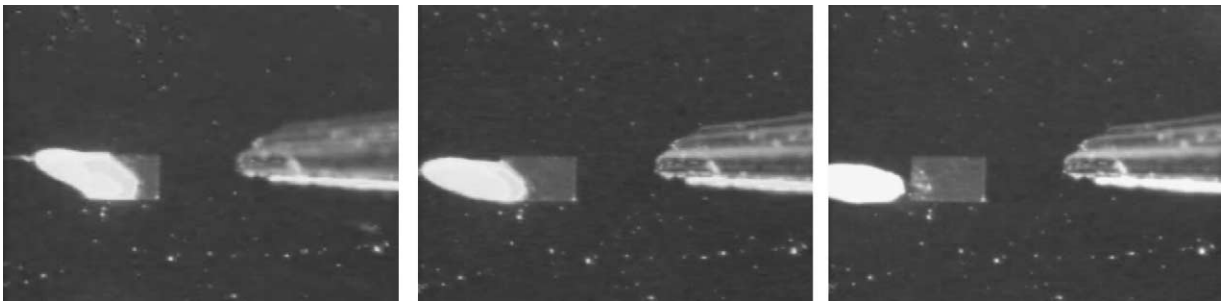

Fig. 2. A picture series illustrating the method used for measuring the adhesion force of the immobilized embryos. A water flow was imposed at an immobilized embryo by a micropipette. The flow rate at which detachment of the embryo took place was measured and a rough estimate of the drag force can be calculated using Eq. (1).

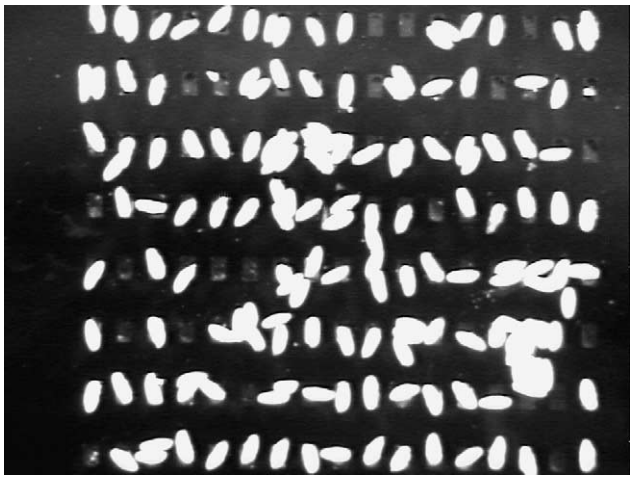

Fig. 3. Embryos immobilized at a sample patterned with an array of rectangular 250 μm × 400 μm sized immobilization sites. A substantial amount of clustering is observed.

In the initial experiments oxidized silicon chips patterned with rectangular 250 μm × 400 μm Cr/Au pads with a pitch of 500 μm × 1000 μm were used as a starting point for the assembly process. An image of a sample from the first immobilization experiments is shown in Fig. 3. A substantial amount of clustering was observed. With the chosen pad size and pitch, more than one embryo can immobilize at one pad, and a single embryo can "bridge" between two pads. In addition embryos tend to stick to each other. As a result, close to 25% of the embryos were misplaced and would not be injected by a matching microneedle array.

In order to reduce the clustering and investigate possible rotational alignment of the embryos new samples with 200 μm × 200 μm cross-shaped and 200 μm × 100 μm truncated cross-shaped pads with a pitch of 800 μm × 800 μm were fabricated. An image of embryos immobilized at one of these samples is shown in Fig. 4. This figure shows that the clustering is reduced substantially. The immobilization yield is, however, somewhat lower since the contact area between the embryo and the pad is reduced. The experimental results are summarized in Table 1.

Although their orientation varies, the embryos tend to position and align themselves to the shape of the gold pads. Images of embryos immobilized at cross-shaped pads (a), and truncated cross-shaped pads (b) are shown in Fig. 5. In order to evaluate the degree of self-alignment an "alignment factor" was defined as $(N(\pm 9^\circ) - N_{\text{random}})/(N_{\text{total}} - N_{\text{random}})$, where $N(\pm 9^\circ)$ is the actual number of embryos aligned within $\pm 9^\circ$ of the symmetry axes of the immobilization sites, N_{random} is the number of embryos that would align within $\pm 9^\circ$ in a situation of completely random rotational positioning, and N_{total} is the total number of immobilized embryos. An alignment factor of 0 represents random rotational alignment, whereas a factor of 1 corresponds to a situation with 100% of the embryos aligned within $\pm 9^\circ$ of the symmetry axes of the immobilization sites. Alignment factors were calculated by inspection of three samples containing arrays of both cross-shaped and truncated cross-shaped pads. One sample with circular-shaped immobilization sites was used for control purposes. In total the evaluation included 760 immobilized embryos.

The average alignment factors of the embryos immobilized at cross-shaped pads were approximately 0.24 for both axis directions. The fact that the alignment factor is nearly equal in both axis directions is expected since the immobilization sites are four-fold symmetric. In the case of the truncated cross-shaped pads the total alignment factor is measured at 0.26. As expected for these asymmetric pad shapes the alignment factor for the longer axis direction is more than twice the one along the shorter axis. For one sample an alignment factor as high as 0.48 was observed. Immobilization on a sample with circular-shaped pads

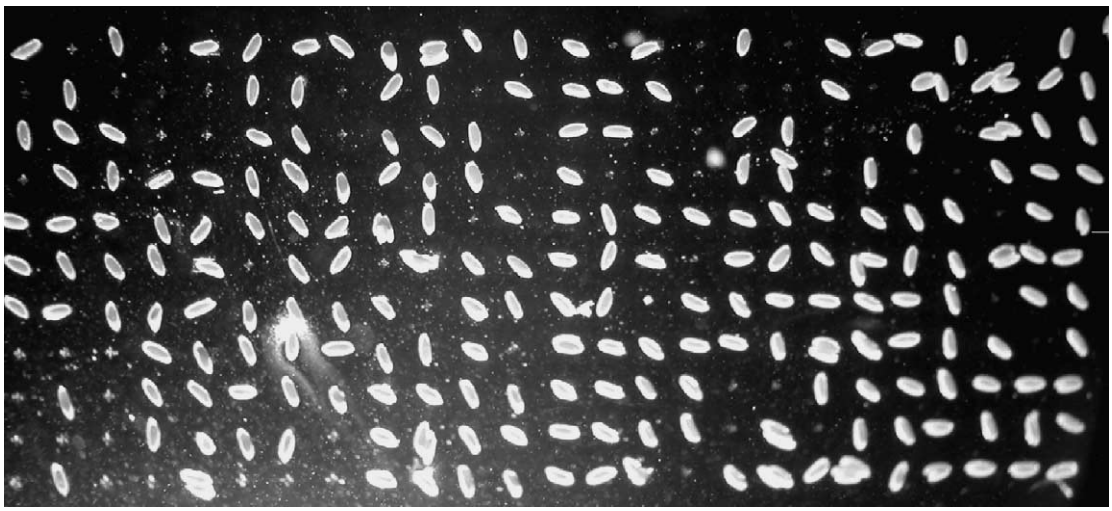

Fig. 4. An image of embryos immobilized at a sample patterned array with 200 μm × 200 μm cross-shaped and 200 μm × 100 μm truncated cross-shaped sites with 800 μm × 800 μm pitch. The clustering is reduced substantially.

Table 1
Measurements results for immobilization of *Drosophila* embryos onto samples with different pad geometries

Pad geometry	Immobilization yield (%)	Misplaced embryos (%)	Adhesion force (μN) estimated by Eq. (1)
Rectangles: 250 μm × 400 μm Pitch: 500 μm × 1000 μm	85	25	36 ± 22
Cross: 200 μm × 200 μm and truncated cross: 200 μm × 100 μm Pitch: 800 μm × 800 μm	72	<5	14 ± 5.5
Non immobilized embryos Penetration force [8]			Max.: 6 ± 3 52.5 ± 7

Immobilization yield: number occupied of sites/number of possible sites; misplaced embryos: number of additional embryos in clusters/number of immobilized embryos.

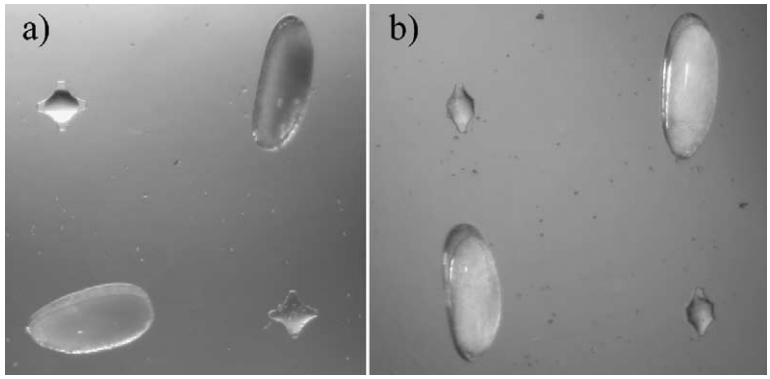

Fig. 5. Close-up images of (a) oil-covered 200 μm × 200 μm cross-shaped; and (b) 200 μm × 100 μm truncated cross-shaped pads with and without immobilized embryos. An example of an immobilization site only partly covered by the oil film can be discerned in the upper left corner of (a).

exhibits an alignment factor of 0.07. For completely random positioning, which would be likely with circular immobilization sites, the alignment factor is expected to be 0.

The results show that the fluidic microassembly facilitates positioning, and that the pad shape influences the rotational self-alignment of the embryos. In average around 40% of the immobilized embryos were aligned within ±9° of the symmetry axis of the immobilization sites. This corresponds to a preferential self-alignment of only ∼20%, since 20% of the embryos would already be aligned within ±9° of the symmetry axes of the immobilization sites in the case of entirely random positioning.

There are several explanations for these results. In a number of cases it was observed that the oil film did not cover the pads completely. One example of such a situation can be discerned in the upper left corner of Fig. 5a. The shape of the oil film at this pad is close to circular symmetric, and an embryo immobilized at this site will not experience a deep local minimum of the potential energy when aligned along one of the symmetry axes of the pad. The fact that the both the oil films and the embryos are curved is also a possible explanation of the low alignment factors observed in the experiments. The observed alignment of embryos on circular pads indicates that the rinse process also influences the alignment of the embryos. This is not unexpected since the rinse includes vertical movement of the sample along the symmetry axis while the sample is kept submerged in water.

The adhesion force is a key parameter in the tuning of the rinse step in the fluid assembly process and sets the limits of acceptable alignment errors during penetration and injection experiments. Estimates of the drag force, F_D, acting at the embryo were calculated using [7]:

$$F_D \approx \tfrac{1}{2} C_D A_D \rho_F v_A^2 \quad (1)$$

$$v_A = \left(\frac{Q}{A_T}\right) \quad (2)$$

where C_D is the drag coefficient, A_D is the cross-section of an embryo, ρ_f is the density of water, v_A is the average flow velocity, Q is the flow rate and, A_T is the cross-sectional area of the pipette measured by scanning electron microscopy (SEM). For one of the pipettes used in the experiments the diameter, 140 μm, is smaller than the diameter of an embryo which is typical 250 μm. In this case, A_D in Eq. (1) is assumed to be equal to A_T. C_D is usually determined experimentally. In the following calculations, we have adopted a statistical curve fit for the drag coefficient for spheres and disks [7].

According to [7], an estimate of C_D can be calculated using the empirical expression:

$$C_D = 18.5 Re\, e^{-0.6} \quad (3)$$

which is valid for Reynolds numbers, Re, $2 < Re < 500$. The estimates for the adhesion forces are given in Table 1. The result for embryos left at sample surface between the immobilization sites is also included in Table 1. The calculated adhesion force for these "non immobilized" embryos is well below the value for the immobilized ones. Proper design of the rinse process will hence ensure that no excess embryos will reside at the surface.

The force required to penetrate *Drosophila* embryos has been reported to be of the order of 50 μN [8]. The estimated adhesion force is smaller than this measured penetration force for all the pad geometries that were investigated. This indicates that lateral injection of immobilized embryos would be difficult to accomplish, but injection with microneedles oriented vertically to the silicon chip surface will be possible. In such injection experiments a certain amount of misalignment of the injectors and embryos is expected. This could cause movement of the embryos before injections takes place and in worst case reduce the injection yield.

4. Conclusion

A technique for positioning and immobilization of *Drosphila* embryos in 2-D arrays for use in high throughput microinjection experiments has been characterized. Immobilization yield, fraction of misplaced embryos and adhesion force of the embryos has been measured for samples with two different pad geometries. For samples with 250 μm × 400 μm pads with a pitch of 500 μm × 1000 μm, a substantial amount of clustering is observed. By reducing the pad size and changing the pitch and shapes <5% of misplaced embryos was observed. The immobilization yield for embryos at these smaller pads is also somewhat lower than that for samples with larger pad sizes.

The assembly process facilitates rotational self-alignment, and only 30–60% of the immobilized embryos were aligned within $\pm 9^\circ$ of the symmetry axis of the immobilization sites. We expect, however, that improved self-alignment could be achieved with proper design of the immobilization sites and by optimizing the sample preparation process. This could include more sophisticated pad patterns or etching of groves in the substrate in order to obtain a more pronounced minimum of the potential energy for the immobilized embryos.

The adhesion force of embryos immobilized at the smaller immobilization sites was estimated at 14 ± 5.5 μN, which is substantially lower than the required penetration force. Preliminary injection experiments indicate, however, that adhesion forces in the measured range are sufficiently high to allow injection with properly designed microinjectors. This demonstrates that the described immobilization technique is suitable for use in high throughput microinjector systems.

Acknowledgements

The project was kindly sponsored by DARPA program [Bio:Micro:Info] program (MDA972-00-1-0032), the Research Council of Norway, SensoNor ASA, and Norchip ASA.

References

[1] A. Scmid, B. Schindelholtz, K. Zinn, Combinatorial RNAi: a method for evaluating the functions of gene families in *Drosophila*, Trends Neurosci. 25 (2) (2002) 71–74.
[2] J.C. Lacal, R. Perona, J. Feramisco (Eds.), Microinjection, Birkhauser, Basel, 1999.
[3] R. Bernstein, X.J. Zhang, S. Zappe, M. Fish, M. Scott, O. Solgaard, Positioning and immobilization of *Drosophila* embryos in 2-D arrays for drug injection, in: Proceedings of the μTAS 2002 Symposium, vol. 2, Kluwer Academic Publishers, 2002, pp. 793–795, ISBN 1-4020-1010-9.
[4] S. Zappe, X.J. Zhang, I.W. Jung, R.W. Bernstein, E.E.M. Furlong, M. Fish, M. Scott, O. Solgaard, Micromachined hollow needles with integrated pressure sensor for precise calibrated injection into cells and embryos, in: Proceedings of the μTAS 2002 Symposium, vol. 2, Kluwer Academic Publishers, 2002, pp. 682–684, ISBN 1-4020-1010-9.
[5] K. Chun G. Hashiguchi, H. Toshiyoshi, H. Fujita, Fabrication of Array of Hollow Microcapillaries Used for Injection of Genetic Material into Animal/Plant Cells, Jpn. J. Appl. Phys. 38 (1999) L279–L281.
[6] U. Srinivasan, D. Liepmann, R.T. Howe, Microstructure to Substrate Self-Assembly Using Capillary Forces, J. Micromech. Syst. 10 (2001) 17–24.
[7] C.D. Cooper, F.C. Alley, Air Pollution Control: a Design Approach, second ed., Waveland Press, January 1994, pp. 91–93, ISBN 0-8813-3758-7.
[8] X.J. Zhang, S. Zappe, R.W. Bernstein, O. Sahin, C.-C. Chen, M. Fish, M. Scott, O. Solgaard, Integrated optical diffractive micrograting-based injection force sensor, in: Proceedings of the Digest of Technical Papers of the 12th International Conference on Solid-State Sensors and Microsystems, 2003, pp. 1051–1054, IEEE catalog number 03TH8664C/ISBN 0-7903-7732-X.

Biographies

Ralph W. Bernstein received his doctoral degree in physical electronics in 1990 from the Norwegian Institute of Technology (NTH) in Trondheim, Norway. He is now Research Director at SINTEF Electronics and Cybernetics, Department of Microsystems. He started at SINTEF as a Senior Scientist from 1992 and became Research Director in 1996. Before that he was employed as a Research Scientist at Norwegian Telecom Research from 1990 to 1992. He was Associate Professor II at the University of Tromsø from 1996 to 2001. Bernstein was on leave from SINTEF as a Visiting Scholar at Ginzton Laboratory, Stanford University, CA, USA in 2001–2002. Ralph W. Bernstein's fields of expertise are within III–V semiconductor technology, microsystem technology (MST) and MEMS design. His recent research interests have been focused on Bio-MEMS for high throughput injection of biological material into embryos and cells. He is a member of the technical committee for the European EUREKA-program, EURIMUS, and is an evaluator within the thematic area IST of the 6th EU framework program.

Xiaojing (John X.J.) Zhang received his B.Sc. degree in precision electronic instrumentation from Shanghai Jiao Tong University, China in

1995 and his M.Sc. degree in electrical engineering from University of Maine, Orono in 1998. His industrial experience includes working at Hewlett-Packard (later, Agilent Technologies) on the design of parallel optical interconnects and the evaluation of IEEE802.3ae optical transceivers for Cisco 10 Gb/s LAN-switching equipment. He is currently a Ph.D. candidate in electrical engineering at Stanford University. His dissertation research includes diffractive optical microelectromechanical systems (Optical MEMS) and Bio-MEMS design, fabrication and characterization, with focus on developing integrated microphotonic sensing platform for in vivo single cell and embryo manipulation, force microscopy, and near-field imaging.

Stefan Zappe received his diploma degree in electrical engineering from the Berlin University of Technology, Germany, in 1996. From 1996 until 2001 he worked as a Ph.D. student at the Microsensor and Actuator Center at the Berlin University of Technology. In February 2001, he joined the Stanford Microphotonics Laboratory at Stanford University, CA, USA as a Postdoctoral Researcher. His research activities include microfluidic systems for cell- and embryo-handling, sorting, and microinjection; biology of fruit-fly development; gene silencing by means of RNAi (RNA interference); micro-orifices for DNA shearing; microfluidic systems based re-usable arrays for DNA sequencing; integration of active and passive optical components into microsystems.

Matthew P. Fish received his B.Sc. degree in biology from San Jose State University, San Jose, CA, in May 1991 and his M.Sc. degree in molecular biology from San Jose State University in May 1999. He is currently employed by the Howard Hughes Medical Institute, working at Stanford University, Stanford, CA in the Department of Developmental Biology. His research work focuses on the function of a novel, developmentally regulated gene in *Drosophila melanogaster*.

Matthew P. Scott received his B.S. and Ph.D. degrees in biology from M.I.T. He did postdoctoral research at Indiana University and then joined the faculty at the University of Colorado at Boulder. In 1983 he moved to Stanford University School of Medicine where he is now Professor of Developmental Biology and of Genetics. He has published more than 130 papers and three patents. His research areas are developmental genetics and cancer research, particularly the roles of signaling systems and transcriptional regulation in embryonic development. His research employs genetics, genomics, cell biology, and molecular biology in exploring how cells acquire their fates and are patterned. He is an editor of 'Current Opinion in Genetics and Development' and of the 'Proceedings of the National Academy of Sciences'. He is a past president of the Society for Developmental Biology, a member of the American Academy of Arts and Sciences, and a member of the National Academy of Sciences. He is presently chairing Stanford's Bio-X program, which is designed to accelerate the coming together of engineering, physics, and chemistry with biology and medicine.

Olav Solgaard received the B.S. degree in electrical engineering from the Norwegian Institute of Technology and his M.S. and Ph.D. degrees in electrical engineering from Stanford University, California. He held a postdoctoral position at the University of California at Berkeley, and an assistant professorship at the University of California at Davis, before joining the faculty of the Department of Electrical Engineering at Stanford University in 1999. His research interests are optical communication and measurements with an emphasis on semiconductor fabrication and MEMS technology applied to optical devices and systems. He has authored more than 100 technical publications, and holds 13 patents. He is a co-founder of Silicon Light Machines, Sunnyvale, CA, and an active consultant in the MEMS industry.