# Miniature Bioprocess Array: A Platform for Quantitative Physiology

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|---|---|
| **Total Cost of Project:** | $ 3,802,606 |
| **Total Government Funding Requested:** | $ 3,802,606 |
| **Proposed Start Date:** | July 1, 2000 |
| **Proposed End Date:** | June 20, 2003 |
| **Date Submitted:** | March 30, 2000 |

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# C. STATEMENT OF OBJECTIVES

The recent completion of several microbial genomes allows the development of novel antibacterial and antifungal drugs targeted to specific genes. While a multitude of drug candidates can be synthesized using combinatorial chemistry and biology, there are few convenient high-throughput screening methods for assessing the efficacy of these candidates. Further, screening all possible gene deletions against all possible drugs makes this exercise nearly insurmountable using available screening technologies.

Similar high-throughput screening problems are faced by the pharmaceutical and biotechnology industries in optimizing microbial fermentations. Several parameters (pH, temperature, medium formulation, mutations in specific genes) must be assessed to determine the key combination of parameters for optimal product formation. As shake-flask culture techniques are not reliable predictors of large-scale fermentation conditions, evaluating even a fraction of the combinations of culture conditions in bioreactors can be prohibitively expensive, time-consuming, and tedious.

We propose here an integrated research program to develop a high-throughput miniature bioprocess array and complementary models of microbial metabolism to assess to effects of changes in culture parameters or drug targets on metabolism. The specific aims are as follows:

1. To develop a high-throughput miniature bioprocess array. This micro-system will be based on an array of 150-μl wells, each one of which incorporates MEMS for the closed-loop control of cell culture parameters such as temperature, pH, and dissolved oxygen.
2. To develop approaches to self-assemble the MEMS components and the CMOS chiplets into the bioassay array.
3. To develop models of cellular metabolism and genetics from the completed genomes of *Escherichia coli* and *Saccharomyces cerevisiae* that can be used to determine the genetic or metabolic effects of a particular culture parameter or drug candidate.
4. To test the effect of changes in culture conditions on the production of metabolic by-products by *E. coli* and *S. cerevisiae*. We will measure cell density, substrate consumption, production formation, etc. and use these measurement in the metabolic model to assess those pathways most affected by changes in culture conditions.
5. To test the effect of specific genetic mutations on the ability of an organism to survive specific culture conditions. Those pathways most affected by the mutations will be determined using measurement of key culture parameters and the metabolic models.
6. To develop a training program for undergraduate, graduate and post-doctoral students in a new area that integrates biology, informatics, and microelectronics.

# D. STATEMENT OF THE APPROACH

The miniature bioprocess array will be based on an array of 150-μl wells, each one of which incorporates MEMS for the closed-loop control of cell culture parameters such as temperature, pH, and dissolved oxygen. The wells will also incorporate a suite of sensors, including interdigitated capacitors for cell density, thermopile temperature sensors, and oxygen sensors. Deep reactive-ion etched (DRIE) capacitive sensors will enable new capabilities, such as the measurement of cell density in the bulk of the solution. Oxygen will be generated by electrolysis, which also provides a means of mixing the solution in the well. Data acquisition, communication, and control will be implemented in foundry CMOS. A four-wire bus will connect the electronic interface at each well to each other and to a battery, a clock, serial input/output, and ground.

Initial research will explore the precision possible with a plastic assembly substrate that can be laminated between two microtiter plates. This plastic layer is intended to provide dimensional stability that is superior to that of the injection molded plastic used for the microtiter plate. A liquid handling system will be placed at the bottom of the miniature bioprocess array for sampling of cultures and will potentially interface with high-throughput capillary electrophoresis, DNA chips, etc. A gas-phase analytical system on top of the miniature bioprocess array will allow one to quantify gasses consumed and produced. Key issues are strategies for multi-step or simultaneous multi-part assembly, planarization processes that are compatible with the plastic substrate, and the interconnect metallization process. As this device would be most useful if it were disposable, we will explore methods to produce inexpensive arrays using fluidic self-assembly (FSA) technology to self-assemble the MEMS components and the CMOS chiplets.

The miniature bioprocess array will be tested with two well-known organisms, *Escherichia coli* and *Saccharomyces cerevisiae*. We will develop metabolic and genetic models for these organisms using information from the genome sequencing projects, various databases of metabolism and genetic regulation, and the vast literature on these two organisms. The models will be of two types: steady-state models of metabolism and dynamic models of genetic regulation and metabolism.

We will use this miniature bioprocess array to analyze the effects of genetic mutations on observable physiological variables – such as growth, product synthesis, substrate consumption, etc. – and changes in gene expression. These variables will then be used in conjunction with metabolic and genetic models, formulated from the various sequencing projects, to predict the effects of various genetic mutations on cell physiology.

Finally, we will use the miniature bioprocess array to screen bioactive compounds, i.e., potential pharmacological agents, antimicrobials, and toxins, to examine their effect on metabolism and physiology.

The research necessary to develop this very complex system is inherently multi-disciplinary. Electrical engineering is needed to develop the microelectronic analytical and control systems. Mathematical modeling coupled to bioinformatics is necessary to analyze and control the various operations in each well of the miniature fermenters. Biotechnology/bioengineering is needed to understand the physiological data collected for each microorganism and to design the various operations in the miniature bioreactors to avoid historical problems with bioreactors. As such, we have assembled a team of electrical engineers, biosystems engineers, and biotechnologists to develop this complex system.

Finally, we will train a new generation of bio:info:micro engineers who will integrate all of these research areas and lead industry and academics in this new discipline.

# E. STATEMENT OF SIGNIFICANCE

The ability to screen antibacterial and antifungal drugs has been hampered, in part, by a lack of high-throughput culturing systems that offer a very controlled environment to monitor and control metabolism. The ability to screen large numbers of chemicals is essential for the rapid development of new therapeutics.

Similarly, bioprocess optimization for the production of therapeutics is a tedious exercise. Large numbers of combinations of medium composition, temperature, pH, energy sources, etc. must be tested to find the optimal combination to maximize product formation. Unfortunately, simple and inexpensive shake-flask cultures are unreliable predictors of cell physiology in bioreactors.

The miniature bioprocess array offers new capabilities to meet these needs. First, the organism of interest can be grown in a controlled, reproducible environment in which temperature, pH, dissolved oxygen, nutrient consumption, and product secretion can be monitored simultaneously and controlled. Second, coupling the analytical capabilities of the microelectronics to mathematical models of metabolism and genetic control will allow one to more accurately determine the effects of the environmental conditions on cell physiology. This, in turn, will lead to better models to predict the effect of new drug and environmental toxicants on the growth of cells. Finally, this system will be engineered so that it can be easily interfaced with many of the recently-developed analytical techniques – DNA microarrays, protein chips, capillary electrophoresis, etc. – to allow sophisticated analysis of gene expression and metabolism.

Many of the lab-on-a-chip systems that are currently available are prohibitively expensive for most teaching and research laboratories and for small companies. The use of fluidic self-assembly to place the microelectronics devices in the plastic could lead to an inexpensive analytical device that could be purchased even under the tight budgetary constraints of an academic research or teaching laboratory.

For DARPA, this complete system could lead to the generation of new methods for screening molecules intended to treat or resist biological warfare agents, cancer, and infectious diseases. This system could also be used to optimize bioprocesses for the generation of antibiotics, polymer precursors, or biofuels. Similar benefits could be derived by the U.S. pharmaceuticals industry.

# F. BODY OF PROPOSAL

## 1. General concept

The recent completion of several microbial genomes allows the development of novel antibacterial and antifungal drugs targeted to specific genes. While a multitude of drug candidates can be synthesized using combinatorial chemistry and biology, there are few convenient high-throughput screening methods for assessing the efficacy of these candidates. Further, screening all possible gene deletions against all possible drugs makes the problem nearly insurmountable using available screening technologies.

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We propose here an integrated research program to develop a high-throughput bioreactor system and complementary models of microbial metabolism to assess to effects of changes in culture parameters or drug targets on metabolism. We propose to develop a high-throughput miniature bioprocess array. This micro-system will be based on an array of 150-μl wells, each one of which incorporates MEMS for the closed-loop control of cell culture parameters such as temperature, pH, and dissolved oxygen. The wells will also incorporate a suite of sensors, including interdigitated capacitors for cell density, thermopile temperature sensors, and oxygen sensors. Deep reactive-ion etched (DRIE) capacitive sensors will enable new capabilities, such as the measurement of cell density in the bulk of the solution. Oxygen will be generated by electrolysis, which also provides a means of mixing the solution in the well. A liquid handling system will be placed at the bottom of the miniature bioprocess array for sampling of cultures and will potentially interface with high-throughput capillary electrophoresis, DNA chips, etc. A gas-phase analytical system on top of the miniature bioprocess array will allow one to quantify gasses consumed and produced. Data acquisition, communication, and control will be implemented in foundry CMOS. A four-wire bus will connect the electronic interface at each well to each other and to a battery, a clock, serial input/output, and ground.

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The miniature bioprocess array will be tested with two well-known organisms, *Escherichia coli* and *Saccharomyces cerevisiae*. We will develop metabolic and genetic models for these organisms using information from the genome sequencing projects, various databases of metabolism and genetic regulation, and the vast literature on these two organisms. The models will be of two types: steady-state models of metabolism and dynamic models of genetic regulation and metabolism.

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Finally, we will use the miniature bioprocess array to screen bioactive compounds, i.e., potential pharmacological agents, antimicrobials, and toxins, to examine their effect on metabolism and physiology.

The development of such a system requires an integration of biotechnology, bioinformatics, and microelectronic devices. As such, we have assembled a team of Arkin (biotechnology and bioinformatics), Howe (microelectronic devices), Keasling (biotechnology), and Smith (microelectronic devices). Besides integrating the research, we will develop an interdisciplinary education plan for undergraduate and graduate students choosing to do research in this area.

# 2. Introduction and Background

First, we review the state-of-the-art in bioprocess detection and analysis. Next, we review methods to assess cellular physiology and models to capture these measurements and better understand the overall physiology of an organism. Finally, we describe a method that will be used to assemble the miniature bioprocess array.

## Devices for screening and culturing microorganisms

The last several years have seen extensive research in the areas of micro Total Analysis Systems (μTAS) and biomonitoring devices (Shoji 1999; Manz 1990). A number of researchers have explored the complexities of multi-chamber, multi-sensors systems for chemical assaying and reaction control/monitoring (Bergveld 1994; van den Berg 1998; Richter 1996). Systems have been implemented which take advantage of lateral assembly of several "microfluidic" platforms to reduce interconnect density and fluid handling complexity (Mensinger 1995; Fettinger 1993). Commercial biomonitoring systems such as i-STAT's blood clotting detector have employed microfluidics along with several key biosensors to successfully detect changes in a biofluid (Bisson 1998). These systems, however, are all based on the detection or control of single reactions, whether between reagents or within biofluids. Even systems employing multiple reactors in parallel, such as the Affymetrix GeneChip® (Anderson 1997) modules, employ either single reactions followed by analysis or a series of sequential reactions with distinct chambers and dwell times.

With the exception of biofluid screening (e.g. blood, urine, etc.) very little attention has been paid to sophisticated closed-loop control and monitoring of live cell cultures at the micro-scale. Such monitoring should be able to detect a much broader span of stimuli than standard chemical or biological sensor modules. Kim (Drexel University MS 1997) patterned glucose, pH, DO, and density sensors into silicon wells; monitoring of *E. coli* and yeast parameters was done over the course of several days. Aycliffe et al. (1997) fabricated a Teflon® chamber 2.5 cm on a side which contained a thermocouple and heating wire for closed-loop control of temperature; the reactor was used to study the migration of polymorphonuclear-leukocytes in chemotactant gradients. Of course, with certain exceptions, most eukaryotic cell cultures require much larger vessels than those commonly employed with μTAS. Culturing of neurons and heart cells (Pine 1980; Kovacs 1994; Stenger and McKenna 1994; Novak and Wheeler 1986; Israel 1990) using micro-electrodes for sensing and stimulation has been demonstrated several times, however.

The development of micro-sensors for the relevant parameters in live cultures (Clark cell oxygen sensor, ISFETs, glucose, etc.) has an extensive literature (Madou 1989, Kovacs 1997). Integration of micro-optical systems for fluorescence analysis has recently emerged. Hsueh (1997) showed an integrated silicon p-i-n diode for detecting electrochemiluminescence in a

micro-sample of labeled DNA. Simpson (1998) integrated a fluorescence detector at the circuit board level to detect fluorescence levels in *P. putida* TVA8. Reese et al. (1994) showed novel analysis techniques using simple electrode sensors; they found that a wealth of information about cell motility, viability and stimulation could be gained merely by analyzing the local fluctuations in an electrode submerged in live cell culture. Ebina et al. (1989) studied how the resistance across a yeast culture and its media changed during growth. In the realm of chemical screening, Fryder et al. (1995) developed a system with 15 different sensors; using a neural network they could train the system to distinguish between different alcohol vapors. Similarly, Wilson and DeWeerth (1995) showed that with non-linear pre-processing, using batches of five types of sensors they could discriminate between five major reducing chemicals across a broad range of concentrations (see also Kovacs (1997)).

## Methods to assess changes in physiology

Cellular physiology may be assessed at many levels including the rate of secretion of known compounds and ions, the growth rate under different conditions, and more cellular measures. These latter measures include quantitation of gene and protein expression, specific metabolite levels, cell morphology, and molecular localization, and even population heterogeneity. Such data are necessary if a full molecular understanding of cellular physiology sufficient for it's re-engineering is to be achieved and have already provided powerful insights into cellular function. However, in order for these data to be optimally informative, well-controlled, reproducible culture conditions must be obtainable. The bioprocess array provides a platform for precise control of small culture conditions. A number of physiological parameters can be measured in culture; however, in order to understand the basis of the changes in these observables more molecular data is necessary in to form a model.

In the last few years the techniques for measuring the average concentrations of biochemicals in cell cultures has improved dramatically. Also, microscopic measurements for tracking individual protein levels and cellular locations have become increasingly precise. Some of the most exciting work in this regard is the development of techniques that allow monitoring of tens, hundred and even full genetic complements of molecule types to be measured simultaneously. On a modest scale there has been a rapid increase in the resolution of rapid separation techniques such as capillary electrophoresis that can be used for measuring metabolite levels in bulk (Cohen 1997). Two-dimensional protein gels have been becoming easier to produce, more reproducible and higher resolution. In combination with advanced gel sampling and mass spectroscopy and phosphor imaging methodologies hundreds of proteins may be identified on the gel and their concentrations and even their post-translational modification state inferred (VanBogelen 1999; VanBogelen 1999). Gene expression microarrays able to measure the average concentration of even mRNA transcript in a population of cells are already in use for the study of metabolic and physiological state (DeRisi 1997). However, without precisely controlled culture conditions, well-defined and measured perturbation and without at least some pathway knowledge such data is difficult to analyze extraction of new information is not optimal. It is difficult to say whether the observed responses are expected based on past pathway knowledge or if it is unexpected. Not only that, but when only one change in condition is tested it is difficult to parse what exactly is the physiological cause of the cellular response. The bioprocess array and the modeling proposed below will go a distance to solving these problems.

Another approach to teasing apart the causal pathways for different physiological responses is the genetic modification of the organism. One of the most exhaustive and interesting approaches is so-call haploinsufficiency trials (Giaever 1999; Winzeler 1999). In this case, strains of the budding yeast, *Saccharomyces cerevisiae,* were constructed, each deleted for one copy of a particular gene. In its diploid state, these "haploid" mutants are less stable than their wild-type parent strains (due to loss of redundancy) but are more often viable than the double deletions. In place of the deleted gene is a resistance cartridge and a molecular bar code (DNA sequence) that uniquely identifies this strain. The haploid strains often survive when the complete deletion does not. The trials are run by growing thousands of these strains of yeast

strain in a batch culture, exposing the culture to a bioactive compound, then tracking the difference in growth rates by measuring the relative amount of each genomic "bar code" in the population over time. Again, reproducible culture conditions are extremely important to this technique, there may be interference among strains (cross talk, chemistry induced by cell death, etc.), and it is often difficult to interpret results without a knowledge of the regulatory pathways. The bioprocess array would allow well-defined culture conditions and may be used to run a separate reactor for each strain. The data analysis and modeling framework could put this data into functional context.

Finally, for many physiological behaviors, there is a great deal of heterogeneity in single cell behavior. In one extreme is the sporulation reponse of *Bacillus subtilis* (an industrial important organisms closely related to anthrax) in which the population bifurcates under metabolic stress in sporulating and non-sporulating forms. In less dramatic examples there is functionally important metabolic heterogeneity in almost any population of cells (Elsner 1993; Naito 1993; Cossarizza 1996; Kaprelyants 1996). The heterogeneity can be a marker of physiological state.

There has been some early work on trying to deduce regulatory networks from such microscopic data as just described (Liang 1998; Michaels 1998; Szallasi 1999) some of which has been developed (Arkin 1995) and experimentally demonstrated by (Arkin 1997). However, such techniques are in their infancy and are much aided by have pre-existing models of large pieces of the pathway.

# Metabolic and Genetic models

A number of mathematical modeling strategies have been developed over the years to capture the dynamics of metabolism, gene expression, and growth. These models tend to be either dynamic (time-dependent) or steady state. While the steady-state models tend to be simpler to formulate and solve, they give no information about the response of a biological system to a particular perturbation. We describe below two modeling techniques that have been used in the Arkin and Keasling laboratories.

## *Dynamic models*

The Arkin laboratory has developed methods for analysis of full dynamic models of metabolic and genetic reaction networks (Arkin 1994; Swanson 1996; McAdam 1997; Arkin 1998; McAdams 1998; Arkin 1999). These tools have been applied to understanding the glycolytic/gluconeogenic switch in mammalian liver glycolysis and the TCA cycle, control of early embryonic cell cycle in *Xenopus laevis*, and stochastic kinetic analysis of the lysis lysogeny decision in phage λ. These models allow a detailed molecular understanding of particular physiological control systems and, in this previous work, has lead to critical reassessment of how external signals are processed by these networks and has allowed the physiological effects of different mechanistic hypotheses to be distinguished. Such full dynamical models, with their detailed descriptions of regulation, are necessary in three cases: 1) when the perturbations around a stationary state are large enough that the linear stationary state assumptions are violated, 2) when the stationary state itself is dynamic (i.e., at a steady-state growth rate, all cell cycle related processes are oscillating), and 3) when one is trying to understand the control and dynamics of switching of cellular physiology between two states. When these full models are of sufficient quality, rational re-engineering of the cell has the best chance of working. However, in order for these models to be predictive, the foundational data must be detailed and of extremely high quality.

The bioprocess array technology goes a good distance in creating a highly controlled environment in which to collect data about many physiological parameters of the cell. Much of this data is of use in validating (rather than building) models in that the model should reproduce the physiological states measured. However, these in-process measurements are not of sufficient microscopic detail to be used in the model building procedure. For this task, quantitative

concentration and rate measurements are the most direct and useful. Low-resolution techniques such as expression microarrays can be very helpful in this regard. Thus the follow-on measurements from cultures in the bioprocess array will be critical to this work.

### *Steady-state models*

The Keasling laboratory has developed a method to predict how the introduction of a new pathway or the enhancement of an existing pathway will affect cell growth and metabolism (Pramanik 1997; Pramanik 1998). To predict how the heterologous pathways should be balanced with the pathways necessary for growth, we have developed a steady-state mathematical model to predict fluxes through the various metabolic pathways. This model has been formulated from the known stoichiometry of the metabolic pathways in bacteria:

$$\frac{d\mathbf{x}}{dt} = \mathbf{S} \cdot \mathbf{v} - \mathbf{b}$$

where **x** is the vector of metabolites, **S** is the stoichiometric matrix, **v** is the vector of fluxes through enzymes, and **b** is the demand vector. Since many metabolites participate in multiple metabolic pathways and since there are cyclic pathways in the cell (*i.e.*, TCA cycle), there are more reactions than metabolites (more equations than unknowns). At steady state, one can solve for **v** using linear programming by maximizing (and/or minimizing) a combination of the fluxes and by placing constraints on the variables. Since the model predicts how the fluxes through the metabolic pathways must be distributed in order to achieve a specific growth rate and product, one can compare the fluxes through the metabolic pathways under various growth conditions and predict which metabolic pathways will be regulated. In addition, one can predict how alterations in metabolic pathways will affect growth of the cell.

## Fluidic Self Assembly for Inexpensive Devices

A key aspect of the fabrication of these bioprocess arrays is large number of sensors and devices that will be distributed over the array. Even a small number of elements for each of the hundreds of cells would result in thousands of interconnects if no processing were done on the array. Preprocessing, however, requires the integration of VLSI electronic controllers with the bioprocess array. We propose to accomplish the integration of VLSI foundry silicon devices and other sensors and actuators by fluidic self-assembly (FSA).

Self-assembly is an assembly technique by which parts are made in such a way that they will attract, align and dock into an assembly without external directive mechanisms. In contrast to serial, “pick and place” assembly methods, self-assembly has advantages in the assembly of large numbers of micron-to-mm sized components.

The FSA technique will be shown to have unique advantages in the following areas:

- Inexpensive, massively parallel assembly of millions of components
- Handling of very small components (as small as 10 microns)
- High positioning accuracy. (+/- 1 micron or better)
- High density interconnections (5 micron interconnection spacing)
- Very low parasitic electrical interconnections (two orders of magnitude less than the best ball grid arrays)
- Robust interconnections through direct metalization of planar structures.
- Direct, seamless integration of otherwise incompatible technologies, such as single crystal III-V's and VLSI silicon on injection molded plastic substrates.
- Separate, optimized fabrication on native substrates for each individual process.

Small assemblies are preferable for several reasons, cost per area as well as form factor considerations. Mechanical difficulties such as thermal coefficient of expansion differences become less troublesome in for small assemblies, because of the small distances involved. Accurate placement and small components lend themselves to low parasitic structures. For example, just the pads for solder bumps or ribbon bonds are at least 50 microns square, and make a major contribution to the parasitic capacitance. Conventional assembly is also costly, both in

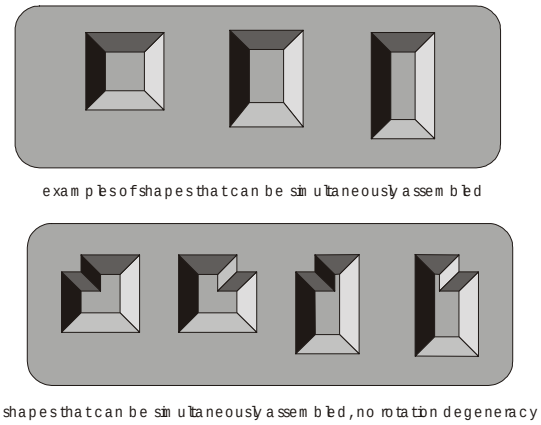


**Figure 1.** Mechanical shapes for specific orientation.

terms of economics (0.7 cents per pick and place, and 0.5 cents per wire bond), and in terms of parasitics (nanohenries of series inductance and picofarads of parallel capacitance). Conventional assembly techniques also run into difficulties when surface forces become important, with parts sticking to each other or to tool surfaces.

Our fluidic self-assembly process starts with micromachined components and matching micromachined sites on a flat substrate. The parts for the assembly are handled as a slurry, and dock into there matching sites. Millions of components can be placed in minutes, each with high accuracy (99.99% yield). The assembled items are then coplanar with the original substrate, and we have demonstrated that the self-assembled structures can be planarized and interconnected through a conventional metalization and photolithographic definition. The accuracy of the assembly allows the wiring traces and vias to be comparable to those used within the chips themselves, resulting in **high performance, seamlessly integrated systems.**

Fluidic Self Assembly can efficiently handle very large numbers of these very small (and therefore very inexpensive) components. An assembly rate already achieved of tens of thousands of components per minute with high placement accuracy is far beyond the capabilities of serial assembly methods. It is possible that massively parallel deterministic methods may get into these assembly rate ranges, but they are much more complicated and have their own risks.

The molecular regime offers many examples of efficient self-assembly processes. This is proof that self-assembly can make very sophisticated systems! The challenge is to design self-assembly processes in which design and manufacturing tools can be controlled and directed to form desired systems accurately and economically. For example, it is essential to take advantage of pholithograpic replication over planar surfaces, itself a massively parallel process, for both implementation of the self-assembly features and the active elements which are being assembled.

Fluidic self-assembly has several major stages. First, the assemblies must have some gross motion to randomly carry parts to the vicinity of where they are to be assembled. For chemical and small biological systems, this is driven by Brownian motion and diffusion. It is important that components not prematurely stick together in incorrect ways. Then comes a phase of changing into the proper orientation. In biological systems this is driven by hydrodynamic steering based on shape, and relatively long range electrical forces from ionization. The attractive forces must not be strong enough to bind up the part before it is correctly oriented. After it is in the correct place, relatively strong and selective chemical forces bind in into place. These chemical forces also usually perform the function of the system, delivery of energy, enzymatic action, etc.

*Why self assemble in a fluid?*

For objects on the scale of a millimeter and smaller, surface forces dominate over all other forces. For example, if a larger object is gripped with a tool, when the grip is released, it falls. But smaller objects stick to the gripper. The object can be scraped off the tool with another surface, but then it is stuck to that surface. Manipulating small objects successfully thus requires control of surface interactions. Even if objects are manipulated in air, they will have a layer of water molecules on their surfaces that will cause them to bond to other surfaces. The formation of a meniscus will result in a high bonding force. In a vacuum, clean metals will cold weld to each other. In an aqueous solution, the hydrophilic/hydrophobic interactions of the surfaces can be manipulated and the forces controlled. For example, two hydrophilic surfaces will repel at short distances, and develop a water lubrication layer between them.

The fluidic self-assembly techniques that we are developing are, of course, simplified in comparison to biochemical processes. Brownian motion being negligible for parts in the size of

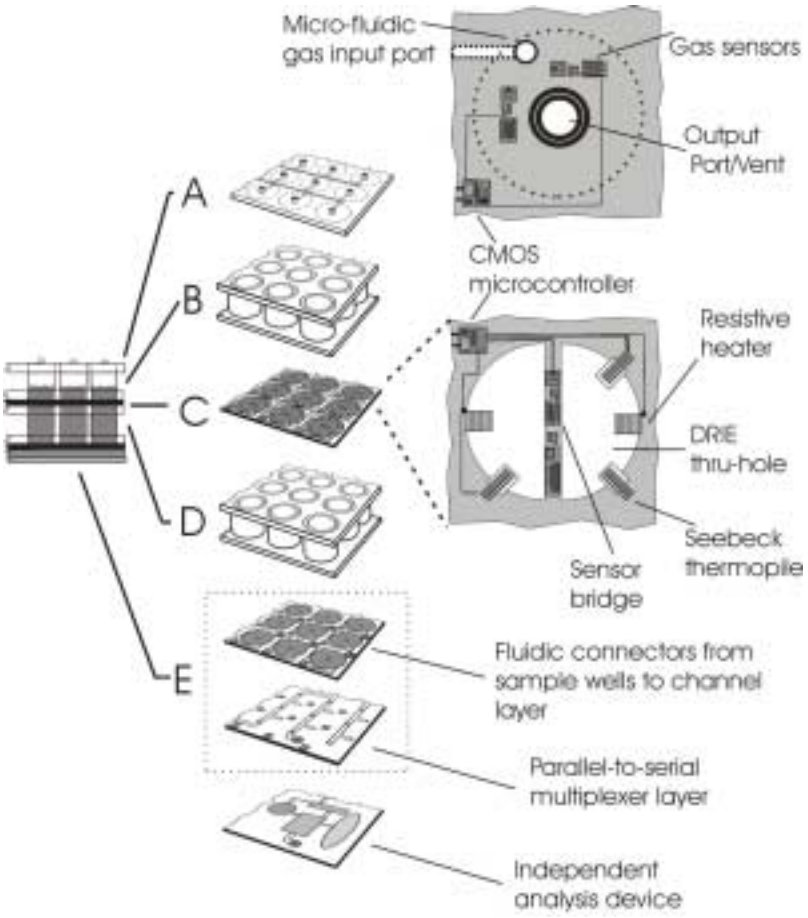


**Figure 2.** Schematic diagram of the miniature bioassay array.

10 microns or larger, we use fluid motion by pumping or stirring of a slurry containing the components. In the first self-assembly technique that we developed, the alignment phase is accomplished by hydrodynamic steering using micromachined tapered sites in a flat substrate, and assembles parts which match those sites (Figure 1). Hydrodynamic steering orients the parts, and the alignment can proceed to the accuracy of the site. The forces holding the parts in place are Van Der Waals forces and gravity. Finally, the top surface of the part ends up planar with the surface of the substrate, so that electrical contacts can be made by conventional metalization.

# 3. Technical Approach

We propose here a focused research program toward the demonstration of a miniature bioprocess array. This microsystem is based on an array of 150 μL-sized wells, each one of which incorporates MEMS for the closed-loop control of cell culture parameters such as temperature, pH, dissolved oxygen. The wells incorporate a suite of sensors, including interdigitated capacitors for cell density, thermopile or CMOS PTAT temperature sensors, dissolved oxygen sensors, pH sensors, and $O_2$ and $CO_2$ gas sensors in the head space. Deep reactive-ion etched (DRIE) capacitive sensors also enable new capabilities, such as the measurement of local fluctuations in cell density. Oxygen can be generated independently in each well by electrolysis, which might also provides a means of mixing the solution in the well. Data acquisition, communication, and control are implemented in foundry CMOS. A four-wire bus connects the electronic interface at each well to each other and to a battery, a clock, serial input/output, and ground.

The miniature bioprocess array shown in Figure 2 incorporates the biotechnology industry-standard 96 (or 384)-well microtiter plate format for the growth wells. In order to embed the silicon MEMS in the fluid chamber, we adopt a laminated structure. Two standard micro-titer plate arrays (B, D) are sandwiched between (A) a molded plastic front-end with micro-fluidic gas inlet ports and gas sensors, (C) the primary sensory/control platform with silicon MEMS and CMOS components, and (E) a silicon microfluidic parallel-to-serial multiplexer. The latter enables the contents of a selected well to be output to a serial bio-MEMS analysis system, which is shown schematically at the bottom of Figure 2.

The insets in Figure 2 illustrate an initial concept currently in fabrication for the MEMS sensing and control structures. Layer A includes a microchannel manifold connected to a global gas source; individual microchannels route into each well from this manifold, providing a

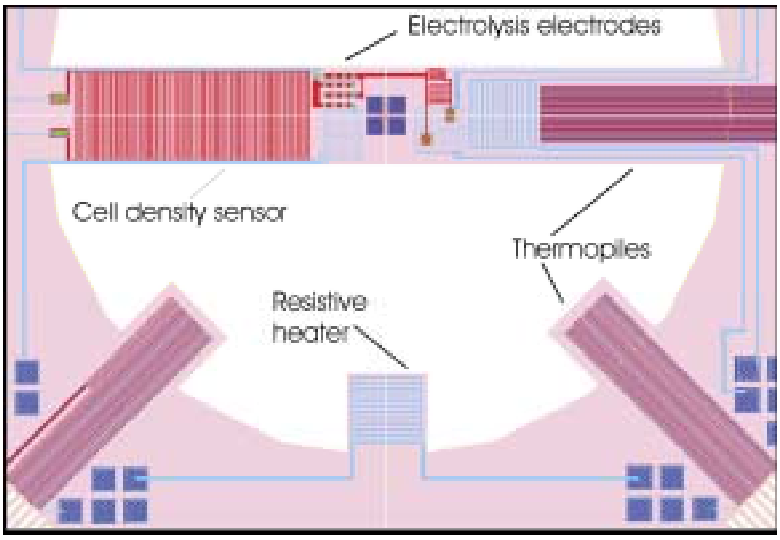


**Figure 3.** Mask layout for initial design for MEMS sensor/actuator layer C in bioassay array.

constant and well-defined gas content into the head space of each well. An output port vents directly to ambient from each well (optionally, these ports could be sealed with a laminate membrane to prevent gas/moisture diffusion to ambient). Layer A also includes thin-film gas sensors routed to a CMOS microcontroller; these allow for determination of certain gas species ($O_2$, $CO_2$, etc.) concentrations during operation. Layer C includes capacitive cell density sensors, temperature sensors, resistive heaters, electrolysis electrodes for oxygen generation and mixing, and pH sensors. Layer E of Figure 2 is the interface between the miniature bioprocess array and conventional analytical microsystems. It selects and samples the contents of a well, minimizes cross contamination between samples of wells, and delivers a fluid "plug" of sample fluid to the downstream analytical tool. We plan to develop an 8 parallel channel output version of layer E in this proposal; however, time separation of the samples could enable output from any well in the 96- or 384-well array.

## Task 1: MEMS and CMOS design and fabrication

Instrumenting each well is feasible using MEMS technology. Over the past two decades, a variety of semiconductor structures have been explored for monitoring a wide range of biological parameters. Figure 3 is the mask layout of an initial version of layer C in Figure 2. Interdigitated capacitors have been used to monitor the cell density in cultures through the permittivity difference between cell and solution. We propose a new type of capacitive cell density sensor, in which deep reactive ion etching (DRIE) of the silicon wafer is used to form the capacitor fingers. These fingers are located on the horizontal bridge across the microtiter well in Figure 3 and the intra-finger spacing is selected to allow cells to pass through the capacitor. As a result, this sensor should provide a better measurement of the cell density in the bulk of the fluid. Combined with the output of the "surface" interdigitated capacitor, we will have a more reliable measure of the cell density in the well.

Thermopile-based temperature sensors are attractive for stable, robust sensing of temperature. A reference junction for the "cold" side of the junction is conveniently located at the perimeter of the array. Clark cell dissolved oxygen microsensors, consisting of platinum electrodes covered by an oxygen permeable membrane, are distributed on the surface of the silicon bridge. Fluorescence marker techniques may be used as well, for which integrated photodetectors would be indicated to enable local detection of light emission. Other parameters, such as pH, glucose concentration and cell motility may be useful for assessing the health of the cell culture and sensors for these will be investigated.

The two variables that are critical to the growth environment are the temperature and the dissolved oxygen concentration in the medium. The control of these variables is the goal of this subtask. Resistive heating of silicon regions at the perimeter of the well, as well as portions of a microbridge suspended in the well, can be used for temperature control. The placement of the heaters will be optimized for minimizing thermal gradients in the well during normal operation.

In addition, the heaters can be driven with phased voltage waveforms for generating thermal gradients, which could aid mixing of the growth medium.

The inset in Figure 2 shows a CMOS microcontroller that has been flip-chip assembled onto the MEMS wafer. This chip contains the interface circuits to the various sensors and actuators, which include capacitive sense circuits for the cell density sensors. In addition, it implements a communication protocol on a four-wire serial bus that interconnects all the wells on the array, as well as an external host. The system partitioning between the local and external computer remains to be determined; however, it is likely that the microcontrollers will assess the response of sections (e.g., 4 x 4 or 8 x 8) of the array.

CMOS interface, control, and communication circuit design are also the subject of this task. A first generation layout will focus on signal acquisition from the MEMS sensors and strategies to implement closed-loop control of well parameters. The second-generation design will, in addition, implement a serial communication bus for well-well and well-host communication.

## Task 2: Miniature bioprocess array fabrication and evaluation

In this task, the MEMS sensors and actuators and first-generation CMOS interface electronics are laminated with a microtiter plate to validate and optimize their functionality with microbial cultures. The central goal of this task is to optimize the use of the MEMS sense and control capability developed under task 1 to achieve the goal of efficient analysis of microbial growth and metabolism. Experimental results will be used to modify the growth conditions and the design of the MEMS-based cell density sensors, temperature control, mixing, and oxygen control functions. Mixing can be verified by observation of microspheres. Through this task, the MEMS and CMOS will be fine-tuned to function optimally for the cell growth monitoring and control function.

We will test the miniature bioprocess array by growing *E. coli* and *S. cerevisiae* in it and monitoring the cell density, pH, dissolved oxygen, and other culture parameters. As many organisms secrete acids or alcohols during growth on rich media, it should be possible to observe large changes in pH at high cell densities. The secretion of these metabolites will also increase as dissolved oxygen concentrations decrease. In addition to measuring changes in the environment brought about by steady cell growth, we will effect rapid changes in the environment (temperature, dissolved oxygen, etc.) using the devices incorporated on the bioassay array and observe changes in cell growth.

## Task 3: Interface to microfluidic analytical instruments

The bioprocess array consists of a number of active microbial populations cultured in parallel. Response to a change in environmental conditions or to bioactive compounds is prescreened by monitoring cell density, temperature, pH, oxygen consumption, as well as selective markers released by the cells in response to specific stimulus. When pre-determined "trigger conditions" are met in a specific well, a sample is taken from that culture and output to more sophisticated analytical micro-instrumentation. Delivery of samples from wells of interest to the analytical columns may require different schemes depending on the type of cell culture and the parameters of interest.

We propose a micro-channel system (layer E in Figure 2) that utilizes silicone oil (or a similarly inert, hydrophobic substance) and patterned hydrophobic/hydrophilic regions to implement this parallel-to-serial sampling function. The system will allow for systematic and repeated testing of multiple wells. The goal is to implement this function without the need for fluidic microvalves or pumps. Vacuum is used to withdraw plug samples into the channel. Each of the samples is delivered in sequence to the analysis column; each is isolated into its own discrete partition by partitions of silicone oil. A simple manifold is fabricated that interconnects the wells, so that a sample from a particular well of interest can be directed to the analysis column.

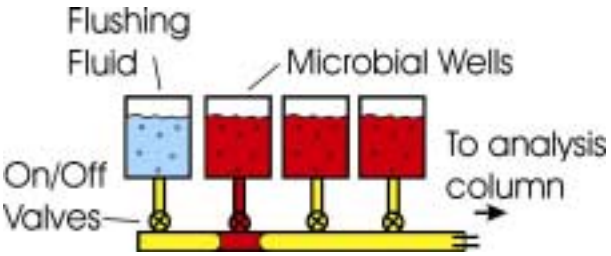


**Figure 4.** Ideal well-sampling microfluidic system.

For analytical techniques or processes that can be sampled through flushed columns, we envision a microchannel that has sampling ports into different wells. Ideally, each sample port would be provided with a valve that isolates the sample manifold until that well is selected (allowing one to choose which well is run through the column). One or more reservoirs of flushing fluid would be provided to sequentially clean the manifold and prepare for reuse. An example of such a scheme is shown in Figure 4. However, as valves with an absolute off state are still problematic for MEMS structures, we propose to explore an alternative scheme.

We propose instead a micro-channel system that utilizes silicone oil (or a similarly inert, hydrophobic substance) and patterned hydrophobic/hydrophilic areas to accomplish the sampling of individual wells. Figure 5 shows a schematic view of this system in operation.

As shown in Figure 5(a), a set of the sample wells is connected to a microchannel via a hydrophobic feedthrough. If necessary, flushing fluid reservoirs can be connected in similar fashion between sample well feedthroughs. A manifold also connects to this microchannel at regular intervals, interspersed with the sample feedthroughs. Initially, the entire sample is flooded with silicone oil as shown in Figure 5(a). The hydrophobic areas readily accept the oil up to the lip of each well. In Figure 5(b), when samples of this well group are desired a vacuum is applied to the manifold; as the same vacuum is used for all samples, the vacuum is assumed to come from a single external (i.e., macro) source. The vacuum in effect overcomes the meniscus forces and draws a slug from each well into the primary channel. Once these slugs have been taken, the vacuum is released.

At this point (Figure 5(c)), silicon oil is drawn down the primary channel, pushing the samples along. As the feedthroughs are hydrophobic, sample material is not allowed to leak into them (either into the manifold or from adjacent wells). Additionally, the silicon oil readily moves into refill the feedthroughs, thereby providing an effective plug against contamination and cross-talk. The samples are then fed sequentially into the downstream analysis suite. By adding one additional feedthrough downstream, unnecessary samples can also be discarded (Figure 5(c)). In effect, this system maintains isolation between wells and between micro-samples without the use of fluidic microcomponents

## Task 4: Integration of Electronic sensors, actuators, and control chips into the array by self assembly

A key aspect of the fabrication of these bioprocess arrays is large number of sensors and devices that will be distributed over the array. Even a small number of elements for each of the hundreds of wells of the array would result in thousands of interconnects if no processing were done on the array. Preprocessing, however, requires the integration of VLSI electronic controllers with the bioprocess array. We propose to accomplish the integration of VLSI foundry silicon devices and other sensors and actuators on the miniature bioprocess array by fluidic self assembly (FSA).

The current "microengineering tool kit" is capable of producing a great range of sensor and actuator devices (Boser 1997). This set of fabrication methods consists mainly of bulk and surface silicon micromachining, laser micro-machining, and LIGA. In the next generation of MEMS, micromechanical sensors and actuators will be integrated with electronic and optical components to give powerful and complex microsystems (Payne 1995). At present, however, this remains a significant challenge since the fabrication sequences and material requirements of the different components are often incompatible. The development of efficient wafer-scale assembly

techniques can be used to overcome this hurdle and combine a diversity of materials on a single chip.

In order to be successful, a microassembly technique must be able to position a large number of components simultaneously with microscale precision. Once the parts are assembled, reliable mechanical bonds and electrical connections to the substrate may be furnished. In the self-assembly approach, a solution of microcomponents flows over a target wafer patterned with binding sites, resulting in part-substrate attachment. This technique can easily give different donor and target substrate layouts, thus reducing materials costs. In addition, since the parts may be fabricated and tested separately, this approach can reduce the yield losses associated with monolithic processes (Payne 1995).

We intend to use the selectivity of the hydrophillic/hydrophobic interactions combined with the planarity and robustness of the assembly with micromachined sites to achieve new functionality in the assembly process.

This will involve designing mechanical features of sites to achieve orientational and site selectivity. This will initially include the mechanically unsymmetrical sites and nanochips, and keying protrusions from the bottom of the sites. It will also involve designing chemical features of sites to achieve orientational and site selectivity. This includes matched hydrophillic/hydrophobic keyed patterns into mechanically recessed sites.

Many high performance MEMS depend on very low parasitic interconnects. For example, capacitive interfaces are used in many accelerometers or gyroscopes to sense the displacement of a proof-mass as a result of an inertial force. Typically these displacements are well below 1 Angstrom (0.001 Angstrom is typical for 1deg/second surface micromachined gyroscopes), thus necessitating extremely sensitive pickup electronics.

Typical sense capacitors in surface micromachined devices are 100fF or less, limited by the size of the structure and pickup electrodes. Often, and particularly when off-chip circuits are used, the interface parasitic capacitance exceeds this value by a large factor. For example, typical solder-bump flip-chip processes add 1pF or more parasitic capacitance to each interconnect. The result is an order-of-magnitude or more signal reduction. Although the electronics can of course amplify the signal, the sensitivity of the device is still reduced since in such an arrangement the noise (e.g. from the amplifier) is gained up also. Hence, for a given amplifier with set noise characteristics, a low-parasitic interface results in a more than an order-of-magnitude better instrument sensitivity.

Interconnect resistance is also important. Long polysilicon runners between the mechanical device and the pads or electronics often add up to several kilo-Ohm. When this resistance exceeds the inverse of the transconductance of the sense amplifier as is the case when a sub-micron IC process is used, the noise from this resister dominates over that of the amplifier