Title: **Physiomics Array: A Platform for Genome Research and Cultivation of Difficult-to-Cultivate Microorganisms**
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Final Report

Executive Summary

A scalable array technology for parametric control of high-throughput cell cultivations is demonstrated. The technology makes use of commercial printed circuit board (PCB) technology, integrated circuit sensors, and an electrochemical gas generation system. We present results for an array of eight 250 μ l microbioreactors. Each bioreactor contains an independently addressable suite that provides closed-loop temperature control, generates feed gas electrochemically, and continuously monitors optical density. The PCB technology allows for the assembly of additional off-the-shelf components into the microbioreactor array; we demonstrate the use of a commercial ISFET chip to continuously monitor culture pH. The electrochemical dosing system provides a powerful paradigm for reproducible gas delivery to high-density arrays of microreactors. We have scaled the technology to a standard 96-well format and have constructed a system that could be easily assembled.

Introduction

There is a pressing need for reliable, high-throughput cell cultivation technology. The growing library of genomic data is expanding the number of possible modifications to cultured species. Additionally, recent estimates indicate a large number of microorganisms found in the environment are unculturable using current techniques or conditions (Cowan 2000; Gao and Moore 1996; Moter and Gobel 2000; Pillai 1997). Increasingly, the researcher will explore an extensive cultivation parameter space when optimizing metabolic activity or bioconversions. Despite this, bioprocess experimentation and cultivation has remained largely unchanged for decades, relying mostly on shake flasks and 1-10 l bioreactors (Schugerl 2001; Stanbury, et al. 1995).

The miniaturization of cultivation technology has been hampered by the lack of suitably durable, compact and relevant sensors and by the limited ability to control parameters within small volumes (Harms, et al. 2002; Scheller, et al. 2001; Schugerl 2001; Walther, et al. 1994). Applicable sensor technology has been progressing steadily; methods employing microfabricated silicon-based sensors or optochemical sensing allow for the monitoring of parameters in many simultaneous experiments (Pons 1993; Scheller, et al. 2001; Zanzotto, et al. 2002; Zhang, et al. 2002). Parameter control, however, is still relatively primitive. For example, even the more successful miniaturized bioreactor designs utilize tiny, hand assembled tubing to provide oxygen to the culture (Kostov, et al. 2001). Several commercial cell culture plates (e.g. www.synthecon.com;

www.opticell.com; BD Biosciences CellLine System; Integra Bioscience Tecnomouse System) employ gas permeable membranes to allow gas transfer between a plenum and culture, but these do not allow for the independent control of a large number of individual experiments.

The miniaturization of traditional methods of gas supply for use in dense arrays is not straightforward. To control the amount of gas in a miniature bioreactor by scaling down conventional techniques, a complicated microfluidic system including pumps, valves, a gas reservoir (or filter) and a network of microfluidic channels would be needed. Electrolysis presents a compact, scalable approach for gas delivery to small volume cultures. The *in-situ* electrochemical generation of gas in each microbioreactor eliminates the need for complicated microfluidics by converting an electrical current directly into a given rate of gas generation. The gas dosage can thus be controlled precisely and independently in each bioreactor.

We developed electrolytic gas generation as a key component in a miniaturized, scalable bioreactor array. The array is assembled from commercial microplates, printed circuit boards (PCB) and microfabricated components. Oxygen, carbon dioxide or hydrogen can be generated by noble metal electrodes in an electrolyte chamber below each reactor and transferred to the culture via a gas permeable membrane. Each of eight 250- μ L wells continuously reports optical density, temperature, oxygen input, and has a modular area to add additional sensors (e.g. pH, dCO₂, electrodes for impedance spectroscopy, etc). Temperature is controlled via buried thick film thermistors and heaters under each well. We demonstrated the modularity of the system by integrating a commercially available solid-state pH sensor chip and using it to monitor cell cultures.

First generation physiomics array

Microbioreactor array fabrication. Each array was assembled from two eight-well plastic microplate strips (Costar R.I.A./E.I.A. 8 Well Strip), a commercially fabricated PCB, gold wire and a silicone membrane. The PCB was designed in-house and fabricated by Hughes Circuits (www.hughescircuits.com). One-eighth inch holes were drilled into the bottom of each well of a microplate strip. The perforated microplate strip was adhered to a silicone membrane (see below) using silicone sealant (Dow Corning 734 RTV Sealant) and allowed to cure overnight. Gold wire was cut into 1.2 cm pieces and soldered onto pads on the PCB. The second microplate was adhered to the bottom of the PCB with silicone (as above). This bottom microplate strip was then filled with the appropriate electrolyte. Additional sensors, such as an ISFET chip (Sentron Europe, E2310000, 0.5mm x 1.5mm), were wire-bonded onto the PCB board; wirebonds and exposed pads were coated with RTV silicone to insulate them from the biological medium (Fig. 1b). The top microplate strip with membrane was then adhered to the PCB using silicone (as above).

Prior to each experiment, each bioreactor array was sterilized with 250 μ l of 70% ethanol, flushed twice with DI water and dried under a UV sterilization lamp. Microreactors containing the ISFET pH sensor chip had to be filled with 10% hydrogen fluoride solution for 60 sec., then quickly rinsed several times with DI water.

Silicone membrane. A 30- μ m thick silicone membrane was formed by spinning two part silicone elastomer (Gelest OE-41) onto a 4" silicon wafer coated with 2- μ m thick photoresist (Shipley OCG Oi R-897 10I, 30 min. 150°C bake) at 4000 rpm and curing the silicone at 55°C for 4 hours. After the silicone cured, a plastic microplate was adhered to it using silicone. The membrane was released from the wafer by immersing in methanol for 20 minutes, then rinsing with DI water.

Equipment and Instrumentation. The various bioreactor array components were controlled using custom-built electronics, an on-board BASIC STAMP microcontroller (Parallax Inc.) and a PCMCIA data acquisition card (National Instruments DAQCard-6062E) run by an IBM Thinkpad laptop. The STAMP microcontroller sequenced the oxygen generation by pulse width modulation of a constant voltage through relays. The use of relays was crucial to avoid electrode corrosion as noble metal electrodes can corrode during the cyclic evolution of hydrogen (Hoare 1964 ; Maharbiz, et al. 2002). The STAMP microcontroller also sequenced sensor interrogation of each bioreactor well. Data was taken through the DAQ card. All data acquisition code was written in Matlab (v5.3, Mathworks, Inc.). In order to assure an anaerobic ambient during experiments, the housing for the bioreactor array was continuously flushed with dry, sterile nitrogen and a fresh oxygen scavenger pouch (AnaeroPack, Mitsubishi Gas Chemical America Co.) was introduced into the chamber before each experiment. The bioarray housing was mounted on a shaker plate.

Optical system. Optical density was measured on-board using an array of 580 nm LEDs (AND, AND50A ultra-bright orange surface mount LED) and detectors (Silicon photodiodes, PDB-C154SM, Photonic Detectors, Inc.). Pin-holes were mounted between detectors and wells to screen scattered light. For each well, the on-board optical system was calibrated using known concentrations of 1 μ m polystyrene spheres and known concentrations of stationary phase *E. coli*. Sensor response was linear with bead density up to an optical density of 4 at a wavelength of 600 nm.

Results with the first generation arrays

The bioreactor array consists of a PCB strip with batch assembled components sandwiched between two commercial microplates (Fig. 1a). The bottom microplate wells contain electrodes immersed in electrolyte; the top wells hold the cell culture. Each array contains eight microreactors. For each reactor, the PCB contains a buried thick-film thermistor/heating resistor pair, underside solder pads for the attachment of electrolysis electrodes, and a 16 mm² perforated area for gas transfer (Fig. 1b). The gas transfer area is covered by a thin, gas permeable silicone membrane. The eighth well in each strip contains free space and eight electrical connection points to mount additional sensors. A molded fixture containing eight light-emitting diode/photodetector pairs fits over the assembled array and takes continuous optical density readings for each culture.

Figure 2. Cell growth as a function of oxygen generation. Oxygen supplied: (1) 10 mmol O₂/hour, (2) 6 mmol O₂/hour (3) 3 mmol O₂/hour (4) 0 mmol O₂/hour, (5) 6 mmol O₂/hour (lower time axis), (6) 0 mmol O₂/hour

Multiple curves are shown for curves (2) and (3) to illustrate reproducibility. Cultures 1-4 were grown in LB medium. Cultures 5-6 were grown in M9 minimal medium with 1% glycerol (lower time axis). Optical density measurements were taken every 30s during fermentations.

The specifics of electrolytic gas generation are discussed elsewhere (Maharbiz, et al. 2003; Maharbiz, et al. 2002). Briefly, by controlling the current flowing between the two noble metal electrodes in each electrolyte chamber, the amount of gas generated can be precisely controlled. This gas generation builds pressure in the electrolyte chamber, which leads to gas transfer into the cultivation chamber. Gas transfer is dependent on the thickness and area of the silicone membrane between the upper and lower microplates. As the parameters of the membrane are constant and repeatable, the gas transfer rate into the culture is very well defined. The electrolyte is chosen to generate the desired gas (Ebersson 1969; Gileadi and Piersma 1966;

Petrucchi 1982). The only gas sources for the microreactor cultures are the electrolysis chambers.

As examples, 0.5M phosphate buffer (NaH₂PO₄·2H₂O, Na₂HPO₄, pH 7.2) is used to generate oxygen and hydrogen via water electrolysis, while the oxidation of formic acid is used to generate carbon dioxide and hydrogen (Fig. 1c, d) (Ebersson 1969; Gileadi and Piersma 1966; Petrucchi 1982). An electrolyte solution of 1M HCOOK (potassium formate) in methanol was also considered but the permeation of evaporated methanol into the culture via the silicone membrane was a concern.

Microaerobic cultivations of wild-type *E. coli* were performed while controlling the oxygen generation rate in each microreactor of the array (Fig. 2, 3). An increased gas generation rate resulted in increased growth rate and final cell culture density at the corresponding microreactor. The open circles in Figure 3 show the variability of culture growth across the array without oxygen input. The mean final OD₆₀₀ was 0.75 with a variance of 0.001; the mean final pH was 6.3 with a variance of 0.012. The reproducibility of gas feed conditions across multiple microreactors is excellent; experiments 2 and 3 in Figure 2 show examples of growth curves generated in different

Figure 4. Temperature control. Each reactor contains a buried resistor heater and thermistor for closed-loop temperature control. This plot shows the temperatures in each well of the microarray for three separate setpoints. The error bars show the temperature variation over an hour; maximum variation was less than ± 2 °C. Only the heater in reactor 3 was controlled. Due to the thermal conductance across wells, one heater is sufficient to establish a temperature gradient across the array.

microreactors with identical oxygen inputs. Blue circles in Figure 3 show culture growth results for microreactors given the same oxygen supply (10 $\mu\text{mol O}_2/\text{hour}$). The mean final OD_{600} was 1.30 with a variance of 0.02 the mean final pH was 7.1 with a variance of 0.03.

Figure 3 shows optical density and pH changes for experiments in different media with varying oxygen input. *E. coli* growth in complex medium showed a strong dependence on the oxygen generation rate (Figure 3, blue and green circles). Growth rates for cells grown in complex

medium with no oxygen input were significantly reduced and the culture acidified as result of fermentation (Figure 2, curve 4; Figure 3, red circles). As *E. coli* cannot metabolize glycerol without oxygen as an electron acceptor, cultures in M9 minimal medium supplemented with 1% glycerol showed growth only in the presence of oxygen (Figure 2, curves 5 and 6; Figure 3, orange circles).

The temperature in each well can also be actively measured and controlled using the resistive components. Figure 4 shows temperature gradients established across a microreactor array using the temperature control system. The thermal resistance between wells ranges from 50 - 75 °C/W and each well temperature can be maintained within $\pm 2^\circ\text{C}$.

To demonstrate the modularity of the PCB platform, a commercial pH sensor chip and a platinum reference electrode were assembled into the array. Figure 5 shows the growth curve and culture pH for an anaerobic *E. coli* incubation in complex medium supplemented with 1% glucose. A drop in culture pH matched an increase in cell density as expected for *E. coli* in a glucose-rich anaerobic environment.

Next Generation Physiomics Array

This first version had a limited feature set compared to the most recent version of the 8-microbioreactor arrays. The only parameter that could be set was the oxygen generation rate. In order to make use of existing control electronics, the 96-element array has 8 groups of 12 replicates, such that 8 oxygen generation rates can be set and all 12

microbioreactor arrays was difficult and time consuming because the electrolysis chamber, PCB, gas exchange membrane, and culture chamber all had to be carefully glued together (Fig. 6). In order to simplify the production and use of the 96-microbioreactor arrays, a clamping and gasket system has been designed to hold the components together without the use of glues. Initial attempts to clamp the microtiter plates along two parallel edges failed, as the plates would bend and the gasket in the middle of the array would not seal. It was determined that the clamps needed to completely cover the two microtiter plates to get complete sealing of all microbioreactors. Two metal plates were machined to align and hold a pair of 96-well microtiter plates in compression while still allowing pipette access to the culture chambers (Figs. 8 & 9). These two plates are connected with 4 bolts. By simply loosening the bolts, it is possible to swap out the culture chamber and gas exchange and then reuse the remainder of the system over many experiments. Several tests have shown that this system prevents both liquid and gas exchange between microbioreactors.

The 8-microbioreactor arrays used gold wire for the electrodes. This wire would corrode over time and alter the gas generation rate. In the 96-microbioreactor arrays platinum is

replicates within a group have the same oxygen generation rate. This setup can either be used to have 12 replicates of the same experimental conditions, or another parameter, such as media composition, can be varied across these 12 microbioreactor with equal oxygen input. The initial version of the 96-microbioreactor arrays has no sensors.

Assembly of the 8-

Figure 7. PCB for the first version of the 96-microbioreactor arrays. The only signals on this board are power and ground for electrolysis.

used as the electrode to prevent corrosion related problems. Initially bronze pins with a thin coating of sputtered platinum were used. Experiments showed these pins have a limited lifetime before pinholes develop in the platinum causing the underlying bronze to rapidly corrode. Use of solid platinum wire for the electrodes is now being explored.

The gas transfer membrane used in the 8-microbioreactor array were 20 μm thick silicone sheets fabricated in

the Berkeley Micro-fabrication Laboratory. These sheets are elastic and under high rates of gas generation, they would expand into the culture chamber. The bowing of the membrane is problematic, as it limits the maximum gas generation rate because there is limited room to displace the culture. To combat the expansion problem and reduce dependence on custom fabricated parts, a thicker (100 μm) commercially available silicone membrane was used. Adequate oxygen transfer was not achieved with the thicker membrane. Reinforcing the custom fabricated silicone membranes with a mesh of metal or fibers is being pursued. Additionally, non-silicone commercial membranes are being investigated.

Integration of an optical density (OD) measurement system into the 96-microbioreactor arrays is significantly more difficult than for the 8-microbioreactor arrays. This difference is primarily due to the additional space constraints present in the 96-microbioreactor design, which prevent placing of LEDs and photo detectors on the sides of the culture chambers with enough room for effective pinhole layers. To deal with these new constraints, the orientation of the OD measurement system will

Figure 8. End view of the assembled first version of the 96-microbioreactor array.

Figure 9. View of the top of the assembled first version of the 96-microbioreactor array. Note the pipette holes in the top metal plate.

the gaskets and will act as the pinhole. The photo detectors and associated amplification circuitry will be on a second PCB that will be placed on top of the culture chambers. A second version of the PCB that holds the electrodes has been fabricated to include the addition of the LEDs for OD measurement (Fig. 11). The design of the photo detector PCB is in progress. Testing of this OD measurement system will occur in the Fall.

With minor modification, it may be possible to use the OD measurement system to perform green fluorescent protein (GFP) based measurements (Fig. 12). The LED for performing OD measurements can be replaced with a blue LED. Most LEDs emit over a narrow range of wavelengths. The output of some blue LEDs nicely matches the excitation range of GFP such that no additional filtering of the excitation light source is needed. The photo detectors currently in use detect at the GFP emission wavelengths, however the excitation light must be filtered out. Filtering will be performed by a thin plastic light filter (intended for coloring theater lights) placed between the culture chambers and the photo detectors. Because this filter will be a high-pass filter, with a cut off wavelength below 600nm, it may be possible to develop a system

be rotated such that the light source is below the culture chamber and the photo detector is above the culture chamber (Fig. 10). The light source will remain a surface mount LED, but it will now be mounted on the same PCB as the electrodes. A metal layer with a small drill hole will be added between the gas transfer membrane and one of

Figure 10. Schematic cross section of one microbioreactor from the second version of the 96-microbioreactor arrays. This version adds the OD measurement system.

Figure 11. PCB for the second version of the 96-microbioreactor arrays. This board has power and ground for both electrolysis and 600nm LEDs.

with two LEDs that can measure OD and GFP in the same experiment. Testing of the GFP measurement system is scheduled for the late Fall.

Integration with other DOE programs

We have set up collaborations with Derek Lovely to cultivate *Geobacter*, Hoi-ying Holman to use IR in our device, and Margaret Romine (PNNL) to cultivate *Shewanella*. We have received several strains of *Shewanella* containing GFP and will begin to cultivate

them in the device.

Publications and Presentations:

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Figure 12. Schematic cross section of one microbioreactor from the proposed third version of the 96-microbioreactor arrays. This version adds the dual OD/GFP measurement system.

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Patents

M. Maharbiz, W. Holtz, R. T. Howe, & J. D. Keasling. 2003. Method and apparatus for gas generation and novel biological reactors. Provisional patent number 60/474,979. Filed: May 30, 2003.

Other

A small start-up company, Microreactor Systems, has formed around this technology. In collaboration with Gener8, a prototyping company, Microreactor Systems hopes to have a prototype of the system running and being tested in our laboratory within 9 months.

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