Modification of the Environmental Sample Processor (ESP) to Enable Remote Detection of Marine Microbes and Genes They Express

ERG #731

Annual Report for the Gordon and Betty Moore Foundation

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Introduction

The overall goal of this project is to enhance the Environmental Sample Processor (ESP) to make it a better tool for detecting marine microbes and the genes they harbor and express. The "analytical module" concept is a centerpiece of this project. Analytical modules are envisioned to be stand-alone devices that can be added to or removed from the existing "core ESP". The core ESP currently supports a generic suite of sample collection and processing operations (e.g., cell concentration and lysis; Greenfield et al. 2006). With analytical modules new and varied functions could be imparted downstream of that core, creating new possibilities for interrogating activities and genomic content of microbial communities in a remote sensory context.

Key activities proposed for Year-1 of this project (December 2005-November 2006) were to:

1. Define a suite of molecular analytical analyses that are desired for use with the ESP;
2. Scope the engineering developments required for migrating those assays from laboratory use to the ESP;
3. Design a test fixture that provides connectivity (electrical/fluidic) between the existing core ESP and candidate analytical functions identified.

Following in Year-2 (December 2006-November 2007), we then proposed to:

1. Select a limited set of new analytical functions/modules for incorporation into the ESP;
2. Create a detailed design for integrating those assays into an ESP;
3. Compare results of assays that employ traditional laboratory equipment to their equivalent as embodied within the "modified ESP";
4. By the end of Year-2, demonstrate a complete analytical cycle – from live sample collection, to processing, to reporting of results – using a modified ESP in the laboratory.

Project Activities and Strategic Choices, March 2006-07

Science requirements

Detection and quantification of specific sequences of DNA and RNA have been deemed the most important functions to focus on in the near-term. In particular, we are using some of the work Jon Zehr and Ed DeLong are spearheading as examples of the investigative themes we aim to augment. Meeting the generic requirements of DNA and RNA analysis requires development of techniques and technologies that support nucleic acid purification and amplification, and that improve options for applying probe arrays. Moreover, the amplification technology must support real-time PCR. As described in greater detail below, a significant portion of the work to develop these capabilities is being leveraged from separate grants.

Direct detection of target molecules, particularly mRNA, is also desirable (i.e., detection of target sequences without invoking an intermediate amplification step). Relative to what researchers are used to doing now, there is some doubt as to whether that approach will provide a sufficiently sensitive technique for assessing the expression of some (most?) genes of interest, especially for organisms that may be a minor component of an assemblage. Nonetheless direct detection of RNA is currently what the ESP was designed to do (e.g., Greenfield et al. 2006), so is worth exploring further.

We have therefore decided to pursue a two-pronged approach to meeting the objectives of this project. The first involves developing the means for the ESP to carry out "traditional" analyses that require PCR. The second aims to refine and further evaluate direct capture of target sequences with an emphasis on RNAs known to be abundant in marine waters. At MBARI, targets for that work include rRNA/rDNA of major groups of marine bacteria and archaea, and a gene involved in production of bacteriochlorophyll *a* (pufM) that is found in aerobic anoxygenic phototrophs (e.g., Kolber et al. 2000, Koblizek et al. 2005). Researchers within Jon Zehr's group will pursue direct detection of mRNA associated with production of RuBisCO (a ubiquitous enzyme involved in fixation of CO2) from marine cyanobacteria (e.g., Paul et al. 2000). Zehr's group will also explore options for using PCR in the context of the ESP as that capability emerges.

As was reported last year, the time and resources required for carrying out analyses aboard the ESP brings the notion of "adaptive sampling" to the fore. The instrument should be capable of sampling in response to external sensors providing high frequency physical, chemical and biological measurements. In this fashion one could maximize opportunities for collecting samples when conditions are most appropriate for addressing specific hypotheses (e.g., 'when the concentration of oxygen, nitrate and ammonia are *x, y, z*, then take a sample ….'), as opposed to simply collecting samples at a fixed time intervals. In addition to adaptive sampling, it is also desirable that the ESP be capable of an "adaptive response," where one or more analytical techniques are applied in a variable cascade to interrogate a given sample at different levels (e.g., 'if gene *x* is present, then perform analysis suite *y*; if gene *x* is absent, then archive the sample and ….'). Developing adaptive sampling and adaptive response capabilities are outside the scope of this project. We will pursue adaptive sampling under the auspices of the NASA grant.

Engineering developments

Our assumption at the start of this project was that the analytical techniques identified would correspond closely to discrete analytical modules – standalone devices that take a refined sample from the ESP and perform all subsequent reactions autonomously. That in mind, we foresaw a need to create some kind of a fixture for testing analytical modules in the lab and later merging individual modules with the ESP. With clearer definition of the techniques and technologies that we will actually pursue, however, it obvious those concepts need refinement. The set of candidate analytical modules identified are not entirely self-contained. Instead, they place a requirement on the ESP to prepare reactions and pump reagents on a microfluidic scale.

PCR

We are currently meeting the requirement for nucleic acid purification and PCR aboard the ESP by working with researchers at Lawrence Livermore National Laboratory (LLNL), leveraging a grant from NASA (Figure 1). The LLNL module is 4-color, real-time flow-through PCR device. It is based on a system that is currently used for detection of airborne pathogens (http://www.eurekalert.org/features/doe/2002-01/dlnl-rfd062402.php). The development of the PCR module, a multi-year and $600K+ effort, comes at no cost to this project. The prototype PCR module is nearing completion. At least five copies of the device will be built at LLNL and delivered to MBARI later this year. One or more of those copies will be dedicated to this project.

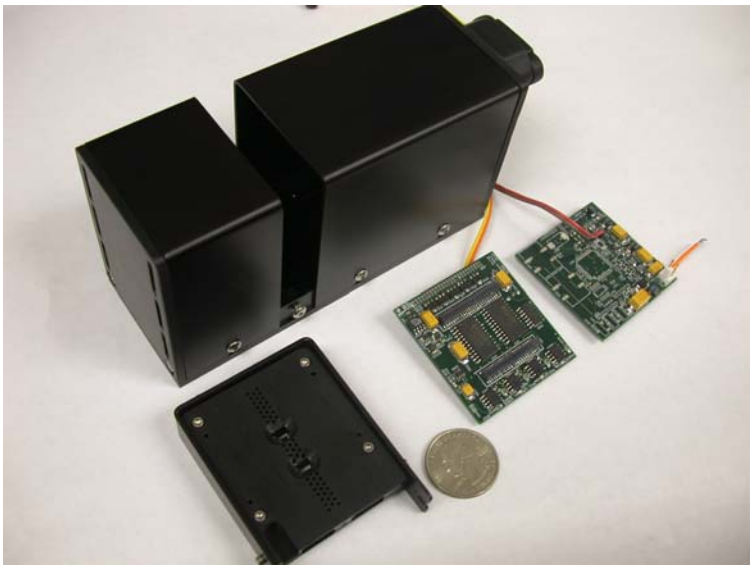

Figure 1. Some of the major components of the prototype ESP flow-though PCR module developed at LLNL (as shown Feb 07). Bottom left to right: heater module, U.S. quarter for scale, analog board, digital board. Top: enclosure. .

Probe arrays

We are pursuing two options for enhancing probe array chemistries in the ESP. The first involves refinement of our current arrays that are printed on nitrocellulose membranes (e.g., Figure 2). At present the arrays are printed using a custom-built contact pin printer. For a variety of reasons this printer will not meet the needs of this project in the near-term, primarily since the printing pins are no longer available in the U.S. and there are difficulties with depositing a constant amount of probe ink to replicate spots on the array. We have identified and tested a commercially available, non-contact (piezo) printer from

Perkin Elmer. This machine will meet our needs, and a demo model is available locally at a substantial discount (~$60K with spares and service kits versus >$100K new). An ability to produce arrays that can be used in the ESP is essential to this project. Using commercially available equipment to meet that requirement reduces the need for engineering developments – a reasonable trade. Therefore, we have requested permission to purchase this printer with this grant by shifting funds from salary to equipment. Final approval of this request is now pending.

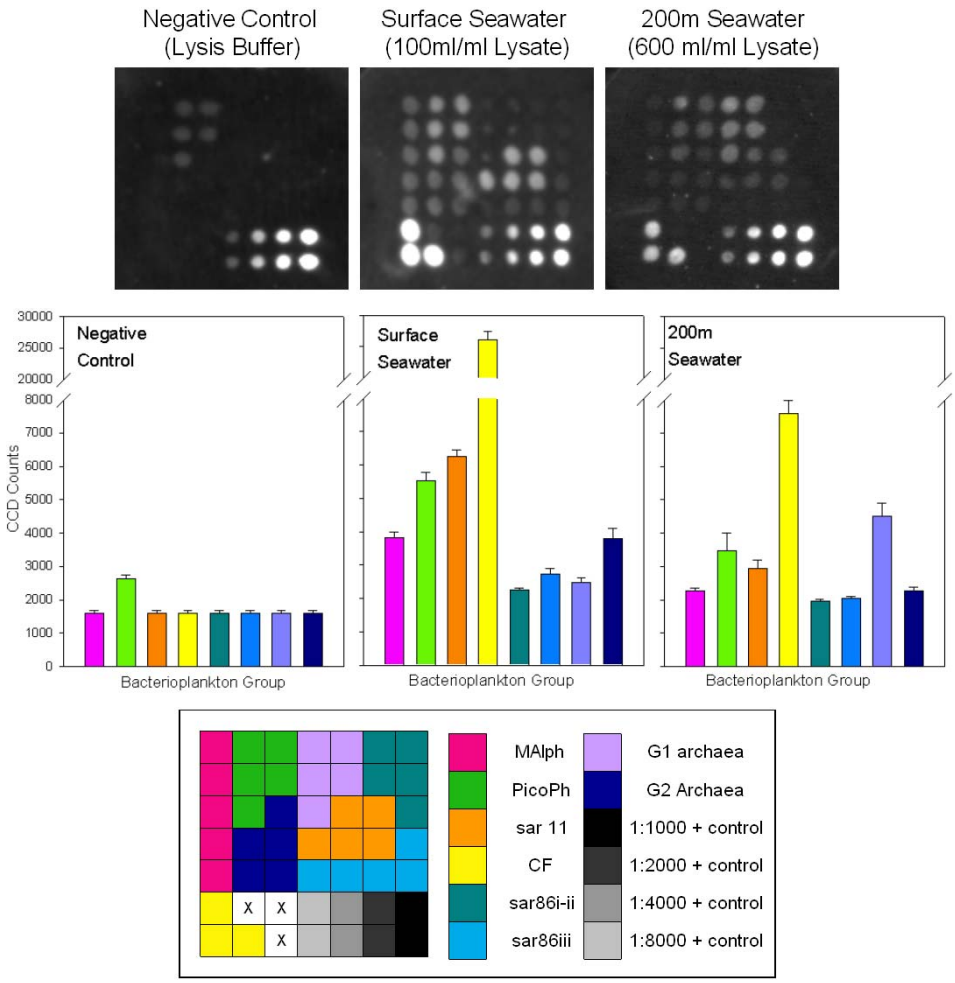

Figure 2. DNA probe arrays printed with probes for marine microbial groups developed using the ESP supplied with different samples. The bottom panel shows the pattern of probes and an abbreviation of the group targeted. The top panel shows the actual arrays exposed to (left to right) lysis buffer only, sample collected near the surface, and a sample collected at 200m. The arrays demonstrate change in the microbial community as a function of depth, quantified as mean pixel intensity in the middle panel. The actual size of the arrays show are ~15mm square. Images and data provided by Chris Preston.

As reported last year, we identified a reusable microbead array that also appears consistent with meeting the requirement for probe array-based chemistries (e.g., Ahn et al. 2006). The primary advantages of the microbead array over the printed array are that

it is reusable, compact, and requires smaller volumes of reagents. With support from this project we initiated a pilot study with David Walt (Tufts University http://ase.tufts.edu/chemistry/walt/index.htm) to evaluate the prospects of using this technology in concert with the ESP. Work has progressed slowly. There have been a number of delays in acquiring test arrays (production of the arrays and beads is being done by a graduate student in Walt's lab). Despite the slow start, we have begun tests of these arrays using a simple flow cell (e.g., Figure 3). The pace of this work is now accelerating.

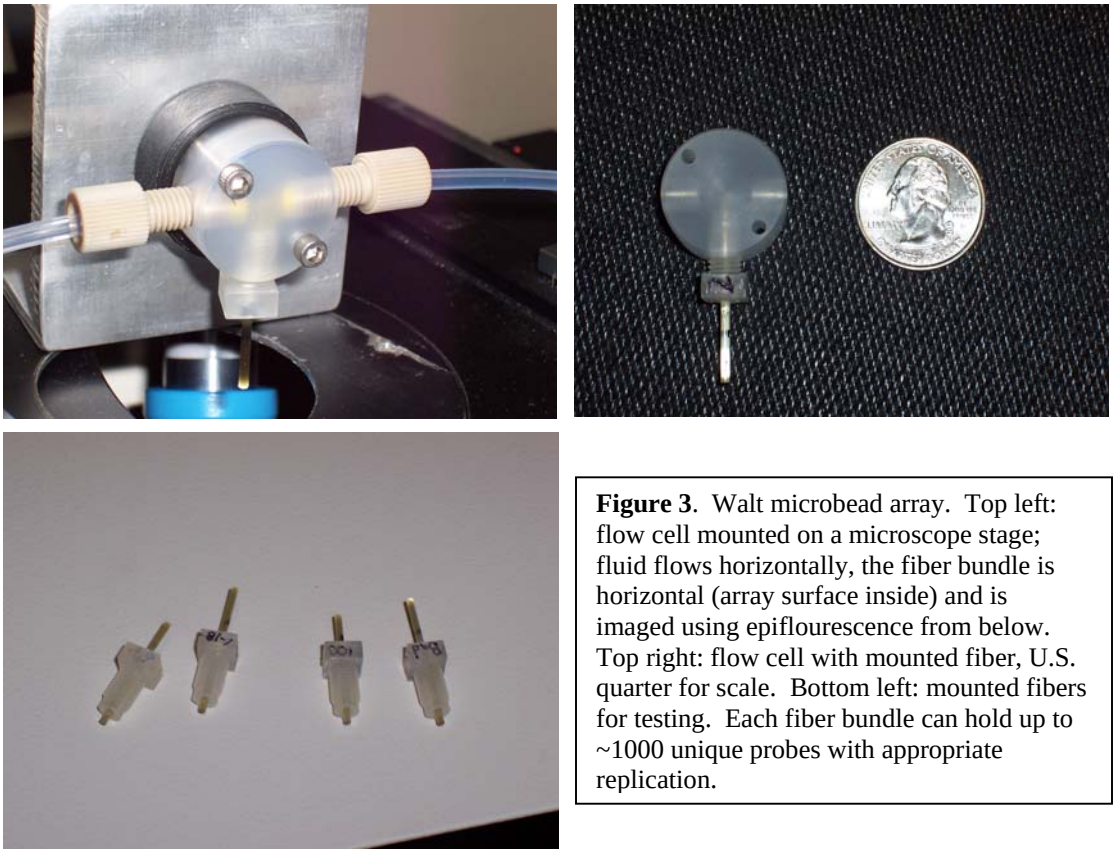

Figure 3. Walt microbead array. Top left: flow cell mounted on a microscope stage; fluid flows horizontally, the fiber bundle is horizontal (array surface inside) and is imaged using epiflourescence from below. Top right: flow cell with mounted fiber, U.S. quarter for scale. Bottom left: mounted fibers for testing. Each fiber bundle can hold up to ~1000 unique probes with appropriate replication.

Although the microbead array is very appealing in many respects, it is reportedly not well suited to distinguishing between small changes in target molecule concentration. The microbead array seems best suited to discriminating order of magnitude differences in the target strength. In contrast, our printed arrays easily discriminate two-fold differences in target concentration. So at this time it is not clear which approach offers the best solution for meeting the objectives of this project and the potential end users. For that reason, we continue to explore both options (another impetus to acquire the new array printer).

The microfluidic block

Both the PCR module and microbead array place a requirement on the ESP to prepare reactions and pump reagents on a microfluidic scale. In discussions with other research groups that could be classified as developing "analytical modules," this same theme continues to emerge. While the analytical module concept is still valid, we realized that

it is somewhat ahead of its time. What is needed, therefore, is a more generic interface with ESP – an "analytical bus," so to speak – a take-off point for distributing samples and reagents to one or more detection modules. This unit could also be used to gang one or more analytical functions in a cascade (e.g., employ probe arrays down stream of PCR).

To meet that need we have designed a separate fluid handling station that can be added to the core ESP. This work was carried out with support from a NASA grant and in collaboration with workers at LLNL. This unit is referred to as the "microfludic block" (Figure 4). The microfludic block was designed primarily to support the PCR module. It includes provisions fo a reusable solid phase extraction column for purifying nucleic acids, and reagents for a variety of PCR master mixes, primer/probe combinations and control templates. This same sample handling and distribution block will support the microbead array, as well other devices that may emerge in the future.

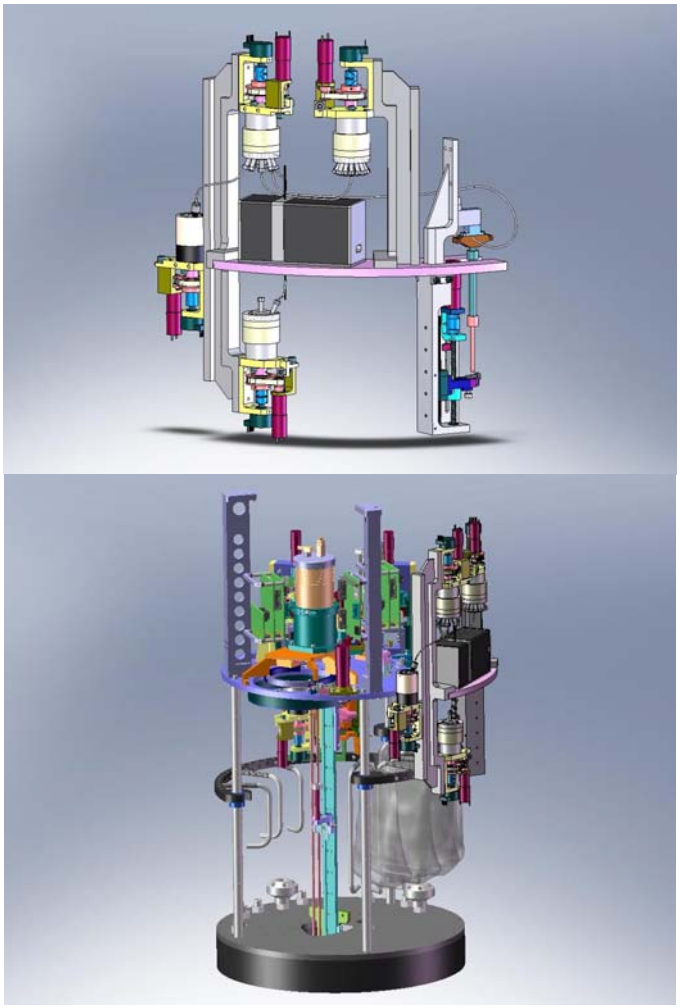

Figure 4. Solid model showing the microfluidic block. Top panel: standalone unit with the LLNL PCR module inserted at correct scale Bottom panel: the same block and PCR module attached to a core ESP. Note, tubing runs between valves are not accurate, and some parts of the ESP have been removed to improve clarity. For scale, the ESP is approximately 16" in diameter, 30" tall.

With support from this project, we are now acquiring parts for and will soon initiate the construction of the microfludic block. The design is such that it will function as a standalone, benchtop unit and will use the same electronics, valves, motors, and control software (etc) as the core ESP. The idea is that this unit can be added to or removed from

the ESP as a single module, with its attendant detection devices, without disassembly. Once a particular block is ready for use aboard the ESP, it can be moved as a single unit. This will also allow us to produce a number of copies of the block that can be distributed to multiple laboratories for testing, configuration and protocol development/validation. Investigators can then explore detection chemistries as would be deployed in the ESP, without necessarily having the core ESP to provide upstream sample collection and initial processing (sample prep can be carried out manually). Once assays are "certified" for use with the ESP and/or are ready for deployment in situ, then that particular block can either be fitted to a core ESP, or the essential configuration translated to copy of the hardware and then fitted to a dedicated ESP. The advantage of this approach is that it makes it less cumbersome and much cheaper to distribute the essential analytical functions to interested users. Another reason for developing a generic interface is to spur collaborations with groups that are developing novel molecular detection chemistries and technologies that may be appropriate for use with the ESP. By making this interface available, such groups could proceed with development efforts at their home institutions more easily and efficiently than if they had to come to MBARI (or elsewhere) to sort out integration details at the prototypical, "back end" level.

Construction of a core ESP

The original project plan called for production of an ESP in Year-3that would be modified and made available for testing. To meet that schedule we must begin acquiring parts no later than the end of Year-2 (this fall). Therefore we have asked that the budget be modified to reflect that fact. Actual construction of the unit will take place early in Year-3, ahead of proposed field operations scheduled for the end of Year-3.

Budget comments

To date we have been fortunate to so extensively leverage off other grants, and have made significant progress towards meeting our objectives at a substantial savings to this project. For example, expenditures associated with developing the PCR module and the designing the microfluidic block over the past ~1.5 years exceed the total budget of this project. In coming months expenditures charged against this grant will accelerate dramatically. We are hiring a dedicated engineer to focus on building and refining the microfluidic block, and to help facilitate integration of the PCR module. We will also begin acquiring parts for a dedicated ESP. We will then call on additional engineering resources to fabricate and calibrate that machine, and modify it as per our project plan. As noted earlier, purchase of a new probe array printer will also expedite work substantially and also be a significant expenditure.

Achievement of Grant Purposes

Output 1

- Functional requirements refined.
- Prototypical, candidate 'analytical modules' identified:
  - 4-color, flow-through real-time PCR
  - Walt microbead array
- Acquisition of a commercially available array printer consistent with ESP applications will advance this project.

Output 2

- Concept for modifying the ESP formulated
  - Microfludic block design completed
  - Parts on order
  - Hiring dedicated engineer to focus on refining the design and fabricating the hardware.
- Initiating purchase of components necessary for building an ESP that will be dedicated to this project (for construction in Year-3 as proposed).
- Significantly leveraging off of other projects to further stated objectives.

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