

Chapter 2

Development of a Capillary Waveguide Biosensor Analytical Module for Use with the MBARI Environmental Sample Processor

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Abstract

The Monterey Bay Aquarium Research Institute's Environmental Sample Processor (ESP) is well established as an innovative sampling and instrument platform for sensors designed for in situ monitoring of microorganisms in the ocean. The platform can be deployed for periods up to 3 months and real-time data can be remotely downloaded at any time. A daughter platform, the microfluidic block (MFB), is used as an interface between the ESP and analytical modules to permit rapid deployment of new sensor technologies. We have developed a capillary waveguide biosensor (CWB) which employs nucleic acid hybridization for detection and quantification of specific microorganisms, and have integrated it with the MFB for use with the ESP. An important aspect of the CWB is the use of a combined capillary waveguide/hybridization surface that can be regenerated for repeated use. This chapter describes design issues related to the integration of the CWB and MFB and the development of coating protocols to maximize the operational life of the capillary.

Key words: Capillary waveguide, Fluorescence, Hybridization, Nucleic acid, RNA, MBARI environmental sample processor, Capillary waveguide biosensor, Microfluidic block, Capture probe, Ocean sensing

1. Introduction

Many biological processes occur on temporal and spatial scales that require near-continuous monitoring to fully understand their dynamics. Although a few biological variables (e.g., chlorophyll concentration) can be monitored via satellite or moored instruments, for many others no methods are available to collect data at appropriate temporal and spatial scales. The dynamics of microbial processes are particularly difficult to study except by intensive, ship-based studies. However, shipboard studies are expensive and

therefore occur too infrequently to capture more than snapshots of the true variability of microbial dynamic processes. In recent years, increasing attention has been focused on the need to develop automated, in situ methods to address the need for long-term, cost-effective measurements of microbial properties and processes, e.g., microbial abundance, biomass, productivity, and metabolism. Some of these properties are amenable to direct measurements (e.g., cell counts, concentrations of the biochemical products of metabolic pathways), while others can be examined through proxy variables such as the presence, abundance, and expression of specific genes. Automated, in situ sampling has enormous potential benefit for water quality management, human health, and the seafood industry, both economically and with respect to food safety. Applications include detection of human, fish, and shellfish pathogens in seawater, including water at aquaculture facilities, and detection and monitoring of harmful and nuisance species in a diverse range of environments. In conjunction with physical and chemical monitoring, perhaps based on the same monitoring platforms, real-time information on microbial populations will expand our understanding of (and ability to predict) microbially mediated processes in the ocean and their responses to stresses such as climate change or hypoxia. To provide this type of information, researchers at the Monterey Bay Aquarium Research Institute (MBARI) have developed the Environmental Sample Processor (ESP), which is now being commercially sold by McLane Laboratories (<http://www.mclanelabs.com>). The ESP's capabilities include large volume water sample collection, biomass concentration, and extraction of nucleic acids. Samples may be stored for later retrieval, or delivered to downstream analytical modules developed by other researchers as well as by MBARI.

Several analytical modules are already in development, including a filter-based sandwich hybridization assay to detect toxic microalgae, and an ELISA assay for the algal toxin domoic acid (1, 2). MBARI scientists have also developed a PCR module to amplify specific gene targets from a DNA sample delivered by the ESP core (1). For in-depth description of the ESP the reader is directed to the MBARI URL at <http://www.mbari.com/>.

Detection methods based on nucleic acids are often employed because they can be used to infer the presence and activity of specific organisms and metabolic pathways. Most such methods are based on nucleic acid hybridization, and those techniques involving direct detection of target nucleic acids (rather than amplification) generally rely upon the use of surface-bound probes to which the targets hybridize. The hybridized products may be detected by a variety of techniques, including fluorescent labeling, chemiluminescence, and plasmon resonance. Fluorescent labeling is perhaps the best established and is the mainstay of microarray-based systems. We have developed a capillary waveguide biosensor (CWB)

which draws upon microarray technology in its use of surface-bound oligonucleotide capture probes and fluorescence-based detection. Because direct chemical labeling of the target with fluorophore is not amenable to use in the field, we employ a secondary hybridization with a fluorescently labeled detection probe. To eliminate some of the instrument-dependent fluctuations inherent in a fluorescence measurement, we (3) proposed an instantaneous normalization of the fluorescent signal by the unfiltered excitation signal, overcoming difficulties attendant on extracting concentration data from photon counts.

Recognizing that in the early stages of instrument development a complete ESP is not necessary, MBARI engineers designed an intermediate platform, the Micro Fluidic Block (MFB), to encourage development of novel analytical modules. The MFB provides functions for full control of rotary valves and syringes needed for moving reagents through the reaction chamber, as well as the computer platform needed for developing the software codes for control of the new instrument. This chapter focuses on the procedures necessary for integrating the CWB with the MFB for use on the ESP platform. We also describe a crucial part of the CWB development process, the attachment of capture probes to the interior surface of a capillary. In addition, we have optimized probes and hybridization conditions, and demonstrated the ability of the capillary to be regenerated for sequential measurements.

1.1. The Micro Fluidic Block

The MFB (shown as delivered in Fig. 1) is modified to be operated independently but is otherwise identical to the ESP's fluid handling system subsequent to sample collection. It is powered by a 12 V 15 A sealed lead acid battery (Powersonic PS-12120), which is continuously charged using a Xenotronics HPX-30 charger. The MFB is designed around the ARM host board, which provides a serial port and an Ethernet connection to the outside environment. The host board supports the Linux operating system, and the programming language is Ruby v1.6.8. Control software for the MFB and user-developed codes for the analytical module reside on the host board. A 2 GB compact flash card provides additional data storage; however, data can be downloaded from a remote computer at any time via a Web-based interface. The host board also supports two USB 1.1 peripherals. A gateway from the host board to the Dwarf stack is established through the use of a core board, which connects to the former via the RS232 cable and to the latter through the I2C bus connector. The Dwarf stack comprises a core board, rotary valve board and a sensor board. The host board uses the I2C network to issue instructions to the core board, which directly controls the solenoid valves (eight on the current MFB), as well as two heaters. The core board also passes RS232 from the host board to the analytical module being developed. A connection between the host board and the analytical module can also be

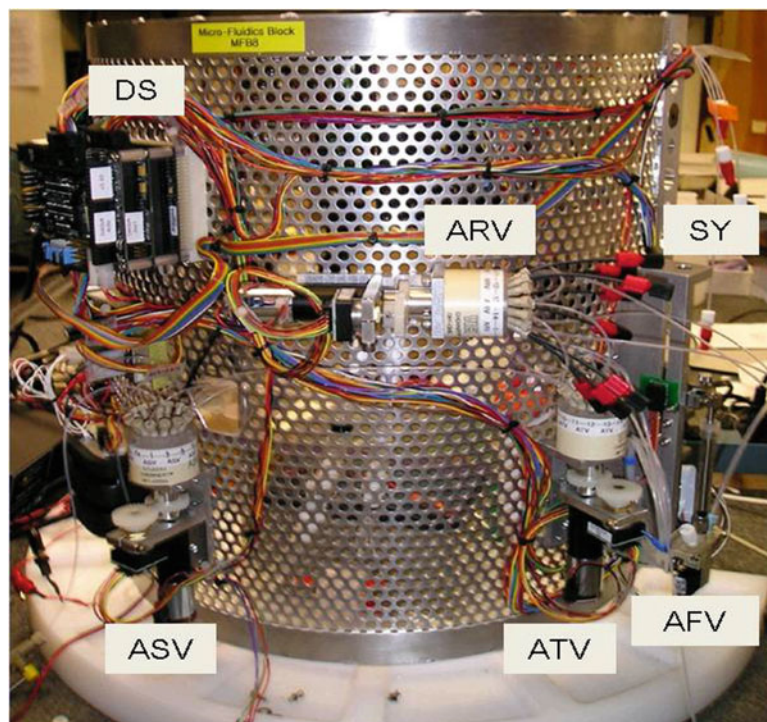


Fig. 1. Unmodified microfluidic block (MFB). *DS* Dwarf stack, *ARV* analytic reagent valve, *SY* syringe, *SPE* solid phase extraction, *ASV* analytic sample valve, *ATV* analytic top valve, *AFV* analytic fluid valve.

established through the USB port. The rotary valve can control up to four DC brushed motors and their encoders. The sensor board can also process data from two temperature sensors and one pressure sensor.

Figure 2 shows the complete fluidics pathway available on the MFB. Reagents can be pumped into the reaction chamber through various ports of the analytic reagent valve (ARV). These valves are controlled by Ruby functions supplied with the MFB and resident on the ARM host board. Use of these functions from user-developed software code will be described later. The input port of the capillary reaction chamber is connected to port 14 of the analytical top valve (ATV) and the output to the center port of the analytical sample valve (ASV). The analytic fluidic valve (AFV) is used to switch between the carrier fluid and reagents. Use of the carrier fluid, rather than air, for shuttling reagents and samples through the fluid system is recommended, as it allows purging of air bubbles from the fluid lines. In order to prevent mixing of neighboring fluids, small air pockets can be introduced in the fluid pathway.

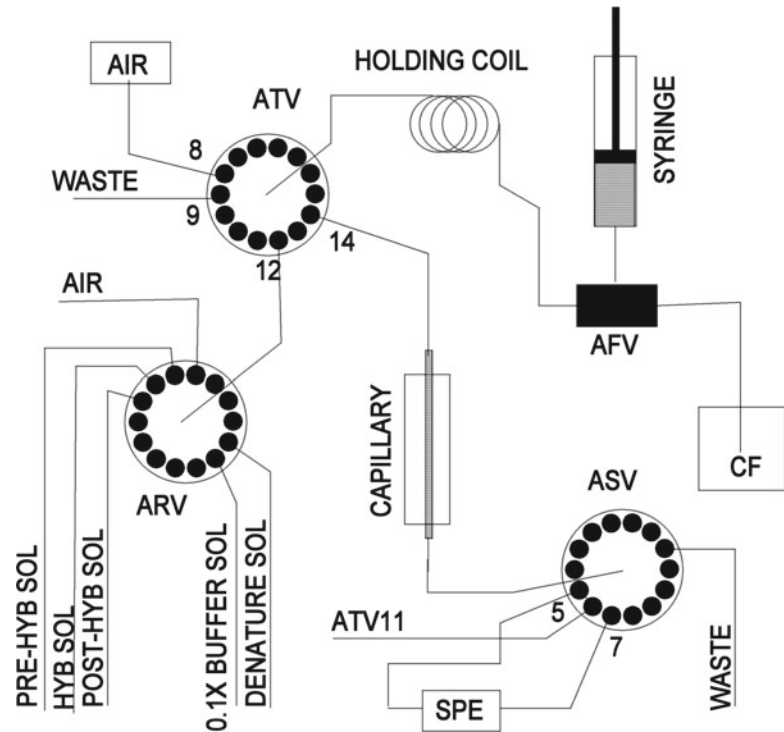


Fig. 2. Fluid pathways of the MFB. *ATV* analytic top valve, *ARV* analytic reagent valve, *AFV* analytic fluid valve, *ASV* analytic sample valve, *SPE* solid phase extraction, *CF* carrier fluid.

In the actual ESP system, a nucleic acid extract is supplied from the lysate coil, which is connected to the output of the solid phase extraction heater. The analytical module will draw the DNA slug from the lysate coil and move it into the capillary for detection.

Figure 3 illustrates the various interconnects between the MFB and CWB. During the development phase, the local host computer connects to the ARM host on the MFB via a hardwire Ethernet cable. This connectivity is used to upload user Ruby script files to the MFB for execution and for downloading the data files back to the local machine. However, the code can also be developed on the local machine by creating the **esp** environment on the hard drive. The RS232 port on the AM can be connected to the MFB through either the emulated serial port on the Dwarf stack or the USB1.1 connector on the host board using a RS232 to USB dongle.

1.1.1. MFB Installation

The first step upon delivery of the MFB is to establish communication between the local host machine and the MFB core board, as directed in the accompanying user manual. This is achieved by using a wired Ethernet connection from the local computer to the ARM processor host board. The host board will integrate itself into any network that supports both DHCP and DDNS. The pre-configuration file assigns a hostname to the MFB, for example,

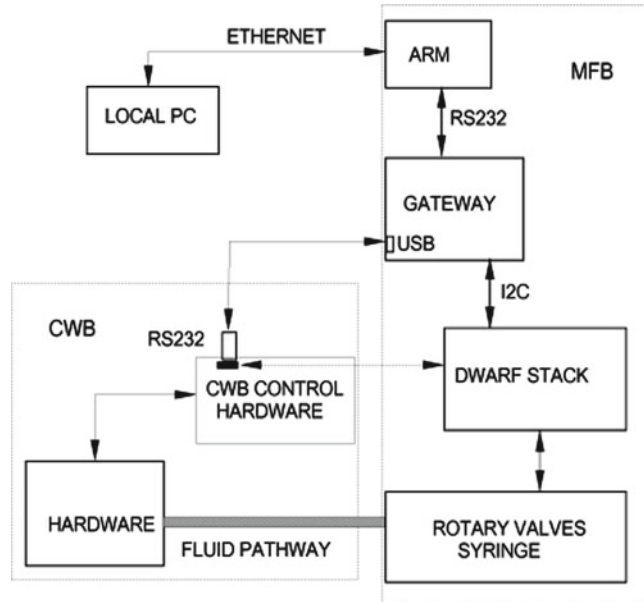


Fig. 3. Schematic of the CWB-AM, showing the interconnects between the MFB and CWB.

MFB8. At start-up a unique IPv4 address is assigned to this hostname. From the local machine running the Linux OS, the command `ssh mfb@MFB8` will prompt for a password (supplied by the vendor). For networks supporting DDNS, the MFB can only be identified by its numeric IP address of the form 192.168.1.204. This address is typically assigned by the network router and may change when the MFB is powered down. It should also be noted that some routers assign the IP on a lease arrangement. In other words, at the expiration of the lease period the IP will have to be reassigned. Occasional problems with connectivity may be traced back to this issue.

The MFB is accessed by running the **esp** application that controls the MFB from the host's (*MFB8*) Linux command prompt. Typically, a new terminal window is opened and the following sequence of commands sets up the **esp** environment from which the MFB's various valves and syringes can be tested.

<code>ssh ~dir@192.168.1.104</code>	[~ is a space character, dir: folder on MFB]
<code>cd ~esp2/bin</code>	[change directory]
<code>./~ESPenv~mfb~MFB8</code>	[opens the esp environment]
<code>su</code>	[login as super user]
<code>AFV.to~: holdingcoil</code>	[MFB command to move AFV to holding coil]

Operation of various valves, as described in the MFB user manual, can be verified in the **esp** environment.

1.2. The Capillary Waveguide Biosensor

Figure 4 shows a desktop version of the CWB, prior to integration with the MFB. The heart of the CWB is a silica capillary, which serves as the reaction chamber for target detection, and as an optical element for delivering the excitation energy to the surface-bound hybridized products and for collecting the fluorescence emission. For details on the CWB the reader is referred to our earlier papers (3, 4) and a recent chapter (5). The essential features include an opto-fluid connector, which serves as an input fluid port and also as a laser delivery system. Light from a laser source is launched directly into the annulus wall of the capillary, which has the optical properties of a multimode optical fiber. A small fraction of the guided optical modes penetrate into the core region of the liquid-filled capillary, creating an evanescent wave field, which excites the fluorochrome label attached to immobilized hybridization products. The penetration depth is typically smaller than the excitation wavelength. Some of the photons emitted from the excitation volume are trapped within the capillary wall, through the mechanism of tunneling, and can be collected at the capillary ends, while some are emitted into the lumen of the capillary and still others are radiated out of the curved outer surface. As fluorescence detection is noncoherent, theoretically the signal could be enhanced through a collection of all possible emissions; however, in practice the optical system for achieving this task is not easily realizable (see Note 1).

The CWB uses a 1 mm silica optical fiber to collect photons emitted from the curved surface. The distal end of the pickup fiber

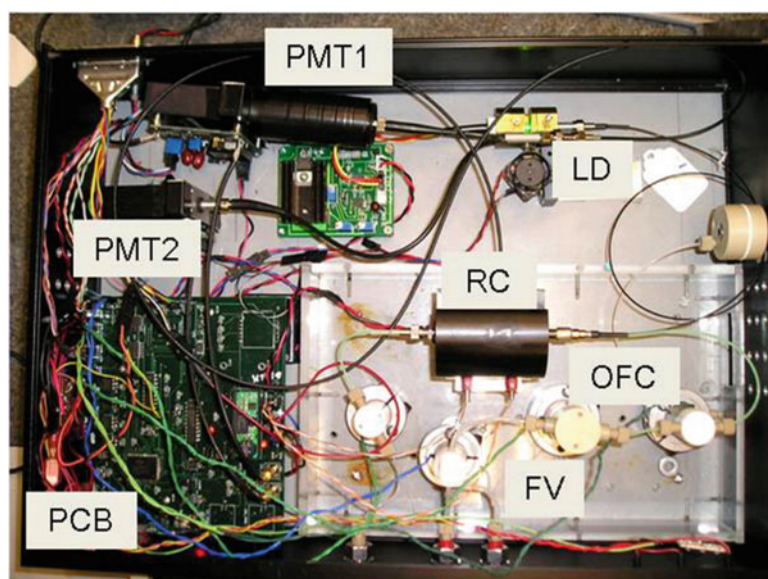


Fig. 4. Earlier (desktop) version of the capillary waveguide biosensor (CWB). *FV* fluid valves, *PMT1(2)* photomultipliers, *RC* reaction chamber, *OFC* opto/fluid connector, *PCB* printed circuit board, *LD* laser diode.

is mated with a 1×2 fiber optic multiplexer, having a power splitting ratio of 200:1. Photon fields emanating from the two fiber arms are filtered to separate the emission and excitation components before being detected by two single photon counting photomultiplier modules.

2. Development of the Capillary Waveguide Biosensor Analytic Module

As discussed in Sect. 1.1, the MFB platform allows the user to adapt an existing (or new) sensor in order to create an analytic module (AM) that can be merged with the ESP at the front (upstream) end. Design of the AM must accommodate constraints imposed by the ESP platform, of these, autonomous functioning is critical to its success. It is expected that the AM will be activated by a single function call from within the ESP application. Size and energy constraints will fundamentally limit the scope of the AM's functionality. For example, heaters, being the most energy consuming components, will require special attention, and consideration of the data transfer rate over the RS232 serial interface will limit overly ambitious imaging-based sensors.

As depicted in Fig. 5, the capillary waveguide biosensor analytic module (CWB-AM) has three distinct functional subsystems: (1) a fluid management system, (2) a data acquisition system, and (3) a temperature controller. The MFB platform takes care of the first. Design of the remaining two subsystems is central to an efficient merging of the two technology platforms.

2.1. Data Acquisition System

2.1.1. Microcontroller

The first design decision concerns the selection of the control platform for the modified CWB, and involves a trade-off between functionality, cost, and complexity of development. Solutions based on the use of a single-board computer or a microprocessor-based embedded system are unnecessary for the CWB-AM. We selected the Silicon Labs microcontroller (#634-C8051F120DK), a fully integrated mixed-signal system on a chip. It has eight 8-pin I/O ports, one 12-bit 9-channel analog-to-digital converter, three 12-bit digital-to-analog converters, 128 kB of programmable flash memory for in-system programming, 8,448 bytes of RAM, and two 24-bit counters with a minimum sample time of 15.625 ms. An external 1 MB RAM (IDT #IDT71V124) was added to the system to give the capability to store count data for a 100 s duration. An in-depth design discussion is not appropriate here. Briefly, the microcontroller forms the hub of the CWB-AM platform. Firmware for controlling and checking the status of the subcomponents resides on the master controller's flash RAM, and can be upgraded in the system at any time. For interested readers the microcontroller code can be obtained from the corresponding author.

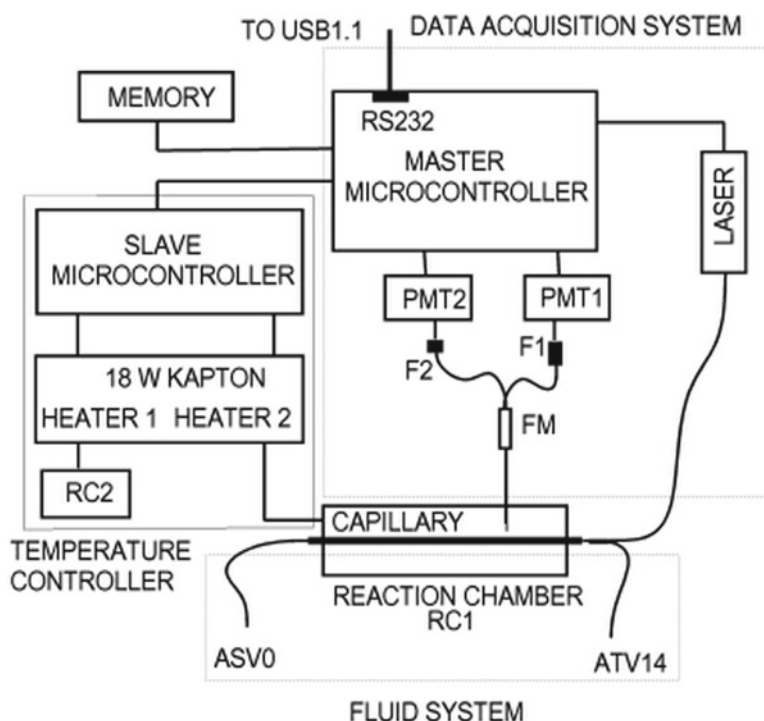


Fig. 5. Schematic of the CWB. *PMT1(2)* single photon counting modules, *F1* emission filter set, *F2* neutral density filter, *RC2*: second reaction chamber, *FM* 1 × 2 fiber optic multiplexer.

2.1.2. Detection System

The detection system comprises both electronic and optical components. A well-designed optical system will have an optimal filter set that matches the excitation and emission spectra of the fluorescent dye complex being used with the detection probe. However, angular sensitivity of these components is usually overlooked. A few degrees off normal incidence can significantly broaden the filter bandwidth, resulting in an increased background signal. For fiber-based detection schemes such as this one, care should be taken in designing the excitation and emission optics, particularly when the numerical aperture exceeds 0.2. The 1 mm collection fiber, with a NA of 0.5, requires collimation optics, which increases its physical size (see Note 2).

An earlier model of the CWB used a compact DPSS laser, with a footprint of less than 200 mm²; however, we later sacrificed size for better power stability and the capability for digital control. It was replaced by a 30 mW, temperature-stabilized, solid-state 532 nm laser (World Star Tech TECGL-05-TTL), with power stability better than 2 %. For single photon counting systems, there are two possible solutions, one based on a photomultiplier (PMT) and the other on an avalanche photodiode detector (APD). The latter is more costly but has a higher quantum efficiency at the

operating wavelength. However, it requires active cooling to keep the dark counts at ~150 cps, whereas the PMT has a dark count less than 50 cps at room temperature. APD-based systems have an active area of 0.3 mm² compared with PMTs with an area of 5 mm². The detector size is not an issue for small diameter fibers, but for the 1 mm pick-up fiber, PMT-based systems are the only choice. The CWB-AM uses two PMT-based single photon counting modules, Hamamatsu H8259-01 (see Note 3).

2.2. Temperature Controller

The temperature controller presented a major challenge, as it consumes the most power and its design is critical for extended battery life. The size of the temperature housing, the insulation material, the resistive heating element, and the control circuitry all play a critical role in the final design. The size of the temperature housing is determined by the length of the capillary, which is fixed at 65 mm for the current design. The diameter of the housing is a trade-off between thermal mass and the higher energy required to heat it. The mass is needed as the hybridization times could be of the order of 60 min. The CWB uses custom designed 12 V 18 W Kapton flat resistive heaters. A system requiring rapid thermal cycling (e.g., PCR) can use a much smaller thermal mass and thereby use smaller heating elements.

Most constant temperature controllers use a PID feedback loop to obtain the desired temperature profile. The PID coefficients are empirically tuned to the thermal load. The feedback loop can be implemented with either analog or digital signals. Digital feedback is more robust and flexible and allows downloading of new PID coefficients for remote applications, but requires additional electronic components. We decided on a digital PID algorithm, modified to be run on a microcontroller that did not have a floating point processor. The PID algorithm was further modified by turning off the feedback loop until the measured temperature was within 5 °C of the set point. This modification reduced the thermal time constant. A slave microcontroller was added to the system for independent temperature control. Typically, one temperature-controlled reaction chamber is needed for the CWB; however, a second temperature controller was added in case thermal denaturation of dsDNA in sample extracts was required. As this may require temperatures close to 90 °C, it cannot be performed in the capture probe coated capillary. Both temperature housings are controlled by the slave microcontroller using a 2 × 1 multiplexer, and each chamber has a steady-state stability of ±1 °C with a maximum attainable temperature of 100 °C. The two chambers can be operated at different set temperatures.

2.2.1. Energy Usage Considerations

In a tethered deployment with cabled power, the duration of deployment of the CWB will be determined primarily by probe and hybridization chemistry, i.e., by the longevity of capture probes and reagents. However, long-term untethered operation under

battery power requires a detailed exploration and understanding of energy usage in the coupled MFB/CWB system. The entire MFB and CWB system is powered by a 12 V, 15 Ah rechargeable lead acid battery. Because developers of new analytical modules have no control over the MFB's energy consumption, analytical modules must be designed to be as energy efficient as possible. The CWB requires the use of alternating hybridization and regeneration (target removal) steps; in the current system the former typically is performed at 52 °C for 15 min and the latter at 65 °C for 5 min. Elevated temperature increases the specificity of hybridization and the efficiency of capillary regeneration, but comes at the cost of increased energy consumption. The optimal hybridization temperature varies with the length and sequence of probes as well as the composition of the buffer fluid in which hybridization takes place.

A Hall current sensor with a 1 ms response time was designed into the hardware to allow instantaneous measurement of energy consumption under different usage scenarios. For example, a measurement cycle with a 30-min hybridization at 60 °C followed by regeneration at 80 °C gave a battery consumption of 0.5 Ah (data not shown). Under these extreme conditions, the 15 Ah rechargeable battery powering the MFB and CWB would support up to 30 target detection cycles. Clearly, it is desirable to decrease energy consumption as much as possible. Strategies for achieving the lower energy budget include reducing the mass of the reaction chamber, lowering the hybridization and regeneration temperatures, and finding more efficient methods of heating the fluid inside the capillary. The latter could be done by employing integrated heating elements close to the surface of the capillary. We are already using a very efficient insulator, Pyrogel 2250 (www.aerogel.com), which reduced the heating time from 120 s (1/4 in. rubber) to 15 s. However, the use of a low thermal conductivity insulator meant that rapid temperature reductions in turn became difficult. Since forced cooling was not a viable option due to its energy cost, we added cooling channels to pump relatively cool water (e.g., ambient temperature water in temperate or colder environments) through the housing.

As a final test, the entire CWB-AM system, running with maximum load, was placed inside a refrigerator to replicate the temperature environment of an immersed ESP in cold waters. Figure 6 shows the temperature profile of the two temperature-stabilized reaction chambers (these data was taken with 1/4 in. rubber insulation). All other electronic components, including the laser and the PMT, continued to operate as normal. Such tests are crucial if considerable downtime and cost are to be avoided during field trials.

2.3. Integration of the CWB and MFB

Figure 7 shows a photograph of the completed MFB-CWB platform, ready for testing with the ESP. In its present configuration, the CWB comprises a number of distributed components mounted on the aluminum curved plate. These components include two

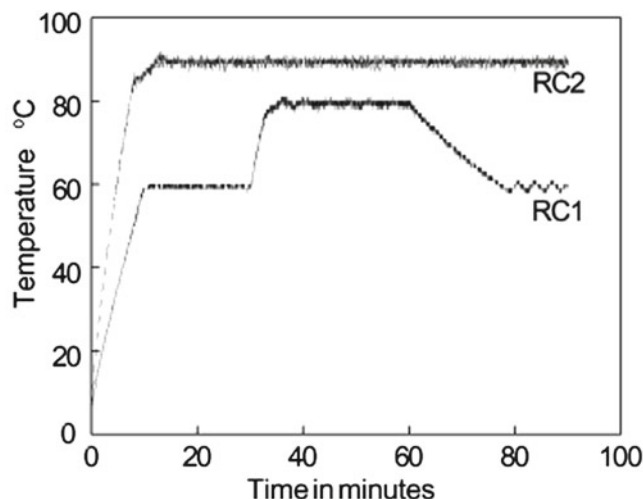


Fig. 6. Environment temperature test. The complete CWB was placed inside a refrigerator at 4 °C. Temperature profiles of the two reaction chambers RC1 and RC2 are shown.

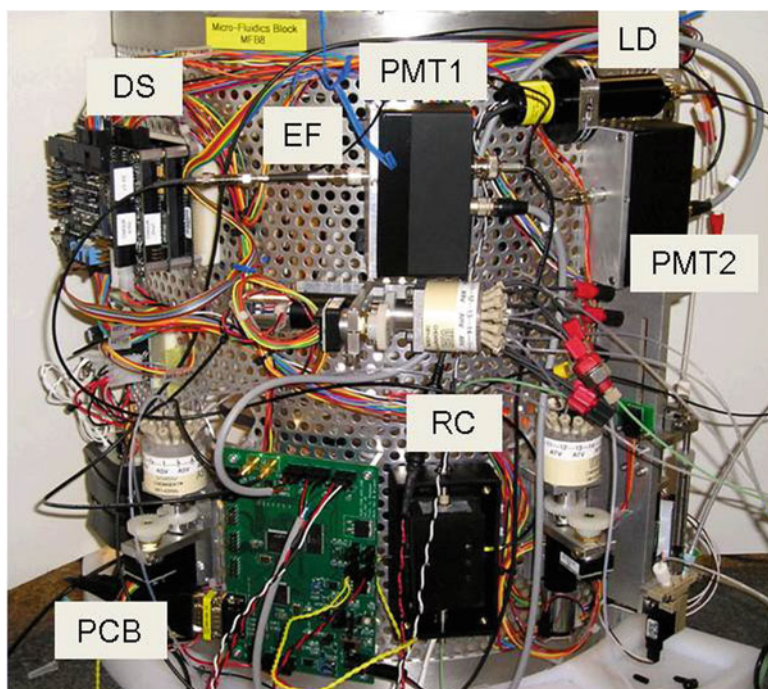


Fig. 7. MFB-CWB integrated platform. *PMT1(2)* single photon counting modules, *EF* emission filters, *RC* reaction chamber with capillary, *OFC* opto/fluid connector, *PCB* printed circuit board, *DS* Dwarf stack, *LD* laser diode assembly.

photomultipliers with optical filters, a DPSS laser source, the hardware control board, and the capillary reaction chamber.

Each target detection cycle in the CWB involves a complex sequence of tasks. As a part of the integration process, the MFB

rotary valves and syringes have to be controlled from the user-written Ruby script files. This feature required that the MFB function commands be wrapped using pipes. For example, the MFB command for moving the ARV to port 12 is “ARV.to 12”, this command can be called from the user script by opening a pipe and sending commands as follows:

```
espClient=IO.popen "espclient", "a+"    [once at the top of the
                                           program]
...
espClient.puts "ARV.to 12"
puts espClient.gets("\0").chomp "\0"
...
espClient.close                        [at the end of the script
                                           file]
```

At the end of the measurement cycle, a data file containing the raw fluorescent photon counts for each cycle is stored on the MFB ARM board memory. The files can be downloaded to the local machine at any time.

3. Capillary Preparation, Probe Selection, and Hybridization Protocol Development

The capillary's inner surface must allow a firm attachment of oligonucleotide probes and be capable of regeneration, i.e., the bound target must be removable without altering capture probes or their attachment to the surface. These considerations will determine the capillary's usable lifespan. In order to better understand the attachment chemistry of the capture probe to the silica surface, an extensive study was undertaken using a microarray reader.

3.1. Capillary Preparation

Hybridization/waveguide chambers for the CWB were constructed from TSP fused silica capillary tubing (1 mm ID, 1.3 mm OD; Polymicro, Inc.). The construction process involved cleaning, surface activation, silanization, final assembly, and capture probe application. Adequate cleaning and activation of the silica surface are especially critical to achieving good results.

3.1.1. Cleaning

The outer polyimide coating was scraped from capillary tubing and the tubing was cut into 65 mm lengths. Capillaries were cleaned by soaking them in anhydrous ethanol (Cat# E1028-500MLGL, Spectrum Chemical, Gardena CA) for 20 min to overnight. Alcohol was blown out of the capillaries with filtered, compressed oil-free nitrogen (see Note 4). After drying (approximately 30 s per capillary), capillaries were flushed with ultraclean (18 MΩ or equivalent) water for a few seconds and drying was repeated.

3.1.2. Activation

Two methods of preparing the capillary surface for silanization have been tested. Method A was to bake the capillaries by wrapping them in cleaned aluminum foil (previously cleaned by heating to 130 °C for 30 min in an oven) and heating them to 100 °C for 2 h or longer. Higher temperatures may be beneficial, but 100 °C is the minimum to remove a “bound water” layer from the surfaces to be treated. In Method B, an ozone generator was used and a vacuum pump was employed to suction ozone through the capillaries. This removes not only the water, but also any organic contamination on the surfaces.

3.1.3. Silanization

Immediately after activation, capillaries were filled with a solution of 1–2 % 3-glycidioxypropyltrimethoxysilane (Gelest, SIG 5840.0) in anhydrous ethanol. After 1–5 min, liquid was removed by blowing with compressed nitrogen. Silanized capillaries were placed into a clean container for 24 h at 35 °C. Care was required to keep atmospheric debris away from the surfaces, although some reaction with atmospheric water vapor occurs.

3.1.4. Assembly

A half-inch stainless steel ferrule was mounted, using a 5 min epoxy, on each end of the capillary. The ferrules permit polishing of the capillary end surfaces and are necessary for ease of handling. Capillary end surfaces were manually polished to an optical flatness of $\lambda/2$ and polishing byproducts were removed by blowing with a gas duster. Capillaries were stored at room temperature under low vacuum (vacuum-sealed freezer bags) to help prevent contamination of surfaces.

3.1.5. Capture Probe Application

To remove residual particles from the polishing process, a syringe and custom-built injection fitting were used to flush capillaries 3× with 1 ml of spotting solution ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3.4 mM; Na_2HPO_4 , 146.5 mM; SDS, 5 % w/w). Capillaries were then injected with spotting solution containing 20 μM amino-modified capture probe. Capture probes modified with a fluorophore were used in tests of coating evenness and quality. Filled capillaries were incubated at room temperature overnight in a horizontal position over saturated $\text{NH}_4\text{Cl}/\text{KNO}_3$ to maintain humidity (6). After 16–18 h, capillaries were flushed with approximately 10 ml of 0.1× SSC, (saline sodium citrate; prepared from sterile 20× SSC; (7)). They were then dried with a gas duster and stored in vacuum-sealed freezer bags until they were used.

3.2. Probes

Two types of oligonucleotide probe are needed to bind and detect rRNA or rDNA in the CWB. These are as follows:

1. Capture probe. Capture probes bind specifically to targeted DNA or RNA; for example, to rRNA or rDNA from an organism

being monitored. For best adhesion to the capillary's silane layer, they should contain a functional group (amino group in this instance) that reacts with a functional group of the silane being used. A spacer (e.g., C₁₂) between the amino group and the oligonucleotide is desirable to facilitate free access of target molecules to the surface-bound probe.

2. Detection probe. These bind to a different site on the target nucleic acid and incorporate a fluorophore. They need not be specific, since specificity is conferred by the capture probe. Detection probes may also be used in the absence of sample RNA or DNA ("background controls") to evaluate background levels of fluorescence due to nonspecific binding between capture and detection probes.

Two other categories of oligonucleotide were needed for process development:

3. Positive control. A fluorescently labeled oligonucleotide complementary to the capture probe was used to evaluate capture probe performance (independently of the detection probe) under various conditions.
4. Labeled capture probe. A capture probe with an additional fluorophore modification was used to visualize capture probe binding to a silane layer, allowing comparison of coating methods.

Although oligonucleotide sequences can be obtained from published sources or designed *in silico*, empirical testing is essential. Oligonucleotides and probes used in the study are shown in Table 1. Sequences were derived from ProbeBase (8) except where noted, and probes were custom synthesized by OligosEtc Inc. or Integrated DNA Technologies. We designed two new detection probes (FVdet1 and FVdet2) based on published *Vibrio* sp. sequences and information derived from screening candidate probes using Integrated DNA Technologies' online utility OligoAnalyzer 3.1 (<http://www.idtdna.com/analyzer/applications/oligoanalyzer/>). The new detection probes were designed for close binding site proximity to capture probe Amn-GV, and for low tendency to form a heterodimer with the capture probe. The former allows detection even if RNA is partially fragmented (either intentionally or as a result of extraction and processing), while the latter is an important consideration in preventing high background control values. Typical concentrations in hybridization solutions were 100 nM for positive controls, and 200 nM for detection probes used in background controls or for labeling of RNA. All probes were diluted in 0.1 µm filtered, RNase-free (1 h autoclaved), deionized and distilled water.

Table 1
Oligonucleotide capture probes, detection probes, and targets used in the study

Name	Sequence	Target region	Notes	References
Amn-Vibcap	5'-Amino-Cl2-ACC.ACC TGC ATG CGC TTT-3'	<i>Vibrio</i> sp., 16s rRNA bases 572–589	Modified “VIB572a”; <i>Vibrio</i> capture probe; ProbeBase # pB-01188	Huggett et al. (13)
Amn-Vibcap-Cy3	5'-Amino-Cl2-ACC ACC TGC ATG CGC TTT-Cy3-3'	<i>Vibrio</i> sp., 16s rRNA bases 572–589	Capture probe labeled with Cy3 for testing probe attachment; ProbeBase # pB-01188	Huggett et al. (13)
Amn-GV	5'-Amino-Cl2-AGG CCA CAA CCT CCA AGT AG-3'	<i>Vibrio</i> sp., 16s rRNA bases 841–860	Modified “GV” <i>Vibrio</i> capture probe; ProbeBase # pB-00611	Eilers et al. (14), Giuliano et al. (15)
Amn-Vibr1	5'-Amino-Cl2-CGC TGG CAA ACA AGG ATA AG-3'	Vibrionaceae, 16s rRNA 1113–1132	Modified “Vibr1” <i>Vibrio</i> capture probe; ProbeBase # pB02258	Kyselkova et al. (16)
A532-Vibtar	5'-Alexa Fluor 532-AAA GCG CAT GCA GGT GGT-3'	N/A	Complementary to Amn-Vibcap; used for positive controls	This study
Cy3-G Vtar	5'-Cy3-CTA CTT GGA GGT TGT GGC CT-3'	N/A	Complementary to Amn-GV; used for positive controls	This study
A532-Vibdet	5'-Alexa Fluor 532-AGG CCA CAA CCT CCA AGT AG-3'	<i>Vibrio</i> sp., 16s rRNA bases 841–860	Modified “GV”; <i>Vibrio</i> detection probe; ProbeBase # pB-00611	Eilers et al. (14), Giuliano et al. (15)
Cy3-EUB338	5'-Cy3-GCT GCC TCC CGT AGG AGT-3'	Most bacteria, 16s rRNA bases 338–355	Modified “EUB338”; <i>Vibrio</i> detection probe; ProbeBase # pB-00159	Amann et al. (17)
FVdet1-A546	5'-CTA ATC CTG TTT GCT CCC CAC-Alexa Fluor 546-3'	<i>Vibrio</i> sp., 16s rRNA bases 788–808, likely others	Detection probe designed for proxim- ity to Amn-GV	This study
FVdet2-A546	5'-CGT TTA CGG CGT GGA CTA C-Alexa Fluor 546-3'	<i>Vibrio</i> sp., 16s rRNA bases 817–836, likely others	Detection probe designed for proxim- ity to Amn-GV	This study

3.3. Growth of Bacteria and Preparation of RNA

3.3.1. Cultivation and Harvesting

Vibrio harveyi BAA-1116 (American Type Culture Collection) was maintained on Difco Marine Broth 2216 (Becton Dickinson, 279110) solidified with 1.2 % agar (Becton Dickinson, 214010). Stock cultures were maintained at 30 °C and transferred monthly. Cell slurries were obtained by inoculating spread plates on the same medium and incubating overnight at 30 °C. Cells were scraped from the plates with an alcohol-dipped, flamed 25 × 40 mm cover slip and washed into a few ml of liquid Difco Marine Broth. The suspension was washed by centrifugation, resuspended in sterile 2 % NaCl, and its cell concentration determined by the epifluorescence method of Hobbie et al. (9) as modified by Watson et al. (10). After being pelleted by centrifugation and the supernatants discarded, aliquots (usually on the order of 10⁹ cells) of packed cells were stored frozen at –80 °C.

3.3.2. RNA Extraction

An RNeasy Mini Kit (Qiagen, 74104) was used to extract RNA from cell pellets. The extraction procedure was modified to incorporate lysozyme digestion according to procedures described for bacteria by Qiagen (11). Quantity and quality of extracted RNA was evaluated by means of a Nanodrop ND-1000 (Thermo Scientific) and by agarose gel electrophoresis. RNA was aliquoted, immediately stored at –80 °C, and typically used at a concentration of 20–22 µg/mL hybridization solution (see Note 5). Total RNA extracted from bacterial cells is generally considered to contain 90–95 % ribosomal RNA.

3.3.3. RNA Fragmentation

Secondary structure of rRNA/rDNA affects its accessibility to probes (12); this problem might be exacerbated when the short probes are bound and the ca. 1,500 nt RNA is free in solution. To help eliminate secondary structure, RNA was chemically fragmented in some experiments according to the ZnCl₂ protocol described by Kretech, Inc. (http://www.kretech.com/Portals/kretech/downloads/labeling/40_RNA%20fragmentation_v2.0doc.pdf). This method is claimed to yield fragments averaging 60–200 nt.

3.4. Optimization of Hybridization and Denaturation Protocols

3.4.1. Use of Slides as a Model System

3-Glycidoxypropyltrimethoxysilane-coated microarray slides (Corning, CLS40041) were used to optimize probes and hybridization conditions, since they allowed a large number of hybridizations to be performed simultaneously and their use enabled the concurrent optimization of 3-glycidoxypropyltrimethoxysilane coating protocols for capillaries. The spotting and hybridization procedures described by the slide manufacturer (8) were modified by the use of the same nonproprietary spotting solution used for capillaries, and by the omission of distilled water and 1× SSC washes (which proved unnecessary in preliminary tests). Slides were spotted using a manual glass slide replicator (V&P Scientific, VP478A) and a custom-built single-position indexing unit.

Hybridizations were carried out under lifter slips (Thermo Scientific, 25X401-2-4772). Elevated incubation temperatures were maintained for slides by means of a humidified multislide chamber (Genetix Ltd.) and a hybridization oven (Hybaid H9270), and for solutions by means of a water bath (GeneMate HSP1000). Hybridization times of 15 min—overnight at optimal temperature showed no consistent differences in fluorescence intensity (data not shown). For convenience, slides were hybridized for 1 h and capillaries for 15 min. All solutions were prepared using deionized, distilled water that had been 0.2 μm filtered and autoclaved for 1 h to inactivate RNases. Glassware and disposable supplies were selected to be RNase-free or were autoclaved for 1 h, while care was taken to select RNase-free reagents for all stages of hybridization. Slides were visualized and fluorescence data were acquired using a GenePix 4000B microarray scanner (Molecular Devices).

3.4.2. Hybridization in the CWB

Solutions, temperatures, and times for a typical hybridization cycle are shown in Table 2. The hybridization solution contains 15 % formamide (see Note 6) and therefore the effective hybridization temperature is 61 °C. Buffer rinses entail flushing the previous solution from the feed line and capillary using 0.1 $\times$ SSC, then refilling with 0.1 $\times$ SSC. Fluorescence data are gathered after refilling the capillary (i.e., after Rinse 1 and Rinse 2). Hybridization of RNA with detection and capture probes is carried out simultaneously, while positive control hybridizations are carried out with a labeled target complementary to the capture probe, and background control hybridizations receive the detection probe but no RNA. It should be noted that each cycle of target detection requires two measurements, i.e., before and after hybridization. This is necessary to prevent false-positive results.

Table 2
Hybridization and denaturation protocol used in the CWB

Step	Solution	Temperature (°C)	Incubation (min)
Denature	0.1 $\times$ SSC + urea (50 % w/w)	65	5
Rinse 1	0.1 $\times$ SSC	52	0
Prehybridize	5 $\times$ SSC, 0.01 % BSA (w/w), 0.1 % SDS (w/w)	52	15
Hybridize	5 $\times$ SSC; 15 % formamide (v/v); 0.1 % SDS (w/w); labeled target oligo, labeled detection probe, or RNA + detection probe as required	52	15
Posthybridize	2 $\times$ SSC, 0.1 % SDS (w/w)	52	5
Rinse 2	0.1 $\times$ SSC	52	0

4. Results of Capillary Tests and Hybridization Optimization Studies

As a first step in preparing the CWB for target detection, it was necessary to check the system under controlled conditions using a standard reference material (SRM). For this purpose an uncoated capillary was mounted into the reaction chamber and loaded with a solution of Alexa Fluor 532-labeled positive control solution (A532-Vibtar; Table 1) at an arbitrary concentration. The CWB-AM was run remotely from a Linux host machine. A Ruby script file with an abbreviated measurement cycle was uploaded into the MFB's ARM processor for execution. The CWB was instructed to load a buffer solution into the capillary and record the background fluorescence signal N_B for 30 s. Subsequently, the SRM was loaded into the capillary and the fluorescent signal N_S was recorded for 30 s. The difference between these two signals provides an indication of the operational status of the CWB. A difference below the preset limits (5) indicates that the CWB should not be operated. Figure 8 shows a summary of the results from 20 cycles

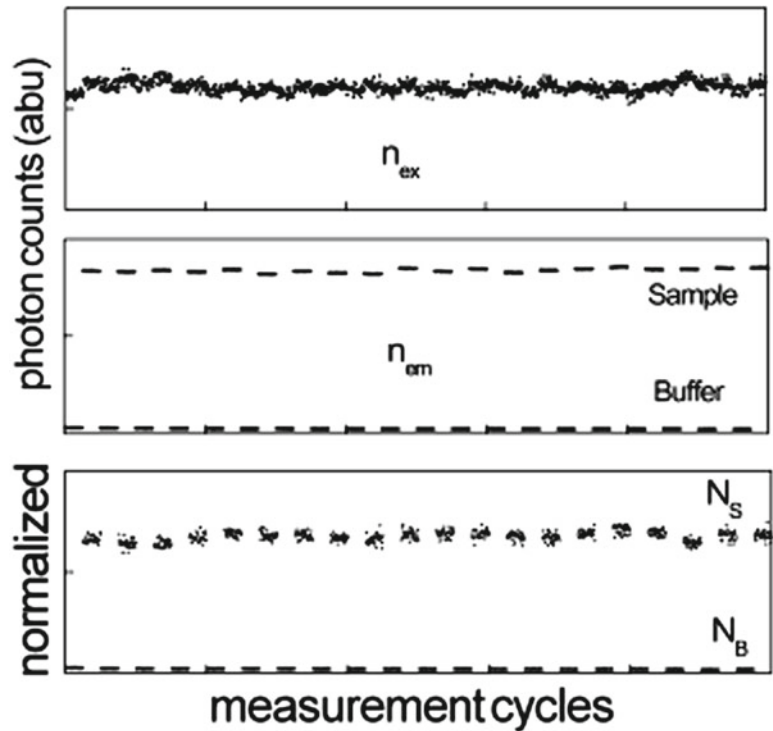


Fig. 8. System test with an uncoated capillary, loaded with a standard reference material (Alexa532 dye solution). *Top panel:* raw photon count data measured with PMT2. The signal count n_{ex} is proportional to the excitation energy; *middle panel:* raw photon count data at the output of the emission filter, measured with PMT2. This is the fluorescence emission signal n_{em} ; *bottom panel:* normalized data ($=n_{em}/n_{ex}$). N_S and N_B correspond to sample and background, respectively.



Fig. 9. Visualization of probe attachment inside capillaries, silanized after baking (a) or ozone treatment (b). Capillaries were coated with Cy3-labeled capture probe (Amn-Vibcap-Cy3) and were visualized using a microarray scanner and a slotted polycarbonate holder.

of measurements. The top panel is the raw (scaled) photon counts corresponding to the optical excitation power n_{ex} , at the point of measurement. The middle panel is the raw photon counts corresponding to the fluorescence emission n_{em} . The bottom panel is the normalized signal, $n_{\text{em}}/n_{\text{ex}}$ (N_{B} for the background solution and N_{S} for the dye solution). As discussed by Dhadwal et al. (4), this normalization eliminates the machine to machine variability that plagues fluorescence measurements. The results show that the system is working correctly and within specifications. From a practical view, the background fluorescence signal determines the limit of detection (see Note 7).

In order to stably immobilize a capture probe on the interior surface of the capillary, the surface of the capillary needs to be silanized. Proper cleaning and activation of the silica surface proved critical to this procedure. Figure 9 shows capillaries in which surface activation was carried out by Method A (baking) and Method B (ozone treatment) as described in Sect. 3.1.2. Visualization of the coating by application of a labeled capture probe clearly showed that the probe attached more densely and evenly to capillaries prepared by Method B, which was adopted for use with the CWB.

Hybridization temperature and buffer composition both influence the specificity of hybridization, and can be conveniently expressed as an “effective temperature,” a function of the actual temperature and the effects of formamide concentration. The effective temperature must be high enough to minimize mismatches and nonspecific binding. Too high an effective temperature will reduce the number of target molecules bound to probes, including both binding of targets to capture probes and binding of detection probes to immobilized targets. Figure 10 shows a typical

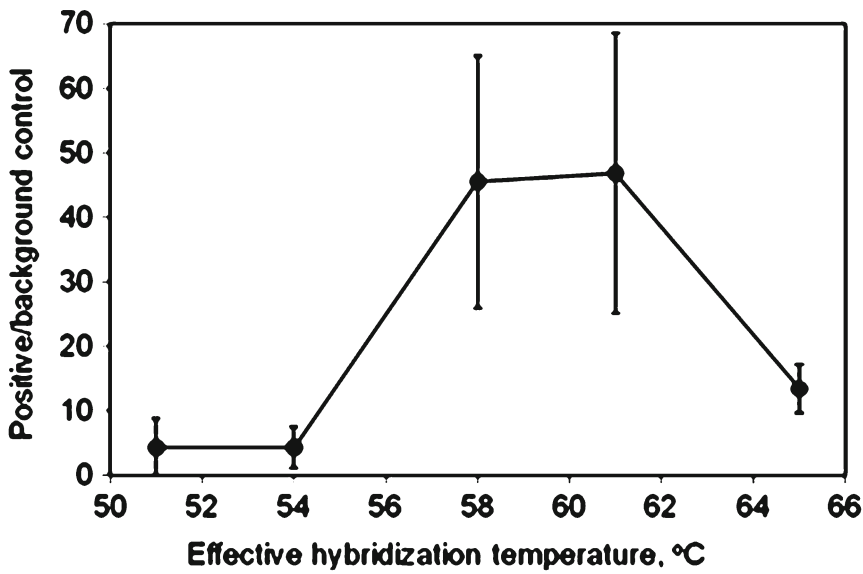


Fig. 10. Optimization of hybridization temperature. Effective hybridization temperature was varied by varying actual temperature (45, 52, or 59 °C) and formamide concentration (10 % or 15 %; 1 % formamide is equivalent to a 0.6 °C temperature increase). Overnight hybridizations were performed on microarray slides spotted with capture probe Amn-Vibcap. Values represent the ratio between fluorescence intensity of positive and background controls, where positive control slides received a complementary labeled oligonucleotide (A532-Vibtar) and background controls received detection probe A532-Vibdet in the absence of RNA as a measure of nonspecific hybridization (noise). *Error bars* represent standard deviations.

experiment in which hybridization temperature was optimized by maximizing the ratio between fluorescence intensity of positive and background control slides. The results show an optimal hybridization temperature range between 58 and 61 °C. The rather high standard deviation in the data points is due to spot variability associated with the use of a manual arrayer and normalization of the data with the low background control signal.

The use of microarrays expedited the testing of candidate probe pairs for the detection of *Vibrio*. Figure 11 shows tests of two capture probes and three detection probes. The best results (lowest background control values combined with highest detection of RNA) were achieved using capture probe sequence GV and detection probe FVdet2. Little difference was observed between fragmented and intact RNA. This finding suggests that access to both binding sites is not markedly impeded by RNA secondary structure, a conclusion supported by the effectiveness of GV as a FISH probe (10, 11) and the close proximity of the two binding sites. Our findings also indicate that, as expected from their proximity, the GV and FVdet2 binding sites are seldom separated by fragmentation of the RNA.

Methods tested for the removal of positive control oligonucleotide (to regenerate the capture probe for subsequent hybridizations)

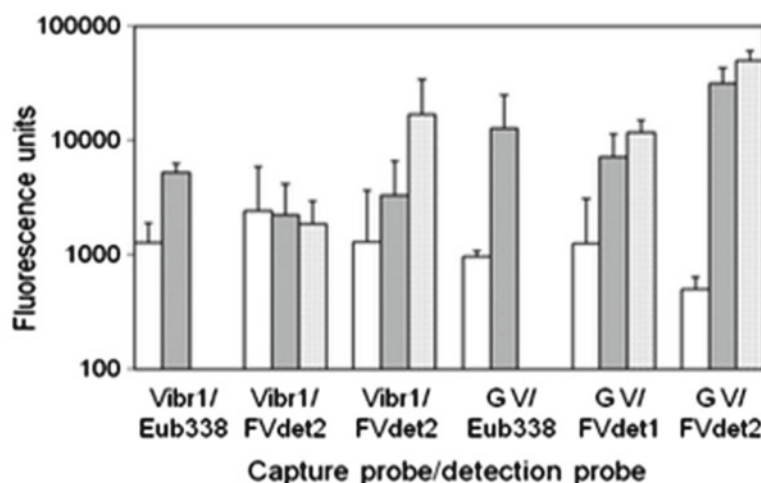


Fig. 11. Effect of capture and detection probe sequences on fluorescence signal from *Vibrio* RNA. Hybridizations were performed for 1 h on microarray slides. *Unshaded bars*: background control with detection probe but no RNA, *hatched bars*: detection probe plus unfragmented RNA, *stippled bars*: detection probe plus ZnCl_2 -fragmented RNA. Fragmented RNA bound to our capture probes was not detected with detection probe Eub338 because of the large distance between binding sites (data not shown). 2.35–2.55 $\mu\text{g}/\text{slide}$ total RNA was used. *Error bars* represent standard deviations of 60 spots. Data for the Cy3-labeled Eub338 are corrected for comparison with the two Alexa Fluor 546-labeled probes.

included formamide (up to 90 %), $0.1\times$ SSC at temperatures up to 95°C , and urea (Fisher Scientific BP169-500; 50 % w/w in $0.1\times$ SSC) (data not shown). A 50 % urea treatment at 65°C gave best results and was selected for use in the CWB (see Note 8).

The optimized probe system and hybridization/denaturation protocol (Table 2) were then tested using the CWB. Figure 12 shows sample data obtained over four cycles using a labeled target oligonucleotide. The capillary had been previously used and filled with $0.1\times$ SSC for storage. One cycle was required to reestablish stable performance, after which the difference between normalized fluorescence values before and after hybridization remained constant. To date, over 20 cycles have been achieved using a single capillary, without apparent loss of performance (see Note 9). We are confident that we have found a coating procedure that will preserve the integrity of the capture probe for many more cycles. The final number will be established after the complete system is fully operational.

Development of a field-deployable biosensor draws upon fields ranging from optical engineering, to chemical engineering, to molecular biology. Using an interdisciplinary approach we have achieved reproducible and automated detection of an oligonucleotide target. The system is currently in final testing stage and the next step will be to demonstrate detection of extracted RNA in the CWB, as has already been done using our probes and protocols on microarrays.

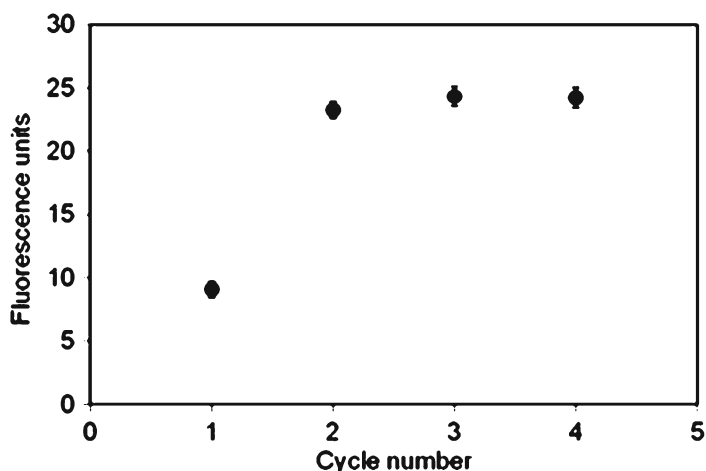


Fig. 12. Consecutive hybridizations in the CWB. Repeated hybridizations and denaturations were performed in automated mode, using a capillary coated with Amn-GV capture probe and Cy3-GVtar as the target. The capillary had been previously used for approximately 20 hybridization cycles and filled with $0.1\times$ SSC for storage. Values represent the difference between mean normalized fluorescence intensity measured before and after hybridization at each cycle. Sixty sets of data points were acquired at each measurement time; error bars represent standard deviations for the difference between hybridized and unhybridized mean values. One cycle was needed to restore normal functioning of the capillary, after which values stabilized.

5. Notes

1. The detection limit of a fluorescence instrument is determined by the background signal, which is minimized by carefully selecting compounds that make up the solution at zero concentration and by reducing stray reflections through creative optical design and by the use of a holographic notch filter to exclude excitation energy from bleeding into the emission band.
2. Spectral response of the emission filters is based on normal incidence. A spread of a few degrees will lead to suboptimal filtering, causing an increase in the background signal. Thus, for fiber-based optical receivers, care must be taken to collimate the output of the optical fiber.
3. As discussed in Note 1, the detection limit is determined by the background signal, which will be at its lowest value when it approaches the dark count of the single photon counting system. Photomultipliers have dark counts of the order of 30 cps at room temperature, whereas avalanche photodiodes can achieve dark counts of 150 cps when actively cooled.

4. Any oil contamination in the compressed gas will coat and contaminate the interior surfaces of the capillaries.
5. RNA is exceedingly susceptible to degradation, and precautions should be taken to minimize exposure to room temperatures and to RNases. If partially used, an RNA aliquot should be discarded rather than refrozen. RNA solutions should contact only RNase-free solutions and equipment.
6. Deionized formamide is best aliquoted and stored at -20°C , preferably under an inert gas. It should not be used if a yellowish color develops.
7. Dhadwal (5) described a target detection error rate, which computes the probability of detection based on the signal-to-noise ratio of both the zero concentration and the target solutions. He showed that it was possible to further reduce the detection limit by increasing the data accumulation time and using time-averaged signals for comparison.
8. We do not recommend this use of microarray slides for tests of probe regeneration, since drying of slides for examination in the microarray scanner makes subsequent denaturation of the probe–target complex exceedingly difficult (Eli Hatchwell, personal communication). The number of cycles that can be tested using slides is thus limited by the number of replicates that can be run (since slides must be sacrificed at each hybridization/denaturation cycle), and some air exposure is nonetheless unavoidable.
9. The CWB allows the entire process, including data acquisition and storage of the capillary between experiments, to be carried out with liquid inside the capillary, avoiding the problems described in Note 8.

6. Future Directions

We intend to explore the dynamic range of the system and its ability to quantify *Vibrio*, a genus which includes numerous human and animal pathogens, against a background of other organisms. The development of robust probe attachment capabilities, capillary regeneration methods, and field-deployable instrumentation provides a foundation upon which probe systems for other target organisms of economic and ecological importance can be constructed and optimized according to the procedures we have outlined.

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