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Real-time *In-situ* Detection of Microbes

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Abstract

Currently, no methods exist for the real-time detection and quantification of microbes in the environment or for the detection and identification of pathogenic organisms in clinical specimens. We have developed technologies which overcome these limitations and provide detection limits as low as a ten microbial cells per cm^2 on abiotic surfaces, and per mL in fluids.

The detection and quantification of microbes [total microbial load] is based on the intrinsic fluorescence of microbial metabolites and protein cofactors, and provides an estimate of the total microbial load as well as the relative distribution of live cells, dead cells, and endospores. Unlike existing methods, no additional reagents or sample contact is needed. This technology has been applied to the *in-situ* measurements of two sub-glacial microbial communities at sites in the Svalbard Archipelago, Norway, and to the efficacy of disinfection of contact lenses.

In the rapid spread of a life-threatening infection, early diagnosis is of great importance. In such situations, pathogen counts will be very low, which also presents a significant challenge to diagnostic methods. We have developed a point-of care disposable diagnostic based on the en masse capture of blood-borne microbes from 1 mL of fresh whole blood with surface-tethered, small molecule ligands. Quantification is based on the intrinsic fluorescence of captured cells.

Keywords: Microbe detection; Fluorescence, *In-situ* measurements; Point-of care diagnostics

Abbreviations: Em: Fluorescence Emission; Ex: Fluorescence Excitation; LED: Light Emitting Diode; RPN: Reduced Pyridine Nucleotides

Introduction

There is considerable interest in the real-time assessment of the microbial load in a sample. Outbreaks of food-and waterborne disease highlight the need for robust instrumentation that does not suffer from the need for prior outgrowth of target microbial cells. Any method that relies on cellular outgrowth prior to physic-chemical interrogation potentially suffers from differential growth kinetics of various cells in the original sample. In particular, the viable-but-not-culturable phenomenon [1] constitutes a serious drawback for many current technologies used to assess bacterial loads in complex sample matrices that contain heterogeneous populations of cells where they no longer grow out on standard growth media. In addition, any outgrowth step necessarily increases the total sample analysis time, which can be critical in a variety of situations, e.g. diagnosis of bacterial meningitis, sterilization of medical implements, or the shipment of freshly packed produce to retail markets.

At present, the determination [2] of total bacterial load is qualitative and largely relegated to the laboratory, with the main alternatives including direct microscopic examination of concentrated suspensions (> 107 cells/ml), PCR amplification of a range of genes, simple enzyme-based assays [3], and fluorescence measurements using DNA-intercalating dyes (e.g. epifluorescence microscopy or flow cytometry). A cell-counting method that is broadly applicable to microbes, and applicable to a variety of sampling problems (thin films of aqueous cell suspensions, ice, soil, rock, food surfaces, medical implements, etc.), would be highly useful in guiding physical sampling efforts, decontamination protocols, forensic investigations, and medical assays where the real-time determination of total bacterial loads is needed.

A variety of optical techniques have been applied to the detection and quantification of microbes, particularly involving scattering or spectroscopy. Simple light scattering (culture turbidity) in the visible region is the classical method for the determination of the concentration of cells in liquid suspension. Advances in instrumentation and data processing have enabled IR and Raman measurements on microbial cells [4-6], and commercial instruments (e.g. River Diagnostics and Brucker) are available for specific microbial identification based upon the comparison of 'fingerprint' regions in spectra with reference spectra in a database. However, prior outgrowth on manufacturer-specified media is required. Current commercial instruments, including field-portable ones (e.g. Ocean Optics systems), are suitable for obtaining spectra of millimeter-sized colonies of cells that are visible to the eye, but not low numbers of cells. The addition of a microscope to the optical path of an instrument (as in a Raman microspectrometer) solves this problem, but renders the equipment more expensive and the high intensity excitation can photobleach the sample.

Fluorescence methods provide the most sensitive optical detection of biomolecules. High fluorescence sensitivity, coupled with a short-collection-time requirement and the ability to monitor large areas/volumes continuously are very attractive for the *in-situ* investigation of microbes in the environment. We developed a multiwavelength fluorescence instrument to distinguish live cells, dead cells, and endospores, based on the intrinsic fluorescence of a number of metabolites and protein cofactors [7-13]. Importantly, our approach does not rely on 260-280 nm excitations because this excites

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aromatic amino acids, purines, and pyrimidines; these are ubiquitous in real-world samples. More than fifty years ago, it was noted that the fluorescence due to NAD[P]H decays upon cell death as the oxidized forms (NAD[P]+) accumulate [14,15]. Pyridine nucleotide fluorescence occurs at Ex 365 nm/Em 440 nm, with small contributions from other fluorophores. Bacterial sporulation is accompanied by the production of a distinctive, fluorescent calcium dipicolinate complex that is densely packed in the endospores [16]; this fluorescence (Ex 635 nm/Em 770 nm) disappears as the compound is expelled when the spores germinate. Nonviable cells, those not respiring but maintaining some level of cellular integrity, exhibit fluorescence (Ex 590 nm/Em 675 nm) from flavins, cytochromes and apo forms of porphyrins. We report field an intrinsic bacterial fluorescence detector that contains notable improvements (particularly field portability and improved detection limit) over our earlier instruments [7]. This methodology was specifically tailored for the determination of total cell counts, apportioned among live cells, dead cells, and endospores, in colonies *in situ* (i.e., without sample contact or the use of added reagents such as fluorescent dyes). Applications to several practical problems are presented to illustrate the capabilities of the detector.

Methods

Detection

Over the past several years, we have developed a method and hand-held prototype devices for the detection of microbial life on surfaces (e.g. foods, glass, plastics, cloth, stainless steel, etc.), in water/liquids [7,17-19]. This technology has been shown to have a sensitivity of 10-20 cells/cm^2 [cm^3] in these conditions. These prototypes employ multiple intrinsic fluorescence markers and are based upon a patented multi-wavelength intrinsic fluorescence methodology [12]. The method detects microbes using intrinsic fluorescence with signals from more than one emission wavelength arising from excitation with one or more wavelengths. This fluorescence is directly proportional to the concentration of the metabolite, e.g., RPN, or, equivalently, the number of 'live' (metabolizing) cells. Figure 1 shows the kinetics of fluorescent spore core components and cell metabolites with spore germination. Taken together, these can be used to distinguish viable cells from dead cells from spores [20].

To distinguish the presence of microbes from environmental interferences, we use adaptive algorithms [21] that are trained using data sets taken under standardized test conditions consisting of a variety of microbial environments and conditions for each optical instrument and sample presentation configuration. In particular, the Neyman-Pearson test receiver-operating characteristic [7] gives a probability of false alarm of 10-4 for a probability of detection of 0.998. It is important to note that viable cells, dead cells, spores, and media can be distinguished by these methods [10,11,17]. Since we are measuring several fluorescence signatures whose ratios must fall within narrow physiological bounds, interference at any one of them can easily be detected and corrected. In particular, we note that other microbial components also fluoresce: bacteriochlorophylls, phycocyanins and phycoerythrins, siderophores such as pyoverdins and deferrioxamines, and some B-vitamins [22].

Identification

The capture and concentration of microbial (bacterial, viral, fungal) pathogens and proteinaceous toxins from fluids occurs via molecular recognition: specific binding between the target and surface-tethered custom ligands [23,24] (Figure 2a). In brief, a peptide, polysaccharide, or other small biomolecule that is specific for the target pathogen is covalently tethered by an organic linker to a substrate surface. Concentration of the pathogen from fluids is a result of the capture of the microorganism by the surface-tethered ligand through a kinetically rapid interaction; captured pathogens are retained on the surface for possible reaction with other diagnostic reagents or subsequent detection with intrinsic fluorescence as described above.

The technological innovation and benefits of this methodology are shown in Figure 2. They include: 1) faster binding kinetics which reduce assay times (tethered-ligands bind as much analyte in one minute as many antibodies bind from identical solutions in ca. 30 minutes, Figure 2b); 2) improved retention of captured analyte during washing steps allows tethered-ligand surfaces to be used to concentrate analyte from solution (resulting in lower detection limits for assays, Figure 2c); 3) lowered assay reagent costs; 4) small ligands have been shown to capture target analytes from complex solutions (including mud); 5) improved stability and less sensitive storage conditions (small molecule ligand surfaces have been shown to retain activity after storage for one year at room temperature); and 6) increased binding capacity (ca. 10,000 tethered peptide ligands can occupy the same area or 'footprint' occupied by one antibody molecule adsorbed or chemically bound to a surface) on an optimally conjugated surface.

We have shown [24] that an oligopeptide ligand, tethered to a glass chip, can be used to capture *Staphylococcus aureus* cells from aqueous solutions for mass spectrometric detection (detection limit <3 cfu/mL, equivalent to what was observed using a monoclonal antibody for cell capture). We have used (see below) this same tethered ligand to capture *S. aureus* cells from whole blood, with intrinsic fluorescence as the basis of *S. aureus* detection.

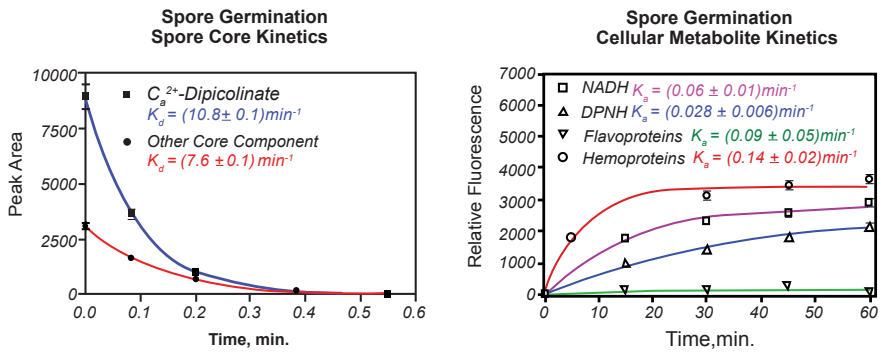

Figure 1: Kinetics of *Bacillus megaterium* Spore Germination in LB Media at 37°C. DPNH is other reduced dipyridine nucleotides [20].

Instrument

Descriptions of this fluorescence instrument and its application to the determination of total microbial load are given in References [7,12]; hence, only a brief description is given here. Figure 3 shows a detection instrument for surfaces that was used in the studies described below. Excitation of the desired fluorescence which contains contributions from live cells, dead cells, and spores is provided by three interference-filtered LED light sources (~10 nm FWHM). Assuming equal amounts of the three components (live cells/dead cells/spores), Table I shows these contributions to each excitation/emission pair we used, [7,12]. These light sources are amplitude-modulated at different frequencies [25]. All three fluorescence emissions can be observed simultaneously with a single photomultiplier detector and the individual fluorescence contributions separated by Fourier transformation, since the resultant fluorescence also has the same amplitude modulation as its excitation. This approach significantly narrows the noise bandwidth, which dramatically enhances the signal-to-noise ratio. Together with the use of a custom filter to reject excitation photons, this makes the instrument useful in detecting microbial populations that vary by up to nine orders of magnitude (limited by quenching when the cell density is too large). The total microbial load (and also the relative amounts of live cells, dead cells, and spores) is determined based on calibrations of the instrument with a variety of microbes (Table 1).

The multiwavelength fluorescence instrument was calibrated using laboratory-prepared samples of *Bacillus thuringiensis* and *Escherichia coli* (reference strains available from the American Type Culture Collection, ATCC) containing known amounts of live cells, dead cells, and spores before field use. Although the instrument had been calibrated to compare data collected in the field with those collected in the laboratory, optically clear plastic-laminated samples of *B. thuringiensis* spores were also used to verify the instrument calibration immediately before and after measurements in the field. We also collected samples in the field, isolated pure bacterial strains by standard methods and used a mixture of these field-derived cells for further instrument calibration.

Results and Discussion

We have previously [17,18] used similar instruments and methods noted above to address issues involving microbial contamination (surface, water, air), as well as point-of-care diagnostics. Here we will discuss applications to life in extreme environments, disinfection of contact lenses, and the development of a point-of-care diagnostic for the detection of pathogens at ID50 levels in small amounts of fresh whole blood.

Life in extreme environments

Currently, there are no methods to determine the total microbial load on an abiotic substrate (e.g., rock) in real-time. This capability could allow *in-situ* microbial assessments in the field without the need for added reagents or physical contact with the microbial cells, and greatly enhance sampling efforts. We have used a hand-held, fluorescence detection device (Figure 3) for *in-situ* studies and demonstrated its applicability to sub-glacial microbial communities at sites in Palander, Svalbard, Norway, during AMASE 2008 (Arctic Mars Analog Svalbard Expedition) [26]. Such extreme environments may provide models for viable habitats for life elsewhere in the universe.

While the fluorescence contributions of live cells, dead cells, and endospores to each of the emission channels (Table 1) were determined by calibration of two reference strains in the laboratory as described above, these were calibrated again after the expedition using Svalbard-collected bacteria and the same rocks (after cleaving to expose fresh surfaces) that were investigated *in-situ*. This calibration was in good agreement with the prior one. For each site, measurements of the ice/snow surface were taken about every 1 cm using a small sterile shovel to remove the top layer until a rocky bottom was reached. No physical contact by the instrument was made with the section being measured. Site 1 was at the base of the ice cap and consisted of a gully,

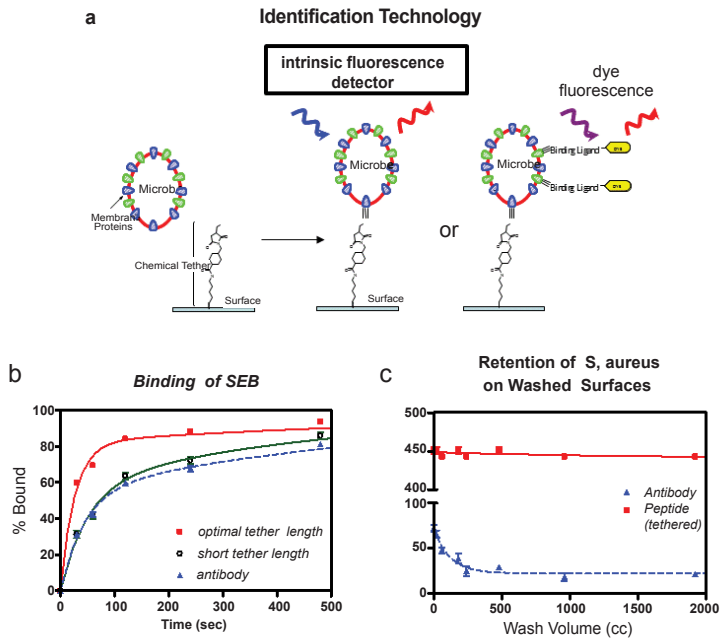

Figure 2: Identification Technology. (a) Capture of specific microbes by tethered ligands for receptors and surface proteins. (b) Binding and (c) retention comparison of tethered ligands and antibodies.

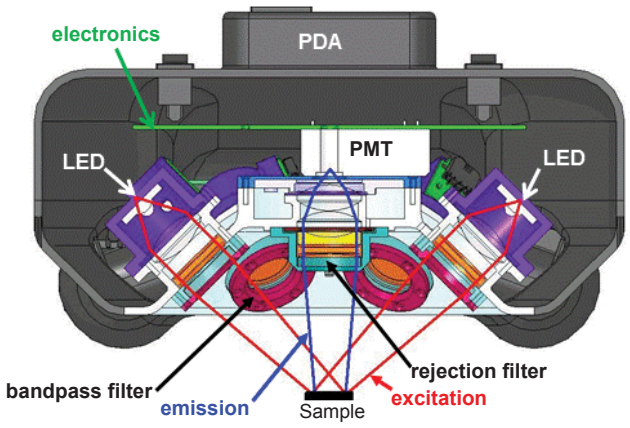

Figure 3: Schematic diagram of the fluorescence instrument used in these studies.

Excitation/Emission	Contributions
365 nm/440 nm	0.7 [live cells] + 0.15 [dead cells] + 0.15 [endospores]
590 nm/675 nm	0.5 [live cells] + 0.5 [dead cells]
635 nm/770 nm	0.5 [dead cells] + 0.5 [endospores]

Table 1: Source of Contributions to Each Fluorescence Channel.

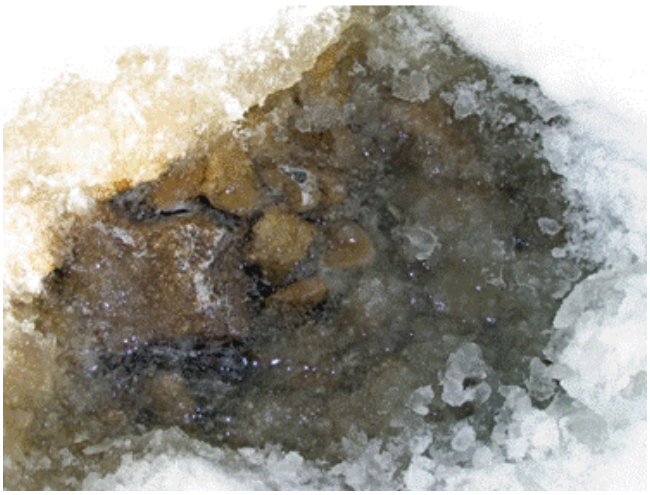

Figure 4: Palander Ice Cap, Svalbard, Norway, site 2 having approximately 15 cm granular snow on top and 32 cm base ice.

approximately 10 cm of granular snow, and approximately 3.5 cm base ice on top of a rocky bottom while site 2 was near the top of the ice cap and had approximately 15 cm of granular snow, and approximately 32 cm base ice before the rocky bottom. Figure 4 shows a picture of site 2. Rocks from the bottom of both sites were collected by aseptic methods and stored in sterile containers for transport to our laboratory.

The excitation (Em) data collected at both sites had only very small 635nm emission (Ex) values, which were within the measurement error. This meant that there were no endospores or non-viable cells detected. Furthermore, the measurements for 590 Ex and 365 Ex gave the same value for the live cell density within the error, 2X104 ± 6 X102 live cells/cm^2 for site 1 and 7.6 ± 0.08 X106 live cells/cm2 for site 2. A *Bacillus* sp. was cultured from a rock sample taken from site 2, but no fluorescence characteristic of endospores was detected on the rock. This was also verified by light microscopy of rock scrapings after staining for bacterial endospores. Bacteria isolated from site 2 were strains not capable of sporulating. Our fluorescence detector located thriving colonies of bacteria in a thin film of water at the ice-rock boundary in both sites, but not in the snow and ice above.

Disinfection of contact lenses

The disinfection of contact lenses is essential to lens wearers, but determination of the efficacy of the various available protocols and products is difficult and time-consuming. Classical methods, such as microbial outgrowth, can not only prejudice the growth of specific species, but cannot detect the presence of microbes that are viable-but-nonculturable [1]. We have developed a method and protocol to determine the total microbial load on contact lenses which distinguishes viable and nonviable cells and spores in real-time and to compare various disinfection procedures and products. This is described in detail elsewhere [27].

Acuvue™ (Johnson & Johnson Vision Care, Inc., 7500 Centurion Parkway, Jacksonville, FL 32256) contact lenses were chosen for investigation and were measured with the set-up shown in Figure 5 using the intrinsic fluorescence instrument shown in Figure 3. Lenses were measured both before and after incubation with *Pseudomonas aeruginosa* (ATCC 10145). Lenses were then cleaned and disinfected with AMO Easy Rub™ (Abbott Medical Optics, Inc., 1700 East St. Andrew Place, Santa Ana, CA 92705-4933) and Alcon No Rub™

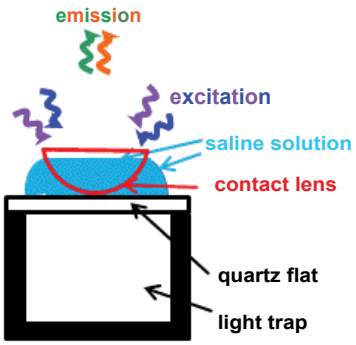

Figure 5: Set-up for measurement of biofilms on contact lenses [27].

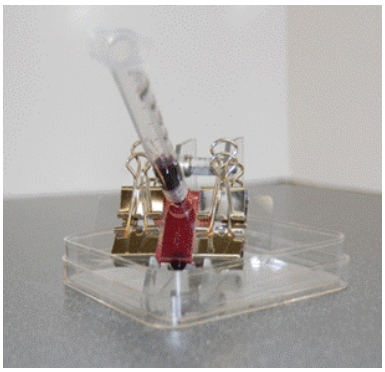

Figure 6: Prototype point-of-care disposable diagnostic for the detection of *Staphylococcus aureus* in fresh whole blood.

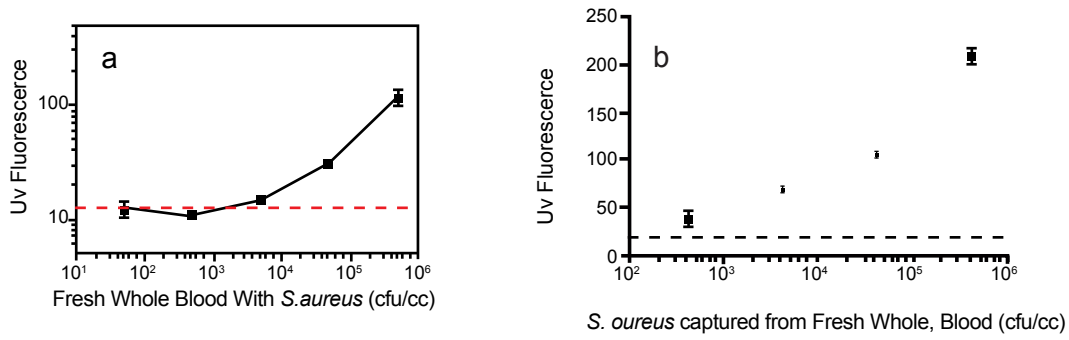

Figure 7: Comparison of the limit of detection using fluorescence measurements of the reduced pyridine nucleotides of *Staphylococcus aureus* in fresh whole blood (a) captured in a 0.2 mm thick quartz cell with that achieved on (b) a tethered-ligand quartz surface and rinsed to remove the blood. The dashed line is the uncontaminated fresh whole blood control.

(Alcon, Inc., Bösch 69, CH-6331, Hünenberg Switzerland) according the respective manufacturer's instructions. As a control, lenses were also rubbed and rinsed with CVS saline for contact lenses. Lenses were then measured for the effectiveness of the procedure. As a control, both disinfected sample lenses and control lenses were placed in media for outgrowth and cells were counted by standard methods.

The background fluorescence of the contact lenses varied widely. *P. aeruginosa* formed biofilms on the contact lenses that varied in concentration from 106 - 1013 cells/mL and all lenses yielded no colony growth on culturing following cleaning and disinfection. Nevertheless, the intrinsic fluorescence protocol showed that a medically significant amount of bacteria, 10^5 - 10^9 cells/mL, on the treated lenses remained viable. Such viable-but-nonculturable cells are not detected by current methods for verification of contact lens decontamination. The cleaning/disinfection procedures injure the cells to the point at which they cannot grow in microbial media used in standard protocols. However, it is known that such cells can be resuscitated via a variety of means to resume normal growth and virulence traits. Our result points to need for further refinement of contact lens decontamination protocols.

Detection of pathogens at ID50 levels in whole blood

We have also developed prototype disposable point-of-care diagnostics based on this technology. An example, presented below, is a diagnostic for the capture of *Staphylococcus aureus* from spiked fresh whole blood. In order to capture only *S. aureus* cells, we attached the anti-protein A peptide discussed above [24] to a quartz surface by a long (ca. 50 Å) tether. An experimental set-up is shown in Figure 6. A small amount (eg. 1 mL) of fresh whole blood was allowed to flow over the coated glass surface from a syringe. The limit of detection of *S. aureus* in whole blood using a very thin ligand-coated quartz cell (0.2 mm) (Figure 7a) is compared with *S. aureus* cell capture from whole blood onto a peptide-coated quartz glass surface after a rinse step [24] to remove the blood (Figure 7b). Cell capture and rinsing improves the detection limit by ca. two orders of magnitude. The use of very thin cells should enable the detection of other pathogenic agents at ID50 levels in very small amounts of fresh whole blood.

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