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Taxonomic identification of microorganisms by capture and intrinsic fluorescence detection

Hea-Young Mason, Christopher Lloyd, Margaret Dice, Robert Sinclair, Walther Ellis, Jr, Linda Powers *

National Center for the Design of Molecular Function, Department of Electrical Engineering, Utah State University, Logan, UT 84322-4155, USA

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Abstract

Quick and accurate detection of microbial contamination is accomplished by a unique combination of leading edge technologies described in this and the accompanying article. Microbe capture chips, used with a prototype fluorescence detector, are capable of statistically sampling the environment for pathogens (including spores), identifying the specific pathogens/exotoxins, and determining cell viability where appropriate.

Keywords: Microbe sensor; Microbe identification; Molecular recognition

1. Introduction

Microbial contamination of foods, water, and air have been the focus of much attention recently, including *Escherichia coli* O157:H7 in ground beef products, vegetables, and juice, *Salmonella typhi* in chicken, eggs, and mayonnaise-based salads, *Cryptosporidium parvum* and *Vibrio cholerae* in water, *Legionella pneumophila* in air, and *Bacillus anthracis* spores in mail. In addition to the re-emergence of old microbial-based diseases such as tuberculosis (*Mycobacterium tuberculosis*) and nosocomial *Staphylococcus aureus* infections, other maladies such as heart disease and stomach ulcers have been ascribed to microbial infections (*Chlamidia pneumoniae* and *Heliobacter pylori*, respectively) during the last decade. Finally, the threat of bioterrorism/biowarfare looms at any large public event or gathering. These occurrences emphasize the importance of quick and accurate assessment of microbial contamination in a variety of settings. This and the accompanying contribution *Reagentless detection of microorganisms by intrinsic fluorescence* couple leading-edge technologies to detect and identify the nature of microbial contamination in food, water, air, and on surfaces.

Many current diagnostic technologies (Hobson et al., 1996; Henchal et al., 2001) require cellular outgrowth in detection protocols. While restricting our further comments and observations to bacteria, it is important to note that much of the information in this contribution can also apply to viruses and eukaryotic microbes). This step requires hours–days, and the selected growth medium can favor the growth of bacteria with specific phenotypes, with the result that the bacterial population after outgrowth is not representative of the population in the original sample. Indeed, the problem of viable-but-not-culturable cells in samples is one of the key problems in environmental microbiology (Barer and Harwood, 1999; Colwell and Grimes, 2000).

High sensitivity, short collection time requirements, lack of sample contact, and capability of scanning large areas/volumes render fluorescence methods an attractive alternative for microbial detection, as discussed in the accompanying article *Reagentless detection of microorganisms by intrinsic fluorescence*. However, fluorescence signatures rarely provide a clear identification of the type of contamination (e.g., microbial species). In order to differentiate among the microbes, particularly bacteria, we have developed (Powers et al., 2001) capture

* Corresponding author. Tel.: +1-435-797-2033; fax: +1-435-797-3328.
E-mail address: lsp@biocat.ece.usu.edu (L. Powers).

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Table 1
Bacterial cell capture using tethered hemin

Pathogens and enterics (captured)		Nonpathogens (not captured)
Alcaligenes faecalis	*Listeria monocytogenes*	*Achromobacter cyclolastes*
Alcaligenes denitrificans	*Mycobacterium avium*	*Bacillus globigii*
Bacillus cereus	*Neisseria gonorrhoeae*	*Bacillus megaterium*
Bordetella bronchtseptica	*Neisseria meningitidis*	*Bacillus subtilis*
Bordetella pertussis	*Pseudomonas aeruginosa*	*Bacillus stearothermophilus*
Brevundimonas diminuta	*Pseudomonas aurofaciens*	*Bacillus thuringiensis*
Campylobacter jejuni	*Salmonella typhi*	*Clostridium pasteurianum*
Chlamydia pneumoniae	*Salmonella typhimurium*	*Halobacterium sodomense*
Ctostridium difficile	*Serratia marescens*	*Lactobacillus plantarum*
Clostridium perfringens	*Shigella flexneri*	*Lactobacillus rhamnosus*
Corynebacterium diphtheriae	*Staphylococcus aureus*	*Micrococcus luteus*
Enterobacter aerogenes	*Staphylococcus pyogenes*	*Pseudomonas oleovorans*
Escherichia coli (EIEC strains)	*Vibrio cholerae*	*Rhodospirillum rubrum*
Escherichia coli (serotype 0157:H7)	*Vibrio fluvialis*	*Thiobacillus ferrooxidans*
Haemophilus influenzae	*Vibrio vulnificus*	*Vibrio carchariae*
Helicobacter pylori	*Yersinia enterocolitica*	*Vibrio hollisae*
Klebsiella pneumoniae	*Yersinia pestis*	

technology that exploits small biomolecules, having molecular weights less than ca. 1500 Da, that recognize specific outer membrane components (e.g., receptor proteins, lipopolysaccharides).

2. Experimental

All reagents were of the best grade obtainable, and were used as received unless indicated otherwise. Water was distilled, deionized, and then redistilled prior to use.

2.1. Bacteria

Growth media were Difco or BBL products unless indicated otherwise. *V. cholerae* El Tor was a gift from Dr John Ezzell, and grown at 30 °C in marine broth. All other bacterial strains were purchased from the ATCC (American Type Culture Collection). *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus globigii*, *Enterbacter aerogenes*, *Pseudomonas aeroginosa*, and *S. aureus* were grown at 37 °C in nutrient broth. *Lactobacillus plantarum* was grown at 37 °C in *Lactobacillus* MRS broth. *Streptococcus pyogenes* was grown at 37 °C in trypticase soy broth. *V. carchariae*, *V. fluvialis*, *V. hollisae* and *V. vulnificus* were grown at 30 °C in marine broth. *Yersinia enterocolitica* was grown at both 28 and 37 °C in brain infusion medium. All other species noted in Table 1 were grown at 37 °C in Luria–Bertani broth. All cells were grown past mid-log phase, centrifuged at $3750 \times g$ for 5 min, then washed once with a minimal medium (see below). The pellet was centrifuged again (same conditions) and re-suspended in the minimal medium.

A defined, minimal medium having a negligible fluorescence background (provided that extremely pure reagents are used) can be prepared by the following method. In 1 l of water, add 0.5 g of NaCl, 1.0 g $(NH_4)_2SO_4$, 10 g Tris–Cl, 0.3 g $MgSO_4$, 2 g glucose, 5 mg $CaCl_2$, 10 ml of 20% glycerol, and 0.5 g alanine. In addition, 5 mg each of the following amino acids are added: cysteine, methionine, glycine, glutamic acid, serine, proline, and threonine. After adding trace amounts (ca. 1 mg each) of zinc sulfate, ferrous sulfate, and copper sulfate, the pH of the resulting solution is adjusted to 7.2, and it is then filter-sterilized. This medium maintains cell viability without giving the cells enough nutrients to enable them to begin to actively divide.

2.2. Tethered ligands

In order to capture an analyte of interest, we have used glass microscope slides as surfaces to which ligands believed useful in capturing cells or free proteins (soluble bacterial toxins) can be attached. Typically, the slides are first cleaned in a bath composed of 3:1 (v/v) fuming sulfuric acid and 35% hydrogen peroxide, followed by extensive rinsing with water. This must be done with great care, as this so-called 'piranha etch' solution is extremely corrosive. After baking at 110 °C for 3 h, the slides are then treated with a 2% (v/v) solution of *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride (50% in methanol; available from United Chemical Technologies, Inc.) in dry, HPLC-grade toluene for 45 min., followed by extensive rinsing with toluene and air-drying. Once this stage is reached, a variety of ligand attachment schemes, all involving incubations at room temperature, are possible. Hemin (ferriprotoporphyrin IX) can be covalently attached by overnight incubation of the silanized slides with a toluene solution saturated with the *N*-hydroxysuccinamide diester of hemin (Frontier Scientific, Logan, UT), followed by washing with toluene and a air-drying. Alternatively, the slides can be treated with a solution of 50% glutaraldehyde (v/v) in phosphate buffer (pH 8.0) for 30 min, rinsed with copious amounts of water, allowed to dry, and then a variety of amine-containing

ligands can be attached by overnight incubation. For example, an overnight incubation with a 2% (w/w) solution of desferrioxamine B, followed by a water rinse, and then a 2 min incubation with 10 mM ferrous sulfate (pH 7.0), another rinse with water and air-drying, produces slides containing the attached siderophore ferrioxamine B. A third alternative, illustrated in Fig. 1, involves the use of a coupling reagent (e.g., SMCC or DCC) to attach other types of ligands, including peptides and glycoconjugates. Ligand attachments to other surfaces are also feasible, including mylar film, glass beads, and magnetic beads.

2.3. Cell-capture protocol

All bacteria reported in this paper were captured from aqueous solutions using the following protocol, A specific culture was grown out to a density of ca. 10^{11} cells per ml, quantitated using a Petroff–Hauser counting chamber and a Zeiss microscope, and then diluted to obtain desired concentrations. Typically, 20 ml of cell suspension was applied to a ca. 1 cm diameter spot on a glass slide, and allowed to stand for 10 min. The liquid was then rinsed off using 5 ml of phosphate-buffered saline solution (pH 7.2) containing 0.3% Tween-20. Adhering cells were then detected by intrinsic cellular fluorescence, as described in the previous contribution, and additionally inspected by phase-contrast microscopy.

A sample pretreatment, using Novagen BugBuster and lysozyme, can be used to lyse bacterial cells, thereby making their soluble contents also available for sampling with a coated chip.

Fluorescence measurements reported herein were made using a prototype fluorescence detector, and focus on bacterial NADH/NADPH fluorescence (Dalterio et al., 1987; Duysens and Amesz, 1957; Powers, 1999).

3. Results and discussion

The capture technology described below does not rely on antibodies, nor are auxiliary reagents, other than buffered solutions used in rinse steps, needed for cell detection. Three general approaches can be used in developing ligands, other than antibodies, for microbial cell capture; (1) use iron-containing reagents (e.g., hemin, ferric siderophores) that target bacterial membrane receptors used in iron acquisition, (2) exploit the recognition of outer-membrane antigens involved in adhesion to 'natural ligands' (extracellular matrix (ECM) proteins or portions thereof, host cell glycoconjugates, etc.), and (3) select small biomolecules in combinatorial biopanning protocols using carefully chosen microbial antigens as the targets. The first two approaches rely upon important bacterial virulence determinants (Roth et al., 1995; Salyers and Whitt, 2001).

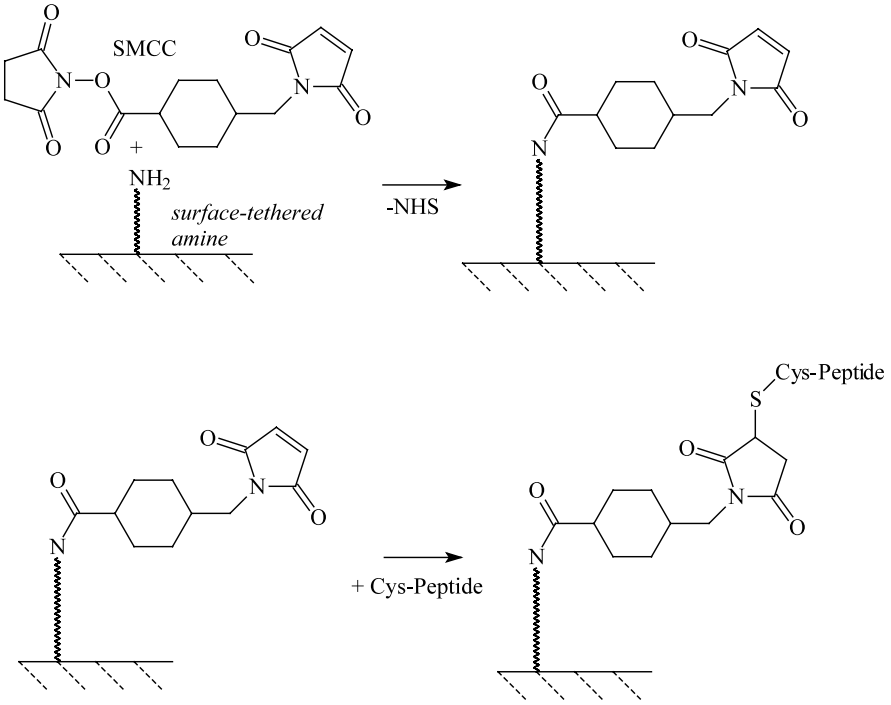

Fig. 1. Attachment of a synthetic peptide to a glass surface.

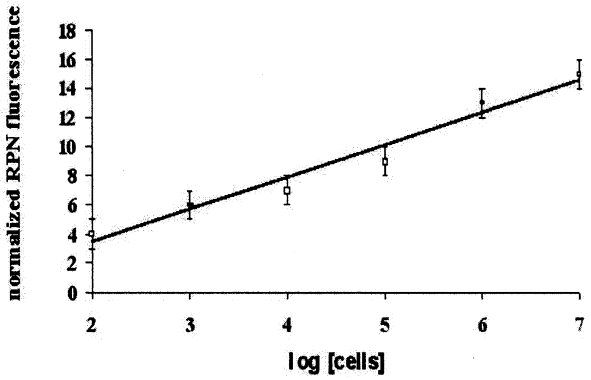

Fig. 2. Capture of live *S. typhi* using tethered hemin. RPN is reduced pyridine nucleotide (NADH, NADPH).

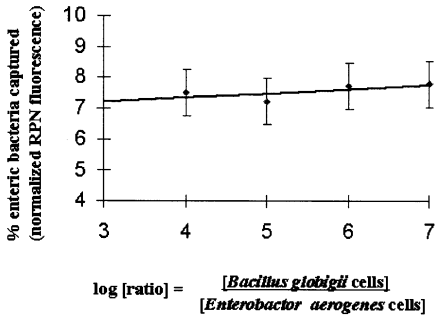

Fig. 3. Capture of *E. aerogenes* cells, using tethered hemin, from solutions containing large excesses of *B. globigii*. RPN is reduced pyridine nucleotide (NADH, NADPH).

3.1. Bacterial receptors involved in iron uptake

With the possible exception of lactobacilli, all bacteria require iron for growth. Most iron in mammals is intracellular and stored as hemin (in hemoglobin and myoglobin), but iron is also contained in non-heme proteins. Extracellularly, iron is held by transferrin and lactoferrin, two high-affinity, iron-binding glycoproteins. Once a microorganism (other than a virus) colonizes a host, it must rely on the host for its source of critical nutrients, particularly iron. Hence, enteric and pathogenic bacteria typically have receptors for iron-containing host proteins and 'free' hemin (Perry et al., 1993; Izadi et al., 1997), produce water-soluble iron chelating agents (siderophores) to strip nonheme ferric/ferrous ions out of host proteins, and produce membrane receptors for these ferric siderophore complexes (Neilands, 1984; Winkelmann et al., 1987). Most pathogens use more than one strategy for iron acquisition. We have shown that hemin, chemically tethered to a surface by an organic linker, can be used to capture mammalian pathogens and enteric bacteria (i.e. bacteria that normally colonize the gastrointestinal tract) *en masse* from solutions (Table 1). Hemin-based ligand capture is demonstrated for *S. typhi* in Fig. 2 with a

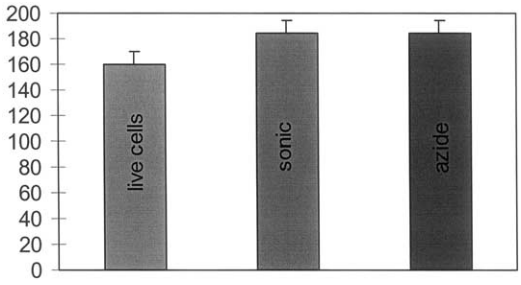

Fig. 4. Capture of *K. pneumoniae* using a tethered ferric siderophore complex, ferrioxamine B. Two hundred cells in 20 μl were applied to a chip, followed by a 5 ml water rinse. 'Sonic' and 'Azide' refer to cells killed by sonication or addition of sodium azide, respectively.

sensitivity of $\sim 10^2$ cells. Cells containing hemin receptors (*E. aerogenes*) are captured even when the sample is flooded with cells not displaying hemin receptors (*B. globigii*) as illustrated in Fig. 3. Note that the ligand sites on the illuminated surface have been saturated at all ratios.

Ferric siderophore complexes are excellent ligands for fluorescence-based cell detection because the ferric iron quenches the near-UV fluorescence of the siderophore itself. Fig. 4 illustrates the capture of *Klebsiella pneumoniae*, a food pathogen. Dead cells, produced by addition of sodium azide or sonication, are also captured as long as the cellular receptor for the ligand used as the 'bait' is intact. In favorable situations (i.e. when the sample matrix is not inordinately complex) the detection limit is 20–50 cells. While ferrioxamine B is not discriminatory in that it is recognized by many species of bacteria, there are a few instances when binding to a tethered siderophore complex can be used to infer more detailed information. These include alcaligin (*Bordetella* spp.), yersiniabactin (*Yersinia* spp.), and vibriobactin (*V. cholerae*). Interestingly, the tethered ferric vibriobactin complex is only recognized by *V. cholerae*; related vibrios, including *V. fluvialis*, *V. vulnificus*, *V. carchariae*, and *V. hollisae*, do not bind to surfaces coated with this siderophore complex.

A limiting condition for the satisfactory use of many siderophores and hemin as ligands in the capture of many bacterial species is the degree to which the cells are iron-starved. Cells taken from most infected body fluids and environmental samples will be iron-starved and thus have abundant outer-membrane receptors for iron-containing ligands. However, many types of cells (e.g., *V. cholerae*) utilize biosynthetic pathways for various receptors that recognize iron-containing species (e.g., ferric siderophore complexes, hemin) that are strongly repressed by available iron. Hence, cells taken from rich growth media (i.e., some laboratory-derived cultures) may not bind well, owing to a paucity of the relevant receptor molecules in the outer envelope of the organism sought.

3.2. Bacterial adhesins

Bacterial adhesion is an important early step in infection (Hultgren et al., 1993; Patti and Hook, 1994; Law, 1994). The organism must adhere to the host tissue, possibly to provide resistance to mechanical clearance defense systems and to provide easier access to nutrients provided by the host, and multiply to create a colony. Pathogens and enteric bacteria appear to have an abundant repertoire of adhesins, microbial surface proteins that bind to a defined host cell molecule. Most bacterial strains are capable of producing more than one type of adhesin. Typically, these outer envelope proteins recognize carbohydrate components of host glycoconjugates (glycolipids, glycoproteins). In particular, glycosphingiolipids (Hansson et al., 1985; Karlsson, 1989) at the host cell surface have been shown to function as sites for in vitro bacterial cell attachment.

Bacterial cells can also interact with ECM proteins, particularly the plasma glycoprotein fibronectin (Yamada, 1991; Westerlund, Korhonen, 1993; Olsen et al., 1993; Doig and Trust, 1993). The repetetive arginine-glycine-aspartic acid motif in fibronectin is considered to be crucial in microbial recognition of this protein, as well as other ECM proteins. Other ECM targets for bacteria include laminin, collagens, and vitronectin. Synthetic peptides mimicking motifs recognized by bacterial adhesins are also attractive ligands for applications that do not require species-specific cell capture.

3.3. Other diagnostic proteins

The key issue in developing ligands for cell capture, whether antibodies or small molecules, concerns the target antigen. It normally must be accessible to the immobilized ligand, and ideally should be present on the cell surface in many copies to enable polyvalent binding of a given cell to the coated surface. Some types of ligands, indicated above, can be used to capture microbial cells/proteins *en masse*, and uses for these include testing potable water supplies and testing for bacterial contamination in the food industry. In other cases, more detailed information is desired, particularly speciation. Detailed searches of the literature (including GenBank) are needed to arrive at good targets for ligand development. A considerable problem, as is the case with antibodies, concerns possible ligand cross-reactivity. This is a key motivation for the development of multiple ligands against a given bacterial species, and including soluble toxins as additional markers.

We have used combinatorial peptide libraries (Burritt et al., 1996; Yu and Smith, 1996; Barbas et al., 2001) to discover peptides that recognize selected antigens that are good markers for specific microorganisms (bacterial cells, viral particles, microbial toxins). For example, we have developed a 7-mer peptide specific for Protein A of *S. aureus* which, when tethered to a surface, specifically captures *S. aureus*. A second peptide, identified by another group (Sato et al., 1996), specifically captures the toxic-shock syndrome toxin-1 produced by *S. aureus*. A third peptide (Goldman et al., 2000) recognizes staphylococcal enterotoxin B (SEB). The sequences of these peptides are noted in Table 2. Using the latter two proteins as *S. aureus* markers requires that the cells in a sample be first broken open.

Table 2
Peptide sequences useful in recognizing *S. aureus* proteins

Sequence	Target	Reference
Asp-Arg-Ser-Tyr-Leu-Ser-Phe-lle-His-Leu-Tyr-Pro-Glu-Leu-Ala	TSST-1	Sato et al., 1996
Trp-His-Lys	SEB	Goldman et al., 2000
His-His Lys-His-His-His	Protein A	This work

In order to demonstrate multi-ligand capture of a single bacterial species and thus give more certainty to its identification, we produced a *S. aureus* 'chip' containing hemin, the siderophore staphyloferrin A (Konetschny-Rupp et al., 1990), the anti-Protein A peptide, and the anti-toxic-shock-syndrome toxin-1 (anti-TSST-1) peptide. No cells bind to the sector containing the latter peptide, but TSST-1 does and this toxin contains three tryptophan residues that serve as the basis for fluorescence detection. Tests with a variety of Gram-positive and Gram-negative bacteria, and cells suspended in dirty media (e.g., aqueous air filter extracts, agricultural waste water) show that this technology is capable of distinguishing *S. aureus* in a variety of conditions. The use of four independent ligands ensures that the degree of statistical certainty is much higher than with only one ligand. It is important to note that after capture, any viable cells can be grown out by simply adding a nutrient medium.

4. Conclusions

While the accompanying article focuses on the detection of microbial contamination, this article describes capture technology for the identification of microbial contamination. The capture technology provides a method of concentrating specific microbes, or specific microbial proteins, in a well-defined area on a surface. Identification is based on what sectors of the surface contain bacterial cells or soluble toxins. The fluorescence detector, described in the accompanying paper, monitors the binding of biomaterial in each sector containing a ligand and determines the nature of the

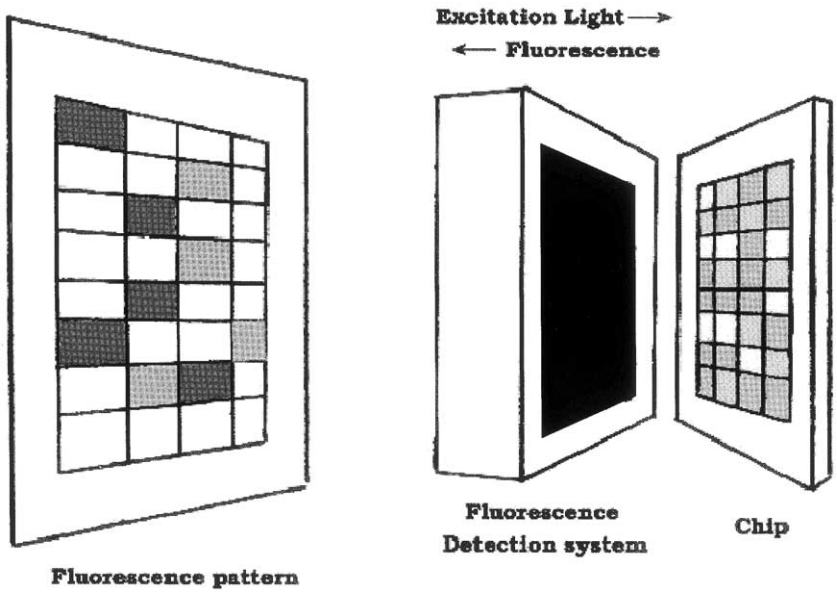

Fig. 5. Sensor for microbial contamination combining both the capture and detection (see the accompanying article) technology. Ligands are laid down in arrays on a disposable chip. Cell/toxin binding to particular sectors, and the resulting fluorescence pattern, suffices to determine what is captured.

biomaterial (e.g., live bacterial cells, dead cells, sporulated cells, or tryptophan-containing protein molecules). A simplified cartoon of a prototype capture chip and detection system is shown in Fig. 5.

Ideally, the materials used in producing chips should not fluoresce. We have noted, however, that even glasses containing small amounts of fluorescent impurities and peptides containing a single tryptophan pose little problem, provided that the detection algorithm takes these underlying signals into account.

A considerable virtue of small molecules; vis-à-vis antibodies, concerns thermal stability. Chips containing sectors coated with siderophore complexes, small peptides (<20-mers), and hemin, can be stored dry for periods of at least 6 months without refrigeration. As procedures for the chemical synthesis of oligosaccharides improve, many such oligomers will likely find use as ligands for microbial cell capture as well. Finally, we note that the orientation of small molecules with respect to a surface is more easily controlled than is the case with antibodies.

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