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# Methods for Whole Cell Detection of Microorganisms

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**Abstract**
Microbes are ubiquitous, and can be found occupying nearly every imaginable niche on Earth. These include organic and inorganic surfaces, interfacial boundaries and within macroscopically solid matrices, such as the pore space of rocks. Because phylogenetically divergent microbes may be visually indistinguishable, understanding the species distribution and ecological significance of environmental microbes requires diagnostic tools that extend beyond simple phenotypic description. Methods for microbial diagnostics can be divided into two broad categories: cellular and acellular. Acellular techniques, such as the polymerase chain reaction or certain immunoassay formats may be effective at detecting molecular, structural or biochemical targets associated with specific cell types, but this information is provided out of its "natural", and arguably most meaningful context — that of the individual microbial cell. In contrast, cellular methods have the potential to preserve an abundance of valuable information. Apart from molecular identity, this includes information regarding cell morphology and other physiological characteristics, cell number and distribution within a sample, and physical or spatial associations with other cell types. The aim of this chapter is to provide an overview of whole cell methods for microbial detection, including both existing approaches and those still in development. The tools described here are expected to find wide application for the detection of microbes on surfaces or within complex matrices across a number of parallel or allied fields, including environmental, food and clinical microbiologies.

**Disciplines**
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## Chapter 3

# Methods for Whole Cell Detection of Microorganisms

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Microbes are ubiquitous, and can be found occupying nearly every imaginable niche on Earth. These include organic and inorganic surfaces, interfacial boundaries and within macroscopically solid matrices, such as the pore space of rocks. Because phylogenetically divergent microbes may be visually indistinguishable, understanding the species distribution and ecological significance of environmental microbes requires diagnostic tools that extend beyond simple phenotypic description. Methods for microbial diagnostics can be divided into two broad categories: cellular and acellular. Acellular techniques, such as the polymerase chain reaction or certain immunoassay formats may be effective at detecting molecular, structural or biochemical targets associated with specific cell types, but this information is provided out of its "natural", and arguably most meaningful context – that of the individual microbial cell. In contrast, cellular methods have the potential to preserve an abundance of valuable information. Apart from molecular identity, this includes information regarding cell morphology and other physiological characteristics, cell number and distribution within a sample, and physical or spatial associations with other cell types. The aim of this chapter is to provide an overview of whole cell methods for microbial detection, including both existing approaches and those still in development. The tools described here are expected to find wide application for the detection of microbes on surfaces or within complex matrices across a number of parallel or allied fields, including environmental, food and clinical microbiologies.





In Microbial Surfaces; Camesano, T., et al.;
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## Introduction

Whether our interests lie in preventing or diagnosing disease, ensuring the productivity of biotransformative processes such as industrial and food fermentations or monitoring the quality of basic natural resources such as surface waters or soil, detection of microorganisms is an essential process. Microbes inhabit almost every surface niche imaginable, including soil particles, mineral veneers on desert rock surfaces, the worn stone surfaces of Roman catacombs and other monuments, the pore space *within* rocks, liquid inclusions within Arctic sea ice, plant surfaces or structures such as stems, leaves and roots, animal surfaces such as skin and teeth, food surfaces or digesta particulates within the gut, or manmade structures such as indwelling medical devices (*1-8*).

Although it has been over a century since Robert Koch first described the use of solid media for isolating microorganisms in pure culture (*9*), it has really only been within the last 20 years that microbiologists have been able to begin stepping outside the primarily phenotypic, "plate and see" framework developed by Koch and his contemporaries (*10, 11*). In this time, the rapid growth of the field of molecular microbiology has fueled an explosion of new methods and capabilities for detection of microbes (*10, 12*). These include methods for *in situ*, culture-independent detection of specific microbial cells and the tools of microbial forensics, which enable epidemiological tracking of pathogens from foodborne disease outbreaks and identification of natural (or deliberate) sources of environmental contamination (*13-15*). Such molecular techniques typically test for nucleic acids (rDNA, other genomic DNA sequences, mRNA, etc.) extracted in bulk from a sample, obviating the need for first isolating the target organism. Although the capacity to detect cell-specific nucleic acids without the need for culture may be considered an advantage of such acellular techniques, it is not without cost. The individual cell is the "...fundamental unit of biological organization..." (*16, 17*). Additional layers of information are therefore intrinsically linked to the "granular" or corpuscular nature of microbes. These include cell number, position or distribution within a sample, co-localization with other cell types, attachment to or interaction with specific substrates, as well as more ephemeral data such as biochemical activities or behavioral properties of individual cells (*12, 17*). This chapter seeks to provide a basic overview of the methods available for whole cell microbial detection and their use in the allied fields of environmental, food and clinical analysis. Special emphasis will be given to fluorescence in situ hybridization (FISH), but additional whole cell approaches and their merits or limitations will also be discussed. Apart from providing convenience, rapidity is not necessarily an essential feature of methods focused on environmental microbiology. However, in clinical and food applications, timely results are paramount, as the health of a patient or the safety of the food supply are at stake. Because of its importance to these disciplines, rapidity will be a recurrent subtext in this chapter.

# Microbial Habitats – The "Environment"

Microorganisms are indispensable coinhabitants of our ecosphere, responsible for driving the basic global geochemical cycles upon which all life depends (*18*). The field of environmental microbiology is tasked with investigating and describing the interactions between microbes and the physical spaces they inhabit. Typically, the word "environment" evokes familiar images from the natural world – surface waters, soils or sediments, the phyllosphere, hot springs and deep sea thermal vents, for example. Given microbial proclivities for filling and thriving in just about any exploitable space available, the word "environment" can also be extended to include such man-made environments as municipal wastewater treatment facilities (activated sludge), metalworking fluids, tannery effluents and acid mine drainages. As hosts to naturally-occurring assemblages of microbiota, our bodies may also be considered from an environmental perspective, with infectious disease representing an example of an ecosystem out of balance. Ecological concepts have also been applied to food systems (*19, 20*). As with our bodies, the foods we eat may be populated with a "natural flora" – the expected successions and end compositions of microbial populations in fermented foods, for example. These foods may also contain unwanted, or "invasive" species such as pathogens or spoilage flora.

These conceptual parallels between the fields of environmental, food and clinical microbiologies are mirrored in the practical features of these disciplines, with many similarities in the goals, tools and protocols existing between them (*19*). Of course, there are differences – environmental microbiology is typically more basic in nature, approaching the "big problems" of what large assemblages of microorganisms (potentially *thousands* of different species) are doing *in situ*, and how these activities impact on basic global processes such as geochemical cycling (*12, 18*). Thus, environmental microbiology incorporates methods for both microbial detection and ecophysiological characterization (*12*). Food and clinical microbiologies usually deal with more applied problems, such as detection of a relatively few cell types within foods or clinical specimens. Still, certain themes or phenomena cut across the boundaries of these disciplines. Biofilms, for example, are the most prevalent mode of microbial growth in nature (*21*). As such, they are a recurrent theme not only in the natural world, but also impact the food processing and health fields. Biofilms present challenges to effective cleaning of food processing environments and equipment, contribute to the establishment and persistence of oral infections or cystic fibrosis and may be important reservoirs of infectious disease through colonization and growth on indwelling medical devices (*22, 23*). Recognition of the overlaps between these three branches of microbiology can allow the advantageous adaptation of techniques for detection or characterization developed for one field for use in another. Examples include the adaptation of culture-independent approaches for

studying the *in situ* population composition and diversity originally developed for environmental microbiology for use in studying microbial populations in foods (*19, 24*).

## Nucleic Acid-Based Methods: Generating Sequence-Specific Fluorescence Signals Within Whole Microbial Cells

The attraction of whole cell diagnostic methods is that they link detection of pathogen-specific markers to their cellular context, providing additional layers of information beyond simple "presence/absence" determinations. Over the past decade, several distinct methods for generating sequence-specific fluorescence signals within intact bacterial cells have been developed, primarily for environmental microbiology applications. These include *in situ* PCR (*25-29*), *in situ* reverse transcription-PCR (*30-32*), *in situ* reverse transcription (*33*), chromosomal painting (*34, 35*), *in situ* rolling circle amplification (*36, 37*), *in situ* loop-mediated signal amplification (*38*) and fluorescence *in situ* hybridization (FISH) (*39-43*). Descriptions of each method are given below, followed by a discussion of their potential benefits and drawbacks for use in routine diagnostics in environmental, food and clinical microbiology applications. Although presented together, these methods differ widely in assay complexity, sensitivity of detection and other factors, such as their abilities to localize signals within microbial cells. Additionally, many of these approaches are "homebrew" assays, rather than being commercially available in kit form, and are therefore realistically accessible only to specialist laboratories. However, some technologies such as DNA or PNA-FISH and rolling circle amplification (RCA) are now available commercially. The assay validation, reagent quality control and technical support that are available with these commercial kits will make these technologies more accessible to a wider user base.

At its simplest, FISH is likely the best candidate for robust, routine application in simple molecular diagnostics for environmental, food and clinical applications. This is because it does not require complex, multi-step, multi-component protocols involving potentially capricious reagents such as enzymes. Given the intrinsic physical restrictions of microscopic examination, only a relatively few cells can be examined on the surface of a microscope slide (*36, 44*). Therefore, any method that is restricted to a slide-based format may face substantial limitations in detection sensitivity. In-solution methods, such as FISH, can be combined with higher-throughput methods for liquid-phase analysis such as flow cytometry. This enables a larger portion of the sample to be screened, allowing increased detection sensitivities, even in the presence of high backgrounds of non-target cells or debris. However, as with any approach, FISH does have its limitations. Typically, FISH is used to target relatively high copy-

number targets within the cell. For cells containing limited copies of the target molecule, such as starved or dormant cells, standard FISH protocols may not be sensitive enough to ensure detection of all cells. However, it has been nearly 20 years since its first description for use in detecting bacteria (*39*), and in recent years, several variant FISH techniques have been developed to target lower copy-number targets, thereby pushing the resolution of this approach toward low-copy and single copy (e.g. genomic) sensitivities (*43*). These adaptations and improvements of the FISH approach will be described briefly in a later section. Other key technologies for generating sequence-specific fluorescence signals within whole microbial cells are discussed below. Typically, these have been developed for detection of bacteria, but in principle may also be used for detection of other microorganisms, with few, if any modifications.



## *In Situ* PCR and *In Situ* Reverse Transcription-PCR

For *in situ* PCR (ISPCR) or *in situ* reverse transcription-PCR (ISRT-PCR), amplification of a target sequence using appropriate primers and fluorescently-labeled dNTP's results in the production of a labeled PCR product within the cell (*25*). Alternatively, digoxigenin-labeled dNTP's or biotinylated primers may be used and the amplicon detected with fluorescently-labeled or enzyme-labeled antibodies specific for digoxigenin or biotin (*29, 30, 32*). Although these methods can be used for the detection of specific microorganisms at the genus, species or subspecies level (*25, 27*), their true strength lies in their abilities to detect low-abundance targets, such as single-copy genes or mRNA, which are below the sensitivities of methods such as (conventional) FISH (*29, 32*). From a standpoint of practicality, these analyses are complex and time-consuming, and involve multiple steps in addition to cell fixation, including cell immobilization on glass slides, permeabilization with lysozyme, protease and/or RNase digestions, the PCR reaction itself, and post-PCR detection of labeled amplicons with fluorescently-labeled antibodies or enzyme-based fluorescent signal amplification steps (*29, 30, 32*). In light of this, ISPCR and ISRT-PCR are much better suited to fine-structure analyses such as examining the distribution of a gene in representative samples of a population or following the expression of specific mRNA's than for routine identification of bacterial species (*29, 30, 32*).

## *In Situ* Reverse Transcription

*In situ* reverse transcription (ISRT) (*25*) is an isothermal method for amplification of rRNA or mRNA targets within target cells for fluorescence detection. In this method, cells are fixed, spotted onto microscope slides, permeabilized and hybridized with unlabeled DNA primers specific for the

desired target within the cell. After hybridization, reverse transcriptase, RNase inhibitors, and a mixture of unlabeled and fluorescently-labeled dNTP's are added. Reverse transcriptase activity results in primer extension and the incorporation of fluorescently-labeled dNTP's into the resulting extension products. Cells in which successful extension has taken place are fluorescent and can be detected by fluorescence microscopy or other suitable methods.

Although ISRT does not result in the exponential amplification of target RNA, this method can still yield bright fluorescence without thermal cycling because multiple fluorophores are incorporated into the extension product (*25*). ISRT may be especially useful for the detection of multi-copy targets such as rRNA where target levels are below the detection limits of methods such as FISH, as may be the case with dormant cells. However, under conditions where rRNA levels were high enough to yield adequate FISH signals, Hodson et al. (*25*) reported that cells labeled through ISRT were not substantially brighter than those labeled using FISH. Although ISRT is considerably less complex than ISPCR or ISRT-PCR, the relatively modest gains in signal strength vis-à-vis FISH argue against its routine use for detection of target cells under conditions where they are expected to be actively-growing (and therefore detectable via FISH) (*25*).



## Bacterial Chromosome Painting

Another method for generating a sequence-specific fluorescence response within whole cells is bacterial chromosome painting (BCP) (*34, 35, 43*). In this method, chromosomal DNA from the organism to be targeted is randomly digested with restriction enzymes, yielding an undefined mixture of fragments ranging from 50 to 200 bp. This complex pool of DNA fragments is then enzymatically labeled with fluorescent reporter molecules and the resulting fluorescent probes are hybridized against formalin-fixed, RNase-treated cells spotted onto microscope slides. Because the target, chromosomal DNA, is present in all living cells, BCP should be applicable to fast-growing, as well as dormant cells (*34*). BCP is able to differentiate closely-related bacteria, but prehybridization with unlabeled chromosome digests of non-target *Salmonella* (chromosome *in situ* suppresson) is required to differentiate between *Salmonella* species (*34*). A major drawback of BCP is the time required to achieve a sufficient signal. Reassociation rates for long nucleic acid probes are inversely proportional to the sequence complexity of the probes being hybridized (*45*). Because BCP relies on a large set of probes generated from random genomic digestion, the sequence complexity is high and the reassociation rate is very low, requiring 2 days to yield adequate signals from target cells (*34, 43*).

## *In Situ* Rolling Circle Amplification

Rolling Circle Amplification (RCA) is an isothermal method for nucleic acid amplification that targets short sequences (~ 40 or fewer bases) and generates long, single-stranded amplicons comprised of tandemly repeated sequences that can subsequently be detected via hybridization (*36, 37, 46*). In standard RCA techniques, amplicon production proceeds in a linear fashion; however, modifications of this technique variously termed "ramification", "cascade" or "hyperbranched" RCA have been developed to yield geometric amplicon production (*46*). In the RCA reaction, a circulizable probe is hybridized to its target on a plasmid or on the chromosome, then the circle is closed via ligation. A primer then directs DNA polymerase to extend the circular sequence progressively around the circle, leading to the formation of the long linear product of tandem repeats (*36, 37, 46*). The population of products formed is generally distributed over a wide range of lengths, typically appearing as a continuous smear of high molecular weight DNA when viewed on an electrophoretic gel (*46*). Detection of the tandemly repeated sequences in the amplicon is then facilitated using fluorescently-labeled detector or "decorator" probes (*36, 37, 47*). For *in situ* RCA, this entire process is carried out within the context of whole bacterial cells. As with the other *in situ* amplification processes mentioned above, this entails using a complicated protocol involving multiple steps (washes, enzyme digestions, hybridization, ligation, etc.). Therefore, to avoid cell loss, cells are first attached to a membrane filter, then embedded in agarose (*36*). Next, cells are permeabilized with lysozyme and proteinase K and RNA is removed via digestion with DNase-free RNase (*36*). Ligation reagents are added, the sample is incubated on ice and given time (15 min) to diffuse into the cells, followed by the ligation reaction (90 min) to form circulized probes within the cells (*36*). After buffer and distilled water rinses, the cell-containing membranes are then dehydrated in an ethanol series and dried. Next, RCA reagents are added and again incubated on ice (15 min) to allow perfusion into the cells, followed by the isothermal RCA reaction at temperatures appropriate to the amplicon (55°C - 63°C in the example provided by Maruyama et al., 2005, reference 36) for 90 min, followed again by rinsing and final detection of the amplicon via a FISH reaction utilizing a 16 h incubation at 46°C (*36*). Because the RCA conditions are relatively mild (compared to those of *in situ* PCR, for example), additional whole cell analyses, such as antibody-based staining may also be performed on cells after RCA, enabling correlation of immunophenotypic and genomic data (*36*). Although complicated, *in situ* RCA provides a method for detection of low-copy gene sequences within whole bacterial cells. The ability to detect single-copy genes carried on plasmids or on the chromosome provides additional discriminatory power, allowing target cells to be distinguished from closely-related, non-target cells. For example, Maruyama et al. (*36*) were able to detect the single-copy Shiga-like toxin ($stx_1$)

gene on the chromosomes of enterohemmoragic *E. coli*. Techniques such as this, which are able to detect pathogen-specific virulence factors, will enable detection of pathogenic variants of these bacteria, distinguishing them from "garden variety" strains of little or no public health impact.

## *In Situ* Loop-Mediated Isothermal Amplification

Maruyama and colleagues have also adapted another isothermal sequence amplification method for the *in situ* detection of low-copy sequences within whole, permeabilized bacterial cells. As originally described, loop-mediated isothermal amplification of DNA (LAMP) is a method capable of quickly amplifying select DNA sequences, enabling production of up to $10^9$ copies within one hour (*48*). The LAMP procedure relies on a self-sustaining strand displacement reaction carried out by a DNA polymerase having high strand displacement activity, in this case the *Bst* large fragment from *Bacillus stearothermophilus* (*38, 48*). The technique uses up to six distinct primers that hybridize with the target sequence to create the topologically unique nucleic acid structures used for amplification (*38, 48*). As adapted for *in situ* detection of target genes within bacterial cells, only four LAMP primers were required and specific amplification products could be detected either via FISH with amplicon-specific probes, or via incorporation of fluorescently-labeled dCTP during amplicon formation (*38*). As with *in situ* RCA, *in situ* LAMP has no requirement for complicated, expensive cycling equipment and the relatively low temperature used (63°C) is potentially less destructive to delicate cell structures, preserving cell morphology and diagnostic epitopes (*38*).

## Fluorescence *In Situ* Hybridization

The last method considered here is fluorescence *in situ* hybridization (FISH), first described for bacteria in 1989 (*39*). The FISH technique (also aptly termed "phylogenetic staining") uses fluorescently-labeled nucleic acid probes targeted to complementary rRNA targets located on ribosomes within intact cells. An advantage of targeting rRNA is that it is a multi-copy target - several thousand copies are typically present within active microbial cells (*44*). The cumulative signal from multiple probe-target binding events after hybridization provides the basis for sequence-specific fluorescence of target cells. Of all the techniques discussed here, FISH is by far the most straightforward and least complex. As described above, unlike ISPCR, ISRT-PCR, ISRT, *in situ* RCA or *in situ* LAMP, FISH does not depend on enzymatic activity (e.g. DNA polymerase or reverse transcriptase) to generate a detectable product. Unlike BCP, FISH uses well-defined probes or probe sets and hybridizations can be

carried out in a fraction of the time needed for BCP. Additionally, FISH can be performed in solution, allowing the subsequent analysis of a large number of cells, especially if hybridization results are analyzed by flow cytometry (*49, 50*). A typical FISH protocol involves the following steps: harvesting of cells (via centrifugation or filtration), fixation (this step has multiple purposes, including permeabilization of cells to probes, preservation of cell morphology, inactivation of endogenous enzymes, prevention of target molecule leakage from cells), hybridization (heating of cells in the presence of probe(s) in a simple buffer system containing salt and detergent), washing (used to remove excess, non-specifically bound probe – not always required, depending on the properties of the probe used and the sample being tested), harvesting cells again via centrifugation, and detection of hybridized cells via microscopy or another appropriate method. Because of its simple and streamlined nature, FISH can be used to achieve rapid molecular detection of target cells in complex matrices such as natural waters, food or bodily fluids (*51-54*).

Although most DNA-based FISH reactions are typically carried out for several hours at relatively low temperatures (e.g. 3 h at 46°C) (*42, 44, 51*), these parameters can be adjusted to enable more rapid detection of bacteria, particularly with Gram-negative bacteria, which are relatively easy to permeabilize (*53, 54*; Brehm-Stecher and Johnson, unpublished data, **Figure 1**), although this approach has also been used successfully for rapid on-slide hybridizations of certain Gram-positive bacteria, such as the 5 min hybridization of *Streptococcus pneumoniae* reported by Jansen et al. (*55*).

FISH has been shown to be remarkably robust in its ability to detect bacterial cells exposed to stressors typical of food processing environments, including high salt, low pH and low or freezing temperatures (*51*). In some circumstances, however (e.g. environmental applications), FISH-based detection of target cells may be complicated by poor signal quality. This can occur with poorly-permeabilized cells, with cells having an intrinsically low rRNA content, with starved or stressed cells containing fewer or degraded ribosomes (*42, 44, 56*) or in cells displaying low signal-to-noise ratios due to sample autofluorescence (*57*). In these situations, a more efficient means of permeabilization, an enrichment or repair step, or some means of signal amplification may be needed in order to detect FISH signals from target cells. Alternatively, probes having improved properties of penetration or binding, such as peptide nucleic acid probes (PNAs) may be used to enhance the sensitivity of the FISH approach.

### *Specialized Applications of FISH*

A key drawback to FISH, as it is typically used, is its inability to detect low-copy or single-copy targets. As discussed above, this does not pose a problem

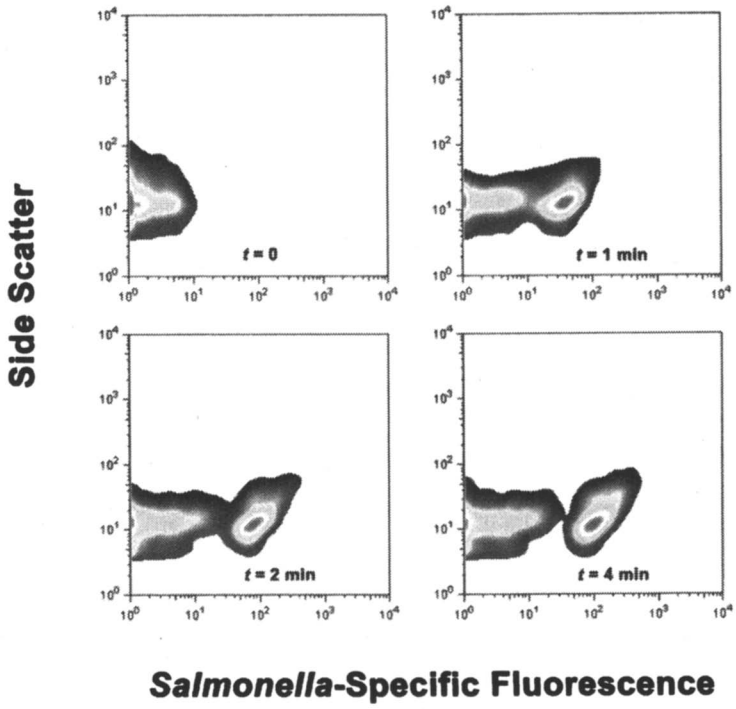


*Figure 1. Detection of Target Cells From Within a Complex Mixture Via Rapid DNA-FISH and Flow Cytometry. A complex mixture containing* Salmonella typhimurium *and several strains of closely-related non-target bacteria (*Escherichia coli, Citrobacter freundii, Proteus vulgaris *and* Shigella dysenteriae*) was fixed with 10% buffered formalin and hybridized at 55 °C for up to 4 min with an rRNA-targeted DNA probe (Sal3-Cy5, 5ng* $\mu l^{-1}$*). The progress of the hybridization reaction was examined via flow cytometry (FACSCalibur, BD Biosystems) at the following intervals: prior to hybridization [*t *= 0 min] and after 1, 2 or 4 min hybridization. After only one minute, the subpopulation of* Salmonella *was easily resolved from the background flora (geometric mean fluorescence of* Salmonella *population = 46.3). At two minutes, the probe-conferred fluorescence of the* Salmonella *population increased further (geometric mean fluorescence of* Salmonella *population = 85.6);* Salmonella*-specific staining with this probe reached near-maximal value after four minutes (geometric mean fluorescence of* Salmonella *population = 111). These data highlight the rapidity of DNA-FISH as a means for genotypic detection of specific microbial cells within complex mixtures.*

for use of this technique to detect rRNA-encoded targets, as the ribosome is a naturally-amplified target molecule. However, not all microorganisms can be identified successfully on the basis of "signature" 16S or 23S rRNA sequences. For example, typical FISH formats cannot be used to discriminate toxigenic *E. coli* from non-pathogenic *E. coli*, as the virulence determinants carried by this pathogen are chromosomally encoded. Apart from rRNA, additional targets for FISH analyses have been explored, including other forms of RNA (e.g. tmRNA, mRNA) and plasmid DNA (*43*). Although these are multicopy targets, the low abundance of these species requires the concurrent use of some method for signal amplification, such as catalyzed reporter deposition (CARD) (*58, 59*). Recent breakthroughs in FISH technology using polynucleotide probes now allow the detection of low copy (< 10 copies per cell) targets within individual cells (*43*). These probes not only hybridize to their targets within the cells, but also apparently interact with each other to form networks outside of the cell. With such networks "anchored" to the cell via specific hybridization with internal targets, excess, non-specifically bound probe can be removed via washing. With each probe containing multiple fluorophores, this external "mesh" of probes serves as a means for signal amplification, allowing detection of targets present at very low copy number (*43, 56*).

Additional modifications and variations that have expanded the capabilities of the FISH technique for environmental and clinical applications include combined microautoradiography and FISH (MAR-FISH) (*12*), which enables correlation of substrate uptake with cell type, and FISH-based detection of antibiotic susceptibility (*60*). This latter approach takes advantage of the fact that in certain pathogen-antibiotic pairs (*Helicobacter pylori* and clarithromycin, for example), antimicrobial resistance can be traced to point mutations on the 23S ribosomal subunit, which can be detected via FISH using mismatch-sensitive probe sets (*60*). Similar possibilities may also exist for other pathogen-antibiotic pairs, and a searchable database (the Ribosomal RNA Mutation Database) has been described, which could facilitate development of similar tests (*61*).

These innovations have pushed the envelope of what is possible using the FISH technique. Outside of environmental applications, though, most microbial testing requirements do not extend beyond presence/absence testing or detection and enumeration of a single target cell type. Although "classic" or traditional FISH is not as exquisitely sensitive as some of the other available methods for generating sequence-specific fluorescence signals within whole microbial cells, it is arguably the simplest and most robust method for doing so. As such, it can be readily used for routine detection of target cells within environmental samples, foods or clinical specimens, providing a rapid and sensitive means for molecular detection of pathogens in these samples. For more detailed background on the FISH technique, readers are referred to several excellent reviews, which cover everything from the basics of the process, to food, clinical and environmental and other specialized applications of the method (*40-44*).

*Combining FISH & Flow Cytometry*

A problem central to environmental, food and clinical microbiologies is the detection of specific microorganisms within physically and microbiologically complex sample matrices. Environmental samples, contaminated foods, infected tissues or bodily fluids or liquid dilutions made from such samples may contain high loads of particulate matter or non-target microflora, both of which present challenges to the direct detection of target cells. Flow cytometry is a general detection platform that enables the rapid, multiparametric analysis of complex microbial populations. In flow cytometric analysis, liquid samples are taken up and hydrodynamically focused to form a laminar flow within a surrounding sheath fluid (usually phosphate buffered saline). Within this stable stream, the cells (ideally) form a single file line and tumbling or other potentially interfering movements are minimized. The cells are then passed through the "heart" of the flow cytometer - the flow cell – where they are illuminated with a high-intensity light source, typically a laser or laser diode. Detectors and appropriate filter sets are then used to collect data on cellular responses, including forward angle light scatter (providing information on cell number and some size information), side angle scatter (providing information on the internal content, or opacity of the cells) and probe or dye-conferred fluorescence characteristics. As a multiparameter technique, flow cytometry can be used to distinguish between cellular and acellular particles, and when combined with an appropriate molecular probe, to detect specific microbial cells.

The combination of FISH and flow cytometry (FISH-FC) has found wide use for the analysis of complex samples in environmental, food and clinical applications. An example of this for environmental analysis is combined FISH and Fluorescence Activated Sorting (FISH-FACS) for the detection and sorting-based enrichment of specific cell types from natural samples. Kalyuzhnaya *et al.* (*62*) used this approach to examine methanotrophic bacteria from lake sediments and Sekar *et al.* (*63*) used similar methods to investigate the composition of marine bacterioplankton communities. Post-sorting genomic techniques were used in both cases for additional sample characterization (*62, 63*). Food applications of FISH-FC include the use of a *Pseudomonas*-specific DNA probe to detect this spoilage organism in milk (*64*) and detection of *Salmonella* spp. in raw, cubed pork using DNA probes specific for this genus (Bisha and Brehm-Stecher, unpublished data, **Figure 2**). Perhaps the ultimate test of this technique's performance in a "noisy" environment (and linking both food and clinical analyses) is the use of FISH-FC to quantify specific cell types in feces (*65, 66*). As FISH is a culture-independent technique, this approach may be used to study the ecology of unculturable gut microflora (*66*). In the clinical realm, FISH-FC using both DNA and PNA probes has been explored for rapid detection of pathogens in blood cultures, including Gram-negative rods, *Staphylococcus aureus* and *Candida* spp. (*52, 67*). Access to rapid, robust and

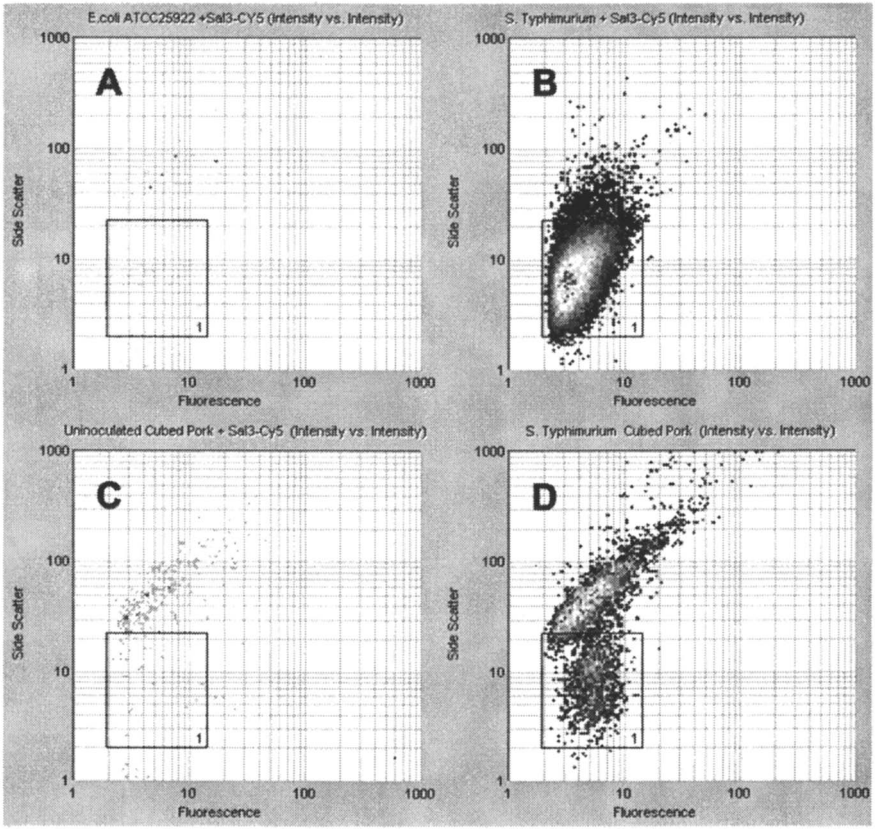


*Figure 2. Direct Cytometric Detection of* Salmonella *spp. in Raw, Cubed Pork Meat Using Combined FISH and Flow Cytometry. This series demonstrates the specificity of fluorescence in situ hybridization (FISH) combined with flow cytometry for the rapid detection of* Salmonella *spp. in raw meat. Hybridization conditions were similar to those used in Figure 1. Panel A shows a high load of* Escherichia coli *hybridized with a* Salmonella*-specific DNA probe (control for non-specific staining of non-target cells). Panel B shows a pure culture of* S. typhimurium *hybridized with the probe (positive control for target cells). Panel C shows uninoculated cubed pork hybridized with the probe (control for non-specific staining of food particles). Panel D is a sample of cubed pork containing* $10^5$ *CFU/ml* S. typhimurium *hybridized with the probe. These data demonstrate that although there is some interference from non-specific binding of the probe to food particles, target cells can still be easily discriminated from such background on the combined basis of scatter and fluorescence signals. An RBD-3000 cytometer (Advanced Analytical, Ames, IA) was used for these analyses.*

culture-independent methods for pathogen detection or surveillance is expected to be important to this sector, as the benefits of rapid clinical diagnostics have been clearly identified in terms of both cost savings and improved patient outcome (reduced mortality) (*68, 69*).

With applications in environmental, food and clinical microbiologies, FISH-FC is a powerful combination, allowing the study of complex natural populations in the environment, as well as rapid detection of pathogens in both foods and clinical samples. Harmonization of FISH-FC protocols between the latter two areas should enable the detection of foodborne pathogens throughout the consumption-to-disease continuum, potentially improving food safety through disease intervention and patient outcomes through more timely diagnoses of disease. The availability of a new generation of smaller, task-dedicated cytometers designed specifically for microbial detection may also help speed the adoption of FISH-FC for more routine use by food and clinical microbiologists (*52*).



## Biomimetics

Literally defined, "biomimetics" implies the direct copying of biology. More precisely defined, the term refers to nature-inspired design of molecules, materials or devices (*70*). Biomimetic approaches have been successfully applied for the development of antifouling "superglues" based on mussel adhesion strategies (*71*), artificial antibodies and enzymes ("plastibodies" and "plastizymes") based on molecular imprinting techniques (*72*) and synthetic antimicrobial polymers that mimic the structure and function of host defense peptides (*73*). Using examples from nature as templates, new and advantageous synthetic analogs can be made, ranging in scale from molecules to materials. In molecular biology, biomimetic bioaffinity reagents are synthetic molecules that interact with natural ligands, including cell surface structures, proteins and nucleic acids. Because they may incorporate "unnatural" or artificial elements of design, such as use of a synthetic polymer backbone or scaffold, rather than naturally-occurring biopolymeric scaffolds, these reagents may be engineered to have very different and advantageous properties. Examples may include chemical or physical robustness vis-à-vis their biopolymeric counterparts, different charge densities or chiralities and the ability to modulate and "fine tune" biological activity using combinatorial approaches, ultimately yielding novel synthetic biorecognition reagents having distinct advantages over their natural counterparts.

Peptide nucleic acids (PNAs) have emerged as key biomimetic reagents for use in nucleic acid-based diagnostics. PNAs are synthetic DNA mimics made by grafting either natural or non-natural nucleobases onto a repeating backbone of

amide-linked *N*-(2-aminoethyl) glycine (AEG) units (*74*). Like DNA probes, PNAs hybridize to complementary DNA or RNA sequences via Watson-Crick base pairing, but their uncharged, hydrophobic backbones confer several advantageous properties over DNA-based probes. These include intrinsic resistance to enzymatic degradation (they are not natural substrates for either nucleases or proteases), faster hybridization kinetics, higher binding affinities for their targets, and the ability to penetrate "difficult" biological structures such as the exosporium of freshly-germinated bacterial endospores, the cysts of parasites such as *Cryptosporidium* and *Giardia* (Jens Hyldig-Nielsen, personal communication) and the thick cell walls of bacteria such as *Mycobacterium* and *Listeria* spp. (*49, 75, 76*). An additional advantage of PNA probes is their capacity for binding to portions of the ribosome that are physically inaccessible to traditional DNA probes, enabling detection of organism-specific diagnostic sequences that are otherwise "buried" in the higher-order structure of the ribosome (*49, 50, 74, 77*). This latter property stems largely from the fact that PNA probes are typically hybridized under low salt (0 – 100 mM NaCl), high temperature (55°C or higher), high pH (pH 9.0) conditions that destabilize the higher order structures of target nucleic acids. Together, these properties give PNA probes unique advantages as FISH probes over conventional DNA-based probes for whole cell detection of several important classes of microorganisms. As an interesting aside, Nelson et al., (*78*) have demonstrated the formation of AEG from methane, nitrogen, ammonia and water in electric discharge reactions designed to simulate conditions present in Earth's primordial atmosphere. From these data, PNA has been suggested as being the first molecule of heredity, serving as a potential bridge from the pre-RNA world to the RNA world (*78*)

Additional nucleic acid mimics having applications in whole cell diagnostics include locked nucleic acids (LNAs). LNAs are conformationally-restricted ("locked" via 2′-*O*,4′-*C*-methylene linkages) ribonucleotide derivatives that have been developed in recent years (*79, 80*). Advantages of LNAs include rapid hybridization, very high thermostabilities for LNA-DNA or LNA-RNA hybrids (~ 8 - 10°C increase in melting temperature per LNA residue), high water solubility (often problematic with PNAs) and the ability to combine DNA and LNA monomers to form chimeric "mixmer" molecules (*80*). This latter capability enables greater control of probe melting temperature and allows placement of LNA moieties within a probe where they can potentially be used to confer position-specific effects (*80, 81*). Like PNAs, LNAs have been used advantageously in acellular diagnostics as capture probes and reporter probes in real-time PCR, but recent reports have also focused on their use in whole cell detection as FISH probes (*81, 82*). In this regard, they have been reported for use to detect sequences on lymphocyte metaphase chromosomes and interphase nuclei (*82*) and for increasing the hybridization brightness of probes directed against Gram-negative bacteria such as *Escherichia, Anaerolinea and Comamonas* spp. (*81*). As charged molecules, however, it will be interesting to

see if LNAs can be used to target "difficult" microbes with the same facility as is currently done using PNAs.

Several approaches to the development of antibody-like (and therefore biomimetic) binders have also been described in recent years. These include bioimprinted sol-gel polymers for binding whole yeast cells (*83*), peptide-based binders (*84, 85, 86*) and "affibodies" – non-antibody binders derived from randomizable small protein scaffolds (e.g. staphylococcal protein A) (*87, 88*).

Biomimetic affinity reagents such as these are expected to play an important role in future molecular detection strategies, as they have the capacity to mimic or improve upon "natural" ligand-receptor interactions, can be engineered for unique physicochemical properties or target specificities and may be combinatorial in nature, enabling "biopanning" or other similar discovery strategies, leading to rapid generation of new pathogen-specific binders or disease diagnosis without the need for prior knowledge of target sequence, or for emerging diseases, of the pathogen responsible for symptoms (*89*).



## High-Throughput Methods for Reagent Discovery

Combinatorial or library-based methods for discovery of binding partners have several advantages over manual, "hunt and peck" approaches. First, a comparatively large dataspace can be searched for bioaffinity reagents that bind specifically to the target cell or desired cell surface feature. Second, libraries containing combinatorially synthesized variants of a parent compound can be screened to identify those variations that contribute to a desired property (binding specificity or avidity, for example) and these features can be incorporated into subsequent molecular design in order to refine or focus the activity of the compound. Bioinfomatics tools can also be used to mine large virtual dataspaces (e.g. the continually growing rRNA sequence dataspace) to assist in or automatically identify group- or species-specific oligonucleotide probes or primers, speeding what once was a fully manual or database-assisted process (*90-93*). Multiple layers of *in silico* mining and *in vitro* selection processes can be combined using high throughput wet chemistry evaluation to create a discovery pipeline for microbial signature sequences. In this way, many of the same informational tools developed for use in the Human Genome Project can also be marshaled to feed a discovery pipeline leading to new molecular diagnostics for bacteria and viruses (*94*).

## Antimicrobials as Detection Reagents; Advantages of External Binders

In order for antimicrobial peptides (AMPs) to exert their actions on microbial cells, they must first bind to structures on the surfaces of target cells

(*85*). With this in mind, several researchers have exploited the selective binding properties of naturally-occurring AMPs for use in bacterial capture or immobilization (*84, 85, 95*). For example, Kulagina et al. (*85*) used magainin I as a capture reagent for a proof-of-concept silanized glass array detector targeting *Salmonella* and *E. coli* O157:H7. Blais et al. (*95*) used the AMP polymyxin B, which has been well-characterized for its interactions with lipopolysaccharide (LPS) of the Gram-negative outer membrane, to selectively bind Gram-negative bacteria in a microtiter-based assay. A subsequent immunochemical reaction with serotype-specific antibodies enabled detection of *E. coli* O111 and O26 serotypes in foods. Benefits of using polymyxin as an adsorbant in this type of application include its high-binding capacity for the LPS of Gram-negative bacteria, its chemical stability, low cost and ready availability (*95*). The availability of combinatorially synthesized biomimetic antimicrobial polymers (*73*) opens the exciting possibility for their development as next-generation binders. If this approach proves feasible, the potential plasticity in chemical, physical and binding properties offered by such reagents could be substantial. As fully synthetic compounds, such reagents may also have the added advantages of relatively simple and scalable production and the ability to incorporate surface-binding chemistries, facilitating their attachment to sensor surfaces. Apart from detection, the use of AMPs to selectively bind bacteria and even discriminate pathogenic and non-pathogenic cell types may also be very useful in such wide-ranging applications as cell-based immobilized bed reactors for industrial fermentations, water purification and bioremediation of contaminated materials (*84*).

Bioaffinity reagents that bind to cell surfaces, such as recombinant and natural antibodies, peptide binders, or the AMPs discussed above, have advantages in live cell applications over binders that interact with intracellular targets. Cell-surface binders do not require cell permeabilization to access their targets and can be reacted with target cells under physiological conditions, enabling the detection of living cells. The ability to detect living cells adds another dimension to the detection process, as it may facilitate more rapid and streamlined detection assays and target cells may be subsequently grown for additional characterization.

## Cell-Based Sensors

Thus far, this chapter has primarily focused on the use of exogenous molecular reagents (probes, primers, enzymes, AMPs, etc.) to detect whole microbial cells. In other work, intact microbial cells have themselves been used as detection reagents in chemical or environmental sensing applications. These applications involve whole cell biosensors consisting of engineered microbial

strains containing stress-regulated promoters (*recA*, *uvrA*, etc.) upstream of suitable reporter genes (e.g. *gfp* or *lux*). In the presence of genotoxic agents such as formaldehyde, these "cellular canaries" produce a detectable product (e.g. green fluorescent protein or luciferase), effectively reporting the presence of environmental toxins (*96*). Whole (non-microbial) cells can also be made to serve as pathogen-specific detectors. For example, Rider et al., (*97*) engineered a line of murine B cells expressing both cytosolic aequorin and pathogen-specific, membrane-bound immunoglobulin M (IgM). Contact with the target pathogen causes cross-linking of surface-displayed IgM's, which triggers an intracellular biochemical cascade (release of calcium). In the presence of calcium, the aequorin emits light, providing a detectable output (*97*). To perform the assay, the live-cell sensor is mixed with the test sample, allowed to interact briefly, the mixture centrifuged, and the tube placed in a luminometer for reading. The system has been examined with foot-and-mouth disease virus and several bacterial pathogens including *Yersinia pestis*, *Bacillus anthracis* spores and *E. coli* O157:H7, and is claimed to be capable of detecting as few as 50 colony-forming units within minutes (*97*).



## Conclusions

Microbes are ubiquitous on Earth and occupy surfaces and microniches in a variety of complex environments, both organic and inorganic. Sophisticated diagnostic tools are needed in order to characterize the species distribution and ecological significance of the organisms present in and on these matrices. Methods for microbial diagnostics can be divided into two basic categories: cellular and acellular. Although acellular techniques can provide rapid and actionable information on the presence of specific microorganisms in various samples, including foods and clinical specimens, microbes are fundamentally cellular in nature, and additional layers of information are available within the context of the whole cell. This has spawned the development of a number of methods for detection and characterization of microbes at the level of the individual cell. Although such methods are sensitive, most are presently still too complex for use in routine analysis. At it simplest (basically a "shake and bake" approach), FISH provides a straightforward, robust and sensitive (enough) method for the molecular detection of individual microbial cells. It can be combined, to great effect, with single cell detection platforms such as flow cytometry to facilitate the sensitive detection of specific cells within microbially and physically complex matrices such as environmental samples, foods and clinical specimens.

Although they are often considered separately, many parallels exist between the disciplines of environmental, food and clinical microbiologies. These include

commonalities in goals, reagents, detection hardware and target organisms. Protocols developed for the detection of organisms in one area may often be adapted for use in another area, sometimes with minimal need for adjustment. Further harmonization and cross-pollination across the somewhat artificial boundaries between these disciplines will advance the state of the art for practical single cell detection in general.

This review has been focused primarily on the reagent side of the detection equation. This is an exciting time - new advances in biomimetic reagents, methods for combinatorial and bioinformatics-based reagent discovery are converging to push the envelope of whole cell diagnostics development. Synergies between next-generation reagents and newer, faster (and smarter?) detection platforms will continue to explore the limits of what is possible for whole cell detection of individual microorganisms.



## References

1. Caracciolo, A. B.; Grenni, P.; Cupo, C.; Rossetti, S. *FEMS Microbiol. Lett.* **2005**, *253*, 55.
2. Kuhlman, K.R.; Fusco, W. G.; La Duc, M. T.; Allenbach, L. B.; Ball, C. L.; Kuhlman, G. M.; Anderson, R. C.; Erickson, I. K.; Stuecker, T.; Benardini, J.; Strap, J. L.; Crawford, R. L. *Appl. Environ. Microbiol.* **2006**, *72*, 1708.
3. La Cono, V.; Urzi, C. *J. Microbiol. Methods* **2003**, *55*, 71.
4. Walker, J. J.; Spear, J. R.; Pace, N. R. *Nature* **2005**, *434*, 1011.
5. Junge, K.; Eicken, H.; Deming, J. W. *Appl. Environ. Microbiol.* **2004**, *70*, 550.
6. Shinkai, T.; Kobayashi, Y. *Appl. Environ. Microbiol.* **2007**, *73*, 1646.
7. Diaz, P. I.; Chalmers, N. I.; Rickard, A. H.; Kong, C.; Milburn, C. I.; Palmer R. J. Jr.; Kolenbrander, P. E. *Appl. Environ. Microbiol.* **2006**, *72*, 2837.
8. Macfarlane, S.; Macfarlane, G. T. *Appl. Environ. Microbiol.* **2006**, *72*, 6204.
9. Koch, R. In *Milestones in Microbiology*; Brock T. D., Ed.; ASM Press: Madison, WI, **1999**; p. 101.
10. Cebula, T. A.; Jackson, S. A.; Brown, E. W.; Goswami G., LeClerc, J. E. *J. Food Prot.* **2005**, *68*, 1271.
11. Woese, C. R.; Kandler, O.; Wheelis, M. L. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 4567.
12. Wagner, M.; Nielsen P. H., Loy, A.; Nielsen J. L.; Daims, H. *Curr. Opin. Biotechnol.* **2006**, *17*, 83.
13. Fletcher J.; Bender, C.; Budowle, B.; Cobb, W. T.; Gold, S. E., Ishimaru, C. A.; Luster, D.; Melcher, U.; Murch, R.; Scherm, H.; Seem, R. C.; Sherwood, J. L.; Sobral, B. W.; Tolin, S. A. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 450.

14. Gerner-Smidt, P.; Hise, K.; Kincaid, J.; Hunter, S.; Rolando, S.; Hyytia-Trees, E.; Ribot, E. M.; Swaminathan, B.; Pulsenet Taskforce *Foodborne Pathog. Dis.* **2006**, *3*, 9.
15. Simpson, J. M.; Santo Domingo, J. W.; Reasoner, D. J.; *Environ. Sci. Technol.* **2002**, *36*, 5279.
16. Harold, F. M. *Microbiol. Mol. Biol. Rev.* **2005**, *69*, 544.
17. Brehm-Stecher, B. F.; Johnson, E. A. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 538.
18. Schmidt, T. M. *Int. Microbiol.* **2006**, *9*, 217.
19. Giraffa, G.; Neviani, E. *Int. J. Food Microbiol.* **2001**, *67*, 19.
20. Gould, GW. *Soc. Appl. Bacteriol. Symp. Ser.* **1992**, *21*, 59S.
21. Shapiro, J. A. *Annu. Rev. Microbiol.* **1998**, *52*, 81.
22. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. *Science* **1999**, *284*, 1318.
23. Donlan, R. M. *Emerg. Infect. Dis.* **2002**, *8*, 881.
24. Ercolini, D.; Hill, P. J.; Dodd, C. E. R. *J. Microbiol. Methods* **2003**, *52*, 267.
25. Hodson, R. E.; Dustman, W. A.; Garg, R. P.; Moran, M. A. *Appl. Environ. Microbiol.* **1995**, *61*, 4074.
26. Porter, J.; Pickup, R.; Edwards, C. *FEMS Microbiol. Lett.* **1995**, *134*, 51.
27. Tani, K.; Kurokawa, K.; Nasu, M. *Appl. Environ. Microbiol.* **1998**, *64*, 1536.
28. Tolker-Nielsen, T.; Holmstrøm, K.; Boe, L.; Molin, S. *Mol. Microbiol.* **1998**, *27*, 1099.
29. Hoshino, T.; Noda, N.; Tsuneda, S.; Hirata, A.; Inamori, Y. *Appl. Environ. Microbiol.* **2001**, *67*, 5261.
30. Holmstrøm, K.; Tolker-Nielsen, T.; Mølin, S. *J. Bacteriol.* **1999**, *181*, 1733.
31. Chen, F.; Binder, B.; Hodson, R. E. *FEMS Microbiol. Lett.* **2000**, *184*, 291.
32. Lange, M.; Tolker-Nielsen, T.; Molin, S.; Ahring, B. K. *Appl. Environ. Microbiol.* **2000**, *66*. 1796.
33. Chen, F.; González, J. M.; Dustman, W. A.; Moran, M. A.; Hodson, R. E. *Appl. Environ. Microbiol.* **1997**, *63*, 4907.
34. Lanoil, B. D.; Giovannoni, S. J. *Appl. Environ. Micobiol.* **1997**, *63*, 1118.
35. Lanoil, B. D.; Carlson, C. A.; Giovannoni, S. *J. Environ. Microbiol.* **2000**, *2*, 654.
36. Maruyama, F.; Kenzaka, T.; Yamaguchi, N.; Tani, K.; Nasu, M. *Appl. Environ. Microbiol.* **2005**, *71*, 7933.
37. Maruyama F.; Tani, K.; Kenzaka, T.; Yamaguchi, N.; Nasu M. *Appl. Environ. Microbiol.* **2006**, *72*, 6248.
38. Maruyama F.; Kenzaka, T.; Yamaguchi, N.; Tani, K.; Nasu M. *Appl. Environ. Microbiol.* **2003**, *69*, 5023.
39. DeLong, E. F.; Wickham, G. S.; Pace, N. R. *Science* **1989**, *243*, 1360.
40. Amann, R.; Ludwig, W. *FEMS Microbiol. Rev.* **2000**, *24*, 555.

41. Amann, R.; Fuchs, B. M., Behrens, S. *Curr. Opin. Biotechnol.* **2001**, *12*, 231.
42. Moter, A.; Göbel, U. B. *J. Microbiol. Methods* **2000**, *41*, 85.
43. Zwirglmaier, K. *FEMS Microbiol. Lett.* **2005**, *246*, 151.
44. Amann, R. I.; Ludwig, W.; Schleifer K. –H.; *Microbiol. Rev.* **1995**, *59*, 143.
45. Anderson, M.L.M. *Nucleic Acid Hybridization*; BIOS Scientific Publishers: Oxford, UK, 1999; pp. 23-25.
46. Demidov, V. V. In *Encyclopedia of Diagnostic Genomics and Proteomics*, Fuchs, J., Podda, M., Eds. Marcel Dekker, Inc., New York, **2005**; pp. 1175-1179.
47. Zhong, X.; Lizardi, P. M.; Huang, X.; Bray-Ward, P. L.; Ward, D. C. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3940.
48. Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. *Nucleic Acids Res.* **2000**, *28*, E63.
49. Brehm-Stecher, B. F.; Hyldig-Nielsen, J. J.; Johnson, E. A. *Appl. Environ. Microbiol.* **2005**, *71*, 5451.
50. Fuchs, B. M.; Wallner, G.; Beisker, W.; Schwippl, I.; Ludwig, W.; Amann, R. *Appl. Environ. Microbiol.* **1998**, *64*, 4973.
51. Fang, Q.; Brockman, S.; Botzenhart, K.; Wiedenmann, A. *J. Food Prot.* **2003**, 66, 723.
52. Hartmann, H.; Stender, H.; Schäfer, A.; Autenrieth, I. B.; Kempf, V. A. J. *J. Clin. Microbiol.* **2005**, *43*, 4855.
53. Ootsubo, M.; Shimizu, T.; Tanaka, R.; Sawabe, T.; Tajima, K.; Ezura, Y. *J. Appl. Microbiol.* **2003**, *95*, 112.
54. Tang, Y. Z.; Hoong Gin, K. Y.; Lim, T. H. *Appl. Environ. Microbiol.* **2005**, *71*, 8157.
55. Jansen, G. J.; Mooibroek, M.; Idema, J.; Harmsen, H . J. M.; Welling, G. W.; Degener, J. E. *J. Clin. Microbiol.* **2000**, *38*, 814.
56. Zwirglmaier, K.; Ludwig, W.; Schleifer, K. –H. *Mol. Microbiol.* **2004**, *51*, 89.
57. Hammer, B.; Moter, A.; Kahl, O.; Alberti, G.; Göbel, U. B. *Microbiology* **2001**, *147*, 1425.
58. Pernthaler, A.; Amann, R. *Appl. Environ. Microbiol.* **2004**, *70*, 5426.
59. Schönhuber, W.; Guenhael, L. B.; Tremblay, J.; Amann, R.; Kulakauskas, S. *BMC Microbiol.* **2001**, *1*, 20.
60. Rüssmann, H.; Adler, K.; Haas, R.; Gebert, B.; Koletzko, S., Heesemann, J. *J. Clin. Microbiol.* **2001**, *39*, 4142.
61. Triman, K. L.; Peister A.; Goel, R. A. *Nucleic Acids Res.* **1998**, *26*, 280.
62. Kalyuzhnaya, M. G.; Zabinsky, R.; Bowerman, S.; Baker, D. R.; Lidstrom, M. E.; Chistoserdova, L. *Appl. Environ. Microbiol.* **2006**, *72*, 4293.
63. Sekar, R.; Fuchs, B. M.; Amann, R.; Pernthaler, J. *Appl. Environ. Microbiol.* **2004**, *70*, 6210.

64. Gunasekera, T. S.; Dorsch, M. R.; Slade, M. B.; Veal, D. A. *J. Appl. Microbiol.* **2003**, *94*, 936.
65. Vaahtovuo, J.; Korkeamaki, M.; Munukka, E.; Viljanen, M.K.; Toivanen, P. *J. Microbiol. Methods* **2005**, *63*, 276.
66. Zoetendal, E. G.; Ben-Amor, K.; Harmsen, H. J. M.; Schut, F.; Akkermans, A. D. L.; de Vos, W. M. *Appl. Environ. Microbiol.* **2002**, *68*, 4225.
67. Kempf, V. A. J.; Mändle, T.; Schumacher, U.; Schäfer, A.; Autenrieth, I. B. *Int. J. Med. Microbiol.* **2005**, *295*, 47.
68. Alexander, B. D.; Ashley, E. D.; Reller, L. B.; Reed, S. D. *Diagn. Microbiol. Infect. Dis.* **2006**, *54*, 277.
69. Barenfanger, J.; Drake, C.; Kachich, G. *J. Clin Microbiol.* **1999**, *37*, 1415.
70. Bar-Cohen, Y. *Bioinsp. Biomim.* **2006**, *1*, P1.
71. Fan, X.; Lin, L.; Dalsin, J. L.; Messersmith, P. B. *J. Am. Chem. Soc.* **2005**, *127*, 15843.
72. Bruggeman, O. *Adv. Biochem. Eng. Biotechnol.* **2002**, *76*, 127.
73. Tew, G. N.; Liu, D.; Chen, B.; Doerksen, R. J.; Kaplan, J.; Carroll, P. J.; Klein, M. L.; DeGrado, W. F. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5110.
74. Stender, H.; Fiandaca, M.; Hyldig-Nielsen, J. J.; Coull, J. *J. Microbiol. Methods* **2002**, *48*, 1.
75. Ferrari, B. C.; Veal, D. *Cytometry A* **2003**, *51*, 79.
76. Lefmann M.; Schweickert B.; Buchholz P.; Göbel, U.B.; Seiler, P.; Theegarten, D.; Moter, A. *J. Clin Microbiol.* **2006**, *44*, 3760.
77. Lehtola, M. J.; Loades, C. J.; Keevil, C. W. *J. Microbiol. Methods* **2005**, *62*, 211.
78. Nelson, K.E.; Levy, M.; Miller, S. L. *Proc. Natl. Acad. Sci.* USA **2000**, *97*, 3868.
79. Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 13252.
80. Petersen, M., Wengel, J. *Trends Biotechnol.* **2003**, *21*, 74.
81. Kubota, K.; Ohashi, A.; Imachi, H.; Harada, H. *Appl. Environ. Microbiol.* **2006**, *72*, 5311.
82. Silahtaroglu, A. N.; Tommerup, N.; Vissing, H. *Mol. Cell Probes* **2003**, *17*, 165.
83. Dickert, F. L.; Hayden, O. *Anal. Chem.* **2002**, *74*, 1302.
84. Gregory, K.; Mello, C. M. *Appl. Environ. Microbiol.* **2005**, *71*, 1130.
85. Kulagina N. V.; Lassman, M. E.; Ligler, F. S.; Taitt, C. R. *Anal. Chem.* **2005**, *77*, 6504.
86. Petrenko, V. A.; Sorokulova, I. B. *J. Microbiol. Methods* **2004**, *58*, 147.
87. Fernández, L. A. *Curr. Opin. Biotechnol.* **2004**, *15*, 364.
88. Renberg, B.; Shiroyama, I.; Engfeldt, T.; Nygren, P. –Å.; Karlström, A. E. *Anal. Biochem.* **2005**, *341*, 334.
89. Kouzmitcheva, G. A.; Petrenko, V. A.; Smith, G. P. *Clin. Diagn. Lab Immunol.* **2001**, *8*, 150.

90. Ashelford, K. E.; Weightman, A. J.; Fry, J. C. *Nucleic Acids Res.* **2002**, *15*, 3481.
91. Emrich, S. J.; Lowe, M.; Delcher, A. L. *Nucleic Acids Res.* **2003**, *31*, 3746.
92. Xing, E. P.; Wolf, D. M.; Dubchack, I.; Spengler, S.; Zorn, M.; Muchnik, I.; Kulikowski, C. *J. Theor. Biol.* **2001**, *212*, 129.
93. Zhang, Z.; Willson, R. C.; Fox, G. E. *Bioinformatics* **2002**, *18*, 244.
94. Fitch, J. P.; Gardner S. N.; Kuczmarksi, T. A.; Kurtz, S.; Myers, R.; Ott, L. L.; Slezak, T. R.; Vitalis, E. A.; Zemla, A. T.; McCready, P.M., *Proc. IEEE* **2002**, *90*, 1708.
95. Blais, B. W.; Bosley J.; Martinez-Perez, A.; Popela, M. *J. Microbiol. Methods* **2006**, 65, 468.
96. Sørensen, S. J.; Burmølle, M.; Hansen, L. *Curr. Opin. Biotechnol.* **2006**, *17*, 11.
97. Rider, T. H.; Petrovick, M. S.; Nargi, F. E.; Harper J. D.; Schwoebel, E. D.; Mathews, R. H.; Blanchard, D. J.; Bortolin L.. T.; Young, A. M.; Chen, J.; Hollis, M. A. *Science* **2003**, *301*, 213.