

Multispectral Bacterial Identification

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ABSTRACT

A multispectral optical technique was developed to simultaneously classify individual bacterial cells within mixed populations. Multispectral Bacterial Identification (mBID) combines innovations in microscopy with a software analysis program to measure and categorize the fluorescence signals from multiplexed 16S ribosomal RNA probes hybridized to populations of different bacteria. Software was developed to identify individual bacteria at the level of species within these mixed populations. To test the feasibility of mBID, we examined the fluorescence emissions from a mixture of probes specific for individual species of known bacteria from the American Type Culture Collection (ATCC). Currently, up to seven species can be detected simultaneously by fluorescence microscopy. An eighth signal was reserved for a universal probe to control for fluorescence intensity. mBID can also be used to identify uncultured microorganisms. We plan to couple this new multispectral technology to existing identification technologies that utilize 16S rRNA sequence alignment. Using this integrated identification protocol, bacteria that may be associated with chronic conditions (e.g., prostatitis and vaginosis) will be identified first by analyzing their 16S rDNA sequences and then by visualizing them with fluorescent probes hybridized to their 16S rRNA *in situ*.

Keywords: 16S rRNA, *Bacteria*, fluorescence microscopy, phylogeny, probes, vaginosis.

1. INTRODUCTION

Bacterial identification by a variety of methods has become an essential diagnostic tool in areas such as healthcare, food and water quality testing, and enzyme discovery (Amann *et al.*, 1992; O'Hara *et al.*, 1993; Vandamme, E.J., 1994; Birnbaum *et al.*, 1994; Vandamme, P., 1996; Relman, 1998; Schrenk *et al.*, 1998). Traditionally, microbiologists performing bacterial identification have relied on cultivation of organisms, despite the realization that most of them (>99%) are not cultivable by standard methods (Amann *et al.*, 1995; Pace, 1997; Head *et al.*, 1998; Hugenholtz *et al.*, 1998). More recently, molecular methods have been developed to examine the diversity of microorganisms without the need to isolate or culture them. One class of methodology takes advantage of the conserved nature of protein synthesis in all cellular organisms. With about 10,000 partial or complete sequences now available for comparison, the small subunit 16S ribosomal RNA (rRNA) is currently the molecule of choice for identifying bacteria and other microorganisms at the species level. Molecular strategies based on PCR, cloning, sequencing, and probing have enabled biologists to examine the total bacterial community in a sample without any *a priori* knowledge of the species present in the mixture (Amann *et al.*, 1995). Although rRNA-based ID is only accurate to approximately the level of species, its tremendous versatility makes it extremely valuable for high-throughput screening and identification of microorganisms.

The information gained from 16S rRNA gene sequence comparisons can be used to deduce detailed phylogenetic relationships based on evolution. An evolutionary distance map generated from 16S rRNA gene sequence data highlights the major lineages of *Bacteria* and *Archaea* (Figure 1). The highly conserved portions of 16S rRNA genes are ideal for designing primers that will amplify small subunit rRNA genes from all three domains of life (*Bacteria*, *Archaea*, and *Eucarya*). At the other extreme, primers can be designed to highly variable regions of 16S rRNA genes and thus amplify only a particular species or genus in a mixture of microorganisms. Likewise, fluorescent DNA hybridization probes based on 16S gene sequencing can be constructed to identify organisms in a large group (e.g., phylum) or in a localized group (e.g., genus), depending on whether the probe sequence is complementary to a conserved or variable region of the 16S rRNA, respectively. Probes can be covalently labeled with fluorophores to enable *in situ* hybridization and identification by fluorescence imaging microscopy (Amann *et al.*, 1990). Ribosomal RNA is a particularly convenient and attractive

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hybridization target for quantitative microscopy because a typical *E. coli* cell contains approximately 20,000 ribosomes (Neidhardt, 1987), and thus ~20,000 copies of the target sequence.

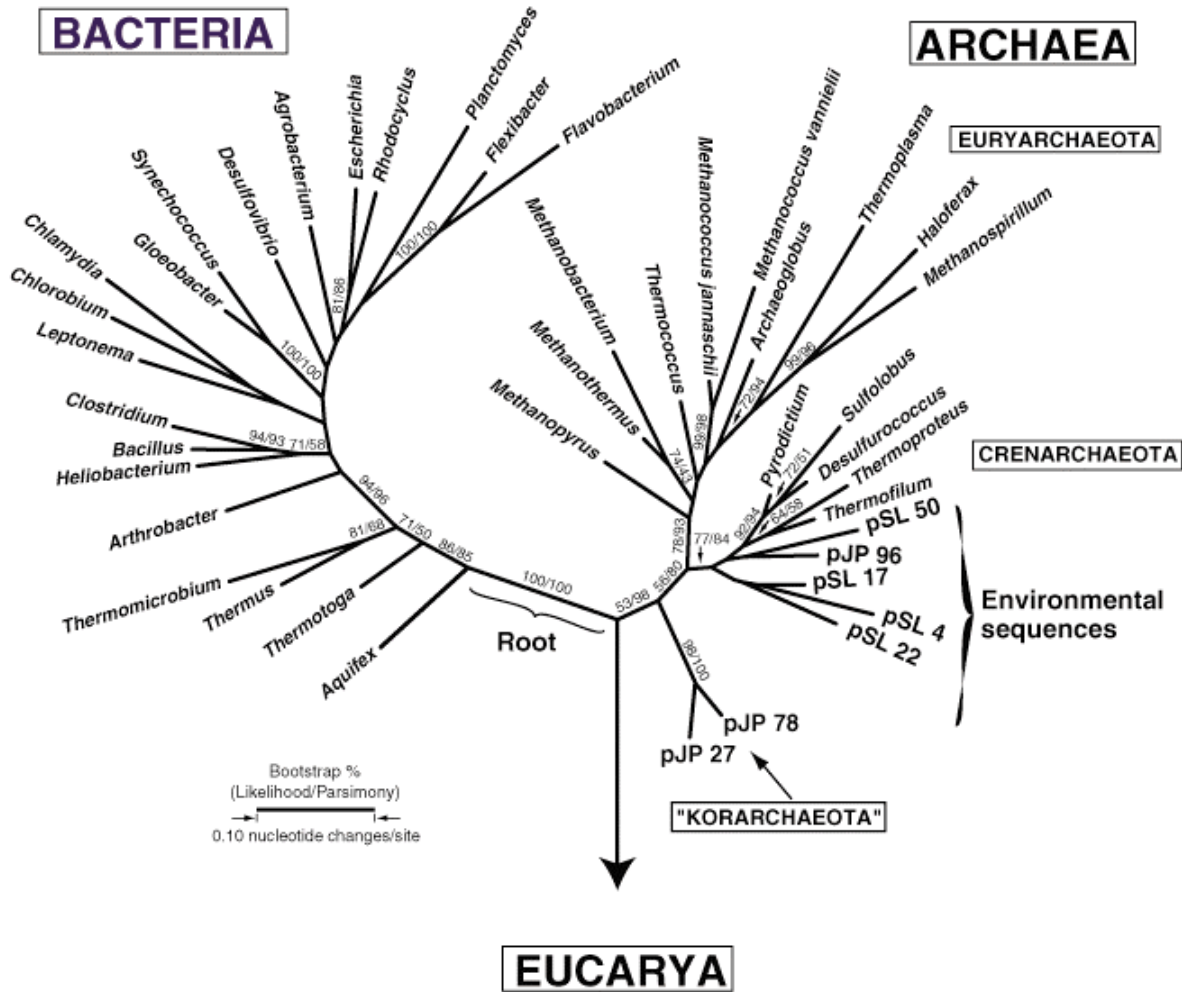


Figure 1. Phylogenetic tree of the three domains of life (*Bacteria*, *Archaea*, and *Eucarya*) based on the evolutionary distance of the 16S rRNA molecule (adapted from Barns *et al.*, 1996). Members of the *Eucarya* are omitted for clarity. The genera listed are representative of major lineages of the two domains. Bootstrap values indicate the percentage of trees that resulted in the branching order shown. Two different treeing algorithms, maximum likelihood and maximum parsimony, generated these values. Environmental sequences are known only from their 16S rRNA gene. Note that although the arrangement of the branches on the tree may change as new information is gathered, this does not affect the accuracy of using a unique 16S rRNA gene sequence to identify a particular organism.

PCR has been an extremely powerful tool for analyzing samples and constructing databases of sequences. It has been used to amplify the 16S rDNA from microorganisms isolated from highly diverse and extreme environments, as well as from clinical sources (Hugenholtz *et al.*, 1998; Relman, 1998). Unknown organisms are being identified at the level of new phyla, expanding on the bacterial line of descent. Many of these new phyla do not have cultured representatives, and yet PCR analysis indicates that they are abundant in the environment. These organisms are completely novel, and they may be a rich source of new antibiotics, enzymes, and other bioactive compounds for medicine and biotechnology (Short, 1997; Rondon *et al.*, 1999). Recently, attempts have been made to reduce the sequencing load and to increase the screening throughput by employing restriction fragment length polymorphism (RFLP) analysis to examine the diversity of these

microbial populations. To design actual probes however, a full length 16S rRNA sequence is needed, and it must be aligned into an existing database.

As we noted above, the etiology of human infections has historically relied on cultivation to identify the responsible microorganisms. Isolation and inoculation of cultured microbes is the conventional means to link causation of disease to a particular pathogen – i.e., Koch's Postulates. However, microbes that are difficult or impossible to culture with current techniques can cause some human clinical syndromes that were originally thought to be nonmicrobial. Indeed, a number of pathological conditions are known to be the result of uncultivated bacteria (Fredricks & Relman, 1996; Lorber, 1996). A few examples in which molecular methods have been used to identify uncultured bacteria have been reported. The causative agent of Whipple's disease, for instance, is resistant to culture, but can be identified using PCR and 16S rRNA gene sequence analysis (Relman *et al.*, 1992). Additionally, the PCR approach has been used to identify the Whipple bacillus (*Tropheryma whippelii*) in the eye and mononuclear cells of blood (Rickman *et al.*, 1995; Müller *et al.*, 1993). Similarly, the etiologic agents of cat scratch disease and bacillary angiomatosis were identified using PCR and 16S rRNA analysis (Adal *et al.*, 1994). Sequence analysis identified the agent as a member of the genus *Rochalimaea* (*Proteobacteria*; alpha subdivision).

The difficulty of cultivation also has led to the realization that many human infections are more complex than originally thought. The common expectation of syntrophy, where one organism is dependent on the metabolism of another – frequently observed in the environment – may be prevalent in the diseased state as well. Biofilms are a good example of microbial communities, and they have caught the attention of biomedical science, since microbial communities existing as biofilms play a role in both human health and disease. Bacteria that form these biofilms are well known in tooth decay and artificial implants, and are now implicated in other diseases, including kidney, urinary tract and ear infections. Individual species of bacteria in a community may be dependent on other species for survival, which makes isolation in culture a formidable task. Likewise, analyzing such communities *in situ* using the currently available methods is a difficult undertaking.

Evidence for a complex bacterial population in a disease condition was recently described for prostatitis, a common disease in adult men of all ages (Tanner *et al.*, 1999). Frequently, patients are diagnosed with 'nonbacterial' prostatitis, but some of these patients respond to antibiotic treatment and show evidence of distinct bacterial species by molecular techniques, despite the absence of cultivable bacteria (Tanner *et al.*, 1999). These species were identified by phylogenetic analyses of their 16S rRNA gene sequences from mixed populations. Prostatitis is an appropriate model to study bacterial identification by multispectral imaging because various bacterial species are present, including some that are uncultured, and samples are easy to acquire and process. A second disease amenable to study by mBID is bacterial vaginosis, a prevalent disorder that is probably the result of an imbalance in the various bacterial populations that comprise the vaginal flora. Interestingly, biofilms may play a significant role in the pathology of prostatitis and vaginosis, contributing to the lack of cultured microorganisms in many patients (Potera, 1999).

For *in situ* analysis of these bacterial species, the information gained from PCR and sequencing can be advantageously exploited to synthesize fluorescently labeled oligonucleotide probes for direct hybridization against the 16S rRNA. Up to now, however, these *in situ* hybridization experiments have utilized no more than one or two probes per sample because of the lack of adequate instrumentation. The ability to employ multiple probes simultaneously and to analyze them with a radiometrically-calibrated system will increase the amount of information that can be obtained from a given sample and substantially increase the overall throughput. In addition, the analysis can be performed on single cells, without extracting, purifying, and amplifying the nucleic acids each time. A whole-cell *in situ* method such as mBID has numerous advantages over other bacterial ID techniques, including:

- Non-cultivable organisms can be detected
- Cells need not be viable
- DNA/RNA amplification is not necessary after the sequence is known
- Processing is rapid
- High throughput is achievable
- Unknowns can be tentatively identified via phylogeny
- Single cells can be individually analyzed
- Rare cells can be detected in complex backgrounds

Once the PCR-based analyses have enabled species-specific hybridization probes for known populations of bacteria, one can take full advantage of the speed and simplicity of a fluorescence-based method (i.e., mBID) to streamline the process. Moreover, even if sequence information is lacking, mBID still enables a level of bacterial ID that becomes increasingly accurate as more spectroscopic channels are used to quantitate the hybridization levels of the targeted probes. Unlike ID that is based solely on PCR, mBID is a highly parallel, imaging-based technique that enables *in situ* analyses and ID of many individual cells within a single field of view. We anticipate that the mBID system, which has been developed with the goal of obtaining FDA approval as a medical device, will be extremely useful to clinical research laboratories that wish to study bacterial communities.

2. METHODS AND RESULTS

2.1 Selecting 'test targets' for instrument development

After searching the scientific literature and the rRNA phylogenetic databases, we selected a set of approximately two dozen well-characterized, nonpathogenic bacterial and archaeal species that were used in the initial mBID hybridization studies. This particular set of species provides diverse 'targets' from many different taxa, including the *Archaea*, several members of the *Proteobacteria*, the Low G+C gram-positive bacteria, and *Actinobacteria* (High G+C gram-positive bacteria). In Table 1, we list specific probe sequences and fluorescent dye labels used to ID one particular set of seven bacterial species. Sixteen other species-specific probes have also been designed (including probes against members of the domain *Archaea*), and a series of analogous species-specific ID experiments have been successfully performed (data not shown).

Probe Sequence	Species Specificity / Phylum or Subdivision	Channel	Dye
CCACTGCCTTTTACACCAGA	<i>Lactococcus lactis</i> / Low G+C gram positive	1	Alexa 350
AACTTTACTCCCTTCCTCC	<i>Escherichia coli</i> / γ <i>Proteobacteria</i>	2	Pacific Blue
CCGCGGC(T/G)GCTGG	All (Universal)	3	Bodipy 493/503
GCCCTTTGTTCTGT	<i>Bacillus subtilis</i> / Low G+C gram positive	4	Bodipy R6G
ACCGCCCCAAAAGGAGAAACCACA	<i>Arthrobacter globiformis</i> / <i>Actinobacteria</i>	5	Bodipy 564/570
ACCAGTTGACATCGTTTAGGGC	<i>Leptothrix discophora</i> / β <i>Proteobacteria</i>	6	Bodipy 581/591
ATCCAGGTACCGTCACCTTAA	<i>Corynebacterium flavescens</i> / <i>Actinobacteria</i>	7	Cy5
ACAACCCATAGGGCAGTCT	<i>Flexibacter maritimus</i> / *BCF	8	Cy5.5

*BCF is the *Bacteroides*, *Cytophagales*, *Flavobacterium* phylum.

Table 1. Probe sequences for selected bacteria used to test species-specific hybridization in an eight-channel experiment. One channel (Channel 3) was reserved for a universal probe, whereas the remaining seven channels are each designed to identify a particular species with the corresponding probe.

2.2 Imaging Spectroscopy and mBID

One of the most challenging engineering problems in mBID is to spectrally separate and quantitate many different fluorescent tags within a complex mixture while maintaining pixel-resolution (spatial) image registration. In addition, the mBID imaging spectrophotometer requires the spatial resolution of an epifluorescence microscope (<1 μ m) and the spectral resolution of a conventional fluorimeter (~5 nm). To meet these engineering goals, we have replaced the fixed-wavelength excitation and emission filters of a conventional epifluorescence microscope (Olympus AX80) with a large number of discrete excitation filters and a fully tunable emission filter, respectively. This tunable filter consists of a computer-interfaced

Circular Variable Interference Filter (CVIF) to select the emission wavelength, and thus it allows us the flexibility of choosing any desired wavelength between 400 and 700 nm. Because of the extreme surface parallelism of the CVIF, we have been able to achieve sub-pixel image registration – which is not usually feasible with a set of conventional emission filters. In addition, signal-to-noise considerations in fluorescence microscopy demands an epifluorescence configuration; therefore, we incorporated a programmable 8-position turret (containing dichroic mirrors) into the mBID instrument. To achieve intense monochromatic illumination, we have also adapted a stepper-driven, dual-wheel excitation filter set (carrying 18 bandpass filters) to the epi-illumination optics of the AX80.

2.3 Fluorophores and Optical Filters

Fluorescent dyes were selected so as to minimize spectral overlap among channels, provide simple coupling reactions to DNA probes, and maintain high quantum yields with low photodestruction. Each fluorophore / channel combination was optimized with respect to the wavelength of maximum transmission and bandpass for the excitation and emission filters, and the cut-on for the dichroic beamsplitter. Typically, the excitation filter for a particular dye is placed close to the dye's excitation maximum while minimizing the excitation of the next bluest and next reddest dyes. The dichroic filter is set slightly to the blue of the midpoint between the excitation and emission maximum of the dye, while the narrow bandwidth CVIF is positioned as close as possible to the emission maximum.

The optimization of mBID's filters-to-fluorophore set is essential in reducing light dose and photodestruction – as well as spectral overlap. Our original Fluorescence Imaging MicroSpectrophotometer (FIMS) configuration (Youvan *et al.*, 1997) is not optimized for any particular set of dyes, since it performs full-spectrum excitation and emission scans. Thus the mBID configuration, using discrete excitation filters, a set of 8 dichroic filters, and a full spectrum CVIF, is unique. Five optical parameters were optimized simultaneously for each channel summarized in Table 2: two parameters are specified for each excitation filter (wavelength of maximum transmission and bandwidth); two parameters are specified for each dichroic filter (inflection point of the transmission cut-on and out-of-band light transmission characteristics); one parameter is specified for the wavelength of maximum transmission of the CVIF.

	Channel 1	Channel 2	Channel 3	Channel 4	Channel 5	Channel 6	Channel 7	Channel 8
Fluorescent Dye	Alexa 350	Pacific Blue	Bodipy 493/503	Bodipy R6G	Bodipy 564/570	Bodipy581/591	Cy Dye 5	Cy Dye 5.5
Excitation Filter	365/30	420/20	490/10	530/10	560/10	590/10	630/10	680/10
Dichroic Filter	400DCXU	450DCXU	500DCXU	Q545LP	Q578LP	Q608LP	645DCXU	Q690LP
Emission Filter (CVIF)	442 nm	465nm	520nm	555nm	585nm	615nm	665nm	700nm

Table 2. Optimization of mBID filter parameters to an 8-dye set.

2.4 Probe Hybridization

Whole cell *in situ* hybridization is typically carried out by immobilizing cells on gelatin-coated slides after they have been fixed and permeabilized. The probe solution is then added to the slide and incubated for 2-15 hours at a temperature that is determined by the melting temperature (T_m) of the oligonucleotide DNA probe / rRNA duplex. After hybridization, slides are washed and then mounted in an antifade reagent (Amann *et al.*, 1990; DeLong *et al.*, 1989; Rice *et al.*, 1997). To facilitate high throughput of mBID samples, we have also developed a micro-volume hybridization protocol that can be performed in microcentrifuge tubes or microplate trays. This procedure is analogous to conventional methods with the exception that the cells are fixed, permeabilized, hybridized, and washed in suspension. [Bacteria are collected by a brief, low speed centrifugation step.] In addition to being more amenable to automation and robotic handling, another advantage of hybridization in suspension is that it prevents the loss of cells, such as coccoid bacteria, which adhere poorly to slides. This will be critical for accurately enumerating cells within complex bacterial populations. Micro-volume hybridization is also essential for constructing calibration slides, where it is important to combine bacteria, which have been hybridized in separate reactions, onto a single slide.

2.5 mBID Graphical User Interface

The mBID graphical user interface (GUI) includes multiple window types, wizards, and a number of options pertaining to file management, data acquisition, image processing, and display. It is not a simple operation to visualize and extract useful information from massive amounts of multidimensional data. A schematic of the ultimate mBID GUI – which coordinates the display of both spatial and spectral information – is depicted in Figure 2.

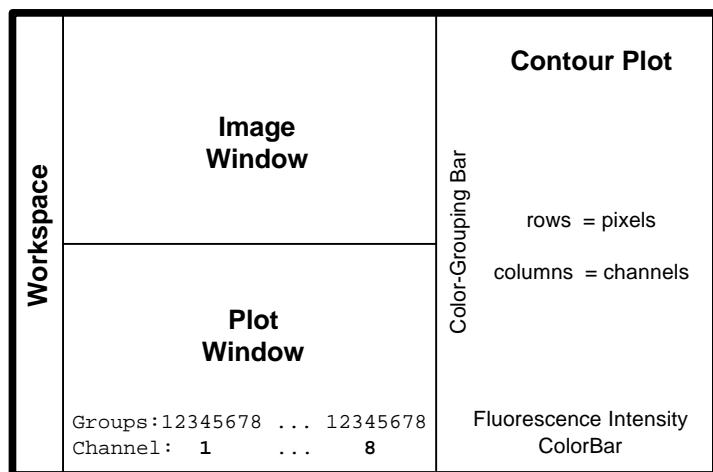


Figure 2. Schematic of the mBID Graphical User Interface.

The **Workspace** Window on the far left is used to organize projects, files, and spectral data. It maintains point-and-click functionality very much like the Microsoft Explorer. An **Image** Window is provided for the user to display processed images at different magnifications. Traditionally, the **Plot** Window has been used to display line spectra similar to that of a conventional spectrophotometer. In mBID, we have written code to display histogram or bar-graph style data. To the right, a **Contour** Plot Window with multiple components is depicted. The Contour Plot was pioneered by KAIROS as a tool to visualize and extract information from as many as 1,000,000 individual spectra simultaneously. In an unsampled display mode, each row of the contour plot corresponds to a single pixel or feature in the image. In mBID, each column of the contour plot corresponds to a spectral channel. The fluorescence intensity of the feature in that spectral channel is encoded by a scheme that is defined by the horizontal **ColorBar** directly below the Contour Plot. Accordingly, fluorescence intensities are color encoded using black for the lowest intensity and a rainbow of warmer hues to depict higher fluorescence intensity values, while reserving white for highest intensity. All three windows are interactive, and mappings between the windows are maintained via the vertical “**Color-Grouping Bar**”. For example, clicking on a single row in the Contour Plot results in the appearance of a colored tick mark next to this row while simultaneously updating the Plot Window and highlighting the associated feature in the Image Window – all using the same ‘marking’ color. Multiple rows can be associated into a color group by dragging the computer’s mouse down the side of the Contour Plot, thereby invoking a different color for each group selected.

In terms of condensing and visualizing massive amounts of spatial and spectral information, algorithms associated with menus ‘behind’ the Contour Plot can be employed to associate rows into like groups using a number of different criteria, including: similarity by sum-of-the-square-of-the-differences, maximum intensity, channel of maximum intensity, etc. After the grouping operation is performed, the average spectrum of each group can be calculated and displayed. Using this option, all features or pixels belonging to the same group are mapped to one pseudocolor (as set by the interactive Color-Grouping Bar) and the average spectrum of each group is displayed in the Plot Window. In mBID, the vertical axis of the Plot Window corresponds to fluorescence intensity. However, the horizontal axis is more complex, and is comprised of multiple group intensities repeated for each channel. We used the results of a seven-species mBID experiment (shown in Figure 3) for further explanation of this unique type of grouping operation and histogram display.

2.6 Probes to seven cultured bacteria

Figure 3 shows a pseudocolored image of the hybridization pattern of eight probes to the seven species that were described in Table 1. The Contour Plot has been sorted by the channel of maximum intensity into seven groups. The average spectrum for each group has been calculated, and an assigned pseudocolor is associated with the rows in the Contour Plot by the vertical Color-Grouping Bar. Using these pseudocolors, pixels in the image are back-painted. Starting from the leftmost histogram bar in the Plot Window, the intensity of Group 1 in Channel 1 appears as a full-height purple bar corresponding to the upper-left high intensity region of the Contour Plot, where the fluorophore indicative of Channel 1 shows highest intensity. Groups 2 through 7 show no intensity (black) in Channel 1; hence, no other histogram bars are present except for a short bar associated with Group 8 (encoded in red). Moving further to the right in the Plot Window, we see a total of eight groupings of seven vertical bars for each of the eight Channels – a total of 56 histogram bars.

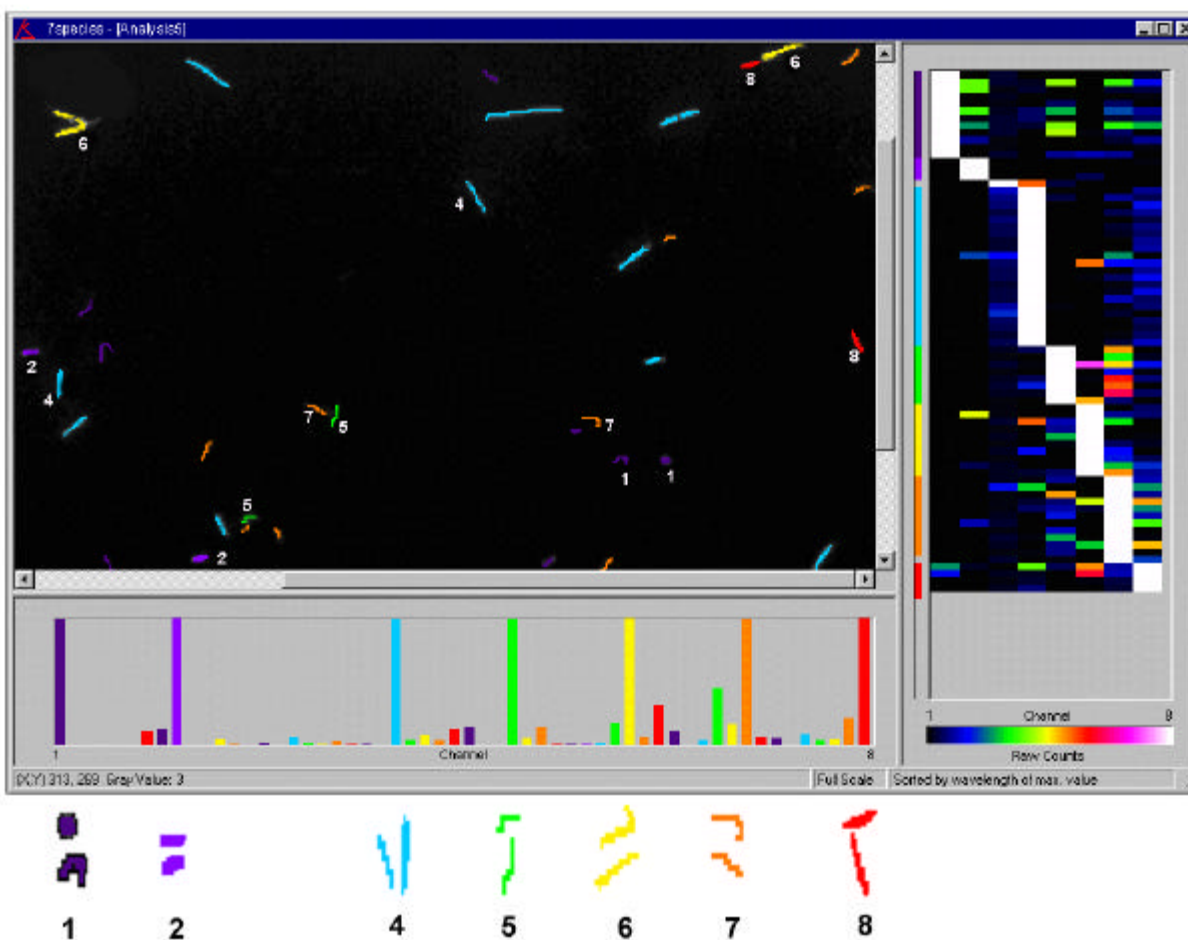


Figure 3. Graphical user interface for the mBID instrument demonstrating the identification of 7 bacterial species using multispectral detection of fluorescent probes. The mBID instrument measures the fluorescence intensity in all eight channels for every pixel in the image (Channel 3 was reduced to facilitate sorting). These spectra are sorted, grouped, assigned a pseudocolor, and then used to back-paint the image and identify the species that are present. Below the GUI, we have enlarged 2 bacteria from each of the seven groups (against a white background) simply to avoid some color degradation that occurs in the RGB to CMYK printer conversion. Numbers correspond to the probes and species listed in Table 1. Channel 3 would normally be used for the universal probe. A 24-bit RGB image of this figure can be viewed through the online journal *Biotechnology et alia* at <www.et-al.com>.

3. SUMMARY

Using mBID technology, we have demonstrated the feasibility of simultaneously identifying seven different bacterial species in the same sample. In combination with control experiments that used single probes (not shown), the results shown in Figure 3 demonstrate that it is possible to separate the spectral signals from eight different fluorescent probes, correct for spectral overlap, and back-color each different species. Data presented in Figure 3 have also been normalized based on a universal probe (Channel 3), which corrects the channels for any differences in the number of ribosomes per cell. Successful demonstration of probe specificity and sensitivity was achieved with general and specific probes to bacteria. Finally, it should be noted that the spectra have been automatically sorted using the Contour Plot, and this enabled us to 'backpaint' the bacteria in the image based on their multispectral fingerprint. Thus, the mBID prototype can be used to identify a particular species. Unlike conventional diagnostic procedures that rely on cultivation, mBID has the capacity to analyze unculturable microbes, including those found in clinical samples.

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