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Scanning Electron Microscopy Imaging of Bacteria Based on Nucleic Acid Sequences

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1. Introduction

Scanning electron microscopy (SEM) has been widely used in environmental microbiology to characterize the surface structure of biomaterials and to measure cell attachment and changes in morphology of bacteria. Moreover, SEM is useful for defining the number and distribution of microorganisms that adhere to surfaces. Traditionally, inability to provide phylogenetic or genetic information about microorganisms has been one limitation of SEM in environmental microbiology.

Modern molecular studies based on DNA and RNA sequence analysis have led to an understanding of the microbial diversity and composition of bacterial communities in various environments. Introduction of the concept of in situ hybridization (ISH) with RNA- or DNA-targeted fluorescent probes has led to important research regarding the identification and quantification of individual cells and has demonstrated great potential in the analysis of the composition of bacterial communities in the environment. Combining morphological study with SEM and ISH techniques (SEM-ISH) has provided new insights into the understanding of the spatial distribution of target cells on various materials.

2. Application of ISH techniques to SEM

2.1 Concepts of ISH

ISH is a method of detecting and localizing specific nucleic acid sequences in morphologically preserved tissues or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. To detect target cells, the permeability of the cell and the visibility of the nucleotide sequence to the probe must be increased without destroying the structural integrity of the cell. The type of probe to be used and how to label it to yield better resolution with the highest accuracy should be taken into consideration.

In environmental microbiology, fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has provided information about the absolute abundance, morphology, and cell size of bacteria with defined phylogenetic affiliations and had been applied to the investigation of community composition in lakes, river, oceans, activated sludge, drinking water, etc. (Amann et al., 1995). FISH analysis of bacterial communities with mRNA- or DNA-

targeted probes presents a unique challenge because of its low sensitivity and resolution (Moraru et al., 2010; Pernthaler & Amann, 2004). The concepts of ISH can be incorporated into scanning electron microscopy imaging of microorganisms (Kenzaka et al., 2005a, 2009).

2.2 Metal labeling of target cells for SEM-ISH

To identify cells of interest by SEM, cells carrying specific DNA or RNA sequences must be labeled with a metal instead of fluorescent molecules. Target cells that were hybridized with oligonucleotide or polynucleotide probes can be labeled with gold or platinum (Fig. 1). In case of gold labeling of the target sequence, hybridization was performed with biotin-labeled oligonucleotide probes, and then target microbes were identified by nanogold-labeled streptavidin (Hacker, 1998; Fig. 1a). In case of the platinum probe complex, hybridization was performed with platinum-labeled oligonucleotide probes (Fig. 1b). Platinum-labeled probes can be prepared by allowing the platinum complex to bind to the probe at guanine-N7 atoms (Brabec & Leng 1993; Dalbiès et al., 1994). To amplify the signal in the both cases, gold enhancement was performed to enlarge the gold particles. In this reaction, gold ions in solution are catalytically deposited onto nanogold particles as metallic gold (Au^0). These particles grow in size as development time elapses. Consequently, hybridized cells contain gold up to tens of nm in size inside the hybridized cells. These hybridized cells release a strong backscatter electron signal (BSE) because of the accumulation of gold atoms inside cells (Kenzaka et al., 2005a, 2009).
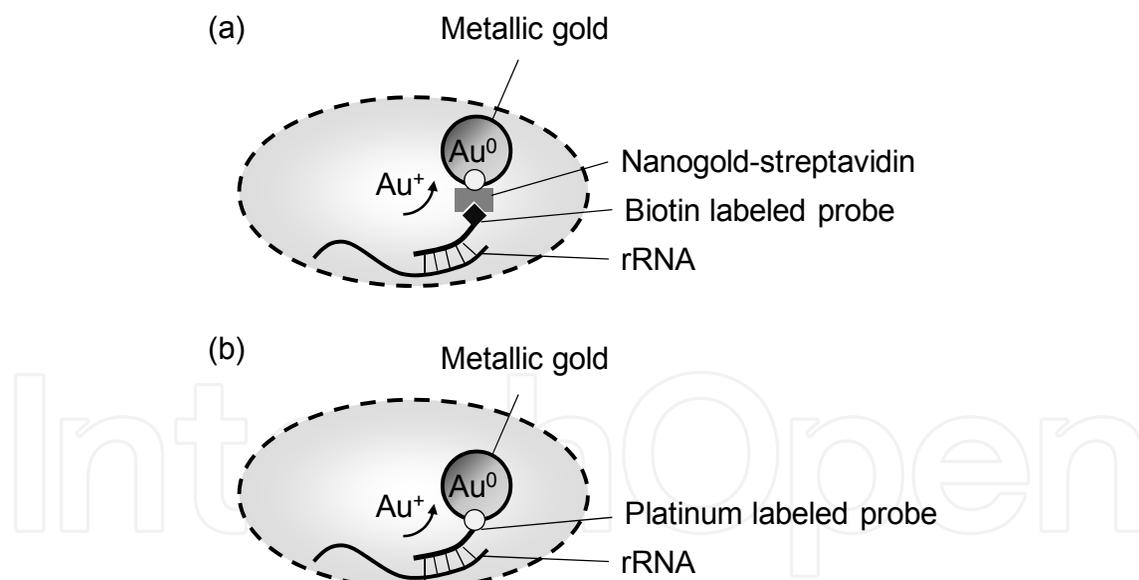

Fig. 1. Systematic representation of metal labeling for SEM-ISH. (a) Biotin labeled probes are hybridized with rRNA, followed by streptavidin-gold labeling, gold enhancement.
(b) Platinum labeled probes are hybridized with rRNA, followed by gold enhancement.

High-vacuum SEM images of a mixture of bacterial strains after ISH with an rRNA-targeted probe and labeling with gold are shown in Fig. 2. Secondary electron (SE) image demonstrate the surface topography of all cells (Fig. 2a). In BSE image, *Escherichia coli* cells hybridized with the ES445 probe which was targeted for *Escherichia-Shigella* (Kenzaka et al., 2001) were identified as bright spots because of the enhanced signal as a

result of gold labeling (Fig. 2b). In hybridized cells, the amount of gold in the cells was greater than that on the cell surface; thus, this labeling resulted in a higher BSE signal. In the same microscopic field, both images could be viewed side by side.
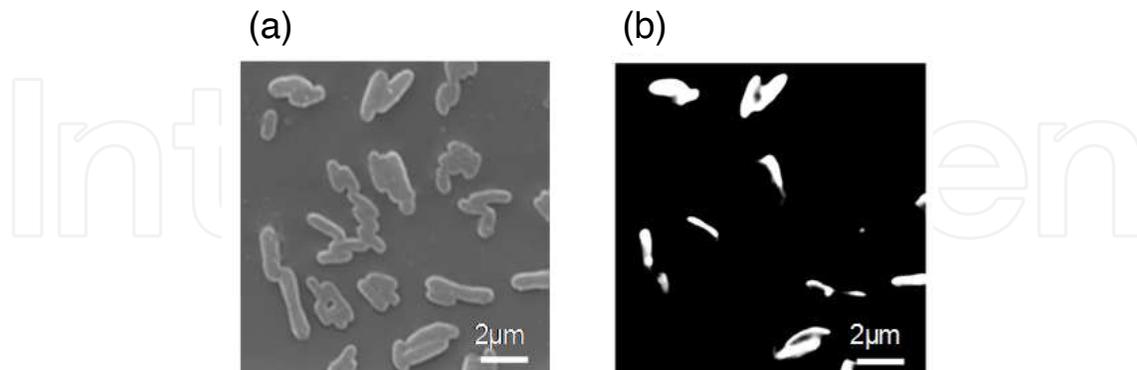
(a)   (b)

Fig. 2. High-vacuum SEM images of bacterial cells after ISH: Mixture of *Escherichia coli* and *Aeromonas sobria* cells hybridized with ES445 probe (targeted for *Escherichia-Shigella*). The same microscopic fields are shown with SE (a) and BSE images (b).

3. Experimental protocols for SEM-ISH

Experimental protocols for SEM-ISH are similar to FISH except metal labeling, and both techniques have the same challenges and limitations. For success in ISH, several issues need to be considered before proceeding with experiments: permeabilization and pretreatment, hybridization condition (composition of the hybridization solution, temperature, sodium concentration, and presence of organic solvents), washes, controls, etc. Here we discuss the general protocols for ISH in comparison to FISH.

3.1 Preparation of materials for ISH

Before processing for ISH, the specimens must be fixed and permeabilized to allow the penetration of the probes and reagents into the cells and protect the target RNA or DNA from degradation by nucleases. Fixation conditions may vary according to the target bacteria and type of sample. Optimal fixation results in good material penetration as well as the maintenance of cell integrity and morphological detail. For FISH, 3%–4% (v/v) formaldehyde or paraformaldehyde is generally suitable for gram-negative bacteria. In case of gram-positive bacteria, cells were fixed with 50% ethanol (Amann et al., 1995).

Before or after fixation, cells are usually prepared on glass slides or trapped on a polycarbonate filter. For aggregated samples or biofilms, species are fixed in formalin and then embedded in paraffin before being sectioned (Sekiguchi et al., 1999). For SEM-ISH, similar protocols with paraformaldehyde can be employed.

For better attachment of the specimens to the slide or polycarbonate filter, the surfaces were treated with a coating agent such as gelatin (Amann et al., 1990b), poly-L-lysine (Lee et al, 1999), or agarose (Pernthaler et al., 2002a). If peroxidase-labeled molecules were used

for signal enhancement, an additional enzymatic treatment with lysozyme was required (Pernthaler et al., 2002b; Schönhuber et al., 1997). Some cases require further enzymatic treatment to open the peptidoglycan layer. Lysozyme and pancreatic lipase for enterococci (Waar et al., 2005), lysozyme and lysostaphin for staphylococci (Kempf et al., 2000), and lysozyme and achromopeptidase for actinobacteria (Sekar et al., 2003) have been previously employed. When peroxidase-labeled probes and antibodies were used, diethyl pyrocarbonate treatment or other additional treatments were required to inactivate intracellular peroxidase (Pernthaler et al., 2002a). Microbial communities in the natural environment are complex, and the permeability of their cell walls is not uniform. The application of mixed enzymes or other chemical treatments may be required (Pernthaler et al., 2002b), but it remains difficult to sufficiently permeabilize the cell walls of all complex bacterial communities.

3.2 Probes types

Probes are sequences of nucleotide bases complimentary to the specific DNA or RNA sequence of interest. These probes can be as small as 15–30 nucleotides or up to 1000 nucleotides. The strength of the binding between the probe and the target molecules is crucial in hybridization. This strength is affected by the various hybridization conditions described below.

Several types of probes can be used in performing ISH: oligonucleotide DNA probes, polynucleotide DNA probes, polyribonucleotide probes, peptide nucleic acid (PNA) probes, locked nucleic acid (LNA) probes, etc. Stability, availability, speed, expense, ease of use, specificity, cell wall penetration ability, and reproducibility should be considered for selecting a probe type.

3.2.1 Oligonucleotide DNA probes

An oligonucleotide DNA probe is a short sequence of nucleotides that are synthesized to match a target. They are synthetically produced, commercially available, and economical. The target nucleotide sequence must be known. The probes used to detect bacteria are small, generally approximately 15–30 bp. A small-sized probe allows for easy penetration into the bacterial cells of interest. For effective hybridization, the thermodynamics of nucleic acid hybridization based on Gibbs free energy change should be considered. The affinity of the probe to the target site is defined as the overall Gibbs free energy change for intramolecular DNA and RNA interactions that take place during ISH (Yilmaz & Noguera, 2004).

3.2.2 Polynucleotide DNA probes

Polynucleotide DNA probes have similar advantages to oligonucleotide DNA probes, but they are much larger, typically 50–1000 base long. These are synthetically produced by reverse transcription of RNA, or by PCR, or by fragmentation of the PCR product (Niki & Hiraga, 1998). Fragmented chromosomal DNA can be used as a probe (Lanoil & Giovannoni, 1997). However, synthesizing this type of probe requires time and expensive reagents. Polynucleotide DNA probes can be labeled at multiple sites with fluorescent dyes, digoxigenin, or biotin and thus are used to amplify probe-derived signals.

3.2.3 Polyribonucleotide probes

RNA probes have the advantage that RNA–RNA hybrids are considerably thermostable and resistant to digestion by RNases. In vitro transcription of plasmid DNA with RNA polymerase can be used to produce RNA probes (Delong et al., 1999; Pernthaler et al., 2004; Zwirglmaier et al., 2004). These probes, however, can be very difficult to work with as they are highly sensitive to ubiquitous RNases.

3.2.4 Peptide nucleic acid (PNA) probes

PNAs are the synthetic analogs of DNA. DNA and RNA have deoxyribose and ribose sugar backbones, respectively, whereas the backbone of a PNA backbone comprises repeating N-(2-aminoethyl)-glycine units that are linked by peptides (Nielsen et al., 1991). The backbone of PNAs contains no charged phosphate groups. Less electrostatic repulsion occurs when the PNA probe hybridizes to DNA or RNA sequences. The PNA–DNA or PNA–RNA complex is more stable than the natural nucleic acid complexes. PNA is not easily identified by either nucleases or proteases, making them resistant to enzyme degradation. Because of their higher binding strength, it is not necessary to design long PNA oligomers. Such oligomers are chemically synthesized and commercially available. PNA-FISH also has broad applications in clinical microbiology (Stender, 2003).

3.2.5 Locked nucleic acid (LNA) probes

LNAs are a class of analogs that contain an extra bridge connecting the 2' oxygen and 4' carbon (Obika et al., 1997). LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. The LNA–DNA heteroduplex is thermostable. DNA probes with LNA have the advantage of higher affinity and specificity than normal DNA probes, and greater design flexibility and lower costs than PNA probes (Silahtaroglu et al., 2003). Such oligomers are chemically synthesized and commercially available. LNA nucleotides are used to increase the sensitivity and specificity of expression in DNA microarrays, FISH probes, real-time PCR probes, and other molecular biology techniques.

3.3 Hybridization

Hybridization must be performed under stringent conditions to allow the binding of the probe to the target sequence. The representative components in a hybridization buffer are shown in Table 1. For hybridization of oligonucleotide DNA probes to rRNA, sodium chloride (NaCl), tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), sodium dodecyl sulfate (SDS), and formamide were the major components. For hybridization of polyribonucleotide probes to rRNA, the components were slightly modified. For polynucleotide DNA probes, NaCl, sodium citrate and formamide were major components. A buffer solution containing NaCl, Tris-HCl, and SDS or SSC buffer (containing NaCl and sodium citrate) was used as the wash solution.

The preheated hybridization buffer was applied to the sample containing probes complementary to the target sequence. In case of rRNA-targeted ISH, stringency can be adjusted by varying either the formamide concentration or the hybridization temperature. Formamide decreases the melting temperature by weakening the hydrogen bonds, thus

enabling lower temperatures to be used with high stringency. The salt immobilizes hybrid molecules and is used instead of formamide to reduce toxic waste. The general hybridization conditions used for ISH with rRNA-targeted probes are shown in Table 2. The concentration of NaCl ranged from 0 to 900 mM and that of formamide ranged from 0% to 50%, and the hybridization temperature ranged from 37° to 55°C. Hybridization time ranged from 30 min to 16 h. After hybridization, the slides or filters are rinsed with the appropriate buffer to remove the unbound probe.

Components in hybridization buffer	Probe type	Target molecule	References
NaCl, Tris-HCl, SDS	Oligonucleotide DNA	rRNA	Amann et al., 1990a, 1995
NaCl, Tris-HCl, SDS, formamide	Oligonucleotide DNA	rRNA	Amann et al.,1995; Manz et al., 1996
NaCl, Tris HCl, dextran sulfate, SDS, formamide, blocking reagent	Oligonucleotide DNA	rRNA	Pernthaler et al., 2002b
NaCl, Tris-HCl, EDTA, poly(A), formamide, dextran sulfate	Polyribonucleotide	rRNA	Delong et al., 1999
NaCl, Tris-HCl, dextran sulfate, SDS, formamide, *E.coli* tRNA, salmon sperm DNA, blocking reagent	Polyribonucleotide	rRNA	Pernthaler et al., 2002a
NaCl, sodium citrate, formamide, dextran sulfate, blocking reagent, Denhardt's solution, yeast RNA, salmon sperm DNA	Polyribonucleotide	mRNA	Pernthaler & Amann, 2004.
NaCl, Tris-HCl, SDS, formamide	Polyribonucleotide	DNA	Zwirglmaier et al., 2004
NaCl, sodium citrate, formamide, salmon sperm DNA	Polynucleotide DNA (fragmented PCR product)	DNA	Niki & Hiraga, 1998
NaCl, sodium citrate, formamide, dextran sulfate	Polynucleotide DNA (fragmented genomic DNA)	DNA	Lanoil & Giovannoni, 1997

Table 1. Representative components of hybridization buffer.

	Formamide (%)	NaCl (mM)	Temperature	Time (h)
Condition	0-50	0-900	37-55	0.5 -16

Table 2. General hybridization conditions for rRNA-targeted ISH.

Bouvier & del Giorgio (2003) investigated factors that influenced the detection of bacterial cells in rRNA-targeted FISH. They collected experimental conditions for FISH and environmental factors based on published reports and found that both NaCl and formamide in the hybridization buffer and wash solution significantly influence the performance of rRNA-targeted FISH. These two chemicals are used to adjust the stringency conditions of hybridization and wash steps.

Appropriate controls play an important role in optimizing hybridization and wash conditions. In general, target bacteria include perfect match sequence as positive control. As a negative control, non-target bacteria include a known mismatched sequence.

4. Problems and pitfalls in ISH

4.1 False positive results

The accuracy and reliability of ISH is highly dependent on the specificity of the probe. The sequence design and evaluation of the new probe are essential. Appropriate positive and negative control strains should be included in every ISH experiment. Probe sequences should be carefully examined using the latest version of sequencing data. The stringency conditions of hybridization and wash steps affect both false positive and negative results The mild conditions result in nonspecific probe binding to mismatched sequences or cell structures. Newly designed probes should be evaluated in laboratories by molecular microbiological methods such as ISH, dot blot hybridization, melting curve analysis etc.

4.2 False negative results

Low signal intensity may be a result of insufficient penetration of the probe into the target cells. It depends on the structure of the cell wall or membrane of the bacterial cells. Permeabilization conditions need to be optimized so that all reagents can penetrate the cell. The permeability of the bacterial cell wall structures is not uniform, and different permeabilization procedures have been employed for different cells. In general, gram-negative bacteria tend to be permeable under well-known permeability conditions. For gram-positive bacteria, special fixation and pretreatment is required as described above.

In case rRNA is the target molecule, loop and hairpin formation of target RNA hampers hybridization, leading to differential accessibility of these probes. Self-annealing and self-hairpin formation of the probe itself can also lead to low signal intensity (Fuchs et al., 1998). In addition, the rRNA content of bacterial cells may vary considerably within species as well as strains depending on the physiological state in the given environment. Low rRNA content may result in low signal intensity or false negative results. Various strategies have been used to overcome this difficulty, including FISH combined with direct viable counting, use of multiply-labeled polynucleotide DNA or RNA probes, enzymatic signal amplification, and in situ DNA amplification (see 5. Enhancement of signal intensity).

To test false negative results because of methodological problems, universal probes such as EUB338 (Amann et al., 1990a) have been commonly used as positive control probes. If the control with the universal probe yields good results in ISH, then fixation, probe permeabilization, and rRNA content of the target cells are not the problem. A commonly used negative control probe is NON338, which is complimentary to EUB338 (Wallner et al.,

1993). Using this probe, the non-specific binding of the probe to cell structures other than target nucleic acids can be evaluated.

5. Enhancement of signal intensity

The signal from hybridized cells is highly dependent on the content of target molecules. Despite its potential, the application of ISH in targeting rRNA of resident bacteria in oligotrophic environments is hampered by the low copy number of target molecules. To increase signal intensity, two major environmental microbiological approaches were employed, signal amplification and target nucleic acids amplification. The representative methods are shown in Table 3.

Category	Methods	References
Signal amplification	Tyramid signal amplification	Schönhuber et al., 1997
	Two-pass Tyramid signal amplification	Kubota et al., 2006
	HNPP/Fast Red	Yamaguchi et al., 1996
	Multiply-labeled polyribonucleotide	Delong et al., 1999
Target DNA amplification	In situ PCR	Hodson et al., 1995
	LAMP	Maruyama et al., 2003
	CPRINS	Kenzaka et al., 2005b
	In situ RCA	Maruyama et al., 2005
Target RNA amplification	Direct viable counting	Kenzaka et al., 2001

Table 3. Signal amplification and target nucleic acids amplification methods.

5.1 Signal amplification

Enzymatic signal amplification using a tyramide signal amplification system (TSA) or HNPP/Fast Red was combined with oligonucleotide/polynucleotide probes to increase the sensitivity of ISH (Kenzaka et al., 1998; Kubota et al., 2006; Schönhuber et al., 1997). In the alkaline phosphatase-HNPP/Fast Red system, a digoxigenin-labeled oligonucleotide probe was detected by an alkaline phosphatase-conjugated anti-digoxigenin antibody. Fluorescent molecules accumulate in the target cells because of the activity of alkaline phosphatase (Yamaguchi et al., 1996). In horseradish peroxidase (HRP)-TSA system, oligonucleotide probes were labeled with HRP that generates fluorescent molecules in cells when fluorescent tyramide was used as a substrate (Schönhuber et al., 1997). These enzymatic amplification systems resulted in an 8–20-fold amplification of signal intensity. In case of a two-pass TSA, tyramide tagged with dinitrophenyl was generated around the probe/target site by the activity of HRP, and then HRP-labeled anti-dinitrophenyl antibody further accumulated in cells. Tyramide-Cy3 was deposited by the activity of accumulated HRP (Kawakami et al., 2010).

The signal in SEM–ISH can be amplified using a similar system to detect low copy number target DNA sequences in individual cells (Kenzaka et al., 2009). In the study, digoxigenin-

labeled polynucleotide probes were hybridized with plasmid DNA. Peroxidase-labeled anti-digoxigenin antibody was bound to digoxigenin. By using tyramide signal amplification, biotin molecules were accumulated inside target cells. Target cells were identified by streptavidin bound to a gold immunoprobe. Gold particle enhancement was performed to amplify probe signals from hybridized cells. Low vacuum SEM images of a mixture of *E. coli* JM109 harboring plasmid pT7GFP (tagged with *gfp* and *bla* genes) cells and *E. coli* Okayama O27 cells after ISH with *gfp* and *bla* probes are shown in Fig. 3a, 3b, respectively. *E. coli* JM109 harboring pT7GFP were approximately 5-μm long and rod shaped. The long rod-shaped cell with the target gene showed a strong signal because of the high density of gold in one portion of the cell (shown as arrows in Fig. 3a and 3b).
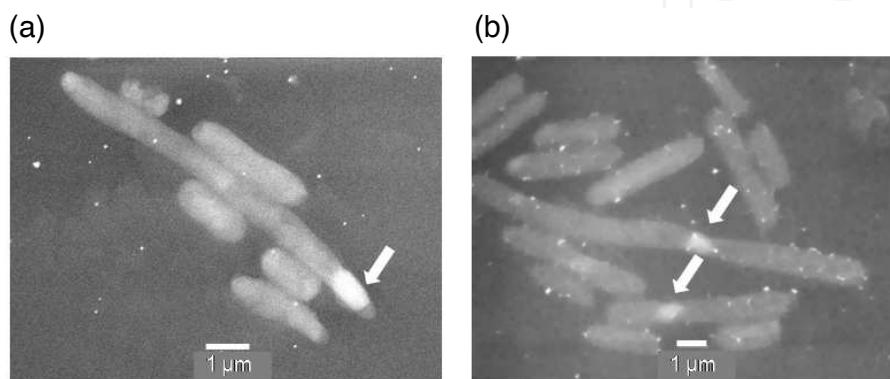
(a)   (b)

Fig. 3. Low-vacuum SEM images of a mixture containing *E. coli* JM109 harboring pT7GFP cells and *E. coli* Okayama O27 cells after ISH with *gfp* probe (a) and *bla* probe (b). Arrow indicates target *E. coli* JM109 cells harboring pT7GFP.

5.2 Target nucleic acids amplification

In situ DNA amplification techniques based on fluorescent labeling have been successfully applied to identify individual genes in a single bacterial cell. The basic approach is in situ PCR in which target DNA sequences are amplified inside cells (Hodson et al., 1995; Tani et al., 1998). The application of in situ PCR with functional probes provides a powerful tool for detection of genes or gene products in individual cells. This method, however, cannot be applied to diverse species in the natural environment mainly because of permeability, the leakage of amplified products, and less effective concentration of target cells. Longer amplified products after in situ DNA amplification would give better results because these products are less likely to leak out from the cell.

In situ loop-mediated isothermal amplification (LAMP) generates long tandem repeats of the target sequence, preventing amplicons from leaking outside the cell (Maruyama et al., 2003). The mild permeabilization conditions and low isothermal temperature used in the in situ LAMP method causes lesser cell damage than in situ PCR.

Cycling primed in situ amplification (CPRINS) uses a single primer and results in linear amplification of the target DNA. The amplicons are long, single-stranded DNA and are thus retained within the permeabilized microbial cells. ISH with a multiply labeled probe set enabls significant reduction in a nonspecific background while maintaining high signals of target bacteria (Kenzaka et al., 2005b).

In situ rolling circle amplification (RCA) require one short target sequence (less than 40 mer) and generate large, single-stranded, and tandem repeats of target DNA as amplicons. The circularizable probes are approximately 90 mers, comprising short complementary sequences of the target DNA at the 3' and 5' ends, respectively, with an arbitrary sequence in the middle. These probes are labeled with a phosphate group at the 5' end and circularized by ligation when hybridized to the target sequences. The RCA primers amplify the complementary sequence of the circularized probe by hybridization to a specific region of the probe. The amplicons are a single-stranded tandem repeats of the circularized probe sequence. It can be detected with labeled oligonucleotide probes. (Maruyama et al., 2005). CPRINS and in situ RCA can be performed on polycarbonate filters, which allow the effective concentration of target cells from aquatic samples and enhance the quantitative analysis.

Target rRNA molecules can be increased by the direct viable counting (DVC) method (Kenzaka et al., 2001). This method is based on the incubation of samples with antimicrobial agents and nutrients. The antibiotic cocktail acts as a specific inhibitor of DNA synthesis and prevents cell division without affecting other metabolic activities. The resulting cells can continue to metabolize nutrients and elongate and/or become fattened after incubation. By employing these techniques, SEM-ISH will lead to further improvements in sensitivity.

6. Applicability of SEM-ISH to complex microbial communities

Fluorescence microscopy and confocal laser scanning microscopy are important tools in effectively examining complex microbial communities attached to various materials in the natural environment. Potential problems with these fluorescent techniques include autofluorescence, which results from natural substances within plant tissue, organic debris, soil particles, etc. This may hamper the observation of target microbes in complex microbial communities.

SEM allows the visualization of cells attached to materials (e.g., sediment particles) without the interference of autofluorescence and without requiring the ultrathin sectioning of materials. In our experiments, *E. coli* JM109 cells expressing green fluorescent protein (GFP) were introduced into natural river water samples and subjected to FISH with an ES445 probe. Figs. 4a and 4b show the fluorescence micrographs of bacterial cells hybridized with a Cy3-labeled probe. Under blue excitation, the inoculated cells expressing GFP were identified (Fig. 4a).

Although these cells were expected to show bright Cy3 fluorescence under green excitation, the *E. coli* cells attached to organic debris in river water samples were masked by the nonspecific binding of the probe to organic debris in river water samples (Fig. 4b). Consequently, this hampered the identification and accurate enumeration of target cells by FISH.

To enhance the reliability of enumerating target cells, SEM-ISH was employed for the same river water samples containing inoculated *E. coli* JM109 cells. Fig. 4c shows the SE image of organic debris with *E. coli* cells on the surface. The advantage of SEM-ISH is to enable clear observation of the cell surface structure, detritus, etc., under higher magnification. Fig. 4d shows the portion of Fig. 4c that was magnified. Inoculated *E. coli* cells were approximately 5-μm long and rod shaped. The magnification of the SE image allowed clear differentiation between the target cells (shown as arrow in Fig. 4d) and the other cells. By comparing the

BSE image from the same microscopic field, the probe signal was detected from the hybridized cells (Fig. 4e). Even when SEM-ISH was applied to the river water samples, the problem of nonspecific binding of probes to organic debris as shown in Fig. 4b was not completely resolved. However, high magnification allowed target cells to be detected and distinguished from others, even with a high background. As a result, SEM-ISH proved better than fluorescence-based methods in discriminating between target cells and others in the water samples (Fig. 4d and 4e).
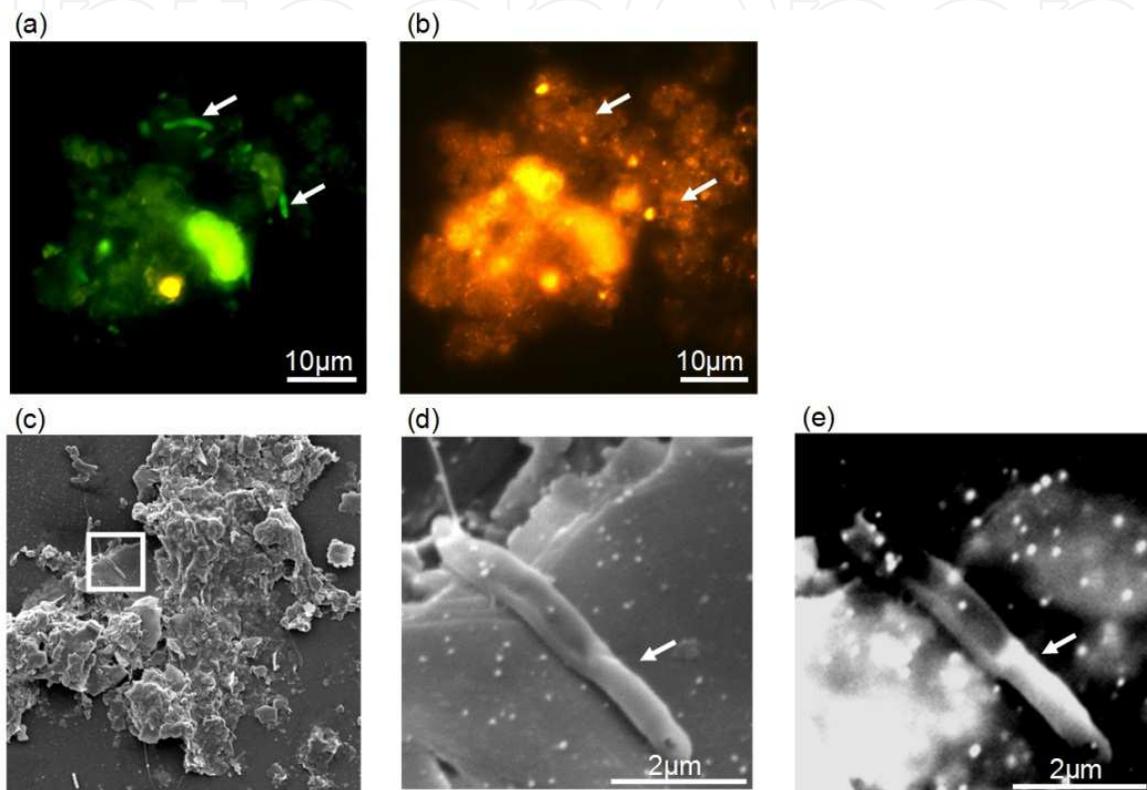
Fig. 4. Organic debris masks the probe signal in river water. *E. coli* cells expressing GFP were inoculated into river water samples, and subjected to FISH with Cy3 labeled probe ES445 (a, b) or SEM-ISH with biotin labeled probe ES445 (c, d, e). *E. coli* cells expressing GFP became attached to organic debris in the river water sample and delineated under blue excitation (a), but probe signals were masked under green excitation (b). High vacuum SEM imaging of *E. coli* cells attached to organic debris in the river water sample (c) was magnified (d). The topographic information was obtained with SE images (c and d), and cells hybridized with ES445 probe were detected with the BSE image (e). Arrow indicates introduced *E. coli* cell.

Certain bacterial cells happen to be buried in the surface of materials such as sediments or soil particles, and cell boundaries were unclear under SEM. In this case, it was difficult to distinguish individual cells within the particle structure using only SE signals. BSE images provided the probe signals from the hybridized cells in the same microscopic fields and clarified the existence of buried cells. We applied SEM-ISH with rRNA-targeted probes to examined bacteria communities on surface of sediment samples (Kenzaka et al., 2005a). SEM-ISH revealed the significant abundance of the *Cytophaga–Flavobacterium* cluster (detectable by probe CF319) on the surface of sediment particles and confirmed a wide distribution over the particle surface. When observed at high magnification, certain bacterial

cells were found to be buried in the particles (Fig. 5a and 5b). SEM-ISH with rRNA-targeted probes identified the buried cells based on rRNA sequence.
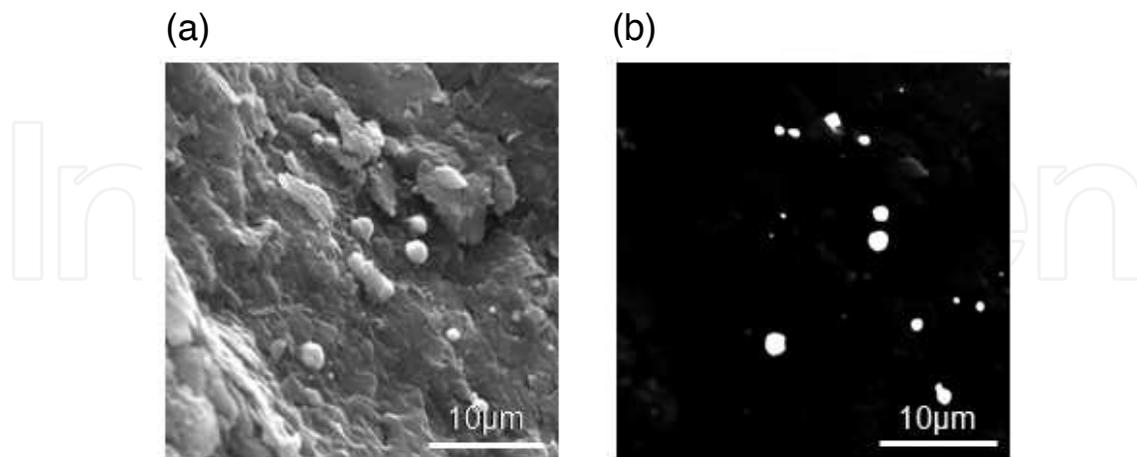

Fig. 5. High-vacuum SEM images of bacteria attached on surface of river sediment particles detected by ISH with the probe CF319 (targeted for *Cytophaga-Flavobacterium* phylum). The same microscopic fields are shown with SE image (a), BSE image (b). The topographic information was obtained with SE images (a), and cells hybridized with the probe CF319 were detected with the BSE image (b).

7. Access to yet-to-be-cultured bacteria with ISH

In the environment, more than 90% of the bacterial communities cannot be cultured by standard techniques, and the yet-to-be-cultured fraction includes diverse microorganisms that are only distantly related to the cultured ones. Culture-independent methods are essential to understand the genetic diversity, population structure, and ecological roles of the bacterial communities. The use of PCR-based clone libraries or metagenomics of an assemblage of microorganisms has great potential for the exploring novel species and sequences and for the understanding the composition and function of microbial communities and their dynamics in the environment (Handelsman, 2004).

Once novel RNA or DNA sequences are obtained by such approaches, ISH with newly designed probes targeting the novel sequences would be a valuable tool for the investigation of their distribution and abundance in the given environment. To validate probe specificity, ISH of clones with target sequences as inserts into plasmids (clone-ISH) would play important roles (Schramm et al., 2002).

8. Conclusion

SEM has been widely used in environmental microbiology to study cell and biofilm morphology. Combining morphological study with SEM and ISH with RNA- or DNA-targeted probes has demonstrated great potential in visualization of cells attached to materials without interference by autofluorescence and requiring no ultrathin sectioning of materials. SEM-ISH addresses some of the limitations of FISH alone and enhances the reliability of monitoring target cells in environmental samples in which the application of fluorescence-based methods is limited. The concepts of ISH in electron microscopic studies

can lead to a new understanding of the spatial distribution of target cells as well as of the extent of cell heterogeneity on plant, metal, alloy, bioreactor, or the three-dimensional structures of the attachment matrix.

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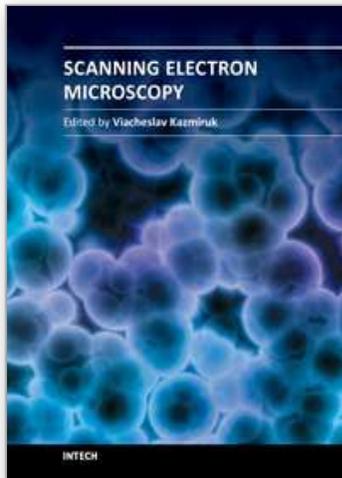
Scanning Electron Microscopy

Edited by Dr. Viacheslav Kazmiruk

Today, an individual would be hard-pressed to find any science field that does not employ methods and instruments based on the use of fine focused electron and ion beams. Well instrumented and supplemented with advanced methods and techniques, SEMs provide possibilities not only of surface imaging but quantitative measurement of object topologies, local electrophysical characteristics of semiconductor structures and performing elemental analysis. Moreover, a fine focused e-beam is widely used for the creation of micro and nanostructures. The book's approach covers both theoretical and practical issues related to scanning electron microscopy. The book has 41 chapters, divided into six sections: Instrumentation, Methodology, Biology, Medicine, Material Science, Nanostructured Materials for Electronic Industry, Thin Films, Membranes, Ceramic, Geoscience, and Mineralogy. Each chapter, written by different authors, is a complete work which presupposes that readers have some background knowledge on the subject.

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