FLUORESCENCE MICROSCOPIC OBSERVATIONS
OF MICROORGANISMS IN SOIL

A Thesis
Presented in Partial Fulfillment of the Requirements for
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Graduate School of The Ohio State University

By
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*****

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ABSTRACT

Microscopic observation of soil thin sections is potentially an important approach to trace the fate of external bacterial cells following soil inoculation and to determine the spatial distribution of indigenous bacterial populations in the soil. Various fluorescence microscopy techniques and their applications are reviewed with special emphasis on microbial distributions within soil microenvironments. Commonly used fluorochromes and factors affecting the staining of soil microorganisms are discussed.

The distribution of inoculated E. coli cells and indigenous bacteria in a silt loam soil was studied using conventional epifluorescence microscopy and confocal laser scanning microscopy (CLSM). A sandy soil was used for comparison of the influence of soil texture on staining quality. Seven fluorochromes and three resins were tested for their suitability for preparing high quality soil thin sections. Scotchcast resin 3™ was chosen as the best embedding medium after a comparison of important features with LR white resin and Nanoplast™ resin. Inoculated bacterial cells, stained with fluorescein isothiocyanate, 5-(4,6-dichlorotriazinyl)aminofluorescein and eosin Y, were distinguished only with difficulty from background fluorescence in the silt loam soil, but were distinguished clearly in the sandy soil due to the non-fluorescence of sand particles. Ethidium bromide, 4',6-diamidino-2-phenylindole and calcofluor white M2R stained inoculated cells clearly...
against the soil and resin background, although different levels of primary and induced fluorescence existed. Inoculated cells were mainly distributed among soil aggregates and some were located inside soil aggregates. Few indigenous cells were seen in the microscopic field of investigation and they were low in fluorescent intensity. This result was not unexpected due to the fewer and smaller cells (compared to inoculated cells), possible capsular layers around the cells or blocked contact of the fluorochromes with bacterial cells in uninoculated soil. Changing of the solution composition and pH during staining did not significantly improve image quality. Incubation of soil with 0.2% glucose for 24 h before fixation was not successful in enhancing the observation of indigenous cells. Satisfactory results were obtained from both epifluorescence microscopy and CLSM, with the best stain and resin combination being ethidium bromide and Scotchcast resin.
DEDICATION

To my husband and my family, for their love and support.
ACKNOWLEDGMENTS

I am very grateful to my advisor, Dr. Warren Dick, for providing me the opportunity of studying here, for his instruction in my research work and his guidance in my spiritual growth. Especially I thank him for driving hundreds of miles to Columbus when we had meetings during the past two years. His enthusiasm in research influenced me and I respect him as a model in both scientific study and daily life.

Special thanks to Dr. Olli Tuovinen for his kindness opening the door to me so I could have a home lab to use in Columbus apart from Dr. Dick’s lab in OARDC at Wooster. I express my thanks to him for his generous help throughout my research – from the starting proposal to the final version of my thesis – even though he could not continue to serve as a committee member due to his recent sabbatical in Japan. I thank him for his patient listening and sincere advice when I had difficulties. I learned a lot from him in many aspects.

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I also sincerely thank the Environmental Science Graduate Program for supporting me financially for more than a year so that I could finish my degree.
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Soil Microbiology

Fluorescent Microscopy
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>VITA</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>NON-STANDARD ABBREVIATIONS</td>
<td>xiv</td>
</tr>
</tbody>
</table>

1. FLUORESCENCE MICROSCOPY FOR VISUALIZATION OF SOIL MICROORGANISMS — A REVIEW

1.1 Abstract ............................................................................. 1
1.2 Introduction ......................................................................... 2
1.3 Principles of Fluorescence Microscopy .......................................... 4
    1.3.1 Fluorescence and Fluorochromes ........................................... 4
    1.3.2 Fluorescence Microscopy ....................................................... 6
    1.3.3 Confocal Laser Scanning Microscopy ....................................... 9
1.4 Soil Thin Section Fluorescence Microscopy ..................................... 11
    1.4.1 General Overview ............................................................... 11
    1.4.2 Fluorochrome Types Based on Binding Targets ............................ 15
        1.4.2.1 Nucleic Acid Stains ...................................................... 15
        1.4.2.2 Protein Stains ............................................................ 17
        1.4.2.3 Polysaccharide Stains — Fluorescent Brighteners ............... 18
        1.4.2.4 Other Fluorescent Stains ............................................ 19
    1.4.3 Ionic Characteristics ......................................................... 19
    1.4.4 Resin Embedding .................................................................. 20
    1.4.5 Staining Order and Counterstaining .................................... 22
    1.4.6 Stain Concentration, Solution pH and Soil Clay Content ........... 23
1.4.7 Primary Fluorescence of Soil ................................................................. 23
1.4.8 Other Factors .......................................................................................... 25
1.5 Concluding Remarks .................................................................................. 25

2. EXPERIMENTAL EVALUATION OF FLUOROCHROMES AND RESINS FOR MICROSCOPIC VISUALIZATION OF SOIL BACTERIA

2.1 Abstract ...................................................................................................... 28
2.2 Introduction ................................................................................................ 29
2.3 Materials and Methods .............................................................................. 34
  2.3.1 Soil and Bacterial Inoculum .................................................................. 34
  2.3.2 Fluorochromes ..................................................................................... 34
  2.3.3 Staining Effect of Fluorochromes on Cell Cultures and Mixture of Cells with Soil Particles ......................................................... 34
  2.3.4 Staining Effect of Selected Fluorochromes in Soil Column Experiment .......................................................................................... 38
    2.3.4.1 Inoculation, Fixation, Staining and Destaining ........................... 38
    2.3.4.2 Sample Dehydration .................................................................... 39
    2.3.4.3 Resin Embedding ........................................................................ 39
    2.3.4.4 Thin Sectioning ........................................................................... 41
    2.3.4.5 Ionization and pH of Solutions .................................................... 41
    2.3.4.6 Glucose Addition ....................................................................... 42
    2.3.4.7 Cells Removed from the Soil Column during Thin Section Preparation .................................................................................. 42
  2.3.5 Microscopy and Photography .................................................................. 45
  2.4 Results and Discussion ............................................................................ 45
    2.4.1 Evaluation of Fluorochromes Used ................................................ 45
      2.4.1.1 Cell Culture and Cell: Soil Mixture ....................................... 45
      2.4.1.2 Soil Thin Sections .................................................................. 48
    2.4.2 Resin .................................................................................................. 49
      2.4.2.1 Scotchcast Resin 3 ................................................................ 49
      2.4.2.2 LR White Resin ..................................................................... 49
      2.4.2.3 Nanoplast Resin ..................................................................... 51
      2.4.2.4 Adhesives .............................................................................. 51
    2.4.3 Imaging Inoculated Bacterial Cells with Epi-FM and CLSM .......... 52
      2.4.3.1 Visualization of Inoculated Bacterial Cells ............................ 52
        2.4.3.1.1 Ethidium Bromide-Stained Soil ..................................... 52
        2.4.3.1.2 Soil Stained with Other Fluorochromes ...................... 60
      2.4.3.2 Visualization of Indigenous Bacterial Cells ........................... 65
    2.4.4 Primary Fluorescence of Soil Components ....................................... 65
    2.4.5 Effect of Solution Ions and pH ......................................................... 68
    2.4.6 Other Factors Affecting Thin Section Quality .................................. 68
    2.4.7 Cells Removed from the Soil Column during Thin Section Preparation .................................................................................. 69
2.5 Conclusions and Suggestions ........................................ 70

APPENDIX
  Molecular formula and structures of selected fluorochromes......... 73

REFERENCES................................................................. 77
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>General overview of previous studies on direct microscopic observation of soil microorganisms</td>
<td>13</td>
</tr>
<tr>
<td>1.2</td>
<td>Major properties of commonly used fluorochromes for soil microbial studies</td>
<td>16</td>
</tr>
<tr>
<td>1.3</td>
<td>Summary of primary fluorescence of soil components based on previous studies</td>
<td>24</td>
</tr>
<tr>
<td>2.1</td>
<td>Common names and chemical names of the fluorochromes used in this study</td>
<td>35</td>
</tr>
<tr>
<td>2.2</td>
<td>Major properties of the fluorochromes used in this study</td>
<td>36</td>
</tr>
<tr>
<td>2.3</td>
<td>Positive and negative qualities of the fluorochromes used in this study</td>
<td>47</td>
</tr>
<tr>
<td>2.4</td>
<td>Evaluation of important properties of the three resins used in this study to prepare thin sections</td>
<td>50</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>State changes of an electron in an atom or molecule after it absorbs a photon. The electron can be excited to different excitation states but only the first excitation state is shown. Fluorescence occurs when the electron emits a photon of longer wavelength and returns to the ground state.</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Light paths of (A) a transmitted-light microscope, and (B) an incident-light fluorescence microscope</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Light path of a confocal fluorescence microscope. Out-of-focus light rays will be blocked by the detector pinhole and only the in-focus light rays can reach the detector.</td>
<td>10</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic experimental design for using a fluorochrome to stain cells, cell:soil smears and soil thin sections. B = bacteria, F-fixed = formaldehyde-fixed, G-fixed = glutaraldehyde-fixed, PBS = phosphate buffer saline, G⁻ = gram negative, G⁺ = gram positive</td>
<td>43</td>
</tr>
<tr>
<td>2.2</td>
<td>Scheme of (A) slide preparation for soil smears and thin soil sections and (B) collection of bacteria in the leachates of five washings in a parallel soil column study.</td>
<td>44</td>
</tr>
<tr>
<td>2.3</td>
<td>Bacterial cells in a silt loam soil inoculated with <em>E. coli</em> and stained with ethidium bromide. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation</td>
<td>53</td>
</tr>
<tr>
<td>2.4</td>
<td>Bacterial cells in a silt loam aggregate inoculated with <em>E. coli</em> and stained with ethidium bromide. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation</td>
<td>55</td>
</tr>
<tr>
<td>2.5</td>
<td>Photomicrograph of epifluorescent <em>E. coli</em> in phosphate buffer. Epifluorescence microscopy, blue excitation</td>
<td>56</td>
</tr>
</tbody>
</table>
2.6 Projection image of 25 confocal serial sections (taken at 400 nm increments) of ethidium bromide-stained silt loam soil that had been inoculated with E. coli. Excitation wavelength was 488 nm .................. 58

2.7 Photomicrograph of inoculated E. coli in the silt loam soil stained with ethidium bromide. Epifluorescence microscopy, blue excitation .......... 59

2.8 Photomicrograph of sandy soil inoculated with E. coli and stained with DAPI. Epifluorescence microscopy, UV excitation ......................... 61

2.9 Projection image of 25 confocal serial sections (taken at 400 nm increments) of FITC-stained sandy soil inoculated with E. coli. Excitation wavelength was 488 nm. The green color represents bacteria and the red color represents the sand ................................................. 63

2.10 Projection image (top) and reconstructed 3-D image (bottom) of 25 confocal serial sections (taken at 400 nm increments) of FITC-stained sandy soil inoculated with E. coli. Green color represents bacteria and red color represents the sand ......................................................... 64

2.11 Indigenous bacteria in an aggregate of ethidium bromide-stained silt loam soil. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation ........................................... 66
## GLOSSARY AND NON-STANDARD ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>CFW</td>
<td>calcofluor white M2R</td>
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<tr>
<td>CLSM</td>
<td>confocal laser scanning microscope</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTAF</td>
<td>5-(4,6-dichlorotriazinyl)aminofluorescein</td>
</tr>
<tr>
<td>EFM</td>
<td>epi-fluorescence microscope</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>eosin yellow; 2',4',5',7'-tetrabromo-3',6'-dihydroxyspiro-[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one disodium salt</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polysaccharides</td>
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<td>Ethidium bromide</td>
<td>3,8-diamino-5-ethyl-6-phenyl-, bromide</td>
</tr>
<tr>
<td>Eu(TTA)$_3$</td>
<td>europium chelate, or europium(III) thenoylethylfluoroacetate</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanates</td>
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<tr>
<td>LP</td>
<td>long pass filter</td>
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<tr>
<td>Mg-ANS</td>
<td>magnesium salt of 8-aminole-1-naphthalene sulfonic acid</td>
</tr>
<tr>
<td>OM</td>
<td>organic matter</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline solution</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SFDA</td>
<td>5-(and 6-)sulfofluorescein diacetate</td>
</tr>
<tr>
<td>SP</td>
<td>short pass filter</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>Thiazol yellow G</td>
<td>2,2'-(1-triazene-1,3-diyl-di-4,1-phenylene)bis[6-methyl-7-benzothiazolesulfonic acid] disodium salt</td>
</tr>
</tbody>
</table>
CHAPTER 1
FLUORESCENCE MICROSCOPY FOR VISUALIZATION OF SOIL MICROORGANISMS -- A REVIEW

1.1 Abstract

Fluorescence microscopic techniques used for characterizing microbial distributions in soil are discussed in this review. Principles and applications of epifluorescence microscopy and confocal laser scanning microscopy are reviewed for visualization of soil microorganisms. Several fluorochromes have been used for soil microbial studies. These include dyes that bind to specific cellular components, such as acridine orange, ethidium bromide, fluorescein isothiocyanate, 5-(4,6-dichlorotiazinyl)aminofluorescein, 4′,6-diamidino-2-phenylindole, europium chelate, magnesium salt of 8-anilino-1-naphthalene sulfonic acid and calciofluor white M2R. Both cationic and anionic dyes have been used based on their cellular specificity and their affinity for charged soil particles. There are many other factors that influence the staining of soil thin sections such as resin embedding and soil texture. In situ microscopic observations of soil organisms have focused on plant roots, protozoa, fungi, inoculated bacteria, and rhizobial and rhizobacterial cells. Few studies have focused on indigenous bacteria and their spatial relationship within various microhabitats. General limitations of applying fluorescence microscopy to soil ecological studies are the non-specific binding of dyes to the soil
matrix and the autofluorescence of some soil components. The development of fluorescent in situ hybridization and confocal laser scanning microscopy techniques provides new potential for microbial distribution studies.

1.2 Introduction

The soil is a heterogeneous system where the behavior of soil microorganisms is affected by their immediate surrounding microenvironment. Soil aggregates are considered to be the basic microhabitats of soil microorganisms (Hattori, 1988). Therefore, the structure and composition of soil aggregates are closely related to microbial distribution. There are two approaches to study microbial distribution in soil aggregates: (1) fractionation of outer and inner layers of soil aggregates to distinguish microbial cells, and (2) direct observation of soil microorganisms in situ by means of microscopic techniques.

In the fractionation approach, microbial analyses (e.g., isolation or enumeration) were conducted on water-washed fractions of soil aggregates and the results showed a trend of microbial distribution from the outer to inner layers of the aggregates (Hattori, 1988). In traditional soil microbial analyses, microorganisms are first extracted from the soil, and then cultured in different media (plating techniques). Isolates can be purified if identification is needed. Conventional culture techniques can provide information only on the culturable species in the soil. The extraction of soil microorganisms is not complete due to the rapid and strong sorption of some microbial cells to clay particles. Not knowing what cultural conditions to use also causes many species to be undetected. As a
result, methods based on cultural recoveries underestimate the actual microbial population by at least one or two orders of magnitude (Hartmann et al., 1997).

Direct microscopic observations of soil microorganisms have been performed with fluorescence microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM). Transmission electron microscopy and SEM can provide high resolution images and therefore are very useful in ultrastructural studies of soil particles and microorganisms (Foster, 1988). For situations where submicron resolution is adequate, fluorescence microscopy is appropriate since artifacts from sample preparation are not evident in its images but are noticeable in SEM or TEM images (DeLeo et al., 1997). Fluorescence microscopy has been applied for microbial enumeration or spatial distribution of soil microorganisms in their microhabitats (Tippkötter et al., 1986; Altemüller and Van Vliet-Lanoe, 1990; Postma and Altemüller, 1990; Pickup, 1995; DeLeo et al., 1997; Fisk et al., 1999).

The introduction of confocal laser scanning microscopic (CLSM) technique provides a new potential for soil microbial distribution studies. Confocal laser scanning microscopy has gained popularity over the past ten years and originally was used primarily in the biomedical and life sciences. With its many advantages, this technique is attracting the attention of researchers in microbial ecology studies involving biofilms, compost processes, and biofouling (Lawrence et al., 1991; Caldwell et al., 1992a; Surman et al., 1996; Swope and Flickinger, 1996; Chalmers et al., 1997).
1.3 Principles of Fluorescence Microscopy

1.3.1 Fluorescence and Fluorochromes

Fluorescence is defined as the property of some atoms and molecules to emit light at longer wavelengths after absorbing light of a particular and shorter wavelength (Herman, 1998). Each fluorescent atom or molecule can absorb light of only certain wavelengths. An electron of a fluorescent molecule absorbs a photon of energy and is excited from the ground state to a higher electronic energy and vibrational state (Figure 1.1). The energized electron then returns to the ground energy state with a loss of vibrational energy to the environment. During the return of the molecule to the ground state, a photon of longer wavelength is emitted. This is referred to as fluorescence (Herman, 1998; Ploem and Tanke, 1987). The emitted light is always of a longer wavelength and of a lower intensity than that of the absorbed light because of the energy loss (Ploem, 1993).

Excitation (or absorption) spectrum can be obtained by scanning the absorption wavelengths at a constant emission wavelength. Similarly, emission spectrum can be obtained when the excitation wavelength is constant and emission wavelengths are measured. Some molecules are autofluorescent, emitting fluorescence without any prior treatment. This phenomenon is called primary fluorescence. Secondary fluorescence represents the fluorescence produced by application of certain stains, which are known as fluorochromes (Altemüller and Van Vliet-Lanoe, 1990).
Figure 1.1. State changes of an electron in an atom or molecule after it absorbs a photon. The electron can be excited to different levels of excitation states but only the first excitation state is shown here. Fluorescence occurs when the electron emits a photon of longer wavelength and comes back to the ground state.
1.3.2 Fluorescence Microscopy

Fluorescence microscopes can be categorized into two types based on their optical paths: transmitted-light fluorescence microscopes and incident-light or epi-illumination fluorescence microscopes. The former requires very careful alignment of the condenser and objective lenses and can not be used for simultaneous fluorescence/phase or fluorescence/differential interference contrast imaging. With the epi-illumination set-up, the condenser is also the objective. Therefore, only one optical axis exists and perfect alignment is not required. Other advantages of epifluorescence microscopy are the effective use of a dichromatic beam splitter (whose function will be explained later in this section) and the easy change-over between fluorescence microscopy and transmitted light microscopy. All these advantages make incident-light the most commonly employed illumination system (Ploem, 1993; Herman, 1998). The optical paths of these two illumination set-ups are shown in Figure 1.2.

The key components of epifluorescence microscopy include an excitation light source, wavelength selection devices (a set of well-balanced filter combinations), objectives, and detectors (Ploem, 1993; Herman, 1998). Only the first two components will be discussed in this paper. A light source (e.g., tungsten halogen lamps, high-pressure Hg, Xe, Hg/Xe combination arc lamps, or lasers) emits excitation energy of different wavelengths. The maximal excitation of fluorescence occurs when the light wavelength peak is close to the absorption peak of the fluorescent molecule (Herman, 1998).
Figure 1.2. Light paths of (A) a transmitted-light microscope, and (B) an incident-light fluorescence microscope.
Maximization can be reached by using an excitation filter that only transmits light below a specific wavelength corresponding to the maximal absorbance of the molecule (Herman, 1998). The excitation filter also reduces the amount of background light that has the same wavelength range as the emitted fluorescent light.

The second filter in epifluorescence microscopy, a dichromatic beam splitter, plays a critical role in the function of the epifluorescence microscope. The dichromatic beam splitter is also known as a dichromatic mirror (Ploem, 1993) and is designed to reflect light of shorter wavelength while transmitting light of longer wavelength (Herman, 1998). The excitation light is reflected by the dichromatic beam splitter and condensed by the objective before reaching the specimen. In theory, only the fluorescence emitted from the specimen passes through and reaches the detector (the eyepiece or a camera in the microscope). The development of the dichromatic beam splitter is one of the major advantages of epifluorescence microscopy over transmitted-light fluorescence microscopy (Ploem, 1993). To further limit the unabsorbed excitation light from reaching the detector, a barrier filter is installed between the chromatic beam splitter and the detector.

The various types of filters are defined by their wavelength selection characteristics: short pass filter (SP), long pass filter (LP), dichromatic beam splitter, wide pass filter, narrow band pass filter and SP-LP combination (Ploem and Tanke, 1987; Herman, 1998). Details of these filters are outside the scope of this review.
1.3.3 Confocal Laser Scanning Microscopy

The principle of confocal microscopy is the same as that of epifluorescence microscopy, except for the introduction of two pinholes (or apertures) into the system, instead of the excitation and emission filter, to eliminate the “out-of-focus” light. The first pinhole is placed in front of the light source and the second is located in front of the detector. These two pinholes have the same focus, so-called “confocal,” which allows only the point of light in focus to be observed (Sheppard and Shotton, 1997; Paddock, 1999).

Currently the major application of confocal microscopy is fluorescence imaging. The basic configuration of a confocal fluorescence microscope is shown in Figure 1.3. The point of light from the source pinhole is reflected by a dichromatic beam splitter and focused by the objective lens onto the specimen. The emitted fluorescent light passes through the detector pinhole via the dichromatic beam splitter, and is then focused onto a photomultiplier, which produces a signal related to the brightness of the light. Light from out-of-focus specimen parts and unwanted scattered light are all efficiently filtered out by the detector pinhole (Sheppard and Shotton, 1997; Paddock, 1999). Unlike conventional epifluorescence microscopy, where the image can be viewed directly from the fluorescence of an entire specimen, confocal fluorescence microscopy can obtain a translated image (not a real image). This image is built up by a computer from the output of the photomultiplier as the focused spot of light scans across the specimen.

A light beam from a laser is a commonly used light source for scanning across the specimen, so the confocal microscope is commonly referred to as a confocal laser
Figure 1.3. Light path of a confocal fluorescence microscope. Out-of-focus light rays will be blocked by the detector pinhole and only the in-focus light rays can reach the detector.
scanning microscope. The 2-D images obtained from the microscope are more accurate than images obtained from a conventional epifluorescence microscope due to the improved resolution, detection sensitivity and elimination of out-of-focus light (Caldwell et al., 1992b). By changing the focus in the depth direction, confocal laser scanning microscopy can obtain a series of 2-D images (xy) along the z axis, from which 3-D images can be reconstructed with the aid of some special graphic software. These features make confocal laser scanning microscopy particularly advantageous over conventional epifluorescence microscopy (Caldwell et al., 1992b). In a way, confocal laser scanning microscopy is a bridge between conventional light microscopy and scanning electron microscopy.

1.4 Soil Thin Section Fluorescence Microscopy

1.4.1 General Overview

To study the in situ spatial distribution of bacteria, intact soil samples must be used. This can be accomplished using resin-embedding techniques that maintain both the soil structure and the spatial relationship between the microorganisms and soil matrix. Thin sections of the resin-embedded soil sample make the microscopic observation of fluorochrome-stained microorganisms optically possible. Common procedures for preparation of soil thin sections include fixation, staining and destaining, dehydration, resin embedding, and thin sectioning of the soil sample (Altemüller and Van Vliet-Lanoe, 1990). Strictly speaking, these preparation treatments disturb the soil sample to a certain extent. No reports have been published, however, on how much disturbance these procedures may cause.
Selection of a suitable fluorochrome is the most important step in imaging of bacteria in thin sections. Fluorochromes can be classified into two groups according to their functional characteristics. The first group specifically stains cell components such as nucleic acids, proteins, lipids, or the cell membrane. The second group of fluorochromes will not fluoresce, but can become fluorescent as a result of metabolic cell processes (Tsuji et al., 1995; Riis et al., 1998). In microbial distribution studies, viability or activity of bacterial cells is often not a concern. Hence, commonly employed fluorescent dyes belong to the first group. Some effort has been made using a stain that combines these two dye types to differentiate between living and dead cells in soil (Bhupathiraju et al., 1999; Maeda et al., 1999).

There are thousands of fluorochromes in the first group, but only a few are routinely employed for soil microbial studies. Commonly used dyes in soil microbial studies are acridine orange (AO), ethidium bromide, fluorescein isothiocyanate (FITC), 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF), 4',6-diamidino-2-phenylindole (DAPI), the magnesium salt of 8-anilino-1-naphthalene sulfonic acid (Mg-ANS), and europium chelate (Postma and Altemüller, 1990; Bottomley, 1994; Bloem et al., 1995; Maeda et al., 1999). The fluorescent brightener, calcofluor white M2R, has been used mainly to measure fungal biomass (Morgan et al., 1991; Bloem et al., 1995). Although direct microscopic bacterial counting does not use resin embedding and thin sectioning techniques, it does provide useful information on the suitability of a fluorochrome for staining bacteria against an optically opaque soil background. Table 1.1 gives a general overview of the studies on direct microscopic observations of soil microorganisms with
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Inoculum</th>
<th>Stain</th>
<th>Resin type</th>
<th>Microscopy</th>
<th>Soil type</th>
<th>Soil treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi and Bacteria</td>
<td><em>Pythium debaryanum</em>  <em>Bacillus cereus</em>  <em>Bacillus megaterium</em>  <em>Verticillium alboatrum</em></td>
<td>Mg-ANS</td>
<td>No resin</td>
<td>EFM</td>
<td>Many types</td>
<td>Autoclaved soil aggregates</td>
<td>Mayfield (1975)</td>
</tr>
<tr>
<td>Plant roots and protozoa</td>
<td>Ryegrass, barley  <em>Engelmaniella halseyi</em></td>
<td>Many types for plants Mg-ANS for protozoa</td>
<td>Polyester resin</td>
<td>EFM</td>
<td>Sandy loam</td>
<td>Sieved (&lt;5 mm) and air dried</td>
<td>Tippkötter et al. (1986)</td>
</tr>
<tr>
<td>Bacteria: inoculum and indigenous</td>
<td><em>Rhizobium leguminosarum</em>  biovar trifolii</td>
<td>CFW/AO counterstaining</td>
<td>Vestopal 160 (polyester)</td>
<td>EFM</td>
<td>Loamy sand silt loam</td>
<td>Air dried and sieved (&lt;2 mm)</td>
<td>Postma and Altemüller (1990)</td>
</tr>
<tr>
<td><strong>Microbial groups:</strong></td>
<td><strong>Bacteria</strong></td>
<td>Fuchsin (acid &amp; basic)</td>
<td>Polyester resin</td>
<td>EFM</td>
<td>Chernozem (Loess)</td>
<td>Sieved (&lt;2 mm) and sterilized</td>
<td>Tippkötter (1990)</td>
</tr>
<tr>
<td></td>
<td><strong>Actinomycetes</strong></td>
<td>Eosin Y  Acriflavin  Morin  CFW  Auramine  Uvitea  P-rosaniline  AO  Congo red</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Indigenous bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. General overview of previous studies on direct microscopic observation of soil microorganisms (to be continued).
Table 1.1. (continued)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Inoculum</th>
<th>Stain$^2$</th>
<th>Resin type</th>
<th>Microscopy$^3$</th>
<th>Soil type</th>
<th>Soil treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td><em>Pseudomonas fluorscens</em></td>
<td>CFW</td>
<td>Crystic resin</td>
<td>EFM</td>
<td>Sandy loam</td>
<td>Sieved (2 – 3 mm) and air dried</td>
<td>White et al. (1994)</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus sp.</em> <em>Bacillus megaterium</em></td>
<td>AO</td>
<td>Scotcheast resin (epoxy)</td>
<td>CLSM</td>
<td>Fine sand</td>
<td>sieved (63 – 150 µm), carbonates, OM and iron oxides removed</td>
<td>DeLeo et al. (1997)</td>
</tr>
<tr>
<td>Fungus</td>
<td><em>Mucor racemosus</em></td>
<td>SFDA/ Mg-ANS</td>
<td>Polyester resin</td>
<td>EFM</td>
<td>Clay loam</td>
<td>Air dried and sieved (1 – 2 mm)</td>
<td>Maeda et al. (1999)</td>
</tr>
<tr>
<td>Bacteria: inoculum indigenous</td>
<td><em>Arthrobacter crystallopoies</em></td>
<td>Thiazine red R</td>
<td>Scotcheast resin (epoxy)</td>
<td>EFM</td>
<td>Loamy fine sand</td>
<td>Soil cores, autoclaved</td>
<td>Fisk et al. (1999)</td>
</tr>
<tr>
<td>Indigenous bacteria and EPS</td>
<td>-</td>
<td>DAPI and FITC- Concanavalin A</td>
<td>Nanoplast resin (melamine)</td>
<td>CLSM</td>
<td>Marine stromatolite sediments</td>
<td>Sections from fresh sample</td>
<td>Decho and Kawaguchi (1999)</td>
</tr>
</tbody>
</table>

$^1$EPS = extracellular polysaccharide.

$^2$AO = acridine orange, CFW = calcifluor white M2R, DAPI = 4',6-diamidino-2-phenylindole, FITC = fluorescein isothiocyanate, Mg-ANS = magnesium salt of 8-anilino-1-naphthalene sulfonic acid, OM = organic matter, SFDA = 5-(and 6-)sulfofluorescein diacetate.

$^3$CLSM = confocal laser scanning microscopy, EFM = epifluorescence microscopy.

$^4$- = information not available.
biological stains (some are not fluorescent dyes). Some characteristics of major fluorochromes are listed in Table 1.2.

1.4.2 Fluorochrome Types Based on Binding Targets

Cell component-binding fluorochromes are discussed in groups according to their targets (nucleic acids, protein, cellulose and others) in the following paragraphs.

1.4.2.1 Nucleic Acid Stains

Acridine orange is one of the oldest, most studied, and commonly employed nucleic acid stains used in microbial studies. Acridine orange has two peculiar characteristics. The first is its concentration effect. Postma and Altemüller (1990) reported that the color of acridine orange-stained bacterial cells was weak green at low acridine orange concentration (0.05 g L\(^{-1}\)) and intensely red at high concentration (1.0 g L\(^{-1}\)). The second feature is that acridine orange-bound DNA fluoresces yellow-green to green and acridine orange-bound RNA appears orange to red (Handbook of Fluorescent Probes and Research Chemicals, 2000).

Although acridine orange is still used to stain inoculated bacteria in sandy soil (DeLeo et al., 1997), staining soil microorganisms with acridine orange before resin embedding is not recommended. A major disadvantage of acridine orange is its non-specific staining of microorganisms and soil particles (Altemüller and Van Vliet-Lanoe, 1990).
<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Molecular weight</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Target</th>
<th>Color of stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange (AO)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>301.82</td>
<td>500 (DNA bound)</td>
<td>525 (DNA bound)</td>
<td>Nucleic acids</td>
<td>Green or red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>460 (RNA bound)</td>
<td>650 (RNA bound)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcofluor white M2R (CFW)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>936.95</td>
<td>UV</td>
<td>-&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Polysaccharides</td>
<td>Violet</td>
</tr>
<tr>
<td>4',6-diamidino-2-phenylindole (DAPD)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>350.25</td>
<td>358</td>
<td>461</td>
<td>Double stranded DNA</td>
<td>Violet</td>
</tr>
<tr>
<td>5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>495.28</td>
<td>492</td>
<td>516</td>
<td>Protein, carbohydrates and polysaccharides</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>Ethidium bromide&lt;sup&gt;1&lt;/sup&gt;</td>
<td>394.31</td>
<td>518</td>
<td>605</td>
<td>Nucleic acids</td>
<td>Red</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate (FITC)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>389.38</td>
<td>494</td>
<td>519</td>
<td>Protein</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>Magnesium salt of 8-anilino-1-naphthalene sulfonic acid (Mg-ANS)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>311.00</td>
<td>385</td>
<td>485</td>
<td>Protein and membrane</td>
<td>Blue</td>
</tr>
<tr>
<td>Thiazine red R&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>599.58</td>
<td>UV</td>
<td>-</td>
<td>-</td>
<td>Red</td>
</tr>
<tr>
<td>Thiazol yellow G&lt;sup&gt;2,4&lt;/sup&gt;</td>
<td>695.73</td>
<td>402</td>
<td>-</td>
<td>-</td>
<td>Yellow to greenish</td>
</tr>
</tbody>
</table>

<sup>4</sup> Lillie (1977).
<sup>5</sup> - = information not available.

Table 1.2. Major properties of commonly used fluorochromes for soil microbial studies.
Ethidium bromide and DAPI are two other common nucleic acid stains. They are normally used for bacterial enumeration or biomass measurements in soil smears. Roser (1980) reported a better contrast of soil and bacteria in ethidium bromide stained soil smears than acridine orange treated soil smears. Although the intensity was lower, the fluorescence of ethidium bromide stained cells fades slowly. Very little information is available on the use of ethidium bromide or DAPI to stain soil microorganisms either before or after resin embedding. DAPI and ethidium bromide were used to stain a loamy soil with and without an *E. coli* inoculum (see Chapter 2 of this thesis). Inoculated cells stained with both stains were readily discerned under epifluorescence microscopy. The fluorescent intensity of the indigenous bacterial cells, however, was very low and essentially indistinguishable from autofluorescent soil particles.

Europium(III) thienoyltrifluoroacetonate (europium chelate or Eu(TTA)_3) has been used to stain soil microorganisms combined with calcofluor white M2R (Postma and Altemüller, 1990). Since acetone dehydration was used in the study, acetone was used to rinse the soil smear for testing the stability of the adsorbed stains. The authors found that the intense red fluorescence of Eu(TTA)_3-stained bacterial cells disappeared.

1.4.2.2 Protein Stains

Fluorescein isothiocyanate is normally used in conjugated forms with proteins. Postma and Altemüller (1990) obtained clear, brightly stained rhizobial cells after applying fluorescein isothiocyanate-conjugated serum to a soil smear. However, the stain intensity decreased when polyester resin was used. Decho and Kawaguchi (1999)
employed fluorescein isothiocyanate-concanavalin A (concanavalin A - a type of lectin) to stain extracellular polymeric secretions of stromatolite sediments and obtained good images using confocal laser scanning microscopy.

Fluorescein isothiocyanate is considered one of the best stains for soil work (Bloem et al., 1995) and DTAF, a fluorescein isothiocyanate analogue that stains carbohydrates and proteins, is gaining more applications for enumeration of soil bacteria (Bloem et al., 1995; Schumann and Rentsch, 1998). An improved image of DTAF-stained bacteria in soil smears was reported by Bloem et al. (1995) when compared with ethidium bromide-stained cells. DTAF has not been used to stain bacterial cells in soil thin sections to date.

Mayfield (1975) used a magnesium salt of 8-anilino-1-naphthalene sulfonic acid (Mg-ANS) to stain bacteria and fungi in soil aggregates on a microscope slide. In photographs, the stained microorganisms appeared bright against a dark soil background. Mg-ANS was used as a vital stain for protozoa by Tippkötter (1990). The ciliate, *Engelmaniella halseyi*, was stained before addition to soil samples and embedding in resin. Mg-ANS can also be used to stain polished resin blocks (Gray, 1990). Combined staining with 5-(and 6-)sulfofluorescein diacetate and Mg-ANS has been successfully applied to differentiate living and dead microbial cells in soil thin sections (Maeda et al., 1999).

1.4.2.3 Polysaccharide Stains — Fluorescent Brighteners

Fluorescent brighteners have an affinity for cellulose and related substances (Altemüller and Van Vliet-Lanoe, 1990). They normally stain cell walls producing
intense light blue or bright violet-blue coloration with violet excitation or UV excitation, respectively. Calcofluor white M2R, also called cellufluor, is one of the fluorescent brighteners commonly used. This stain binds to polysaccharide structures of microbial cell surfaces (Hartmann et al., 1997) and is primarily used to investigate plant root hair and fungal hyphae in soil thin sections (Tippkötter et al., 1986; Postma and Altemüller, 1990). Inoculated bacterial cells in soils were stained brightly according to Postma and Altemüller (1990). Calcofluor white pre-stained cells were also used as an inoculum to study bacterial distribution in soil pores (White et al., 1994). All of these studies gave satisfactory results using calcofluor white.

1.4.2.4 Other Fluorescent Stains

The binding mechanisms of thiazol yellow G, primulin, and thiazine red R are unclear, so they are classified into this group. In a cooperative study by Altmüller and Postma (1989), thiazol yellow G and primulin were applied to soil samples after fixation but prior to resin embedding and thin sectioning in an effort to stain plant roots and soil microorganisms. Acceptable results were obtained from both of these yellow stains. Less effective staining was reported for thiazine red R by Postma and Altemüller (1990), but applying thiazine red R into intact cores of Freehold fine sandy loam gave satisfactory results for fluorescence microscopy of soil thin sections (Fisk et al., 1999).

1.4.3 Ionic Characteristics

Some stains are cationic in solution. They are also called basic stains because of the positive charge on the chromophore. The positive charge is problematic in soil microbial
studies because fluorochromes not only stain the microbes but also sorb to clay particles of soil, which have negative charges associated with their double-layer structure (Altemüller and Van Vliet-Lanoe, 1990). Many fluorochromes, such as acridine orange, ethidium bromide, DAPI, acriflavine and auramine, belong to this category.

Stains may also be anionic in solution. With a negative charge on the chromophore, these stains are also called acidic stains (Altemüller and Van Vliet-Lanoe, 1990). This type of fluorochrome is less likely to sorb on soil particles. Both fluorescein isothiocyanate and DTAF carry a negative charge at neutral pH. Fluorescein is very pH sensitive and its ionization state changes as pH changes (Klonis and Sawyer, 1996). Calcofluor white M2R is also anionic in solution (Altemüller and Van Vliet-Lanoe, 1990). Other anionic dyes include thiazol yellow G, primulin and thiazine red R.

1.4.4 Resin Embedding

Resin embedding of soil is used to maintain soil structure and microbial location in the soil matrix. Three types of resin are widely used for embedding purposes: polyester resin, epoxy resin, and acrylic resin. Good quality thin sections depend on resin characteristics such as viscosity, completeness of impregnation, attainable thickness of thin sections, and surface hardness. Tippkötter and Ritz (1996) tested different types of resins for use in in situ studies of plant roots, indigenous fungi and actinomycetes in soil. Two polyester resins proved most suitable for their purposes.

Resin can be polymerized (or cured) by heat, curing agents (hardeners, catalysts or accelerators), or UV irradiation (Hayat, 1989). An ideal situation is to cure resin after a
thorough resin infiltration through the soil sample. For fast-curing resins (such as when
accelerators are used), the resin polymerization may be done before the resin penetrates
the soil thus causing problems (see Chapter 2 of this thesis). This is, however, not
problematic for heat-cured resins because the resin will remain unpolymerized until the
required temperature condition is reached. The required curing temperature varies
between resins. The temperature needed for curing should be considered if the target
biological sample might be altered by high temperature. Polymerization by UV
irradiation is poorly understood and therefore not widely used for resin curing (Hayat,
1989).

Resin autofluorescence greatly interferes with the visualization of stained bacterial
cells in soil thin sections. Some types of resin are strongly autofluorescent, thus
quenching the fluorescence of the stained cells. Resin fluorescence decreases with

Information about fluorescence of different types of resin is very limited. Nanoplast
resin has almost no fluorescence when used as an embedding medium for marine
stromatolite sediments (Decho and Kawaguchi, 1999). This makes this melamine resin
attractive for in situ soil microorganisms observations. However, the cost, brittleness, and
lengthy curing time (Hayat, 1989) hinder its broad application in soil ecology studies.

An ideal thin section thickness is 15 μm or less. This thickness produces a good
image not only from the reduced out-of-focus specimen fluorescence, but also reduces
resin autofluorescence. Common procedures to obtain soil thin sections include cutting
and polishing of resin blocks. The resin block is trimmed to a suitable size with a
diamond saw, then one face of the block is polished with a diamond wheel and affixed to
the smooth surface of a microscope slide with adhesive resin. Excess resin is trimmed
and the remaining 1 to 2 mm resin section on the slide is further ground with a diamond
wheel and then finally hand polished with grinding powder. The thickness can be
determined optically by the interference color of quartz or from the ultramicrotome
setting (Hayat, 1989; FitzPatrick, 1993). Confocal microscopy can also be used to
measure thin section thickness because it can scan in the direction of thickness and the
scanning depth will be shown on the computer screen in μm (see Chapter 2 of this thesis).

1.4.5 Staining Order and Counterstaining

Staining the sample after fixation but before embedding and sectioning was
recommended by Altemüller and Van Vliet-Lanoe (1990). Staining of resin-impregnated
samples may not be effective because staining would be mainly a surface effect. Bacterial
cells far below the surface may not be stained if the section is 15-30 μm thick. Direct
staining of thin sections or blocks normally is used to counterstain soil in thin sections.
Counterstaining is applicable mainly for improving the contrast of soil microorganisms
and the soil matrix. It combines pre-staining of microorganisms (with anionic dyes)
before resin embedding with staining of the soil in the thin section (with cationic dyes)
(Altemüller and Van Vliet-Lanoe, 1990). Successful counterstaining was reported with
calcofluor white M2R (a fluorescent brightener) and acridine orange (Postma and
Altemüller, 1990). Thiazol yellow or primulin counterstained with acridine orange was
also suggested by Postma and Altemüller (1990).
1.4.6 Stain Concentration, Solution pH and Soil Clay Content

A higher concentration of fluorochromes normally increases fluorescence of the stained cells until all the binding sites of cell components are filled. The increased level of fluorochrome may also lead to more fluorochrome molecules sorbed to soil particles, making the destaining step more difficult. Hence an optimal stain concentration should be chosen based on balancing cell fluorescence intensity and ease of destaining.

Many fluorochromes require a specific pH range. For example, fluorescein isothiocyanate and DTAF are very pH-sensitive and carry a negative charge under neutral pH conditions. The charge may change if the pH changes (Klonis and Sawyer, 1996). Acridine orange staining also depends on the pH range. Current protocols for soil thin sections call for acridine orange in 10% HCl to get a better contrast between organisms and the soil (Altemüller and Van Vliet-Lanoe, 1990).

Clay content affects the fluorescent background in thin section because cationic dyes tend to bind to negatively charged clay surfaces. For example, acridine orange stains many types of clays (FitzPatrick, 1993). Background fluorescence, which consists of soil autofluorescence and excess stain adsorbed on the soil, will interfere with observations of stained cells.

1.4.7 Primary Fluorescence of Soil

Many soil components are autofluorescent (Table 1.3). This primary fluorescence interferes with the secondary fluorescence emitted by stained cells in the soil thin section. Soil primary fluorescence remains a problem for in situ soil microbial studies.
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Fluorescent components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minerals</td>
<td>Aluminum oxide hydroxide&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Apatite minerals&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Carbonates&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Clay and colloidal materials&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Feldspars and other weathering silicate minerals: strong light greenish (UV excitation)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fluorite, zircon, or some feldspars&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Newly formed aluminum polymers&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Phosphate: greenish yellow (blue excitation)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Phytolites and other biogenic opal: strong fluorescence&lt;sup&gt;1,2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Organic substances</td>
<td>Chitin, tannic acid, cellulase, protease, and gum xanthan&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Chloroplasts: red fluorescence&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cutinized and suberized bark cell walls: whitish fluorescence (UV excitation)&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lignified parts: blue fluorescence (UV excitation) to whitish, yellowish and brown as decomposition continues&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Parenchymatic tissues in needle litter: brownish to reddish fluorescence (blue excitation)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Polysaccharide material such as the callose plugs of pollen tubes&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Undecomposed fragments of cellulose&lt;sup&gt;1,3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Young cell walls around root tips, cap cells and root hairs: blue (UV excitation), change over time and decomposition&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>FitzPatrick (1993).
<sup>4</sup>Preece (1971).

Table 1.3. Summary of primary fluorescence of soil components based on previous studies.
1.4.8 Other Factors

Good quality thin sections can only be obtained when every treatment step is compatible with the others (fixatives, fluorochromes, dehydration methods and resin types) (Tippkötter and Ritz, 1996). For example, the embedding resin is also an organic solvent and may react with the stains. This could lead to reduced fluorescence intensity of stained cells because some stains (1) are degraded during the embedding procedure (Hayat, 1989) or (2) cause increased resin fluorescence that masks the identification of stained cells (Altemüller and Van Vliet-Lanoe, 1990). If a chemical dehydration method is employed to remove water after staining but before the embedding process, ethanol or acetone may release the stain binding to the microbial cells as it replaces water in the soil sample.

1.5 Concluding Remarks

Early thin section fluorescent observations of soil organisms used plant roots, protozoan cells, or fungal hyphae. These organisms are all of relatively large size and easy to discern under epifluorescence microscopy (Tippkötter et al., 1986). The techniques were then extended to visualize inoculated bacteria including *Rhizobium* cells in high cell densities (White et al., 1994; DeLeo, 1997). Very few studies of indigenous cells in thin sections have been reported (Tippkötter, 1990; Decho and Kawaguchi, 1999; Fisk et al., 1999). The biological stains used in these studies changed over time from non-fluorescent to fluorescent dyes.
The size and color of cells in cultures may be used as a reference for inoculated cells in soil. Postma and Altemüller (1990) distinguished inoculated cells from native cells by cell color and location. They reported the added cells stayed in larger soil pores, usually with high fluorescence intensity, while indigenous cells tended to remain in small pores and with weak staining color. However, this differentiating method is too arbitrary to be accepted as a general approach.

Due to soil autofluorescence, bacterial cells are difficult to discern using fluorescence microscopy. To date, there is no satisfactory method to differentiate unambiguously bacterial cells from other stained particles with similar size distribution. Because of the many factors involved in soil thin section preparation, there is no universal method for all biological materials in soil.

An alternative for general staining of bacterial cells is to specifically stain certain bacteria with fluorescence-labeled molecular probes. Fluorescent in situ hybridization (FISH) has been adopted to fill this role in soil microbial studies (Pickup, 1995; Macnaughton et al., 1996). By using a specific oligonucleotide probe, only the target organisms are stained by hybridization with the probe, allowing in situ observation of soil microorganisms. Information about microbial community structure in microhabitats may be obtained by using this technique, but the difficulty of identifying selectively stained cells from autofluorescent soil background is still a concern.

Spatial distributions of microorganisms on a soil microstructural scale can be characterized by confocal laser scanning microscopic (CLSM) techniques. One advantage of CLSM is that the out-of-focus fluorescence, common in epifluorescence microscopy,
can be eliminated. Another important advantage is that 3-D images of specimens can be reconstructed with graph processing software and hence the spatial location of soil microorganisms can be viewed using a computer. Problems due to soil autofluorescence, however, cannot be totally eliminated in CLSM. With the development of novel fluorescent dyes and more functional adaptations of CLSM, it may be possible to minimize the interference of primary fluorescence and improve the imaging of stained microbial cells.
2.1 Abstract

Microscopic observation of soil thin sections is potentially an important approach to trace the fate of external bacterial cells following soil inoculation and to determine the spatial distribution of indigenous bacterial populations in the soil. The distribution of inoculated E. coli cells and indigenous bacteria in a silt loam soil was studied using conventional epifluorescence microscopy and confocal laser scanning microscopy (CLSM). A sandy soil was used for comparison of the influence of soil texture on staining quality. Seven fluorochromes and three resins were tested for their suitability for preparing high quality soil thin sections. Scotchcast resin 3™ was chosen as the best embedding medium after a comparison of important features with LR white resin and Nanoplast™ resin. Inoculated bacterial cells, stained with fluorescein isothiocyanate, 5-(4,6-dichlorotriaziny1)aminofluorescein and eosin Y, were distinguished only with difficulty from background fluorescence in the silt loam soil, but were distinguished clearly in the sandy soil due to the non-fluorescence of sand particles. Ethidium bromide, 4',6-diamidino-2-phenylindole and calcofluor white M2R were found to stain inoculated cells clearly against the soil and resin background, although different levels of primary
and induced fluorescence existed. Inoculated cells were mainly distributed among soil aggregates and some were located inside soil aggregates. Few indigenous cells were seen in the microscopic field of investigation and they were low in fluorescent intensity. This result was not unexpected due to the fewer and smaller cells (compared to inoculated cells), possible capsular layers around the cells or blocked contact of the fluorochromes with bacterial cells in uninoculated soil. Changing of the solution composition and pH during staining did not significantly improve image quality. Incubation of soil with 0.2% glucose for 24 h before fixation was not successful in enhancing the observation of indigenous cells. Satisfactory results were obtained from both epifluorescence microscopy and CLSM, with the best stain and resin combination being ethidium bromide and Scotchcast resin.

2.2 Introduction

Bacteria are presumably distributed unevenly but not randomly in the soil due to the complex nature of structural aggregates. Pores between and within soil aggregates form a network that is filled with water and/or air depending on environmental conditions, and this network becomes the habitat for soil microorganisms. Based on electron microscopy studies, soil bacteria are more likely to reside in small, inner-aggregate pores while larger microorganisms (e.g., fungi and protozoa) tend to live in the outer part of aggregates (Hattori, 1988). This may be due to the fact that small pores retain water longer than larger pores, and may also facilitate bacterial escape from predation by larger microorganisms. Bacterial cell numbers in small pores were normally high and constant.
in density and cells were grouped mainly as single cells or in microcolonies. The density of cells in larger pores within the outer part of the aggregates often fluctuated (Foster, 1988).

Investigating the distribution of a soil bacterial population in its immediate microenvironment will help people understand the relationship between the population and its environment. Microscopic imaging techniques provide the most direct way to study microbial distribution in situ (Brock, 1987). Fluorescence microscopy is one of the techniques commonly used. It can confirm the location of indigenous bacterial populations (Tippkötter et al., 1986; Tippkötter, 1990) or follow the fate of introduced microorganisms in the soil (Postma and Altemüller, 1990; White et al., 1994; DeLeo et al., 1997; Fisk et al., 1999).

Since bacterial distribution may be determined by direct observation of thin sections, sample preparation is critical to obtain good quality microscopic images. Common procedures for fluorescence microscopy of soil microorganisms include fixation, staining, destaining, dehydration (if a water immiscible resin is used), resin embedding and thin sectioning of soil samples. Most previous studies applied an organic aldehyde (formaldehyde or glutaraldehyde) to fix the soil, which helped preserve the structure and shape of biological samples (Tippkötter and Ritz, 1996). Staining was typically performed after sample fixation, but sometimes stain was dissolved in the fixative solution and the two procedures were done simultaneously (Fisk et al., 1999). Acetone or ethanol was commonly employed to dehydrate the sample. Air-drying and freeze-drying were also considered appropriate (Hayat, 1989; Fisk et al., 1999).
One of the important steps in sample preparation is staining. In earlier studies, biological samples were stained with fluorochromes after soil thin sections had been made. In a study by Tippkötter et al. (1986), fungi, bacteria and soil organic matter in both soil thin sections and soil resin blocks were stained and observed with an epifluorescence microscope. It was found that stains could penetrate 2-10 μm into the section or block when applied to the surface. Basic fuchsin and methylene blue were found satisfactory although they did not show satisfactory results for plant roots. Altemüller and Van Vliet-Lanoe (1990) suggested that staining soil before resin embedding would be more effective than staining the soil thin section. In a later study by Tippkötter (1990), soil was stained prior to resin embedding. Of the many fluorochromes tested, auramine, acridine orange, acriflavin, eosin Y and morin resulted in satisfactory results for bacteria while calcofluor white M2R was better for fungi staining.

Compared with indigenous populations, inoculated bacterial cells are more easily observed. Fisk et al. (1999) studied both indigenous bacteria and inoculated *Archromobacter crystallopoietes*. Thiazine red R (1:10,000) was applied on top of the soil and allowed to penetrate the soil. Thin sections of 10-20 μm were made after the soil was embedded in epoxy resin. Orange cells were observed in inoculated soil under an epifluorescence microscope, but only a few indigenous bacteria could be located. In the study of Postma and Altemüller (1990), rhizobial cells were inoculated into three soils which were packed individually in glass cylinders. In the soil thin sections, inoculated cells stained with calcofluor white appeared bright blue and could be seen clearly against a brown and black soil background. Indigenous cells were described as smaller cells located in smaller
pores with lower staining intensity. A general approach to differentiate indigenous bacteria from inoculated cells is not currently available unless the inoculated cells are labeled with a fluorescently-labelled molecular marker.

Resin embedding is another crucial step in obtaining high quality soil thin sections. An ideal resin should have low reactivity and viscosity, provide complete soil impregnation, and yield minimal autofluorescence with sufficient hardness (Tippkötter and Ritz, 1996). Commonly employed resins are epoxy resins (DeLeo et al., 1997; Fisk et al., 1999) and polyester resins (Tippkötter et al., 1986; Altemüller and Vliet-Lanoe, 1990; Postma and Altemüller, 1990; Maeda et al., 1999). Other resin types, like melamine resins and acrylic resins, have also been used to address specific requirements of particular studies (Hayat, 1989; Decho and Kawaguchi, 1999).

Despite the obvious advantages of applying the fluorescence microscopy technique in soil ecology, there are limitations in the currently used methods. First, the primary fluorescence (or autofluorescence) of soil components (Altemüller and Vliet-Lanoe, 1990) strongly interferes with the observation of stained microorganisms. Second, the resin used for embedding and mounting may also fluoresce and mask the fluorescence of cells (Altemüller and Vliet-Lanoe, 1990). The third limitation is the decreased color contrast of cells and background due to retention of stains on soil, especially for cationic dyes (Altemüller and Vliet-Lanoe, 1990).

The introduction of digital microscopy, particularly confocal laser scanning microscopy (CLSM), offers new potential for the observation of microbial distributions in natural microhabitats. The development of image processing technology makes it
possible to reconstruct 3-D images reflecting the spatial distribution pattern of fluorescent targets. These techniques have been increasingly used in biofilm and bioaggregate studies (Lawrence et al., 1991; Caldwell et al., 1992a; Swope and Flickinger, 1996). Confocal laser scanning microscopy can also produce a more accurate 2-D image compared with epifluorescence microscopy since the blur originating from unfocused light is eliminated. Improved image resolution and detection sensitivity make digital microscopy one of the most promising new methods to study the spatial heterogeneity of microenvironments (Caldwell et al., 1992b; Hartmann et al., 1997). Confocal laser scanning microscopy was considered a better choice than scanning electron microscopy (SEM) for situations where micron or larger resolution is adequate because artifacts resulting from the sample preparation process are noticeable in SEM images but are less evident or absent in CLSM images (DeLeo et al., 1997).

The purpose of this study was to conduct an assessment of various combinations of thin section/staining/microscopic methods to visualize and study inoculated and indigenous bacteria in soil. Digital imaging was employed in epifluorescence microscopy and CLSM to obtain a better view of the bacterial distribution in microhabitats. An attempt to avoid the limitations of fluorescence microscopy was made by screening fluorochromes and resin and finding the primary fluorescence range of the tested soil. The goal was to provide information to the soil microbiologist and microbial ecologist to help them obtain optimum results from microscopic studies.
2.3 Materials and Methods

2.3.1 Soil and Bacterial Inoculum

Sandy soil and silt loam soil samples were used in this study. The sample of sandy soil (>95% sand) was received from Dr. J.M. Bigham (School of Natural Resources, The Ohio State University). The sample of silt loam soil (top 3-4 cm) (organic C – 20.5 g kg⁻¹, sand – 240 g kg⁻¹, silt – 535 g kg⁻¹, clay – 225 g kg⁻¹) was from a continuous corn field (Ostrofsky et al., 1997) in Ohio. The silt loam soil sample was stored in a sterile glass bottle at 4°C. Escherichia coli and Bacillus megaterium were both used for cell culture staining, but only E. coli was used for preparing cell:soil mixtures and soil column inoculations. E. coli and B. megaterium cultures were grown in Trypticase Soy Broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C and 30°C, respectively, for 24 h before use.

2.3.2 Fluorochromes

The common and chemical names of the stains used in this study and their properties are listed in Table 2.1 and Table 2.2, respectively. For the molecular formula and structure of each stain, refer to Appendix A.

2.3.3 Staining Effect of Fluorochromes on Cell Cultures and Mixture of Cells with Soil Particles

A 1 ml aliquot of the E. coli or B. megaterium cell suspension in trypticase soy broth was transferred to a 1.5 ml micro centrifuge tube. The supernatant was discarded
<table>
<thead>
<tr>
<th>Common name</th>
<th>Chemical name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcofluor white M2R (CFW)</td>
<td>2,2'-(1,2-ethenediy)bis[5-[[4-[bis(2-hydroxyethyl)amino]-6-(phenylamino)-1,3,5-triazin-2-yl]amino]-benzenesulfonic acid, disodium salt</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole, dihydrochloride</td>
<td>Polysciences, Warrington, PA</td>
</tr>
<tr>
<td>Thiazol yellow G</td>
<td>2,2'-(1-triazene-1,3-diyldi-4,1-phenylene)bis[6-methyl-7-benzothiazolesulfonic acid], disodium salt</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanates</td>
<td>Polysciences, Warrington, PA</td>
</tr>
<tr>
<td>DTAF</td>
<td>5-(4,6-dichlorotriazinyl)aminofluorescein</td>
<td>Molecular Probes, Eugene, OR</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>2',4',5',7'-tetrabromo-3',6'-dihydroxyspiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, disodium salt</td>
<td>Fisher Scientific, Pittsburgh, PA</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide</td>
<td>Polysciences, Warrington, PA</td>
</tr>
</tbody>
</table>

Table 2.1. Common names and chemical names of the fluorochromes used in this study.
<table>
<thead>
<tr>
<th>Property</th>
<th>CFW</th>
<th>DAPI</th>
<th>DTAF</th>
<th>Eosin Y</th>
<th>Ethidium bromide</th>
<th>FITC</th>
<th>Thiazol yellow G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation (nm)</td>
<td>UV</td>
<td>358</td>
<td>492</td>
<td>515-518</td>
<td>518</td>
<td>490</td>
<td>402</td>
</tr>
<tr>
<td>Emission (nm)</td>
<td>Not available</td>
<td>461</td>
<td>516</td>
<td>Not available</td>
<td>605</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td>Ionization</td>
<td>Fluorescent brightener (anionic)</td>
<td>Cationic</td>
<td>Anionic (neutral pH)</td>
<td>Anionic</td>
<td>Cationic (neutral pH)</td>
<td>Anionic</td>
<td>Not available</td>
</tr>
<tr>
<td>Affinity</td>
<td>Polysaccharides</td>
<td>Double stranded DNA</td>
<td>Protein, carbohydrates, polysaccharides</td>
<td>Not available</td>
<td>Nucleic acids</td>
<td>Protein</td>
<td>Not available</td>
</tr>
<tr>
<td>Color of stained cells</td>
<td>Violet</td>
<td>Violet</td>
<td>Yellow-green</td>
<td>Yellow-Green</td>
<td>Red</td>
<td>Yellow-green</td>
<td>yellow to greenish</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>936.95</td>
<td>350.25</td>
<td>495.28</td>
<td>692.00</td>
<td>394.31</td>
<td>389.38</td>
<td>695.73</td>
</tr>
</tbody>
</table>

1CFW = calcofluor White M2R, DAPI = 4',6-diamidino-2-phenylindole, DTAF = 5-(4,6-dichlorotriazinyl)aminofluorescein, eosin Y = eosin yellow, FITC = fluorescein isothiocyanate.

Table 2.2. Major properties of the fluorochromes used in this study. Data retrieved from http://www.probes.com (08/14/2000), Lillie (1977), and Gurr (1971).
after the suspension was centrifuged at a speed of 12,000 rpm for 10-15 sec. To study the staining effect of fluorochromes on fixed cells, the cell pellet was resuspended in 1 ml 2.5% glutaraldehyde (Polysciences, Warrington, PA) or 4% formaldehyde freshly made by dissolving paraformaldehyde (Polysciences) in deionized water. Glutaraldehyde- and formaldehyde-fixation continued for 6 h. The cell pellet was then stained in 1 ml of stain solution. A series of concentrations was tested for selected stains (abbreviations are explained in Table 2.1):

- **CFW**: 0.01, 0.1, 0.5, 1.0, 2.0 and 5.0 mg ml\(^{-1}\) in sterile deionized water for 1 h,
- **DAPI**: 0.1, 1 and 10 μg ml\(^{-1}\) in phosphate buffer (Atkin, 1999) for 1 h,
- **Thiazol yellow G**: 0.003, 0.03, 0.3 and 3 mg ml\(^{-1}\) in deionized water for 2 h,
- **FITC**: 4, 40 and 400 μg ml\(^{-1}\) in phosphate buffer (Bloem et al., 1995) for 2 h,
- **DTAF**: 20 and 200 μg ml\(^{-1}\) in phosphate buffer (Bloem et al., 1995) for 2 h,
- **Eosin Y**: 0.04% in water for 1 h,
- **Ethidium bromide**: 0.01% in water (Dutrieux et al., 1994) for 15 min.

Deionized water and all the phosphate buffers were sterilized using filters with 0.2 μm openings. The calcofluor white solution was also filtered before use.

The cell suspension was centrifuged and the pellet was washed three times in the same solution used for the staining (i.e., phosphate buffer or deionized water). After the pellet was resuspended in 1 ml of solution, 10 μl of the cell suspension was placed on a glass microscopic slide and air-dried. To reduce photobleaching, a drop of Vectashield™ mounting medium (Vector Laboratories, Burlingame, CA) was applied on top of cell smear before a cover slip was added and sealed with nail polish.
In a parallel experiment, 0.3 g soil was mixed with E. coli cell suspension before fixation to investigate the effect of staining on the cell and soil mixture.

2.3.4 Staining Effect of Selected Fluorochromes in Soil Column Experiments

2.3.4.1 Inoculation, Fixation, Staining and Destaining

A glass cylinder (1.4 cm inner diameter, 4 cm long) closed at the bottom with cheese cloth was filled with 5 g soil and loosely place in a 50 ml conical polyethylene tube. Soil aggregates were assumed to remain relatively intact because the silt loam soil was not air-dried and sieved before being placed into the cylinder. The soil column was inoculated with $10^9$ to $10^{10}$ E. coli cells per gram of soil. The E. coli cell culture in trypticase soy broth was centrifuged and rinsed with phosphate buffer saline, then resuspended in 1 ml phosphate buffer and applied to the top of the soil column over a period of 10 min. Filter-sterilized deionized water (0.5 ml) was added after inoculation to rinse the cells downward into the column. The inoculated soil column was equilibrated at room temperature for at least 6 h.

Formaldehyde (4%) or glutaraldehyde (2.5%) was used to fix the inoculated and uninoculated soil columns to keep the shape of bacterial cells by crosslinking proteins in the cells. The fixatives were poured into the space between the glass cylinder and the polyethylene tube so the soil column was immersed from the bottom. The sample was fixed for about 12 h. The soil column was then treated with stain solution for 12 h (1 mg ml$^{-1}$ of CFW, 0.1 μg ml$^{-1}$ of DAPI, 40 μg ml$^{-1}$ of FITC and 0.2 mg ml$^{-1}$ of DTAF, 0.04% eosin Y and 0.01% ethidium bromide) followed by three washings. The washings were
conducted using the same solution used to prepare the stain. The washings rinsed the soil column from the top and the solutions which emerged from the bottom were removed.

2.3.4.2 Sample Dehydration

Following staining, the samples were freeze dried. A freeze-drying method was chosen to avoid (1) a long dehydration time, (2) further disturbance of soil aggregates during washing, and (3) extraction of soluble soil organic matter that may be caused by acetone or ethanol dehydration (Tippkötter and Ritz, 1996). After freeze-drying, the soil column was embedded in one of the three resins as described below.

2.3.4.3 Resin Embedding

Three types of resin were tested for their suitability as embedding media in this study. One was an epoxy resin (Scotchcast Resin 3™, Industrial Electrical Products Division/3M, St. Paul, MN), one was an acrylic resin (LR White, London Resin Company Limited, Berkshire, England) and the third was a melamine resin (Nanoplast™ resin, Polysciences).

Scotchcast Resin has two parts — resin (part A) and catalyst (part B). Two parts A and three parts B (by weight) were mixed thoroughly. The mixture was heated in an oven at 60°C to reduce the viscosity and to remove air bubbles before being poured into a container to immerse the soil column in the polyethylene tube. Vacuum (about 400 mm Hg) was applied periodically for 2-3 h to aid penetration of the soil and to remove air
bubbles. The sample was left overnight to allow complete resin infiltration. Then the resin was cured at 95°C in an oven for 12 h.

LR White can be polymerized (or cured) with either accelerator curing or heat curing. In accelerator curing, we mixed the resin and accelerator thoroughly at designed ratios (10 ml, 20 ml, 30 ml, 40 ml and 60 ml resin to 1 drop accelerator) before pouring the resin into a tube and immersing the soil. The resin mixture was allowed to penetrate the soil and was cured at room temperature. Curing times varied from 10 min to 2 h when the ratio of resin to accelerator was changed from 10 ml resin to 1 drop accelerator to 60 ml resin to 1 drop accelerator.

Heat curing of LR White does not need an accelerator but requires anaerobic conditions. Resin was poured into a tube to immerse the soil sample and was left overnight to penetrate the soil. Helium gas was flushed through the tube for about 5 min with a cap placed loosely on the tube to release air. The cap containing a syringe needle (to provide a means for gas to escape) was then tightened on the tube and the tube was placed in an oven at 60±2°C overnight to cure the resin.

Nanoplast resin needs the addition of a catalyst for curing. The resin and the catalyst were mixed completely in a ratio of 10 g of resin to 0.20 g of catalyst and the resin mixture was allowed to penetrate the soil overnight. The sample was placed in a desiccator containing silicagel at 40°C for 2 d to dry the sample. The sample was then removed from the desiccator and placed in an oven at 60°C for 2 d for curing.
2.3.4.4 Thin Sectioning

After the resin was polymerized, the outer polyethylene tube was removed. The resin block was then cut into sections with a diamond saw (Hillquist, Inc. Seattle, Washington) and polished before being glued on a glass microscopic slide with Hillquist™ epoxy resin (Hillquist) or superglue (cyanoacrylate adhesive, Midwestern Home Products, Wilmington, DE). The sample was trimmed and the remaining 1 to 2 mm resin section on the slide was further ground with a diamond wheel followed by hand polishing using decreasing sizes of grinding powder. The thin section (about 10 – 15 μm thickness) was covered with a glass coverslip and sealed with colorless nail polish. The thickness was initially assessed based on experience and later confirmed by CLSM when the microscope laser scanned the soil thin section along the z-axis.

2.3.4.5 Ionization and pH of Solutions

Clay has a negatively charged double layer structure. Cationic dyes will be adsorbed to clay particles. Phosphate buffer saline provides an even higher negative charge to the soil matrix, which is undesirable in the destaining process. A lower pH provides more hydrogen ions (H+) and may neutralize some of the negative charge on the clay surface, and therefore may result in easier removal of excess stain on soil particles. To explore the ion charge effect on staining and destaining of bacteria in the soil, KCl solutions of pH 3.0 and pH 7.0 were used to dissolve FITC and DAPI which initially used phosphate buffer saline (with a higher pH value) as the dilution solution. The KCl solutions were also used to dissolve ethidium bromide instead of distilled water. Fluorescein
isothiocyanate, an anionic dye, was examined because it is very pH-sensitive. This study was done using an *E. coli* inoculated sample.

2.3.4.6 Glucose Addition

To improve our ability of observing indigenous bacterial cells in soil thin sections, a parallel study was conducted. 1 ml of 0.2% (w/v) sterile glucose was added to the soil column that did not contain bacterial inoculum and the soil was incubated at room temperature for 24 h. The purpose of the glucose was to provide carbon and energy for the native bacteria and to promote their metabolism.

2.3.4.7 Cells Removed from Soil Column During Thin Section Preparation

Parallel soil columns with and without bacterial inoculum (in duplicate) were washed with filter-sterilized deionized water at the five specific steps corresponding to preparation of the thin sections (fixation, staining and three times of washing). This study was to investigate how many bacteria would be flushed out by washing during the sample preparation process. Cell numbers in phosphate buffer saline before inoculation and in each leachate collected after washing were obtained through counting on Trypticase Soy Agar (TSA) (Difco Laboratories, Detroit, MI) colony plates. All the procedures used to prepare the soil thin sections and for plate counting are summarized in Figure 2.1 and Figure 2.2, respectively.
Figure 2.1. Schematic experimental design for fluorochromes staining of cell cultures, cell:soil smears and soil thin sections. B = bacteria, F-fixed = formaldehyde-fixed, G-fixed = glutaraldehyde-fixed, PBS = phosphate buffer saline, G⁻ = gram negative, G⁺ = gram positive.
Figure 2.2. Scheme of (A) slide preparation for soil smears and soil thin sections and (B) collection of bacteria in the leachates of five washings in a parallel soil column study.
2.3.5 Microscopy and Photography

Cell smears, cell-soil smears and soil thin sections were first examined using a Zeiss Standard Universal epifluorescence microscope equipped with a HBO-200 mercury lamp and filter set of G365/FT395/LP420, G436/FT510/LP520 and 450-490/FT510/LP520. Later a Zeiss Axioskop 2 epifluorescence microscope with a HBO 103 mercury lamp and DAPI and FITC filter sets was used. An AxioCam charge-coupled device (CCD) camera was used to capture digital images. Soil thin sections were also examined with a Leica TCS SP Confocal Laser Scanning Microscope (CLSM) equipped with lasers (458 nm, 488 nm, 568 nm and 633 nm lines).

Photographs were made using Fuji 1600 ASA film. Digital images were captured and converted to TIF format. Leica TCS SP image analysis software supplied with the microscope was used for projecting images. Final images were processed with Adobe PhotoShop 5.5.

2.4 Results and Discussion

2.4.1 Evaluation of Fluorochromes Used

2.4.1.1 Cell Culture and Cell:Soil Mixture

The observed results of cell smears and cell: soil smears were used to determine the optimum fixative, stains and stain concentrations for preparation of soil thin sections. Both *E. coli* and *B. megaterium* could be stained with all of the fluorochromes tested regardless of the status of the cells (living or fixed) except thiazol yellow G. These six stains (CFW, DAPI, FITC, DTAF, eosin Y and ethidium bromide) could be used for both gram-negative and gram-positive bacteria. Bacterial cells stained with thiazol yellow G
fluoresced too weakly to be seen with either UV or blue excitation. None of the concentrations of thiazol yellow G tested could stain cells efficiently despite the acceptable results reported (with 0.3 mg ml\(^{-1}\)) by Altemüller and Van Vliet-Lanoe (1990). Hence, this stain was not used in the subsequent cell: soil mixture study and the soil thin section study. Further information about thiazol yellow G must be obtained before the dye can be used for soil microbial studies. Advantages and disadvantages of the seven stains used are summarized in Table 2.3 based on the results from previous studies and this study.

From the observation of living and fixed cells, glutaraldehyde fixation was found to cause autofluorescence of cells since unstained glutaraldehyde fixed cells appeared yellow green under blue excitation while unfixed cells were not fluorescent. This induced autofluorescence may add an extra absorption peak for the stained cells. Living cells and formaldehyde fixed cells showed no such effect and the stained cells looked similar in both cases in color and color intensity. Due to the relative incompleteness of formaldehyde fixation, glutaraldehyde was mainly used for soil thin section studies.

Since using a higher stain concentration can cause difficulty in destaining the soil, minimum stain concentrations resulting in strong fluorescent intensity of cells were chosen for soil thin section studies. Bright, fiber-like particles were found in samples stained with unfiltered CFW, which obscured the target cells. After CFW was filtered through a glass filter with 0.2 \(\mu\)m pores, satisfactory images were obtained without the interference of fiber-like particles. The concentration of CFW after filtration may have changed, but was not measured in the study.
<table>
<thead>
<tr>
<th>Fluorochromes&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Positive quality</th>
<th>Negative quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFW</td>
<td>Behaves like anionic dye, not adsorbed on soil particles&lt;sup&gt;2&lt;/sup&gt;</td>
<td>None observed, but can not be used with confocal laser scanning microscope without a UV laser source&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>DAPI</td>
<td>Stains cells bright blue under UV excitation, distinguishable from autofluorescence of soil components and resin&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Cationic dye, easily adsorbed on soil matrix&lt;sup&gt;2&lt;/sup&gt;, can not be used with confocal laser scanning microscope without a UV laser source&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>DTAF</td>
<td>Satisfactory for soil smear&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Fluorescence color too close to the soil autofluorescence&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Anionic dye, not adsorbed on soil particles&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Fluorescence color too close to the soil autofluorescence&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Less emission wavelength overlap with that of soil autofluorescence&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Cationic dye, easily adsorbed on soil matrix&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>FITC</td>
<td>Satisfactory for soil smear&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Fluorescence color too close to the soil autofluorescence&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thiazol yellow G</td>
<td>Anionic dye, not adsorbed on soil particles&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Very weak fluorescence&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>CFW = calcofluor white M2R, DAPI = 4′,6-diamidino-2-phenylindole, DTAF = 5-(4,6-dichlorotriazinyl)aminofluorescein, eosin Y = eosin yellow, FITC = fluorescein isothiocyanate.

<sup>2</sup>Based on available literature.

<sup>3</sup>Based on the present study.

Table 2.3. Positive and negative qualities of the fluorochromes used in this study.
Although calcofluor white M2R and DAPI behave similarly in terms of fluorescent color under UV excitation, they bind to different cell components with different binding mechanisms. Calcofluor white M2R associates specifically with polysaccharides while DAPI binds to nucleic acids (Schumann and Rentsch, 1998). Ethidium bromide intercalates between the base pairs of the double helix of DNA and cells stained with ethidium bromide appeared orange to red under UV and blue excitation.

The presence of soil particles in the cell: soil smear study caused quenching of light. As a result, fluorescence intensity of the stained cells decreased when they were mixed with soil particles, but they still could be seen in soil pores or loosely attached on soil aggregates.

2.4.1.2 Soil Thin Sections

In the soil thin section study, many yellow-green particles were observed in green fluorochrome (FITC, DTAF and eosin Y) treated loamy soil under blue excitation. Inoculated cells could hardly be distinguished from soil particles due to the similarity in color and fluorescence intensity. In sandy soil thin sections, cells could be seen clearly because quartz particles do not fluoresce. Calcofluor White, DAPI and ethidium bromide stained cells remained distinguishable from fluorescent soil particles due to more satisfactory color contrast. This will be discussed later in section 2.4.3.
2.4.2 Resin

Some important relevant properties of the three resins used are listed in Table 2.4. The choice of resin that provided optimum results was based on balancing these properties.

2.4.2.1 Scotchcast Resin 3

Scotchcast resin had satisfactory resin block quality, ease of handling and cost, but it autofluoresced. Thin sections of this resin alone fluoresced slightly blue and green under UV and blue excitation, respectively. When soil was embedded in the resin, the resin that occupied the pores between the soil aggregates fluoresced more strongly than resin alone. Thickness of the thin section affected the fluorescent intensity of the resin. When cell fluorescence was strong enough and the thin section was thin enough, the resin autofluorescence did not interfere with obtaining a satisfactory microscopic image. Scotchcast resin was chosen as the appropriate embedding resin for the soil thin section study.

2.4.2.2 LR White Resin

When the catalyst curing method was employed, resin blocks of good quality could be obtained using the resin and accelerator ratios of 10 ml resin to 1 drop accelerator and 20 mg resin to 1 drop accelerator. However, the polymerization time (from 10 min to 1 h) was too short to allow the resin to completely penetrate the soil column. This resulted in air bubbles trapped inside the soil aggregates that deteriorated the thin section quality.
<table>
<thead>
<tr>
<th>Relevant property</th>
<th>Scotchcast Resin 3</th>
<th>LR White</th>
<th>Nanoplast resin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accelerator curing</td>
<td>Heat curing</td>
<td></td>
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<tr>
<td>Resin penetration of soil</td>
<td>***\textsuperscript{1}</td>
<td>*</td>
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<tr>
<td>Resin block quality</td>
<td>***</td>
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<td>Curing time</td>
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<tr>
<td>Autofluorescence</td>
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</table>

\textsuperscript{1}Ranking: *** very suitable, ** suitable, * not suitable.

Table 2.4. Evaluation of important properties of the three resins used in this study.
Increasing the resin amount resulted in a longer curing time, but the surface of the block was not completely cured. Resin blocks of both good quality and poor quality were obtained from heat curing. Poor quality blocks may be a result of aerobic conditions that may occur during curing. Helium gas was released from the polyethylene tube via the syringe needle and air might get into the tube; thus, an anaerobic condition may not be maintained during curing. Thin sections of LR White fluoresce more strongly than Scotchcast resin. Based on the unsatisfactory results of the LR white resin, it was not deemed acceptable.

2.4.2.3 Nanoplast Resin

Nanoplast resin was reported as being non-fluorescent (Decho and Kawaguchi, 1999), but it was found to have numerous problems. Shrinkage occurred in the Nanoplast resin block after curing. In addition, if the water content of soil sample was too high, e.g. if the soil column was saturated with water or solution after the destaining step, the internal areas of the resin block would not be cured completely. A pure resin layer on the slide itself did not fluoresce or fluoresced very weakly under UV, violet or blue light excitation. However, resin around soil particles in the thin section was fluorescent. The fluorescence intensity was higher than that of pure resin but lower than that of Scotchcast resin.

2.4.2.4 Adhesives

The Hillquist epoxy resin used for gluing the resin block to the slide fluoresces strongly. Hence non-fluorescent superglue was employed as the adhesive. Superglue
hardened very quickly, but the thin sections obtained tended to fall from the slide after contact with water. When the resin block was trimmed to a smaller size (contact area with slide < 1 cm²), good quality soil thin sections were obtained. It seems superglue is a good choice for small samples because of its quick curing and non-fluorescence. Scotchcast resin was tested as an adhesive but turned out not suitable for binding purpose.

2.4.3 Imaging Bacterial Cells in Soil Thin Sections with Epi-FM and CLSM

Images obtained using the epifluorescence microscope reflect the true fluorescence color of the soil sample but CLSM images contain pseudocolors assigned by the user. Therefore, all the color descriptions below were based on epifluorescence microscopic images.

2.4.3.1 Visualization of Inoculated Bacterial cells

2.4.3.1.1 Ethidium Bromide-Stained Soil. The best quality thin sections were obtained using ethidium bromide and epoxy resin. Ethidium bromide is a dye commonly used for bacterial enumeration. In the inoculated samples, many cell aggregates and single cells were observed between soil particles (Figure 2.3). These cells appeared yellowish-green under blue excitation with the FITC filter set and orange with the rhodamine filter set. There are two absorption peaks for ethidium bromide-stained cells. This was confirmed by confocal laser scanning microscopy, i.e., the best images of cells were obtained when detector wavelength ranges were set at 500-540 nm and 600-640 nm. The first peak was the combined fluorescence of cell autofluorescence induced by glutaraldehyde treatment and ethidium bromide fluorescence. The second emission peak was only from ethidium
Figure 2.3. Bacterial cells in a silt loam soil inoculated with *E. coli* and stained with ethidium bromide. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation.
bromide emission. Some literature reports the excitation wavelength for ethidium bromide-bound DNA is in the UV range (Roser, 1980). In this experiment, it was found that cell images in soil thin sections under UV light were not as good as those obtained from blue excitation. Besides blue excitation (488 nm), ethidium bromide-stained cells could also be excited by yellow light (568 nm) when tested with CLSM.

Figure 2.4 shows a soil aggregate about 0.1 mm in diameter that had been inoculated with *E. coli*. Black quartz particles were surrounded by brown fluorescent clay particles and organic matter. The space around the soil aggregate was filled with resin and appeared green under blue excitation. Inoculated cells were located on the surface of quartz particles or scattered in the clay particles and organic matter. Most bacterial cells were similar in size (ca. 0.3 μm × 0.9 μm). It is evident that the observed cells were inoculated *E. coli* because they were constant in shape, size and color intensity although they looked smaller compared with pure *E. coli* cells in culture (ca. 1.3 μm × 2.5 μm) (Figure 2.5).

To determine the cell concentrations in the soil thin section, the following calculation was conducted with an assumption that only cells in the top 1 μm of the thin section can be observed. Assuming 10 cells are observed over an image area of 10⁴ μm² and with a thin section thickness of 10 μm, then

\[ \text{volume of thin section over 10}^4 \text{ μm}^2 \text{ area} = 10^4 \text{ μm}^2 \times 10 \text{ μm} = 10^5 \text{ μm}^3 = 10^{-7} \text{ cm}^3, \]

\[ \text{density of cells in this volume of thin section} = 10 \text{ cells} / 10^{-7} \text{ cm}^3 = 10^8 \text{ cells} \text{ cm}^3. \]

Therefore, a concentration of 10 cells in a microscopic image area of 10⁴ μm² corresponds to a concentration of 10⁸ cells per cm³ of soil. This ratio can also be used in
Figure 2.4. Bacterial cells in a silt loam aggregate inoculated with *E. coli* and stained with ethidium bromide. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation.
Figure 2.5. Photomicrograph of epifluorescent *E. coli* in phosphate buffer. Epifluorescence microscopy, blue excitation.
later sections to calculate the cell concentrations in soil. Based on this calculation, cell numbers observed in Figure 2.3 and Figure 2.4 represented a cell concentration of $10^8 - 10^9$ per cm$^3$ of soil. This is similar to the result of Altemüller and Van Vliet-Lanoe (1990) and Postma and Altemüller (1990). Very little information is available on the extent to which sample preparation disturb the bacterial microenvironment.

The color contrast was good for the digital epifluorescence microscopic images, while digital images obtained from CLSM looked somewhat different. Figure 2.6 shows the CLSM projection image derived from 25 slices of 2-D x-y images at 0.4 μm intervals along the z-axis. The number of fluorescent particles in the CLSM image seemed higher than in epifluorescence microscopic images over a thin section area of about 10,000 μm$^2$. One reason is the image was combined from 25 slices, so all cells in the whole volume (100 μm × 100 μm × 10 μm) could be seen while only cells on the specimen surface could be observed clearly with EFM images. Another reason for this higher number is that the fluorescent particles with similar excitation and emission wavelengths appeared on the computer screen in monocolor (such as white, green or yellow, according to the users' requirements) while the non-fluorescent materials were black. Therefore, fluorescent non-bacterial particles were difficult to distinguished from fluorescent bacterial cells. If different fluorescent materials have no overlap with their excitation and emission wavelengths, they can be assigned a different color and thus be distinguished from each other.

Figure 2.7 shows a bacterial (E. coli) colony in the silt loam stained with ethidium bromide using epifluorescence. The photomicrograph was taken with a conventional 35
Figure 2.6. Projection image of 25 confocal serial sections (taken at 400 nm increments) of ethidium bromide-stained silt loam soil that had been inoculated with *E. coli*. Excitation wavelength was 488 nm.
Figure 2.7. Photomicrograph of inoculated *E. coli* in the silt loam soil stained with ethidium bromide. Epifluorescence microscopy, blue excitation.
mm camera. The cells appeared orange under blue excitation. This was because the sample was fixed with formaldehyde rather than glutaraldehyde so that only the fluorescence of ethidium bromide showed up. The image resolution was not as good as the digital images described previously (Figure 2.3 to 2.6).

Both soil aggregates and resin may turn yellow if the soil column is not destained thoroughly before embedding. The cationic characteristic of ethidium bromide is responsible for the difficulty in destaining the soil. Although not tested, it seemed likely that the resin reacted with ethidium bromide adsorbed on the soil during resin infiltration and curing. When this occurs, destaining becomes a very important step, especially for cationic stains. Further development of techniques to effectively remove the excess stain needs to be completed if cationic stains are to be used.

2.4.3.1.2 Soil Stained with Other Fluorochromes. Satisfactory results were also found in samples treated with two UV-excitable fluorochromes, calcofluor white M2R and DAPI. In both cases, bright blue inoculated cells were distributed in pores between soil aggregates as single cells or cell aggregates. The cells could be distinguished although some soil components and resin fluoresced blue under UV excitation. When the DAPI filter set was changed to a FITC filter, those cells could no longer be seen but the autofluorescence of soil components and resin remained. The cell numbers observed in an inoculated sandy soil (Figure 2.8) were significantly lower than those in a silt loam soil. Calcofluor white and DAPI treated samples could not be examined with CLSM because the available CLSM did not have a UV laser source required for the excitation.
Figure 2.8. Photomicrograph of sandy soil inoculated with *E. coli* and stained with DAPI. Epifluorescence microscopy, UV excitation.
Inoculated cells stained with green fluorochrome (FITC, DTAF and eosin Y) could be seen clearly only in sandy soil thin sections (Figure 2.9). The images of stained bacteria and sand particles were collected with the FITC detector and with transmitted light, and assigned green and red, respectively. The two images were then combined using software that comes with the CLSM. Inoculated cells were found clearly in the depression areas of the sand particles, either in aggregates, or as single cells. The locations of the bacterial cells could be discerned by their fluorescent intensity – stronger intensity of cells indicated a surface location on the sand particle compared with cells with weaker fluorescent intensity. Autofluorescent materials in silt loam soil made it difficult to distinguish cells from the soil background. Sand particles might be coated with a thin layer of organic matter or clay and therefore fluoresced slightly using epifluorescence microscopy after the staining treatment.

3-D visualization of the inoculated bacteria can be achieved using the software supplied with the CLSM (Figure 2.10). Figure 2.10A and 2.10B are the projection image and the reconstructed 3-D image of the same specimen, respectively. The resolution of the 3-D image is much lower than that of the projection image. The color assignment of the sand particles was inactivated in Figure 2.10B to improve the visualization of bacteria. The angle of the 3-D visualization can be rotated as needed. A spatial distribution of bacterial cells in their microhabitats can thus be viewed directly from the reconstructed 3-D image.
Figure 2.9. Projection image of 25 confocal serial sections (taken at 400 nm increments) of FITC-stained sandy soil inoculated with *E. coli*. Excitation wavelength was 488 nm. The green color represents bacteria and the red color represents the sand.
Figure 2.10. Projection image (top) and reconstructed 3-D image (bottom) of 25 confocal serial sections (taken at 400 nm increments) of FITC-stained sandy soil inoculated with *E. coli*. Green color represents bacteria and red color represents the sand.
2.4.3.2 Visualization of Indigenous Bacterial Cells

Cells (2 - 6 cells per field) observed in soil thin sections without any inoculation (Figure 2.11) indicated a cell density of $10^7$ to $10^8$ per cm$^3$ of soil for the fluorochromes used. The fluorescence intensity of these cells was much lower than that of inoculated bacterial cells. This was not unexpected due to the following reasons.

(1) Indigenous soil bacteria were probably coated with extracellular polysaccharide material which slows down the diffusion of the stain into the cells.

(2) Most indigenous bacteria reside in small soil pores while the inoculated bacteria tend to collect in the bigger soil pores.

(3) Saturated moisture conditions after fixation may have caused the soil to swell and thus blocked the small soil pores so that the stain did not reach the native bacteria in the succeeding staining treatment.

(4) Inoculated bacterial cells may be too small to be visualized compared to the larger, introduced bacterial cells. This observation is in agreement with findings reported in previous studies (Postma and Altemüller, 1990; Fisk et al, 1999).

Adding glucose, which was designed to help indigenous cells grow larger, did not result in significant size change of cells in the soil thin section. This could be due to insufficient amount of carbon added and insufficient time for incubation.

2.4.4 Primary Fluorescence of Soil Components

To find the optimal fluorescence wavelength range to use for the silt loam soil, an unstained soil thin section was scanned with all four excitation wavelengths (458 nm, 488
Figure 2.11. Indigenous bacteria in an aggregate of ethidium bromide-stained silt loam soil. A CCD camera was used to capture the image. Epifluorescence microscopy, blue excitation.
nm, 568 nm and 633 nm) and different detector (emission in epifluorescence microscopy) wavelengths under CLSM. Based on the corresponding digital images obtained, the strongest primary fluorescence occurred with an excitation at 488 nm and a detector wavelength range of 490-560 nm. With excitation at 488 nm and detector wavelength at 580 - 660 nm, fluorescent particles could also be observed. No fluorescence was observed with excitations at 458, 568 and 633 nm. Primary fluorescence could also be detected with UV excitation using an epifluorescence microscope.

This indicated that most autofluorescent components in the silt loam soil fluoresce with UV excitation (340 nm – 400 nm) and blue excitation (430 nm – 500 nm), and the emission wavelength range is quite broad. To reduce the interference of primary fluorescence with target cell observation, a knowledge of the existing autofluorescent materials in soil is necessary. If most of the primary fluorescence in the soil matrix requires a certain range of wavelengths for excitation (e.g., UV excitation and blue excitation) and if emission is also restricted to certain wavelengths, it should be possible to remove the interference with a specially designed filter such as a bandpass filter set on the epifluorescence microscope or an adjustable detector filter for CLSM. The important thing when selecting optimum fluorochromes for soil bacteria is to find fluorochromes whose excitation and emission wavelengths have minimal overlap with those of the primary fluorescence in soil.

Tippkötter (1990) studied the primary fluorescence of many types of soil organic matter, minerals and microorganisms. These materials were investigated individually and it was found that many organic substances such as chitin, lignin, tannic acid, cellulase,
protease, cellulose and mannose had a strong to moderate primary fluorescence. Minerals such as calcite, illite and kaolinite and the organic components of microorganisms may be visible by primary fluorescence. However, Tippkötter (1990) did not mention the colors or the fluorescence wavelength ranges of the above substances and minerals. Soil components vary based on soil type, and the primary fluorescence of soil components still remains poorly characterized to date.

2.4.5 Effect of Solution Ions and pH

Changing solutions used to prepare stains from phosphate to chloride and from a neutral pH (7.0) or higher pH to a lower pH (3.0) was hypothesized to release cationic stain molecules from the anionic clay double layer and reduce fluorescence of soil matrix. These changes did not result in better images of stained cells in soil thin sections for any of the four fluorochromes examined. These attempts indicated that solution composition and pH are not the dominant factors determining the quality of the soil thin section image; alternatively, the method used was not sensitive enough to detect the expected effect.

2.4.6 Other Factors Affecting Thin Section Quality

There are many other factors affecting the quality of soil thin sections besides the basic factor of the stain itself. The bacterial inoculum was applied on the top of the soil column, which may not have distributed the cells evenly to different depths. This contributed to the variation of cell numbers observed in thin sections. Although a rather
even cell distribution was obtained in a pre-test by mixing inoculum with soil before packing the soil in the cylinder, this procedure increased the difficulty of handling and caused more disturbance of the soil aggregates.

The staining method developed may not effectively stain all cells in the soil if the soil volume is larger than 2 cm³. Cationic stains like ethidium bromide tend to be adsorbed on the top layer of soil and are difficult to destain. A similar situation may occur with the pH-sensitive anionic stains, like FITC or DTAF, if the staining solution pH is optimized. The top soil layer tends to be stained heavily by these dyes. The soil layer 1 cm below the surface is a good choice to make thin sections. Some anionic stains like eosin Y can be destained effectively, and this can be discerned by the colorless effluent of the destaining solution.

Staining the surface of soil thin sections instead of the entire soil column before resin embedding was unsuccessful due to the strong affinity of stains to the resin and soil. The resin and soil in this case fluoresced much more strongly than that of a pre-stained soil column. Thin section cracking or detachment of the thin section from the slide described by Tippkötter et al. (1986) was not observed in this experiment.

2.4.7 Cells Removed from the Soil Column during Thin Section Preparation

Plate counting of cell suspensions before inoculation showed that the number of *E. coli* cells inoculated into the soil column was $10^{10}$. The plate counts from the sequential washings indicated that about $10^7$ cells were removed from the soil column, which was about 0.1% of the cells in the soil column. Since inoculated *E. coli* was the dominant
bacterium in the soil, most of the cells removed were *E. coli* cells; i.e., the 0.1% may reflect that a relatively low number of introduced bacteria from the soil column were removed with sequential washings. The number of indigenous bacterial cells may be ignored due to the much higher number of inoculated bacterial cells.

Plate counting for soil columns without inoculum showed that about $10^4$ to $10^5$ cells were present in the leachates. The plate counts may only account for 1% or less of total cells leached from the soil column (Hartmann *et al.*, 1997) as already mentioned in Chapter 1. Hence the actual number of the cells lost in the leachate could be $10^6$ to $10^7$ cells. Assuming the silt loam soil column contains $10^8$ to $10^9$ cells per cm$^3$ of soil (not determined in this study — the assumption was made based on experience), there should be still about at least $10^7$ to $10^8$ cells left in the silt loam soil. This means enough cells exist in the soil column to be observed under microscope.

If further studies are conducted, direct microscopic counting of bacteria in the leachate and soil are recommended since results from microscopic enumeration can provide an accurate total bacterial concentration (both live and dead cells).

### 2.5 Conclusions and Suggestions

Universal fluorescent staining with ethidium bromide, calcofluor white and DAPI could be used to trace the fate of introduced bacteria and confirm the location of indigenous bacteria in soil. The best combination of stain and resin found in this study was ethidium bromide and Scotchcast resin 3, with superglue as an adhesive for binding the resin block to the glass slide. In ethidium bromide-stained silt loam soil, the external
bacterial cells introduced to the soil were stained intensively in the soil thin sections and could be distinguished from the background, while stained indigenous bacteria were relatively low in numbers and fluorescence intensity. Cells were located either on the surface of quartz particles or scattered in the matrix of clay particles and organic matter in the soil.

Stain selection should be based on the excitation and emission wavelength ranges of fluorochromes and soil autofluorescence. The ideal situation is that they have little to no overlap in their excitation and emission. The use of red fluorochromes resulted in less overlap of emission with those of most soil primary fluorescence as compared to green fluorochromes. Most red fluorochromes are cationic, and the soil can be heavily stained if destaining is not thorough. Novel anionic red fluorochromes would be desirable for soil bacteria staining as they could lead to a better contrast of cells and the soil. Further studies of soil primary fluorescence are essential and will certainly aid in the visualization of soil microorganisms when using fluorescent microscopy.

When soil was autofluorescent, EFM produced better images than CLSM because the color difference of cells and other fluorescent particles could be discerned more easily with the filter set used. The CLSM projection images provided accurate 2-D images at any depth of the thin section and provided 3-D images of these cells against the background when soil was not autofluorescent. These attractive features confirm the potential use of CLSM in soil microbial ecology studies. 3-D images can also be reconstructed by rendering and surfacing 2-D image slices with PC software to produce a spatial relationship of soil microorganisms in their microhabitats. Epifluorescence
microscopy and CLSM both have their own advantages and disadvantages, but these two techniques can be used together to compensate for the limitations of each other.

Fluorescent in situ hybridization (FISH) techniques which use fluorescence-labelled molecular probes to specifically stain certain bacteria have been increasingly used in recent years. The stain and resin combinations developed in this study may be used for FISH application in soil microbial distribution studies. Engineered bacteria that are introduced into contaminated soil to remediate a site may also be visualized using a method of pre-staining the bacteria before inoculation.

The development of microdissector techniques for biomedical studies provides the possibility of picking up a single cell or a cluster of cells upon which biochemical analysis can be conducted (Schütze and Lahr, 1998). Cells captured by such precise instruments will not be affected by the unwanted cells left in the surrounding areas. This technique looks promising in soil microbiology studies to characterize bacterial cells in their microhabitats.
APPENDIX

MOLECULAR FORMULA AND STRUCTURES OF SELECTED FLUOROCHROMES

CFW (C_{44}H_{34}N_{12}O_{6}S_{2}Na_{2})

DAPI (C_{22}H_{27}N_{5}O_{6})
DTAF (C_{23}H_{12}Cl_{2}N_{4}O_{5})

Eosin Y (C_{20}H_{6}O_{5}Na_{2}Br_{4})
Ethidium bromide ($C_{21}H_{20}BrN_3$)

FITC ($C_{21}H_{11}NO_5S$)
Thiazol yellow G (C₂₈H₁₉N₅O₆S₄Na₂)
REFERENCES


