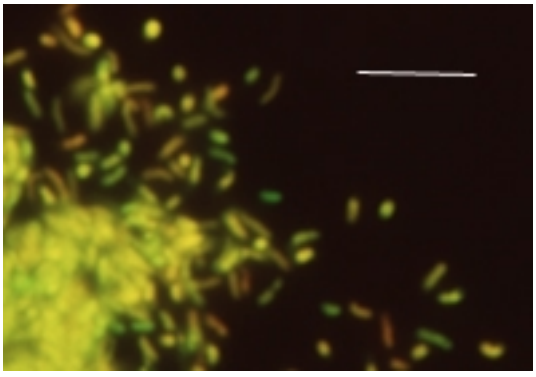
Experiment	**Characterizing Phenotypes of Bacteria by Staining Method**
Advisor	NN
Reading	Chapters 3.1, 3.7, 3.8, 16.5 in BBOM 9th Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, (BBOM, International Edition), Prentice Hall, 1999. ISBN: 0-13-085264-3.
Objectives	• How do typical bacterial stains work? • How are bacterial stains used? • Perform stainings using DAPI, Gram stain and Live/Dead stain • Determine ratio of alive to dead cells by fluorescent staining
Background	Many properties of bacteria can neither be seen under the microscope nor in culture, because bacteria look too similar, though they might perform very different metabolic tasks and belong to different phylogenetic groups. A simple and rapid tool to find out more about an isolated organism or a community of organisms is to apply **specific stains** (BBOM Fig. 1.10b, and chapter 16.5). The basic principle of staining cells is to have a microorganism take up „colored molecules", called **chromophores** (if they are absorbing light) or **fluorophores** (if they are emitting fluorescent light), which remain attached to the organism after rinsing off unbound stain (BBOM 9th ed. Fig. 3.3). Stains are ionic or lipophilic, e.g., Methylene blue chloride → Cl^- + methylene blue$^+$ (chromophore) The chromophore (or fluorophore) is normally incorporated into the bacterial cell or it adheres by ionic interactions to the cell wall, so that responding bacteria are stained with a characteristic color. The sample may be viewed under the **fluorescence microscope** if a fluorophore is used or in the **bright-field** mode under the **transmission light microscope** if the stain contains a chromophore. Fluorescent dyes are also essential for Confocal Scanning Laser Microscopy (BBOM 9th ed. pg. 55), for fluorescence-activated cell sorting (pg. 874), for fluorescence in situ hybridization of genes (FISH, pgs 436, 655) and for fluorescence antibody techniques (pg 654). **Gram staining** (BBOM 9th ed. Fig. 3.4) enables one to distinguish between **Gram-positive** and **Gram-negative** bacterial cell walls (BBOM 9th ed. chapters 3.7 and 3.8). The Gram-positive ones contain a thick **murein** (=peptidoglycan) layer. The Gram-negative cell wall consists of a thin peptidoglycan layer and a lipopolysaccharide- and protein-containing outer cell membrane.  **Figure 1** Fluorescent Gram staining. Gram-negative cells fluoresce green, Gram-positive ones red. Yellow fluorescence is an accumulation of Gram-positive and Gram-negative cells. Bar approx. 10 µm Photo Caroline Brunner

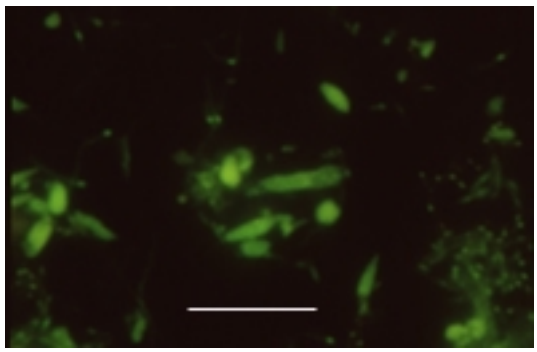


Figure 2
Fluorescent Gram staining of biofilm grown on glass cover slide. Gram-negative cells show green fluorescence. There are no dead cells (red fluorescence) visible in this image
Bar approx. 10 µm
Photo Caroline Brunner

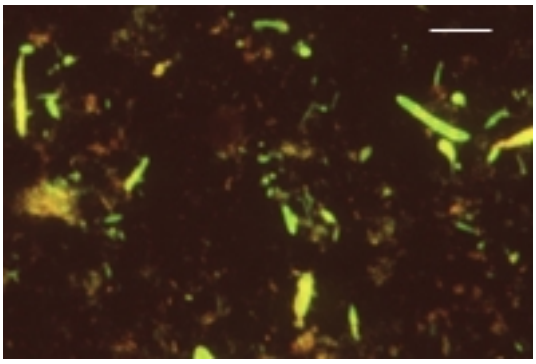


Figure 3
Fluorescent Gram staining of biofilm grown on glass cover slide. Gram-negative cells express green, Gram-positive red fluorescence. Yellow fluorescence is an accumulation of Gram-positive and Gram-negative cells. Bar approx. 10 µm
Photo Caroline Brunner

DAPI (4',6-diamidino-2-phenylindiole) stains intracellularly by binding to AT-clusters in the DNA. DAPI stains all organisms, but it does not interact with inorganic particles.

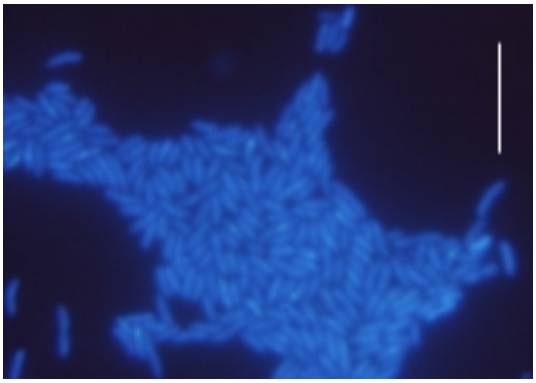


Figure 4
Fluorescent DAPI staining of bacterial isolate from Jöri Lake XIII. Bar approx. 10 µm
Photo Caroline Brunner

Live/dead staining distinguishes cells that have an intact membrane (=alive, green fluorescence), from those with a leaky one (=dead, red fluorescence).

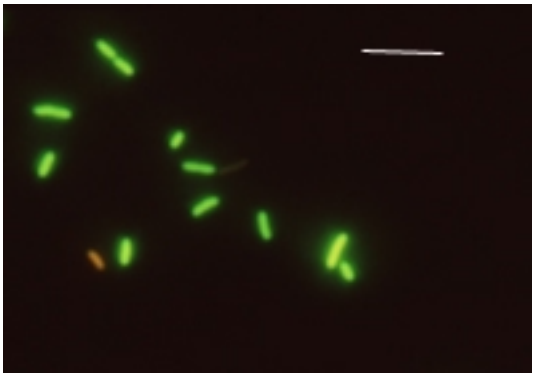


Figure 5
Fluorescent LIVE/DEAD staining of *Geobacter metallireducens*. Live cells show green fluorescence, dead ones appear red. Bar approx. 10 µm
Photo Caroline Brunner

	In **fluorescence microscopy**, the sample is exposed to light of a defined wavelength. Fluorescent substances absorb this light, then emit light with a higher wavelength (= light of lower energy). By aid of filters, which exclude **excitation light**, we can view the **emitting fluorescent light** of the fluorophore which binds to the cells under the fluorescence microscope. Some natural substances such as chlorophyll of phototorphic microorganisms or F_{420} of certain methanogens show a strong red or blue fluorescence, respectively (**autofluorescence**, BBOM 9th ed. Fig 3.6, Fig. 15.45).This must be taken into account when applying fluorescent dyes to chlorphyll- or F_{420}-containing organisms.
Literature	
www. Links	
Practical Work and Experimental Protocols	Samples from last week's enrichmenets and pure cultures isolated from them are stained with the following procedures. **Classical Gram staining** Procedure: 1. Clean a microscope slide 2. Draw three circles on it with a wax pencil 3. Put a loopful of organisms inside each circle and allow to dry 4. Place the slide on a staining rack and flood with crystal violet 5. Rinse gently with water 6. Flood with Gram's iodine, wait a minute and wash again 7. Add 95% alcohol and allow to decolorize for about 30 seconds 8. Wash again with water 9. Counterstain with safranin; wash and dry 10. Inspect under the transmission light microscope in the light field mode **Fluorescent Gram-staining** For fluorescent Gram-staining ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V-7023, Molecular Probes) is used. The kit contains three reagents: DAPI, SYTOX® Green and Texas Red-X. DAPI stains bacteria with intact cell membranes blue fluorescent, whereas bacteria with damaged cell membranes stain fluorescent green with SYTOX® Green nucleic acid stain. The Texas Red-X conjugate of wheat germ agglutinin labels the surface of Gram-positive bacteria fluorescent red. Procedure: 1. Fill bacterial sample into an Eppendorf tube 2. Wash sample carefully with BSA-saline solution (0.25 % bovine serum albumin [BSA], 0.15 M NaCl; sterilized by filtration) 3. Centrifuge and remove supernatant 4. Add 100 µg/ml Texas Red-X conjugate to the sample in the Eppendorf tube 5. Place in the dark for 20 minutes 6. Wash with BSA-saline solution 7. Stain with 3 µl of DAPI stain/SYTOX Green working solution and keep sample at room temperature in the dark for one hour (working solution consists of 3 µl DAPI stain and 3 µl SYTOX Green nucleic acid stain in 54 µl sterile deionized water) 8. Prepare slide and examine under the fluorescence microscope

	Fluorescent Live/Dead staining To determine whether bacteria are alive or dead the Live/Dead-staining is applied. LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy* (Molecular Probes) is used. The two-color fluorescent assay is based on the different abilities of the stains to penetrate healthy bacterial cells and the different spectral characteristics. SYTO® 9 green fluorescent nucleic acid stain, generally labels all bacteria, those with intact and those with damaged cell membranes. In contrast, propidium iodide penetrates only bacteria with damaged cell membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. Procedure: 1. Fill bacterial sample into an Eppendorf tube 2. Wash sample carefully with BSA-saline solution (0.25 % bovine serum albumin [BSA], 0.15 M NaCl; sterilized by filtration) 3. Centrifuge and remove supernatant 4. Add 3 µl staining mix (SYTO®9 + propidium iodide in a 1:1 ratio) to the sample in the Eppendorf tube 5. Place in the dark and keep at room temperature for 20 minutes 6. Wash with BSA-saline solution 7. 5 µl stained and washed culture are placed on microscopy slides covered with a very thin layer of dry 1.5 % agarose. The bacteria are covered with square cover slides and observed under the fluorescence microscope **DAPI staining** Procedure 1. Place 2 ml enrichment culture aseptically in sterile 2 ml Eppendorf tubes 2. Centrifuge for 5 min at 8000 rpm in an Eppendorf microcentrifuge 3. Discard supernatants and resuspend the pellets in 300 µl aqueous 5 % glutaraldehyde-solution 4. Leave the samples for 30 minutes at room temperature 5. Centrifuged again for 5 min at 8000 rpm and discard the supernatants 6. Add 50 µl of a 10 µl/ml DAPI-solution was allow to incubate for 15 minutes in the dark 7. Wash with sterile water to remove the remaining DAPI-stain, centrifuge again and resuspend in a small volume of sterile water 8. Place a drop of the stained suspension on a microscope slide, cover with a square cover slide and placed under the fluorescence microscope
Material	The following is supplied: • Components for classical Gram staining • Fluorescent stains (DAPI, Gram and Live/Dead) • Bacterial samples from own enrichments • Pure cultures of some organisms • Phase Contrast and Fluorescence Microscopes
Laboratory Rules & Precautions	Although the microbes which we will use are not pathogenic, it is still recommended to follow standard microbiological precautions when working with them.
Goals and Experiences gained	To learn more about the composition of microbial communities To follow exact staining procedures To characterize the physiological state of bacteria
Timing	90 minutes

Reporting

Quantifying staining results

Sample	1	2	3
Percentage Alive Percentage Dead			
Percentage Gram-positive Percentage Gram-negative			

Conclusions

Questions to be answered

Can you quantify the ratio of Gram-positive and Gram-negative bacteria in a community?
What percentage of cells in your sample is dead?