A Comparison of Heat Versus Methanol Fixation for Gram Staining Bacteria

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Abstract: Gram staining bacteria is a fundamental technique introduced in general biology and microbiology laboratory courses. Two common problems students encounter when Gram staining bacteria are (1) having a difficult time locating bacterial cells on the microscope slide and (2) over-decolorizing bacterial cells during the staining procedure such that gram-positive bacteria, which should appear purple in color, are pink instead. In this study, we examined whether the method of fixation (heat versus methanol) that is used to adhere bacteria to the slide prior to staining might influence the staining results. We found that significantly greater numbers of *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) cells adhered to slides following methanol fixation compared to slides that were heat-fixed. Additionally, methanol-fixed cells of *Staphylococcus aureus* were consistently stained the correct color (a dark purple) while the staining of heat-fixed cells was more variable with cells ranging in color from purple to pink. Overall, our results indicate that students are more likely to successfully visualize and Gram stain bacteria if the cells are fixed with methanol rather than heat.

Keywords: Gram stain, gram-positive, gram-negative, heat fixation, methanol fixation

Introduction

A fundamental laboratory technique that is introduced in general biology and microbiology courses is staining of bacterial cells on glass slides for visualization and characterization purposes. A common procedure, the Gram stain, differentiates between bacterial species based on the chemical composition of their cell walls. The staining procedure involves applying a primary stain, crystal violet, followed by Gram's iodine, which acts as a mordant, decolorizing with an organic solvent such as ethanol, and counterstaining with safranin. Following the procedure, gram-positive bacteria, which are more resistant to decolorization, appear purple in color while gram-negative bacteria, which are more sensitive to decolorization, appear pink.

Students encounter a number of problems when learning how to Gram stain and view bacterial cells. During the staining procedure, bacterial cells tend to be washed off the slide. Students then have difficulty locating bacterial cells on the slide, particularly the lightly colored (pink) gram-negative cells. Additionally, students often over-decolorize the cells, such that gram-positive cells, which should appear purple, are stained pink instead. This is particularly an issue when older cultures of bacteria are used for the staining procedure (Magee *et al.*, 1975).

Some evidence suggests that the means by which bacterial cells are "fixed" to the glass slide prior to staining may influence the results of the Gram stain (Magee *et al.*, 1975; Mangels *et al.*, 1984). Fixation increases the adherence of bacterial cells, and the most common method employed is heat fixation (Ederer and Lund, 1981). This is completed by passing a slide of bacterial cells through a flame until the underside of the slide is warm to the touch. Chemical methods of fixation have also been described. One is the use of methanol as a fixative agent. A number of studies have shown that methanol fixation gives more reliable Gram staining results than heat fixation (Magee *et al.*, 1975; Mangels *et al.*, 1984). That is, gram-positive bacteria are more likely to be stained purple, and gram-negative bacteria are more likely to be stained pink when cells are fixed with methanol compared to heat. Additionally, gram-positive bacteria fixed with methanol are more resistant to decolorization than cells fixed with heat (Magee *et al.*, 1975; Mangels *et al.*, 1984).