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).

We were surprised, therefore, to find that of six general biology laboratory manuals we examined, five recommended heat fixation of bacteria (Hummer *et al.*, 1983; Dickerman, 2000; Scott and Wachtmeister, 2006; Dolphin, 2008; Vodopich and Moore, 2008), and only one recommended the use of methanol as a fixative agent (Singh and Gunn-Scissum, 2003). The same held true for the microbiology manuals we reviewed. All 15 manuals recommended heat as the preferred method of fixation (Norrell and Messley, 1997; Stukus, 1997; Alexander and Strete, 2001; Bey, 2001; Johnson and Case, 2001; Benson, 2002; Kelley and Post, 2002; Wistreich, 2003; Alexander *et al.*, 2004; Cappuccino and Sherman, 2005; Pollack *et al.*, 2005; Pommerville, 2005; Leboffe and Pierce, 2006; Harley, 2008; Morello *et al.*, 2008), and only two of the 15 (Johnson and Case, 2001; Morello *et al.*, 2008) even mentioned methanol as a possible fixative agent.

Our objective was to examine Gram staining results following fixation of both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria using heat versus methanol as means of fixation. In particular, we were interested in evaluating the number of cells adhering to the slides following the Gram staining procedure. We also assessed the color of the Gram stained bacteria that were heat-fixed versus methanol-fixed. This research was completed exclusively by undergraduate students who were majoring in biology (Roland, Rossi, Weishalla, and Wolf), only one of whom had prior laboratory experience staining bacterial cells. Our overall goal was to determine whether students with little or no experience in completing microbiology laboratory exercises might achieve greater success in Gram staining and viewing bacteria using one method of fixation compared to the other.

Methods

Bacterial cultures. Stock cultures of *S. aureus* and *E. coli* (Presque Isle Cultures, Presque Isle, PA) were maintained on tryptic soy agar (Fisher Scientific, Pittsburgh, PA). Prior to each experiment, a test tube containing 10 mL sterile tryptic soy broth (TSB; Fisher Scientific) was inoculated with *S. aureus* or *E. coli*, placed in a Lab-Line incubator-shaker (Barnstead International, Dubuque, IA), and incubated at 100 rpm and 37^{o}C for 14 hours.

Fixation and Staining of Bacteria. For each experiment, 20 glass slides were cleaned with 95% ethanol, and a circle with a diameter of 2 cm was made on the surface of each slide using a wax pencil. The 14-hour culture of *S. aureus* was diluted 1/100 in TSB, and 20 □L of the diluted culture was spread within the circle on each slide. Alternatively, 20 □L of the 14-hour *E. coli* culture was spread within the circle on each slide. The slides were then allowed to air dry. One set of ten slides was heat-fixed by passing the bottom of each slide through the flame of a Bunsen burner until the slide was warm to the touch. The remaining ten slides were flooded with absolute methanol (200 □L per slide) for 2 minutes (Mangels *et al*, 1984; Singh and Gunn-Scissum, 2003). Excess methanol was decanted off the slides into a waste disposal container, and the slides were allowed to air dry. All 20 slides were randomly numbered 1-20 so the individuals who stained and viewed the slides did not know which slides were heat-fixed versus methanol-fixed. The slides were then stained using Hucker's modified Gram-stain technique (Harley, 2008) and viewed independently by three different individuals using a bright-field light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The experiment was completed three and four times for *E. coli* and *S. aureus*, respectively.

Cell Counts and Statistics. For each slide, bacterial cells were counted in three random fields of view, and the mean number of cells in a field of view was calculated. The Student-t test was completed on the data to determine whether there was a statistically significant difference in the mean number of cells that adhered to heat-fixed slides compared to slides fixed with methanol.

Photographs of Staphylococcus aureus*.* Twenty microliters of a 14-hour culture of *S. aureus* was spread on each of 10 slides, and the slides were allowed to air dry as described above. Five of the slides were heat-fixed, and five were fixed with methanol. The slides were randomly numbered 1-10, Gram stained, and viewed under oil immersion on a Zeiss bright-field light microscope. Photographs of random fields of view were taken with a Canon Powershot G6 digital camera using identical camera settings for photographs of both heat-fixed and methanol-fixed slides. Slides and/or photographs were viewed and independently evaluated by three different individuals.

Laboratory Safety Guidelines and Microbiological Laboratory Techniques. Students were instructed in standard laboratory safety guidelines and proper microbiological laboratory techniques (Norrell and Messley, 1997; Stukus, 1997; Alexander and Strete, 2001; Bey, 2001; Johnson and

Case, 2001; Benson, 2002; Kelley and Post, 2002; Wistreich, 2003; Alexander *et al.*, 2004; Cappuccino and Sherman, 2005; Pollack *et al.*, 2005; Pommerville, 2005; Leboffe and Pierce, 2006; Harley, 2008; Morello *et al.*, 2008) prior to completing this study. Topics discussed included but were not limited to the following: proper handling of bacterial cultures (aseptic technique); proper disposal of microbiological waste; procedures for decontaminating accidental spills; and proper handling and disposal of toxic and flammable chemicals used in this study (e.g., ethanol and methanol).

Results

In all three experiments completed on *E. coli*, slides fixed with methanol had a significantly greater mean number of cells per field of view compared to slides that were heat-fixed ($p < 0.001$, 0.005, and 0.0001, for Experiments 1, 2, and 3, respectively). Representative results from Experiment 3 are shown in Figure 1. In this case, methanol-fixed slides of *E. coli* had more than ten times as many cells per field of view than did slides of *E. coli* that were fixed using heat. All three researchers who independently viewed the slides indicated it was much easier to locate and identify *E. coli* cells on the slides fixed with methanol than on slides fixed with heat.

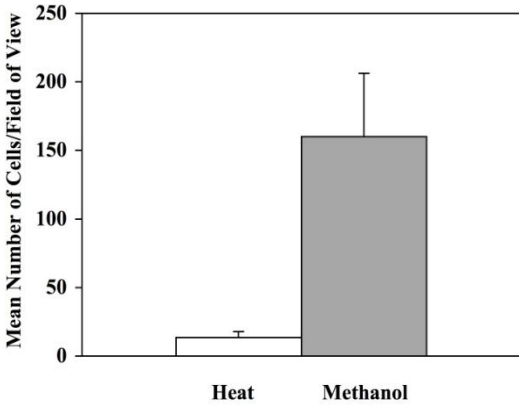


Figure. 1. Mean number of *Escherichia coli* cells per field of view on slides that were heat-fixed (n=10) versus methanol-fixed (n=10). Twenty microliters of a 14-hour culture of *E. coli* was applied to the surface of each of 20 slides and allowed to air dry. Ten of the slides were heat-fixed and the other 10 were fixed with absolute methanol. The slides were Gram stained and viewed under oil immersion with a bright-field light microscope. The number of bacterial cells in three random fields of view was counted for each slide and averaged. Methanol-fixed slides of *E. coli* had a significantly greater mean number of cells per field of view than did heat-fixed slides ($p < 0.0001$).

Similar results were obtained in the four experiments completed on *S. aureus*. In all cases, methanol-fixed slides had a significantly greater mean number of cells per field of view than did slides that were heat-fixed ($p < 0.0005$, 0.0001, 0.0001, and 0.0001 for Experiments 1, 2, 3, and 4, respectively). Representative results from Experiment 4 are shown in Figure 2. In this case, there were 2.5-fold more cells per field of view on slides of *S. aureus* that were methanol-fixed than those slides fixed with heat.

Figure. 2. Mean number of *Staphylococcus aureus* cells per field of view on slides that were heat-fixed (n=10) versus methanol-fixed (n=10). A 14-hour culture of *S. aureus* was diluted 1/100 in TSB, 20 μL of the diluted culture was applied to the surface of each of 20 slides, and the slides were allowed to air dry. Ten of the slides were heat-fixed and the other 10 were fixed with absolute methanol. The slides were Gram stained and viewed under oil immersion with a bright-field light microscope. The number of bacterial cells in three random fields of view was counted for each slide and averaged. Methanol-fixed slides of *S. aureus* had a significantly greater mean number of cells per field of view than did heat-fixed slides ($p < 0.0001$).

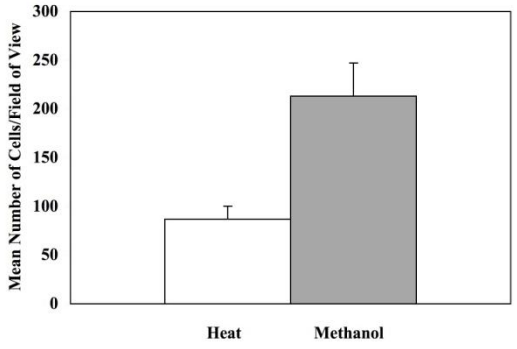
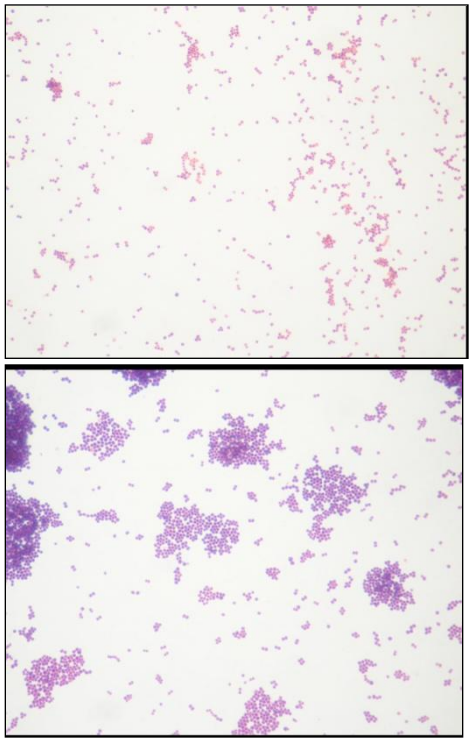


Figure. 3. Photographs of slides of *Staphylococcus aureus* cells that were heat-fixed (A) versus methanol-fixed (B). Twenty microliters of a 14-hour culture of *S. aureus* was applied to the surface of each of 10 slides and allowed to air dry. Five of the slides were heat-fixed and the other five were fixed with absolute methanol. The slides were Gram stained and viewed under oil immersion with a bright-field light microscope. Photographs of random fields of view were taken with a digital camera using identical camera settings for photographs of heat-fixed and methanol-fixed slides.

3A



3B

Methanol-fixed gram-positive bacterial cells were less sensitive to decolorization during the Gram staining procedure than were heat-fixed cells. Shown in Figure 3 are representative photographs of slides of *S. aureus* that were prepared identically except that one slide was fixed with heat (Figure 3A) and the other with methanol (Figure 3B). Clearly, a greater number of *S. aureus* cells adhered to the slide that was fixed with methanol compared to the heat-fixed slide. All three individuals who independently examined the slides agreed that bacterial cells fixed with methanol (Figure 3B) retained the crystal violet (purple) stain more readily than did the cells that were heat-fixed (Figure 3A). Virtually all cells viewed on methanol-fixed slides of *S. aureus* were purple in color. Staining results were variable on the heat-fixed slides where both purple and pink cells were observed. No difference in color was observed in *E. coli* cells fixed with methanol versus heat (data not shown), and therefore the method of fixation did not influence the Gram staining results of this gram-negative bacterium.

Discussion

We found that both gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria that were fixed to slides using methanol as the fixative agent adhered more effectively than did cells that were heat-fixed to slides. Slides of *S. aureus* fixed with methanol had two to four times as many cells present than did slides fixed with heat. In the case of *E. coli*, the difference was even more dramatic with five to ten times as many cells present on methanol-fixed slides compared to slides that were heat-fixed. Methanol fixation prior to Gram staining would clearly allow students to locate and view bacterial cells more easily than heat fixation.

Consistent with previous studies (Magee *et al*., 1975; Mangels *et al.*, 1984), we also found that gram-positive bacteria were less likely to decolorize when fixed to slides with methanol rather than heat. In our experience, *S. aureus* cells that were fixed with methanol consistently were stained a dark purple color. Identically stained cells that were heat-fixed varied in color, ranging from dark purple to pink. The decolorization step is the most critical of the Gram-stain procedure. Students tend either to under-decolorize, leading to gram-negative cells falsely appearing gram-positive (purple), or, more commonly, to over-decolorize, in which case gram-positive bacteria falsely appear gram-negative (pink). Using methanol as a fixative agent would help to eliminate this problem. Gram-positive bacteria fixed with methanol would likely still appear purple in color even if excessive amounts of decolorizing agent were used.

We were curious as to why virtually all of the laboratory manuals we examined recommended heat fixation rather than methanol fixation when staining bacteria. In order to gain insight into this, we contacted a number of the authors of these laboratory manuals and asked them why methanol fixation was not included in their laboratory exercises on bacterial cell staining. Some indicated that they were not aware that methanol fixation was an alternative to heat fixation. Others indicated that since methanol is a toxic and flammable chemical, they were concerned for safety reasons, particularly since Bunsen burners are used when staining bacterial cells. While safety issues are a valid concern, we believe the dangers associated with the

use of methanol (and the ethanol decolorizing agent, for that matter) can be minimized by following certain laboratory safety guidelines. For instance, the experiments completed in this study were carried out in a well-ventilated research laboratory to minimize student exposure to fumes released from the chemicals. Alternatively, one could complete the methanol fixation step under a chemical fume hood to reduce exposure to the vapors. Additionally, the Bunsen burners used to aseptically transfer the bacterial cultures to the slides and to heat-fix the bacteria to some of the slides were turned off prior to methanol fixation and the staining procedure in which ethanol was used. It should also be noted that limited quantities of methanol (and ethanol) were used in this laboratory procedure. For instance, only 200 □L methanol was required to fix bacteria to each slide. If a classroom of 24 students were to complete this exercise, and each student was to Gram stain one slide of bacterial cells, this would result in less than 5 mL methanol being used for the entire class.

The research discussed in this paper was completed by four undergraduate biology students, (Roland, Rossi, Weishalla, and Wolf), who had little to no experience handling bacterial cultures or staining bacterial cells. Results from our study indicate that students are more likely to visualize successfully and Gram stain bacteria properly if the cells are fixed with methanol rather than heat. In light of our findings, it might be useful to reevaluate the method of bacterial fixation used in introductory biology and microbiology laboratory courses.

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