



Micro-staining microbes: An alternative to traditional staining of microbiological specimens using microliter volumes of reagents

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ABSTRACT

Microbial staining techniques are widely employed in clinical and academic laboratories for classifying and identifying microorganisms derived from clinical, food and environmental samples. Staining allows for the rapid visualization and determination of many morphological characteristics of microorganisms, used for their identification and classification. Over the past century, staining techniques such as the Gram stain, the Capsule stain, the Acid-fast stain and the Endospore stain, have seen few advances, and manual staining remains the gold standard. Typical instructions for these staining procedures recommend ‘flooding’ glass slides with milliliter volumes of dye, resulting in large volumes of hazardous waste. Here we present micro-staining, a simple alternative to flooding that utilizes microliter volumes of dye. Micro-staining minimizes the volume of waste generated, leads to significant cost savings for the laboratory, requires limited training, and produces results with equivalent quality to traditional stains.

1. Introduction

Microbial staining with positive and negative dyes remains a fundamental first-step in the identification and classification of all microbes derived from medical, food or environmental samples (Leboffe and Pierce, 2010). This age-old practice is employed in virtually every clinical laboratory and microbiology classroom around the globe. Staining allows for the rapid visualization and determination of many morphological characteristics, including shape, size, and arrangement of the microorganism in question (Cappuccino and Sherman, 2014; Engelkirk and Duben-Engelkirk, 2013). Depending on the stain type and process, many other physical characteristics of the microorganism can be inferred such as peptidoglycan cell wall thickness, presence of one or two membranes, or identification of other specialized bacterial structures (e.g. flagella, endospores, external capsules) (Leboffe and Pierce, 2010). Albeit an imperfect science, given many exceptions to the rule, the elucidation of these physical characteristics can prove to be extremely useful information. However, in today's modern era of molecular biology, a concomitant microbial DNA or 16S rRNA gene sequencing would be most prudent for the proper identification of any unknown microorganism (Barghouthi, 2011). Nevertheless, microbial staining has withstood the test of time as a major tool in the clinical and

academic microbiology laboratory because it is inexpensive, rapid, effective and has results that are relatively easy to interpret (Beveridge, 2001; Coico, 2005; Moyes et al., 2009).

Traditional staining techniques are typically performed by fixing the specimen to a standard-size microscope glass slide, followed by ‘flooding’ the slide with milliliter volumes of dye, and finishing with a washing step under a faucet (Beveridge et al., 2007; Cappuccino and Sherman, 2014; Engelkirk and Duben-Engelkirk, 2013; Leboffe and Pierce, 2010). Our Department at Cal Poly State University, a Primarily Undergraduate Institution (PUI), has recently become interested in replacing this traditional approach of ‘flooding’ slides with a technique that minimizes the volume of liquid waste generated, as well as the cost associated with handling and disposing of this waste. To our knowledge, and surprise, there have been limited published attempts to modify the widely employed practice of ‘flooding’ slides during manual staining. Instead, a review of published articles, published protocols, academic textbooks and online instructional videos revealed that current procedures for manual staining still employ verbs such as ‘flood’, ‘cover’, ‘immerse’, ‘bathe’ or ‘saturate’ with stain to describe the process of applying the stain to the microscope slide, and they all imply the use of milliliter volumes of stain (Bartholomew and Mittwer, 1952; Cappuccino and Sherman, 2014; Engelkirk and Duben-Engelkirk, 2013;

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Leboffe and Pierce, 2010). The most recent published account for reducing the volume of stain waste is a 2010 article by RA Gyure, which suggests employing simple collection vessels (plastic or glass) under each glass slide during the staining procedure. However, milliliter volumes of stain were still employed (Gyure, 2010). The most significant innovation in staining has been the introduction of automated slide staining systems such as the bioMerieux PREVI® Color Gram. These automated systems save time and reduce wasted reagents, while producing quality results as compared to traditional methods (Baron et al., 2010; Wong et al., 2011). However, these staining systems come with a significant upfront cost for the instrumentation itself, the reagents and any downstream cost of repair (Biomérieux Previ-Color Gram, 2019). For the non-clinical laboratory, or for the laboratory with a fixed budget, manual-staining remains the gold standard.

Here we introduce micro-staining, a simple alternative to the age-old procedure of ‘flooding’ slides with unknown (milliliter) quantities of staining solutions. Micro-staining relies on controlled pipetting of microliter volumes of reagents onto the slide. A primary goal in the development of micro-staining was the conservation of reagents to minimize the amount of staining waste generated by our institution. For this technique to be readily employed in a teaching laboratory, it has to be easy to practice and accessible to undergraduate students, low cost, generate minimal volumes of waste, and, most importantly, generate reproducible staining results that are qualitatively comparable to those observed with traditional staining protocols.

2. Materials and methods

2.1. Bacterial specimens

All bacterial specimens are rated Biosafety Level 1 (BSL1) and were obtained from ATCC as follows: *Streptococcus salivarius* (ATCC BAA-1024), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 825D-5), *Mycobacterium phlei* (ATCC BAA-486), *Staphylococcus epidermidis* (ATCC 12228D), *Klebsiella pneumoniae* (ATCC 700603D-5), *Micrococcus luteus* (ATCC 53598D), *Acinetobacter lwoffii* (Cal Poly specimen), *Enterococcus faecalis* (ATCC 47077), *Brevibacterium linens* (Cal Poly specimen), *Serratia marcescens* (ATCC 14756), *Neisseria subflava* (Cal Poly specimen), *Pseudomonas aeruginosa* (ATCC BAA-1744), *Proteus vulgaris* (Cal Poly specimen). All bacterial specimens were grown for 24 h on complex media, including Tryptic Soy Agar (Thermo Scientific Remel) and Tryptic Soy Broth (Thermo Scientific Remel) and incubated at 35 °C with the exception of *M. phlei*, which has an optimal growth at 42 °C and *B. subtilis* which was grown for 5 days to allow for sporulation.

2.2. Stains & dyes

All stains, dyes and other reagents were prepared as follows:

Crystal Violet stain: 2.0 g Crystal Violet 90% (Fisher Chemical) dissolved in 20 mL ethanol (Fisher Chemical). 0.8 g ammonium oxalate monohydrate (Fisher Chemical) dissolved in 80 mL of water. Gram's Iodine: 0.3 g iodine (Sigma Aldrich), 0.6 g potassium iodide (Fisher Chemical), 90 mL water. Safranin stain: 0.5 g Safranin 90% (EMD Millipore), 10 mL ethanol, 100 mL water. Malachite Green stain: 5 g Malachite Green 90% (Fisher Chemical), 100 mL water. Carbolfuchsin: 0.3 g Basic Fuchsin 90% (Fisher Chemical) mixed in 10 mL ethanol mixed with 5.9 mL phenol (Fisher Chemical) mixed in 95 mL water. Acid Alcohol: 3 mL hydrochloric acid (Fisher Chemical) mixed slowly with 100 mL ethanol. Methylene Blue stain: 0.3 g Methylene Blue (Fisher Chemical) dissolved in 30 mL ethanol mixed with 10 mg potassium hydroxide (Fisher Chemical) mixed in 100 mL water. Congo Red: 0.5 g Congo Red (Fisher Chemical) in 100 mL water. Maneval's stain: 0.5 g Fast Green FCF (Fisher Chemical Cat# F99-10), 3.0 g iron (III) chloride (FeCl₃) (ACROS Organics), 95 mL water, 3.9 mL phenol and 5 mL glacial acetic acid (Fisher Chemical).

2.3. Gram stain

The Gram stain control was performed using *S. salivarius* and *E. coli* bacterial strains after 24 h growth in tryptic soy broth. A 25 × 75 mm plain microscope glass slide was cleaned with lens paper and, using a felt-tip permanent marker, three evenly spaced circular markings (~5-mm diameter) were drawn on the slide. The glass slide was flipped over and a loopful of *S. salivarius* was spotted on each marking, followed by a loopful of *E. coli*. The sample was heat-fixed with a Bunsen burner and allowed to cool. 2 µL of Crystal Violet was pipetted onto each of the three samples and incubated for 2 min. The slide was gently placed into a water bath (a 150 mL glass beaker filled with 110 mL of deionized water) and soaked for 2 min with occasional swirling. The slide was lightly pat-dried on a paper towel, followed by dry-compression with bibulous paper. 4 µL of Gram's iodine was pipetted onto each sample and incubated for 2 min, followed by a 2 min wash in a water bath (the wash water does not have to be changed and can be used for the entire experiment) and drying as described before. 5 µL of 90% Ethanol was pipetted onto each sample. Because of the importance of timing of the destaining step, counting was started while pipetting the first sample and continued during the spotting of the subsequent two sample regions with ethanol. At the end of a 15 s count, the slide was immediately placed in a water bath for 2 min, followed by drying as described before. Finally, 3 µL of Safranin was pipetted on to each sample region and incubated for 3 min, followed by a 2 min wash in the water bath and drying as described before. The sample was visualized using a brightfield microscope at 1000 × magnification (Supplementary Video 1).

2.4. Endospore stain, acid-fast stain & capsule stain

The Endospore stain and the Acid-fast stain are prepared as above (Gram stain) and a ~5 mm hole-punched blotting paper disc placed on top of the sample, with dye pipetted on the paper disc. Flaming of the sample for 5 s intervals over the span of 5 min allows for the proper penetration of dye into the bacterial sample. The Capsule stain requires that samples be air-dried (not heat fixed) on to the slide to avoid disruption of the bacterial capsule. Detailed methods can be found in supplemental sections S1.1–3 and Supplementary Videos 2, 3 and 4.

2.5. Safe handling of biological hazards and personal protection requirements

Recommended personal protective equipment include safety glasses (when handling liquid cultures), gloves, closed-toe shoes that cover the top of the foot, and a laboratory coat. Gloves are not required for handling BSL1 microorganisms with standard laboratory procedures if proper hand hygiene is performed, but gloves are recommended if a user's hands have fresh cuts or abrasions and when staining microbes (Emmert, 2013).

2.6. Waste handling and disposal

Safety data sheets (SDS) for information on reagent toxicity, storage, handling and disposal can be found on websites such as www.msds.com, www.msdsonline.com or by entering a search inquiry using online search engines. Waste should be collected in appropriately-labeled waste containers and disposed of in accordance with federal, state and local regulations.

3. Results & discussion

Micro-staining employs readily accessible materials that are commonly present in most microbiology and molecular biology laboratories, including micropipettors and pipette tips, standard glassware (150 mL glass beaker), a timer and all the reagents and materials used

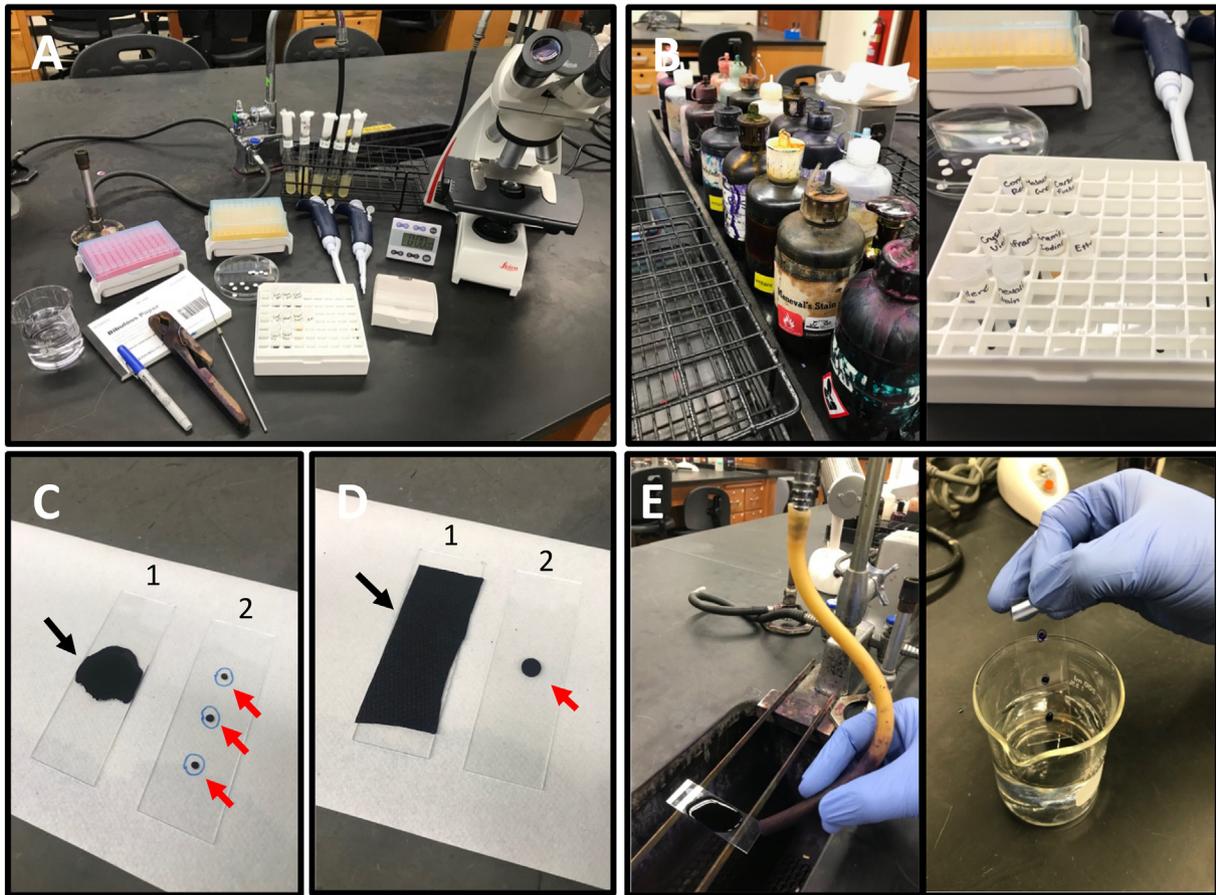


Fig. 1. Traditional staining and micro-staining techniques compared: A) Equipment and materials required for all micro-staining procedures discussed herein. B) Demonstration of the differences in volumes and size of containers between traditional staining (image on left) and micro-staining (image on right). C) Comparison of volumes used in simple and differential stains such as the Gram and Capsule stains, between traditional staining (#1) and micro-staining (#2) techniques. D) Comparison of volumes used in endospore and Acid-fast stains between traditional staining (#1) and micro-staining (#2). E) Demonstration of the differences in wash volumes between traditional staining (image on left) and micro-staining (image on right).

for traditional staining techniques (Fig. 1A). The most striking difference between micro-staining and traditional staining techniques is the volumes of stain used, which is visibly apparent when comparing the sizes of the containers holding the stains and dyes (Fig. 1B). This volume difference is most perceivable when conducting the staining protocol, as can be observed with a primary stain. In micro-staining, multiple samples or repeats can be stained on a single slide (red arrows) in a controlled manner, while traditional ‘flooding’ of stain (black arrow) is generally limited to one or two samples along a glass slide (Fig. 1C). The significant difference in volume of stain can also be appreciated for staining techniques that require the use of high-heat, such as the Acid-fast and Endospore stains, where multiple rounds of stain are used to bathe a piece of paper and thus protect the specimen from the direct flame and prevent desiccation. Traditional staining generally employs large pieces of paper, which require large volumes of stain (Fig. 1D, black arrow), while micro-staining was achieved with small paper discs made from blotting paper (Fig. 1D, red arrow). Another difference between these two techniques can be observed with the wash steps. Traditional staining typically employs a staining rack positioned over a sink fitted with a collection flask or large container, and copious amounts of water from a faucet are used to thoroughly wash microscope slides (Fig. 1E, image on left). Micro-staining makes use of a small water bath (110 mL of water), which can be reused for multiple rounds of staining without affecting the overall outcome (Fig. 1E, image on right). A final observable difference is the overall ‘cleanliness’ of the micro-staining technique. Traditional staining techniques inevitably leave benchtops, workspaces and clothing or lab coats marked and

colored with dyes, while micro-staining generally minimizes the staining of work areas, tools, instruments and hands.

As illustrated in the flowchart of a simple micro-stain (Fig. 2), multiple samples can be processed on a single slide (Fig. 2B, C), microliter amounts of dye or stain are deposited in a controlled fashion (Fig. 2E), and a minimal amount of waste is generated (Fig. 2F), while not compromising on the overall staining quality of the specimens (Fig. 2G, H).

To ensure that micro-staining is a technique that can be applied to many microbiologically relevant stains, side-by-side comparisons of a micro-stain and a traditional stain were performed for a Gram stain (Fig. 3A and E), Endospore stain (Fig. 3B and F), Capsule stain (Fig. 3C & G) and Acid-fast stain (Fig. 3D and H). The vibrancy of colors, distribution and detection of specimens are qualitatively comparable between traditionally stained specimens (Fig. 3A, B, C & D) and micro-stained specimens (Fig. 3E, F, G & H, Supplementary Videos 1, 2, 3 and 4). The Gram stain was further tested on an array of gram-positive and gram-negative microorganisms (Fig. S1), as well as on gram-indeterminate and gram-variable microorganisms (Fig. S2), where no discernable differences in the staining outcomes when comparing the micro-staining to the traditional staining techniques were detected. Additionally, all of these stains were acquired by undergraduate students with limited laboratory experience, highlighting the ease-of-use of this method and its potential for adoption in academic teaching laboratories.

A total volume and cost comparison for micro-staining and traditional staining was prepared based on a 1-year period at our institution,

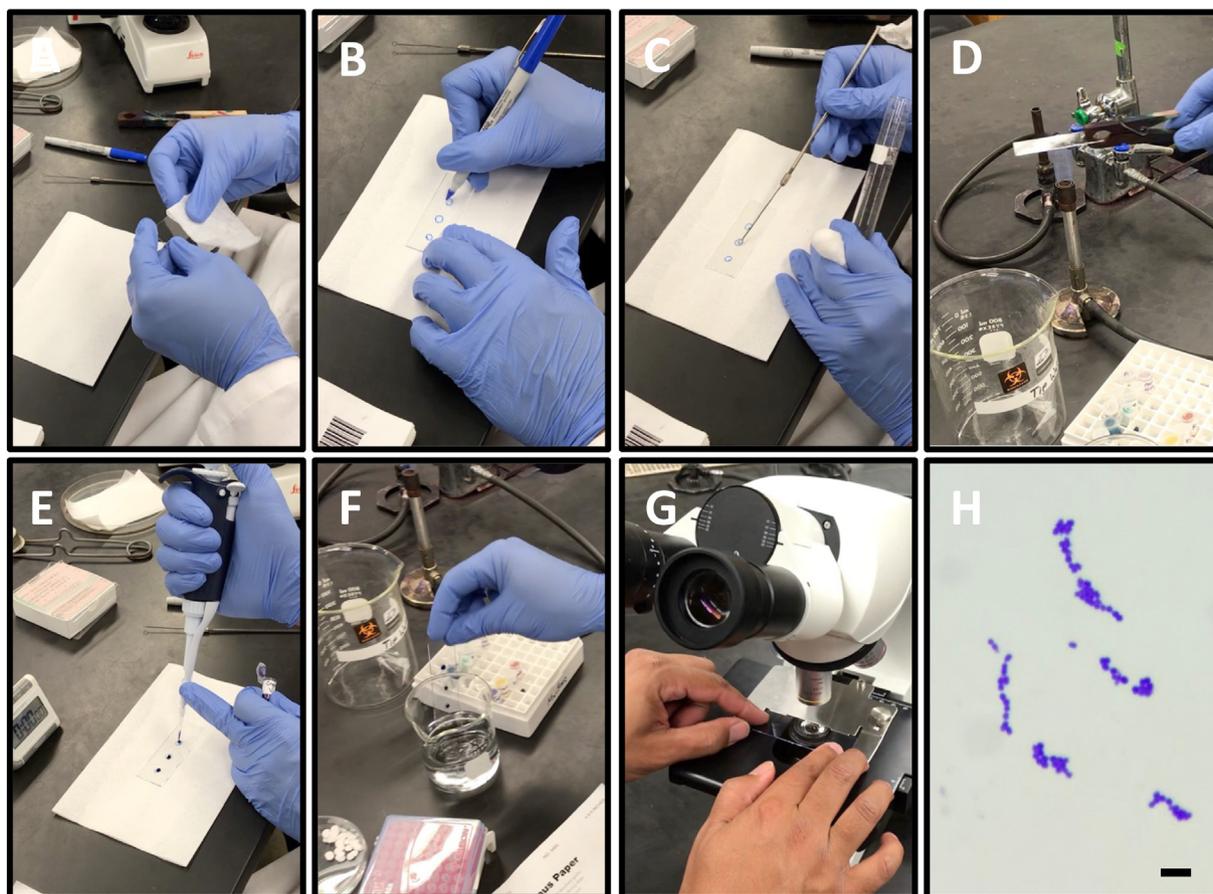


Fig. 2. Overview of a simple stain procedure using the micro-staining technique. A) Thoroughly clean a glass microscope slide with lens paper. B) Mark an inoculation zone(s) on the back of the slide for easy identification of the region of interest (ROI). C) Flip the slide over and inoculate the slide with the specimen. D) Heat fix the specimen by briefly passing the slide over a Bunsen burner flame 2–3 times. E) Inoculate the ROIs with 2 μ L of the chosen stain(s) and incubate. F) Wash the slide in water to remove any excess stain—110 mL of water in a 250 mL beaker fits most conventional microscope glass slides, and the wash water can be re-used multiple times. G) Using bibulous paper, pat the slide dry and view under a microscope. H) Example of a simple stain using crystal violet on a pure culture of *S. salivarius*. (Scale bar: 5 μ m).

where we typically instruct ~800 students in the various staining techniques described above across many levels of microbiology courses. Using very conservative figures, we found that traditional staining requires ~146 L of stain and fixatives for a total cost of USD \$4579.71 per year at our institution (Table 1). These costs were calculated in 2019 prices and reflect our University's corporate discount with reagent suppliers such as, ThermoFisher Scientific. We estimate that when the same number of students use micro-staining performing the average number of stains in duplicate, the total cost of stain and fixatives will be \$17.82 per year, representing a savings of USD \$4561.89 per year compared to traditional staining. If one also factors in the cost of handling and disposing of waste (current rates at our institution are ~\$125 per 20 L of hazardous waste), micro-staining leads to an additional savings of USD \$908.75 per year (Table 1). The total institutional savings for switching from traditional staining techniques to micro-staining techniques are estimated at USD \$5470.64 in 2019 prices (Table 1). We recognize that micro-staining does require the purchase and use of micro-pipettes and pipette tips. However, we found that with a single-year's worth of savings from switching to micro-staining, we were able to stock our laboratories with standard-quality, fixed-volume micropipettes (~\$100) and bulk tips (10,000 units/bag at \$200/bag). Factoring in the cost of pipette tips and the cost of repairing and replacing pipettes on a regular basis, we still estimate a savings on the order of USD \$5000 per year. We acknowledge that the cost-savings may be trivial in the context of a large university's annual operating budget, however this is just an added benefit to the primary benefit of

reducing the amount of waste and the overall hazardous footprint of microbial staining.

4. Conclusions

We introduced a simple, cost-effective and resource-saving method for staining microbial samples in educational and research settings alike. We tested the micro-staining method with a series of widely employed stains, including the Gram stain, Endospore stain, Acid-fast stain and Capsule stain. To ensure that micro-staining gave comparable results to traditional stains, Gram stains were performed on an array of microorganisms, including gram-indeterminate and gram-variable microorganisms. We were unable to discern any significant differences in the staining outcomes when comparing the micro-staining to the traditional staining techniques, however we do acknowledge that further validation of this technique will be required on a greater number and variety of microorganisms for implementation of the micro-staining technique in a diagnostic laboratory setting. Micro-staining is unique in that only microliter amounts of stain are required for obtaining reproducible results that are qualitatively equivalent to traditional staining methods. This unique twist on an age-old practice requires little to no additional training, saves valuable resources and, most importantly, results in reduced quantities of staining waste compared to the traditional staining techniques commonly employed worldwide. We believe this method can be readily adopted by any academic teaching laboratory and is a long overdue shift away from the common practice

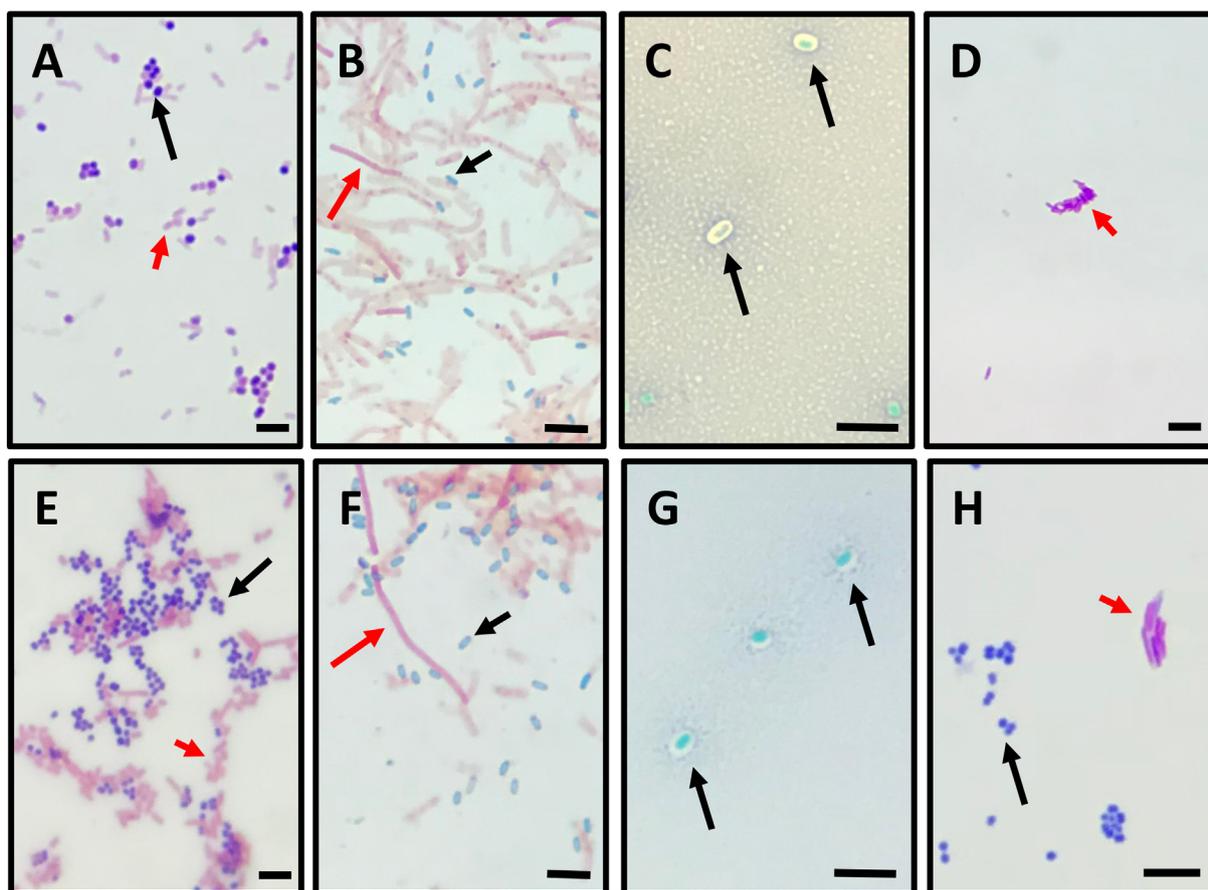


Fig. 3. Traditional staining and micro-staining techniques compared. Traditional Techniques (A-D): A) Gram stain: gram-positive (purple) *S. epidermidis* (long black arrow) and gram-negative (pink) *E. coli* (short red arrow). B) Endospore stain: Green bacterial endospores (short black arrow) and pink vegetative *B. subtilis* (long red arrow). C) Capsule stain: Capsules (clearings) surrounding turquoise colored *K. pneumoniae* (black arrows). D) Acid-fast stain: Fuchsia-colored acid-fast *M. phlei* (short red arrow). Micro-staining Techniques (E-H): E) Gram stain: gram-positive (purple) *S. epidermidis* (long black arrow) and gram-negative (pink) *E. coli* (short red arrow). F) Endospore stain: Green bacterial endospores (short black arrow) and pink vegetative *B.s subtilis* (long red arrow). G) Capsule stain: Capsules (clearings) surrounding turquoise colored *K. pneumoniae* (black arrows). H) Acid-fast stain: Fuchsia-colored acid-fast *M. phlei* (short red arrow) and blue non-acid fast *S. epidermidis* (long black arrow). (Scale bars: 5 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Volume and cost comparison between traditional staining and micro-staining techniques. Volume of stain usage (liters) and cost (USD) comparison of techniques at a California State University over a 1-year period for approximately 800 students.

Stain	Traditional stain		Micro-stain		Hazardous waste disposal
	Total volume used/year	Total \$/year ^a	Total volume used/year	Total \$/year ^a	
Gram stain	24L	\$741	0.1 L	\$3.00	Yes
Endospore stain	36L	\$1752	0.1 L	\$4.90	Yes
Acid-fast stain	10L	\$107.91	0.1 L	\$2.24	Yes
Capsule stain	16L	\$478.82	0.1 L	\$4.52	Yes
95% Ethanol	48L	\$1195.20	0.1 L	\$2.49	Yes
Acid alcohol	12L	\$304.78	0.1 L	\$0.67	Yes
Total materials	146 L	\$4579.71	0.6 L	\$17.82	
Estimated cost of hazardous waste disposal (\$125 per 20 L)		\$912.50		\$3.75	
Total cost (\$)		\$5492.21		\$21.57	

^a Prices indicate 2019 prices found on fishersci.com. These prices reflect a University corporate discount. Prices may vary depending on volumes ordered and geographical location.

of ‘flooding slides’ with stain. Although this technique will most likely prove suitable for clinical laboratory settings, the validity and the sensitivity of this technique on BSL2 clinical specimens, i.e. on AFB sputum smears, remains to be determined and is currently being explored.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2019.105654>.

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