Key 3: Staining it right

How many Gram stain videos are there on Youtube?

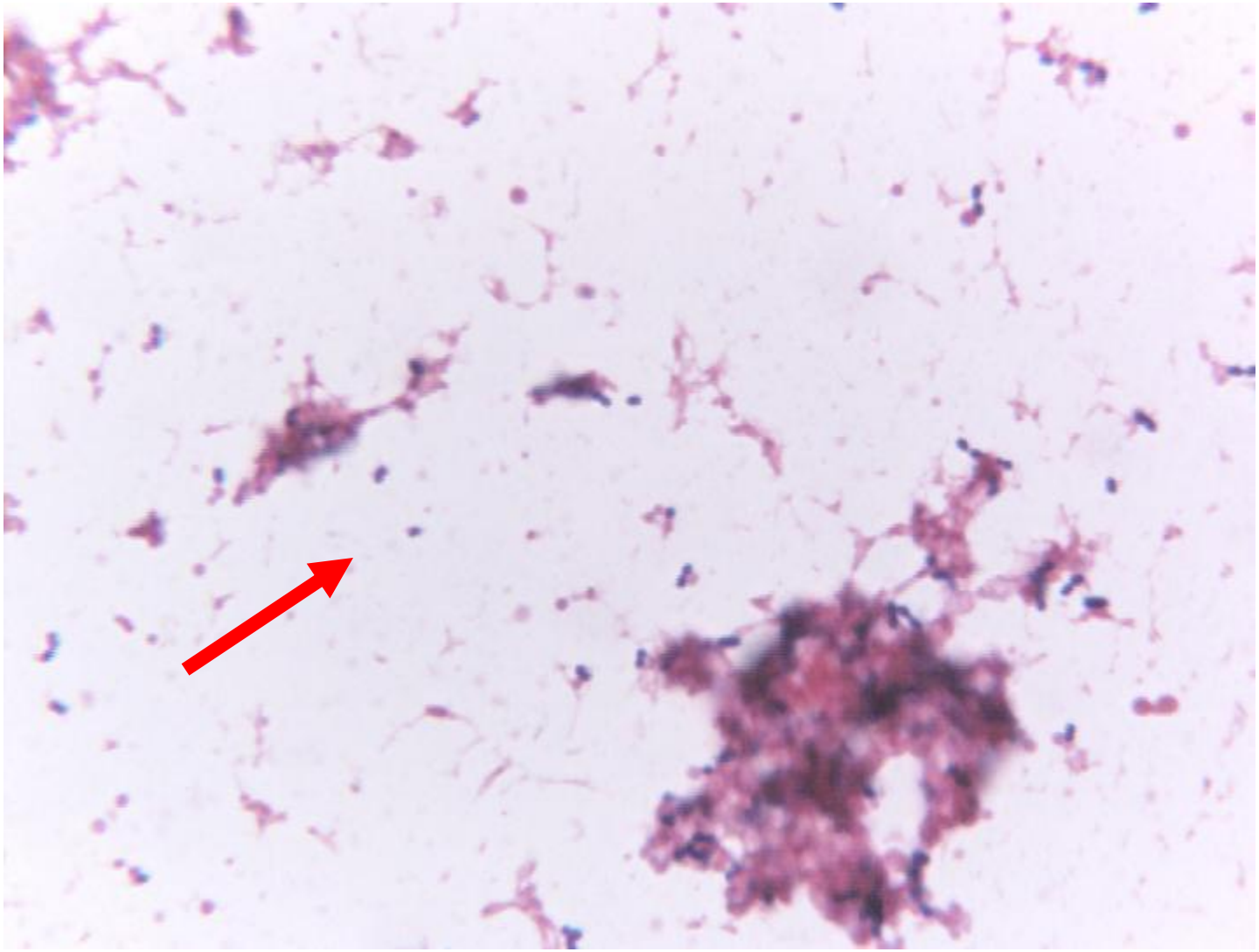
Key 3: Staining it right

- Methanol fixation
- Works best for blood cultures, bloody wounds
- Preserves RBCS
- Can see intracellular bacteria
- Cleaner, whiter background

How to methanol fix

- Air dry the slide completely
- Flood the slide with absolute methanol for 2 minutes
- Tilt and blot the methanol off the slide
- DO NOT RINSE WITH WATER
- Air dry
- Proceed to crystal violet

Blood culture, heat fixation



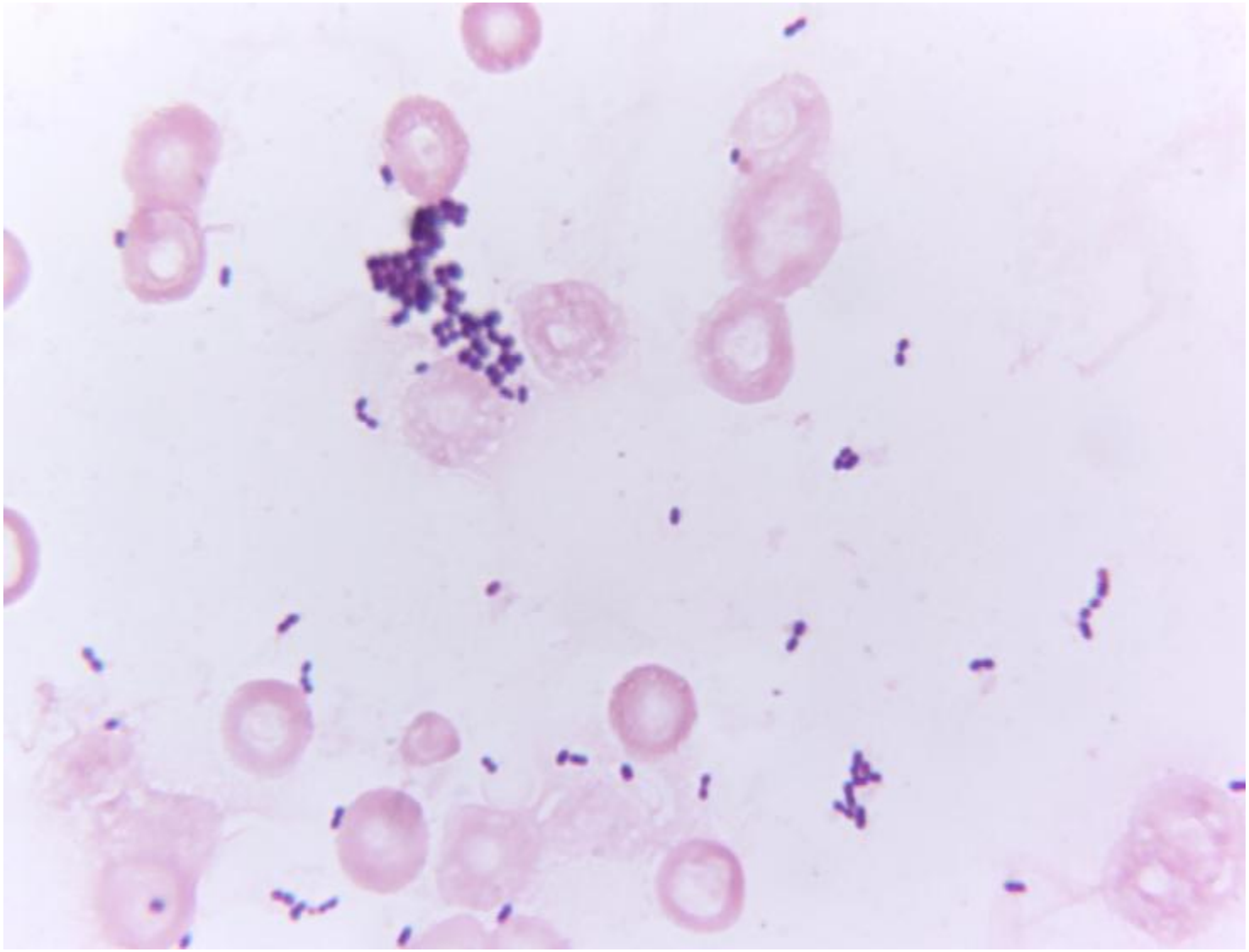
And the answer is?

A. Amorphous material, no organisms seen
B. Gram positive cocci
C. Gram negative rods
D. Gram negative diplococci

This is what happens to human cells when you
heat fix a slide!
RBCs are lysed,
WBCs are distorted

Lubbock, TX

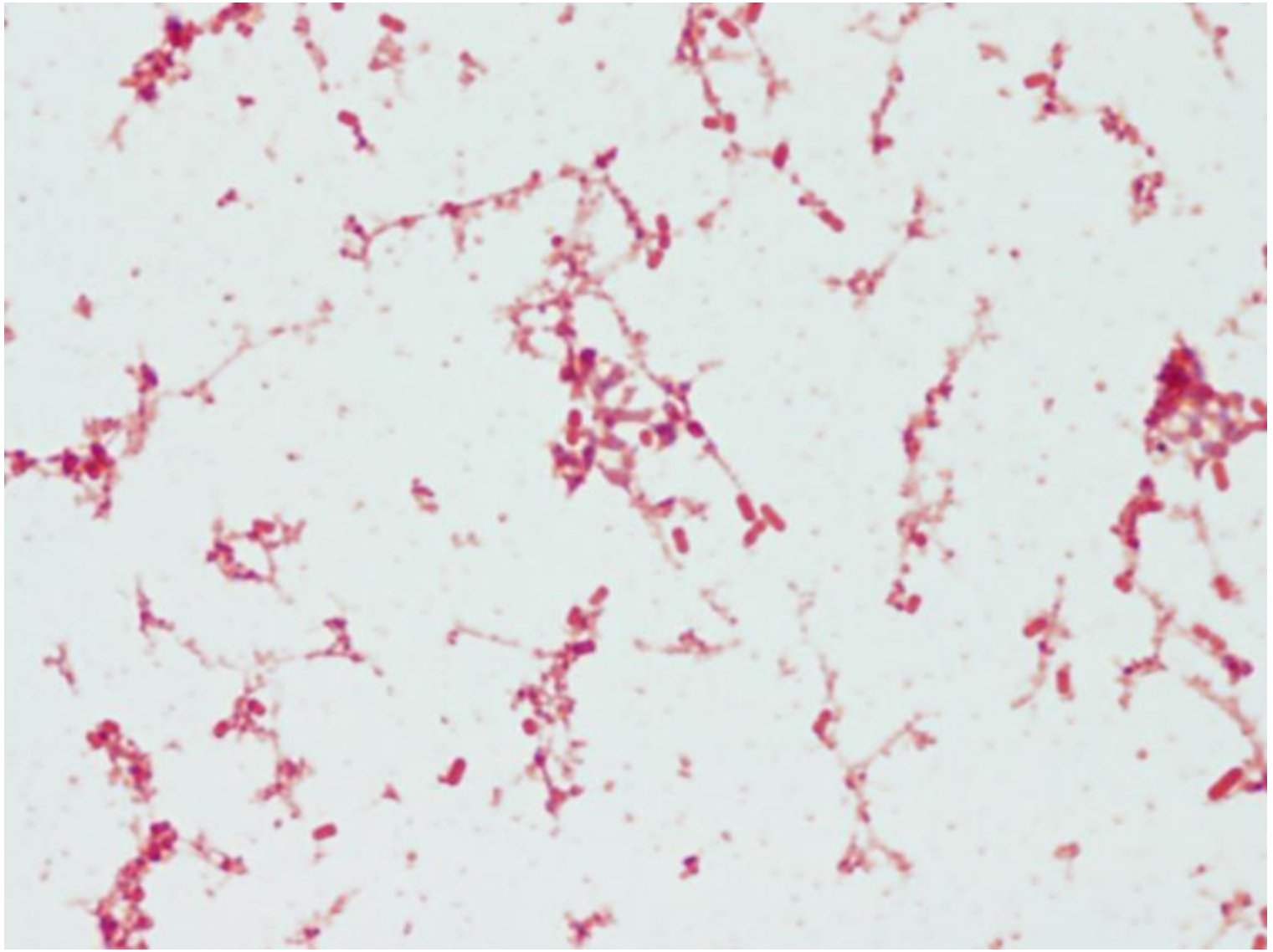
Air dried/methanol fixed, from a duplicate slide made at the same time



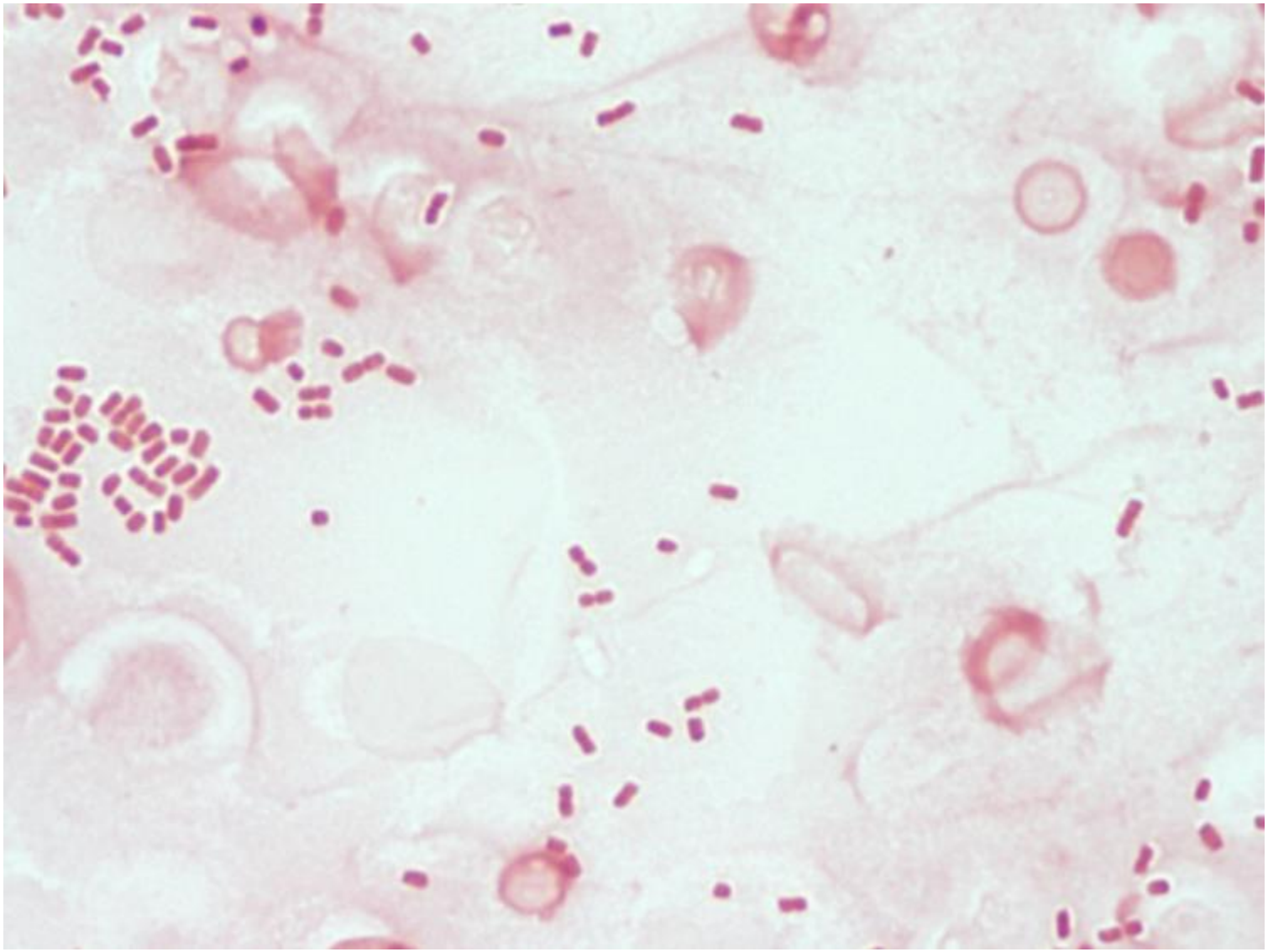
Now that things are cleared up a bit, the answer is?

A. Gram positive cocci in chains
B. Gram positive cocci in clusters
C. Gram positive rods
D. Both gram positive cocci in chains and clusters

Another example heat fixed



Same blood culture stained at same time, methanol fixed



Literature support for methanol fixation

Diagn Microbiol Infect Dis. 1984 Apr;2(2):129-37.

Methanol fixation. An alternative to heat fixation of smears before staining.

Mangels JI, Cox ME, Lindberg LH.

Abstract

Methanol fixation of Gram-stained smears was compared to heat fixation. Smears were prepared in duplicate from direct clinical specimens, blood culture bottles, and bacterial colonies. Results from this study show that methanol fixation is superior in every instance to heat fixation. The morphology of bacteria and tissue cells was not distorted, little or no background debris was observed, and a better Gram reaction was noted.

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.

The actual Gram stain

- Crystal violet 30 seconds
- Gentle water rinse (hold slide vertical and slice through water)
- Gram iodine 30 seconds
- Gentle water rinse
- Decolorize with 50/50 acetone/alcohol (finesse step)
- Gentle water rinse
- Gram safranin 30 seconds
- Gentle water rinse
- Blot edges only
- Air dry – DO NOT USE BIBULOUS PAPER!
- Reference: Clin micro procedures handbook, 3rd ed, ASM press

During which steps of the Gram stain could you go on break for 15-20 minutes and not mess it up?

Decolorizing?

- The hardest part right? Requires finesse!
  - Like knowing when to flip a pancake

You should not **flip** when you see bubbles, but you should **flip** when those bubbles pop and form holes that stay open on the surface of the **pancake**. If a bubble comes to the surface, pops, but is filled in by more **pancake** batter, hold off on **flipping**. Make sure your **pancakes** are hole-y! May 14, 2014

- In my experience, holding each slide in your hand as you decolorize works best
- Have good light and a white background
- Use a 50/50 methanol to alcohol decolorizer

Automated stainers

- Advantage: consistency and automation
- Great for new people, second/third shift
- Good for batch staining
- Disadvantage: maintenance
- The case for automation: Cyto, histo and hem do it, why should we not?

Don’t confuse automated Gram stain makers (WASP, Kiestra) with automated stainers. See automated stainer vendors and weblinks in supplement.

Key 3 summary

- Methanol fix
- Know when and how to flip your pancakes
- Automate the making of slides and actual staining if you can or must.

Random Gram stain challenge: CSF