AFB Smear Microscopy

Terminology

- AFB Smear Microscopy: Microscopic examination of specially stained smears to detect acid-fast organisms such as *Mycobacterium tuberculosis* and non-tuberculous mycobacteria (NTM)

- Acid Fast Bacilli (AFB): organisms (including mycobacteria) that resist decolorization with acid alcohol due to the lipid-rich mycolic acids in the cell wall thereby retaining the primary stain

Terminology

- <u>Processing:</u> digestion, decontamination, and/or concentration of a primary patient specimen prior to setting up culture and smear
- <u>Smear:</u> A small amount of primary patient specimen (direct or processed) is placed on a slide for the purpose of microscopic examination

AFB Microscopy

- Examination of smears is a rapid, convenient and inexpensive test
- All types of specimens can be evaluated
  - *sputum, tissue, body fluids, etc.*
- Positive AFB smear results provide a first indication of mycobacterial infection and potential TB disease
- Must be accompanied by additional testing including culture for confirmatory diagnosis

AFB Microscopy Results Guide Decisions

- Clinical management
  - Patient therapy may be initiated for TB based on smear result and clinical presentation
  - Changes in smear status important for monitoring response to therapy
- Laboratory testing
  - Algorithms for use of nucleic acid amplification tests are often based on smear positivity
- Public health interventions
  - Smear status and grade useful for identifying the most infectious cases
  - Contact investigations prioritized based on smear result
  - Decisions regarding respiratory isolation based on smear result

Smear-positive TB Cases

- Smear-positivity and grade indicates relative bacterial burden and correlates with disease presentation
- Patients that are sputum smear-positive are 5–10 times more infectious than smear negative patients
- Untreated or treated with an inappropriate regimen, a sputum smear-positive patient may infect 10-15 persons/year

Sputum Smear Results

- In 2010, 43% of pulmonary TB cases in the U.S. were sputum smear positive
- Incremental diagnostic yield of examination of three sputum specimens among smear positive cases

First specimen	Second specimen	Third specimen
85.8%	11.9%	3.1%

CDC. Reported Tuberculosis in the United States, 2010. Atlanta, GA: U.S. Department of Health and Human Services, CDC, October 2011.

Mase S, Ramsay A, Ng N, Henry M, Hopewell PC, Cunningham J, Urbanczik R, Perkins M, Aziz MA, Pai M. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. *Int J Tuberc Lung Dis* 2007;11(5):485-95.

Limitations of AFB Microscopy

- Does not distinguish between viable and dead organisms
  - Follow-up specimens from patients on treatment may be smear positive yet culture negative
- Limited sensitivity
  - High bacterial load 5,000-10,000 AFB /mL is required for detection
  - Misses >45% of U.S. TB cases
- Limited specificity
  - All mycobacteria are acid fast
  - Does not provide species identification
  - Local prevalence of MTB and NTM determine the predictive values of a positive smear for MTB

Smear Types

- Direct smear
  - Smear prepared directly from a patient specimen prior to processing
- Concentrated smear
  - Smear prepared from a processed specimen after centrifugation is used to concentrate the material

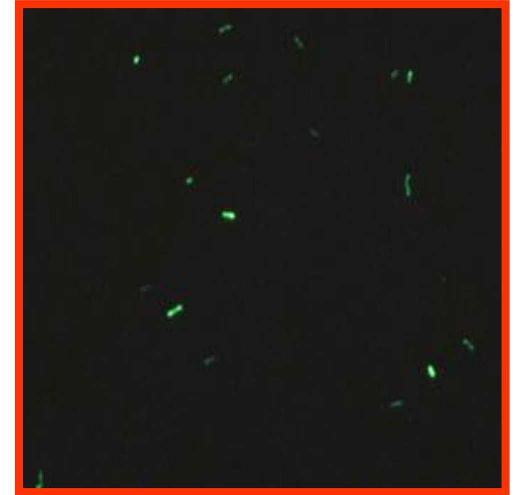
Direct Smears

- Rapid and simple
  - May be performed for quick results
  - Positive results help confirm clinical suspicions
- Not as sensitive as concentrated smear
  - A direct smear *should always* be followed by a concentrated smear

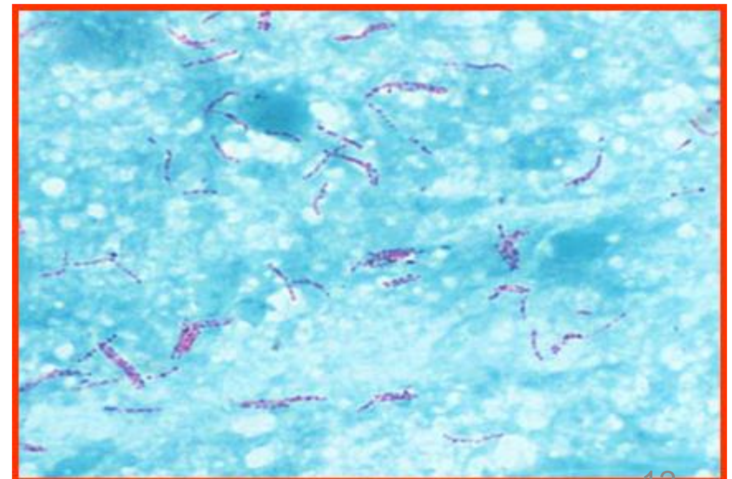
Concentrated Smear

- 3-10 ml of sputum is processed and concentrated by centrifugation
- Smears can be made
  - Directly from processed pellet
    - May increase smear sensitivity
    - However, may result in less material available for NAA testing & culture
  - From re-suspended pellet after the addition of buffer
    - Re-suspended in ~2 ml buffer; one drop for smear

AFB Microscopy Staining Techniques



- Two basic techniques: same principle:
- Fluorescence: Auramine staining
  - Also known as Fluorochrome staining
  - Contrast light & dark
- Brightfield: carbol fuchsin staining
  - Contrast red AFB on blue or green background



Carbol-Fuchsin AFB Staining

- Primary stain is Carbol Fuchsin
  - Ziehl-Neelson (ZN)
    - Requires heat during staining
    - Requires higher magnification
    - More fields examined (e.g. 300 fields at 1000X)
    - Requires more time to read
    - Requires use of oil immersion
    - Stains all NTM well
  - Kinyoun
    - Cold carbol fuchsin method
    - Less toxic fumes
- Neither method is reccomended for staining primary specimens

Fluorescent AFB Staining

- Primary stain is Fluorescent
  - CDC recommends fluorochrome staining for detecting AFB in primary patient specimens
  - Auramine-O, Auramine Rhodamine
    - Read at lower magnification, less fields examined (e.g, 30 fields at 200X)
      - Faster screening of smears than with ZN
      - ~10% more sensitive than ZN
      - Does not require use of oil immersion

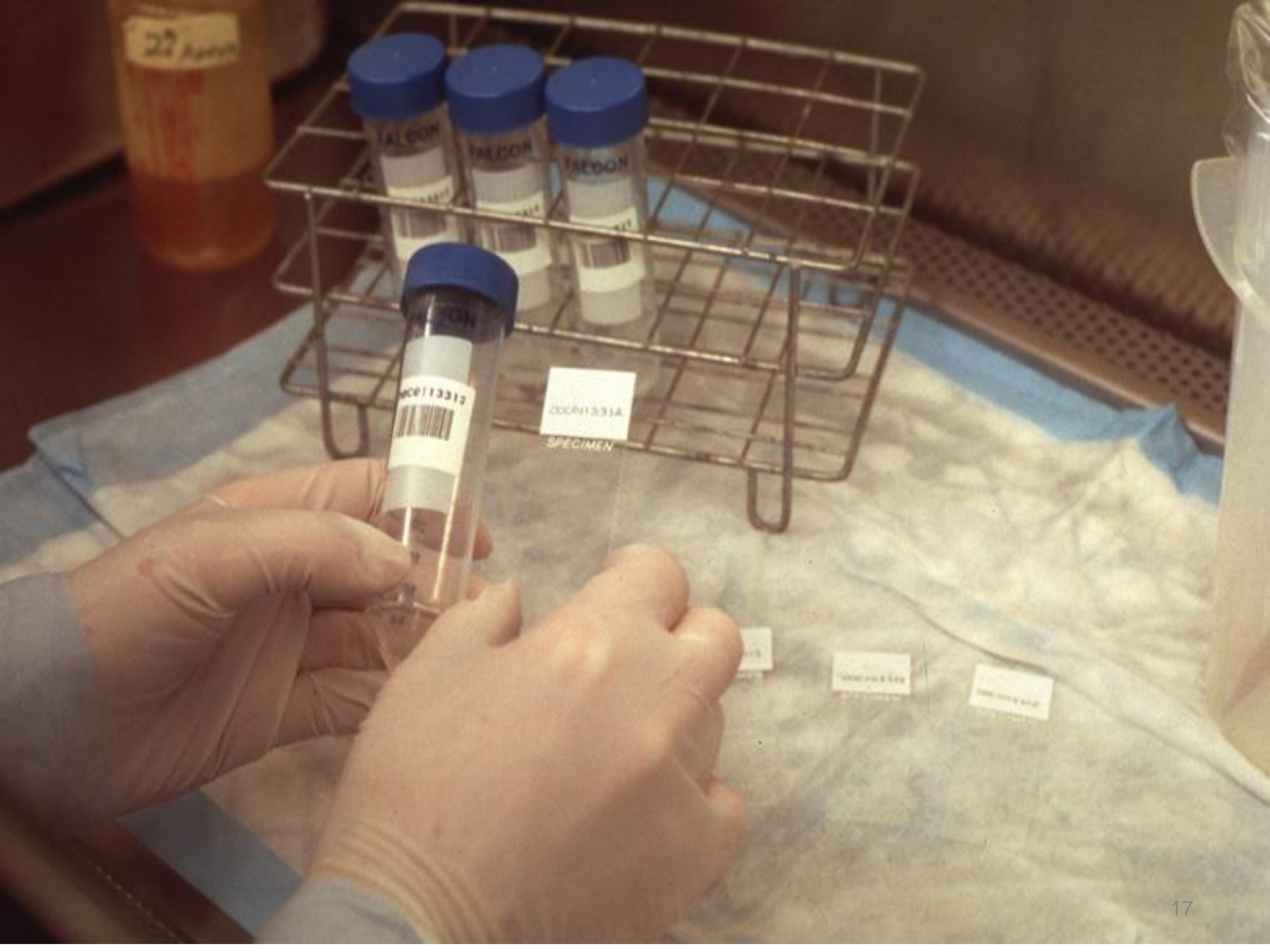
Steps in Performing AFB Microscopy

1. Preparing and Fixing Smears
2. Staining Smears
3. Examining Smears
4. Recording and Reporting Results

Getting Started…

- New, clean, greaseless, and unscratched slides should be used
- Match identifiers on slide with clinical specimens

  - Labeling should be performed with material that stays permanently affixed to the slide during the staining procedure (e.g., graphite or diamond tip pencil)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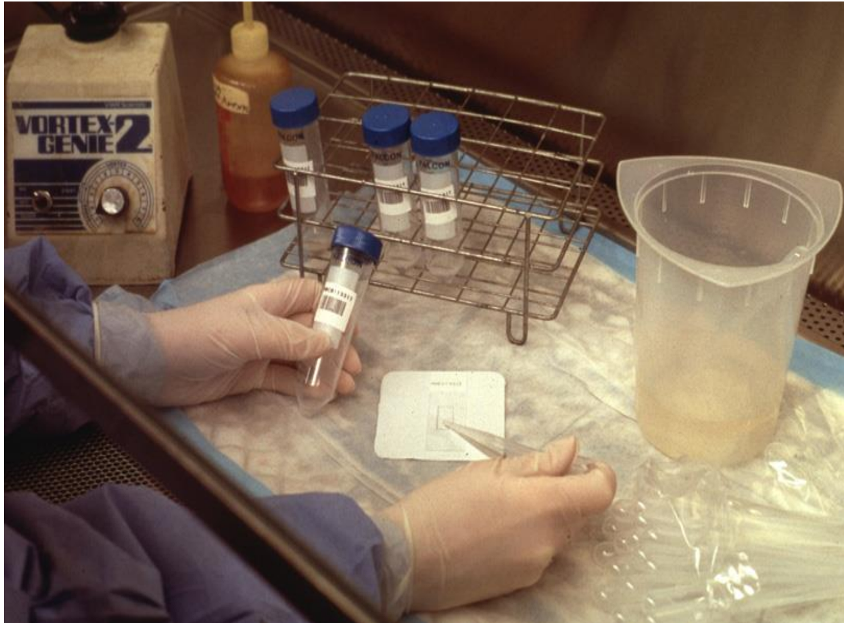
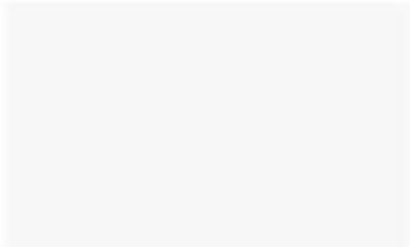
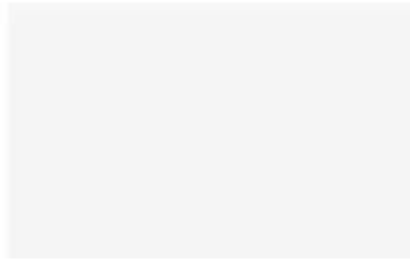
Preparing Smears



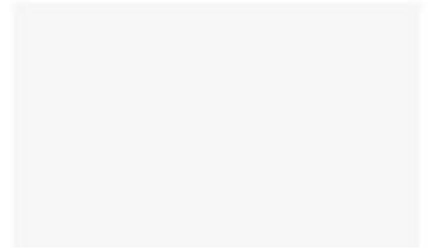
- After processing and concentrating the specimen, 1 to 2 drops of material should be smeared on the slide
- Smear material in an area of approximately 2-cm^2



Too Thick



Just Right



Too Thin

Fixing Smears

- Prior to heat fixing, smears should be allowed to air dry completely within the biological safety cabinet
- Acceptable methods for heat fixing
  - Flame fixing by passing over flame 2–3 times for a few seconds smear side up
    - Avoid scorching
  - 2 hrs minimum at 65-75°C on an electric slide warmer
  - 15 min at 80°C[1]
  - 5% phenol in 70% EtOH for 5 min[2]
    - also kills AFB
- Considerations
  - Flame fixing may aerosolize organisms from smear
  - Insufficient heat or time can lead to smear washing off
  - Slide warmers may not heat evenly
  - Viable AFB remain with some fixing methods

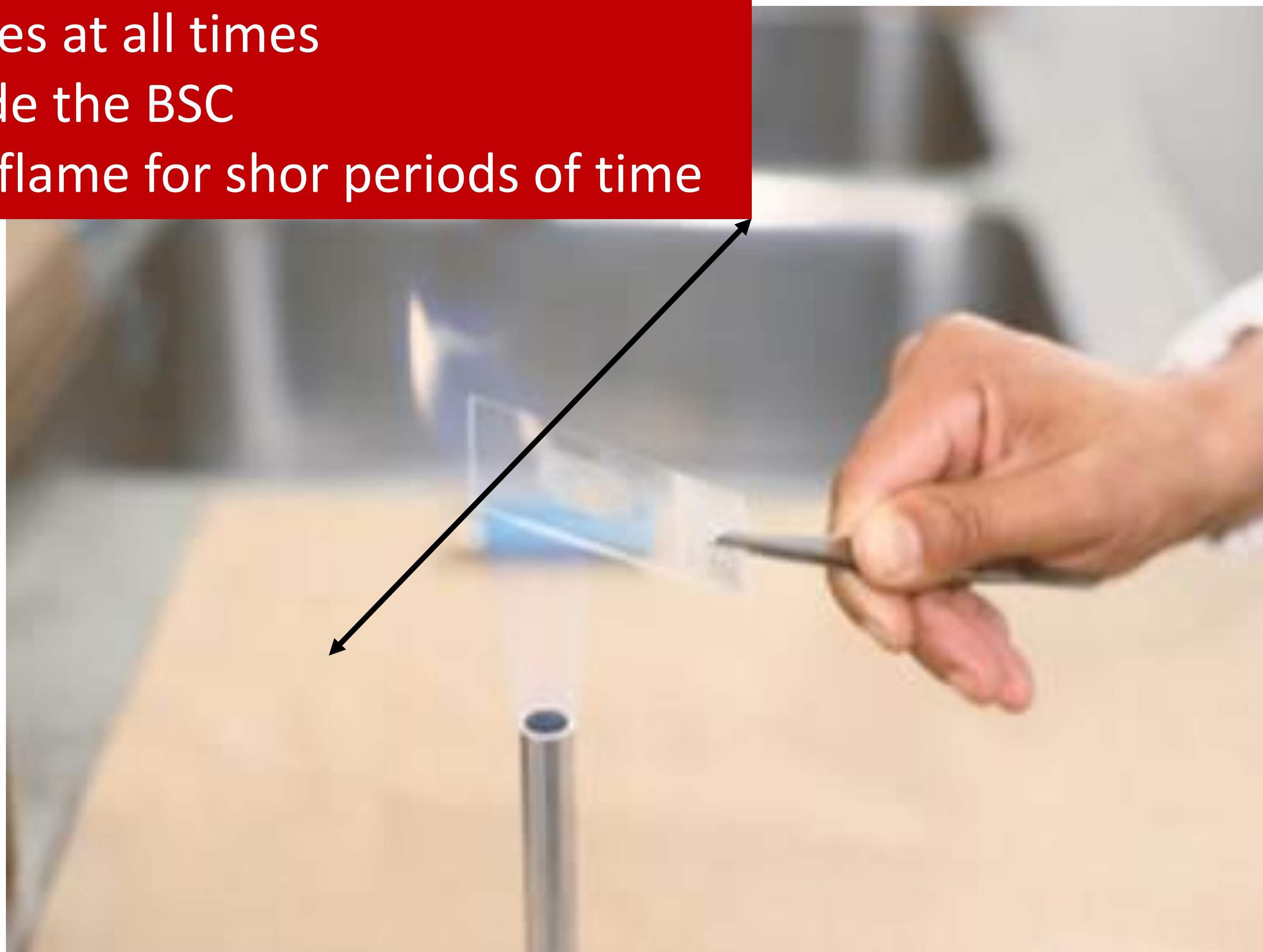
[1] Bailey & Scott's Diagnostic Microbiology 2007 12th ed.
[2] Chedore et al. 2002. J. Clin. Microbiol. 40:4077
CLSI M48-A: Laboratory Detection & Identification of Mycobacteria

65 - 75° C
Keep slides on warmer for 2 hours

Heat Fixing Smear

Safety Concerns:

- Wear gloves at all times
- Work inside the BSC
- Use open flame for shor periods of time



AFB Staining Principles

- Primary stain penetrates cell wall
- Intense decolorization does not release primary stain from the cell wall of AFB
- Color of AFB-based on primary stain
- Counterstain provides contrasting background

Stains Used in Fluorescence Microscopy

- Primary Stains
  - Auramine O
  - Auramine O-Rhodamine-B
- Counter Stains
  - Potassium Permanganate
  - Acridine Orange

Fluorescence AFB Microscopy

- Primary fluorochrome
  - Auramine O
  - Auramine O-Rhodamine B
- Counter Stain
  - Potassium permanganate

Fluorescence AFB Microscopy

- **Primary Fluorochrome**
  - Auramine O
  - Auramine O/ Rhodamine B
  - Acridine Orange

- **AFB Fluoresces**
  - Green
  - Yellow/Orange
  - Yellow/Orange

450x

Water Quality is Key

- AFB microscopy is not specific
  - Acid fast environmental contaminants as well as NTM and MTB presence in the specimen will be detected
- Introduction of an environmental contaminant during wash steps and in reagent preparation must be avoided
- Use of a negative control slide essential for detecting potential environmental contaminants
- Avoid using large containers of reagents and carboys of water
- Use filtered distilled or deoinized water
- Water filtration and distribution systems should be monitored

Staining Reagents

- Commercial products are available or reagents can be prepared in-house
  - If prepared in-house, proper precautions must be taken when handling dyes including appropriate PPE and the use of a fume hood
- Reagents containing fluorescence stains should be stored to protect from light exposure

Steps in the Fluorescent Staining Procedure

1. Place slides on staining rack; slides should not touch
2. Flood slides with fluorochrome stain; no heating. Follow protocol or package insert for timing.
3. Rinse with water; aim flow at edge of slide
4. Decolorize with 0.5% acid-alcohol solution, Follow protocol or package insert for timing.
5. Rinse with water; drain excess
6. Flood slide with counterstain; Follow protocol or package insert for timing.
7. Rinse with water; drain excess
8. Air-dry stained slide; do not blot

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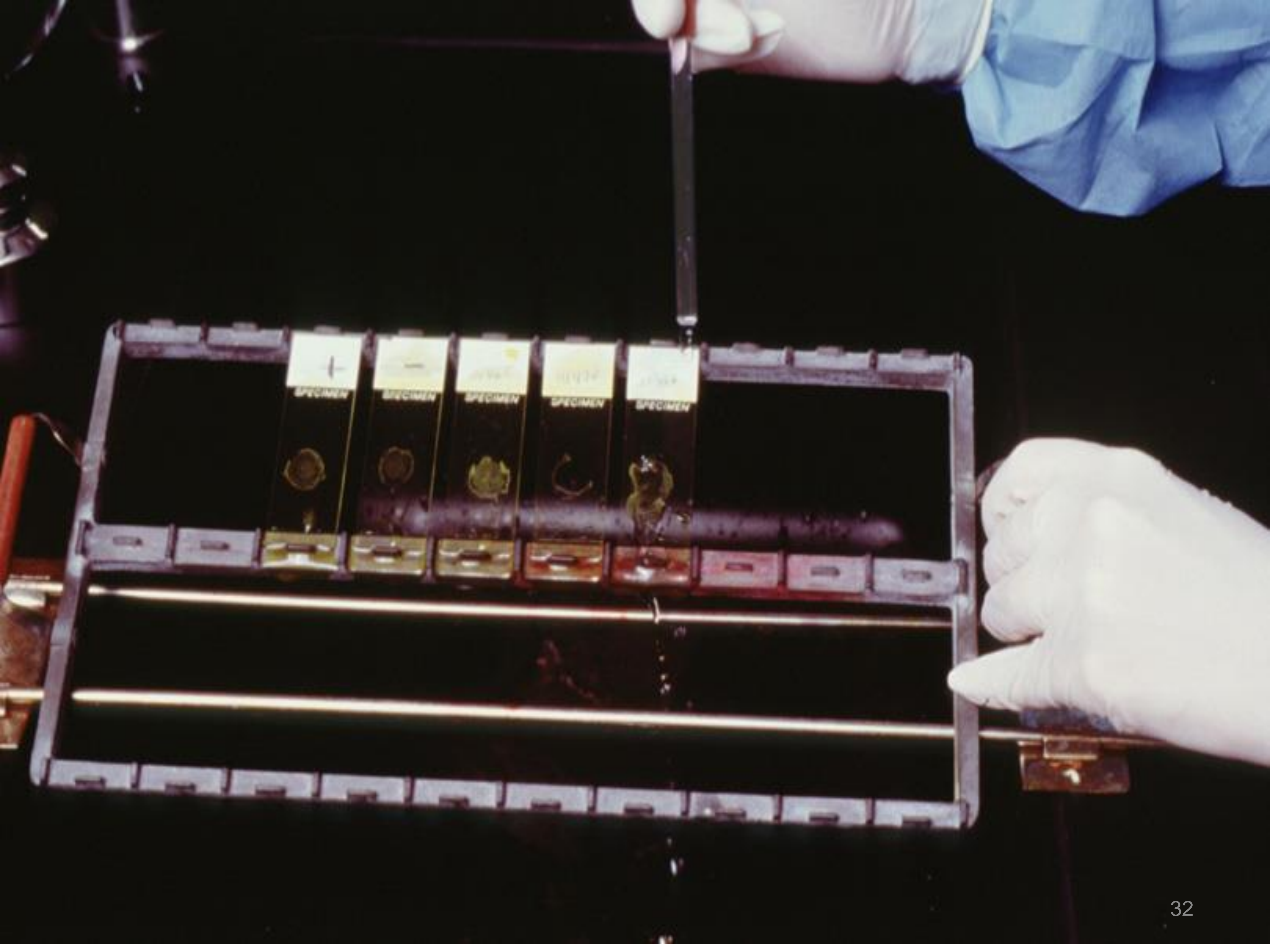
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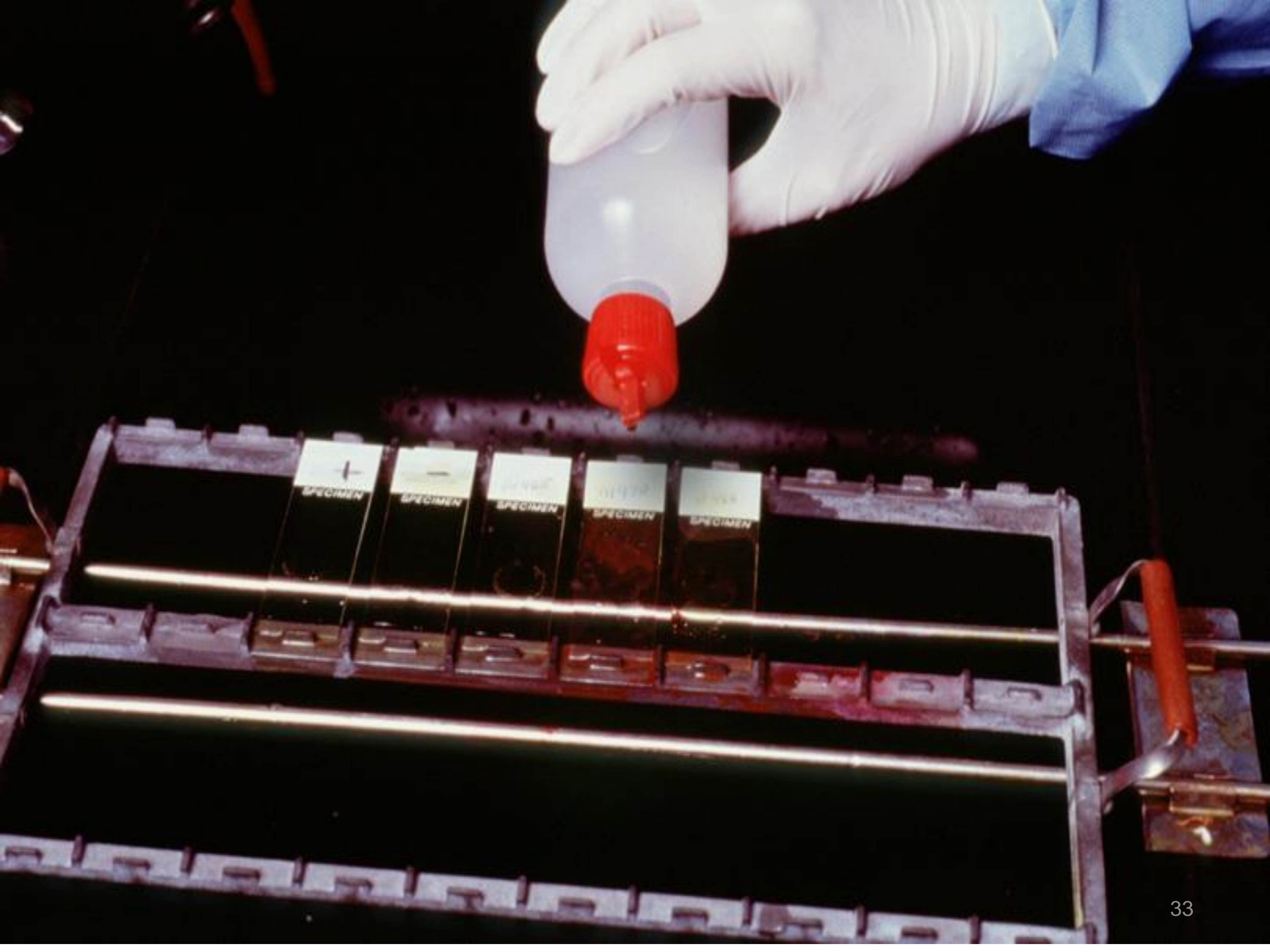
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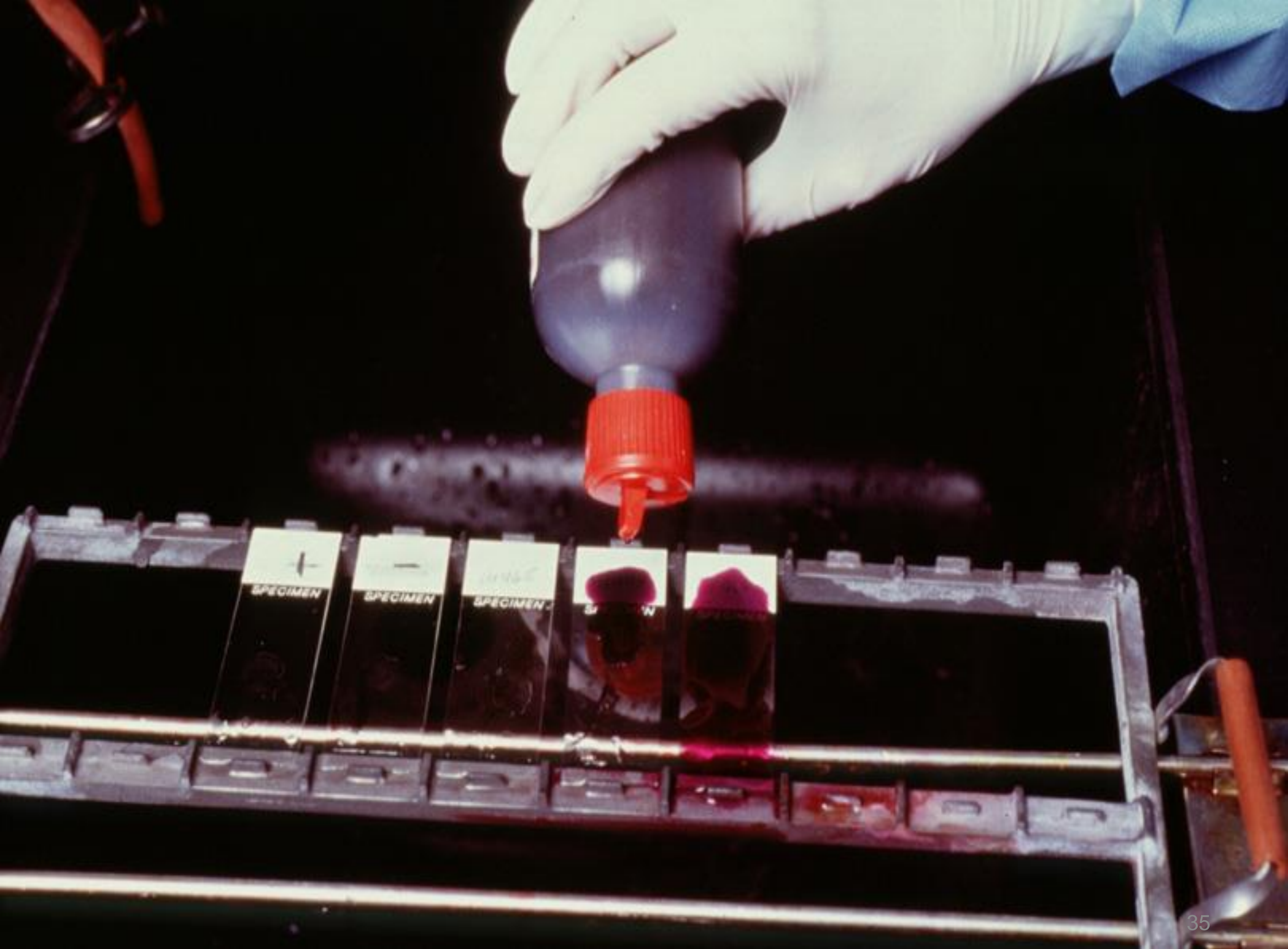
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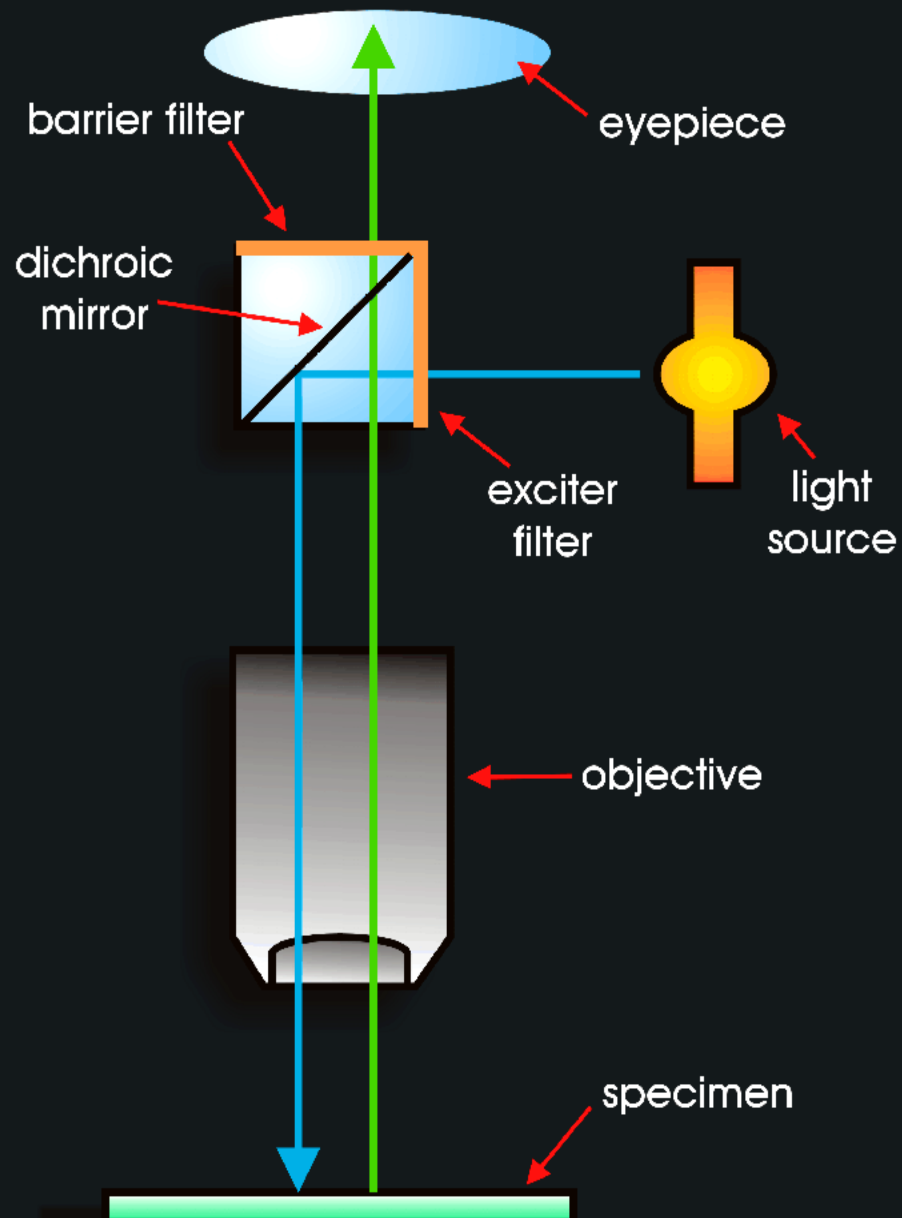
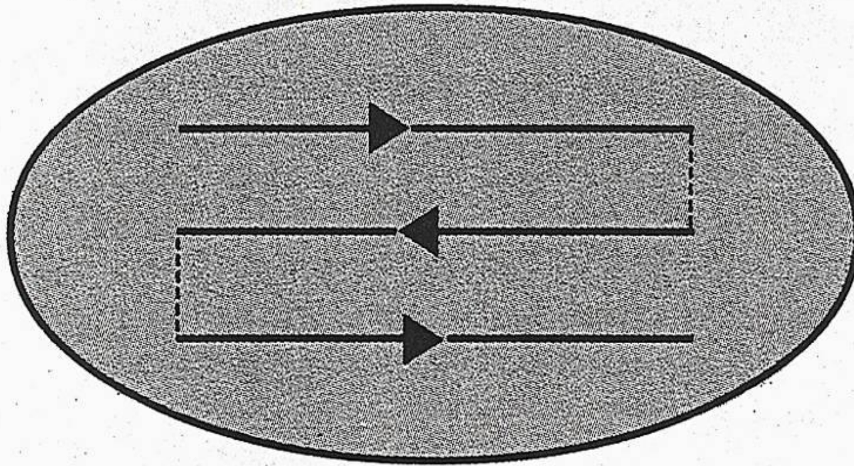
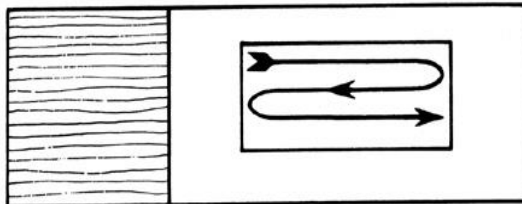

Schematic of an
Incident-Light Fluorescence Microscope
barrier filter
eyepiece
dichroic
mirror
exciter
filter
light
source
objective
specimen

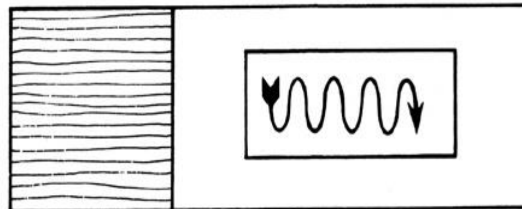
Fluorescence Microscopy

- A fluorescence microscope is required for examining fluorochrome-stained smears:
  - Mercury vapor or halogen bulb light source (about 150 hours of use)
  - Newer mercury bulbs (about 2,000 hours of use)
  - LED Bulbs (about 15,000 hours of use)
  - Excitation and emission (barrier) filters are necessary for visualization of the fluorescently-stained smear (specific to the staining method used. Check package insert)
- LED-based Fluorescent Microscopy
  - LED modules used to adapt light microscopes for reading fluorescently-stained smears
    - May be useful in low income settings
    - More research is needed to evaluate performance

Systematic Examination of Smears







Whichever method you use, BE CONSISTEN!

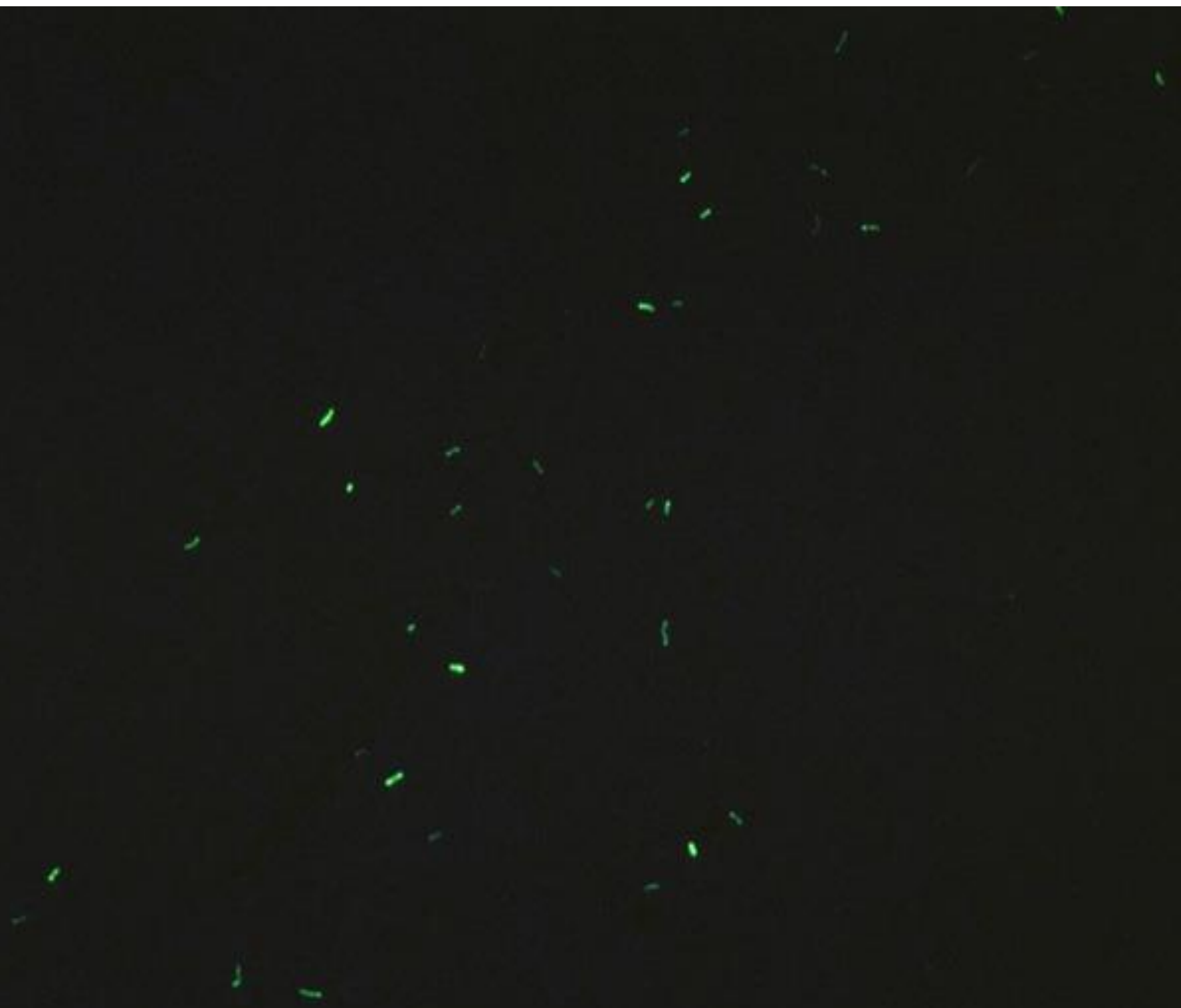
Number of Fields to Examine

Magnification [a]	Number of Fields [b]
200x	30
250x	30
400x	55
450x	70

[a] This final magnification represents the objective lens magnification multiplied by the eyepiece magnification

[b] The minimum number of fields to examine before reporting a smear as negative for acid-fast organisms.

400x



Examining Smears for AFB

- AFB will be rod-shaped, 1–10 µm in length and 0.2–0.6 µm wide
- Appearance is generally long and slender but may also appear bent
- Bacilli may contain heavily stained areas called beads
- Count a clump of bacilli that are touching as one
- Debris, some species of the genera *Nocardia* and *Corynebacterium*, and some fungal spores may appear acid fast

Reporting Smear Results

Fluorescence Microscopy (CDC Scale)			Ziehl-Neelsen Stain (CDC scale)
250X	450X	Report As:	1000X (oil immersion)
0 AFB/ smear	0 AFB/ smear	No AFB seen	0 AFB/ smear
1–2/ 30 fields	1–2/ 70 fields	Report exact count; order repeat specimen	1–2/300 fields
1–9/ 10 fields	2–18/ 50 fields	1+	1–9/ 100 fields
1–9/ field	4–36/ 10 fields	2+	1–9/ 10 fields
10–90/ field	4–36/ field	3+	1–9/ field
>90/ field	>36/ field	4+	>9/ field

CLSI M48

Fluorescence Microscopy		Ziehl-Neelsen Stain		
Number of AFB found at 250x	Number of AFB found at 450x	Number of AFB found at 1000x	Report as:	Or report as:
Negative for AFB	Negative for AFB	0	Negative for AFB	Negative for AFB
Number seen. (Order repeat specimen) **	Number seen. (Order repeat specimen)	1-2 per 300 fields	Number seen. (Order repeat specimen)	Number seen. (Order repeat specimen)
(Number seen/10) per 100 fields	(Number seen/4) per 100 fields	1-9 per 100 fields	1+	Number seen per 100 fields
(Number seen/10) per 10 fields	(Number seen/4) per 10 fields	1-9 per 10 fields	2+	Number seen per 10 fields
(Number seen/10) per field	(Number seen/4) per field	1-9 per field	3+	Number seen per field
>(Number seen/10) per field	>(Number seen/4) per field	>9 per field	4+	>Number seen per field

Manual of Clinical Microbiology.

Acid-fast smear evaluation and reporting[a]

Report	No. of AFB seen by staining method and magnification		
	Fluorochrome stain		Ziehl-Neelsen Stain
	X 250	X 450	X 1000
No AFB seen	0	0	0
Doubtful; repeat	1 – 2/30 F (1 sweep)	1 -2/70 F (1.5 sweeps)	1 – 2/300 F[b] (3 sweeps)[c]
1+	1 – 9/10 F	2 – 18/ 50 F	1 – 9/100 F
2+	1 – 9/F	4 – 36/10 F	1 – 9/10 F
3+	10 – 90/F	4 – 36/F	1 – 9/F
4+	> 90/F	> 36/F	>9/F

[a] Adapted from K/K, 1985

[b] F, microscope fields.

[c] one full sweep refers to scanning the full length (2 cm) of a smear 1 cm wide by 2 cm long.

Reporting smear results

- Use one of the previously recommended or CLSI reporting scales to report and approximation of the number of AFB viewed on the slide using a semi-quantitative scale
- Report smear results within 24 hours of specimen receipt
- Smear positive results are considered critical values and should be reported to the health care provider and public health department as soon as results are known.

Importance of Control Slides

- Assess the quality of the reagents
- Determine if the staining is performed properly
- Determine if the microscope is working properly
- Detect environmental contaminants
- Help find the plane of focus

Quality Assurance of AFB Microscopy

- A known positive and known negative smear should be read with each run and when new reagent used
- QC smears may be prepared in advance, heat-killed, and stored unstained
- Preferable to prepare QC slides using sediment from a clinical specimen
- Records should include stain lot numbers, expiration dates, results of the control slides, and technician name
- Patient smears should only be examined and reported when control slides are acceptable

Monitoring Performance for Smear Status and Smear Sensitivity*

	Numerator	Denominator	Action Thresholds	Investigative actions	
				Potential causes- increases	Potential causes- decreases
Positive smears	Number of AFB smears reported as positive in one month	Total number of AFB smears performed in one month	Patient population dependent; determine baseline and monitor trends	False positive smears (contaminated reagents, tap water rinse, artifacts, technologist issues)	False negative smears (microscope, centrifuge, or technologist problems, insufficient read time)
Smear positive/culture positive rate (sensitivity)	Number of smear-positive specimens** that are culture positive(1.) for MTBC or for (2) any mycobacteria	1)Total number of cultures positive for MTBC* 2)Total number of cultures positive for all mycobacterial sp.**	National averages: More smear positive specimens are MTBC than NTMs. MTBC smear positivity ranges 30-70% nationally	May be due to false positive smears. Patients on treatment may have positive smears and negative cultures. -Cross contamination -Use of poor quality slides -Use of contaminated or poor quality reagents -Technical errors	False negative smears -microscope, centrifuge or technologist problems -Suboptimal specimens submitted to the laboratory -Inadequate staining and evaluation of slides
Correlation culture positive/ smear positive (specificity)	Number of smear-positive specimens inoculated that were culture positive for MTBC or NTM **	Specimens inoculated for culture that were smear positive in one month**	Should be high percent, 90-98%		May be due to false positive smears (see above, left), or false negative cultures, patients on treatment.

***Suggested frequency of monitoring: High volume: monthly. Low volume or low incidence: bi-monthly or quarterly.**
**** Ideally measured for initial diagnostic specimens only; smears from treated patients may be positive, but yield no growth on culture.**

Suggestions for Avoiding False-Positive AFB Smear Results

Cause	Corrective Action
Old, used microscope slides retain material for previous smear	Use only new slides.
AFB transferred from a positive smear to a negative smear	Use a staining rack and keep slides from touching each other; do not use staining jars.
Food particles	Request another specimen.
Precipitated stains	Use only fresh stains, without precipitates, or contaminating organisms. If any precipitate is observed, filter the stain.
AFB transferred in oil on the objective lens	Always wipe oil from the oil immersion lens after each AFB-positive smear is read.

CLSI M48AE: Laboratory Detection and Identification of Mycobacteria; Approved Guideline

Suggestions for Avoiding False-Negative AFB Smear Results

Cause	Corrective Action
Smears that are too thick, causing material to be washed off during staining	Proper digestion of specimen. Avoid making thick smears.
Smear area is too large, making the smear too thin	Apply smear to a 2-cm^2 area.
Non-staining or poorly staining AFB	Protect smear from UV light, direct sunlight, overheating during smear fixation; store stains in dark bottles; high chlorine content in rinse water affects fluorescence stain.
Incorrect slide warmer temperature	Set temperature at 65-75°C and monitor weekly
Incomplete slide reading	Search smear in uniform manner and read suggested number of fields
Insufficient centrifugation	Ensure centrifugation occurs at 3000 x g for at least 15 minutes

CLSI M48AE: Laboratory Detection and Identification of Mycobacteria; Approved Guideline

Maintaining Proficiency in Microscopic Smear Examination

- Smears should be examined by an experienced microscopist
  - Microscopists should meet a level of competency before being allowed to report smear results.
- Mycobacteriology laboratories should participate in an approved proficiency testing program that includes smear microscopy
- To maintain proficiency, laboratories should process at least 15 AFB smears per week
- Other procifiency testing activities
  - Participate in multiple proficiency testing programs
  - Develop an internal proficiency testing program
  - Establish a QA program

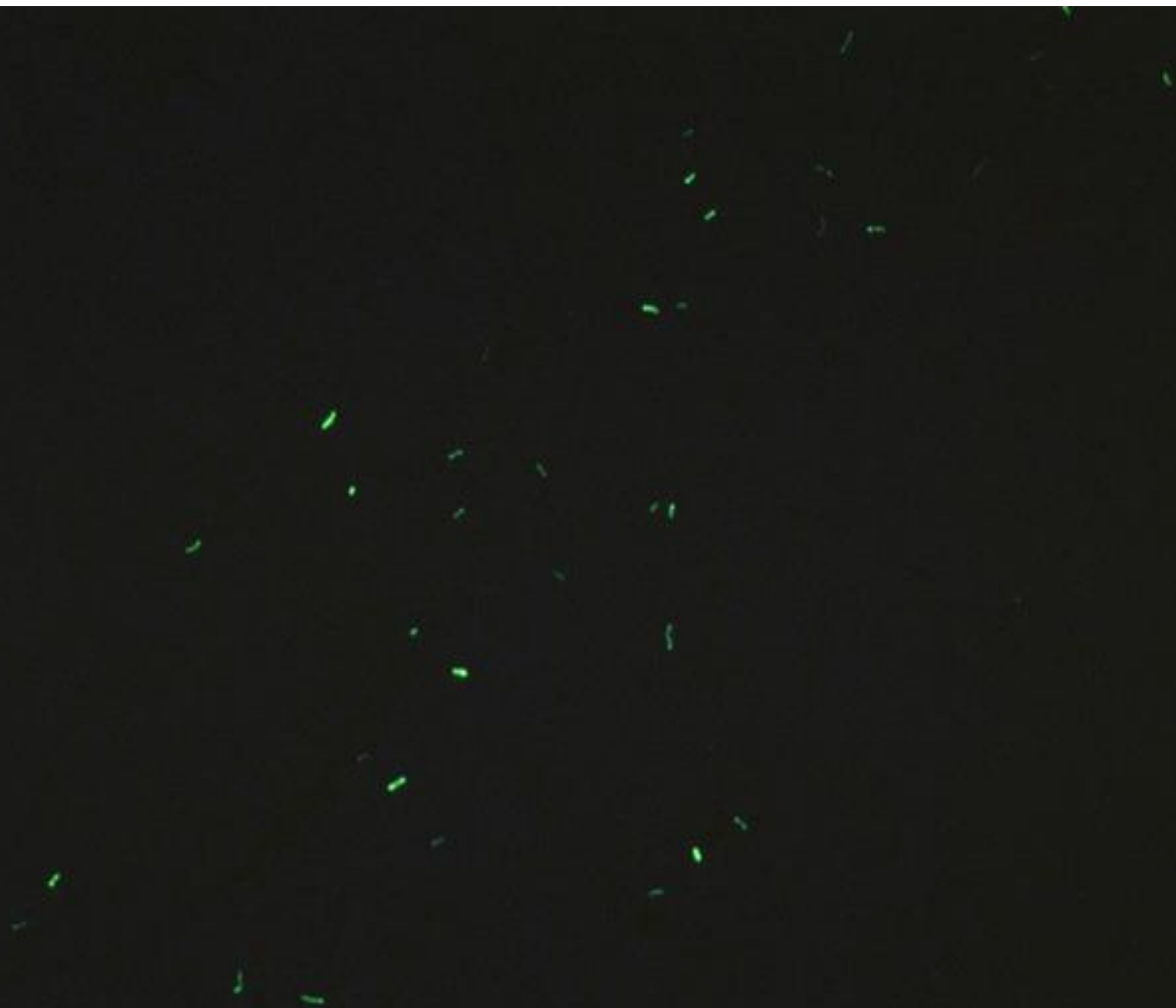
Achieving Reliable Results

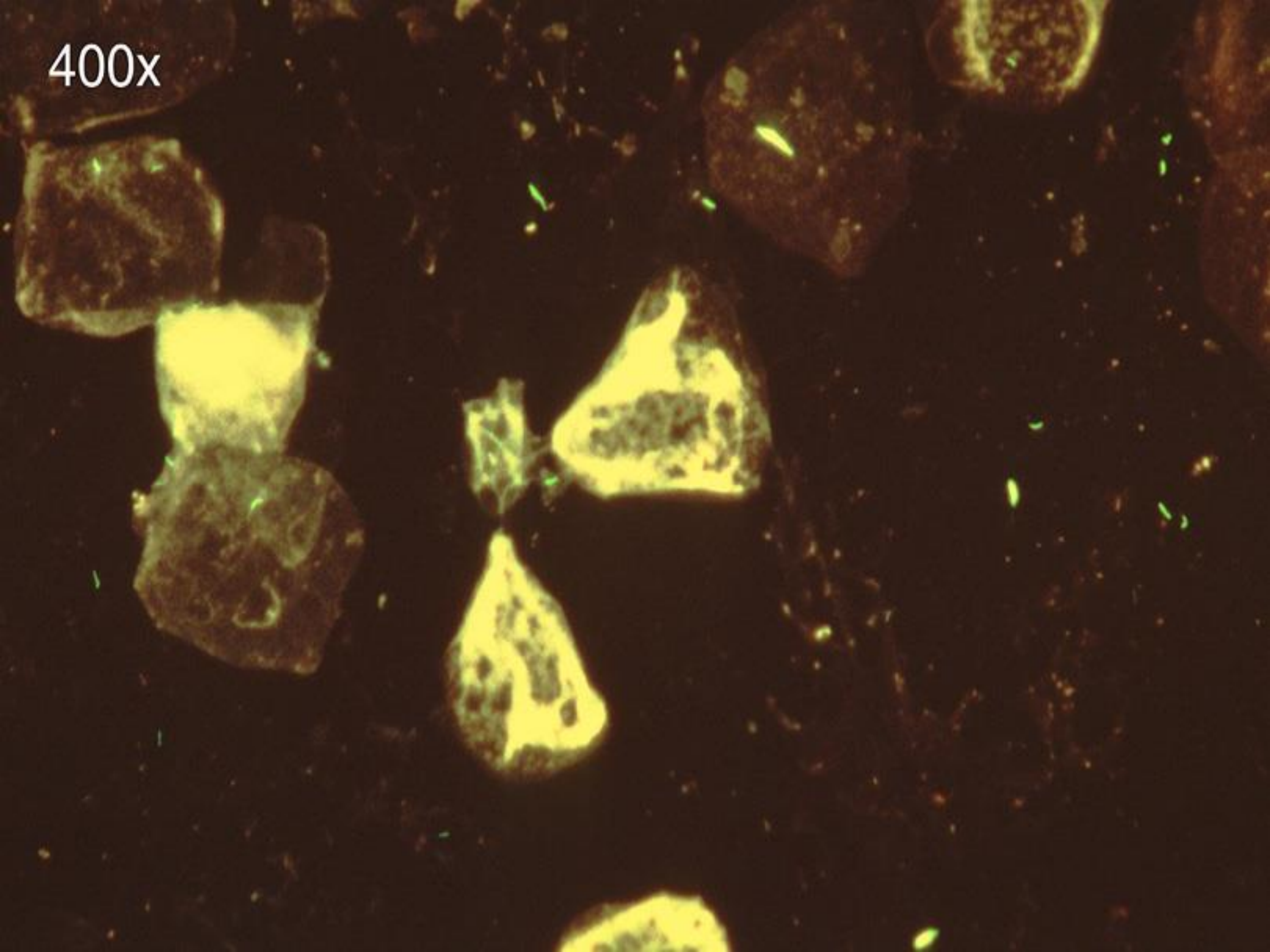
- Obtain good quality specimens is essential
- Prevent contamination of testing reagents and adjacent slides when staining
- Follow established procedures & recommendations
- Ensure accurate reporting and record keeping

AFB Fluorescent Smear Microscopy

Example slides

400x

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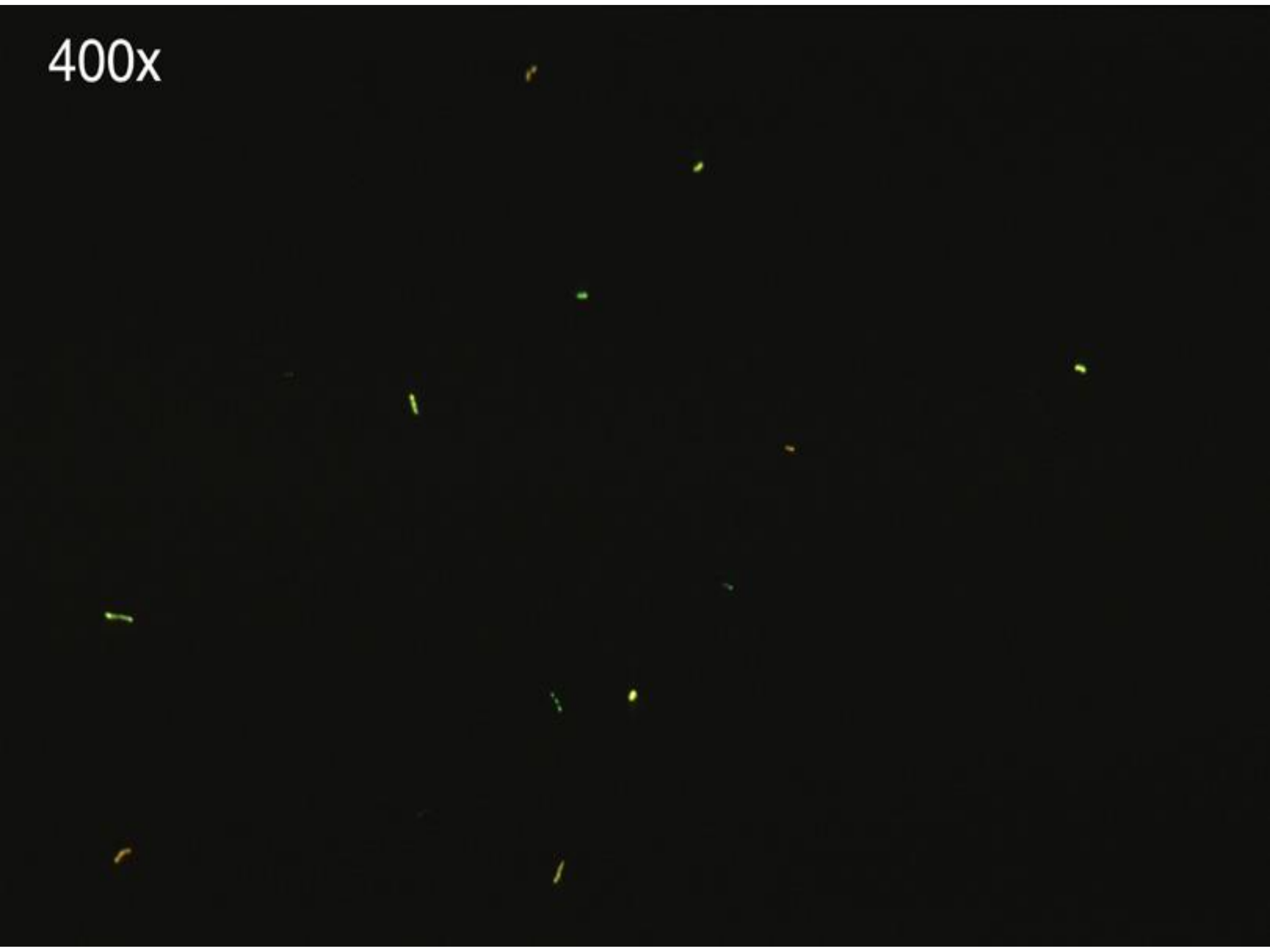
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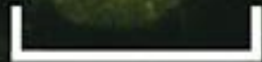
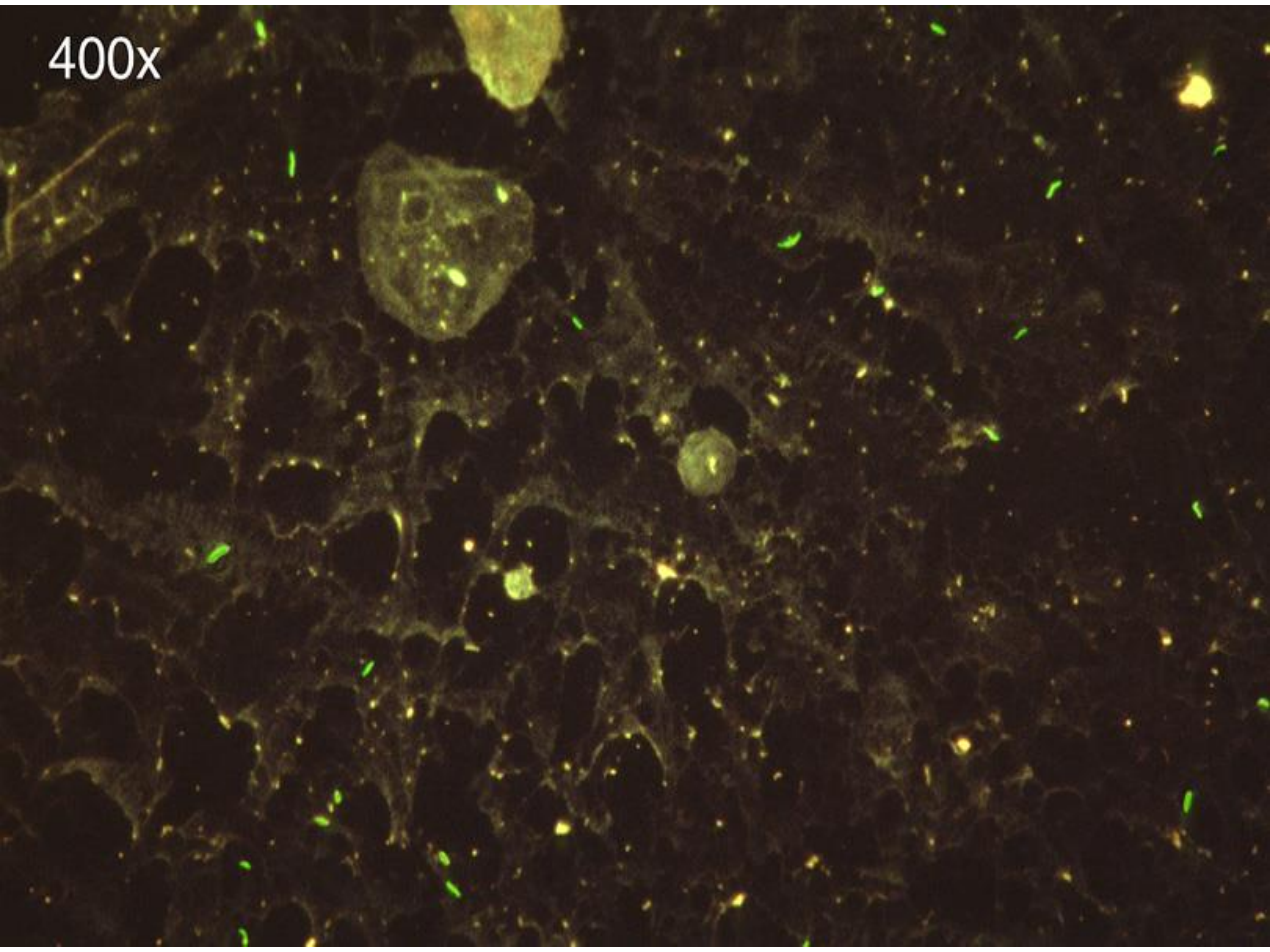

200x

400x

Positive Control - 400x

Negative Control - 400x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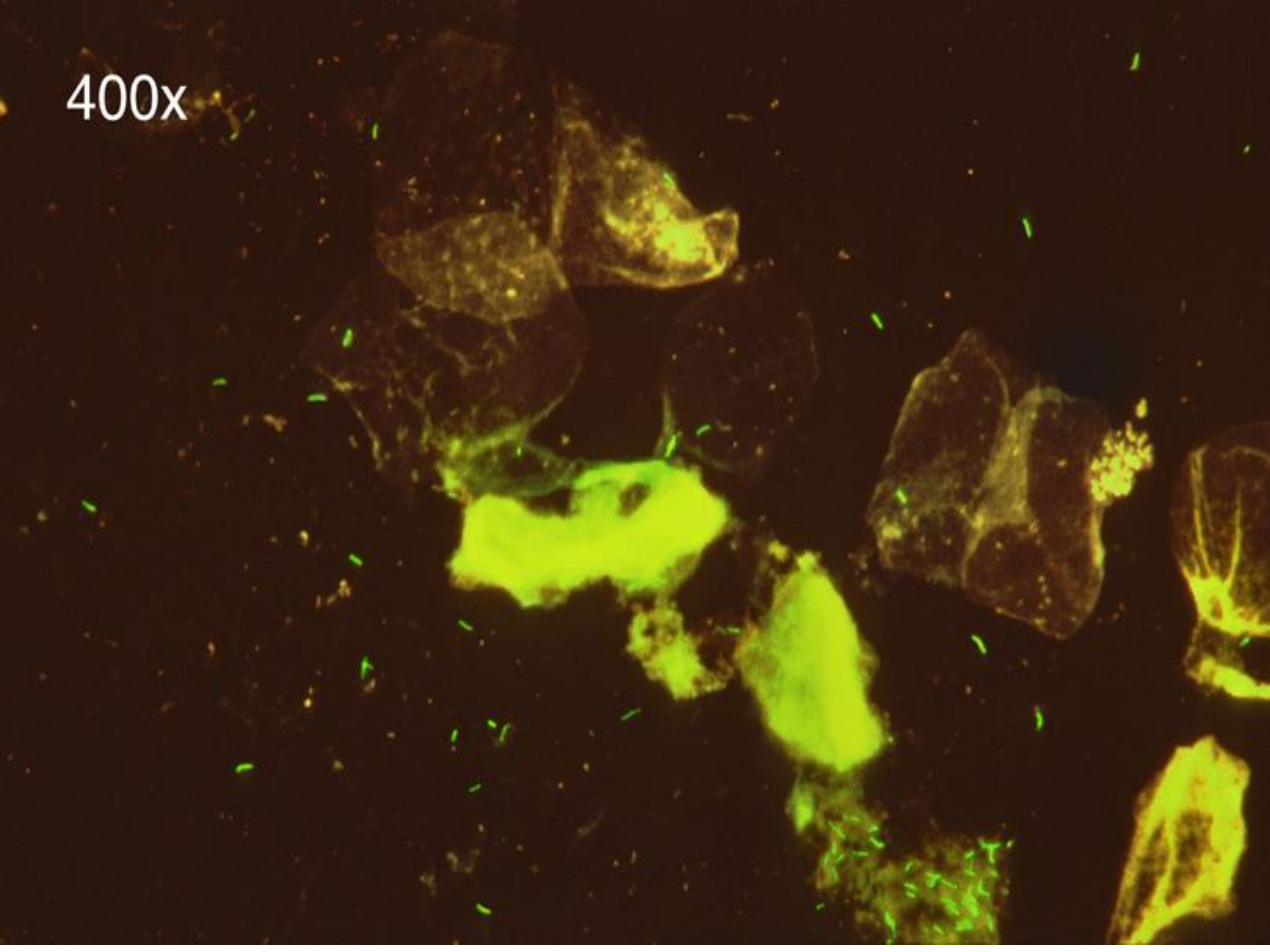
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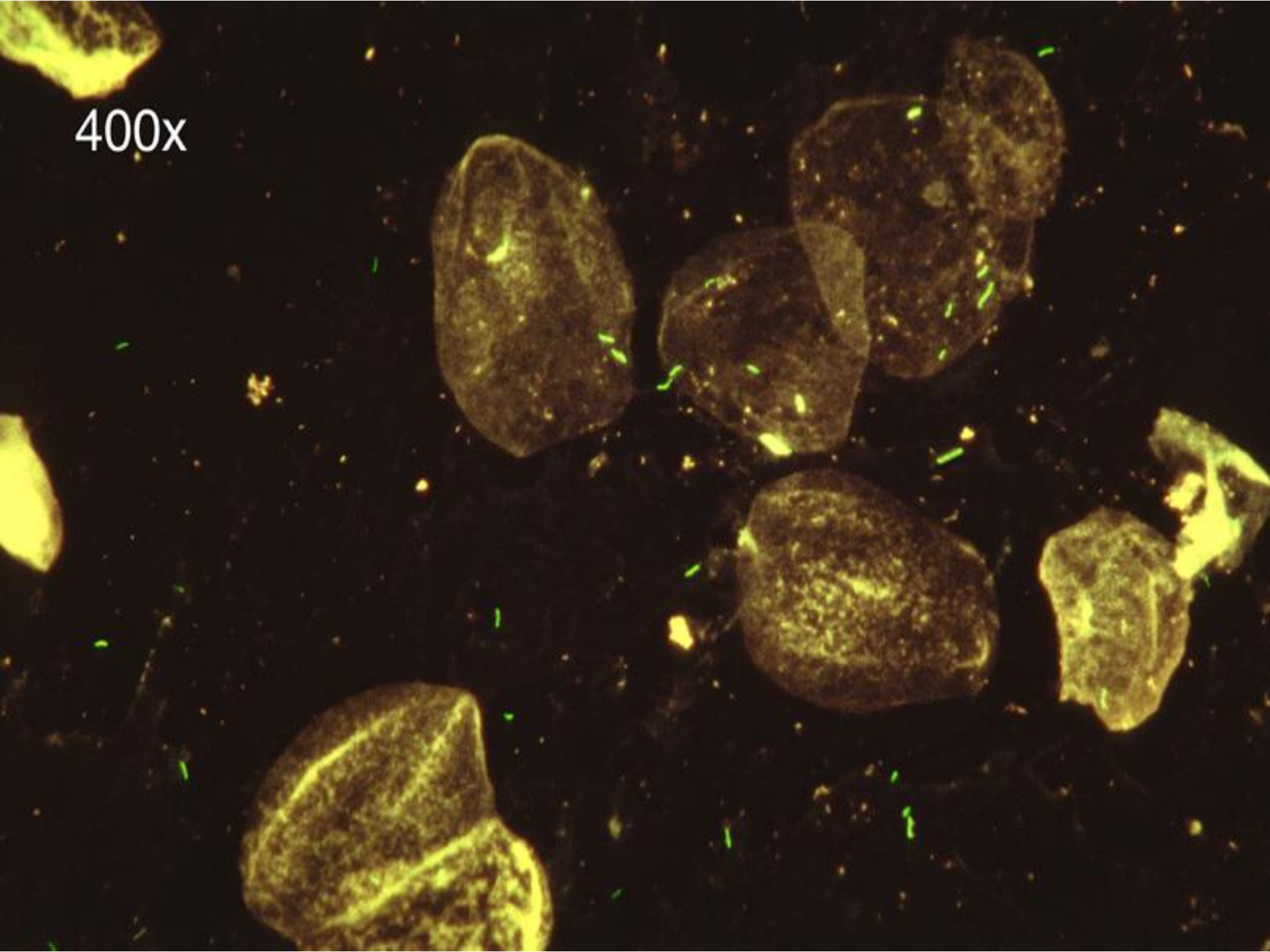
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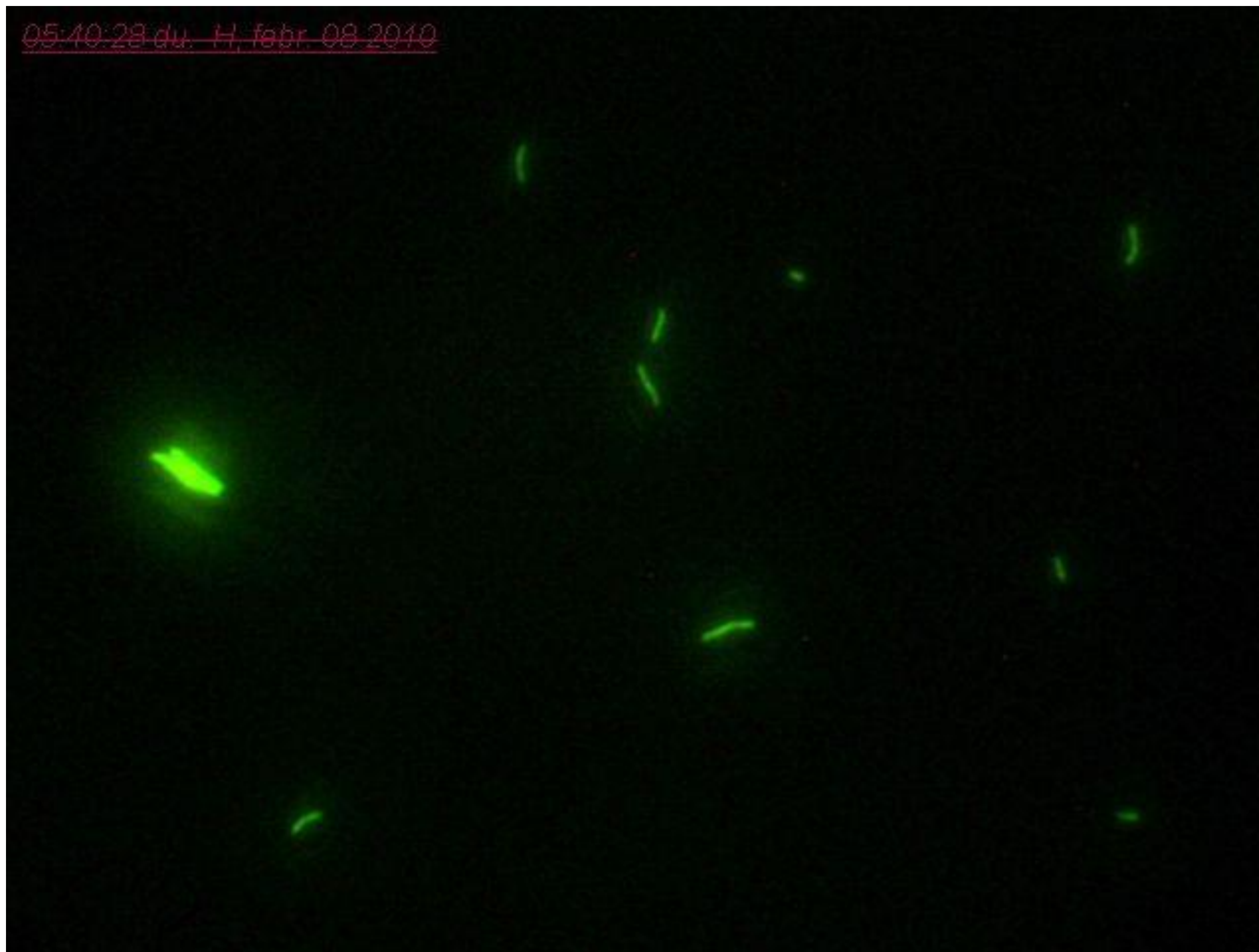
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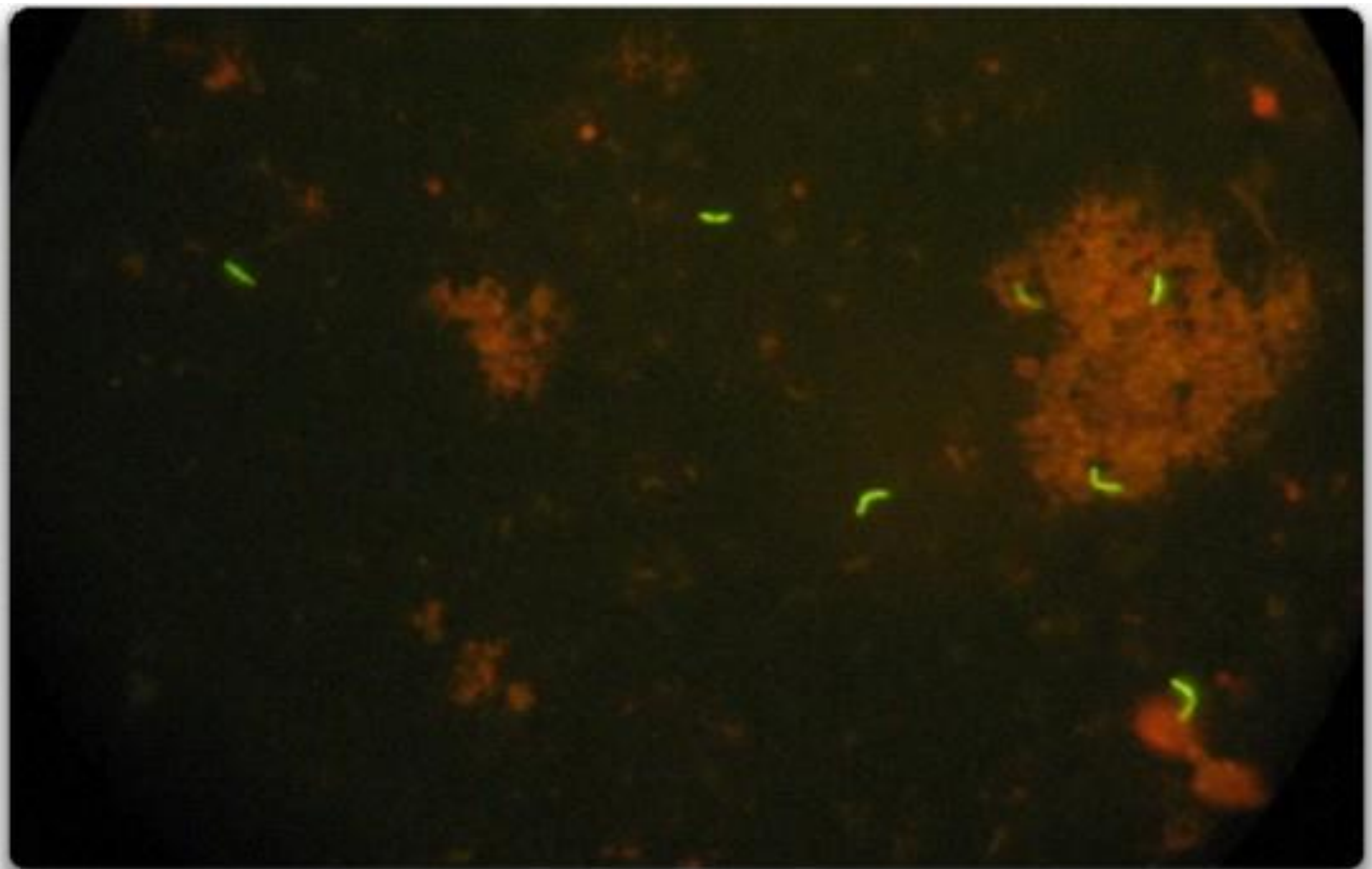

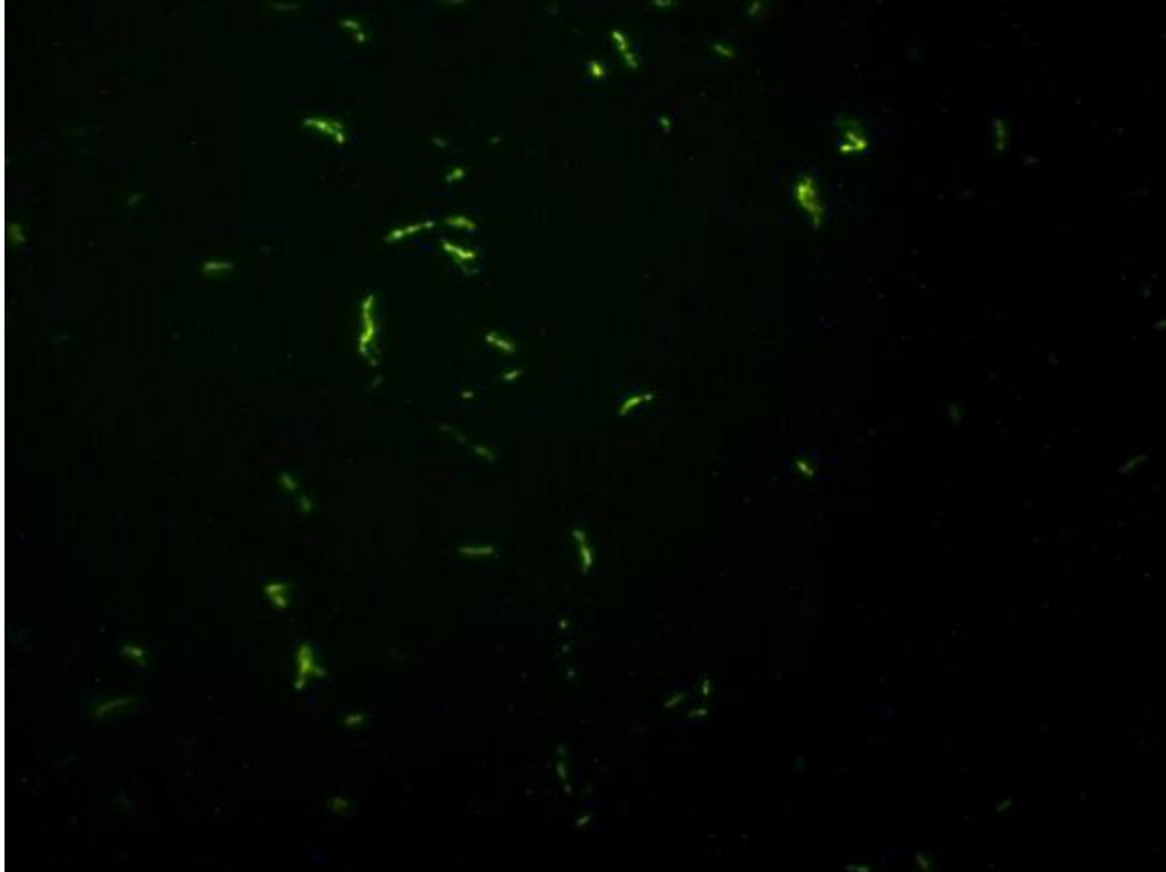

References

- Use of Fluorochrome Staining for Detecting Acid-fast Mycobacteria, CDC
- Manual of Clinical Microbiology, 10th edition
- CLSI M48AE: Laboratory Detection and Identification of Mycobacteria; Approved Guideline
- *Mycobacterium tuberculosis*: Assessing your laboratory (APHL 2009)
- Monitoring the performance of mycobacteriology laboratories: a proposal for standardized indicators; McCarthy, K.D et al.; The International Journal of Tuberculosis and Lung Disease, Volume 12, Number 9, September 2008 , pp. 1015-1020