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

- **Processing**: digestion, decontamination, and/or concentration of a primary patient specimen prior to setting up culture and smear
- **Smear**: 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.

<table>
<thead>
<tr>
<th>First specimen</th>
<th>Second specimen</th>
<th>Third specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>85.8%</td>
<td>11.9%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>


Limitations of AFBMicroscopy

- 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 recommended 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)
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$^2$.
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°C¹
  • 5% phenol in 70% EtOH for 5 min²
    – 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.
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 short 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
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 deionized 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
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 CONSISTENT!
## Number of Fields to Examine

<table>
<thead>
<tr>
<th>Magnification $^a$</th>
<th>Number of Fields $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>200x</td>
<td>30</td>
</tr>
<tr>
<td>250x</td>
<td>30</td>
</tr>
<tr>
<td>400x</td>
<td>55</td>
</tr>
<tr>
<td>450x</td>
<td>70</td>
</tr>
</tbody>
</table>

$a$  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.
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

<table>
<thead>
<tr>
<th>Fluorescence Microscopy (CDC Scale)</th>
<th>Ziehl-Neelsen Stain (CDC scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>250X</strong></td>
<td><strong>1000X (oil immersion)</strong></td>
</tr>
<tr>
<td>0 AFB/ smear</td>
<td>0 AFB/ smear</td>
</tr>
<tr>
<td>1–2/ 30 fields</td>
<td>1–2/300 fields</td>
</tr>
<tr>
<td>1–9/ 10 fields</td>
<td>1–9/ 100 fields</td>
</tr>
<tr>
<td>1–9/ field</td>
<td>1–9/ 10 fields</td>
</tr>
<tr>
<td>10–90/ field</td>
<td>1–9/ field</td>
</tr>
<tr>
<td>&gt;90/ field</td>
<td>&gt;9/ field</td>
</tr>
<tr>
<td><strong>450X</strong></td>
<td></td>
</tr>
<tr>
<td>0 AFB/ smear</td>
<td></td>
</tr>
<tr>
<td>1–2/ 70 fields</td>
<td></td>
</tr>
<tr>
<td>2–18/ 50 fields</td>
<td></td>
</tr>
<tr>
<td>4–36/ 10 fields</td>
<td></td>
</tr>
<tr>
<td>4–36/ field</td>
<td></td>
</tr>
<tr>
<td>&gt;36/ field</td>
<td></td>
</tr>
</tbody>
</table>

Report as:
- No AFB seen
- Report exact count; order repeat specimen
- 1000X (oil immersion)

Order repeat specimen if:
- 1–2/300 fields
- 1–9/ 100 fields
- 1–9/ 10 fields
- 1–9/ field
- >9/ field

For 1000X (oil immersion):
- 1 AFB/ 10 fields
- 1 AFB/ field
### CLSI M48

<table>
<thead>
<tr>
<th>Fluorescence Microscopy</th>
<th>Ziehl-Neelsen Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of AFB found at 250x</strong></td>
<td><strong>Number of AFB found at 1000x</strong></td>
</tr>
<tr>
<td><strong>Number of AFB found at 450x</strong></td>
<td>Report as:</td>
</tr>
<tr>
<td><strong>Negative for AFB</strong></td>
<td><strong>Or report as:</strong></td>
</tr>
<tr>
<td><strong>Number seen. (Order repeat specimen)</strong></td>
<td><strong>Negative for AFB</strong></td>
</tr>
<tr>
<td><strong>Number seen. (Order repeat specimen)</strong></td>
<td><strong>Negative for AFB</strong></td>
</tr>
<tr>
<td><strong>(Number seen/10) per 100 fields</strong></td>
<td><strong>1+</strong></td>
</tr>
<tr>
<td><strong>(Number seen/4) per 100 fields</strong></td>
<td><strong>Number seen per 100 fields</strong></td>
</tr>
<tr>
<td><strong>(Number seen/10) per 10 fields</strong></td>
<td><strong>2+</strong></td>
</tr>
<tr>
<td><strong>(Number seen/4) per 10 fields</strong></td>
<td><strong>Number seen per 10 fields</strong></td>
</tr>
<tr>
<td><strong>(Number seen/10) per field</strong></td>
<td><strong>3+</strong></td>
</tr>
<tr>
<td><strong>(Number seen/4) per field</strong></td>
<td><strong>Number seen per field</strong></td>
</tr>
<tr>
<td><strong>&gt;(Number seen/10) per field</strong></td>
<td><strong>4+</strong></td>
</tr>
<tr>
<td></td>
<td><strong>&gt;Number seen per field 44</strong></td>
</tr>
</tbody>
</table>
## Acid-fast smear evaluation and reporting

<table>
<thead>
<tr>
<th>Report</th>
<th>No. of AFB seen by staining method and magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorochrome stain</td>
</tr>
<tr>
<td>X 250</td>
<td>X 450</td>
</tr>
<tr>
<td>No AFB seen</td>
<td>0</td>
</tr>
<tr>
<td>Doubtful; repeat</td>
<td>1 – 2/30 F (1 sweep)</td>
</tr>
<tr>
<td>1+</td>
<td>1 – 9/10 F</td>
</tr>
<tr>
<td>2+</td>
<td>1 – 9/F</td>
</tr>
<tr>
<td>3+</td>
<td>10 – 90/F</td>
</tr>
<tr>
<td>4+</td>
<td>&gt; 90/F</td>
</tr>
</tbody>
</table>

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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*

<table>
<thead>
<tr>
<th></th>
<th>Numerator</th>
<th>Denominator</th>
<th>Action Thresholds</th>
<th>Investigative actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive smears</strong></td>
<td>Number of AFB smears reported as positive in one month</td>
<td>Total number of AFB smears performed in one month</td>
<td>Patient population dependent; determine baseline and monitor trends</td>
<td>False positive smears (contaminated reagents, tap water rinse, artifacts, technologist issues)</td>
</tr>
<tr>
<td><strong>Smear positive/culture positive rate</strong> (sensitivity)</td>
<td>Number of smear-positive specimens** that are culture positive (1.) for MTBC or for (2) any mycobacteria</td>
<td>1) Total number of cultures positive for MTBC*</td>
<td>National averages: More smear positive specimens are MTBC than NTMs. MTBC smear positivity ranges 30-70% nationally</td>
<td>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</td>
</tr>
</tbody>
</table>
| **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. | ** Ideally measured for initial diagnostic specimens only; smears from treated patients may be positive, but yield no growth on culture.**

* 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

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

<table>
<thead>
<tr>
<th>Cause</th>
<th>Corrective Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smears that are too thick, causing material to be washed off during</td>
<td>Proper digestion of specimen. Avoid making thick smears.</td>
</tr>
<tr>
<td>staining</td>
<td></td>
</tr>
<tr>
<td>Smear area is too large, making the smear too thin</td>
<td>Apply smear to a 2-cm² area.</td>
</tr>
<tr>
<td>Non-staining or poorly staining AFB</td>
<td>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.</td>
</tr>
<tr>
<td>Incorrect slide warmer temperature</td>
<td>Set temperature at 65-75°C and monitor weekly</td>
</tr>
<tr>
<td>Incomplete slide reading</td>
<td>Search smear in uniform manner and read suggested number of fields</td>
</tr>
<tr>
<td>Insufficient centrifugation</td>
<td>Ensure centrifugation occurs at 3000 x g for at least 15 minutes</td>
</tr>
</tbody>
</table>
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 proficiency 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
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