Basics of Mycology

I. Introduction

A. **Mycology** is the study of fungi

B. **Mycoses** are fungal diseases
   1. Superficial & Cutaneous mycoses
      a. Involves only hair, skin and nails
      b. Little or no pathology; main worry is cosmetic effect
      c. Involves destruction of the keratin of hair, skin and nails
      d. Primarily caused by the Dermatophytes
   2. Subcutaneous mycoses
      a. Involves skin, muscle and connective tissue immediately below the skin
   3. Systemic (deep-seated) / disseminated mycoses
      a. Caused by pathogenic fungi that are highly virulent
      b. Involves the deep tissues and internal organs, and has the ability to spread widely throughout the body
      c. Frequently initial site of infection is the lung
   4. Opportunistic mycoses
      a. Caused by ubiquitous saprophytic ("non-pathogenic") fungi and occasionally pathogenic fungi, all of which have limited virulence
      b. Usually see in immunocompromised or debilitated patients
      c. Causes subcutaneous and disseminated infections

C. Patients at risk for fungal infections
   1. Immunosuppressed individuals are at highest risk (i.e. AIDS, decreased PMNs)
   2. Organ transplant patients and others with previous treatment with corticosteroids, cytotoxic agents, or prolonged antibiotic therapy
   3. Patients with malignant neoplasms
   4. Patients with various debilitating immunologic and metabolic disorders (i.e. SLE, diabetes)
   5. Occupations & activities involving direct skin contact with infected animals/materials and ingestion or inhalation of contaminated aerosols/dust

D. Natural habitat is soil and vegetation

E. Taxonomy / Classification
   1. By disease
   2. By class

II. Characteristics of Fungi

A. Eukaryotic
   1. Has a nucleus, nuclear membrane, endoplasmic reticulum, Golgi apparatus, and mitochondria
   2. Rigid cell wall containing **chitin**, mannans, and sometimes cellulose
   3. Lacks chlorophyll

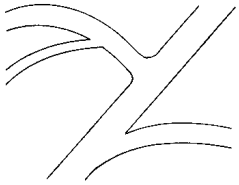
B. Growth Requirements
   1. Nutrients – must absorb from environment since lack chlorophyll
   2. pH – prefer neutral but tolerate wide range
   3. Temperature – optimal growth at room temperature to 30°C, 37°C for dimorphic yeast
   4. Oxygen – most are obligate aerobes
   5. Moisture needed to grow, able to survive dry conditions with spores/conidia

C. Forms
   1. Mould / Mold
      a. Colony – growth of **hyphae** which form a matt of growth called the **mycelium**
      b. Cells – multiple cells forming a filamentous mycelium
      c. Reproduce either asexually (vegetative sporulation or aerial sporulation) or sexually (sexual sporulation)

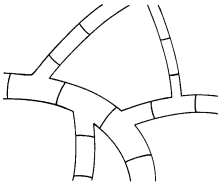
   2. Yeast
      a. Colony – bacteria-like, moist, smooth, creamy colonies
      b. Cells – single, round to oval cells
      c. Reproduce asexually by budding to form **blastoconidia**
         - **Pseudohyphae** – elongation of blastoconidia showing sausage-like constrictions between segments (true hyphae are not constricted at ends)

   3. Dimorphism
      a. Fungi that have the ability to exist in two forms depending on growth conditions
      b. Generally dimorphic fungi have a mould phase and either a yeast or spherule phase
         - Yeast /tissue phase – grows best at 37°C
         - Mould phase – grows best at room temperature or 30°C

D. Structures
   1. **Hyphae**
      a. Long strand of tube-like structures
      b. Types
         - **Aseptate** (or sparsely septate) – without (or very few) transverse walls



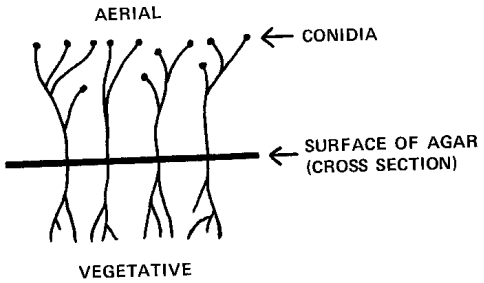
         - **Septate** – subdivided into individual cells by transverse walls



      c. Pigmentation
         - **Hyaline** – Light colored hyphae and/or conidia (fungi with septate hyphae) due to no pigmentation or brightly pigmented
         - **Dematiaceous** – Dark colored (brown-black) hyphae and conidia (fungi with septate hyphae) due to presence of melanin in cell wall

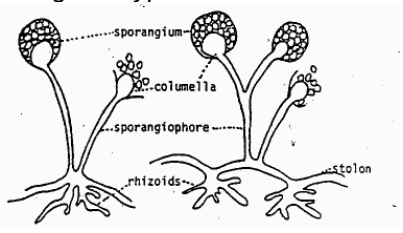
   2. **Mycelium**
      a. Mass of branching intertwined hyphae forming a matt of growth

      b. Types
         - **Aerial mycelium** (also called reproductive mycelium)
            - Portion of mycelium that projects above the agar surface
            - Special **spore** or **conidia**-bearing fruiting bodies derive from this portion
         - **Vegetative mycelium**
            - Extends into substratum of agar and is responsible for absorbing water and nutrients



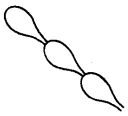
- Structures

Rhizoids – root-like structures that may be located at the base of a sporangiophore or internodally along the hyphae.

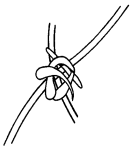


Favic chandeliers – resemble antlers of a deer, ends are blunt and branched

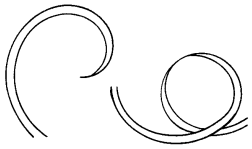
Racquet hyphae – resemble tennis racquets with smaller end attached to large end of an adjacent club-shaped hyphae



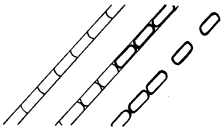
Nodular organs – knots of twisted hyphae



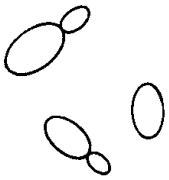
Spiral hyphae – coiled or corkscrew-like turns in hyphae



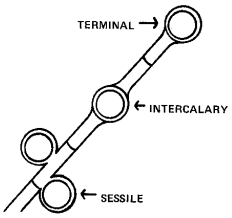
3. Vegetative reproductive structures
   - **Arthroconidia** (arthrospores)
     - Thick-walled barrel-shaped conidia produced by fragmentation of the hyphal strand through the septation points. They may form adjacent to each other or may be separated by alternating empty spaces.



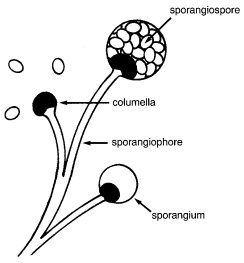
   - **Blastoconidia** (daughter cells)
     - Budding forms characteristically produced by yeast
     - A bud scar (dysjunctor) often remains at point where conidium detached



   - **Chlamydoconidia** (Chlamydospores)
     - Formed from pre-existing cells in the hyphae, which become thickened and enlarged
     - May be found within (**intercalary**), along the side (**sessile**), or at the tip (**terminal**)

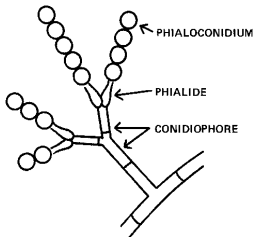


4. Aerial reproductive structures (fruiting bodies)
   - **Sporangiospores**
     - Spores contained in a closed sac called a **sporangium**
     - The sporangium is supported on a base, termed the **columella**, which is located at the tip of the specialized hyphal segment called the **sporangiophore**
     - This type of sporulation is characteristic of the *Zygomycetes*

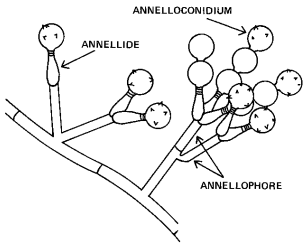


   - **Conidia**
     - Spores produced on the surface of an elaborate fruiting body supported by a specialized hyphal segment called a **conidiophore**
     - The conidiophore can branch into secondary segments called **metulae** which can become conidia-producing segments called **phialides**

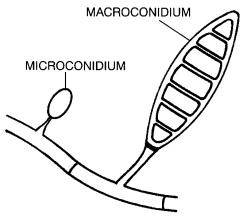
- **Phialoconidia**
  - Conidia which arise from a tube or vase-shaped structure called a **phialide**
  - This type of sporulation is characteristic of the *Penicillium* species



- **Annelloconidia**
  - Conidia which arise from a tube or vase-shaped structure termed a **annellide**
  - The tip of the phialide cyclically extends and retracts when conidia form, leaving a succession of scars
  - Conidia may be formed singly, in long chains, or in tightly bound clusters

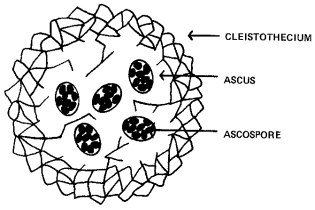


- **Macroconidia**
  - Larger, multi-celled conidia that can vary in size and shape
  - The term “macro” should only be used when smaller conidia are present.
- **Microconidia**
  - Tiny one-celled conidia, usually borne either directly from side of hyphae or supported by a hair-like conidiophore
  - The term “micro” is used only when larger conidia are present

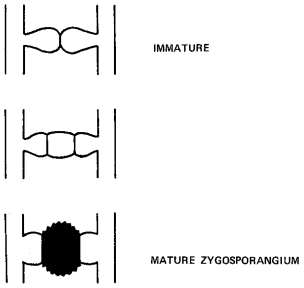


5. Sexual reproductive structures
   a. Fungi having a sexual stage are termed “Perfect Fungi”
   b. Fungi lacking a sexual stage are termed “Fungi Imperfecti”
   c. Sexual sporulation requires 2 specialized fertile cells (having undergone meiosis) to merge and have nuclear recombination occur on the aerial hyphae

- Ascospores
  - Sexual spores (meiotic division) produced in a sac-like structure called an ascus. The **ascus** is the sexual mother cell that forms ascospores inside and may be protected on the outside by an cleistothecium. The **cleistothecium** is a protective sac within which asci and ascospores form.



- Oospores
  - Fusion of 2 morphologically identical cells from different hyphal segments
- Zygospores
  - Fusion of 2 morphologically identical cells from the same hyphal structure
  - *Zygomycetes* reproduce sexually in this manner



III. Specimen Collection & Transport

A. Specimen types and collection
   1. Blood and bone marrow
      a. Acquire by sterile technique
      b. Inoculate biphasic agar-broth bottles designed specifically for fungal cultures

2. CSF
    a. Acquire by sterile technique and transport in sterile container
    b. Centrifuge and use sediment to make slides and inoculate media
    c. Keep at room temperature is culture setup is delayed
3. Cutaneous: Hair, nail and skin scrapings
    a. Hair
        - Use Wood's lamp to see infected areas. Pull out hair by roots with sterile tweezers.
        - If no fluorescence, pull out hairs that are broken and scaly
        - Transport in sterile container
    b. Nails
        - Clean with 70% alcohol
        - Scrape away and dispose of outer layers of nail
        - Sample from beneath the nail plate to obtain softened material from nail bed, or collect shavings from deeper portions
        - Place into sterile container
    c. Skin
        - Clean with 70% alcohol
        - If lesion present, scrape the actively growing edge
        - Scrape areas that look most infected
4. Respiratory: bronchial washings, sputum, throat, transtracheal aspirates
    a. Early morning specimen is best
    b. 24 hour collections unacceptable (bacterial overgrowth)
    c. Transtracheal aspirate should eliminate throat flora
    d. Prepare slides for stains
5. Tissue biopsies
    a. Collected by physician and should be kept moist with sterile saline in a sterile container until processed
    b. Should include normal tissue and tissue from the center and edge of lesion
    c. Inspect tissue for granules and areas of pus and necrosis
    d. Mince tissues for inoculation to media especially if *Zygomycetes* are suspected
6. Urine
    a. Early morning clean catch or catheterized specimen is best
    b. 24 hour collections unacceptable (bacterial overgrowth)
    c. Centrifuge specimen and inoculate media with sediment
7. Vaginal, uterine cervix, prostatic secretions
    a. Acquire by sterile technique and transport in sterile container
8. Wounds, subcutaneous lesions, mucocutaneous lesions, exudates
    a. Acquire by sterile technique and transport in sterile container
    b. From cysts and abscesses, material should be aspirated if possible
    c. Examine for granules

B. Specimen Collection Issues
1. Collect from area most likely to be affected
2. Use sterile technique, avoid contamination with hands
3. Specimen must be adequate, reduces contamination
4. Keep specimen moist
5. Specimen must be properly labeled
6. Exact source/site aids in identification
7. Specimen must be delivered promptly to lab and processed quickly
    a. Prevents overgrowth of bacteria and ubiquitous molds
    b. Pathogenic molds can be slow growers
    c. Yeast multiply quickly so refrigerate if delay in setting up culture

IV. Direct Examination of Specimens

- Provides rapid preliminary report and immediate presumptive diagnosis to guide the physician in treatment
- Special morphological characteristics may give clues to the identity of the causative agent
- Aids in selection of special media to inoculate specimen to
- Direct exam may show evidence of infection despite negative cultures
- Allows for observation of yeast phase of dimorphic organisms
- May indicate need for more than one type of direct examination to be performed

A. Saline wet mount
   1. Phase-contrast microscope is valuable or can use low light
   2. Look for fungal elements such as:
      a. Budding yeast with pseudohyphae (*Candida*)
      b. Broad base budding yeast (*Blastomyces dermatitidis*)
      c. Spherules (*Coccidioides immitis*)
      d. Capsules (*Cryptococcus neoformans*)

B. Lactophenol aniline blue (LPAB) wet mount
   1. Phenol kills organisms
   2. Lactic acid preserves fungal structures
   3. Aniline blue stains the chitin in fungal cell walls
   4. LPAB prep can be made permanent
   5. Look for fungal elements

C. Potassium hydroxide (KOH) preparation (10%)
   1. Hair, skin or nail specimens
      - KOH dissolves the keratin to make fungi more visible
   2. Specimens containing cellular material such as sputum or vaginal secretions
      - KOH dissolves the cells in background to make yeast / fungal elements more visible
   3. Procedure
      a. Add a drop of 10% KOH to specimen on slide. Coverslip.
         - Gentle heating may aid in dissolving debris
         - If specimen is thick, it may take 15-30 minutes to dissolve
      b. Observe under low light or with phase-contrast microscope

D. Gram stain
   1. Fungi stain gram positive
   2. Look for yeast and fungal elements such as pseudohyphae
   3. True fungi are 2-3 times wider than GPR's and will not stain solidly inside
   4. Capsule around yeast can prevent the definitive staining of the yeast itself

E. Acid-fast stain
   1. *Nocardia* is partially positive with a modified Kinyoun acid-fast stain
   2. Ascospores of *Saccharomyces cerevisiae* are acid-fast positive

F. India ink preparation
   1. Used to observe for capsules around yeast (esp. *Cryptococcus neoformans*)
   2. Procedure
      a. Mix small drop of India ink with a drop of specimen and coverslip. (Strive for a thin smear)
      b. Let sit (up to 10 minutes) to allow cells to settle
      c. Observe under microscope with condenser adjusted for maximum light. Look for a clear capsule around yeast. Background is dark.

G. Calcofluor white stain
    1. Binds to polysaccharides in fungal cell walls
    2. Fluoresces when exposed to UV light
    3. 10% KOH can be added to dissolve background
    4. Procedure
        a. Add drop of Calcofluor White stain to specimen on slide. Coverslip.
        b. Allow to sit approximately 3 minutes.
        c. Use a fluorescent microscope and look for apple green fluorescence.

H. Tissue / Histological stains
    1. Wright's stain – look for intracellular yeast in tissue and bone marrow (*Histoplasma capsulatum*)
    2. Gomori Methenamine Silver (GMS) stain – fungi, *Pneumocystis*, and *Actinomyces* stain black against a green background
    3. Periodic Acid Schiff (PAS) stain – fungal elements are magenta against a light pink or green background

V. Selection and Inoculation of Culture Media

A. Safety
    1. Tube media preferred over plate media
        a. Tube media will not dry out over long incubation periods
        b. Reduces chance for fungal reproductive structure to become airborne and contaminate the room and people
        c. Never use plates when suspect *Coccidioides immitis* (extremely infectious and aerosols may be inhaled)
    2. ALWAYS work under a biological safety cabinet
    3. Wear gloves and lab coat
    4. Autoclave specimens and inoculated media when finished
    5. Disinfect work area daily

B. Primary isolation media for fungi
    1. Goal is to isolate all possible pathogens
    2. Generally want 2 types of media – a **nonselective** media and a **selective** media (with antibiotics to inhibit growth of bacteria and enriched for more fastidious fungi)
    3. Ingredients required for fungal growth include carbon, nitrogen, vitamins, minerals and amino acids
    4. **Nonselective Media**
        a. Brain heart infusion (BHI) with/without 5% blood
            - Primary recovery of saprophytic and dimorphic fungi
            - Useful for isolation of *Histoplasma* and *Nocardia* (media containing blood)
            - Useful to convert dimorphic molds from mold to yeast phase when incubated at 35ºC
            - Antibiotics (cycloheximide & chloramphenicol) can be added to make media selective for dimorphic moulds

        b. Inhibitory mold agar (IMA)
            - The best medium to isolate fungal opportunists from a non-sterile site
            - Primary recovery of dimorphic pathogenic fungi and saprophytic fungi that are inhibited by cycloheximide
            - Chloramphenicol & gentamicin inhibit growth of bacteria

        c. Sabouraud's brain heart infusion agar (SABHI)
            - Primary recovery of saprophytic and dimorphic pathogenic fungi, particularly fastidious strains

5. **Selective Media**
    a. Mycosel agar
        - Selective for isolation of dermatophytes
        - Chloramphenicol inhibits bacteria and *Nocardia*
        - Cycloheximide inhibits rapid saprophytes and:
            - *Cryptococcus neoformans*
            - *Candida krusei*
            - *Candida tropicalis*
            - *Candida parapsilosis*
            - *Trichosporon beigelii* (cutaneous)
            - *Aspergillus fumigatus* (25-60%)
            - *Pseudallescheria boydii*
            - *Nocardia asteroids*
            - *Piedraia hortae*

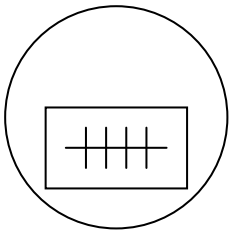
    b. Dermatophyte test medium (DTM)
        - Screening media for dermatophytes
        - pH change causes the phenol red indicator to change from yellow to red
        - Contains antibiotics

6. Incubation temperature
    a. 30ºC is best (room temperature = 25ºC, is acceptable, some fungi may multiply slower at this temperature)
    b. 37ºC may inhibit some fungi, but necessary for yeast phase of dimorphic fungi

7. Incubation time
    a. Hold cultures for 4-6 weeks, examining twice weekly for growth
    b. Dependent on media, temperature and inhibitors in the specimen

8. Incubation atmosphere
    a. Moist – 40-50% relative humidity
    b. Ambient air

C. Subculture and special identification media for fungi
1. Once fungi have grown on primary culture, one frequently needs to subculture for complete isolation and identification
2. Media
    a. Sabouraud dextrose agar (SDA)
        - Supports growth of all fungi (except *Histoplasma* and *Nocardia*)
        - Consists of dextrose, peptone, agar and water
        - pH 5.6 to inhibit bacteria which prefer pH 7.2
    b. Neutral Sabouraud dextrose agar (Emmon's modification)
        - Subculture yeast, allows for better maintenance of yeasts
        - Less dextrose and a neutral pH compared to regular SDA
    c. Cornmeal-Tween 80 agar (CMT)
        - Promotes hyphal and blastoconidia formation
        - Observe pseudohyphae & chlamydoconidia production by *Candida albicans*
        - Enhances pigment of *Trichophyton rubrum* when 1% glucose is added
        - Procedure
            - With loop, make one streak into the agar down the center of an area and 3 or 4 parallel cuts across the first ½ inch or 1 cm apart, holding the inoculating wire at about a 45º angle to dilute inoculum.
            - Incubate 24-72 hours at 30ºC
            - After incubation, place coverslip on surface of the agar, covering inoculation streaks
            - Examine growth through the coverslip with the



microscope using the 10x and 40x objectives. Look for the most characteristic morphology near the outer edges of the coverslip.

d. Niger seed agar or Birdseed agar
   - Used for isolation of *Cryptococcus neoformans* from contaminated specimens
   - *Cryptococcus neoformans* produces phenoloxidase enzymes. These enzymes break down the substrate caffeic acid forming a brown pigment

e. Tween 80 / Oxgall / caffeic acid agar (TOC)
   - Observe brown pigment production by *Cryptococcus neoformans*
   - Can observe germ tube production by *Candida albicans*
   - Better chlamydoconidia development than Cornmeal/Tween 80

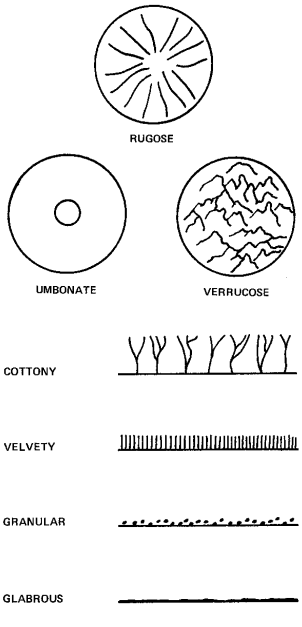
f. Potato dextrose agar
   - Stimulates spore formation and pigmentation
   - Used to subculture fungi for slide culture and observe for colony morphology

VI. Examining the Fungal Culture

A. Differentiating Pathogenic fungi
   1. Growth rate is 10 days or more (slow growers)
   2. Growth on Mycosel agar
   3. Color: dull buff, brown, mousey gray
   4. Dimorphic
      a. Mold phase grows at 30°C (room temperature)
      b. Yeast phase grows at 35°C on BHI agar

B. Identification of fungi
   1. Growth rate
      a. Rapid = 1-5 days
      b. Intermediate = 6-10 days
      c. Slow = 11-28 days

   2. Colonial morphologic features
      a. Appearance (topography)
         - **Rugose** – colonies have deep furrows irregularly radiating from the center
         - **Umbonate** – colonies have a button-like central elevation
         - **Verrucose** – colonies have a wrinkled, convoluted surface
         - **Flat**
      b. Texture
         - **Cottony** (wooly) – very high, dense aerial mycelium
         - **Glabrous** (waxy) – smooth surface due to no aerial mycelium (yeast-like)
         - **Granular** (powdery) – flat and crumbly due to dense conidia production
         - **Velvety** – colonies produce low aerial mycelium
      c. Pigmentation
         - Observe color on both **surface** of colony and on **reverse** side of plate



   3. Microscopic morphologic features
      a. Most definitive means of identification
      b. Evaluate:
         - Shape
         - Method of production

- Arrangement of conidia/spores
- Size and color of hyphae

4. Microscopic techniques for evaluating fungi
   a. Tease mount
      - Procedure
        - Using two sterile teasing needles, transfer a portion of colony (middle third) to a slide
        - Gently tease mycelium apart with teasing needles
        - Add a drop of Lactophenol aniline blue stain
        - Coverslip and observe for fruiting structures under light microscope at 10x and 40x
      - Advantage
        - Perform and examine immediately after maturation
      - Disadvantage
        - Structural morphology is disturbed

   b. Scotch tape preparation
      - Procedure
        - Lightly touch transparent scotch tape, sticky side down, to surface of colony and then removing it
        - Place a drop of Lactophenol aniline blue stain onto a slide
        - Affix tape, sticky side down, into the stain on the slide
        - Observe for fruiting structures under light microscope at 10x and 40x
      - Advantages
        - Perform and examine immediately after maturation
        - Retains juxtaposition of spores and hyphal elements
      - Disadvantages
        - Prep is not easily preserved (view within 30 minutes and then discard slide)
        - Contamination can occur

   c. Slide culture
      - Procedure
        - Place glass slide on 2 wooden sticks in Petri dish (gauze or paper towel under sticks moistened with sterile water)
        - Using sterile scalpel, cut 1 cm x 1 cm square of SAB or Potato dextrose agar and place on slide. Two pieces of agar can be placed on the slide to provide duplicate cultures.
        - Inoculate the 4 sides of the agar with mould using teasing needles or sterile wooden stick
        - Place coverslip on top of agar
        - Tape plate shut and incubate at room temperature (22°C)
        - Examine for growth periodically & add more water as needed to keep moist
        - When conidia / spores are evident, carefully lift coverslip off agar using forceps and place onto slide containing a drop of lactophenol aniline blue stain (coverslip can be sealed with fingernail polish to keep slide permanently)
        - Observe under light microscope at 10x and 40x
      - Advantages
        - Fungal elements are grown and maintained in their original juxtaposition, making identification easier
        - Two mounts from one culture, so you can view one slide and if necessary, leave the other slide to incubate longer

- Disadvantages
  - Technical expertise required
  - Must wait for fungus to mature on inoculated media before identification can occur
  - Zygomycetes grow past coverslip before forming reproductive structures

VII. Serologic Diagnosis of Fungal Disease

- Generally performed only in select reference laboratories

A. Immunodiffusion
   1. *Aspergillus*
   2. *Blastomyces*
   3. *Histoplasmosis*

B. Complement fixation
   1. *Blastomyces*
   2. *Coccidioidomycosis*
   3. *Histoplasmosis*

C. ELISA
   1. *Aspergillus*

D. EIA
   1. *Blastomyces*
   2. *Candida*

E. Latex agglutination
   1. *Cryptococcus* (more sensitive than India Ink Stain in CSF)
   2. *Candida*

F. Fluorescent antibody
   1. *Pneumocystis*

VIII. Molecular Diagnosis of Fungal Disease

A. Probes
   1. Used to identify:
      a. *Histoplasma capsulatum*
      b. *Blastomyces dermatitidis*
      c. *Coccidioides immitis*
      d. *Cryptococcus neoformans*

IX. Antifungal Susceptibility Testing

A. Appropriateness
   1. CLSI has released 3 methods for fungal testing
      a. Yeast testing
      b. Mould testing
      c. Disk diffusion testing (microtiter and Etest)
   2. Concerns
      a. Lack of established breakpoints for most fungal agents
      b. Emergence of antifungal resistance

B. Anti-fungal classes and agents
    1. Polyenes
        a. Amphotericin B (primary antifungal agent used today)
    2. Azoles
        a. Fluconazole (primary antifungal agent in treating yeast infections)
        b. Intraconazole
        c. Voriconazole
    3. Candins
        a. Caspofungin