Chapter 5

DARKFIELD MICROSCOPY FOR THE DETECTION AND IDENTIFICATION OF *TREPONEMA PALLIDUM*

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TEST PRINCIPLES[1,2]

A clinical diagnosis of syphilis is confirmed by using darkfield microscopy to demonstrate *Treponema pallidum* in material from suspected lesions or regional lymph nodes. A positive darkfield result is an almost certain diagnosis of primary, secondary, or early congenital syphilis. For patients with early primary syphilis or for patients with syphilitic lesions and advanced acquired immunodeficiency syndrome (AIDS), the darkfield examination may identify the etiologic agent of syphilis and help diagnose the disease even when antibodies to *T. pallidum* cannot be detected.

Proper equipment, adequately trained personnel, and the examination of several slides may be required to demonstrate the presence of *T. pallidum* in lesion material by darkfield microscopy.

Darkfield Microscopy[3,4]

The standard brightfield microscope may be equipped for darkfield examination by replacing the brightfield, or Abbe, condenser with either a double- or single-reflecting darkfield condenser. Illumination for darkfield microscopy is obtained when light rays strike the object in the field at an oblique angle so that no direct light rays enter the microscope objective, only the rays reflected from the object. Therefore, the object appears self-luminous against a dark background. When a fluid containing particles, including bacteria or treponemes, is placed on a slide, the oblique rays are reflected from the surfaces upward into the barrel of the microscope; these particles appear brightly illuminated against a black background. This type of illumination can be obtained by using a double-reflecting darkfield condenser (Fig 4:1) or a single-reflecting darkfield condenser (Fig 4:2). In the double-reflecting darkfield condenser, two reflecting surfaces produce intense illumination; however, this type of condenser requires precise focusing and accurate centering.

The single-reflecting condenser contains one reflecting surface which does not produce a sharp focusing of the hollow cone of rays. This characteristic makes it easier to manipulate, but less intense illumination is produced. Thus, the single-reflecting condenser is less desirable when high intensity of illumination is required.

Most darkfield condensers require that the numerical aperture (N.A.) of the oil immersion objective be reduced below that of the condenser. This can be accomplished by inserting a funnel stop in the objective or by using an oil immersion objective with a built-in iris diaphragm.

<Insert Figure 4:1(5:1)>

Fig. 5:1.Double-Reflecting Darkfield Condenser

<Insert Figure 4:2(5:2)>

Fig. 5:2.Single-Reflecting Darkfield Condenser

SPECIMEN COLLECTION

To avoid accidental infection when collecting specimens, observe universal precautions (Chapter 2).

Before collecting specimens, always make sure that the darkfield microscope is in good working order.

Specimen

1. The ideal specimen for darkfield examination is a serous fluid that is rich in *T. pallidum* but that contains few blood cells (treponemes may be obscured if many cells are present).

2. Consider every genital lesion in sexually active patients as syphilis until subjected to a darkfield examination and proven otherwise. Other lesions on the skin or mucous membranes should also be examined when syphilis is suspected.

3. Darkfield examination of oral lesions is not recommended. All positive darkfield tests with mouth specimens must be confirmed by a direct fluorescent antibody test. The indigenous flora of the oral cavity frequently contain a spiral organism, *T. denticola*, which is indistinguishable from *T. pallidum*.

4. If topical antimicrobial therapy has been applied to a syphilitic lesion, it may not be possible to demonstrate motile *T. pallidum*, even if several specimens are examined. In this instance, an aspirated sample from an enlarged regional lymph node may be used for diagnosis.

Collection

1. Lesions

a. Remove any scab or crust covering the lesion.

b. Secondary infection exudate, if any, should be removed with a gauze sponge.

c. If necessary, compress the base of the lesion or apply a suction cup to the lesion to promote the accumulation of tissue fluid on the ulcer surface.

d. Apply a glass slide to the oozing lesion or use a sterile bacteriological loop to transfer the fluid from the lesion to the glass slide.

e. Place a cover glass on the specimen and flatten or depress it evenly on the slide, using the blunt end of an applicator stick to remove air bubbles.

f. Examine the slide immediately.

g. To prevent drying, place additional slides with specimens in a moist chamber such as a large plastic petri dish containing a moistened paper towel.

Note: The slide preparations should not contain a large volume of fluid (large volumes cause a rapid liquid flow across the field), nor should the preparation be so thin that it begins to dry before an adequate examination can be made.

2. Dry papulosquamous lesions of the skin

a. Gently remove the superficial layer of skin with a scalpel, needle tip, or mechanical abrader.

b. Try not to cause bleeding. If very little serous fluid appears, compress the lesion.

c. Touch the corner of the surface of a microscope slide to the fluid or use a sterile bacteriological loop to transfer the material to the slide.

d. Material can also be collected by injecting a small drop of sterile saline into the base of the lesion and aspirating the fluid with a small-gauge needle and syringe.

3. Cervical/vaginal lesions

a. With visualization by a bivalve speculum, remove any cervical or vaginal discharge.

b. Obtain serous exudate with a sterile bacteriological loop.

c. Serous exudate, if necessary, can be produced from the lesion by compressing it with a Kelly clamp.

d. Prepare slides as described in no. 1.

4. Mucous patches

a. Using a sterile bacteriological loop, collect some of the mucous material and place it on a clean glass slide.

b. Place a cover glass on the specimen and examine immediately.

5 . Lymph node

a. Disinfect the skin over the node by swabbing it with iodine and alcohol or another suitable agent.

b. Rinse a sterile 20-gauge needle and a 2-ml syringe with sterile physiological saline.

c. Allow 0.2 ml or less of the saline to remain in the syringe.

d. Hold the node firmly and insert the needle well into the node. The ability to manipulate the node freely with the needle tip is a good indication that the capsule of the node has been pierced.

e. Inject the sterile physiological saline into the node.

f. Macerate the tissue by gently manipulating the needle in various directions.

g. Aspirate as much fluid as possible.

h. Discharge the aspirated material onto slides for immediate examination.

Handling

1. Label slide with patient identifier, anatomical site, and time of collection.

2. Examine the slide within 5 -20 minutes of collection, either by bringing the patient to the microscope or the microscope to the patient.

Note: Any appreciable delay in examining a specimen may result in questionable findings, because the motility of the treponemes may be reduced or completely lost.

MATERIALS

Reagents

1. Saline, physiological (0.85%), sterile

2. Disinfectant, 70% alcohol or iodine solution on swabs

3. Control, suspension of nonpathogenic treponemes; i.e., *T. phagedenis* or gingival scrapings containing nonpathogenic treponemes.

Equipment

1. Microscope assembly with a darkfield condenser

    a. Microscope stand with coarse and fine adjustment knobs and a revolving nosepiece for three objectives

    b. Body: an inclined monocular or binocular type

    c. Stage: A plain stage with an attachable graduated or ungraduated mechanical slide carrier

    d. Substage: A rack-and-pinion focusing substage for holding a darkfield condenser

    e. Objectives: Should be parfocal

        10X — low-power used only to focus on specimen and to center condenser; NOT used to search specimen

        40X-45X — high-power used to search the specimen

        90X- I00X — oil-immersion fitted with a funnel stop or built-in iris diaphragm used for final identification of organisms

    f. Oculars: 10X

    g. Condenser: Darkfield oil immersion condenser, either single- or double-reflecting type

    h. Illuminator: The illuminator should be built into the base of the microscope. It should not be attached to the darkfield condenser itself, as the heat generated may be sufficient to cause complete loss of a critical identifying criterion; i.e., the

characteristic motility of the organism. The built-in base illuminator should consist of a 6.0- to 6.5-volt, or equivalent, high-intensity lamp with a variable transformer for regulating light intensity. If a separate external illuminator is used for the microscope, it should be equipped with an iris diaphragm and a 100-watt lamp which, in turn, requires the microscope to have a flat-surface mirror for reflecting the light into the darkfield condenser.

I. Eye shields: These can be obtained for the oculars of some binocular microscope models, thus eliminating the necessity of having to work in a darkened room.

2. Microscope slides, 1 x 3 inches: Using slides of the correct thickness is VERY IMPORTANT in darkfield microscopy. The thickness required by American-made microscopes is usually engraved on the top of the darkfield condensers. For foreign-made microscopes, refer to accompanying literature.

3. Cover glass, size no. 1, 22 x 22 mm: For best results, slides and cover glasses should be scrupulously clean and free of scratches.

4. Oil, immersion, nondrying, Cargille type A or the equivalent **(Cargille code 1248, R. P. Cargille, Inc., Cedar Grove, NJ)**

5. Lens paper and lens cleaner

6. Applicator sticks

7. Gloves, surgical; rubber or plastic

8. Gauze, 2 x 2 inches, sterile

9. Scalpel

10. Loop, bacteriological
11. Pipette, sterile, disposable capillary with safety pipetting device

12. Speculum, bivalve

13. Clamp; Kelly or hemostat

14. Syringe, 1 or 2 ml, with 20 gauge needle, sterile

15. Petri dish, plastic, 150 x 15 mm

16. Discard containers and disinfectant

17. Paper towels

CALIBRATION

Adjustment of the Microscope for Darkfield Examinations[5]

Microscope should be adjusted and in satisfactory working condition BEFORE collecting the specimen for examination.

1. Control slide

    a. Prepare a control slide by placing 1 drop of a suspension of *T. phagedenis* or a drop of saline plus gingival scrapings on a microscope slide of appropriate thickness.

    b. Mount with cover slip.

2. Microscope with built-in base light source

    a. Place a blank glass slide on the stage and raise the substage containing the darkfield condenser to its maximum height. The top of the darkfield condenser should be slightly below the level of the stage, but as close to the glass slide as possible without pushing it up.

    b. Turn on the variable transformer to produce the maximum light intensity.

    c. Lower the substage slightly and place 2-3 drops of immersion oil on the top of the condenser.

    d. Place the control slide on the stage and center the specimen over the condenser with the mechanical slide carrier.

    e. Slowly raise the substage until complete oil contact between the top of the condenser and the bottom of the slide occurs.

    f. Rotate the nosepiece to center the 10X objective over the specimen.

    g. Bring specimen into focus by using the coarse adjustment knob.

    h. At this point, center the light in the field by rotating the two centering screws at the base of the darkfield condenser.

    I. Focus the darkfield condenser by slightly raising or lowering the substage until you observe the smallest diameter of the circular area of intense light.

j. Rotate the nosepiece until the high-dry (40X-45X) objective is in place over the specimen.

k. Bring the specimen into focus by using the fine adjustment knob only.

l. If a satisfactory image is obtained in step k, place a SMALL DROP of immersion oil on the cover glass.

m. Rotate the nosepiece until the oil-immersion objective (90X - 100X) is in place over the specimen and is in contact with the oil on the cover glass.

n. Bring the specimen into focus by using the fine adjustment knob only. Use of the coarse adjustment knob may cause damage to the high dry or oil immersion objective by allowing it to come into contact with the specimen slide. Slightly better contrast may be produced by decreasing the light intensity from the illuminator.

3. Microscope with external illuminator

a. Align the microscope and the external illuminator. The illuminator should be 15-20 cm in front of the plane (flat) side of the microscope mirror.

b. Adjust the iris diaphragm on the front of the illuminator to a diameter of approximately 20 mm.

c. Using a piece of paper placed across the mirror surface, adjust the illuminator so that the image of the filaments of the light bulb is shown in sharp focus on the center area of the plane side of the mirror.

d. Remove paper and adjust the angle of the mirror to direct the light beam into the bottom of the condenser.

e. To complete and verify the microscope adjustment, examine the control slide. Continue through steps c-k in Section A2.

f. After completing steps c-k, open the diaphragm on the external light source until the entire microscope field is illuminated, and then complete steps l-n in Section A2.

QUALITY CONTROL

1. To complete the microscope adjustment and to verify that the microscope is in good working order before examining patient material, prepare a second suspension of gingival scrapings or *T. phagedenis* in a drop of saline on a slide of the proper thickness and mount with a cover glass. Examine under oil immersion as described above.

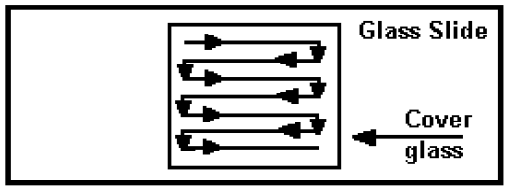
2. Treponemes observed in the patient's specimen must have characteristic morphology and motility described on the following page for *T. pallidum*.

PROCEDURES

Examination of the Specimen for *T. pallidum*

1. Place the slide to be examined on a previously adjusted darkfield microscope.

2. Search the entire specimen methodically with the high-dry objective for spiral organisms that have the morphology and motility characteristics of *T. pallidum*. Search carefully, systematically, exhaustively before making a negative report. A typical systematic scheme for adequately searching the specimen is shown in Fig. 4:3.



3. If a suspected treponeme is observed, center it in the field with the mechanical slide carrier so that it can be examined with the oil-immersion objective.

4. Rotate the nosepiece halfway so that a SMALL DROP of immersion oil can be placed on the cover glass.

5. Continue to rotate the nosepiece until the oil-immersion objective is in place over the specimen and in contact with the oil on the cover glass.

6. Examine the organism carefully, focusing with the fine adjustment knob only.

7. When organisms are found that have the characteristic morphology and motility of *T. pallidum*, report as positive.

8. After examining a slide, discard it into a container of 70% alcohol or other suitable bactericidal solution such as 10% sodium hypochlorite.

9. At the end of the working day, remove the immersion oil from the stage, the darkfield condenser, and the oil-immersion objective of the microscope. Use only lens paper and lens cleaner to clean the oil-immersion objective to avoid scratching the lens. Keep the microscope free of oil and dust and in good working order at all times.

Figure 4:3. Schematic Search Pattern

Differentiation of *T. pallidum* from Other Organisms and Objects[6-8]

1. Characteristics of *T. pallidum*

   a. Morphology

      (1) Shape: A delicate, corkscrew-shaped organism with rigid, uniform, tightly wound, deep spirals.

      Note: Coil appearance is maintained despite active motility of the organism.

      (2) Length: 6-20 micrometers (μm), with an average length of 10 μm. The average organism is slightly longer than the diameter of a blood cell (8 μm).

      (3) Width: Approximately 0.13-0.15 μm; very thin.

      (4) Spiral wave length: Approximately 1.0-1.5 μm.

      (5) Spiral depth: Approximately 0.5-0.7 μm.

   b. Motility

      (1) Translation (uniform movement in a straight line): Slow, may exhibit deliberate forward and backward movements, with occasional erratic movement.

      (2) Rotation: Slow-to-rapid rotation about the longitudinal axis (like a corkscrew); may rotate without changing place.

      (3) Flexion: Rotation is accompanied by soft bending, twisting or undulation from side to side, giving a shimmering, graceful effect. Bending of the organism usually occurs in the middle and is stiffly executed, like the bending of a coil spring, which comes back into place when released.

      (4) Distortion: May occur as a ring, with ends seemingly attached, or in more tortuous convolutions. When attached to or obstructed by heavier objects, vigorous struggling distorts coils.

2. Characteristics of *T. refringens* (formerly *Borrelia refringens*): A member of the normal genital flora.[6,9]

   a. Morphology

      (1) Shape: Spiral organisms that may appear loosely coiled, thick, and coarse.

      (2) Length: Approximately 5-16 μm

      (3) Width: Approximately 0.2-0.3 μm

   b. Motility

      (1) Translation: Rapid movement across or out of the field with a writhing motion.

      (2) Rotation: Active serpentine and rotating motion with marked flexion. The organism may rotate so rapidly that it looks straight.

      (3) Flexion: Marked bending and frequent relaxation of coils.

3. If the material on the slides contain numerous artifacts or refractile objects, the untrained observer may be deceived by miscellaneous pieces of cellular debris, flagella, wavy fibrin strands, cotton fibers, and microscopic scratches on the glass slides. These, and forms similar to treponemes made of spiral fibrin filaments can, because of Brownian movement, be quite deceptive and must be interpreted cautiously.

   In summary, *T. pallidum* is a thin, tightly wound, spiral organism capable of extreme contortions from which it snaps back to its original form in a coiled spring-like manner. It may spin rapidly without translation, move slowly forward and backward without obvious change in direction of rotation or pitch of coils, or the organism may more slowly thread its way corkscrew-fashion in viscous material. A spring-like rigidity is constant and *T. pallidum* does not move rapidly from place to place with a serpentine motion. Any coarsely wound spiral organism exhibiting great flexion and rapid movement from place to place IS NOT *T. pallidum*.

REPORTING OF RESULTS

Reporting Laboratory Findings

Report	*Results*
Darkfield positive	Organisms found that have the characteristic morphology and motility of *T. pallidum*

Darkfield negative	No treponemal organisms found, OR spiral organisms found but without characteristic morphology and motility of *T. pallidum*.
Darkfield unsatisfactory	No *T. pallidum* found, but specimen has too many refractile elements (blood cells, air bubbles, tissue fragments) or specimen is drying.

CALCULATIONS AND RANGES

Because serologic results may not be reactive when the patient is seen initially, the sensitivity of the darkfield examination is usually based on the clinical appearance of the lesion, clinical history, and seroconversion of serologic tests for syphilis.

Sensitivity

$$\frac{TP}{TP + FN}$$

TP = True Positive, the number of persons who test reactive that have syphilis.
FN = False Negative, the number of persons who test negative that have syphilis.

Table 4.1 **Sensitivity of darkfield examination**

Investigators	**Sensitivity**
Romanowski et al[10]	78.8%
Daniels and Ferneyhough[11]	73 .8%.

INTERPRETATION OF RESULTS

The earliest and empirically most specific means of diagnosing syphilis (if yaws, bejel, and pinta are excluded) is by darkfield microscopy. The demonstration of treponemes with characteristic morphology and motility for *T. pallidum* constitutes a positive diagnosis of syphilis in primary, secondary, or early congenital stages, whatever the outcome of serologic testing. False-positive darkfield tests may occur with oral specimens; therefore, such positive specimens must be confirmed by direct fluorescent antibody tests specific for identification of *T. pallidum*. When patients with untreated primary syphilis are positive by darkfield microscopy but are serologically nonreactive, they usually become serologically reactive within several days to several weeks. In other stages, the patient should be seroreactive; if not, the darkfield interpretation and serologic results should be analyzed to decide whether test results may be false-positives or false-negatives, respectively.

Every genital lesion should be considered syphilitic until proven otherwise. Extragenital lesions characterized by indolence, induration, and regional lymphadenopathy should be regarded as possibly syphilitic. Failure to find the organism does not exclude a diagnosis of syphilis.

Negative results may be reported for the following reasons:

1. The number of organisms was insufficient for detection.

2. The patient has received antitreponemal drugs, topically or systemically.

3. The lesion is "fading" or approaching natural resolution or disappearance.

4. The lesion is one of late syphilis.

5. The lesion is not syphilitic.

When the darkfield examination is negative in patients suspected of having primary syphilis, repeated examination (on as many as 3 consecutive days) or aspiration of enlarged regional lymph nodes may be indicated. Theoretically, serologic tests for syphilis should be repeated at 1 week, 1 month, and 3 months. If nonreactive serologic results are obtained for longer than 3 months in untreated patients, syphilis may be excluded as the cause of such lesions. Among patients suspected of having syphilis in other stages, a negative darkfield examination and nonreactive serologic tests suggest that syphilis is extremely unlikely, and follow-up tests are unnecessary.

SOURCES OF ERROR

A summary of the more frequent sources of error in performing the darkfield technique may be helpful as a checklist.

1. Preparation errors

    a. If the specimen contains too many blood cells, air bubbles, or tissue fragments, these refractile elements can obscure the presence of the organism *T. pallidum*.

    b. If cover glasses and slides are dirty or scratched, obtaining a good darkfield will be difficult.

    c. If slides are too thick or too thin, the apex of the cone of light will not coincide with the object being studied.

    d. If the cover glass is too thick, it is impossible to focus on the specimen because of the short working distance of the oil immersion objective.

e. If there is excessive fluid between the glass slide and cover glass, the liquid will flow rapidly across the field of vision, and it will be too deep to scan.

f. If there is too little fluid between the glass slide and cover glass, the specimen will begin to dry and the organisms will lose motility.

2. Microscopy errors

a. If immersion oil is not placed between the condenser and the slide or if immersion oil with an incorrect refractory index is used, no light will reach the specimen.

b. If slides of improper thickness are used, focusing on the lesion material on the slide and illumination of the objects on the slide will be affected.

c. If, while using a paraboloid condenser, the concave side of the microscope mirror is used with an external light source, the light intensity in the field of view will decrease.

d. If the darkfield condenser is not properly centered, the object will be illuminated poorly or not at all.

e. If the darkfield condenser is not properly focused, the most intense illumination of the object will not be obtained.

f. If oil and dust are on the subsurface or reflecting area of the darkfield condenser, the intensity of illumination of the object will decrease.

g. If the high numerical aperture of the oil-immersion objective is not compensated with a funnel stop or an iris diaphragm, undiffracted direct light will enter the oil objective.

h. If immersion oil is on the lens of the low-power (10X) or high-power (40-45X) objectives, the picture will be hazy without sharp definition.

I. If the specimen is inadequately illuminated, it will be impossible to differentiate *T. pallidum* from other spiral organisms.

j. If the microscope is focused on the cover glass instead of on the specimen, a false negative report might be issued.

k. If the search of the specimen is inadequate or unmethodical, a false-negative report might be issued.

3. Errors in differentiating *T. pallidum* from other organisms and objects

a. If one is unfamiliar with the morphology and motility characteristics of *T. pallidum*, a false-positive or a false-negative report might be issued.

b. If one is unfamiliar with the characteristics of nonspecific spiral organisms, tissue debris, fibrin strand, and other extraneous objects, a false-positive report might be issued.

c. If one mistakes the effects of Brownian movement on spiral objects for motility, a false-positive report might be issued.

d. If one sees occasional erratic movement of *T. pallidum* or no movement at all, too much time may have elapsed between making and examining the slide.

TEST LIMITATIONS

1. Oral lesions at or near the gingival margin are unsatisfactory for darkfield examination, as the indigenous flora in this area frequently contains *T. denticola*, a spiral organism that is indistinguishable from *T. pallidum*.

2. The examination of lesion material from patients who have received antitreponemal drugs topically or systemically may produce negative results.

3. Fading lesions of the skin are less likely to yield a positive darkfield because fewer treponemes are present.

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