ParaLens™ Microscope Attachment: Tuberculosis

Background

It is estimated that 1/3 of the world's population is infected with tuberculosis (TB), and that almost two million people die each year from the disease.[1] The World Health Organization (WHO) and the Stop TB Partnership have established Millennium Development Goals of reducing the prevalence of TB by 50% from 1990 levels by the year 2015, and reducing the disease to one per million population by 2050.[2, 3]

The key to these goals is the advocacy of Directly Observed Therapy Short Course (DOTS), a multistage approach to developing TB awareness and support. One of the five elements of the DOTS strategy involves improving the detection of *Mycobacterium tuberculosis*, the acid-fast bacterium (AFB) that causes TB. Traditionally, detection of AFB in suspected pulmonary TB cases has been performed using light microscopy on sputum samples prepared with Ziehl-Neelsen or Kinyoun stains. While this type of stain can be effective, fluorescence microscopy, using auramine stain, is both more sensitive and faster to perform than the traditional methods.[4] Thus, the WHO has recently recommended the use of fluorescence microscopy as an important part of the DOTS strategy. [5]

Until recently, the fluorescence microscopes needed for these tests have been expensive and bulky, using dangerous and often fragile mercury or xenon lamps as light sources. Because of these and other significant limitations, the use of fluorescence microscopy in TB diagnosis has not been fully adopted in many parts of the world hardest hit by the disease. Additionally, despite its improved sensitivity and decreased examination time, conventional auramine stain requires approximately the same amount of preparation time (20 minutes) as Ziehl-Neelsen stain, representing a missed opportunity for faster bacilli detection and decreased workloads.

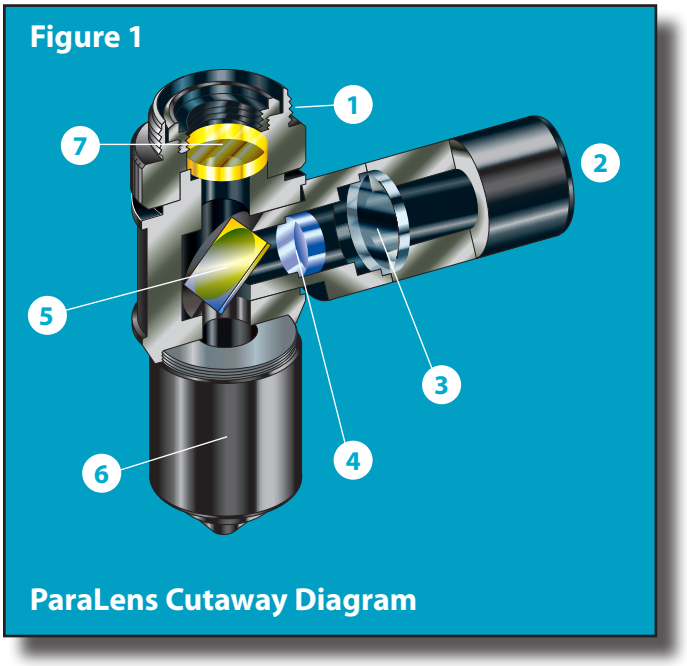
This application note will demonstrate how the QBC ParaLens™ Microscope Attachment and the QBC Fluorescence and Staining Technologies (*F.A.S.T.*)™ TB Kits can work together to help TB programs meet WHO and Stop TB Partnership goals. The ParaLens can attach to any light microscope and, with the use of a durable LED light source, perform fluorescence microscopy comparable to standard fluorescence microscopes at a fraction of the cost. The *F.A.S.T.* TB Kits are complete TB review systems that include a unique *F.A.S.T.* Auramine O stain that can stain slides for review in under 3 minutes. Together, the ParaLens and the *F.A.S.T.* Kits can increase the speed and efficiency of TB review, making it easier and more cost efficient to reduce the prevalence of TB worldwide.

The ParaLens System

The ParaLens Microscope Attachment (as seen in Figure 1) can be attached to any conventional light microscope (1). (Note: Ring transition adapters are available for some microscopes. Please consult the ParaLens Brochure for more details.) The lens contains an extension arm (2), that connects to the light source. The high intensity blue LED produces light with a wavelength of approximately 410-511 nm. The LED is DC powered and can be run on any wall outlet using the AC to DC converter included in the system, by a 12 volt battery using an optional DC connector, through an optional solar powered battery pack, or by most computers using a USB input cable.

Inside the extension arm, light from the LED light source passes first through a focusing lens (3) and then through an excitation filter (4) that allows only light in the 385-480 nm range into the main chamber of the ParaLens. A dichroic beam splitter (5) redirects the light downward to the specimen. Powerful objective lenses (6) allow for the magnification of the fluorescent light from the specimen. When the light returns to the beam splitter, light with a wavelength of ~510 nm or higher is allowed to pass through. Because the specimen has been treated with an Auramine dye, AFB will appear yellow-green or yellow-orange and proceed through to the viewer. An emission filter (7) reduces background noise and optimizes the fluorescence signal transmitted to the observer.

Figure 1



ParaLens Cutaway Diagram

F.A.S.T. TB Kits

The key to the QBC *F.A.S.T.* TB Kits is QBC Diagnostics' revolutionary *F.A.S.T.* Auramine O stain and counterstain. The configuration of this stain is unique

to QBC Diagnostics, and permits sputum staining and counterstaining in just three minutes.

F.A.S.T. TB Kits also feature the unique, patent pending SureFocus™ Microscope Slides. These slides are printed with a fluorescent marker that is visible under the same wavelengths that excite Auramine O stains. This marker can assist users in finding the proper focal plane and maintaining focus throughout review, as well as serving as a reference point during slide review and a control to determine the effectiveness of the stain and microscope used.

F.A.S.T. TB Kits are available in several different configurations suitable to meet each TB lab's unique burdens. The QBC *F.A.S.T.* TB Smear Kit includes supplies for 400 tests, including (see Figure 2) one 120 mL bottle of QBC *F.A.S.T.* Auramine O Stain (1), one 120 mL bottle of QBC *F.A.S.T.* Decolorizer/Quencher (2), 432 SureFocus slides (3), five quality control slides (4), 400 wooden applicator sticks (5), and four bags of 100 sputum cups (120 mL) with lids (6).

Figure 2

***F.A.S.T.* TB Kit Components**

The QBC *F.A.S.T.* TB Smear Kit with Digestion Solution is designed for labs performing culture or PCR. It contains one 120 mL bottle of QBC *F.A.S.T.* Auramine O Stain, one 120 mL bottle of QBC *F.A.S.T.* Decolorizer/Quencher, 432 SureFocus slides, five quality control slides, 400 sterile sputum tubes, 1200 sterile transfer pipettes, and 27 bottles of *F.A.S.T.* Sputum Digestion Solution (with Digestion Buffer packets). The QBC *F.A.S.T.* TB E-Z Smear Kit is designed for portability, as it contains 10 individual packets with supplies to conduct five tests each. Each packet contains a 3 mL bottle of stain, a 3 mL bottle of counterstain, 5 SureFocus slides, 5 applicator sticks, and 5 sputum cups with lids.

Sputum Smear Preparation

If a patient has presented with any of the clinical symptoms of pulmonary tuberculosis, the patient should be asked to give two to three sputum samples over the next few days. The sputum cups contained in the *F.A.S.T.* Kits are designed for this purpose. The patient should be instructed to provide only sputum (consisting of mucus and phlegm), and not saliva. Sputum smear slides and cultures can be prepared from these samples to detect the presence of AFB.

(Note: For further guidelines and standards in the care of patients presenting with TB symptoms, please consult the International Standards of Tuberculosis Care (ISTC) at http://www.tbcta.org//Uploaded_files/Zelf/InternationalStandardsforTuberculosisCare1181209995.pdf and the Practical Approach to Lung Health (PAL) strategy documents at http://whqlibdoc.who.int/hq/2005/WHO_HTM_TB_2005.351.pdf and http://whqlibdoc.who.int/hq/2008/WHO_HTM_TB_2008.410_eng.pdf.)

Once you have collected the patient samples, you can either begin the staining process, or begin the process of processing samples for culture or PCR. For users of the *F.A.S.T.* TB Smear Kit or the QBC *F.A.S.T.* E-Z TB Smear Kit, please skip ahead to the "Staining Process" section, as the next section only pertains to users of the QBC *F.A.S.T.* TB Smear Kit with Digestion Solution.

Preparation of Samples for Culture or PCR

For those interested in culture or PCR, smear slide preparation should be performed in a laboratory setting (BSL 2 or 3). A biosafety hood should be used for work involving culture of AFB or handling that could create aerosols. A good resource for biosafety in TB slide preparation can be found on the Centers for Disease Control website (http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm).

To begin using the Sputum Digestion Solution, pour the contents of the Phosphate Buffer Powder packet into a 500 mL volumetric flask or autoclavable bottle with a 500 mL gradation. Fill with water to the 500 mL line, and mix well. If a flask was used, transfer the contents to an autoclavable bottle. Autoclave the buffer.

Prepare the *F.A.S.T.* Sputum Digestion Solution by first loosening (but not removing) the cap of the plastic bottle. There is a glass ampule within the bottle, containing NALC in dry powder form. Gently squeeze the bottle to break the ampule. Reseal the cap and gently shake to dissolve the NALC, taking care not to foam the solution. Note: Once the ampule has been broken, a bottle of digestion solution is effective for 24 hours.

Once the digestion solution has been prepared, use a transfer pipette to aseptically transfer digestion solution into the sputum tube in a volume equivalent to the sputum sample. Cap the sputum tube tightly and mix well. Allow the tube to incubate at room temperature for 15 minutes. This will digest and decontaminate the specimen while leaving the AFB viable. (Note: If the sample is allowed to incubate for longer than 15 minutes, live AFB bacteria may also be killed.) Quench the digested sputum by pouring buffer into the sputum tube until the mixture reaches the 50 mL line in the tube.

At this point, the sputum tube should be resealed tightly and centrifuged for 15-20 minutes at 3,000 x g. The recommended centrifuge for this procedure is the QBC Horizon Model 755VES, which can achieve the necessary centrifugal force at 4100 RPM. When the centrifuge has finished, there should be a small pellet at the bottom of the tube. Using a second pipette, aspirate most of the water carefully from the tube, taking care not to lose the pellet. Using the third transfer pipette, resuspend the pellet with buffer in a 1-to-1 mixture.

Culture or PCR will result in greater sensitivity, the ability to speciate, and allow for the testing of drug sensitivities. Several culture methods are available and differ based on the media and apparatuses available. Cultures should develop in 7 to 15 days with a liquid medium, and from 3 to 8 weeks with a solid medium. For more information on culture creation, consult the text Essential Procedures for Clinical Microbiology (1998) from the American Society of Microbiology.

Staining Process

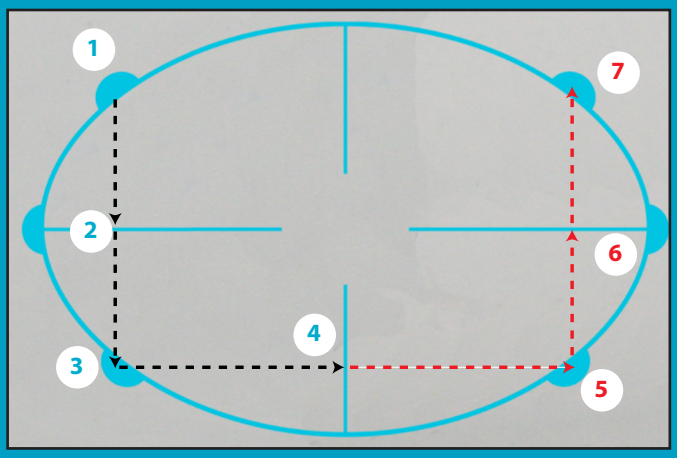
To prepare the slides for staining, use the applicator stick (or, if culture was created, reuse the third sterile transfer pipette) to take a small amount (~ 100 µl) of the sample and apply it to a SureFocus slide. Heat fix the slide to kill any active mycobacteria. The preferred method of heat fixing is a slide warmer set at 65 degrees Celsius for two hours, but heat fixing can be done with an open flame. If a flame is used, do not allow the sample to become charred.

Once the slides have been heat fixed, they are ready for staining. Cover the smear with *F.A.S.T.* Auramine O Stain and let stand for 1 minute. Rinse gently with deionized or tap water and drain. Cover the smear with *F.A.S.T.* Decolorizer/ Quencher, and let stand for 1 minute. Again, rinse gently with water and drain. Once the slide is dry, it can be reviewed using the ParaLens Microscope Adapter.

Set-Up and Focusing the ParaLens

To perform fluorescence microscopy using the ParaLens, place the prepared SureFocus slide on the microscope stage and clamp it into place. To most easily focus the ParaLens, center the objective over the upper left starter circle of the SureFocus fluorescent marker, as demonstrated in Figure 3 (1). This can be accomplished in two ways: by turning on the microscope's light source and finding the starter circle using a low power objective, or by aligning and viewing the descent of the lens from a side perspective.

Figure 3

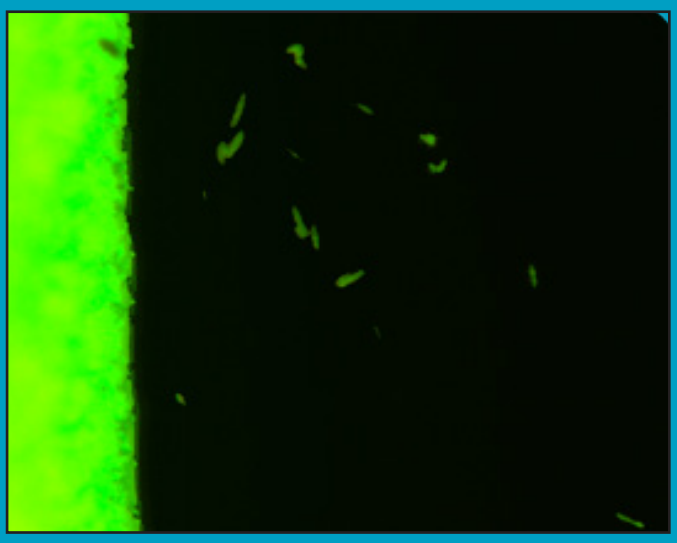


SureFocus Suggested Review Path

Once properly aligned, switch to the 40x or 60x ParaLens objective. If using the 60x objective, apply 2 to 3 drops of immersion oil to the slide. Gradually lower the ParaLens until it touches the oil. From a side perspective, there will be a distinct wicking effect when they touch. Use the eyepieces to focus on the starter circle. Figure 4 shows the SureFocus slide under 600x magnification. (Note: The large green object on the left side is part of the slide marker, while the small green objects on the right are AFB.)

Once the starter circle is in focus, begin viewing the sample at one corner of the smear and work systematically through

Figure 4



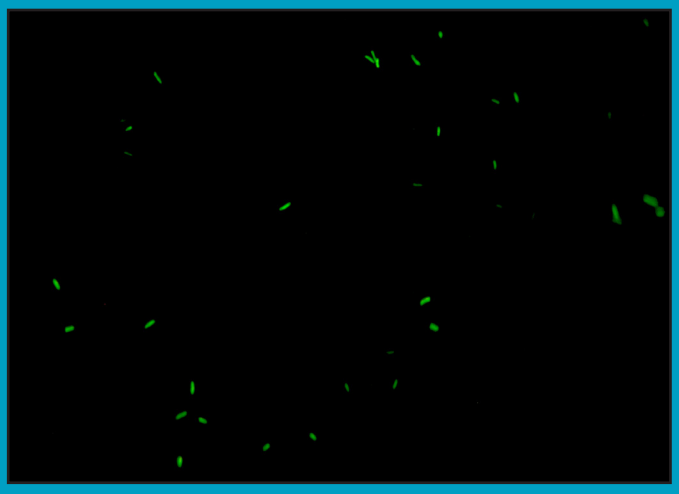
AFB, seen with the SureFocus Marker

the smear. A suggested review path is to move from the upper left starter circle along the Y-axis to the left crosshair (2). Use the crosshair to refocus. Continue down the Y-axis to the lower left starter circle (3). Again refocus. Now move right toward the bottom crosshair (4).

Because of the use of fluorescence microscopy, you will be able to use a lower power objective and still see any mycobacteria present in the path. Thus, you will be able to view the slide in approximately 40 to 60 fields (for 40x and 60x magnification ParaLens, respectively), rather than 100 fields, the standard with a 100x magnification objective. With a 60x objective and using continuous motion, this suggested path ending at the bottom crosshair should contain approximately 77 fields. If further fields are needed for viewing (to confirm a scanty or an unclear result), observe the sample along the path designated by numbers 4-7. Use the same methods as previously described.

For more information on working with the SureFocus microscope slides, please consult the library of materials on the SureFocus slide at www.qbcdiagnostics.com.

Figure 5



AFB, Seen Using the ParaLens and F.A.S.T. Stain

Reading AFB Slides

Under the ParaLens, TB mycobacteria should appear as yellow-green or yellow-orange, rod-shaped objects (as seen in Figure 5). Under the fluorescence microscope, they should stand out in stark contrast to the dark background. Occasionally, artifacts such as crystals, hair, or cells may appear in the sample. To distinguish the difference, it is recommended that you first examine a sample negative for TB under the ParaLens. (See the Quality Control section for more details.)

The World Health Organization has defined a "smear positive" case as "the presence of at least one acid fast bacilli (AFB+) in at least one sputum sample in countries with a well functioning external quality assurance (EQA) system."[6] The sample can also be graded based on the number of mycobacteria seen in 100 fields using a 100x objective. Since the ParaLens makes AFB more visible than with light microscopy, it is possible to use lower magnification and view a larger section of the sample in each field. Use the following chart to determine the proper grade for your objective magnification[7]:

Ziehl-Neelsen Method	Report	Grading for Fluorescent Microscopy	
		40x	60x
0 AFB	No Acid-Fast Bacilli Seen	No Acid-Fast Bacilli Seen	
1-9 AFB per 100 fields	Report Exact Count ("Scanty")	Divide observed count by 5	Divide observed count by 2
10-99 AFB per 100 fields	+		
1-10 AFB per field	++		
10+ AFB per field	+++		

Quality Control

Adequate quality control is critical for the evaluation of evaluation of laboratory staining and review procedures. To facilitate proper quality control, *F.A.S.T.* kits contain 5 quality control slides that contain one non-viable smear of *Mycobacterium tuberculosis* (contained in a circle marked "+") and one non-viable smear of *Escherichia coli* (contained in a circle marked "-"). These smears should be stained and examined as with any patient specimen. QC should be performed regularly and results should be recorded, in accordance with governing regulations.

Change Over

A slide stained for fluorescence microscopy can also be restained for light microscopy using the Ziehl-Neelsen method with no additional preparation. Simply perform the staining using standard methods. One possible complication with this process is that immersion oil on the slide must be removed before beginning the heating process. This can prove difficult with conventional immersion oils, as cleaning could potentially remove the sample from the slide. There are some commercially available immersion oils that can be removed with alcohol which can be effective for this purpose.

Conclusion

The ParaLens microscope adapter is capable of providing the benefits of an expensive fluorescence microscope at a fraction of the cost. It can be used with an existing light microscope, thereby saving precious lab resources. Combined with the increased staining speed of the QBC

F.A.S.T. kits, the system is an important new weapon in the worldwide fight against tuberculosis.

References

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5. *Ibid*.

6. *World Health Organization Website*, n.d.. Web. 3 Sep. 2009. (http://www.who.int/tb/dots/laboratory/policy/en/index1.html)

7. *WHO Laboratory Services in Tuberculosis Control, Part II: Microscopy*. Geneva, Switzerland: WHO, 1998, ppg 31-34.

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