

# 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.<sup>1</sup> The World Health Organization and the StopTB Partnership have the stated goal 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.<sup>2</sup>

The key to this goal 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 treated with Ziehl-Neelsen stain. 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.<sup>3</sup> Thus, the WHO has recently recommended the use of fluorescence microscopy as an important part of the DOTS strategy.<sup>4</sup>

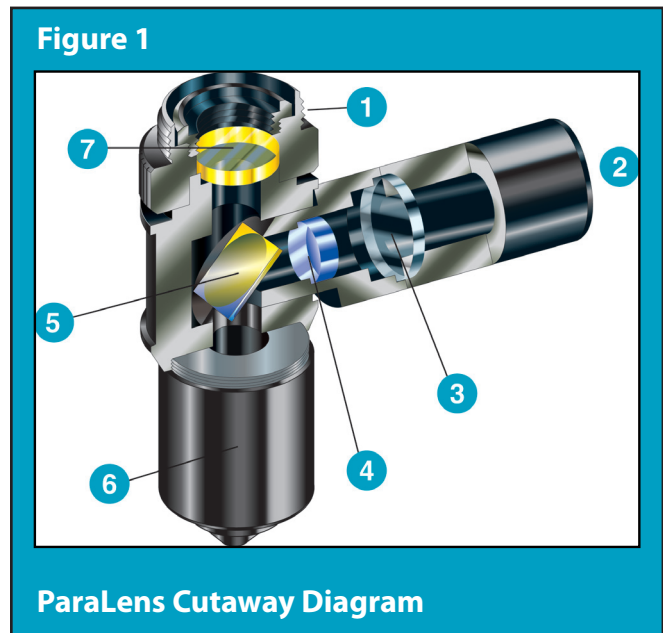
Unfortunately, the fluorescence microscopes needed for these tests are expensive, bulky, and use dangerous and often fragile mercury or xenon lamps as light sources. Because of these and other significant limitations, the use of fluorescence microscopy in TB diagnosis has not been fully adopted in many parts of the world hardest hit by the disease.

This application note will demonstrate how the ParaLens Microscope Attachment, as developed by QBC Diagnostics, can be used with standard Auramine O stains to provide a solution to this problem. The ParaLens is an attachment that can be used with almost any light microscope, thereby saving precious laboratory resources otherwise lost to implementing unnecessary new equipment. With just the use of an LED light source, the ParaLens can perform fluorescence microscopy comparable to expensive, commercially available fluorescence microscopes. By reducing the costs and hassles associated with traditional fluorescence microscopes, the ParaLens can place fluorescence microscopy in the hands of more clinicians and help to improve detection of TB worldwide.

## The ParaLens System

The ParaLens Microscope Attachment (as seen in Figure 1) can be attached to any conventional light microscope with standard Royal Microscopy Society threading (1). (Note: Ring transition adapters are available for non-standard microscopes.) The ParaLens side arm (2) connects to the LED light source. The high intensity LED produces blue 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, or by one of several other power options with the addition of the optional ParaLens Portability Pack, including a 12 volt battery, solar powered battery pack, or a USB cable.

Inside the ParaLens side 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 body 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.



## Reviewing Sputum Samples with the ParaLens

### Preparing the Sputum Smear

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 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.

As a preliminary matter, 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>).

Begin the slide creation process by mixing the sputum sample (usually approximately 5 to 10 ml) with an NALC-NaOH solution (0.25% NALC; 1%NaOH; 0.73% Sodium Citrate) in a 1:1 mixture. Allow the mixture to remain at room temperature for 15 minutes. This will digest the specimen and lyse all of the other cells in the mixture except for AFB. It is also possible to use bleach instead of sodium hydroxide, but bleach will kill the mycobacteria, preventing the creation of cultures. After 15 minutes, add distilled water to the mixture to dilute. If possible, fill with water until the mixture equals a total of 50 ml.

At this point, the tube containing the water and sputum mixture should be sealed and then centrifuged for approximately 20 minutes at 3,000 x g. Using a cold centrifuge is recommended, if possible, as it will increase the thickness of the resulting sample. When the centrifuge has finished, there should be a small pellet at the bottom of the tube. Aspirate most of the water carefully from the tube, taking care not to lose the pellet. Leave about a 1:1 mix of water and sample in the tube. Using a transfer pipette, resuspend the pellet with the remaining water.

Depending on the steps you have taken, you can either create cultures with the digested sputum mixture or begin the process of creating slides. Creating cultures will result in greater sensitivity, the ability to speciate, and allow for the testing of drug sensitivities. However, it is a longer process, lasting anywhere from 1 to 8 weeks, depending on the media used. 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.

To prepare the slides from the sputum pellet or the culture, take a small amount (~ 100 µl) of the sample and apply it to the slide. Allow the slides to air dry for approximately 5-10 minutes, depending on the amount of sample applied to the slide. Once dry, 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 you use a flame, do not allow the sample to become charred. Once the slides

have been heat fixed, they are ready for staining.

## Staining Process

With a conventional auramine-rhodamine stain, begin by flooding the slide with the stain and allow it to set for 15 minutes. Wash off the remaining stain with water (tap water can be used). Next, decolorize the slide with acid alcohol and wash off with water. Repeat this step until no stain is visible to the naked eye. Now counterstain the sample with potassium permanganate for 2 minutes. Rinse again with water, and allow to air dry. This process should take approximately 30 minutes per slide.

As an alternative, QBC Diagnostics offers the *F.A.S.T.* Auramine O Stain, which can speed this process up considerably, allowing for the preparation of slides in as little as 3 minutes. For more information on *F.A.S.T.* stains, please consult the Application Note "Reviewing Sputum Samples Prepared With *F.A.S.T.* AFB Kits" or any of our marketing materials on *F.A.S.T.* AFB Kits.

Regardless of the stain you choose, please consult the manufacturer's instructions for use for more specific instruction.

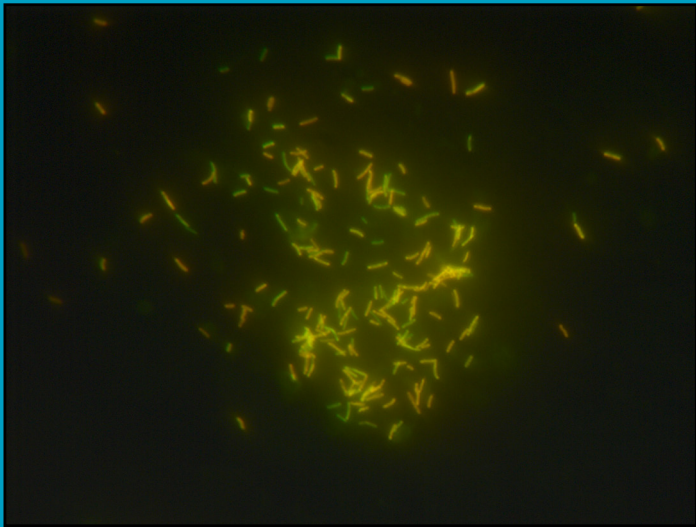
## Set-Up and Focusing the ParaLens

To perform epifluorescence microscopy using the ParaLens, place the slide on the microscope stage and clamp it into place. To most easily focus the ParaLens, center the objective over the area of interest and view the descent of the lens from a side perspective. If using the 60x or 100x objective, apply 2 to 3 drops of immersion oil to the slide. Gradually lower the ParaLens until it touches the oil. There will be a distinct wicking effect when they touch. For the 40x objective, simply lower the lens until it is about 0.5 cm from slide. Now look through the eyepieces, and continue to focus downward.

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

To simplify the focusing and review process, QBC Diagnostics has developed the revolutionary patent pending SureFocus™ Microscope Slide. The SureFocus Slide contains a crosshair-styled smear guide that fluoresces under the same wavelengths that excite Auramine stains. This allows the guide to remain visible throughout review, providing users with confidence that they remain in focus and guidepoints to standardize their review paths.

**Figure 2**



**AFB, As Seen Under the ParaLens**

### Performing a TB Screen

Under the ParaLens, TB mycobacteria should appear as yellow-green or yellow-orange, rod-shaped objects (as seen in Figure 2). 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.

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.”<sup>5</sup> The sample can also be graded based on the number of mycobacteria seen in 100 fields using a 100x objective. The current WHO standard<sup>6</sup> is:

Diagnosis	Number of AFB
Scanty	1-9 AFB per 100 fields
+	10-99 AFB per 100 fields
++	1-10 AFB per field
+++	10+ AFB per field

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. A researcher using a 40x or 60x ParaLens should divide the observed count by two to determine the proper WHO grade.

### 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. Because of these advantages, the ParaLens can be an important new weapon in the hands of TB labs fighting the worldwide fight against tuberculosis.

### References

1. *Centers for Disease Control Website*. Centers for Disease Control, 2009. Web. 3 Sep. 2009. (<http://www.cdc.gov/tb/statistics/default.htm>)
2. *Stop TB Website*. Centers for Disease Control, n.d.. Web. 3 Sep. 2009. ([http://www.stoptb.org/stop\\_tb\\_initiative/](http://www.stoptb.org/stop_tb_initiative/))
3. *World Health Organization Website*, 2005. Web. 3 Sep. 2009. ([http://www.who.int/tb/publications/expert\\_consultation\\_sep05.pdf](http://www.who.int/tb/publications/expert_consultation_sep05.pdf))
4. *Ibid.*
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