Abstract

The QBC ParaLens Advance is a durable, easy-to-use option for providing LED fluorescence microscopy capabilities to any light microscope. Among its many applications, the ParaLens Advance can be used to improve sensitivity in the detection of tuberculosis Acid Fast Bacilli (AFB) when reviewing sputum samples treated with Auramine O stain. This application note demonstrates how to use the ParaLens Advance with Auramone O stain to provide these improved results to more users around the world.

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 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.[2]

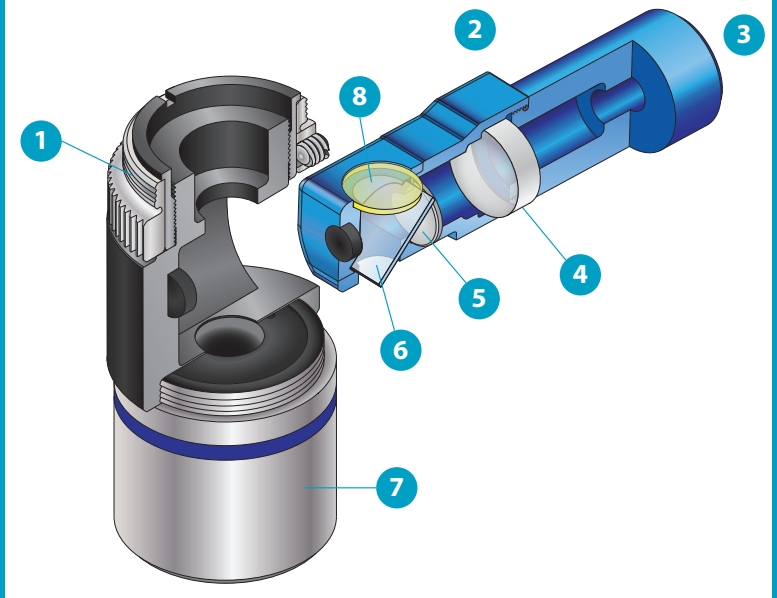
The key to this goal is the advocacy of Directly Observed Therapy Short Course (DOTS), a multistage approach to developing TB awareness and support. One of the five elements of the DOTS strategy involves improving the detection of *Mycobacterium tuberculosis*, the acid-fast bacterium (AFB) that causes 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.[3] Unfortunately, adoption on a worldwide scale has been slowed by the limitations of conventional fluorescence microscopes, which are expensive, bulky, and use dangerous and often fragile mercury or xenon lamps as light sources.

This application note will demonstrate how the ParaLens Advance, an LED fluorescence microscope attachment, can work with auramine stain to improve the sensitivity of AFB detection for more users worldwide. The ParaLens Advance can easily upgrade any compound light microscope for fluorescence microscopy through the use of a bright, durable and easy-to-power LED light source. The WHO has recognized the benefits of LED fluorescence, recommending that users replace light microscopy and standard fluorescence microscopy with the increased sensitivity, durability and portability of LED fluorescence.[4] The ParaLens Advance has been developed to meet this recommendation and ease the adoption of fluorescence microscopy for users conducting TB screening worldwide.

The ParaLens Advance

The main body of the ParaLens Advance (as seen in Figure 1) can be attached as an objective to any conventional light microscope with standard Royal Microscopy Society threading (1). (Ring transition adapters are available for non-standard microscopes.) All filters required for TB detection are contained within a detachable blue filter arm (2), which is inserted into the main body and held in place by a pair of powerful magnets. The blue LED light source attaches to the filter arm (3) and produces powerful blue light with a wavelength of approximately 410-511 nm. The LED is DC powered and can be run using the included AC to DC Power Pack, or by additional power options, such as the ParaLens Advance Portability Pack (including 12 volt battery clips, a solar powered battery pack, a USB cable, and more) or the QBC Mobile Power Station (a 50-hour, 12 volt battery station that can be used to power the ParaLens Advance and other electronic devices).

Figure 1



ParaLens Advance Cutaway Diagram

Inside the filter arm, light from the LED light source passes first through a focusing lens (4) and then through an excitation filter (5) that allows only light in the 385-480 nm range into the ParaLens Advance main body. A dichroic beam splitter (6) redirects the light downward to the specimen. Powerful objective lenses (7) magnify the fluorescent light emitted by the specimen. For AFB detection, the WHO has recommended that specimens be analyzed first under lower magnification, and that suspected AFB should be confirmed under a higher magnification.[5] To facilitate this, the ParaLens Advance is available in a configuration (p/n 424331) that includes two main body assemblies, with 20x and 40x objectives.

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 Auramine dye, AFB will appear yellow-green or yellow-orange and proceed through to the viewer. An emission filter (8) reduces background noise and optimizes the fluorescence signal transmitted to the observer.

Reviewing Sputum Samples with the ParaLens Advance

Sputum Sample Collection

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.

Review of the sputum samples can proceed in two ways. The first is the direct smear technique, in which the unconcentrated sample is smeared onto the slide and heat fixed into place for staining and review. The other involves concentrating the sample, while keeping the bacilli alive, for use in cultures or PCR. Cultures and PCR 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. In most cases, a direct smear of the sputum sample will be sufficient for review.

(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.)

Preparing a Direct Smear Sample

To prepare a direct smear, take a small amount (~100 µl) of the sample and apply it to the slide using a wooden stick. 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 and permanently adhere the smear to the slide. 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.

Concentrating a Sample for Culture or PCR

As a preliminary matter, sample concentration 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 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 subsequent use 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.

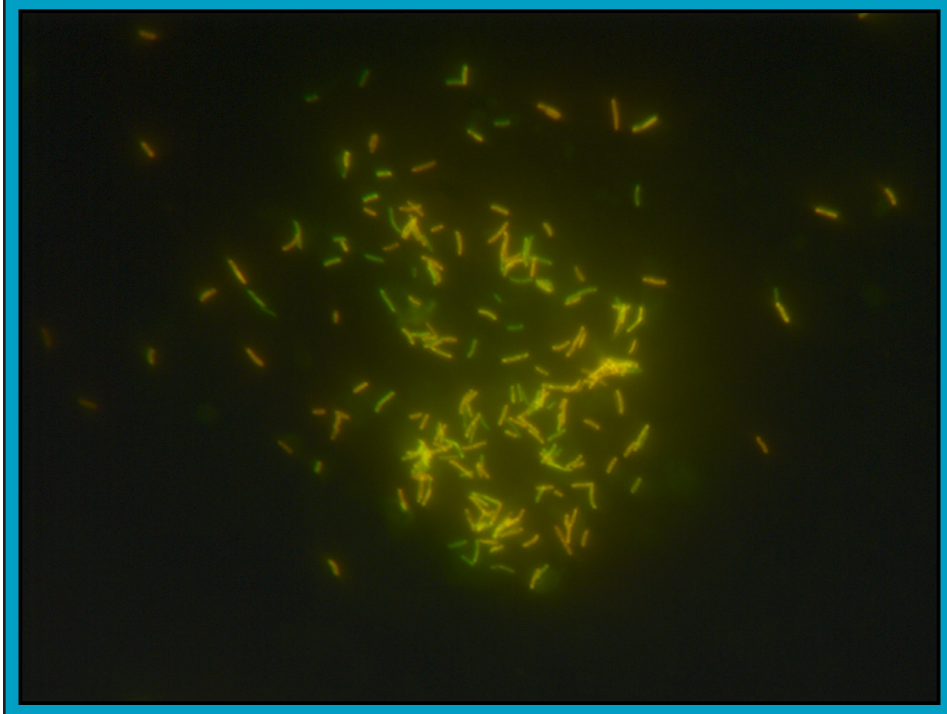
At this point, the tube containing the water and sputum mixture should be sealed and then centrifuged for approximately 20 minutes at 3,000 x g. Using a cold centrifuge is recommended, if possible, as it will increase the thickness of the resulting sample. When the centrifuge has finished, there should 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.

At this stage, you can begin to inoculate cultures or perform PCR with the digested sputum mixture. Several culture methods are available and differ based on the media and apparatuses available. For more information on culture creation, consult the text Essential Procedures for Clinical Microbiology (1998) from the American Society of Microbiology. For information on PCR, consult the text PCR and Diagnosis of Tuberculosis (2004), by Diana Williams.

Staining Process

With a conventional auramine-rhodamine stain, begin by

Figure 2



AFB, As Seen Under the ParaLens

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 Advance

To perform epifluorescence microscopy using the ParaLens Advance, first remove two objectives from the nosepiece of any compound light microscope. Screw the ParaLens Advance 20x and 40x objective main body assemblies into the open slots. Insert the blue filter arm into the 20x assembly. You should feel a distinct pull as the magnetic connection is established.

Place the blue LED light source on the distal end of the filter arm, and manually tighten the small thumb screw. Select the power option of your choosing. If using the included power pack, slide the proper international adapter for your location onto the adapter and press downward to click into place. Insert the plug end of power pack into an outlet, and the cord end into the power input of the LED light source. (Note: For instructions on the use of other power options, see the ParaLens Advance Operator's Manual.) Turn the black intensity control knob to turn on the LED light source.

Place the prepared slide on the microscope stage and clamp it into place. To most easily focus the ParaLens Advance, center the 20x objective over the area of interest and view the descent of the lens from a side perspective. Lower the lens until it is about 0.5 cm from the 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. When using the 20x objective, you should be able to view a sufficient amount of the sample in approximately 26 fields. If you discover what you believe to be AFB, switch to the 40x ParaLens Advance objective to confirm.

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.

Performing a TB Screen

Under the ParaLens Advance, 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 Advance.

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 following recommended grading scale, based on the

current WHO standard, can be used to quantify AFB[7]:

Report	200x (~ 26 Fields)	400x (~ 52 Fields)
No AFB Seen	0	0
Exact Count	1-29 AFB total	1-19 AFB total
+	1-10 AFB per field	20-199 AFB total
++	10-100 AFB per field	5-50 AFB per field
+++	>100 AFB per field	>50 AFB per field

Change Over

A slide stained for fluorescence microscopy can also be re-stained for light microscopy using the Ziehl-Neelsen method with no additional preparation. Simply perform the staining using standard methods.

Conclusion

The ParaLens Advance 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 Advance can be an important new weapon in the hands of TB labs fighting the worldwide fight against tuberculosis.

References

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