



FEASIBILITY STUDY PROTOCOL

Assessment of the operational feasibility of using an LED-based fluorescence microscope prototype for detection of pulmonary TB

STUDY OUTLINE

Technical and Financial Agency:

**Foundation for
Innovative New Diagnostics**

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Feasibility Study Protocol iLED
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Confidentiality statement

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Background

Lack of human resources for health is among the greatest challenges in TB control in many disease endemic developing countries. Staff shortages combined with increasing numbers of requests for sputum microscopy are creating unmanageable workloads with negative effects on TB case finding. The poor sensitivity of light microscopy further aggravates this situation. Simple yet rapid approaches to reduce the laboratory workload and to increase the sensitivity of direct smear microscopy need to be explored urgently. Replacing light microscopy by fluorescence microscopy would be one of the immediate options to improve the situation in high burden countries.

A systematic review by WHO/TDR and FIND [1] has shown that:

- a) Fluorescence microscopy is on average 10% more sensitive than conventional light microscopy. The increased sensitivity is greatest in low grade positives.
- b) The specificity is comparable.
- c) Reading a fluorochrome-stained smear takes only 25% of the time taken to read a ZN stained smear.

To date, the major constraints to the broader implementation of fluorescence microscopy are the high price for fluorescence microscopes and the lack of robustness and sustainability. Furthermore, the acceptability of dark rooms has been generally low in tropical settings.

Ultra-bright light emitting diodes (LED) have been identified as an attractive alternative to mercury vapor lamps since they may allow to overcome the disadvantages of conventional fluorescence microscopy [2]. Conventional fluorescence microscopes use expensive and very fragile gas discharge lamps with high power consumption and a short lifespan of 100-200 hours only (such as Xenon- or Mercury-lamps). Zeiss, in a joint development agreement with FIND, is developing an affordable, but high-quality LED-based fluorescence microscope. The use of a LED as a light source allows to lower the cost of the microscope and at the same time improve the instrument's power consumption (lifespan 10 000 hours) and easily regulate the light intensity. The new microscope needs to be robust (e.g. complete antifungal coating), must allow easy switching from bright light to fluorescence light and should have the option to be battery operated. These innovations, in combination with high-quality optics and the affordable price may allow wide introduction of fluorescence microscopy and gradual replacement of conventional microscopy in the public health sector of resource-limited countries.

The first prototype version of this microscope is now ready to undergo a rigorous performance evaluation. The results of this feasibility study will contribute to the optimization of the microscope design and functionality, but will also help to understand the true potential of LED-based fluorescence microscopy in general.

Study purpose

This is a blinded multicenter feasibility study at 4 reference laboratories to evaluate the performance characteristics of a new LED-based fluorescence microscope for the detection of *TB* in sputum specimens submitted for mycobacterial evaluation. Performance will be compared to conventional fluorescence microscopy and to LJ culture results.

Endpoints

1. Operational Performance:

- Assessment of technicians' appraisal of LED-based prototype microscope in terms of ease of use, maintenance, design, comfort, robustness, contrast, brightness etc.
- Assess necessity of dark room use for LED-based prototype microscope
- Assess suitability of LED-based prototype microscope for Auramine-Rhodamine stain and Methylene blue counterstain.
- Assess speed of fading for different stains and effect of fading on result interpretation.

2. Clinical Performance:

- Sensitivity for direct and concentrated smears in culture positive specimens compared to conventional fluorescence microscopy.
- Specificity in untreated culture negative specimens compared to conventional fluorescence microscopy.
- Inter-reader reproducibility of results (all smears read by 2 readers per site).
- Average field or time to positivity compared to light microscopy and conventional fluorescence microscopy.

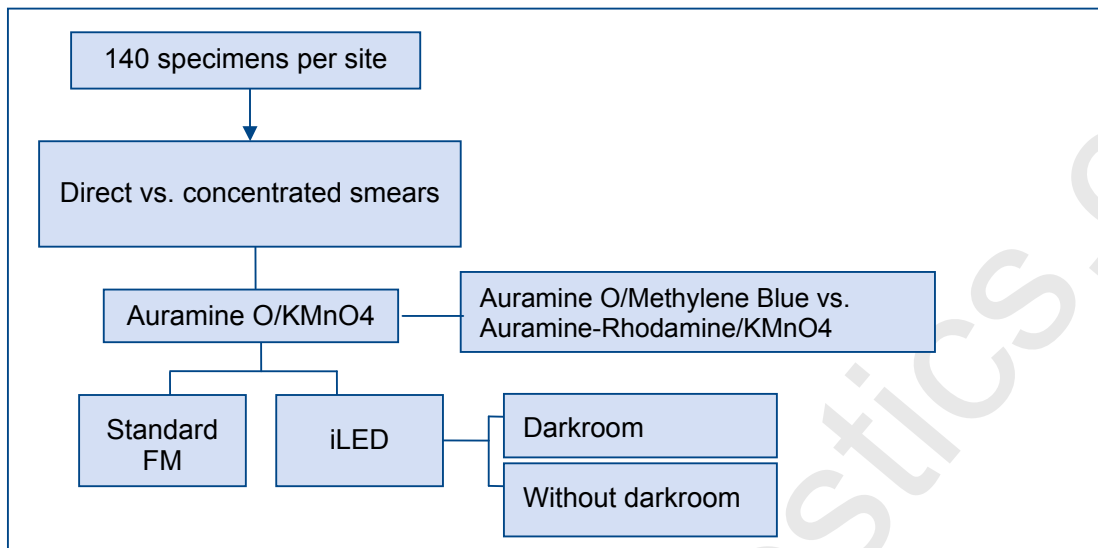
Laboratory sites

Each of the four reference laboratory sites is equipped with culture facilities, a high quality conventional fluorescence microscope, and will be provided by FIND with the LED prototype microscope. Two microscopists experienced in ZN and fluorescence microscopy will be responsible for reading at each of the sites. A lab supervisor will coordinate study activities.

Sample size

800 leftover sputum specimens will be included in the collection in total, 200 samples per site. Out of 200 generated slide sets 140 will be used for the study, applying below described selection criteria.

Study Design



Phase 1: Generation of evaluation slide panel

Specimen left overs will be examined by conventional fluorescence microscope (direct smear) and cultured by LJ. A slide set of 4 slides will be stored unstained per specimen – 1 direct smear and 3 smears from decontaminated and centrifuged pellet – until slide sets of 200 specimens have been stored. Out of 200 generated slide sets, 140 will be selected for the study, applying the following selection criteria: 1) valid culture result (not contaminated), 2) culture positive slide sets are given priority over culture negative slide sets. Each site will target for a culture positivity rate of 30%-50%. Smears will all be labeled with an ID number, for which baseline fluorescence smear result, semi-quantitative culture results, as well as date of culture inoculation and positivity, will be documented in an Excel summary sheet.

Phase 2: Blinded assessment of necessity for dark room use in a subset of 20 slides

Per site, a subset of 20 Auramine/KMnO₄ (10 low positive, 5 high positive, 5 negative in baseline examination) stained slides will be examined in the Primo Star iLED microscope (40 x 10 magnification) by both readers with and without dark room (but no direct sunlight exposure in reading area) in a blinded fashion. In addition to recording results and time to result, lab techs will provide opinion on the necessity of dark room use for the new microscope by completing form “Phase 2”. Should this phase show that a darkroom does not, or only minimally, improve the ease and spread of reading, all following examinations will be done without a darkroom for the LED microscope.

Phase 3: Blinded performance comparison between LED microscope prototype, standard fluorescence microscope & culture by examination of 140 sputum specimens per site stained with Auramine/KMnO₄

The 140 direct and 140 concentrated smears will be stained by Auramine/KMnO₄. Blinded, comparative examination of 140 direct and 140 concentrated Auramine/KMnO₄ stains will be undertaken in with conventional fluorescence microscope 40 x 10 magnification and

Primo Star iLED 40 x 10 magnification), by 2 readers per site. The conventional fluorescence microscope will be used in the darkroom, whereas the Zeiss microscope will be used without darkroom, in an area without direct sunlight exposure (unless phase 2 showed that a darkroom considerably improves ease of reading).

Assessed will be the performance of the Primo Star iLED prototype microscope in direct and concentrated smears in comparison to conventional fluorescence microscopy and the inter-reader reproducibility of results. LJ culture will be used as the gold standard, the smear results will be ultimately compared to. In addition, fields to positivity will be determined and recorded for all readings. For negative and scanty slides, 30 fields for the fluorescence stains. Staining with fluorescence stains must not be done more than 48 h before the slides will be read.

In order to ensure blinding, slides will be overlabeled once in between readings (see described in SOP), labels will be provided by FIND. Forms "Phase 3" will be completed and examination of slides be done according to SOP.

Phase 4: Blinded examination of a subset of 20 Auramine/Methylene Blue and 20 Auramine-Rhodamine/KMnO₄ stained slides

Supervisor selects unstained slides from the remaining slide set to stain 10 low positive slides; 5 high positive slides and 5 negative slide sets by Auramine/Methylene Blue and slides from the same specimen IDs by Auramine-Rhodamine/KMnO₄. Conduct blinded, comparative examination of these slides and compare results to the smear results obtained for the examination of concentrated Auramine/KMnO₄ slides (2 readers per site). For negative and scanty slides, 30 fields will be read. Staining must not be done more than 48 h before the slides will be read. Examination will again take place in the darkroom for examination with the conventional fluorescent microscope and in an area without direct sunlight for the LED microscope.

Phase 5: Blinded assessment of reading time per slide for new prototype compared to conventional fluorescence microscope

Blinded reading of 5x negative, 10 low and 5 high positive slides by 2 readers per site using a conventional light microscope (ZN, concentrated smears, same specimen IDs), a conventional fluorescence microscope and the LED microscope (Auramine slides, concentrated smears, same specimen IDs). For negative and scanty smears, 100 fields will be examined with the light microscope, 30 fields with the fluorescence microscopes. Higher positive smears will be examined until positivity and semiquantitative result have been established. Reading time will be determined for every slide (start time when slide is placed under the microscope and reading starts; stop time when semiquantitative result has been established or for negative and scanty smears until 30 (Auramine) /100 (ZN) fields have been read). It is important, that reading is not interrupted and that readers concentrate during this phase and are not distracted.

Phase 6: Assessment of lab technicians' appraisal of Primo Star iLED fluorescence and bright light function by unblinded assessment of 2 positive Auramine and 2 positive ZN slides per site in comparison to conventional microscopes

Lab techs' appraisal of operational criteria like brightness, contrast, ease of use and robustness will be assessed in comparison to the conventional microscopes. All readers will fill a questionnaire on their appraisal of the new microscopes.

Phase 7: Blinded assessment of fading speed of fluorescence stains

In general, all slides shall be stored in slide boxes and kept after result interpretation. Date of staining shall be recorded on the label and in the Excel sheet "Phase 1". 5 smear-positive Auramine/KMnO₄, 5 smear-positive Auramine/Methylene Blue and 5 Auramine-Rhodamine slides shall be examined once weekly (and later once monthly) in the LED microscope until found to be negative. Slides will be overlabeled in between readings. Speed of fading and effect on result interpretation will be assessed with the help of a report form "Phase 7".

Remaining unstained slides will be stored to answer additional questions that may occur during the study.

Ethical considerations

Human subjects' risks: There are no risks for human subjects. This is a purely laboratory based study. No clinical, demographic, or identifying information about the patients whose left over specimens are being used will be collected, and there are no linkages between study data and identifying information about these patients. No specimen will be stored, no sample bank created. Risks to confidentiality: There are no risks to confidentiality. No personal health information or demographic data will be collected for this laboratory study and linkage between study data and patient records is not possible. According to international guidelines, the study should therefore be exempted from ethical approval. This decision will however be left to local institutions.

Standards

LJ culture will be used as the gold standard. NALC-NaOH-Citrate sputum decontamination procedure will be used. Commercially available staining kits will be provided by FIND and will be standardized for all sites.

30 fields per slide at a magnification of 40 x 10 will be examined by both fluorescence microscopes. Model, serial number and age of the conventional microscope will be documented.

Blinding

1. Slides will be labeled as for routine purposes prior to staining (specimen ID, date, direct or from pellet), but over-labeled according to FIND Excel sheet post-staining.
2. Labeling and over-labeling has to be done by the supervisor who is not involved in slide reading.
3. The fluorescent slides are read on one fluorescence microscope by both readers. These slides will then have to be over-labeled by the supervisor, before they can be examined with the 2nd fluorescence microscope. Half of the slides will be read first in the conventional, then in the LED, the other half first in the LED, then in the conventional fluorescence microscope to avoid bias by fluorescence bleach effects.
4. Reader 1 and reader 2 have to document results independently from each other without exchanging results. It is the task of the supervisor to ensure independent reading. In phase 3, result forms will be given to the supervisor every evening, so that readers have no access to old results.
5. Labels and Excel lists for IDs pre- and post over-labeling will be provided by FIND. These lists shall only be accessible for the person responsible for labeling the slides, not for the lab technicians reading the slides.
6. ID lists for every day and every reader will be provided by FIND, so that the daily workload is standardized and conditions the same for each microscope.

References

1. Steingart, K.R., et al., *Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review*. Lancet Infect Dis, 2006. **6**(9): p. 570-81.
2. Anthony, R.M., et al., *Light emitting diodes for auramine O fluorescence microscopic screening of Mycobacterium tuberculosis*. Int J Tuberc Lung Dis, 2006. **10**(9): p. 1060-2.

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