REPORT FOR WHO

Primo Star iLED
Evaluation & Demonstration

Use of reflected light LED-based fluorescent microscopy for detection of tuberculosis in low and medium income settings

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TABLE OF CONTENT

Table of content 2
Partner institutions 3
Acknowledgements 4
Background 5
Specifications of existing systems for LED-based Fluorescence Microscopy 7
Summary of literature for performance of LED FM 8
FIND study outline 9
Detailed FIND study results – Core Studies 11
  Feasibility study results 11
  Evaluation study results 13
  Demonstration study results 21
  Economic data 40
Detailed FIND study results – Substudies 47
  Comparative studies for existing LED-based FM systems in Uganda and Zambia 47
  Comparison of staining methods and reagents 53
  Assessment of fading speed for Auramine stained slides 57
Other implementation issues 59
  Training 59
  Electrical supply and backup power 60
  Technical and customer support 61
  Use as diagnostic multi-disease platform 62
Summary and conclusions 64
Literature 69

PARTNER INSTITUTIONS

Assessment in reference laboratories

- *Pham Ngoc Thach Hospital, Ho Chi Minh City, Vietnam*
- *National Tuberculosis Reference Laboratory, Bangkok, Thailand*
- *Christian Medical College Vellore, India*
- *Institut für Mikrobiologie, Gauting, Germany*
- *Universidad Peruana Cayetano Heredia, Lima, Peru*
- *Infectious Disease Institute and Mulago Hospital, Kampala, Uganda*
- *Centre for Infectious Disease Research, Lusaka, Zambia*
- *Reference Laboratory for Mycobacteria, Borstel, Germany*
- *Medical Research Council, Fajara, The Gambia*

Implementation in microscopy centers

India (9 demonstration and 3 supervisory sites)

- *Central Tuberculosis Division, Ministry of Health, Government of India*
- *Christian Medical College, Vellore*
- *National Jalma Institute of Leprosy and other Mycobacterial Diseases, Agra*
- *New Delhi Tuberculosis Centre, Delhi*

Vietnam (3 demonstration and 1 supervisory site)

- *National Tuberculosis Programme*
- *Pham Ngoc Thach Hospital, Ho Chi Minh City*
- *KNCV Tuberculosis Foundation*

Peru (3 demonstration and 1 supervisory site)

- *National Tuberculosis Programme*
- *Instituto Nacional de Salud*
- *Universidad Peruana Cayetano Heredia, Lima*

Lesotho (2 demonstration sites and 1 supervisory site)

- *National Tuberculosis Programme*
- *National Tuberculosis Reference Laboratory, Maseru*

Ethiopia (2 demonstration sites and 1 supervisory site)

- *National Tuberculosis Programme*
- *EHNRI National Reference Laboratory, Addis Ababa*

Thailand (1 demonstration site and 1 supervisory site)

- *National Tuberculosis Programme*
- *National Tuberculosis Reference Laboratory, Bangkok*
- *Bamrasnaradura Infectious Disease Institute*

South Africa (3 demonstration sites and 2 supervisory sites)

- *National Health Laboratory Services, Kwa Zulu Natal*
- *National Tuberculosis Reference Laboratory, Johannesburg*

Russia (2 demonstration sites and 1 supervisory site)

- *Samara Oblast Tuberculosis Programme*
- *Samara Oblast Tuberculosis Reference Laboratory*

Cambodia (3 demonstration sites and 1 supervisory site)

- *National Tuberculosis and Leprosy Programme (CENAT)*
- *National Tuberculosis Reference Laboratory, Phnom Penh*

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FIND study team

BACKGROUND

Sputum microscopy remains the cornerstone for the diagnosis of pulmonary tuberculosis (TB) in low-income countries. Indeed case-reporting classifications are currently based on microscopy results. There are an estimated 40,000 microscopy centers with sufficient theoretical capacity to diagnose TB in the developing world, and many years of effort has gone into trying to strengthen this global microscopy network. Thus, although developing simpler tools that might replace microscopy is a priority, improving microscopy by making it more sensitive or more convenient could have an important impact.

For this reason, FIND has assessed a number of potential platforms for improving TB microscopy through advances in instrumentation. Automated microscopy, flow cytometry and other alternative methods have been proposed, but are not yet developed to a stage of proven feasibility. On the other hand, fluorescence microscopy (FM), which takes advantage of the specific staining characteristics of acid-fast organisms with dyes such as auramine and rhodamine, is of proven efficacy and is widely used in industrialized countries. First developed in the 1930s, FM for TB has the primary advantage of allowing slides to be examined at much lower magnification (200 to 400X) than is routinely used with conventional Ziehl-Neelsen (ZN) staining (1000X). This allows a greater portion of the stained material to be examined during a similar period of time, resulting in both more sensitive detection and greater speed. FM also obviates the need for immersion oil and for heat-fixing the slides during staining, which are small, but important additional advantages.

Policy support for expanded use of FM came from an expert meeting at WHO in September 2005. This meeting was organized around three systematic reviews of different aspects of microscopy and was commissioned by the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and FIND. The systematic review of fluorescence microscopy included over 50 publications from the peer reviewed literature and provided convincing data that, in experienced hands, FM was faster and an average of 10% more sensitive than conventional ZN microscopy, without a loss of specificity.[i] Recommendations on expanded use of FM outside of high-throughput laboratories were restrained by lack of information on how it would perform in areas other than reference centers.[ii]

Regardless of WHO recommendations on usage, FM is unlikely to have an impact as long as the equipment required remains expensive and maintenance-intensive. Conventional FM is performed with a sophisticated microscope containing an excitation filter, a dichromatic beam splitter, an emission filter, and a high intensity light source such as a xenon arc lamp or mercury-vapor lamp. New equipment of this type generally costs $US 15,000 to 20,000. Unfortunately, the bulbs used in these conventional systems tend to overheat, requiring a sometimes noisy cooling fan, have a half life of some 200 hours, and are liable to shatter. Bulb replacement is costly (~$200) and often logistically difficult. The requirement of a darkroom is a further practical obstacle to routine use that adds to the cost of installation, and often to a sense of isolation for the technologist. For these reasons, FM has never played a significant role in routine TB detection in disease endemic countries, regardless of its many advantages.

A recent technical advance, the advent of low-cost ultrabright light emitting diodes (LEDs), made feasible the development of inexpensive LED-illuminated microscopes that could be used in place of expensive mercury-vapor bulb solutions. LED-illuminated fluorescence microscopy was first described in 2001,[iii] and an early technical summary of this area was published by Silk in 2002.[iv] The feasibility of using LEDs to adapt existing expensive fluorescent microscopes was shown by Anthony and colleagues, who adapted a conventional FM to use an LED light as described in their 2006 publication.[v]

Since then, FIND has fostered the development of a dual-use microscope that could easily be switched to perform either fluorescent or brightfield applications. Highest quality, affordability - especially for high-burden countries - as well as a strong wide-reaching customer support and distribution capacity was considered essential. Zeiss, a worldwide leader in optics and microscopy, and with high-end LED-based fluorescent systems already in development, was a natural partner for FIND to achieve the following product specifications:

- high-grade optics
- reflected rather than transmitted blue light for fluorescent applications
- battery power on AC failure
- easy switching between fluorescent and brightfield viewing
- utility outside a darkroom
- >10,000 hour bulb life
- inexpensive bulb change
- LED and filter sets that would allow use of Auramine O and acridine orange for TB and parasitologic examinations

The product result of this collaboration, Primo Star iLED, is now commercially available. During the process of development of the iLED, some of the other LED-based options for TB detection with fluorescent microscopy entered development or became commercially available. A summary of technical details has recently been published[vi] and is provided in Table 1 below. Table 2 provides an overview of published feasibility studies for these technologies.

In order to harmonize the strategies for evaluating the performance and impact of these different microscopes or microscope adaptors, FIND and WHO held a meeting on *LED Fluorescence Microscopy* on 26 March 2008 in Geneva with a number of scientists, clinicians and public health specialists working in this field. During the meeting, preliminary plans for Demonstration projects were shared in order to generate a strong and harmonized data set for eventual submission for review by the Stop TB Department of WHO and its expert committees.

SPECIFICATIONS OF EXISTING SYSTEMS FOR LED-BASED FLUORESCENCE MICROSCOPY

Table 1: Specifications of LED-based fluorescence microscopy systems

	Name	Manufacturer	Integrated solution (all- in-one)	Light transmission	LED Intensity regulation	Available objectives	Quick change of magnification	Rapid switch between LM & FM	Battery available
	Primo Star iLED™	Carl Zeiss, Germany	Yes	Reflected light	Yes	10x, 20x, 40x, 100x (oil) Antifungal coating	Yes	Yes	Yes
	FluoLED®	Fraen SRL, Italy	No (clamp-on adaptor only)	Transmitted light§	Yes	Depending on bright field microscope used	Yes	No	Yes
	Lumin™	LW Scientific, USA	No (Add-on module only)	Reflected light	Yes	20x, 40x, 60x, 100x (oil)	No	No	Battery adaptor available
	CyScope®	Partec, Germany	Yes	Reflected light	Yes	20x, 40x, 100x (oil)	Yes	Yes	Yes
	ParaLens™	QBC Diagnostics, USA	No (Add-on module only)	Reflected light	Yes	40x, 60x (oil), 100x (oil)	No	No	No

§ Under certain circumstances, transmitted light is known to generate glare and result in lower FL contrast compared to reflected light transmission.

SUMMARY OF LITERATURE FOR PERFORMANCE OF LED FM

Table 2: Literature overview LED performance

Author (Year)	Sample size	Country (origin of patients)	Study setting	LED device	Gold standard	Sensitivity			Specificity			Reading time/ Nr of fields	Other findings/ Comments
						ZN	FM	LED FM	ZN	FM	LED FM		
Marais BJ (2008)	221 smears	South Africa	Primary health care clinics	Royal Blue Luxeon (Philips Lumileds)	MGIT/LJ Culture	61.1%	73.6%	84.7%	98.9%	99.8%	98.9%	Mean times for negative smears: FM: 1.4 min LM: 3.6 min	Interreader variation lower than in conventional FM or LM
Van Deun A (2008)	461 smears	Thailand	Reference laboratory, Bangkok	FluoLED (Fraen)	FM	_	_	98%	_	_	99%	_	_
	>1000 smears/ month over 2 years	Tanzania	2 Health centres, Dar es Salaam	FluoLED (Fraen)	_	_	_	20% > ZN (data not shown)	_	_	HFP: 21%, 7% HFN: 13, 20% (167 slides)	ZN: 100 fields FM: 40 fields	High user acceptance Rechecking slide suboptimal (storage & unreliable recording)
Nabeta P (2008) (*)	545 smears	Peru, Thailand, Germany, Gambia	4 Reference laboratories	Primo Star iLED (Zeiss)	LJ culture	_	66.6%	72.4%	_	99.2%	98.8%	45-75% faster than LM	Very positive feedback for iLED during users appraisal
Trusov A (2009)	R: 502 M: 205	Russia (R), Macedonia (M)	2 Reference laboratories	Lumin (LW Scientific)	LJ culture	R: 28.5% restained 55.6% direct M: 78.0% restained	R: 52.5% M: 87.8%	R: 72.8% M: 87.8%	R: N/A M: 100%	R: N/A M: 100%	R: 99.4% M: 100%	ZN: 300 fields neg, 100 fields pos FM: 150 fields	Higher sensitivity than ZN in low bacillary load materials such as saliva
Boehme C (**)	Panel of 330 slides per site	Vietnam, India, Peru, Germany, Thailand	5 Reference laboratories	Primo Star iLED (Zeiss)	LJ culture	90.5%	96%	96.3%	100%	94.6%	iLED 40x: 100% iLED 20x: 96.4%	>55% faster than LM	Operational performance rated very high

(*) poster presented at the 39th Union World Conference on Lung Health, October 2008

(**) data submitted for publication

FIND STUDY OUTLINE

Over the last 18 months, extensive LED FM studies focusing on, but not limited to the Primo Star iLED, have been carried out by FIND together with partners. These included analytical studies with experimentally-produced standardized materials, comparative evaluation studies in controlled settings, and implementation projects in a wide variety of settings of intended use.

Feasibility study

The feasibility of using the Primo Star iLED prototype for TB detection was determined in four laboratories experienced in conventional FM. A set of study slides with 30-50% positivity rate was generated prospectively from 140 sputum specimens from patients undergoing diagnostic evaluation for suspected TB. The specimens were cultured on LJ medium, the reference standard for the study. The slides were used in blinded studies assessing several questions, including: the performance of the iLED microscope in comparison to the more expensive standard FM scope, the necessity of a darkroom, the suitability of the iLED excitation wavelength for alternative stains (Auramine or Rhodamine) and counterstains ($KMnO_4$ or Methylene blue), the reading time per slide, the speed of fading of stored slides, and laboratory technician appraisal of the usability and technical suitability of the microscopes.

Evaluation study

Based on the findings of the feasibility study, the microscope design was locked and evaluation studies were initiated to determine the performance in a larger samples size with light microscopy as one of the comparative methods. This study was conducted in four reference laboratories with experience in FM that evaluated panels of slides with specific grading of positivity created by a single facility and shipped to reference laboratories with prior experience in FM. The endpoints of the study were: sensitivity of iLED in smear positive panel slides compared to light microscopy and conventional FM, specificity in smear negative slides compared to light microscopy and conventional FM, assessment of technician appraisal of the iLED FM in terms of ease of use, maintenance, design and comfort, robustness, contrast, brightness, etc., and assessment of the adequacy of 20X vs 40X magnification for slide screening.

Demonstration study

Once performance targets were met through the evaluation study, a Demonstration project was initiated in coordination with National and Regional TB Control Programs in India, Vietnam, Thailand, Cambodia, South Africa, Lesotho, Ethiopia, Russia and Peru. FIND criteria for country selection for the study were: an agreement at National/Regional Levels (MOU) with NTP and/or MOH; a high-burden of TB; a low or middle income ranking; local presence of FIND or an implementing partner; and settings representative of the global TB and HIV situation. There were 28 microscopy centers and 12 supervisory sites chosen for this large study, with site selection based on the rate of smear-positivity, training of microscopists, volume of work, reliability of AC power (sites with intermittent power supply were intentionally selected), interest in the project, and accessibility of study sites for supervisory visits. None of the microscopy centers had prior experience with FM.
The objectives of the project were:

1. To assess the feasibility of implementing Primo Star iLED for TB diagnosis at microscopy centers without prior experience with FM in low- to moderate-income settings and to identify barriers to implementation
2. To determine the false positivity and negativity rate of LED fluorescence reading compared to a ZN baseline and to results from the supervisory site

3. To determine the trend of false positivity and negativity rates of LED fluorescence reading over time (with increasing experience)
4. To assess the impact of this implementation on daily workload and case detection rates for low, middle and high-volume settings
5. To determine lab technicians' appraisal of Primo Star iLED
6. To evaluate detailed costs associated with LED-based fluorescence microscopy in comparison with conventional methods
7. To identify minimal training needs and develop training modules accordingly

Substudies were conducted:

8. To establish comparative performance data for alternative LED-based approaches
9. To compare fluorescence staining methods
10. To assess effects of fading speed on external quality assurance by rechecking

The size and complexity of the Demonstration study reflects the importance of having true performance data from sites without prior experience in FM. LED-based FM is likely to see markedly expanded use in the coming few years, and it will be critical to understand the training and supervisory needs for peripheral microscopy sites using this technology as compared with conventional brightfield examination of ZN-stained slides. Data from these Demonstration projects as well as other published and unpublished documentation will be submitted to WHO expert committees for review in the 3rd quarter of 2009.

DETAILED FIND STUDY RESULTS – CORE STUDIES

Feasibility study results

Methods
Performance of iLED compared with FM was assessed at four reference laboratories experienced in conventional FM (*National Tuberculosis Reference Laboratory, Bangkok, Thailand; Institut für Mikrobiologie, Gauting, Germany; Universidad Peruana Cayetano Heredia, Lima, Peru; Medical Research Council, Fajara, The Gambia)*. A set of study slides with 30-50% positivity rate was prepared prospectively from sputum specimens of patients undergoing diagnostic evaluation for suspected tuberculosis. The sputum specimens were cultured on LJ medium. 140 sputum samples were then examined per site by direct and concentrated methods using standard FM and iLED (both at 400x; 30 fields). In order to ensure blinding between the two methods, smears were overlabelled between readings. The assessment comprised a qualitative performance appraisal based on a questionnaire. Time-to-result was measured for a subset of 40 specimens. We also looked at the suitability of alternative staining solutions in the same subset. The following study questions were addressed:

1. the performance of the iLED microscope in comparison to the more expensive standard FM scope, with LJ culture as reference standard
2. the necessity of a darkroom
3. the suitability of the iLED excitation wavelength for alternative stains (Auramine or Rhodamine) and counterstains ($KMnO_4$ or Methylene blue)
4. the reading time per slide
5. laboratory technician appraisal of the usability and technical suitability of the microscopes.

Results
15 out of 560 specimens had to be excluded because of contamination. 263 out of 545 (48%) specimens were LJ positive; 211 (80%) of these were found to be smear positive by FM or iLED (concentrated); 84/211 (40%) were 3+; 42/211 (20%) 2+; 64/211 (30%) 1+; 21/211 (10%) scanty; and 334 were negative. An initial sub-study comparing iLED use with and without dark room found similar performance. Further examinations with iLED were therefore performed without use of a dark room.

The sensitivity of iLED was $\geq$ conventional FM at all study sites (Tables 3 and 4). For unknown reasons, the sensitivity for both iLED and FM was lower post NALC-NaOH treatment for two of the four sites. The iLED showed an equivalent specificity as seen with conventional FM. The examination time using iLED was equivalent to FM and 45-75% faster than LM (Fig 1). The operational performance characteristics of iLED were rated superior to FM and LM (Table 5). All staining solutions tested (Auramine/KMnO4, Auramine-Rhodamine/KMnO4 and Auramine/Methylene Blue) were found to be similarly suitable for reading with iLED.

Conclusions
This assessment demonstrated good clinical performance of iLED equivalent to conventional FM, even when used without a darkroom. This is a significant advantage with regard to user acceptance. The iLED was found to be user-friendly and well-designed, with users providing enthusiastic feedback regarding the image quality. Equally important was the average gain in reading time of >60%.

Based on the findings of this study, the microscope design was locked and evaluation studies were initiated to determine the performance in a larger samples size with light microscopy as one of the comparative methods.

Table 3: Performance comparison of iLED and FM – direct smears

Direct	**Standard FM**		**Primo Star iLED**	
	Sensitivity (%) 95%CI	**Specificity (%) 95%CI**	**Sensitivity (%) 95%CI**	**Specificity (%) 95%CI**
Site 1	72 (61-80)	96 (87-99)	83 (74-89)	96 (87-99)
Site 2	80 (68-99)	96 (89-99)	82 (70-89)	95 (88-98)
Site 3	57 (44-68)	97 (90-99)	57 (44-68)	99 (93-100)
Site 4	59 (45-71)	95 (88-98)	60 (47-72)	96 (90-99)

Table 4: Performance comparison of iLED and FM – concentrated smears

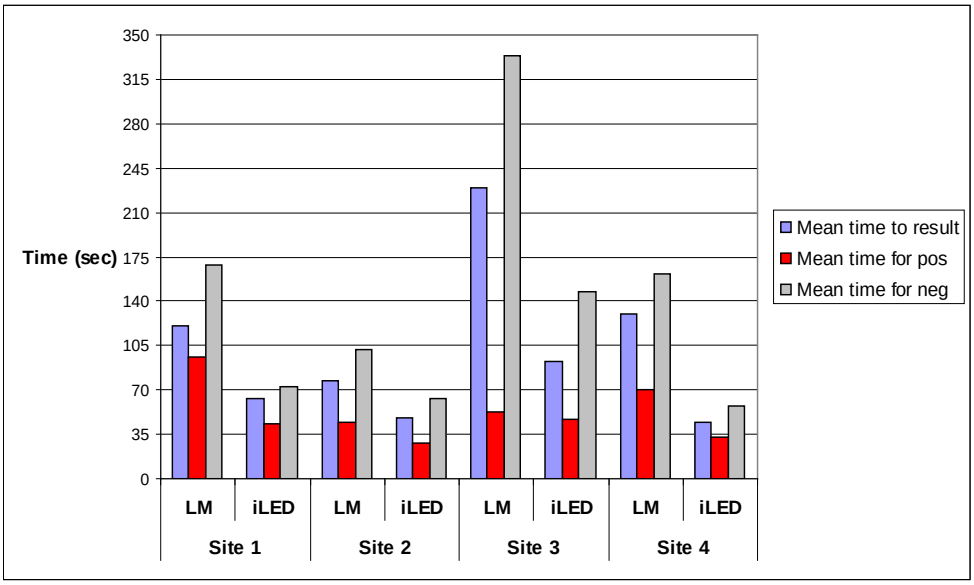
Concentrated	Standard FM		Primo Star iLED	
	Sensitivity (%) 95%CI	Specificity (%) 95%CI	Sensitivity (%) 95%CI	Specificity (%) 95%CI
Site 1	73 (63-81)	100 (93-100)	73 (63-81)	98 (90-100)
Site 2	68 (56-79)	99 (93-100)	72 (59-82)	97 (91-99)
Site 3	52 (40-64)	99 (93-100)	66.1 (54-77)	100 (95-100)
Site 4	74 (60-84)	100 (95-100)	79 (67-88)	99 (93-100)

Table 5: Laboratory technician appraisal

Criteria	**Primo Star iLED (Score 0 to +++)**	**Comments**
• **Contrast** • **Resolution** • **Depths of focus** • **Signal-to-noise ratio** • **Homogeneity of fluorescence illumination** • **Suitability of alternative stains**	**+++** **+++** **+++** **+++** **+++** **+++**	"We have always been rather satisfied with our FM until we used this one… it is less cumbersome to read with the iLED since **fluorescence of AFBs is very bright** and **easy to recognize in the dark background**…" **All +++ (**Au/KMnO4; Au-Rhod/KMnO4; Au/MethBlue) Preferred stain: Au/KMnO4

0 = not satisfied; + =acceptable, but less than expected; ++ = satisfied; +++ = very satisfied, and exceeding expectations.

Figure 1: Mean time to result for light microscopy versus iLED



Evaluation study results

Methods

Once feasibility targets were met, the final Primo Star iLED manufacture series of the microscope was evaluated for performance in the detection of TB in sputum specimens in a laboratory-based, blinded, multi-center study. Panels of slides with specific grading of positivity were produced by a single facility and shipped to 5 reference laboratories (*National Tuberculosis Reference Laboratory, Bangkok, Thailand; Pham Ngoc Thach Hospital, HCMC, Vietnam; Christian Medical College, Vellore, India; Institut für Mikrobiologie, Gauting, Germany; Universidad Peruana Cayetano Heredia, Lima, Peru)* with prior experience in FM that carried out the study. Five quality-assured slide panels were generated from homogenized sputum and distributed to each of the sites, 110 for ZN staining (one per sputum) and 220 for Auramine O/KMnO4 (two per sputum). Sensitivity, specificity and reading-time were assessed by eight readers for iLED (at 20X and 40X, without darkroom, after one week's experience) in comparison to ZN and conventional FM (with darkroom). To ensure blinding, each of the slides was labeled with a different ID number. In-house staining solutions were prepared according to standardized SOPs. Reading-time was determined in two ways for which details were defined in a SOP: a) recording of time-to-result in seconds; and b) recording of results after 30 seconds, 1 min, 3 min and 5 min. The endpoints of the study were:

1. Sensitivity of iLED in smear positive panel slides compared to light microscopy and conventional FM
2. Specificity in smear negative panel slides compared to light microscopy and conventional FM
3. Assessment of technician appraisal of iLED in terms of ease of use, maintenance, design and comfort, robustness, contrast, brightness, etc.
4. Assessment of the adequacy of 200X vs 400X magnification for slide screening.

Data analysis

Sensitivity and specificity (95%CI) were calculated for each method compared with culture as gold standard. The sensitivity and specificity of the methods were compared in a pairwise fashion and McNemar's test for equality of proportions for paired samples was used to determine whether the

proportions of positive and negative results were the same for each method, using a 5% significance level.

Results

The high sensitivity and specificity achieved with iLED across sites is shown in Tables 6, 7 and 8 below. Sensitivity of iLED during this assessment was significantly higher than ZN (overall 6%; 95% CI 94.5-97.6) and equivalent to FM. As expected, a greater difference in sensitivity (25%) was found for very low-positive slides. Specificity of iLED-400X was equivalent to ZN, whereas specificity of iLED-200X was slightly lower and equivalent to FM (see Tables 9, 10a and 10b for details).

Average gain in reading time compared to ZN was 60% for FM and iLED-20X and 55% for iLED-40X. As shown in Table 11, >80% of positive slides had been correctly identified within 30 sec for the fluorescence methods, whereas this was only the case in <50% for ZN. A reading time of 3 to 5 min per slide resulted in a significant additional yield for ZN, whereas this was not the case for iLED or FM.

Operational performance and user acceptance was assessed with a user appraisal questionnaire which was completed by the eight readers (see summary in Table 12). Overall, acceptance was very high and uptake enthusiastic. Based on their findings, three of the five sites decided to adopt iLED for routine work.

Conclusions

This assessment confirmed good operational and clinical performance of iLED at reference centers using a standardized panel of prepared slides. The excellent sensitivity and specificity and the short reading time indicate the potential benefit of making this new generation of microscopes widely available, especially in microscopy centers with a high daily workload and a high burden of TB.

Our findings support previous studies[i] that demonstrated the superior diagnostic sensitivity of fluorescence microscopy compared with conventional light microscopy. As expected, the difference in sensitivity was greatest in low-positive slides. Also, data show that where little time is spent on reading, FM and iLED are far superior compared to ZN. It is therefore likely that the impact of introducing LED would be greatest in settings with a high number of low-positive patients (for example, settings with high HIV prevalence) and in overworked microscopy centers.

The use of 20X rather than 40X did not have any additional benefit with regard to reading time or accuracy in this study. However, several readers indicated that over time, and with more practice, reading became easier and faster for this lower magnification. Artifacts, which could have been confused with mycobacteria by users with little experience in FM, then became more easily distinguishable.

Based on the findings of this study, the next project phase was initiated: demonstration of iLED-40X effectiveness in programmatic conditions at microscopy centers without prior experience in FM.

Table 6: Overall sensitivity and specificity for panel reading at five reference laboratories				
	ZN	**FM**	**iLED 40**	**iLED 20**
Sensitivity	**543/600 90.5% [87.9%, 92.6%]***	**576/600 96.0% [94.1%, 97.3%]**	**578/600 96.3% [94.5%, 97.6%]**	**576/600 96.0% [94.1%, 97.3%]**
Very low pos (scanty)	73/120 60.8% [51.9%, 69.1%]	98/120 81.7% [73.8%, 87.6%]	102/120 85.0% [77.5%, 90.3%]	98/120 81.7% [73.8%, 87.6%]
Low pos (1+)	350/360 97.2% [95.0%, 98.5%]	358/360 99.4% [98.0%, 99.8%]	357/360 99.2% [97.6%, 99.7%]	358/360 99.4% [98.0%, 99.8%]
High pos (2+, 3+)	120/120 100.0% [96.9%, 100.0%]	120/120 100.0% [96.9%, 100.0%]	119/120 99.2% [95.4%, 99.9%]	120/120 100.0% [96.9%, 100.0%]
Specificity	**280/280 100.0% [98.6%, 100.0%]**	**265/280 94.6% [91.4%, 96.7%]**	**280/280 100.0% [98.6%, 100.0%]**	**270/280 96.4% [93.6%, 98.0%]**

***95% confidence intervals are shown in brackets in this and following tables**

Table 7: Site- & reader-specific sensitivity for panel reading at 5 reference laboratories				
	ZN	**FM**	**iLED 40**	**iLED 20**
CMC Vellore, India				
Reader 1 (Experience ZN: 8 years ; FM: 2.5 years)	85.3% [75.6%, 91.6%]	96.0% [88.9%, 98.6%]	96.0% [88.9%, 98.6%]	96.0% [88.9%, 98.6%]
INS, Peru				
Reader 1 (Experience ZN: 9 years ; FM: 9 years)	93.3% [85.3%, 97.1%]	93.3% [85.3%, 97.1%]	94.7% [87.1%, 97.9%]	93.3% [85.3%, 97.1%]
Reader 2 (Experience ZN: 6 years ; FM: 5 years)	96.0% [88.9%, 98.6%]	96.0% [88.9%, 98.6%]	97.3% [90.8%, 99.3%]	96.0% [88.9%, 98.6%]
NRL, Thailand				
Reader 1 (Experience ZN:30 years ; FM: 30 years)	88.0% [78.7%, 93.6%]	97.3% [90.8%, 99.3%]	96.0% [88.9%, 98.6%]	97.3% [90.8%, 99.3%]
Reader 2 (Experience ZN: N/A ; FM: N/A)	88.0% [78.7%, 93.6%]	96.0% [88.9%, 98.6%]	96.0% [88.9%, 98.6%]	94.7% [87.1%, 97.9%]
PNTH, Vietnam				
Reader 1 (Experience ZN: 5 years ; FM:5 years)	94.7% [87.1%, 97.9%]	94.7% [87.1%, 97.9%]	94.7% [87.1%, 97.9%]	94.7% [87.1%, 97.9%]
Gauting, Germany				
Reader 1 (Experience ZN: 10 years ; FM: 4.5 years)	90.7% [82.0%, 95.4%]	97.3% [90.8%, 99.3%]	97.3% [90.8%, 99.3%]	97.3% [90.8%, 99.3%]
Reader 2 (Experience ZN: 16 years ; FM: 1 year)	88.0% [78.7%, 93.6%]	97.3% [90.8%, 99.3%]	98.7% [92.8%, 99.8%]	98.7% [92.8%, 99.8%]

Table 8: Site- & reader-specific specificity for panel reading at 5 reference laboratories				
	ZN	**FM**	**iLED 40**	**iLED 20**
CMC Vellore, India				
Reader 1 (Experience ZN: 8 years ; FM: 2.5 years)	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]
INS, Peru				
Reader 1 (Experience ZN: 9 years ; FM: 9 years)	100.0% [90.1%, 100.0%]	77.1% [61.0%, 87.9%]	100.0% [90.1%, 100.0%]	85.7% [70.6%, 93.7%]
Reader 2 (Experience ZN: 6 years ; FM: 5 years)	100.0% [90.1%, 100.0%]	97.1% [85.5%, 99.5%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]
NRL, Thailand				
Reader 1 (Experience ZN:30 years ; FM: 30 years)	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	97.1% [85.5%, 99.5%]
Reader 2 (Experience ZN: N/A ; FM: N/A)	100.0% [90.1%, 100.0%]	97.1% [85.5%, 99.5%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]
PNTH, Vietnam				
Reader 1 (Experience ZN: 5 years ; FM:5 years)	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]	97.1% [85.5%, 99.5%]
Gauting, Germany				
Reader 1 (Experience ZN: 10 years ; FM: 4.5 years)	100.0% [90.1%, 100.0%]	97.1% [85.5%, 99.5%]	100.0% [90.1%, 100.0%]	91.4% [77.6%, 97.0%]
Reader 2 (Experience ZN: 16 years ; FM: 1 year)	100.0% [90.1%, 100.0%]	88.6% [74.0%, 95.5%]	100.0% [90.1%, 100.0%]	100.0% [90.1%, 100.0%]

Table 9: Pairwise comparison of iLED 40x performance with ZN. The sensitivity of iLED is significantly higher, whereas there is no statistical evidence for a difference in specificity.

Endpoint	Site	LED 40X (a)	ZN (b)	Difference (a) – (b)	95% CI	Pvalue† (a) ≠ (b)	Pvalue†† (a) > (b)-5
Sensitivity	India	96.0%	85.3%	10.7%	[5.3%, 19.7%]	0.005	<.001
	Peru	96.0%	94.7%	1.3%	[-3.6%, 6.5%]	0.564	0.008
	Thailand	96.0%	88.0%	8.0%	[2.0%, 14.6%]	0.011	<.001
	Vietnam	94.7%	94.7%	0.0%	[-7.8%, 7.8%]	1.000	0.085
	Germany	98.0%	89.3%	8.7%	[3.8%, 14.7%]	0.002	<.001
	All Sites	**96.3%**	**90.5%**	**5.8%**	**[3.3%, 8.6%]**	**<.001**	**<.001**
Specificity	India	100.0%	100.0%	0.0%	[-9.9%, 9.9%]	1.000	0.087
	Peru	100.0%	100.0%	0.0%	[-5.2%, 5.2%]	1.000	0.027
	Thailand	100.0%	100.0%	0.0%	[-5.2%, 5.2%]	1.000	0.027
	Vietnam	100.0%	100.0%	0.0%	[-9.9%, 9.9%]	1.000	0.087
	Germany	100.0%	100.0%	0.0%	[-5.2%, 5.2%]	1.000	0.027
	All Sites	**100.0%**	**100.0%**	**0.0%**	**[-1.4%, 1.4%]**	**1.000**	**<.001**

† Two-sided test. Null hypothesis is that the two methods being compared have equal sensitivity or specificity.
†† One-sided test to establish non-inferiority of method (a). Non-inferiority margin is 5 percentage points.

Table 10a: iLED 40x performance compared with FM. The specificity of iLED 40X is significantly higher than for FM, whereas the difference in sensitivity is not significant.

Endpoint	Site	LED 40X (a)	FM (b)	Difference (a) – (b)	95% CI	Pvalue† (a) ≠ (b)	Pvalue†† (a) > (b)-5
Sensitivity	India	96.0%	96.0%	0.0%	(-4.9%, 4.9%)	1.000	0.023
	Peru	96.0%	94.7%	1.3%	(-1.9%, 5.1%)	0.317	0.001
	Thailand	96.0%	96.7%	-0.7%	(-4.1%, 2.5%)	0.564	0.012
	Vietnam	94.7%	94.7%	0.0%	(-4.9%, 4.9%)	1.000	0.023
	Germany	98.0%	97.3%	0.7%	(-1.8%, 3.7%)	0.317	0.001
	All Sites	**96.3%**	**96.0%**	**0.3%**	**(-0.7%, 1.5%)**	**0.480**	**<.001**
Specificity	India	100.0%	100.0%	0.0%	(-9.9%, 9.9%)	1.000	0.087
	Peru	100.0%	87.1%	12.9%	(6.9%, 22.7%)	0.003	<.001
	Thailand	100.0%	98.6%	1.4%	(-3.8%, 7.7%)	0.317	0.015
	Vietnam	100.0%	100.0%	0.0%	(-9.9%, 9.9%)	1.000	0.087
	Germany	100.0%	92.9%	7.1%	(1.6%, 15.7%)	0.025	0.001
	All Sites	**100.0%**	**94.6%**	**5.4%**	**(3.3%, 8.6%)**	**<.001**	**<.001**

Table 10b: iLED 40X performance compared to iLED 20X. iLED 20X appears slightly less specific than iLED 40X.							
Endpoint	**Site**	**LED 40X (a)**	**LED 20X (b)**	**Difference (a) – (b)**	**95% CI**	**Pvalue† (a) ≠ (b)**	**Pvalue†† (a) > (b)-5**
Sensitivity	India	96.0%	96.0%	0.0%	(-4.9%, 4.9%)	1.000	0.023
	Peru	96.0%	94.7%	1.3%	(-2.9%, 5.9%)	0.480	0.004
	Thailand	96.0%	96.0%	0.0%	(-4.0%, 4.0%)	1.000	0.011
	Vietnam	94.7%	94.7%	0.0%	(-4.9%, 4.9%)	1.000	0.023
	Germany	98.0%	98.0%	0.0%	(-3.6%, 3.6%)	1.000	0.007
	All Sites	**96.3%**	**96.0%**	**0.3%**	**(-1.2%, 1.9%)**	**0.637**	**<.001**
Specificity	India	100.0%	100.0%	0.0%	(-9.9%, 9.9%)	1.000	0.087
	Peru	100.0%	92.9%	7.1%	(1.6%, 15.7%)	0.025	0.001
	Thailand	100.0%	98.6%	1.4%	(-3.8%, 7.7%)	0.317	0.015
	Vietnam	100.0%	97.1%	2.9%	(-7.3%, 14.5%)	0.317	0.044
	Germany	100.0%	95.7%	4.3%	(-1.1%, 11.9%)	0.083	0.004
	All Sites	**100.0%**	**96.4%**	**3.6%**	**(2.0%, 6.4%)**	**0.002**	**<.001**

Table 11: Sensitivity of ZN, conventional FM and iLED shown as a function of reading time					
Reading time	**(a) Total pos slides**	**(b) Identified pos slides (cumulative)**	**(b) / (a) Sensitivity (95% CI)**	**(c) New pos**	**(c) / (a) Yield of new pos out of total (95% CI)**
30 sec	600	294	49.0% (45.02%, 52.99%)	294	49.0% (45.02%, 52.99%)
1 min	600	454	75.7% (72.08%, 78.93%)	160	26.7% (23.28%, 30.35%)
3 min	600	532	88.7% (85.88%, 90.96%)	78	13.0% (10.54%, 15.93%)
5 min	600	543	90.5% (87.89%, 92.60%)	11	1.8% (1.03%, 3.25%)
FM sensitivity as function of reading time					
Reading time	**(a) Total pos slides**	**(b) Identified pos slides (cumulative)**	**(b) / (a) Sensitivity (95% CI)**	**(c) New pos**	**(c) / (a) Yield of new pos out of total (95% CI)**
30 sec	600	489	81.5% (78.20%, 84.40%)	489	81.5% (78.20%, 84.40%)
1 min	600	557	92.8% (90.49%, 94.64%)	68	11.3% (9.04%, 14.12%)
3 min	600	576	96.0% (94.12%, 97.30%)	19	3.2% (2.04%, 4.89%)
5 min	600	576	96.0% (94.12%, 97.30%)	0	0.0% (0.00%, 0.64%)
iLED 40x sensitivity as function of reading time					
Reading time	**(a) Total pos slides**	**(b) Identified pos slides (cumulative)**	**(b) / (a) Sensitivity (95% CI)**	**(c) New pos**	**(c) / (a) Yield of new pos out of total (95% CI)**
30 sec	600	511	85.2% (82.10%, 87.79%)	511	85.2% (82.10%, 87.79%)
1 min	600	563	93.8% (91.62%, 95.49%)	52	8.7% (6.67%, 11.19%)
3 min	600	578	96.3% (94.51%, 97.57%)	15	2.5% (1.52%, 4.08%)
5 min	600	578	96.3% (94.51%, 97.57%)	0	0.0% (0.00%, 0.64%)
iLED 20x sensitivity as function of reading time					
Reading time	**(a) Total pos slides**	**(b) Identified pos slides (cumulative)**	**(b) / (a) Sensitivity (95% CI)**	**(c) New pos**	**(c) / (a) Yield of new pos out of total (95% CI)**
30 sec	600	502	83.7% (80.50%, 86.41%)	502	83.7% (80.50%, 86.41%)
1 min	600	566	94.3% (92.19%, 95.92%)	64	10.7% (8.44%, 13.39%)
3 min	600	575	95.8% (93.92%, 97.16%)	9	1.5% (0.79%, 2.83%)
5 min	600	576	96.0% (94.12%, 97.30%)	1	0.2% (0.03%, 0.94%)

Table 12: Operational performance of iLED as rated by reference sites (8 users)	
	User appraisal (8 questionnaires completed by users at evaluation sites)
Installation	Easy with the help of the instruction manual: 100%
Training required for ZN microscopist	3-5 days
Overall handling and features	Very satisfied (superior to microscopes in use at ref lab): 60%; Satisfied (equivalent): 40%
Use of ZN and FM on same system (Ease of switching)	Very convenient: 100% (But reading in ZN mode suboptimal due to shift towards blue light; recommendation to modify filters or use halogen bulb instead if LED for ZN)
Light intensity, background, contrast (FM mode)	Very satisfied: 100%
Resolution and depth of focus	Very satisfied: 80% Satisfied: 20%
Ease of focus	Easy: 100% But initial difficulties expected for users without FM experience
Need for darkroom	Not needed: 100%
Magnification factor for objectives	Suitable: 50% Not ideal: 50% (Reading with 40X only, but need 25X)
Preferred bulb type for ZN mode (available from Zeiss are LED or Halogen bulbs)	80% of users preferred halogen bulbs instead of LED bulbs
Gain in speed	Yes, significant: 100%
Recommendation on implementation	Yes: 100%
Where	In low, medium and high volume labs: 60% Only in high volume labs: 40%
Use of iLED	Use of iLED for ZN & FM: 40% Use for FM only: 60%

Demonstration study results

Methods

Once performance targets were met through the evaluation study, a Demonstration project was initiated in coordination with National and Regional TB Control Programs in several countries. FIND criteria for country selection for the study were: an agreement at National/Regional Level s (MOU) with NTP and/or MOH; a high-burden of TB; a low or middle income ranking; local presence of FIND or an implementing partner; settings representative of the global TB and HIV situation.

There were 28 microscopy centers and 12 supervisory sites chosen for this large study, with site selection based on the rate of smear-positivity, training of microscopists, volume of work, reliability of AC power (sites with intermittent power supply were intentionally selected), interest in the project, and accessibility of study sites for supervisory visits. None of the microscopy centers had prior experience with fluorescent microscopy.

Participating microscopy centers were grouped in clusters. Each cluster consisted of a supervisory site and two to three microscopy centers. The supervisory site was responsible for training, monitoring, rechecking of slides and data management. The selected microscopy centers were intended to be representative for the respective country with regard to location (urban/ rural, power supply), daily workload (low, middle, high) and baseline performance (poor and strong performers).

The study flow is summarized in Table 13: For all sites, baseline performance data were collected using the standard ZN procedures over a month-long pre-study period. Following a one- to five-day training session on the Primo Star iLED microscope, sites initiated a validation phase. During this phase, all slides were screened with the iLED microscope and then confirmed on a daily basis using a conventional FM by a supervisory site. Patient care during this phase was based on the results of FM examination. Proficiency testing panels were also used to assess the study sites.

Sites that showed acceptable performance in the validation phase continued with a three month implementation phase in which the iLED microscope was used as the routine screening tool. Acceptable performance was achieved in case of a) at least 95% result concordance between the demonstration site and the supervisory site, b) acceptable quality of Auramine stain in nearly 100% of rechecked slides, and c) ≤ 2/10 false results in the proficiency testing panel. Rechecking of stored slides by FM continued during implementation phase at a reduced rate (see Table 14).

Demonstration sites for which performance was maintained moved to a six month continuation phase, during which rechecking frequency and intensity was performed as per National TB Program norms. During all study phases, slides with discrepant results (rechecking result deviating from the result at the microscopy center) were reread by an experienced third reader based at the supervisory site. Slide ID numbers for slides requiring a 3rd reading were distributed to the sites by FIND on a monthly basis. As shown in Table 13, regular monitoring visits were conducted using standardized checklists.

At all sites, user acceptance and appraisal was assessed as well as the robustness of the microscope. Logistics and customer support quality were monitored. Several sites looked at specific questions including iLED performance compared to ZN (Peru, India, Thailand, South Africa, Lesotho), fading speed (Vietnam, India, Peru), alternative staining solutions (Peru, India) and reading time reduction (Vietnam, Peru, India).

Table 13: Study flow and standardization during iLED demonstration project										
Study phase	**Duration**	**% slides re-checked**	**Staining reagents**	**Microscope used for reading**	**Microscope used for re-checking**	**Patient management**	**Frequency of retrieving slides /forms**	**Supervisory visits with checklist**	**Forms**	**Data transfer by courier**
ZN Baseline	1 month	100%	Routine Zn stain	Conventional Brightfield (1000X)	Conventional Brightfield (1000X)	Based on ZN result of microscopy center	Once every 2nd week	Monthly	1. Result Form – ZN Baseline 2. Rechecking Form – ZN Baseline	At the end of phase
iLED Training	1-5 days									
Proficiency testing & User appraisal	1 day	100%	For 10 Au and 10 ZN slides	Primo Star iLED (400X) Conventional Brightfield (1000X)	Primo Star iLED (400X) Conventional Brightfield (1000X)	-	-	-	1. Proficiency Testing Result Form; 2. User appraisal questionnaire	Scanned by e-mail following day
Validation	Minimum 1 month. Until targets met.	100% (daily)	Au staining reagents provided by supervisory site once per month	Primo Star iLED (400X)	Conventional FM (200-250X)	Based on conventional FM result from supervisory site	Daily .	Every 2nd week	1. Result Form – Validation 2. Rechecking Form – Validation	Every 2nd week
Proficiency testing & user appraisal	See above									
Implementation	3 months	See Table 14	See validation	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Once every 2nd week	Monthly	1. Result Form – Implementation 2. Rechecking Form – Implementation	Monthly
Proficiency testing & user appraisal	See above									
Continuation	6 months	See Table 14	See validation	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Monthly	Monthly	Same as implementation	Monthly

Table 14: Rechecking intensity during various study phases (NTP decision for implementation and continuation)			
	% Rechecking – Validation (daily)	**% Rechecking – Implementation (monthly)**	**% Rechecking – Continuation (monthly or quarterly)**
South Africa	100% pos, 100% neg	Rechecking as per LQAS (*)	Rechecking as per LQAS (*)
Ethiopia		100% pos, 20% neg	Rechecking as per LQAS (*)
Lesotho		100% pos, 20% neg	Rechecking as per LQAS (*)
Peru		100% pos, 20% neg	Rechecking as per LQAS (*)
Russia		100% pos, 10-30% neg	10-100% pos; 5-10% neg
India - CMC		Rechecking as per LQAS (*)	Rechecking as per LQAS (*)
India – New Delhi		Rechecking as per LQAS (*)	Rechecking as per LQAS (*)
India - Agra		100% pos, 100% neg	Rechecking as per LQAS (*)
Thailand		100% pos, 100% neg	Rechecking as per LQAS (*)
Vietnam		100% pos, 20% neg	Rechecking as per LQAS (*)
Cambodia		100% pos, 20% neg	Rechecking as per LQAS (*)

(*) Lot Quality Assurance System/National TB Programme Guidelines

Results

Scale of the Demonstration project

To date, the implementation phase has been completed by all sites and **more than 60,000 slides** have been examined using iLED, not counting data from Lesotho and Ethiopia because of some missing rechecking results. The significant difference in the average number of specimens examined per day in the 3 phases, as seen in Table 15 for a few sites, such as for microscopy center 1 (MC1) in Vellore, can be explained by restructuring activities undertaken by the NTP during the course of the study. Outstanding continuation data (so far, >9000 slides examined; not shown in Table 15) are expected to provide confirmation of the sustainability of iLED performance only, while all study endpoints could be answered with the large data set obtained so far.

Table 15: Number of slides examined with average daily workload

	Baseline Phase		Validation Phase		Implementation Phase	
	# of specimens tested	Average # of specimens per day	# of specimens tested	Average # of specimens per day	# of specimens tested	Average # of specimens per day
India						
Vellore – MC1	358	13.3	548	12.2	3373	40.2
Vellore– MC2	214	8.6	316	7.7	668	9.3
Vellore –MC3	420	15.6	509	11.3	1395	18.1
Delhi – MC1	1135	42.0	1715	35.7	3233	42.0
Delhi – MC2	432	16.0	680	14.2	929	13.7
Delhi – MC3	111	5.8	247	5.9	354	5.8
Agra – MC1	248	10.3	408	11.0	835	13.0
Agra – MC2	708	29.5	919	26.3	1877	26.8
Agra – MC3	214	8.9	515	9.7	519	8.2
Peru						
Peru – MC1	689	28.7	1047	31.7	1733	24.4
Peru – MC2	375	14.4	572	23.8	1054	18.2
Peru – MC2	232	11.6	277	8.9	590	12.8
Russia						
Russia – MC1	826	27.5	511	39.3	760	13.1
Russia – MC2	187	6.2	308	19.3	404	6.1
Thailand	378	12.6	378	14.0	631	17.1
Vietnam						
Vietnam – MC1	700	31.8	783	34.0	2379	38.4
Vietnam – MC2	212	10.1	286	13.0	725	12.1
Vietnam – MC3	886	36.9	887	40.3	2370	38.9
Cambodia						
Cambodia-MC1	992	45.1	1096	52.2	1471	21.6
Cambodia- MC2	435	21.8	514	21.4	1037	19.9
Cambodia- MC3	399	16.0	391	17.8	1154	17.8
Lesotho						
Lesotho – MC1	987	44.9	826	37.5	(1976)	(32.4)
Lesotho – MC2	1097	49.9	890	38.7	(2726)	(44.7)
Ethiopia						
Ethiopia – MC1	1285	55.9	1942	69.4	(2492)	(55.4)
Ethiopia – MC2	135	5.9	92	5.4	(361)	(4.3)
South Africa						
S. Africa – MC1	1569	40.2	547	45.6	2474	41.2
S. Africa – MC2	1298	31.7	545	36.3	2324	40.8
S. Africa – MC3	697	34.9	566	29.8	1995	36.9
Total	**17219**	**24.3**	**18315**	**22.7**	**34284**	**22.7**

Microscopist experience at validation phase

The experience of participating microscopists with ZN microscopy ranged from one month to 30 years, but most readers had between five and 10 years of ZN experience. As shown below, none of the readers had any prior experience with FM, whereas all of the supervisors responsible for training and rechecking had at least six months prior experience with FM.

Table 16: Experience of laboratory personnel upon start of validation phase

	READERS				SUPERVISORS			
Country	**Nr**	**Average ZN experience (years)**	**Average FM experience (years)**	**Average number of slides read per day (*)**	**Nr**	**Average ZN experience (years)**	**Average FM experience (years)**	**Average number of slides read per day (*)**
South Africa	5	8.1	0	52	2	3.5	3	25
Ethiopia	4	8.3	0	18	2	8	0.5	5
Lesotho	6	4.8	0	20	2	20	2	30
Peru	7	5.8	0	19	2	20	2	30
Russia	5	8	0	30	2	33	17.5	25
India	11	12.2	0	25	5	13.5	3.7	42
Thailand	1	30	0	20	2	27.5	27.5	13
Vietnam	4	23.5	0	21	2	17	5	35
Cambodia	3	10	0	23	2	5	2.5	15

(*) last 12 months

Proficiency of iLED reading during validation phase

We assessed agreement between iLED readings performed by microscopy centers and supervisory sites within the first month and compared the agreement rate to the ZN baseline established prior to iLED introduction. As explained earlier in the report, 100% of ZN results were rechecked by ZN during baseline phase, while 100% rechecking during implementation was done using conventional FM. A high level of agreement during the validation phase was interpreted as evidence that newly trained microscopists could attain proficiency on iLED within a short timeframe and with modest training requirements. This comparison showed us how easily sites adapted to the new technology and reached the same (or higher) proficiency as for ZN.

Statistical methods: Agreement was assessed on a per-specimen basis with adjustment for multiple specimens per patient. Overall agreement was defined as the proportion of specimens for which the reader and supervisor obtained the same result. The labels “relative sensitivity” and “relative specificity” were used to denote estimates of sensitivity and specificity that were obtained by treating the supervisor’s reading as the reference standard. Generalized estimating equations (GEE) methodology was used to calculate 95% confidence intervals for the estimated agreement rates and to test the null hypothesis that agreement remained constant across the baseline and validation phases.

As shown in Table 17 below, the performance target of ≥95% agreement of iLED results compared to FM was met by 27/28 sites in the first month. Only 1 site in South Africa was slightly below targets and had to prolong validation phase by 2 weeks to reach the required accuracy. A high relative specificity for iLED >97% was seen for the majority of sites from very early on, with >99% reached by several sites from week 1 onwards. >95% relative specificity was reached during validation phase by 27/28 sites with only 1 site showing a lower specificity of 88%. Relative sensitivity was variable across sites and ranged between 80% and 100%. As expected, sites with a lower sensitivity during baseline

phase were generally also performing less well for iLED than sites that showed strong performance already during baseline.

The overall agreement rate between readers and supervisors was marginally higher during iLED validation compared to ZN baseline (98.1% vs 96.8%; p = 0.001). Relative sensitivity was also higher during validation (94.2% vs 87.7%; p<0.001). There was no significant difference in relative specificity between the 2 methods (p = 0.85). When looking at sites individually, a significantly lower agreement rate for iLED compared to ZN was only seen for one site in Lesotho, whereas a significantly higher agreement with iLED was seen for a site in Agra, India and a higher relative specificity for sites in Russia, Ethiopia and Vietnam.

Table 17: iLED performance during validation compared to ZN performance during baseline phase

(Baseline: 100% rechecking by ZN; Validation: 100% rechecking by FM; all discrepant slides examined by 3rd reader)

	Baseline phase			Validation phase			p-values
	Overall ZN accuracy (% agreement)	Relative ZN sensitivity (among ZN pos)	Relative iLED specificity (among ZN neg)	Overall iLED accuracy (% agreement)	Relative iLED sensitivity (among FM pos)	Relative iLED specificity (among FM neg)	1. Accuracy 2. Sensitivity 3. Specificity
India							
Vellore							
MC 1	99.7% (357/358) [98.1% - 100.0%]	100.0% (37/37) [90.0% - 100.0%]	99.7% (320/321) [97.8% - 100.0%]	99.4% (490/493) [98.1% - 99.8%]	96.4% (53/55) [86.7% - 99.1%]	99.8% (437/438) [98.4% - 100.0%]	0.497 0.829 0.827
MC 2	98.6% (211/214) [95.8% - 99.5%]	95.7% (45/47) [85.4% - 98.9%]	99.4% (166/167) [95.9% - 99.9%]	100.0% (316/316) [98.8% - 100.0%]	100.0% (55/55) [93.3% - 100.0%]	100.0% (261/261) [98.6% - 100.0%]	0.193 0.488 0.754
MC 3	99.8% (419/420) [98.3% - 100.0%]	95.7% (22/23) [76.4% - 99.3%]	100.0% (397/397) [99.1% - 100.0%]	98.8% (497/503) [96.4% - 99.5%]	95.2% (60/63) [77.7% - 98.5%]	99.3% (437/440) [97.0% - 99.8%]	0.142 0.759 0.354
Delhi							
MC 1	99.3% (1127/1135) [98.1% - 99.8%]	99.2% (121/122) [94.3% - 99.9%]	99.3% (1006/1013) [97.8% - 99.7%]	98.5% (1684/1709) [97.4% - 99.1%]	96.6% (282/292) [91.3% - 98.5%]	98.9% (1402/1417) [97.7% - 99.3%]	0.168 0.454 0.384
MC 2	100.0% (432/432) [99.1% - 100.0%]	100.0% (46/46) [92.0% - 100.0%]	100.0% (386/386) [99.0% - 100.0%]	99.7% (673/675) [98.8% - 99.9%]	98.7% (74/75) [90.7% - 99.8%]	99.8% (599/600) [98.8% - 100.0%]	0.838 0.735 0.754
MC 3	100.0% (111/111) [96.7% - 100.0%]	100.0% (21/21) [82.4% - 100.0%]	100.0% (90/90) [95.9% - 100.0%]	98.4% (243/247) [95.8% - 99.4%]	95.9% (47/49) [84.2% - 99.0%]	99.0% (196/198) [96.1% - 99.7%]	0.601 0.904 0.94
Agra							
MC 1	93.5% (232/248) [88.4% - 96.1%]	75.5% (37/49) [52.2% - 83.4%]	98.0% (195/199) [91.9% - 99.2%]	96.1% (392/408) [92.7% - 97.7%]	90.2% (83/92) [75.7% - 94.2%]	97.8% (309/316) [93.2% - 98.8%]	0.215 0.068 0.958
MC 2	91.7% (649/708) [88.7% - 94.3%]	88.6% (70/79) [75.8% - 94.3%]	92.1% (579/629) [86.2% - 93.3%]	98.0% (901/919) [96.4% - 99.0%]	89.3% (117/131) [78.4% - 94.6%]	99.5% (784/788) [98.1% - 99.8%]	<.001 0.788 <.001
MC 3	99.1% (212/214) [96.4% - 99.8%]	89.5% (17/19) [67.7% - 97.3%]	100.0% (195/195) [98.1% - 100.0%]	98.4% (507/515) [96.6% - 99.3%]	92.0% (80/87) [82.8% - 96.3%]	99.8% (427/428) [98.3% - 100.0%]	0.511 0.698 0.575

	Baseline phase			Validation phase			p-values
Vietnam	**Overall ZN accuracy (% agreement)**	**Relative ZN sensitivity (among ZN pos)**	**Relative iLED specificity (among ZN neg)**	**Overall iLED accuracy (% agreement)**	**Relative iLED sensitivity (among FM pos)**	**Relative iLED specificity (among FM neg)**	**1. Accuracy 2. Sensitivity 3. Specificity**
MC 1	96.4% (675/700) [93.9% - 97.9%]	73.4% (69/94) [49.4% - 77.1%]	100.0% (606/606) [99.4% - 100.0%]	99.0% (771/779) [96.9% - 99.6%]	94.2% (114/121) [82.9% - 98.2%]	99.8% (657/658) [98.9% - 100.0%]	0.039 <.001 0.953
MC 2	98.1% (208/212) [91.5% - 99.4%]	100.0% (31/31) [88.1% - 100.0%]	97.8% (177/181) [90.2% - 99.4%]	99.0% (283/286) [93.9% - 99.9%]	100.0% (46/46) [92.0% - 100.0%]	98.8% (237/240) [92.8% - 99.9%]	0.436 0.743 0.449
MC 3	96.8% (858/886) [94.7% - 97.9%]	89.0% (130/146) [79.1% - 93.1%]	98.4% (728/740) [96.4% - 99.1%]	97.4% (864/887) [95.9% - 98.4%]	89.0% (146/164) [80.1% - 93.1%]	99.3% (718/723) [98.4% - 99.7%]	0.415 0.926 0.105
Cambodia							
MC 1	97.7% (969/992) [96.3% - 98.6%]	91.3% (157/172) [82.9% - 95.0%]	99.0% (812/820) [98.1% - 99.5%]	97.8% (1072/1096) [96.4% - 98.7%]	97.3% (144/148) [92.2% - 99.2%]	97.9% (928/948) [96.0% - 98.6%]	0.881 0.043 0.155
MC 2	97.9% (426/435) [95.5% - 99.1%]	69.0% (20/29) [32.3% - 79.8%]	100.0% (406/406) [99.1% - 100.0%]	98.1% (503/513) [96.5% - 98.9%]	88.6% (39/44) [76.0% - 95.2%]	98.9% (464/469) [97.5% - 99.6%]	0.946 0.074 0.177
MC 3	99.5% (397/399) [98.0% - 99.9%]	97.6% (40/41) [83.4% - 99.7%]	99.7% (357/358) [98.0% - 100.0%]	98.7% (386/391) [95.8% - 99.7%]	95.1% (39/41) [67.2% - 99.2%]	99.1% (347/350) [95.3% - 99.9%]	0.638 0.784 0.936
Thailand							
MC 1	97.6% (369/378) [95.5% - 98.7%]	70.8% (17/24) [45.5% - 83.7%]	99.4% (352/354) [97.8% - 99.9%]	98.9% (374/378) [97.2% - 99.6%]	89.7% (26/29) [71.7% - 96.0%]	99.7% (348/349) [98.0% - 100.0%]	0.175 0.075 0.579
Peru							
MC 1	97.4% (671/689) [95.9% - 98.4%]	84.2% (48/57) [72.0% - 91.5%]	98.6% (623/632) [97.3% - 99.3%]	97.3% (1018/1046) [96.1% - 98.2%]	92.5% (86/93) [83.2% - 96.4%]	97.8% (932/953) [96.5% - 98.5%]	0.943 0.12 0.272
MC 2	99.2% (372/375) [97.6% - 99.7%]	84.6% (11/13) [49.8% - 95.8%]	99.7% (361/362) [98.1% - 100.0%]	97.7% (559/572) [96.1% - 98.7%]	91.3% (42/46) [78.3% - 96.0%]	98.3% (517/526) [96.8% - 99.1%]	0.096 0.601 0.081
MC 3	98.3% (228/232) [95.5% - 99.4%]	55.6% (5/9) [27.9% - 83.1%]	100.0% (223/223) [98.3% - 100.0%]	96.0% (266/277) [92.8% - 98.0%]	81.3% (13/16) [47.9% - 94.9%]	96.9% (253/261) [93.7% - 98.6%]	0.148 0.2 0.003

	Baseline phase			Validation phase			p-values
Russia	**Overall ZN accuracy (% agreement)**	**Relative ZN sensitivity (among ZN pos)**	**Relative iLED specificity (among ZN neg)**	**Overall iLED accuracy (% agreement)**	**Relative iLED sensitivity (among FM pos)**	**Relative iLED specificity (among FM neg)**	**1. Accuracy 2. Sensitivity 3. Specificity**
MC 1	91.5% (756/826) [89.3% - 93.5%]	91.1% (225/247) [86.7% - 94.1%]	91.7% (531/579) [88.4% - 93.5%]	95.0% (485/511) [92.1% - 96.5%]	99.7% (302/303) [97.7% - 100.0%]	88.0% (183/208) [81.4% - 91.5%]	0.047 <.001 0.200
MC 2	100.0% (187/187) [98.0% - 100.0%]	100.0% (13/13) [71.6% - 100.0%]	100.0% (174/174) [97.9% - 100.0%]	96.1% (293/305) [93.1% - 98.1%]	93.8% (30/32) [78.4% - 98.4%]	96.3% (263/273) [92.9% - 98.2%]	0.050 0.899 0.062
Lesotho							
MC 1	99.7% (984/987) [99.1% - 99.9%]	99.3% (139/140) [95.0% - 99.9%]	99.8% (845/847) [99.1% - 99.9%]	95.0% (783/826) [92.2% - 96.4%]	90.1% (109/121) [79.1% - 95.2%]	95.6% (674/705) [92.8% - 97.1%]	<.001 0.034 <.001
MC 2	99.5% (1091/1097) [98.5% - 99.8%]	94.0% (79/84) [84.1% - 97.8%]	99.9% (1012/1013) [99.3% - 100.0%]	98.7% (878/890) [97.3% - 99.3%]	93.8% (76/81) [84.8% - 97.3%]	99.1% (802/809) [97.7% - 99.7%]	0.119 0.740 0.076
Ethiopia							
MC 1	96.0% (1233/1285) [94.3% - 97.2%]	72.7% (96/132) [59.7% - 81.1%]	98.6% (1137/1153) [97.1% - 99.1%]	97.1% (1886/1942) [96.0% - 98.0%]	98.8% (252/255) [96.4% - 99.6%]	96.9% (1634/1687) [94.9% - 97.4%]	0.182 <.001 0.034
MC 2	99.3% (134/135) [95.0% - 99.9%]	100.0% (6/6) [38.5% - 100.0%]	99.2% (128/129) [94.6% - 99.9%]	100.0% (92/92) [96.0% - 100.0%]	100.0% (13/13) [71.6% - 100.0%]	100.0% (79/79) [95.3% - 100.0%]	0.788 0.551 0.731
South Africa							
MC 1	95.9% (1208/1260) [94.3% - 96.8%]	65.4% (51/78) [47.4% - 71.2%]	97.9% (1157/1182) [96.2% - 98.4%]	98.2% (536/546) [96.4% - 99.1%]	85.5% (47/55) [70.2% - 91.8%]	99.6% (489/491) [98.4% - 99.9%]	0.022 0.017 0.04
MC 2	95.9% (1205/1257) [94.4% - 97.0%]	83.9% (130/155) [72.3% - 87.7%]	97.5% (1075/1102) [96.1% - 98.3%]	99.1% (540/545) [97.3% - 99.8%]	96.1% (99/103) [87.6% - 99.2%]	99.8% (441/442) [98.4% - 100.0%]	0.048 0.470 0.035
MC 3	88.2% (612/694) [85.5% - 90.7%]	85.4% (152/178) [78.2% - 90.0%]	89.1% (460/516) [85.1% - 91.3%]	93.4% (520/557) [91.0% - 95.2%]	80.2% (93/116) [68.6% - 85.2%]	96.8% (427/441) [94.7% - 98.1%]	0.003 0.246 <.001
TOTAL	**96.8% (16333/16866) [96.5% - 97.2%]**	**87.7% (1835/2092) [84.6% - 88.4%]**	**98.1% (14498/14774) [97.8% - 98.4%]**	**97.7% (17812/18224) [97.4% - 97.9%]**	**94.2% (2567/2726) [92.2% - 94.6%]**	**98.4% (15245/15498) [97.9% - 98.5%]**	**0.001 <.001 0.850**

The only site with an initially low iLED relative specificity of 88% was discovered to have problems with staining. It is believed that infrequent filtering of the Auramine solution resulted in a higher number of artifacts compared to other sites. Once the site had been re-trained in staining, specificity increased significantly (see implementation data below). Overall, the staining quality was found to be high during rechecking of positive and control slides.

Most microscopists reported that only a short familiarization period of two weeks was required to gain a high degree of confidence with the new staining and reading method. Overall, the introduction and rollout was unproblematic and uptake enthusiastic. All sites passed the proficiency testing at the end of validation phase without difficulties.

Performance of iLED during validation phase

a) Relative sensitivity and specificity of iLED and ZN

During the initial validation phase, some microscopy centers had the capacity to perform ZN in parallel to iLED. This required the preparation of two slides per sputum, one for staining with ZN and one for Au staining. Daily re-checking with conventional FM was the responsibility of the supervisory site. Patient management was based on ZN whereas the daily generated supervisory FM result was used for patient management at the sites that did not have the capacity to perform ZN in parallel.

Statistical Methods: Accuracy of iLED and ZN was compared on a per-specimen basis using conventional FM as the reference standard. Because conventional FM is not perfectly sensitive or specific, the phrases "relative sensitivity" and "relative specificity" are used below to emphasize that the reported sensitivity and specificity results are not absolute but relative to FM. P-values comparing the relative sensitivity and specificity of iLED vs ZN were calculated using the method of Durkalski.[vii].

As shown below, iLED was significantly more sensitive than ZN in this study population (15.5%; p-value < 0.001). There was no significant difference in specificity between ZN and iLED overall or at any of the sites individually.

Table 18: Sensitivity and specificity of iLED and ZN compared to FM during validation

	Relative Sensitivity			Relative Specificity		
	iLED	**ZN**	**P-Value**	**iLED**	**ZN**	**P-Value**
India						
Vellore						
MC 1	97.7% (43/44) [84.5% - 99.7%]	95.5% (42/44) [82.8% - 98.9%]	0.317	100.0% (310/310) [98.8% - 100.0%]	100.0% (310/310) [98.8% - 100.0%]	1.000
MC 2	100.0% (32/32) [88.5% - 100.0%]	87.5% (28/32) [67.0% - 95.8%]	0.109	100.0% (155/155) [97.6% - 100.0%]	100.0% (155/155) [97.6% - 100.0%]	1.000
MC 3	93.3% (42/45) [70.0% - 97.8%]	62.2% (28/45) [40.1% - 77.1%]	0.009	99.0% (284/287) [94.5% - 99.7%]	100.0% (287/287) [98.7% - 100.0%]	0.157
Combined	**96.7% (117/121) [86.8% - 98.6%]**	**81.0% (98/121) [67.5% - 87.1%]**	**0.001**	**99.6% (749/752) [97.9% - 99.9%]**	**100.0% (752/752) [99.5% - 100.0%]**	**0.157**
New Delhi						
MC 1	96.4% (265/275) [91.0% - 98.5%]	83.3% (229/275) [74.2% - 87.1%]	<.001	98.8% (1221/1236) [97.4% - 99.2%]	99.5% (1230/1236) [98.9% - 99.8%]	0.137

	Relative Sensitivity			Relative Specificity		
	iLED	**ZN**	**P-Value**	**iLED**	**ZN**	**P-Value**
MC 2	98.6% (68/69) [90.0% - 99.8%]	89.9% (62/69) [72.4% - 94.9%]	0.140	100.0% (518/518) [99.3% - 100.0%]	98.8% (512/518) [96.8% - 99.5%]	0.034
MC 3	95.5% (42/44) [82.8% - 98.9%]	84.1% (37/44) [61.3% - 92.6%]	0.074	98.6% (137/139) [94.5% - 99.6%]	100.0% (139/139) [97.3% - 100.0%]	0.166
Combined	**96.6% (375/388) [92.1% - 98.1%]**	**84.5% (328/388) [76.6% - 87.2%]**	**<.001**	**99.1% (1876/1893) [98.2% - 99.4%]**	**99.4% (1881/1893) [98.8% - 99.6%]**	**0.741**
Agra						
MC 1	94.1% (48/51) [82.8% - 98.1%]	84.3% (43/51) [62.0% - 92.7%]	0.421	98.0% (199/203) [92.1% - 99.2%]	99.0% (201/203) [96.2% - 99.8%]	0.251
MC 2	90.8% (108/119) [81.6% - 96.6%]	70.6% (84/119) [55.2% - 79.1%]	**<.001**	99.4% (716/720) [97.9% - 99.8%]	99.3% (715/720) [97.8% - 99.8%]	0.602
MC 3	91.1% (72/79) [81.4% - 96.0%]	93.7% (74/79) [75.4% - 97.1%]	0.876	99.7% (374/375) [98.1% - 100.0%]	92.8% (348/375) [88.2% - 96.2%]	0.002
Combined	**91.6% (228/249) [85.5% - 95.0%]**	**80.7% (201/249) [69.3% - 84.6%]**	**0.001**	**99.3% (1289/1298) [98.2% - 99.6%]**	**97.4% (1264/1298) [95.8% - 98.5%]**	**0.073**
Thailand	93.4% (57/61) [83.9% - 97.4%]	60.7% (37/61) [47.6% - 73.0%]	**<.001**	99.5% (729/733) [98.6% - 99.8%]	99.7% (731/733) [98.9% - 99.9%]	0.157
Peru						
MC 1	94.2% (162/172) [88.8% - 97.1%]	79.7% (137/172) [72.2% - 85.2%]	**<.001**	98.3% (1180/1200) [97.3% - 98.9%]	98.6% (1183/1200) [97.6% - 99.1%]	0.505
MC 2	91.2% (62/68) [81.2% - 95.0%]	85.3% (58/68) [75.9% - 91.1%]	0.071	98.3% (737/750) [97.0% - 99.0%]	99.5% (746/750) [98.6% - 99.8%]	0.036
MC 3	90.0% (18/20) [63.5% - 98.9%]	55.0% (11/20) [30.2% - 77.2%]	0.022	96.6% (310/321) [93.5% - 98.1%]	98.8% (317/321) [96.7% - 99.5%]	0.022
Combined	**93.1% (242/260) [89.2% - 96.0%]**	**79.2% (206/260) [73.5% - 84.1%]**	**<.001**	**98.1% (2227/2271) [97.3% - 98.5%]**	**98.9% (2246/2271) [98.3% - 99.2%]**	**0.015**
South Africa						
MC 1	91.4% (32/35) [74.6% - 97.1%]	74.3% (26/35) [57.9% - 86.1%]	0.061	99.5% (441/443) [98.2% - 99.9%]	97.7% (433/443) [95.5% - 98.8%]	0.0499
MC 2	96.1% (98/102) [87.6% - 99.2%]	56.9% (58/102) [45.0% - 67.0%]	**<.001**	99.8% (439/440) [98.4% - 100.0%]	97.5% (429/440) [95.4% - 98.7%]	0.005
MC 3	78.2% (79/101) [65.3% - 83.8%]	68.3% (69/101) [54.9% - 75.4%]	0.077	97.0% (387/399) [94.8% - 98.3%]	92.7% (370/399) [89.2% - 94.8%]	0.007
Combined	**87.8% (209/238) [80.1% - 90.4%]**	**64.3% (153/238) [56.7% - 70.4%]**	**<.001**	**98.8% (1267/1282) [98.1% - 99.3%]**	**96.1% (1232/1282) [94.8% - 97.1%]**	**0.001**
TOTAL	**93.2% (1228/1317) [90.5% - 94.1%]**	**77.7% (1023/1317) [73.6% - 79.4%]**	**<.001**	**98.9% (8137/8229) [98.6% - 99.1%]**	**98.5% (8106/8229) [98.2% - 98.8%]**	**0.900**

b) Increased yield in TB patient detection

The higher sensitivity of iLED compared to ZN in the per-specimen analysis shown in Table 18 should also result in an increased case detection rate. We only considered sites for which we had reliable information on new patients as opposed to patients coming for TB treatment monitoring.
For all countries listed in Table 19 below, two to three smears were performed for new TB suspects, which is why the difference between iLED and ZN appears lower here than in the per specimen analysis. Nevertheless, the difference in detection of new cases was significant with a 14% increased yield of confirmed TB patients compared to ZN.

Table 19: Yield in TB patient detection for iLED compared to ZN during validation phase

Country	A Patients detected with iLED / patients screened	B Patients detected with ZN / patients screened	Difference A-B	P-value
South Africa	15.9% (106/665) [13.4% - 18.9%]	12.6% (84/665) [10.3% - 15.4%]	3.3% (1.8%, 5.1%)	<.001
Peru	9.0% (83/922) [7.3% - 11.0%]	8.4% (77/922) [6.7% - 10.3%]	0.7% (-0.0%, 1.5%)	0.058
India	17.0% (246/1449) [15.1% - 19.0%]	15.2% (220/1449) [13.4% - 17.1%]	1.8% (1.1%, 2.7%)	<.001
Total	**14.3% (435/3036) [13.1% - 15.6%]**	**12.5% (381/3036) [11.4% - 13.8%]**	**1.8% (1.3%, 2.4%)**	**<.001**

Statistical methods for Table 19: Patients were classified as "TB positive" if at least one test was positive. 95% confidence intervals for the case detection rates were calculated using Wilson's score-based binomial confidence interval. 95% confidence intervals for the difference in case detection rates were calculated using Tango's score-based method for paired binary proportions[viii].

Reading time assessment at routine microscopy centers

Methods

A reading time comparison between iLED and ZN was undertaken to confirm that the significant reading time reduction observed at reference laboratories (55%) could also be reached at routine microscopy centers once the microscopists had some experience with iLED.

Fifteen readers from 15 demonstration sites located in Peru (3), India (9) and Vietnam (3) participated in the assessment. At each site, the supervisor selected 50 leftover samples (25 smear negative, 15 low positive, 10 high positive during iLED routine reading) for preparation of ZN and Auramine slides. The prepared slide sets were carefully examined by the supervisor to establish the true result. Then, the readers were each asked to read the prepared slide panels and reading time for each slide was measured by the supervisor. For negative and scanty positive slides, at least 100 fields had to be read with the light microscope and 40 fields with iLED. In general, readers examined the slides until they were confident that they had established the correct result. Focusing was not included in any of the reading times, but a small sub-study showed that there was no significant difference in focusing time for the two methods. When this assessment was done for the first time, readers had about 1 month's experience with iLED and most had several years' experience with ZN). When the assessment was repeated, the readers had been using iLED for approximately three months.

Results

As shown below, a significant reading time reduction of 20% was seen for iLED versus ZN after only one month's reading experience with iLED, although the achieved reduction was still far from the 55-65% seen at reference laboratories. Most readers reported that they had to read more than 40 fields with iLED to establish the result with confidence at this point. After 3 months experience with iLED, the readers reported that they had since gained much more confidence in fluorescence reading and this was reflected in the reading time reduction, which had now reached an average of 45%. The accuracy of results was high for all participants and for iLED and ZN.

Table 20: Reading time assessment – Peru, India, Vietnam
(15 microscopists,50 slides each, 1 and 3 months after iLED introduction)

	ZN	**iLED**	**ZN**	**iLED**
	After 1 month iLED experience		**After 3 months iLED experience**	
Mean reading time (N=50), seconds	**121**	**97**	**128**	**71**
% reduction **p-value* for difference to ZN**		**20%** **<0.0001**		**45%** **<0.0001**
Negative (N=25)	149	126	157	101
Low positive [scanty/+1] (N=15)	119	91	131	82
High positive [+2/+3] (N=10)	45	42	38	34

*calculated using paired t-test

Conclusion

For microscopists without prior FM experience, it takes approximately two to three months to gain sufficient confidence in fluorescence reading and only then does this become a real benefit in terms of reading time reduction.

Operational performance of iLED during demonstration projects

As shown below, the feedback from users during demonstration studies was very positive and, more importantly, remained positive during the later study phases after the "novelty" effect of iLED had worn off. The vast majority of users were in favor of implementing iLED on a wider scale. The questionnaire provided valuable information on training needs and other operational topics.

Table 21: Operational performance of iLED, as rated by users at 28 microscopy centers

	Training & validation (88 questionnaires completed)	**Implementation & continuation (56 questionnaires completed)**
Installation	Easy with the help of the instruction manual: 66% (58/88); Self explanatory (24/88); Rather difficult (6/88)	NA
Installation/first use of battery pack	Easy: 66% (58/88); Needed help of instruction manual: 34% (30/88)	NA
Training required		
1. for trained Zn microscopist (median; range)	Average: 5.2 days (range: 1-15 days)	Average: 5.0 days (range 2-7)
2. for user without prior training (median; range)	Average: 20 days (range 2-90)	Average: 13 days (5-30)
Overall handling and features	Very satisfied (superior to known microscopes): 68% (60/88); Satisfied (17/88); Not satisfied (1/88)	Very satisfied: 73% (41/56) Satisfied: 27% (15/56)
Use of ZN and FM on same system (Ease of switching)	Very convenient: 58% (51/88) Convenient: 42% (37/88)	Very convenient: 78% (44/56) Convenient: 21% (12/56)
Light intensity, background, contrast (FM mode)	Very satisfied: 60% (53/88) Satisfied: 40% (27/88)	Very satisfied: 82% (46/56) Satisfied: 18% (10/56)
Resolution and depth of focus	Very satisfied: 64% (56/88) Satisfied: 36% (32/88)	Very satisfied: 75% (42/56) Satisfied: 25% (14/56)
Ease of focus	Easy: 78% (69/88) Rather difficult: 22% (19/88)	Easy: 91% (51/56) Rather difficult: 9% (5/56) The opening slider is used by many readers to facilitate focusing.
Need for darkroom	Not needed: 94% (83/88) Partial darkroom needed: 6% (5/88)	Not needed: 95% (53/56) Partial darkroom needed (curtains): 5% (3/56)
Magnification factor for objectives	Suitable: 89% (78/88) Not ideal: 11% (10/88) *Suggested 25x* (Reading with 40X only)	Suitable: 89% (50/56) 25x instead of 20x needed: 11% (6/56) (40X remains the most frequently used magnification; but a few readers switched to 20X)
Preferred bulb type for ZN mode (available from Zeiss are LED or	>90% of users preferred halogen bulbs instead of LED bulbs Reason: Blue tones seen with LED bulb	>80% of users preferred halogen bulbs, but several users indicated that they got used to the different color impression

Halogen bulbs)	were found to reduce contrast between AFBs and background	
Technical problems	Defective battery pack version 1 (26/45) Fine Focus needed to be fixed (1/45) Condenser adjustment required (1/45) Loose contact (1/45) 40x lens not working (1/45)	Fine focus needed to be fixed (1/45) Switch to bright light not working (1/45) Stand needed replacement (1/45) Slide holder requiring replacement (1/45)
Multi-purpose or single-purpose	Various: 59% (52/88) TB only: 40% (35/88) Malaria & HAT only: 1% (1/88)	TB only: 53% (30/56) Various: 47% (20/56)
Gain in speed	Yes: 99% (87/88) Yes (but only for neg and low pos): 1% (1/88)	Yes: 98% (55/56) Yes (but only for neg and low pos): 2% (1/56)
Recommendation on implementation	Yes: 94% (83/88) No: 6% (5/88) Comments: Too much effort	Yes: 100.0% (56/56)
Where	In low, medium and high volume labs: 95% (79/83) Only in high volume labs: 5% (4/83)	In low, medium and high volume labs: 80% (45/56) Only in high volume labs: 20.0% (11/56)
Use of iLED	Use of iLED for ZN & FM: 37% (31/83) Use for FM only: 43% (36/83), 20% (16/83) users were convinced that LM would not be needed anymore (total replacement by FM)	Use of iLED for ZN & FM: 59% (33/56) Use for FM only: 21% (12/56), 20% (11/56) users were convinced that LM would not be needed anymore (total replacement by FM)

iLED performance compared to conventional FM over time

This project demonstrated substantial advantages of iLED in routine settings with regard to case detection rate, reading/hands-on time, ease/convenience of use and enthusiasm in uptake. It seemed important to prove that this was not only a temporary effect related to the excitement of using a new technology, but that the performance advantages persisted.

Table 22 shows that iLED performance remained strong throughout the implementation phase and (where available to date) early continuation phase. It is important to keep in mind that the results shown for implementation and continuation phase are only the rechecked data and only represent a fraction of the total number of slides read by iLED (>34000 during implementation phase and >9000 slides during continuation phase).

Table 22: iLED performance over time									
	Validation phase			Implementation phase			Continuation phase		
	Accuracy (% agreement)	Sensitivity (among FM pos)	iLED specificity (among FM neg)	Accuracy (% agreement)	Sensitivity (among FM pos)	Specificity (among FM neg)	Accuracy (% agreement)	Sensitivity (among FM pos)	Specificity (among FM neg)
India									
Vellore									
MC 1	99.4% (490/493) [98.1% - 99.8%]	96.4% (53/55) [86.7% - 99.1%]	99.8% (437/438) [98.4% - 100.0%]	98.9% (1118/1131) [97.8% - 99.4%]	95.7% (156/163) [91.5% - 97.9%]	99.4% (962/968) [98.0% - 99.7%]	98.6% (69/70) [90.5% - 99.8%]	88.9% (8/9) [50.0% - 98.5%]	100.0% (61/61) [94.0% - 100.0%]
MC 2	100.0% (316/316) [98.8% - 100.0%]	100.0% (55/55) [93.3% - 100.0%]	100.0% (261/261) [98.6% - 100.0%]	99.0% (311/314) [95.5% - 99.8%]	95.5% (63/66) [78.8% - 98.5%]	100.0% (248/248) [98.5% - 100.0%]	100.0% (16/16) [76.9% - 100.0%]	100.0% (3/3) [-23.0% - 100.0%]	100.0% (13/13) [71.6% - 100.0%]
MC 3	98.8% (497/503) [96.4% - 99.5%]	95.2% (60/63) [77.7% - 98.5%]	99.3% (437/440) [97.0% - 99.8%]	99.7% (655/657) [98.8% - 99.9%]	98.4% (63/64) [89.8% - 99.8%]	99.8% (592/593) [98.8% - 100.0%]	100.0% (20/20) [81.6% - 100.0%]	100.0% (2/2) [-84.4% - 100.0%]	100.0% (18/18) [79.5% - 100.0%]
Delhi									
MC 1	98.5% (1684/1709) [97.4% - 99.1%]	96.6% (282/292) [91.3% - 98.5%]	98.9% (1402/1417) [97.7% - 99.3%]	98.8% (1062/1075) [97.4% - 99.6%]	96.2% (100/104) [83.7% - 98.9%]	99.1% (962/971) [97.5% - 99.7%]	100.0% (19/19) [80.6% - 100.0%]	100.0% (3/3) [-23.0% - 100.0%]	100.0% (16/16) [76.9% - 100.0%]
MC 2	99.7% (673/675) [98.8% - 99.9%]	98.7% (74/75) [90.7% - 99.8%]	99.8% (599/600) [98.8% - 100.0%]	99.0% (390/394) [96.8% - 99.7%]	100.0% (61/61) [94.0% - 100.0%]	98.8% (329/333) [95.5% - 99.5%]	100.0% (16/16) [76.9% - 100.0%]	100.0% (1/1) [-268.9% - 100.0%]	100.0% (15/15) [75.4% - 100.0%]
MC 3	98.4% (243/247) [95.8% - 99.4%]	95.9% (47/49) [84.2% - 99.0%]	99.0% (196/198) [96.1% - 99.7%]	100.0% (111/111) [96.7% - 100.0%]	100.0% (19/19) [80.6% - 100.0%]	100.0% (92/92) [96.0% - 100.0%]	100.0% (30/30) [87.7% - 100.0%]	100.0% (3/3) [-23.0% - 100.0%]	100.0% (27/27) [86.3% - 100.0%]
Agra									
MC 1	96.1% (392/408) [92.7% - 97.7%]	90.2% (83/92) [75.7% - 94.2%]	97.8% (309/316) [93.2% - 98.8%]	98.7% (778/788) [97.7% - 99.3%]	100.0% (160/160) [97.7% - 100.0%]	98.4% (618/628) [97.0% - 99.1%]	96.6% (56/58) [86.9% - 99.1%]	100.0% (15/15) [75.4% - 100.0%]	95.3% (41/43) [82.7% - 98.8%]
MC 2	98.0% (901/919) [96.4% - 99.0%]	89.3% (117/131) [78.4% - 94.6%]	99.5% (784/788) [98.1% - 99.8%]	95.5% (1611/1687) [93.9% - 96.7%]	89.4% (286/320) [80.5% - 91.5%]	96.9% (1325/1367) [95.1% - 97.8%]	Not ready	Not ready	Not ready
MC 3	98.4% (507/515) [96.6% - 99.3%]	92.0% (80/87) [82.8% - 96.3%]	99.8% (427/428) [98.3% - 100.0%]	100.0% (517/517) [99.3% - 100.0%]	100.0% (92/92) [96.0% - 100.0%]	100.0% (425/425) [99.1% - 100.0%]	100.0% (21/21) [82.4% - 100.0%]	100.0% (5/5) [26.2% - 100.0%]	100.0% (16/16) [76.9% - 100.0%]

	Validation Phase			Implementation Phase			Continuation Phase		
Vietnam	**Accuracy**	**Sensitivity**	**Specificity**	**Accuracy**	**Sensitivity**	**Specificity**	**Accuracy**	**Sensitivity**	**Specificity**
MC 1	99.0% (771/779) [96.9% - 99.6%]	94.2% (114/121) [82.9% - 98.2%]	99.8% (657/658) [98.9% - 100.0%]	98.5% (321/326) [96.4% - 99.5%]	97.8% (88/90) [91.0% - 99.4%]	98.7% (233/236) [96.6% - 99.8%]	97.1% (34/35) [82.1% - 99.6%]	100.0% (2/2) [-84.4% - 100.0%]	97.0% (32/33) [81.1% - 99.6%]
MC 2	99.0% (283/286) [93.9% - 99.9%]	100.0% (46/46) [92.0% - 100.0%]	98.8% (237/240) [92.8% - 99.9%]	98.0% (99/101) [91.9% - 99.8%]	93.9% (31/33) [66.1% - 99.1%]	100.0% (68/68) [94.6% - 100.0%]	100.0% (18/18) [79.5% - 100.0%]	100.0% (10/10) [63.1% - 100.0%]	100.0% (8/8) [53.9% - 100.0%]
MC 3	97.4% (864/887) [95.9% - 98.4%]	89.0% (146/164) [80.1% - 93.1%]	99.3% (718/723) [98.4% - 99.7%]	99.5% (415/417) [98.1% - 99.9%]	99.5% (195/196) [96.5% - 99.9%]	99.5% (220/221) [96.9% - 99.9%]	Not ready	Not ready	Not ready
Cambodia									
MC 1	97.8% (1072/1096) [96.4% - 98.7%]	97.3% (144/148) [92.2% - 99.2%]	97.9% (928/948) [96.0% - 98.6%]	94.2% (326/346) [92.3% - 97.2%]	96.5% (167/173) [91.3% - 98.6%]	91.9% (159/173) [88.2% - 96.3%]	Not ready	Not ready	Not ready
MC 2	98.1% (503/513) [96.5% - 98.9%]	88.6% (39/44) [76.0% - 95.2%]	98.9% (464/469) [97.5% - 99.6%]	93.1% (296/318) [89.3% - 95.6%]	94.9% (112/118) [86.7% - 97.7%]	92.0% (184/200) [87.7% - 95.4%]	Not ready	Not ready	Not ready
MC 3	98.7% (386/391) [95.8% - 99.7%]	95.1% (39/41) [67.2% - 99.2%]	99.1% (347/350) [95.3% - 99.9%]	97.4% (338/347) [96.3% - 99.3%]	95.7% (135/141) [88.3% - 98.7%]	98.5% (203/206) [95.6% - 99.5%]	Not ready	Not ready	Not ready
Thailand									
MC 1	98.9% (374/378) [97.2% - 99.6%]	89.7% (26/29) [71.7% - 96.0%]	99.7% (348/349) [98.0% - 100.0%]	99.3% (607/611) [98.3% - 99.8%]	97.5% (39/40) [84.0% - 99.6%]	99.5% (568/571) [98.4% - 99.8%]	Not ready	Not ready	Not ready
Peru									
MC 1	97.3% (1018/1046) [96.1% - 98.2%]	92.5% (86/93) [83.2% - 96.4%]	97.8% (932/953) [96.5% - 98.5%]	99.1% (444/448) [97.2% - 99.7%]	96.9% (95/98) [89.3% - 99.3%]	99.7% (349/350) [98.0% - 100.0%]	Not ready	Not ready	Not ready
MC 2	97.7% (559/572) [96.1% - 98.7%]	91.3% (42/46) [78.3% - 96.0%]	98.3% (517/526) [96.8% - 99.1%]	97.6% (290/297) [95.1% - 98.8%]	87.5% (21/24) [70.6% - 95.4%]	98.5% (269/273) [96.2% - 99.4%]	Not ready	Not ready	Not ready
MC 3	96.0% (266/277) [92.8% - 98.0%]	81.3% (13/16) [47.9% - 94.9%]	96.9% (253/261) [93.7% - 98.6%]	97.0% (97/100) [91.0% - 99.0%]	100.0% (6/6) [38.5% - 100.0%]	96.8% (91/94) [90.1% - 98.9%]	Not ready	Not ready	Not ready

	Validation Phase			Implementation Phase			Continuation Phase		
Russia	**Accuracy**	**Sensitivity**	**Specificity**	**Accuracy**	**Sensitivity**	**Specificity**	**Accuracy**	**Sensitivity**	**Specificity**
MC 1	95.0% (485/511) [92.1% - 96.5%]	99.7% (302/303) [97.7% - 100.0%]	88.0% (183/208) [81.4% - 91.5%]	98.4% (718/730) [97.1% - 99.1%]	99.2% (471/475) [97.8% - 99.7%]	96.9% (247/255) [93.8% - 98.4%]	95.6% (153/160) [91.1% - 97.9%]	97.0% (64/66) [88.7% - 99.2%]	94.7% (89/94) [87.7% - 97.7%]
MC 2	96.1% (293/305) [93.1% - 98.1%]	93.8% (30/32) [78.4% - 98.4%]	96.3% (263/273) [92.9% - 98.2%]	98.7% (386/391) [97.0% - 99.5%]	98.4% (121/123) [93.8% - 99.6%]	98.9% (265/268) [96.6% - 99.6%]	95.3% (122/128) [89.7% - 97.9%]	93.8% (30/32) [78.1% - 98.4%]	95.8% (92/96) [88.5% - 98.4%]
South Africa									
MC 1	98.2% (536/546) [96.4% - 99.1%]	85.5% (47/55) [70.2% - 91.8%]	99.6% (489/491) [98.4% - 99.9%]	87.9% (123/140) [81.3% - 92.3%]	82.2% (60/73) [70.3% - 89.0%]	94.0% (63/67) [83.2% - 97.5%]	Not ready	Not ready	Not ready
MC 2	99.1% (540/545) [97.3% - 99.8%]	96.1% (99/103) [87.6% - 99.2%]	99.8% (441/442) [98.4% - 100.0%]	92.7% (421/454) [90.4% - 95.3%]	95.3% (222/233) [91.0% - 97.4%]	90.0% (199/221) [87.6% - 94.9%]	Not ready	Not ready	Not ready
MC 3	93.4% (520/557) [91.0% - 95.2%]	80.2% (93/116) [68.6% - 85.2%]	96.8% (427/441) [94.7% - 98.1%]	95.9% (279/291) [92.9% - 97.8%]	97.7% (167/171) [93.8% - 99.1%]	93.3% (112/120) [42.0% - 75.7%]	Not ready	Not ready	Not ready
TOTAL	97.7% (17812/18224) [97.4% - 97.9%]	94.2% (2567/2726) [92.2% - 94.6%]	98.4% (15245/15498) [97.9% - 98.5%]	98.0% (12230/12484) [97.4% - 98.1%]	96.7% (3244/3356) [95.6% - 97.2%]	98.4% (8986/9128) [97.8% - 98.5%]	97.1% (574/591) [95.4% - 98.2%]	96.7% (146/151) [92.2% - 98.6%]	97.3% (428/440) [95.2% - 98.4%]

Conclusions from demonstration study

In collaboration with National and Regional TB Programs, iLED was introduced at 28 routine microscopy centers located in India, Vietnam, Thailand, Cambodia, South Africa, Lesotho, Ethiopia, Russia and Peru.

More than 60,000 iLED results have been analyzed for this report in comparison to a ZN baseline phase. The results showed that:

1. It is feasible to implement Primo Star iLED for TB diagnosis at microscopy centers in low-income settings that had no prior experience with fluorescence microscopy.
2. A high level of agreement between readers and supervisors was observed within the first month of iLED adoption. This high agreement suggests that microscopists could achieve proficiency on iLED within a short timeframe and with accuracy similar or greater than ZN.
3. Since iLED performance was already strong during validation phase for most sites, little further improvement was seen over time with increasing microscopist experience. However, iLED performance was maintained for all sites.
4. Positivity and case detection rates were significantly better than ZN.
5. The decreased workload due to decrease in reading time was perceived by all participating microscopists and reported in the user appraisal questionnaire. A reading time sub-study showed that a significant reduction in reading time >40% is only seen after two to three months when experience and confidence of readers has increased.
6. Lab technicians' appraisal of Primo Star iLED was very positive overall and uptake was enthusiastic. No real barriers to implementation were identified. Valuable input was given with regard to minimal training time required. ZN mode of iLED was much preferred when using the halogen rather than the LED bulb option (Halogen insertion available from Zeiss). The most recent version of the battery pack was found to be functional, but design could be further improved.

Economic data

Rationale

In high burden countries, ZN smear microscopy continues to be the cornerstone in TB diagnosis due to its perceived low cost. Use of more advanced technology such as fluorescent microscopy (FM), while it has several key advantages over ZN, has been very limited due its higher cost (high equipment and maintenance cost) and requirement of more sophisticated laboratory infrastructure (darkroom and consistent electricity). Now, with more than $1 billion USD spent per year on diagnostics for TB globally, there has been considerable effort in developing simpler and cost-favorable FM technology.

In these large-scale studies, ultrabright light-emitting diode (LED) FM has been demonstrated to provide equivalent performance to conventional FM without space and maintenance constraints. LED technology brings significant potential for cost-efficiency as an alternative to ZN. Given the reduced laboratory staff hands-on time and shorter time-to-diagnosis, a cost analysis needs to take into account not only cost of equipment, reagents and infrastructure, but also all relevant costs associated with time and labor.

The primary objective of this analysis was to determine complete economic cost for iLED per test or per patient screened. This included all aspects of costs to perform the test from a health services perspective. Costs associated with time were based on detailed on site time-analysis for participating routine microscopy centers.

Methods and assumptions

Cost analysis is one of many types of economic evaluations that focuses on assessing the cost of providing service, program or intervention.[ix,x] While cost analysis does not directly include the effectiveness aspect of an intervention/program, its main advantage comprehends relative ease in interpreting the results, ability to directly compare costs amongst interventions being considered, and little or no requirement for complicated modeling or assumptions that may have significant influence on the results of an analysis.

For demonstration study purposes, cost analysis was based on "economic" rather than "financial" costing, because the former provides a more complete picture of the costs incurred by the health service when using a particular good or service. Financial costing only reflects the actual flow of money associated with the goods and services purchased and would therefore not be adequate in this case. However, in public health systems in low-income countries, the following factors, 1) a large number of goods and services are donated, 2) 'price' often fails to accurately reflect true value of the goods or services provided, and 3) opportunity cost[xi,xii] , are important components of the comparative assessment of cost and benefits of alternative interventions.

Data on cost of iLED and ZN smear microscopy were collected in three geographically representative demonstration settings: India, Lesotho, and Peru. In each location, data were collected through site visits of 1-2 microscopy centers with low (1-15), 1-2 with medium (16-35), and 1-2 with high specimen volume (greater than 35) per day. Country-specific data were then averaged for the analysis.

Prior to data collection, the work-flow at each microscopy center was evaluated in order to reflect costs associated with unique procedural characteristics of each microscopy center / TB laboratory.

Usage of capital assets (building space and equipment) was quantified as minutes used per square meter of the space. Values of capital assets and equipment were analyzed based on their cost when purchased new. Staff time between TB smear microscopy tasks was not included in the cost analysis since the staff had multiple unrelated responsibilities to perform (mostly for the diagnosis of other diseases). All capital and recurrent costs were collated from the time of specimen arrival at the laboratory until time of test result, hence analyzed for laboratory-only costs. Unit costs were calculated using the 'ingredients' approach, multiplying the quantities used by unit price. General consumables (sputum collection cup, slides, latex gloves, etc.) for both methodologies remained identical; therefore, the costs were the same. All capital costs, mainly laboratory space and building, laboratory equipment, were annualized based on their estimated expected life-years.[xiii] Expenses associated with maintenance and running costs for the building, management and supervision, quality control and assurance, as well as training were summarized as overhead costs.

Transportation costs for specimens was calculated based on the observation that approximately 60% of specimens required transport in Lima, 50% in Lesotho and 40% in India. Transportation methodology differed from country to country. In Lesotho, a specific car was designated for specimen collection for relevant microscopy centers/laboratories from various clinics. In Peru, sputum specimens were delivered to microscopy centers/laboratories by a health worker taking approximately 1-2 hours and utilizing the public transportation system (buses and motor taxis). In India, local microscopy centers mostly examined sputum collected on-site, except at the Intermediate Reference Laboratory level where a significant portion of specimens were delivered via courier service. Regardless of transportation methodology, cost per specimen transported to the laboratory were similar across all sites examined where the cost ranged from US$ 0.31 (India) to US$ - 0.40 (Lesotho).

Costs were expressed in US $ and prices available in the local currency were converted into the 2008 U.S. Dollar value based on the average currency exchange of the local currency[xiv] The majority of the pricing information for assets/reagents was obtained by the laboratory's procurement logs or distributor catalogues and includes cost of procurement at 25% of the unit pricing. Cost parameters and data used for the analysis are listed in the table below.

Table 23: Cost data elements for cost analysis of TB diagnostics and suggested data sources

Data Element	Cost Items	Sources of Data
Physical infrastructure	Construction	*Construction contractors / Government Estates and building planning office / Recent laboratory construction budget*
	Maintenance contracts for all laboratory equipment requiring periodic maintenance	*Laboratory financial records / Laboratory or hospital accounts office / service contractors*
Chemicals reagents and consumables	All types of chemicals and reagents utilized for diagnostic methods evaluated	*Laboratory financial records / manufacturer catalogue (must include all costs associated with procurement, usually at 25% of the catalogue price)*
	All types of general laboratory consumables (e.g. latex gloves, micropipette tips)	*Laboratory financial records / manufacturer catalogue (must include shipping costs)*
Human Resources	Laboratory staff salaries	*Government salary scale / Laboratory or hospital accounts office*
	Laboratory staff allowances and benefits	*Government salary scale / Laboratory or hospital accounts office*
	Staff training off-site	*Laboratory records / interview*
Training and quality assurance	Orientation training for new staff	*Laboratory staff records*
	External QA/QC for various laboratory diagnostic activities	*Pricing/cost available through laboratory/hospital accounts office. List of QA/QC activities was found in the National Quality Assurance guidelines for ZN and in the demonstration project SOP for iLED*
	Internal QA/QC	
Specimen transport *(some sites use courier service in transporting specimen)*	Cost of a vehicle used for specimen transport - evaluated as purchased 'new'	*Accounting office/auto dealer*
	Average distance traveled - annual figure	*List of locations referring specimens to the laboratory*
	Average driver salary	*Accounting office*
	Quantity of fuel used	*Accounting office*
	Fuel Price	*Market research, Accounting office*
	Insurance of vehicle	*Accounting office*
	Other consumables used in specimen transport	*Accounting office*

Table 24: Details on resource cost parameters
Overhead – 1) maintenance & running costs for the operation the laboratory unit • utilities, communications, administrative infrastructure 2) management and supervision • staffing (for general laboratory operation and supervision) • quality control and assurance • general and specific laboratory-related training
Building – cost relating to the use of physical (laboratory) space specific to the laboratory procedure being assessed – calculation based on current real estate quotations and estimates per square meter and approximate building maintenance costs
Equipment – Costs based on annualized cost of equipment and approximate use (in terms of time) of equipment during the diagnostic process (inclusive of 25% procurement cost) • Conventional light microscope (US $ 900 - US $ 1250) • iLED (EUR 1250)
Staff - complete staff hands-on time from the receipt of specimen to dispatch and filling of result forms – based on average salary (cost per minute) of all categories of laboratory staff involved in the diagnostic process at each country evaluated in the study
Chemicals and Reagent – all reagents prepared at the supervising or high volume level site and includes all staffing, use of overhead, building, and chemical/reagents in preparing stock working solutions (calculation based on approximately 5 ml of staining solutions used per slide) • due to significant pricing variation on essential chemicals (manufacturer, distributor, and country specific pricing), chemical unit costs are averaged for all sites
Consumables – cost associated with use of general consumables such as sputum collection cups, latex gloves, glass slides, etc.

Results and Discussion

Complete averaged unit costs, expressed in terms of cost per specimen or slide screened in US $, for ZN and iLED methodology stratified by country and major cost items are summarized in the table below.

Overall, costs per test for iLED were approximately 12% lower than for ZN. Cost per test (sub-total) for ZN ranged between US $ 1.67 to 1.92 and for iLED between US $ 1.49 to 1.72. On average, iLED cost per test was approximately 10% cheaper than ZN. Overall, per test costs were highest in Peru for both ZN and iLED, which was mainly due to higher costs for staff, overhead, reagents and chemicals used for smear microscopy (Table 25).

Table 25: Per test costs for ZN and iLED in selected countries

Input\Test Type	India		Lesotho		Peru	
	ZN	iLED	ZN	iLED	ZN	iLED
OVERHEAD	0.54	0.48	0.57	0.49	0.66	0.55
BUILDING	0.06	0.05	0.04	0.03	0.02	0.01
EQUIPMENT	0.04	0.04	0.05	0.04	0.07	0.04
STAFF	0.42	0.37	0.31	0.27	0.44	0.35
Chemicals and Reagent	0.20	0.13	0.20	0.13	0.13	0.20
CONSUMABLES	0.36	0.36	0.32	0.32	0.35	0.35
Specimen Transport	0.13	0.13	0.20	0.20	0.22	0.22
Total cost per test	1.74	1.55	1.67	1.49	1.96	1.72

A stratification of overall costs in per test costs at low, medium and high volume laboratories (Table 26) shows that the per test costs are lowest at medium size laboratories due to the relatively higher staff costs at low volume laboratories and higher infrastructure and staffing costs at high volume laboratories. However, the daily workload of the laboratory only had a minor influence on the per test costs.

Table 26: Variable per test costs stratified by the daily workload at microscopy centers for low, medium and high volume microscopy centers

Breakdown of variable costs (per test)	ZN			LED		
Slide volume per day	LOW	MED	HIGH	LOW	MED	HIGH
Overhead	0.62	0.57	0.60	0.48	0.47	0.50
Building	0.01	0.02	0.07	0.01	0.02	0.06
Equipment	0.04	0.05	0.07	0.05	0.04	0.06
Staff	0.50	0.36	0.35	0.40	0.31	0.30
Total	**1.17**	**1.00**	**1.09**	**0.94**	**0.84**	**0.92**

Reduced reading time for iLED resulted in an average savings in staff costs of US $ 0.06 per test, when considering that iLED saves 55% in reading time. This would free significant capacity at microscopy centers and/or would allow increased focus on the quality of microscopy.

In addition, we calculated costs per correctly diagnosed patient when screening 1000 TB suspects in India (low HIV prevalence; low number of low positive cases) and Lesotho (high HIV prevalence; high number of low positive cases) assuming an overall sensitivity of iLED and ZN of 96.3% and 90.5% respectively and a specificity of 100% for both methods (see assumptions shown in Tables 27-29 below).

Table 27: Assumptions for simplified cost-effectiveness analysis

Assumptions based on evaluation study results	ZN	iLED 40X
Sensitivity in S-	0%	0%
Sensitivity in S+	90.5%	96.3%
Very low pos (scanty)	60.8%	85.0%
Low pos (1+)	97.2%	99.2%
High pos (2+, 3+)	100.0%	99.2%
Specificity	100.0%	100.0%

Our preliminary (and simplified) cost-effectiveness analysis estimates for both Lesotho and India are shown in the tables below.

Table 28: Cost per new case detected, 2008 US $, screening 1000 TB suspects in India
(assuming 20% smear negative cases (neg in iLED and ZN), 10% very low positive, 30% low positive, 40% high positive)

TB Prevalence	0.05		0.10		0.20		0.30		0.50	
	ZN	iLED	ZN	iLED	ZN	iLED	ZN	iLED	ZN	iLED
Nr of cases detected	38	39	75	78	150	156	226	233	376	389
Nr of cases missed	12	11	25	22	50	44	74	67	124	111
Costs per diagnosed patient	**45.58**	**40.53**	**23.79**	**20.26**	**11.90**	**10.13**	**7.93**	**6.75**	**4.76**	**4.05**

Table 29: Cost per new case detected, 2008 US $, screening 1000 TB suspects in Lesotho
(assuming 40% smear negative cases (neg in iLED and ZN), 20% very low positive, 20% low positive, 20% high positive)

TB Prevalence	0.05		0.10		0.20		0.30		0.50	
	ZN	iLED	ZN	iLED	ZN	iLED	ZN	iLED	ZN	iLED
Nr of cases detected	26	28	52	56	103	112	155	168	258	280
Nr of cases missed	24	22	48	44	97	88	145	132	242	220
Costs per diagnosed patient	**53.98**	**49.81**	**26.99**	**24.91**	**13.50**	**12.45**	**9.00**	**8.30**	**5.40**	**4.98**

On the whole, due to higher overall sensitivity, iLED will capture more true positive cases than ZN regardless of prevalence of TB in the screening group. In the case of higher HIV prevalence settings (Lesotho), more cases will be missed compared to lower HIV prevalent settings (India) for both methodologies. Therefore, cost per new cases detected is higher in Lesotho compared to India. Our preliminary results also indicate that iLED is consistently the less costly method of the two in all parameters evaluated in our analysis. However, the key limitation of our analysis is that it is a pure cost comparison ("cost minimization analysis"); we assumed that clinical outcomes related to the various strategies would be equivalent. This may not be the case, particularly in higher HIV prevalence settings and also in higher TB and MDR-TB prevalence countries where undetected or misdiagnosed patient(s) can have significant consequences when viewed from a societal perspective.

Generally, lower test sensitivity reduces per test/diagnosis cost of the methodology because fewer TB cases are detected. However, our analysis reveals that lower per test cost combined with its higher test sensitivity still makes iLED a cheaper methodology compared to ZN. While our particular analysis has a limited perspective (based only on the laboratory costs) and several analytical limitations, it shows that iLED is a cost-favorable methodology compared to ZN. In 2008, Sohn et al. showed that conventional fluorescent microscopy (CFM) can be cheaper (in terms of per test cost).[xv] However, considering the key requirements (darkroom, constant power, consistent maintenance with light bulb replacement after every 200th use, difficulty in operating, etc.) for CFM, iLED's indifferent costs for maintenance and infrastructural requirements compared to ZN, relative ease of operation, and reduced time for reading, indicated that it has great potential in not only improving diagnostic capacity but also in terms of sustainability when implemented as part of the national TB programs for sputum smear diagnosis of TB. Nonetheless, to appropriately evaluate the full economic and health impact of the use of iLED in high TB burden (as well as in high TB-HIV settings), low income countries, a more detailed cost-effectiveness analysis using epidemiologic and economic modeling analysis is planned.

Conclusion

Based on this costing analysis, iLED represents a cost-efficient alternative to ZN in resource poor, high TB burden settings. While our particular analysis has a limited perspective (based only on the laboratory costs), it shows that iLED is not only cost-favorable compared to ZN, but can easily be implemented into current national TB programs and sustained without having to modify significantly the current budgeting scheme (except for the initial capital investment for purchasing microscopes).

Detailed FIND study results: Sub-studies

Comparative studies for existing LED-based FM systems in Uganda and Zambia

Background

During the process of development of the iLED, some of the other LED-based options for TB detection with fluorescent microscopy entered development or became commercially available. These include adaptations to existing microscopes, or new microscopes with built-in LED FM capacity: FRAEN (Settimo, Italy) makes a product that includes an adaptor that clips onto existing microscopes to provide *transmitted* LED light through the microscope slide, and a set of filters in the barrel of the microscope. Other manufacturers, such as LW Scientific (Lawrenceville, GA, USA), also make equipment to adapt existing microscopes by providing LED powered objectives that provide *reflected* LED light. Though all of these systems have only recently been developed, they have already been used in feasibility studies to detect tuberculosis using conventional FM stains like Auramine O[xvi,xvii,xviii]. Very recently, several replacement microscopes have been developed. Some of these are purpose-built to be as simple and inexpensive as possible. These microscopes from Cytoscience (Fontaines, Switzerland), De-rev (Denver, CO, USA) and Partec (Görling, Germany) have been developed to prototype stage and are now, or soon may be, commercially available. All are monocular and battery-powered.

It seemed very important to determine the clinical and operational performance for commercially available LED systems in comparative studies.

Methods

This substudy sought to compare the performance of three LED-based systems for fluorescence microscopy detection of *M. tuberculosis* with light microscopy (Ziehl-Neelsen staining) and with conventional fluorescence in TB suspects in Kampala, Uganda and Lusaka, Zambia.

The following LED-based systems were evaluated:

1. iLED Primostar (Zeiss)
2. FLuoLED™ add-on device (Fraen Corporation)
3. Lumin add-on device (LW Scientific)

Comparative gold standards used were: a) for Uganda, NIKON Eclipse E200 microscope for conventional fluorescence and iLED for ZN; and b) for Zambia, Olympus BX41 for both methods. Add-on devices were attached to Olympus CX31 in Uganda, and Olympus CX41 in Zambia. Staining was done using quality-controlled in-house Auramine and ZN staining solutions.

The order of reading of the three LED methods was alternated with each batch; e.g. Day 1: read iLED then Fraen then Lumen; Day 2: read Fraen then Lumen then iLED; Day 3: read Lumen then Fraen then iLED, etc. Slides were not re-stained for reading by the different LED-based techniques. Fluorescent smears were read at x40 magnification. 40 fields were read for fluorescence smears and 100 fields for ZN smears.

1. Zambia

A panel slide set composed of 150 positive [100 low positive (scanty and 1+) and 50 high positive (2+ and 3+)] and 150 negative slides was prepared by a participating reference laboratory using 300 sputum samples for which smear and culture results were available. Two slides were prepared from each sputum sample, one for reading by the 4 FM methods and one for ZN reading. The order of

reading of the 3 LED methods was alternated so as to avoid having performance of any one of the FM methods affected due to fading after repeated reading of the same slide. Auramine slides were overlabelled between readings to ensure blinding of the 4 FM methods. Also, ID labels of ZN and Auramine slides were different to ensure blinding between ZN and FM methods. The assessment was carried out by two microscopists who had some experience with FM (< 1 year).

Results

Comparative performance

As shown below, the sensitivity of all 3 LED methods and of conventional FM was significantly higher than for ZN. Although the iLED appeared to be more sensitive than the other LED methods, this was not statistically significant at a 5% level.

The specificity was identical for all LED methods, and slightly lower for conventional FM. There was no statistically significant difference compared to ZN for any of the methods. All false positive results were very low (scanty) by all methods.

Table 30: Sensitivity and specificity of alternative LED methods - Zambia

	FluoLED	**Lumin**	**iLED**	**FM**	**ZN**
Sensitivity in culture positive panel slides	143/150 **95.30%** (CI 90.62%, 98.10%)	139/150 **92.70%** (CI 87.26%, 96.28%)	148/150 **98.70%** (CI 95.27%, 99.84%)	145/150 **96.70%** (CI 92.39%, 98.91%)	128/150 **85.30%** (CI 78.64%, 90.57%)
p-value for difference compared to ZN	0.0026	0.0266	<0.0001	0.0002	-
p-value for difference compared to iLED	0.0625	0.0039	-	0.25	<0.0001
Specificity in culture negative panel slides	149/150 **99.30%** (CI 96.34%, 99.98%)	149/150 **99.30%** (CI 96.34%, 99.98%)	149/150 **99.30%** (CI 96.34%, 99.98%)	145/150 **96.70%** (CI 92.39%, 98.91%)	150/150 **100%** (CI 97.57%, 100.00%)
p-value* for difference compared to ZN	>0.9999	>0.9999	>0.9999	0.0625	-

*p-values were calculated using exact Mc'Nemar test

Examination time

Examination times were measured by the supervisor for all slides. Average examination times for each method are shown below.

Table 31: Average examination time of ZN, FM and three LED FM methods in Zambia stratified by gold standard result

	FluoLED	Lumin	iLED	FM	ZN
No Disease (N=150)	189.44	198.21	185.89	187.04	264.36
Low positive [scanty/+1] (N=100)	153.98	188.36	151.25	142.74	292.42
High positive [+2/+3] (N=50)	55.1	78.68	57.1	55.62	123.46

As shown below, the mean reading time reduction was highly significant for all FM methods when compared to the reading time for ZN slides. The reading time for Lumin was significantly higher ($p < 0.0001$) than for the other 3 fluorescence methods. The same trend was seen in Uganda.

Table 32: Reading time comparison for alternative LED and conventional methods – Zambia

	Fraen	Lumin	iLED	FM	ZN
Overall reading time	155.23	175.01	152.89	150.37	250.23
p-value for difference compared to ZN	<0.0001	<0.0001	<0.0001	<0.0001	-
p-value for difference compared to Lumin	<0.0001	-	<0.0001	<0.0001	<0.0001
Note: p-value was calculated using paired t-test					

2. Uganda

Leftover portions of sputum specimens submitted by TB treatment-naïve patients being investigated for pulmonary tuberculosis at Mulago Hospital complex were utilized in this study. Specimens were first examined by routine direct FM in the Mulago Hospital Microbiology laboratory, which was not quality assured by the study team.

Two additional direct smears were prepared per leftover specimen and stored in a slide box. MGIT and LJ culture were performed and Capilia TB test was used for *M.tuberculosis* identification. Smears were allocated to receive either ZN or fluorescence (Auramine) staining, with the order of staining alternated each day. Staining reagents for ZN and fluorescence staining were prepared according to FIND SOP and were filtered on a weekly basis. Positive and negative control slides were included in each batch. Reading of both smears from one specimen was performed by a single technologist (ZN plus all LED methods). An overlabelling (blinding) system was implemented by the FIND study coordinator to avoid interpretation bias, as the same technologist read all slides from a single specimen. Two technologists read the slides; one had previous experience in both ZN and fluorescence microscopy and the other in ZN only. Readers underwent proficiency testing prior to study initiation. 30 slides were selected for investigating intra- and inter-reader variability using both ZN and LED-based methods. The readers were blinded to the previous results. Discrepant results were re-read to establish the true result.

An average examination time was calculated per reader and per result (negative, very low positive [scanty], low positive [1+] and high positive [2+ and 3+]) for each method using a panel of 40 slides.

Results

Proficiency testing
The two readers both passed proficiency testing for all methods on the first attempt after three days of practical training. In total (combined results from both readers), 1 LFP was obtained for ZN, 0 errors were obtained for iLED, 4 LFPs and 1 LFN were obtained for Fraen and 1 LFP was obtained for Lumin. No QEs or HF results were obtained for any method.

Comparative performance

Table 33: Sensitivity and specificity for detection of TB in slides prepared from routine sputum samples in Uganda

	ZN	Routine FM	iLED	FluoLED	Lumin
Sensitivity in culture positive sputa	33/55 (60.0%; 45.9–73.0)	28/55 (50.9%; 37.1-64.6)	36/55 (66.7%; 51.4–77.8)	38/55 (69.1%; 55.2-80.9)	35/55 (63.6%; 49.5-76.2)
Very low positive (scanty)	2	-	6	7	7
Low positive (1+)	8	-	8	9	7
High positive (2+, 3+)	23	-	22	22	21
Specificity in culture negative sputa	125/127 (98.4%; 94.4-99.8)	125/127 (98.4%; 94.4-99.8)	126/127 (99.2%; 95.7-100.0)	121/127 (95.3%; 90.0-98.2)	126/127 (99.2%; 95.7-100.0)

A different grading scheme was used for routine FM and therefore the grading results are not directly comparable with the other methods and have been omitted.

The sensitivity of the LED FM methods was between 3.6% and 9.1% higher than ZN. However, only the difference between FluoLED and ZN was significant at the 5% level (ZN vs FluoLED™, p=0.025; ZN vs iLED, p=0.083; ZN vs Lumin, p=0.317). There was no significant difference in sensitivity when comparing the three LED methods with each other in a pairwise fashion.

The specificity of FluoLED™ was lower than the other methods. The difference was significant at the 5% level for comparison of FluoLED and Lumin specificity (Lumin vs FluoLED™, p=0.025; iLED vs FluoLED™, p=0.059; ZN vs FluoLED™, p=0.157). All false positive results were very low positive (scanty) results by all methods.

Sensitivity of routine FM was the lowest of all methods, and was 9.1% less sensitive than ZN, although the difference was not significant (p=0.132). Specificity of routine FM was equivalent to the other methods (Table 33).

Examination time
A panel of 40 slides were examined and timed (20 slides per reader) using each method.
Average examination times for each method are shown below.

Examination time was between 2 and 4 times less for LED FM (using any LED method) compared with ZN. It is possible that these results underestimate the benefit in terms of examination time in using

FM compared to ZN since grading charts were used and accurate quantification was required for low positive results. Nonetheless, a substantial reduction in examination time was demonstrated.

Table 34: Average Examination time of ZN and three LED FM methods in Uganda

	ZN	iLED	FluoLED	Lumin
Negative	5.08 (4.51-6.07)	2.35 (2.07-2.90)	2.29 (1.97-3.09)	2.95 (2.62-3.43)
Very low pos (scanty)	6.03 *	2.62 (2.15-3.08)	2.50 (2.40-3.05)	2.97 (2.53-3.83)
Low pos (1+)	8.87*	2.80 *	3.84 (2.55-5.12)	5.47 *
High pos (2+, 3+)	4.07 (3.28 - 6.43)	1.04 (0.74-3.69)	0.82 (0.73-2.45)	1.25 (0.73-1.30)
Overall	5.1 (4.5 – 6.1)	2.3 (2.0 -2.9)	2.38 (1.97-3.05)	2.94 (2.49-3.47)

* 1 slide only

Median examination time (inter-quartile range): Forty fields were examined for LED methods and 100 fields for ZN.

Comparative user appraisal in Uganda and Zambia

A qualitative analysis of user perception of the three LED-based systems was carried out using a semi-structured questionnaire for completion by all microscopists involved in the assessment (two technicians plus head of laboratory in Uganda, and two technicians in Zambia). Whenever the answers were not identical for all users, the number in brackets indicates how many users had this opinion (Table 35).

Table 35: User appraisal – Uganda and Zambia (5 users) Strengths and weaknesses of each technology, as rated by users participating in the comparative studies			
	iLED	**FluoLED**	**Lumin**
Installation	Easy using instruction manual	Difficult to assemble, instruction manual inadequate	Very easy
Training required	2-4 days for ZN-trained microscopists Min 5-7 days for untrained users Adoption believed to be easy	3-5 days for ZN-trained microscopists Min 5-7 days for untrained users Adoption believed to be easy	3-5 days for ZN-trained microscopists Min 5-7 days for untrained users Adoption believed to be easy
Overall handling	Superior to usual microscope	Inferior to usual microscope: add-on is bulky and inhibits slide placement on stage	Inferior to usual microscope: power cable of device interferes with stage movement
Light intensity, contrast and background	Homogeneity of illumination superior to usual microscope, adjustable light intensity	Light intensity sub-optimal; problematic for varying smear thickness	Light intensity too low and not adjustable, poor contrast
Resolution and depth of focus	Very satisfactory	Satisfactory	Unsatisfactory (3) Satisfactory (2)
Need for darkroom	No	No (3) Partial – lights to be switched off & windows covered (2)	Partial – lights to be switched off & windows covered
Magnification	x10, x20, x40 and x100 objectives (for ZN and FM) 40X preferred	Device not attached directly to objective, magnification depends on base microscope. 40X preferred (4); 20X (1)	Device attaches to single objective, magnification chosen when purchasing (x20, x40, x60, x100) 40X preferred
Use of ZN and FM on same system	Easy and convenient to switch between ZN and FM modes; toggle switch seems robust	Difficult to add and remove device, would not use same microscope for FM and ZN on same day	Possible to use for ZN and FM on same day
Technical problems	First released version of battery pack needed redesign	None to date	None to date
Recommendation for implementation	Yes, combination of ease of use and high quality optics make this an attractive tool for TB control	Yes, although some operational limitations were identified	Yes, although some operational limitations were identified (4) No (1)

Conclusions
The objective of this study was to provide a direct comparison of three LED-based systems for fluorescence microscopy for TB detection with the current standard used in developing countries, ZN microscopy. Furthermore, the study also allowed comparison of quality assured LED FM and ZN microscopy with routine FM.

Sensitivity of the LED-based FM was 5.7%, 7.7% and 3.8% higher than ZN (iLED, FluoLED and Lumin), although the difference was only significant for FluoLED.

Routine FM performance was lower than all other methods (although not statistically significant), including quality assured ZN, pointing to issues relating to quality of performance of the routine FM. Routine FM was not controlled as part of this study.

Specificity of the LED methods was not significantly different than the specificity of ZN microscopy. Although specificity of Fraen LED was 3.1% lower than ZN and 3.9% lower than Lumin and iLED, this difference was not significant.

The user appraisal indicated that significant advantages with regard to reading time and performance were seen for all LED systems, but that the operational characteristics and high quality optics of iLED provided significant advantages in terms of ease of use and convenience for the reader (no need for a partial darkroom).

Comparison of staining methods and reagents

Background
Since the introduction of LED-based FM would require that microscopy centers introduce fluorescence staining, guidance should be given on selection, preparation and storage of fluorescence staining reagents/solutions with regard to performance, quality assurance, logistics and cost efficiency.

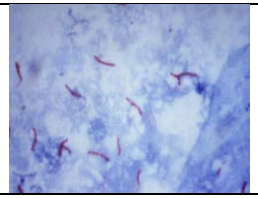
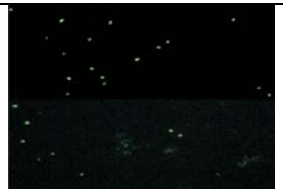
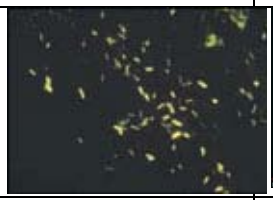
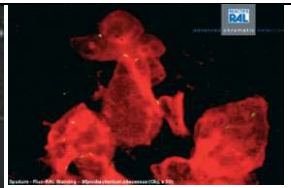
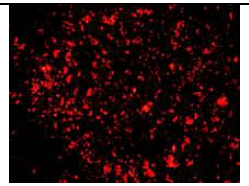
The amount of comparative data for different fluorescence stains is very limited. Based on a small number of studies, there seems to be little difference in sensitivity or specificity for Auramine versus Auramine-Rhodamine[1]. We therefore examined the performance and suitability of staining solutions prepared in-house and kit formats frequently used in Europe and US in a sub-study.

Methods
Our supervisory site located in Peru prepared a panel set of 30 leftover specimens (12 slides per sputum). Eight users from demonstration sites in Peru assessed operational performance (reagent preparation time, hands-on-time, ease of focusing, ease of reading etc; see details in table below) of the 12 staining options listed below. For selected methods (in-house ZN, Auramine, Auramine-Rhodamine, Auramine-Methylene Blue, Fluo Ral, Merck Phenol free), two readers examined the slides that were coded with different IDs to ensure blinding, and documented results and reading time.

Results
Fluorochrome staining was perceived as being simpler and more user-friendly compared with Ziehl-Neelsen staining, whereas the preparation of ZN and FM in-house solutions was found to be comparable. No difference in performance was observed for fluorochrome staining solutions tested during this assessment.

Kit formats had the general advantage of being (almost) ready to use and of requiring less quality assurance. Distribution and storage was also easier from a logistical standpoint. Some of the kits were found to have additional advantages for the user (FluoRal/Acrifluor: lower debris color intensity and less artifacts; Acrifluor: staining simpler and faster; TB-Fluor Phenol free: replacement of toxic solvent). However, there were also some disadvantages (often slightly lower color brilliance than in-house solutions, high cost, small bottle volumes).

Given the (current) higher cost of ready-to-use kits, we have used in-house staining solutions throughout demonstration studies. These were centrally prepared and quality-controlled by the supervisory site, and were distributed on a monthly basis. In addition, positive control slides (and rechecking of slides by supervisors) were used to assess the staining quality at microscopy centers. Given the decreasing price of staining kits, distribution of kits rather than in-house solutions should be considered as a cost-effective option for low volume microscopy centers. Experienced users generally preferred Auramine O staining solutions, whereas users with little experience found it easier to focus and less tiring to read slides with a more lively background (thiazine red, methylene blue counterstains). Only one reader preferred Auramine-Rhodamine over Auramine O, possibly because the participants had been using Auramine O for the preceding six months and were used to the yellow appearance of AFBs. The reading time was significantly shorter for both Auramine O and Auramine Rhodamine (black background with high signal to noise ratio), compared to fluorescence stains with colored background.

Table 36: Performance comparison of most widely used staining solutions					
Staining solution	Ziehl-Neelsen	Auramine/$KMnO_4$	Auramine/ Methylene Blue	Auramine / Thiazine red	Auramine-Rhodamine/ $KMnO_4$
	• In house • BD kit • Merck kit	• In house • BD kit M • Scientific Device Lab. AcriFluor	• In house	• In house • Fluo Ral	• In house • BD kit T • Merck kit TB-fluor Phenol-free
					
Color of AFBs and background	AFBs: pink Background: blue	AFBs: yellow Background: black	AFBs: yellow Background: dark blue	AFBs: yellow Background: red	AFBs: orange Background: black
Toxicity during preparation / staining	Phenol (hazardous if inhaled or absorbed through skin). ***Caveat: Aerosol generation during heating steps***	Auramine (hazardous in case of inhalation; lower health hazard group than Phenol) Phenol (Merck offers a phenol-free kit) ***Advantage: No aerosol generation in absence of heating steps.*** ***Caveat: Central preparation of staining solutions recommended, since use of dust respirator or chemical hood desirable***			
Storage conditions	• Dry reagents: 24 m up to 35°C • Ready to use solutions: 12 m up to 35°C (filter weekly) • Kits: 12 m up to 25°C	• Dry reagents: 24 m up to 35°C. • Ready to use solutions: 6 m up to 35°C, but for Auramine solution better prepare monthly (filter weekly). • Kits: Usually 12 m up to 25°C.			
Preparation time	110	70 Kits are in general ready to use (exception: Fluo Ral requires combining solutions from 6 bottles into 3).			
Hands-on-time during staining / Nr of heating steps	20-25 min Heat fixation of smear and add. heating step	25 min (in-house, BD kit M, Merck kit w/o phenol); 20 min (Acrifluor); 30 min (Fluo Ral); 40 min (BD kit T, Tb-fluor) Only heat fixation of smear			

Staining solution	Ziehl-Neelsen	Auramine/$KMnO_4$	Auramine/ Methylene Blue	Auramine / Thiazine red	Auramine-Rhodamine/ $KMnO_4$
Intensity of AFBs	Solutions with 0.3% carbolfuchsin (for example BD kit) result in lower color brilliance compared to 1%.	In-house stains often show the highest fluorescence intensity as long as Auramine is prepared on a monthly basis and quality assured			
Debris brightness	++	++ Lower debris brightness found for a) Fluo Ral and b) Acrifluor (+)			
Contrast to background	++ (background very lively & distracting)	+++	++	++	+++
Ease of focusing / Ease of reading	++ / ++	+ / +++	++ / ++	++ / ++	+ / +++
Cost	In house: + (see costing analysis for details) Kits: +++	In-house: + (see costing analysis for details) Kits: +++ (some kits available at reduced price for target countries)			
Concordance during panel testing*	Sensitivity 95% (38/40) Specificity 100% (20/20)	There was 100% concordance between fluorescence staining methods in this panel set. Sensitivity 100% (40/40); Specificity 95% (19/20)			
Average reading time expressed as ration of ZN	100%	47%	65%	59%	51%
Comments		For classical staining solution (Au and Au.Rhod) It is not possible to check the smearing and staining quality for negative slides (almost black background). Positive control slides are therefore critical for quality assurance			

*Panel set of 30 ZN and 30 fluorescence slides and 2 readers.

Assessment of fading speed for Auramine stained slides

Rationale
Several National TB Control Programs regarded it as crucial to determine the fading speed of Auramine-stained slides over time. Since the fundament of many TB Quality Assurance Programs is the quarterly rechecking of a randomly selected number of stored slides, impaired reading within three months post staining would mean a drastic change for NTPs.

Methods
Six microscopy centers in India, Russia, Peru and Vietnam conducted a sub-study to assess the fading of slides over a period of 4 months when stored in slide boxes at room temperature (no air-condition) and read with iLED by 1-2 readers each on a monthly basis. A set of 10 routine slides (2 x neg, 2 x scanty, 2 x 1+, 2 x 2+, 2 x 3+) stained with the standard in-house Auramine staining solution was selected by the supervisor for this assessment. Readers assessed the slide set independently from each other and determined the results with fields to positivity and completed a short questionnaire which assessed the severity of reading impairment (0 = none; 1 = slight; 2 = significant; 3 = slide cannot be read anymore) and specifically asked for the fading of AFBs or modification of background.

Results

Table 37: Fading speed assessment for Auramine-stained slides with monthly reading of slides

	1 month post staining		2 months post staining		3 months post staining		4 months post staining	
	Result	Impaired reading	Result	Impaired reading	Result	Impaired reading	Result	Impaired reading
Site 1 (India; 35°C, dry)	No changes in results*	No	No changes in results*	No	No changes in results*	No	Yes, 1 low pos slide was interpreted as neg	Significant fading, reading more difficult
Site 2 (India; 38°C, dry)		No		No		No	No	Mild fading, reading more difficult
Site 3 (India; 35°C, humid)		No		No		No	No	No
Site 4 (Peru; 26°C, dry)		No		No		Mild fading	No	Mild fading
Site 5 (Vietnam, 32°C, humid)		No		No		No	No	No
Site 6 (Russia; 20°C (heating); dry)		No		No		No	No	No

*only minor semi-quantitative changes; no significant change in time to positivity

Max outside temperature and humidity provided in brackets (for Russia, the microscopy center was heated, because the assessment took place Jan-April).

Reading was not impaired at any of the participating microscopy centers over the first three months. Four months after staining (and when the slide was examined for the 5th time), fading of slides was reported at three out of seven sites, but only one site observed a significant impairment in reading and interpreted 1 scanty pos slide as false negative (when reading 40 fields).

Conclusion

The introduction of LED microscopes with the need for fluorescence staining is not expected to require major modifications in quality assurance programs.

OTHER IMPLEMENTATION ISSUES

Training

A training manual, standard operating procedures and standardized result forms were prepared by FIND for the demonstration project to facilitate implementation and standardize processes from ordering of reagents and the preparation of Auramine staining solution to semi-quantitative reading and rechecking of slides. All training materials were shared on the FIND website and made available to WHO.

The training duration was fixed by the NTPs and the local supervisory site. Although someone from the FIND iLED study team was present during training, the lead was given to the local supervisory site. As shown below, the training duration varied between 1 and 5 days, with a great deal of theory and background information being provided in India and a much more practical focus in other countries. Proficiency testing for training participants was standardized across countries and target specifications provided for the validation phase to ensure that patient management was based on iLED results only once a high accuracy and staining quality had been reached (minimum duration of validation phase was 1 month).

As shown below, all microscopists passed the proficiency testing at the end of training and 27/28 sites passed the target specifications after the one-month validation phase.

Table 38: Training provision, proficiency after training and target criteria for validation phase

	Duration of iLED training	Number of practical hours	Proficiency testing post-training*	Target specifications for validation phase		
				Accuracy > 95%	Staining quality acceptable in 100% of rechecked slides	Proficiency testing >80%
South Africa	2 days	6-8	80-100%	Met	Met	Met
Ethiopia	4 days	8-10	100%	Met	Met	Met
Lesotho	4 days	8-10	90-100%	Met	Met	Met
Peru	5 days	10-15	80-100%	Met	Met	Met
Russia	3 days	3-4	90-100%	Met	Met	Met
India	5 days	6-8	80-100%	Met	Met	Met
Vietnam	3 days	6-8	100%	Met	Met	Met
Thailand	1 day	6-8	100%	Met	Met	Met
Cambodia	3 days	6-8	80-100%	Met	Met	Met

The proficiency panel was composed of 10 Au and 10 ZN slides (each 2 very low positive, 3 low positive, 2 high positive and 3 negative). Reading was done by iLED.

The uptake was generally enthusiastic and few difficulties were encountered during the adoption of the new methods. Auramine staining was generally considered easier compared to ZN staining (due to the absence of heating steps). Most errors seen during practice and proficiency testing were due to artifacts being interpreted as AFBs and negative slides therefore being called positive. It required some time for readers to be able to distinguish artifacts from AFBs and it was regarded as important

to have supervisors on site for at least 8 h of practice to provide feedback whenever difficult microscopy fields were seen.

As shown in the user appraisal forms, the majority of microscopists considered five days an optimal duration of training for microscopists with prior ZN experience, while at least 13 days are thought to be needed for microscopists without prior ZN experience. In general, the practical training phase was considered much more essential by microscopists than the theoretical part.

Electrical supply and backup power

An advantage of LED-operated microscopes is the low power consumption which allows for battery operation. Many of the 28 microscopy centers had power cut offs of five min up to 12 h and relied on the iLED battery pack for such occasions. A first version of the battery pack was found not to be robust enough and not to provide a long enough power supply. Zeiss therefore re-designed the battery pack which now has features similar to a motorbike battery. Gradually, battery packs are being replaced at demonstration sites. User feedback for these new battery packs has been provided by sites in India, Vietnam and Peru and is summarized below.

Table 39: User appraisal for iLED battery pack (Vietnam, India, Peru)

1.1 Initial charging time	12 hours (after first use approx. 5-7 hours)
1.2 Battery duration (hours) a) FM mode b) ZN mode (LED bulb) c) ZN mode (Halogen bulb)	 10 – 15.30 hours 7 – 12.40 hours 4 – 5 hours
2.1 Light intensity on FM mode	☒ Satisfied (comparable to electric power supply) ☐ Not satisfied
2.2 Light intensity on ZN mode	☒ Satisfied (comparable to electric power supply) ☐ Not satisfied
2.3 Reading quality on FM mode	☒ Satisfied (comparable to electric power supply) ☐ Not satisfied
2.4 Reading quality on ZN mode	☒ Satisfied (comparable to electric power supply) ☐ Not satisfied
3.1 Ease of connecting & disconnecting & use	☐ Very easy ☒ Easy ☐ Rather difficult ☐ Very difficult
3.2 Design (size (bench space required), appearance)	☐ very convenient ☒ convenient ☐ inconvenient
3.3 Ease of charging (steps & attention required)	☐ very convenient ☒ convenient ☐ inconvenient
3.4 Robustness	Robust, yet has the following disadvantages: 1. The new battery pack is in essence a car battery, and **SHOULD NOT BE LEFT ON ELECTRIC POWER UNLESS FOR CHARGING** or its lifetime will decrease. 2. Integrated cables as provided in the first version of the battery pack would further increase robustness.
3.5 Comments	The new battery pack is relatively bulky and its design less attractive than in the first version. However, it supports the iLED in FM mode for >10 hours without recharging, which was found to be very attractive.

Technical and customer support

FIND kept track of all technical problems which occurred during validation and implementation phase, monitored response time and quality of response from Zeiss MicroImaging and recorded user feedback. This assessment is summarized in the table below.

Table 40: Zeiss response time for technical problems

Technical problems	Response time of customer service	Type / Effectiveness of response
Fine focus needed to be fixed (2/45)	Within 48 h	User instructions provided by telephone & email / Effective
Condenser adjustment required (1/45)	Within 48 h	User instructions provided by telephone & email / Effective
Loose contact (1/45)	Within 48 h	User instructions provided by telephone & email / Effective
40x lens not working (1/45)	2 weeks	Replacement of objective by headquarters / Took longer than expected
Bright light not working (1/45)	4 weeks	Repair by local Zeiss office / NOT effective
Stand needed replacement (1/45)	Within 48 h (but delays due to import /export permissions required)	Replacement by Singapore office / Effective
Slide holder requiring replacement (1/45)	2 weeks	Replacement of slide holder by headquarters / Took longer than expected
Defective battery pack version 1 26/45)	6 months	Redesign of battery pack & replacement of defective battery packs / Took longer than expected

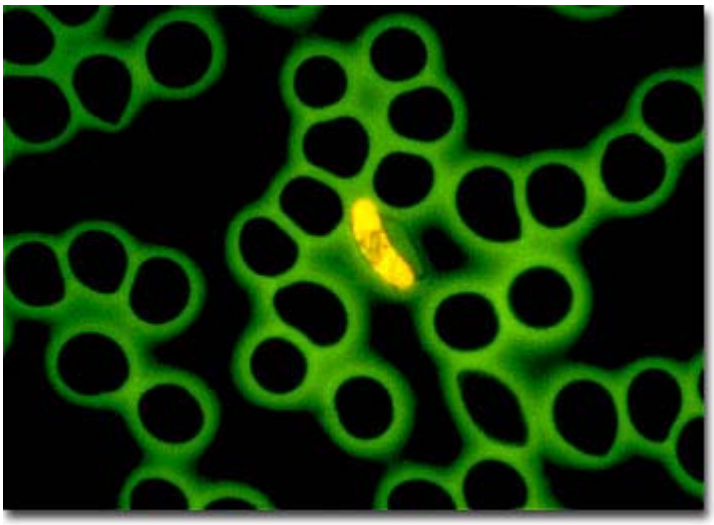
Conclusion:
Overall, customer support provided by Zeiss was satisfactory. Zeiss has a local office or at least a local distributor in most high burden countries, which facilitated interaction with customers enormously and is believed to be crucial for a roll out or widespread implementation.

Use of iLED as diagnostic multi-disease platform

Rationale
Microscopes are by design diagnostic platforms which may be applied for detection of a wide array of pathogens. Microscopists who have been trained and who operate in moderate sized or larger facilities will routinely have expertise detecting malaria, fecal parasites, and tubercle bacilli, urinary tract infections, and sexually transmitted infections in addition to other conditions. Most common applications use brightfield examination only, with or without special stains.

In order to avoid the need for multiple microscopes with dedicated use for specific applications, it seemed important to assess the suitability of iLED for other common applications and to determine any benefits in using iLED rather than brightfield microscopy.

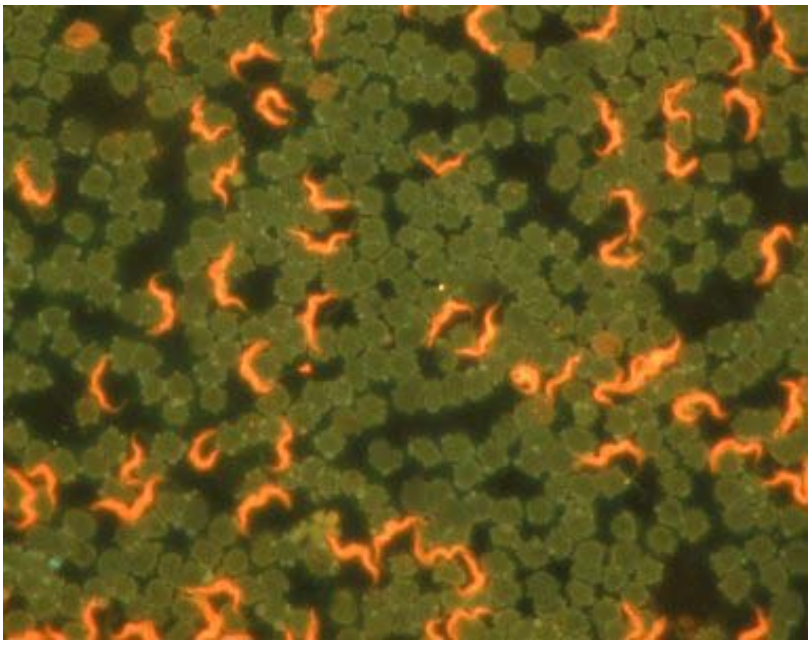
Results
FIND has been evaluating the Primo Star iLED for the detection of malaria parasites and trypanosomes causing sleeping sickness using both non-specific (acridine orange) and specific (labeled aptamers or antibodies) approaches.



Human blood containing a *Plasmodium* gametocyte stained with acridine orange

Preliminary work by FIND with partners at the Hospital for Tropical Diseases in London proved the feasibility of iLED detection of malaria in thick and thin smears stained with acridine orange, and using QBC tubes. Studies are now being planned which will investigate possible gains in sensitivity, speed, or reliability by using fluorescent detection of Plasmodial parasites instead of routine brightfield microscopy with Giemsa staining.

Preliminary studies have also shown substantially improved limits of detection using fluorescent detection of trypanosomes. Though non-specific staining such as acridine orange also stains the DNA of leukocytes, as can be seen from the photomicrograph below of *Trypanosome bruceii rhodesiense* growing in culture, the characteristic shape of trypanosomes makes them readily recognizable.



Trypanosome bruceii rhodesiense stained with acridine orange detected on iLED microscope

Conclusion

LED-based microscopes can be used as multi-disease platforms since they are suitable for other applications such as for the diagnosis of malaria and human African trypanosomiasis (using acridine orange staining).

SUMMARY AND CONCLUSIONS

Examination of sputum specimens for acid-fast bacilli (AFB) by conventional light microscopy (LM) remains the cornerstone for the diagnosis of pulmonary tuberculosis in disease endemic countries. In industrialized countries, AFB fluorescence microscopy (FM) that is more sensitive and faster than LM is widely used. However, the higher costs of FM, as well infrastructure and maintenance requirements, have greatly limited the use of FM in lower-income countries.

A recent technical advance, the advent of low-cost ultrabright light emitting diodes (LEDs), has led to the development of inexpensive LED-illuminated FM that could be used in place of conventional FM. FIND has fostered the development of a dual-use microscope that could easily be switched to perform either FM or LM. Highest quality, affordability - especially for high-burden countries - as well as a strong wide-reaching customer support and distribution capacity was considered essential. Zeiss, a worldwide leader in optics and microscopy, and with high-end LED-based fluorescent systems already in development, was a natural partner for FIND to achieve the following product specifications:

- high-grade optics
- reflected (indirect) rather than transmitted blue light for fluorescent applications (iLED)
- battery power on AC failure
- easy switching between fluorescent and brightfield viewing
- utility outside a darkroom
- preinstalled, ready-to-use
- >10,000 hour bulb life
- inexpensive bulb change
- LED and filter sets that would allow use of Auramine O and acridine orange for TB and parasitologic examinations

FIND has completed feasibility, evaluation and large-scale demonstration studies of the Zeiss iLED FM and believes these will provide support for WHO guidance and recommendations on the use of LED FM in developing countries.

Feasibility study

The feasibility of using a late-stage Primo Star iLED prototype for TB detection was determined in four laboratories experienced in conventional FM. At each site, a set of 140 sputum specimens was prospectively selected from patients with a high risk of TB undergoing diagnostic evaluation, and these were used to generate slides for the evaluation. The specimens were cultured on LJ medium, the reference standard for the study. The slides were used in blinded studies assessing several questions, including: the performance of the iLED microscope in comparison to the more expensive standard FM scope, the necessity of a darkroom, the suitability of the iLED excitation wavelength for alternative stains (Auramine or Rhodamine) and counterstains ($KMnO_4$ or Methylene blue), the reading time per slide, the speed of fading of stored slides, and laboratory technician appraisal of the usability and technical suitability of the microscopes.

The sensitivity of iLED was $\geq$ conventional FM at all four study sites, and showed equivalent specificity. The examination time using iLED was equivalent to conventional FM and 45-75% faster than LM. The operational performance characteristics of iLED were rated superior to FM and LM.

Evaluation study

Based on the findings of the feasibility study, the microscope design was locked and evaluation studies were initiated to determine the performance of manufacture-series Primo Star iLED in a larger samples size with LM as one of the comparative methods. For the study, panels of slide standards with specific grading of positivity were created by a single facility and shipped to four reference laboratories with experience in FM. The endpoints of the study were: sensitivity of iLED in smear positive panel slides compared to LM and conventional FM; specificity in smear negative slides compared to LM and conventional FM; assessment of technician appraisal of the iLED in terms of ease of use, maintenance, design and comfort, robustness, contrast, brightness, etc.; and assessment of the adequacy of 20X vs 40X magnification for slide screening.

Sensitivity of iLED during this assessment was significantly higher than ZN (overall 6%; 95% CI 94.5-97.6) and equivalent to FM. As expected, a greater difference in sensitivity (25%) was found for very low-positive slides. Specificity of iLED-40X was equivalent to ZN, whereas specificity of iLED-20X was slightly lower and equivalent to FM (see Tables 9, 10a and 10b for details). Average gain in reading time compared to ZN was 60% for FM and iLED-20X and 55% for iLED-40X. More than 80% of positive slides had been correctly identified within 30 sec for the fluorescence methods, whereas this was only the case in <50% for ZN. A reading time of 3 to 5 min per slide resulted in a significant additional yield for ZN, whereas this was not the case for iLED or FM. Overall, acceptance was very high and uptake enthusiastic.

Demonstration study

Once performance targets were met through the evaluation study, a Demonstration project was initiated in coordination with National and Regional TB Control Programs in India, Vietnam, Thailand, Cambodia, South Africa, Lesotho, Ethiopia, Russia and Peru. FIND criteria for country selection for the study were: an agreement at National/Regional Levels (MOU) with NTP and/or MOH; a high-burden of TB; a low or middle income ranking; local presence of FIND or an implementing partner; and settings representative of the global TB and HIV situation. There were 28 microscopy centers and 12 supervisory sites chosen for this large study, with site selection based on the rate of smear-positivity, experience and training of microscopists, volume of work, reliability of AC power (sites with intermittent power supply were intentionally selected), interest in the project, and accessibility of study sites for supervisory visits. As the main goal of the study was to demonstrate the feasibility of expanded use of LED-based FM in conventional settings, none of the microscopy centers had prior experience with FM. The supervisory laboratories did have FM experience.

The objectives of the study were:

1. To assess the feasibility of implementing Primo Star iLED for TB diagnosis at microscopy centers without prior experience with fluorescence microscopy in low- to moderate-income settings and to identify barriers to implementation
2. To determine the false positivity and negativity rate of iLED fluorescence reading compared to a ZN baseline and to results from the supervisory site
3. To determine the development of false positivity and negativity rates of iLED fluorescence reading over time (with increasing experience)
4. To assess the impact of this implementation on daily workload and case detection rates for low, middle and high-volume settings
5. To determine lab technicians' appraisal of Primo Star iLED
6. To evaluate detailed costs associated with LED-based fluorescence microscopy in comparison with conventional methods

Prior to iLED introduction, baseline performance data were collected using the standard ZN procedures with supervisory rechecking over a month long pre-study period. This was followed by one- to five-day iLED training for each country (see further information on training in the section "implementation issues"). During a one month validation phase, all slides were screened with the iLED microscope and confirmed using a conventional fluorescent microscope by a supervisory site on a daily basis. Patient care during this phase was based on the results of conventional FM examination. Sites that showed acceptable performance in the validation phase continued with a three month phase in which the iLED microscope was used as the routine screening tool. Rechecking of stored slides by FM continued during this phase. Demonstration sites for which performance was maintained progressed to a six month continuation phase, during which rechecking frequency and intensity was performed as per National TB Program norms. During all study phases, slides with discrepant results (rechecking result deviating from the result at the microscopy center) were reread by an experienced third reader based at the supervisory site. Slide ID numbers for slides requiring a 3rd reading were distributed to the sites by FIND on a monthly basis. Regular monitoring visits were conducted using standardized checklists. At all sites, user acceptance and appraisal was assessed as well as the robustness of the microscope. Logistics and customer support quality was monitored.

More than 60,000 iLED results have been reported from this Demonstration project. The results showed that:

1. It is feasible to implement Primo Star iLED for TB diagnosis at microscopy centers in low-income settings that had no prior experience with fluorescence microscopy.
2. A high level of agreement between readers and supervisors was observed within the first month of iLED adoption. This high agreement suggests that microscopists could achieve proficiency on iLED within a short timeframe and with accuracy similar or greater than ZN.
3. Since iLED performance was already strong during validation phase for most sites, little improvement was seen over time (with increasing microscopist experience).
4. Positivity and case detection rates increased significantly compared to ZN. Overall, there was a 14% increase in case detection (baseline LM vs implementation phase iLED).
5. The decreased workload due to decrease in reading time was perceived by all participating microscopists and reported in the user appraisal questionnaire. A reading time sub-study showed that for readers without prior experience in FM, a significant reduction in reading time (>40%) is only seen after two to three months when experience and confidence of readers has increased.
6. Lab technicians' appraisal of Primo Star iLED was very positive overall and uptake was enthusiastic. No real barriers to implementation were identified. Valuable input was given with regard to degree of training time required. A halogen rather than LED bulb option was much preferred when using the brightfield mode of iLED for ZN (halogen insertion available from Zeiss). The most recent version of the battery pack was found to be functional, but design could be further improved.
7. Overall, using FIND-negotiated pricing for the iLED and stains prepared from powder, the cost per test for iLED was approximately 12% lower than for ZN. The cost per test (sub-total) for ZN ranged between US $ 1.67 to 1.92 and for iLED between US $ 1.49 to 1.72. On average, iLED cost per test was approximately 10% cheaper than ZN. In general, , per test costs were highest in Peru for both ZN and iLED, which was mainly due to higher expenses for staff, overhead, reagents and chemicals used for smear microscopy. Therefore, introduction of iLED is a cost-efficient option.

8. Minimal training needs were determined (primarily for users with prior ZN experience). Training materials were developed and shared with WHO.

LED sub-studies

FIND has also conducted other studies to address several issues relative to implementation of LED FM in developing countries.

Comparison studies of other LED systems

These studies sought to compare the performance of three LED-based systems for fluorescence microscopy detection of *M. tuberculosis* with light microscopy (Ziehl-Neelsen staining) and with conventional fluorescence in TB suspects in Kampala, Uganda and Lusaka, Zambia. In addition, qualitative data on user acceptance were collected. The following LED-based systems were evaluated: iLED Primostar (Zeiss), FLuoLED™ add-on device (Fraen Corporation), and Lumin add-on device (LW Scientific).

In the Zambia study, the sensitivity of all 3 LED methods and of conventional FM was significantly higher than for ZN. Although the iLED appeared to be more sensitive than the other LED methods, this was not statistically significant at a 5% level. The specificity was identical for all LED methods, and slightly lower for conventional FM. The mean reading time reduction was highly significant for all FM methods when compared to the reading time for ZN slides. The reading time for Lumin was significantly higher ($p < 0.0001$) than for the other 3 fluorescence methods.

In the Uganda study, the sensitivity of the LED FM methods was between 3.6% and 9.1% higher than ZN. However, only the difference between FluoLED and ZN was significant at the 5% level (ZN vs FluoLED™, $p=0.025$; ZN vs iLED, $p=0.083$; ZN vs Lumin, $p=0.317$). There was no significant difference in sensitivity when comparing the 3 LED methods with each other in a pairwise fashion. The specificity of FluoLED™ was lower than the other methods. The difference was significant at the 5% level for comparison of FluoLED and Lumin specificity (Lumin vs FluoLED™, $p=0.025$; iLED vs FluoLED™, $p=0.059$; ZN vs FluoLED™, $p=0.157$). All false positive results were very low (scanty) by all methods. Examination time was between 2 and 4 times less for LED FM (using any LED method) compared with ZN.

In both studies, appraisal by the laboratory technicians favored the iLED because of its high quality optics, optimal light intensity and ease of use. Alternative LED-based approaches have significant operational disadvantages compared to iLED. Although, in these studies, performance for Lumin and FluoLED seemed to be slightly lower, this was not statistically significant. This should be confirmed by larger comparative studies and reflected in WHO guidelines /tender specifications.

Comparison of fluorescence staining methods

In-house and ready-made fluorescence staining methods were compared with regard to performance, hands-on time and price. The FIND supervisory site located in Peru prepared a panel set of slides from 30 leftover specimens (12 slides per sputum). Eight users assessed the operational performance (reagent preparation time, hands-on time, ease of focusing, ease of reading etc; see Table 36 above) of 12 different staining options. For selected methods (in-house ZN, Auramine, Auramine-Rhodamine, Auramine-Methylene Blue, Fluo Ral, Merck Phenol free), two readers examined the slides that were coded with different IDs to ensure blinding, and documented results and reading time.

Fluorochrome staining was perceived as being simpler and more user-friendly than Ziehl-Neelsen staining, whereas the preparation of ZN and FM in-house solutions was found to be comparable. No difference in performance was observed for the different fluorochrome staining solutions tested during this assessment. Although some staining solutions in kit format have advantages (for example phenol free solutions), current prices and quality do not justify purchase of pre-prepared solutions for higher volume microscopy centers. Standard in-house solutions (Auramine, Auramine-Rhodamine) showed equivalent or superior color intensity and performance. However, quality assurance is critical and central preparation of solutions highly recommended.

Effects of fading speed on external quality assurance by rechecking

Several National TB Control Programs (NTPs) regarded it as crucial to determine the fading speed of Auramine-stained slides over time. Since the fundament of many TB Quality Assurance Programs is the quarterly rechecking of a randomly selected number of stored slides, impaired reading within three months post staining would mean a drastic change for NTPs.

Six microscopy centers in India, Russia, Peru and Vietnam conducted a sub-study to assess the fading of slides over a period of 4 months when stored in slide boxes at room temperature (no air-condition) and read with iLED by 1-2 readers each on a monthly basis. A set of 10 routine slides (2 x neg, 2 x scanty, 2 x 1+, 2 x 2+, 2 x 3+) stained with the standard in-house Auramine staining solution was selected by the supervisor for this assessment. Readers assessed the slide set independently from each other and determined the results with fields to positivity and completed a short questionnaire which assessed the severity of reading impairment (0 = none; 1 = slight; 2 = significant; 3 = slide cannot be read anymore) and specifically asked for the fading of AFBs or modification of background.

Reading was not impaired at any of the participating microscopy centers over the first three months. Four months after staining (and when the slide was examined for the 5th time), fading of slides was reported at three out of seven sites, but only one site observed a significant impairment in reading and interpreted 1 scanty positive slide as false negative (when reading 40 fields). Thus, it was concluded that the introduction of LED microscopes with the need for fluorescence staining is not expected to require major modifications in quality assurance programs.

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