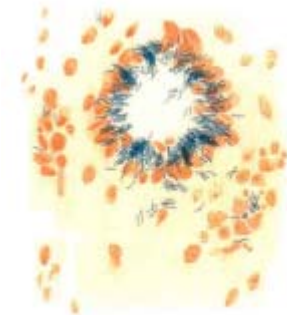


# Microscopy

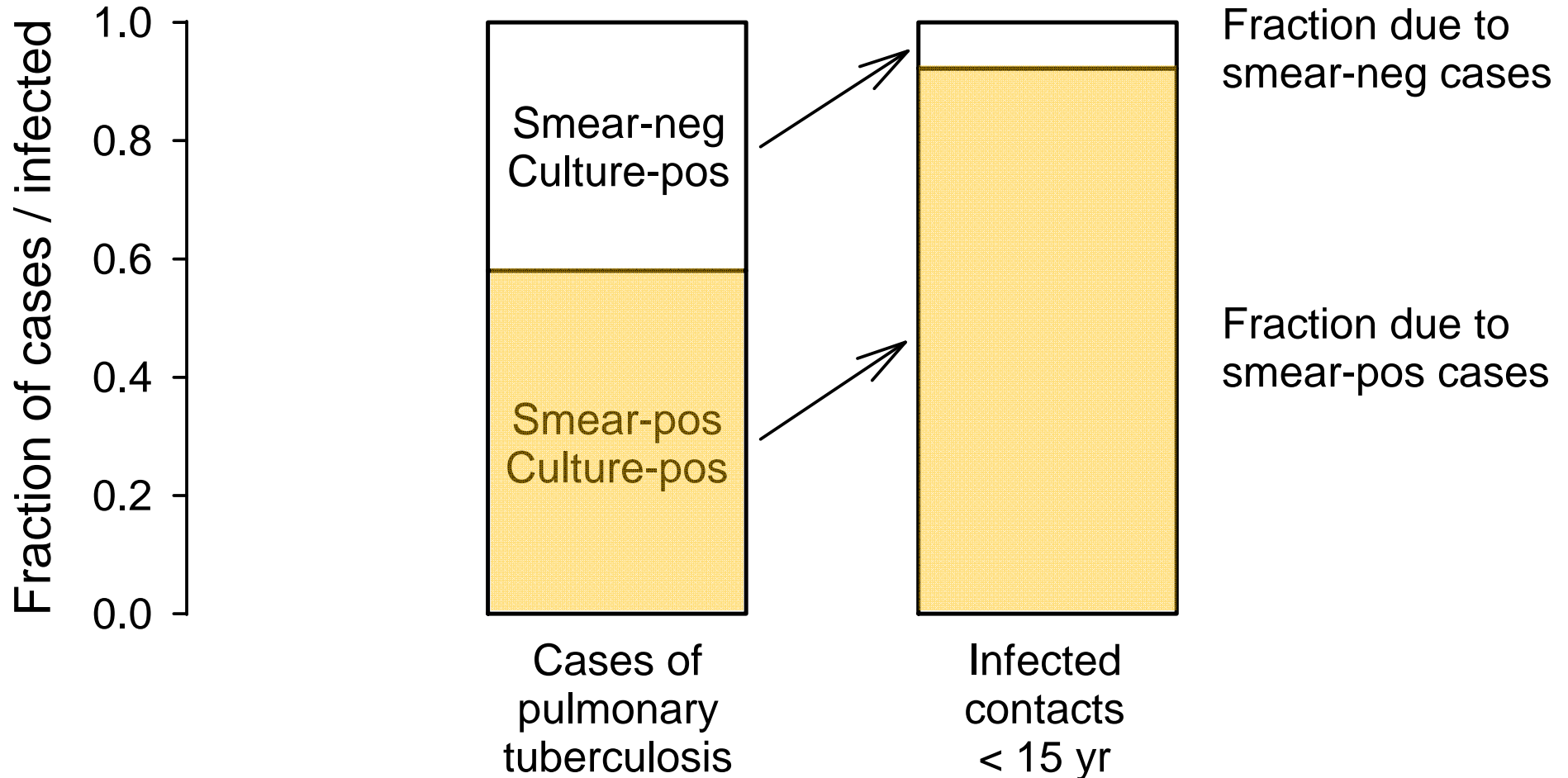
Münchenwiler, March 25, 2010

Hans L Rieder



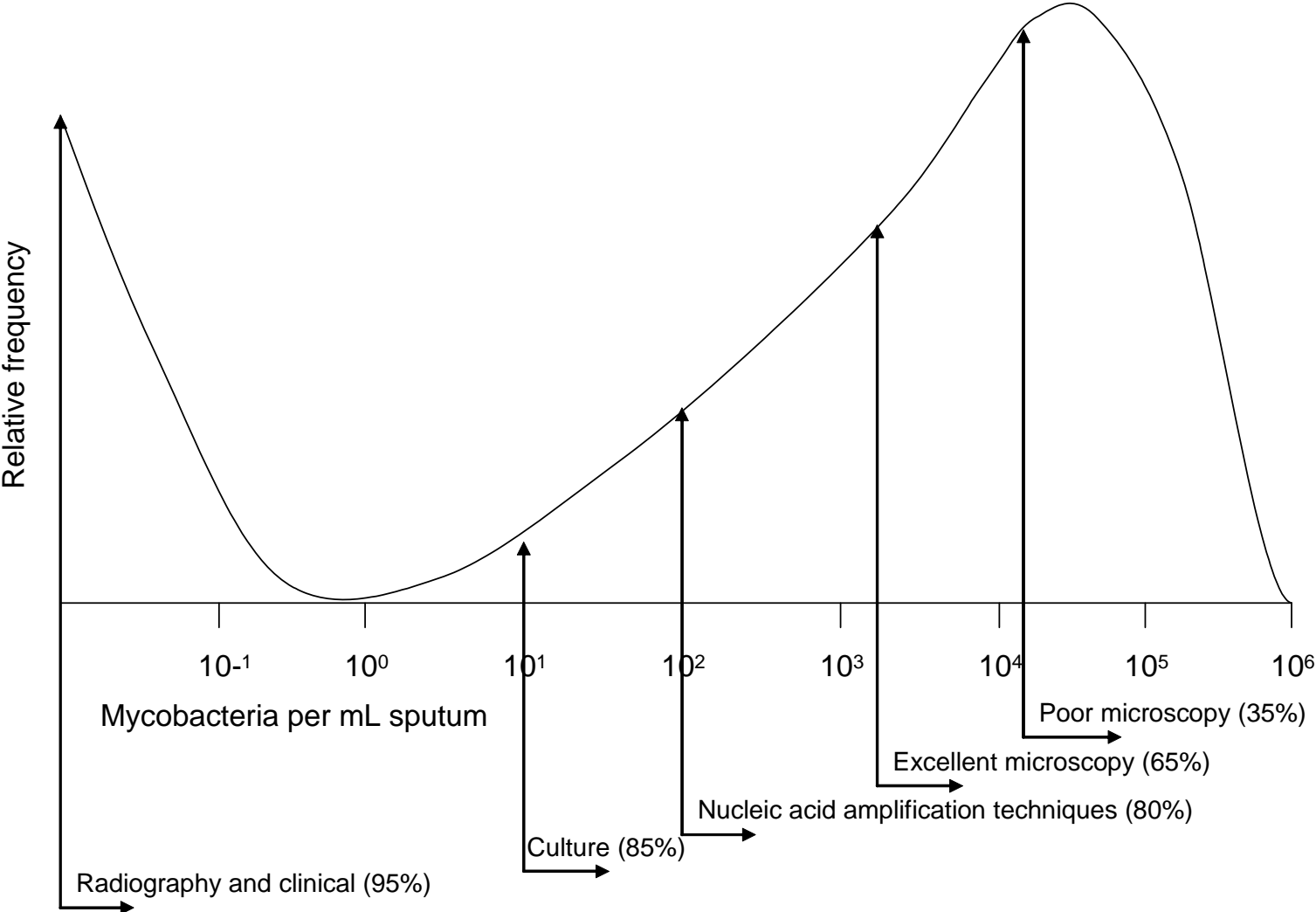
*Picture drawings: Koch R. Mittheil Kaiserl Gesundheitsamt 1884;2:1-88*

# Sensitivity of Direct Sputum Smear Examination in Identifying Pulmonary Tuberculosis and Transmitters



Calculated from data from:  
*Grzybowski S, et al. Bull Int Union Tuberc Lung Dis 1975;50:90-106*

# Schematic Presentation of Relative Frequency of Patients, Number of bacilli, and Available Diagnostic Methods

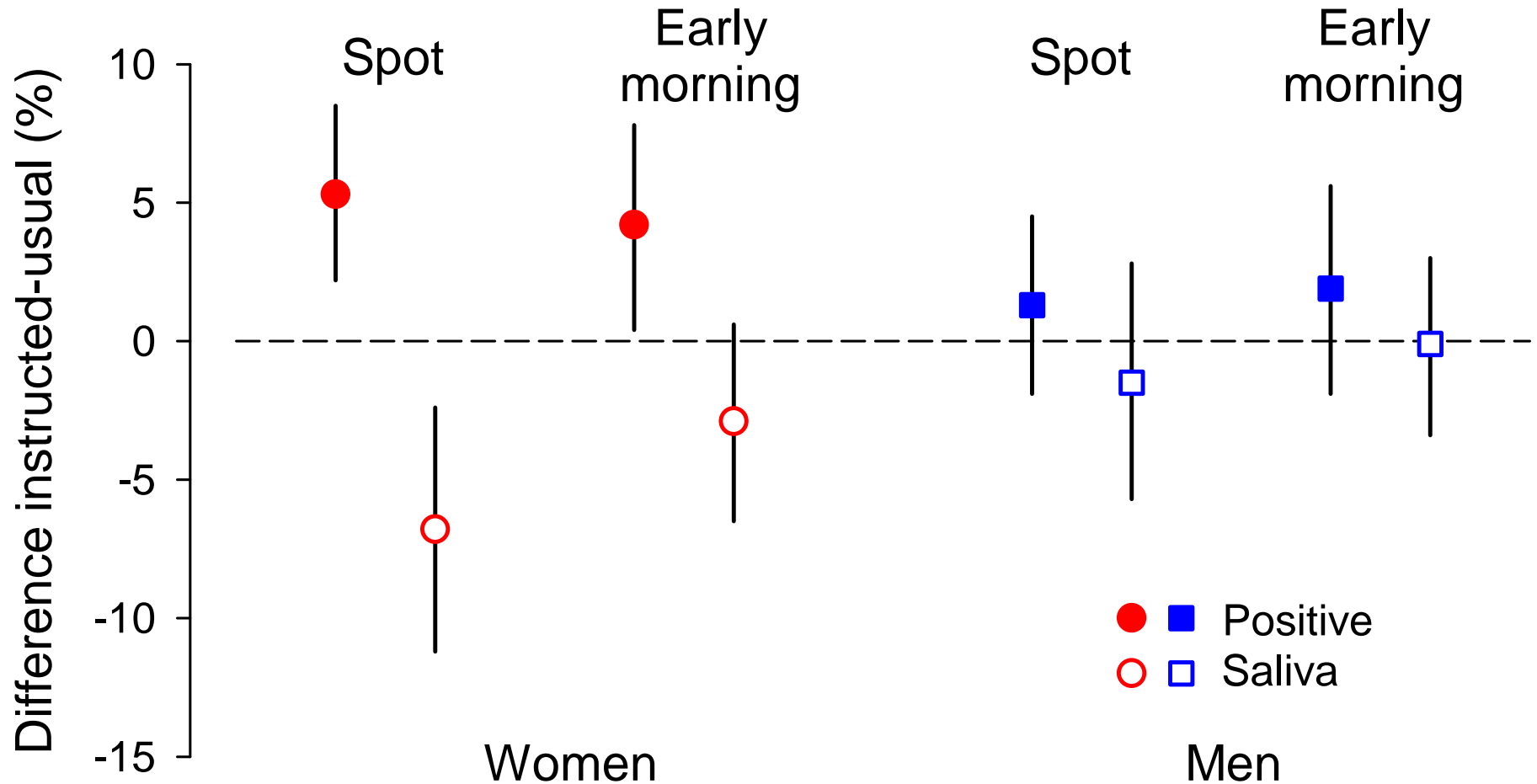


*Rieder H L, Van Deun A, Kam K M, Kim S J, Chonde T M, Trébuçq A, Urbanczik R. Priorities for tuberculosis bacteriology services in low-income countries. Second edition. Paris: International Union Against Tuberculosis and Lung Disease, 2007*

## Where things may not be optimum and adversely impact on sensitivity of the sputum smear microscopy result

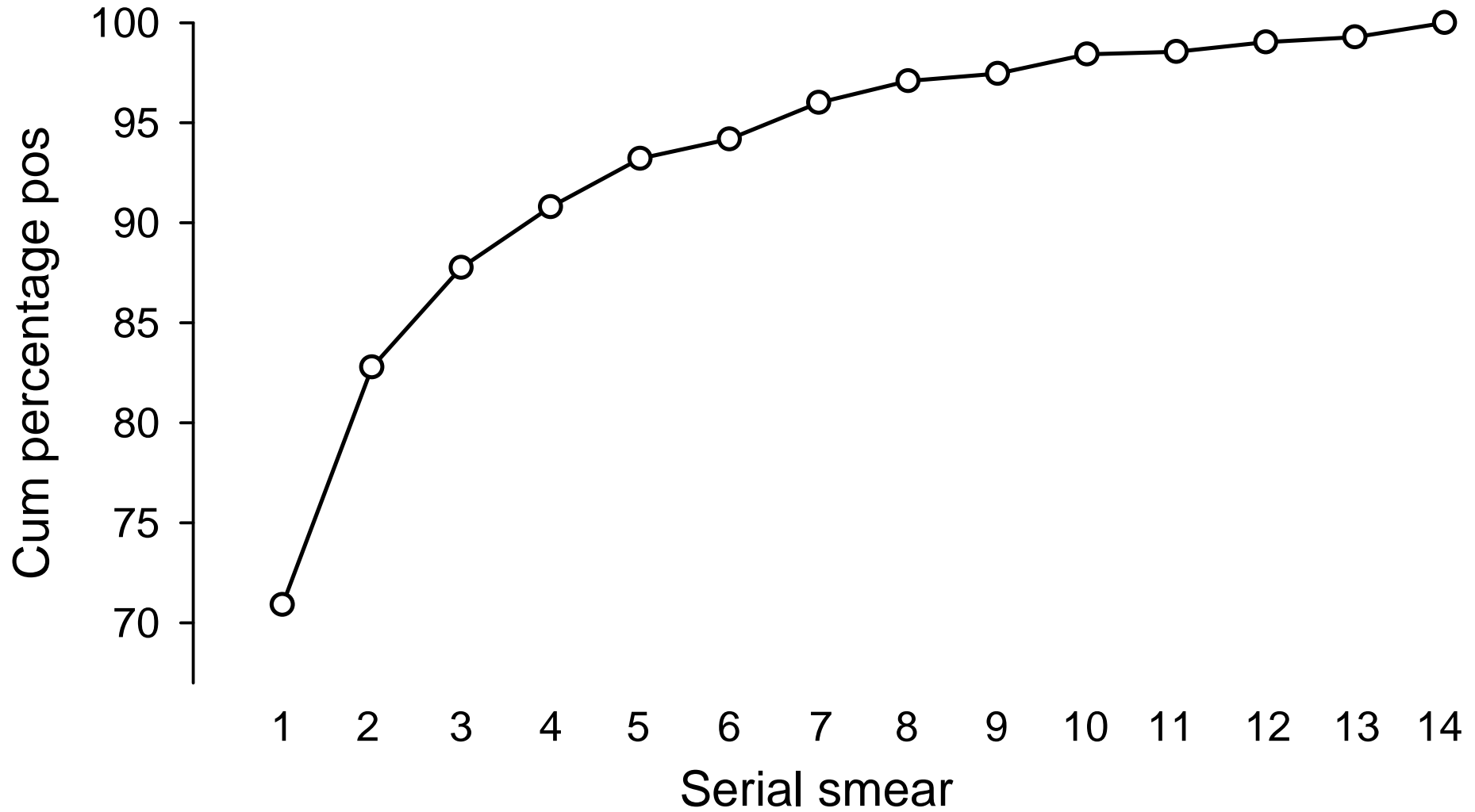
Step	Problem area
Sputum production	Patient instruction on what constitutes a good sputum sample
Sputum processing	Non-homogeneity of sputum, smear preparation
Staining	Quality of fuchsin
Examination	Time spent on examination, actual number of fields properly examined

# Improving yield of sputum smear microscopy by simple sputum-submission instructions, Pakistan, 2005



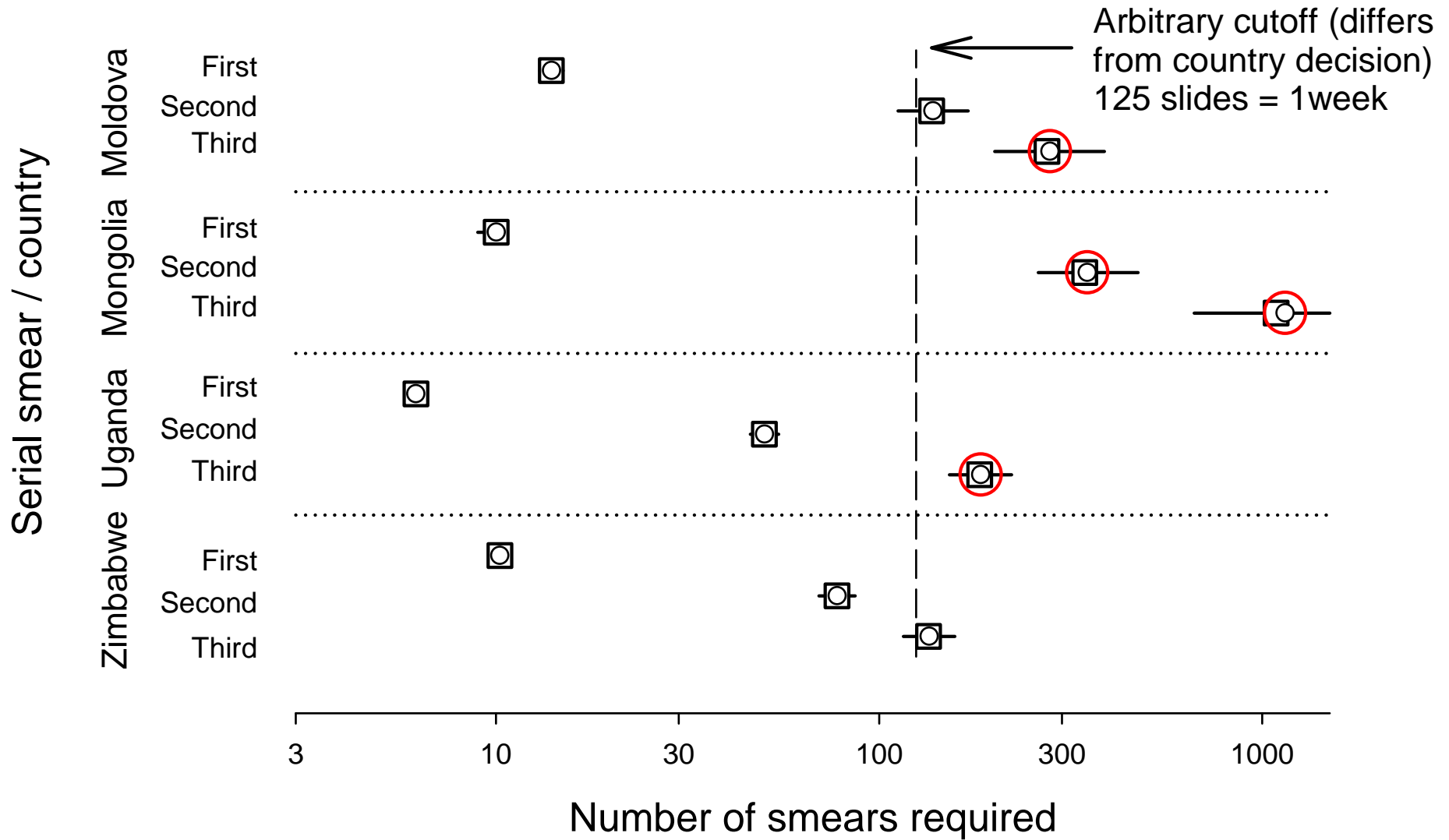
Sameer Khan M, et al. *Lancet* 2007;369:1955-60

# Cumulative Yield of Sputum Smear Examination Among 825 Smear-Positive Patients, United Kingdom, 1930s

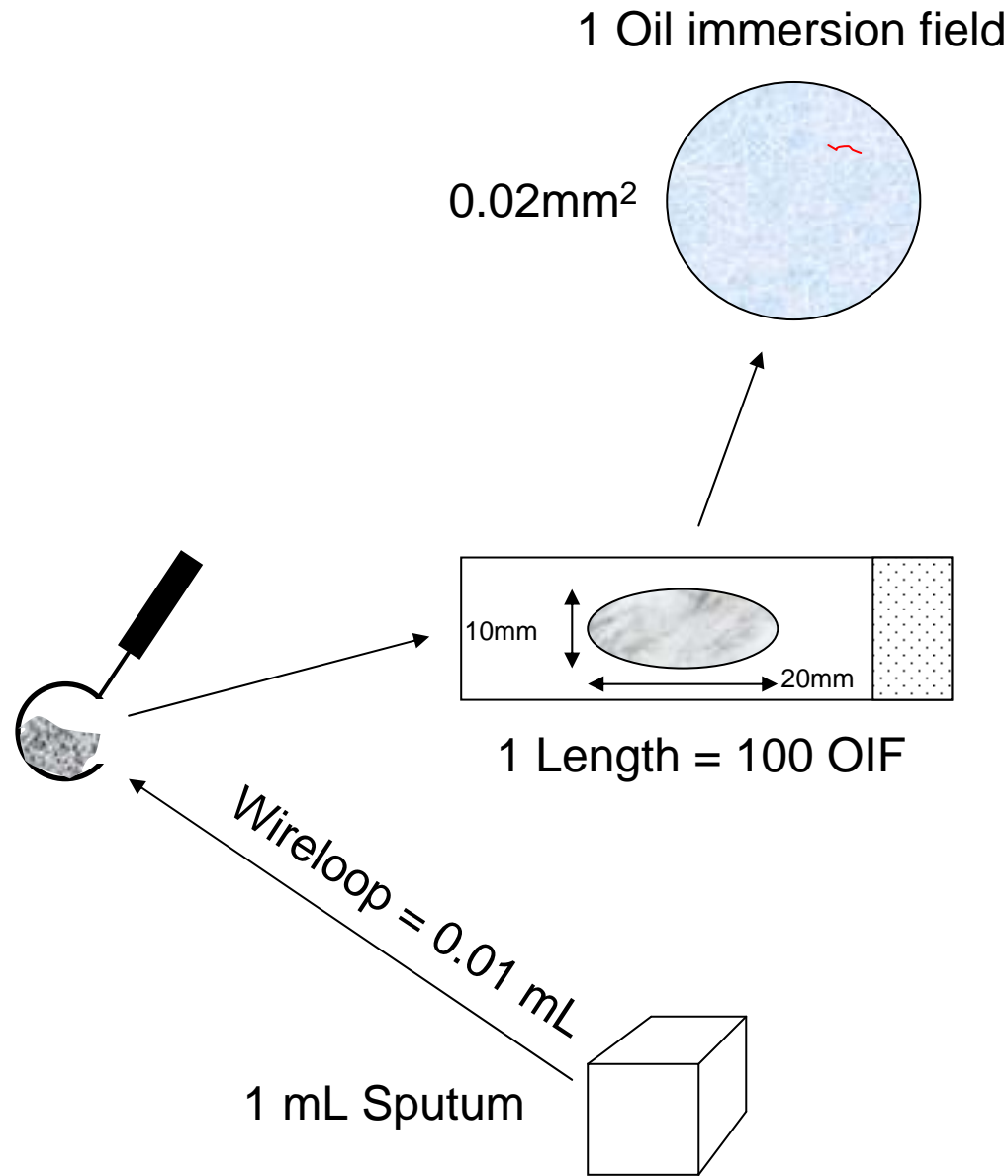


*Hunter RA. Tubercle 1940;21:339-59*

# Number of Examinations Required for Successive Serial Smears to Identify one Additional Case, by Country



*Mabaera B, et al. Int J Tuberc Lung Dis 2006;10:1030-5*  
*Katamba A, et al. Int J Tuberc Lung Dis 2007;11:659-64*



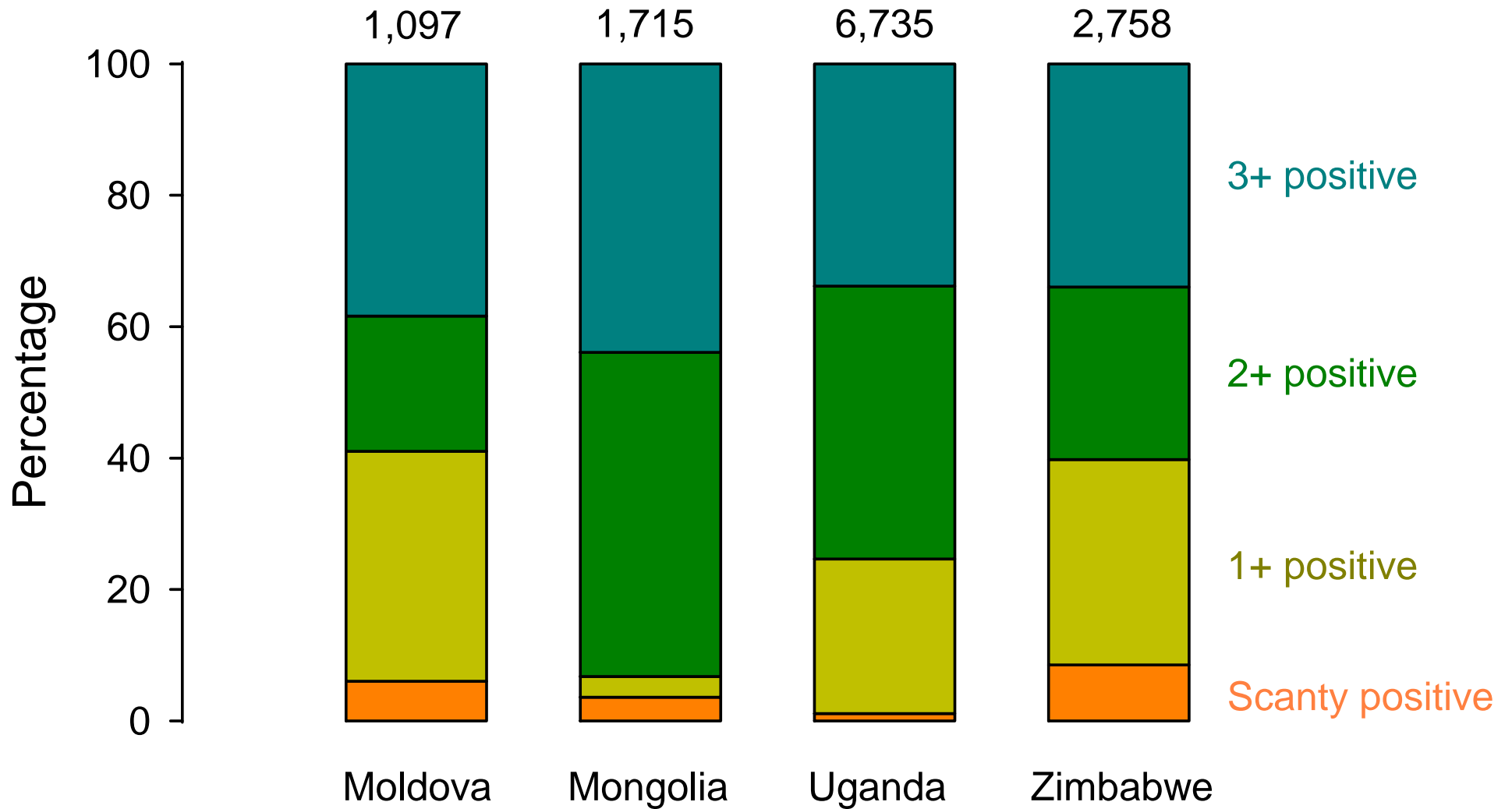
*To see:*  
1 AFB in 100 fields

*requires:*  
smear surface of 200 mm<sup>2</sup>  
containing 100 AFB

*which requires:*  
1 mL sputum  
containing 10,000 AFB

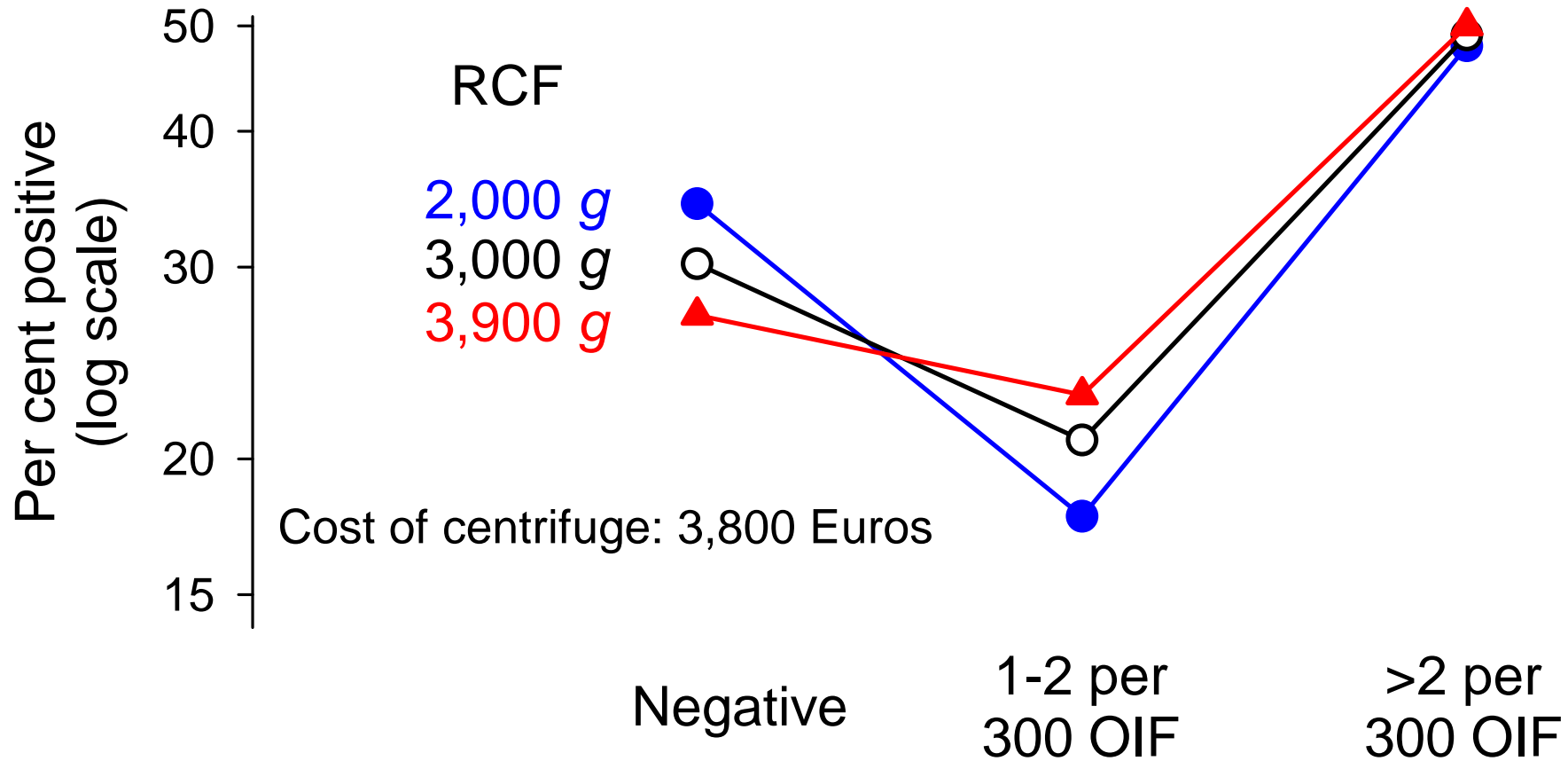
*Toman K. Tuberculosis case-finding and chemotherapy.  
World Health Organization, Geneva, 1979*

# Distribution of Graded Results among Sputum Smear Microscopy Positive Cases, by Country



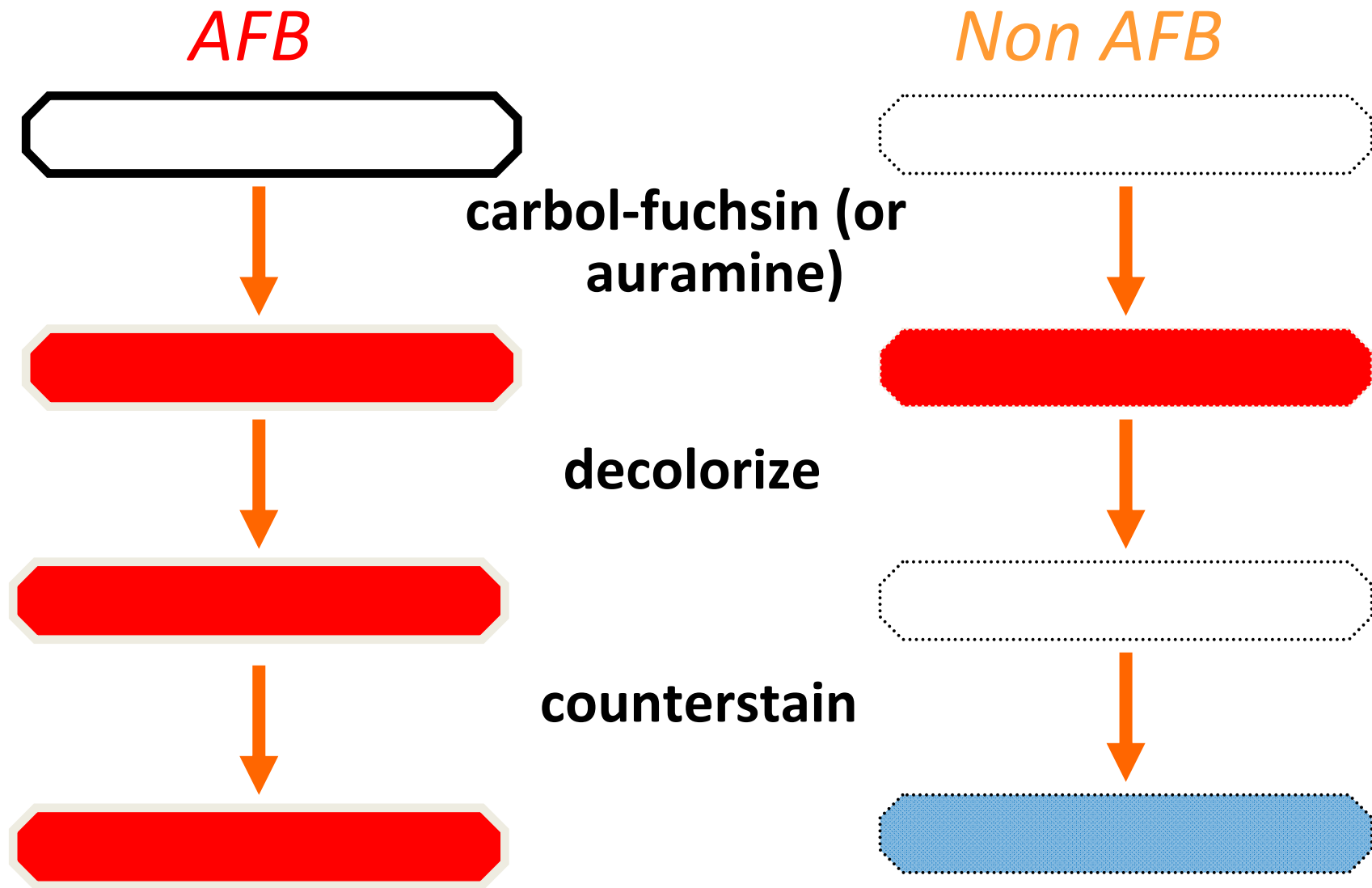
*Unpublished data, Union Tuberculosis Laboratory Collaborative Study*

# Distribution of AFB Smear Results Among Culture-Positive Specimens, by Relative Centrifugal Force, 15 Minutes



*Ratnam S, et al. J Clin Microbiol 1986;23:582-5*

# Principle of staining



*Slide courtesy: Van Deun A, unpublished lecture notes, Union International Tuberculosis Course, Arusha, November 2009*

# Principles in two types of microscopy

Staining	Bright-field microscopy	Fluorescence microscopy
Primary stain (stains everything)	Fuchsin	Auramine
Decolorant (destains everything except mycobacteria)	Hydrochloric acid <i>or</i> Sulfuric acid	Hydrochloric acid
Counter-stain (stains everything except color-saturated mycobacteria)	Methylene blue	Potassium permanganate <i>or</i> Ink

## The “Ziehl-Neelsen” staining technique: an experimental path to optimization, ready and all set since 1882

Contributor	Contribution
Robert Koch	Primary stain: methylene blue, alkaline potassium hydrate as mordant, vesuvium as both decolorant and counterstain
Paul Ehrlich	Fuchsin as primary stain, alkaline alinine as mordant, nitric acid as decolorant, and proposal of a blue counterstain
Franz Ziehl	Replace mordant with phenol
Friedrich Neelsen	Combine the best of all: primary and counterstain from Ehrlich, mordant from Ziehl, and replacing decolorant with sulphuric acid

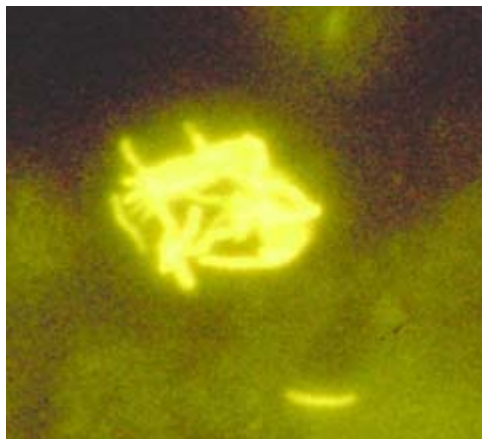
# Technik.

Aus dem Hygienischen Institut der Universität Köln.  
(Direktor: Prof. Reiner Müller.)

## Fluoreszenzfärbung von Tuberkelbakterien mit Auramin.

Von Paul K. H. Hagemann.

Man läßt 15 Minuten eine 5proz. Phenolum liquefactum enthaltende Auraminlösung (1:1000 in dest. Wasser; nach dem Phenolzusatz gut durchschütteln) auf den in der Flamme fixierten Ausstrich einwirken, spült kräftig mit Leitungswasser ab, differenzieret mit HCl-Brennspiritus (1000 cm<sup>3</sup> Brennspiritus; 4 cm<sup>3</sup> konz. reine HCl des DAB; 4 g NaCl) 3 Min., wobei nach 1½ Min. der HCl-Brennspiritus erneuert wird; daraufhin abermaliges kräftiges Abspülen mit Leitungswasser. Eine Gegenfärbung unterbleibt. Die Präparate sehen makroskopisch wie ungefärbt aus.

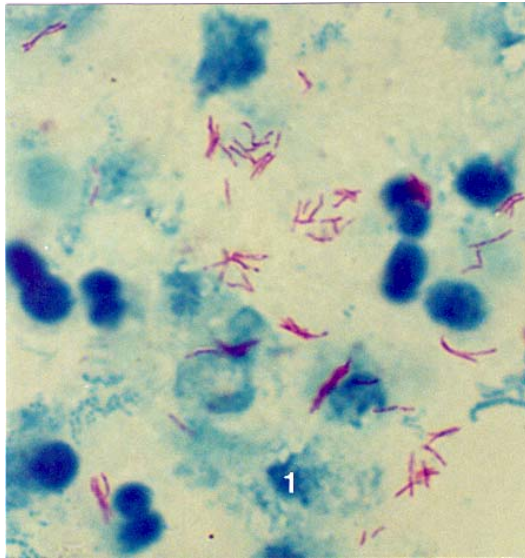


Stained with above recipe (HLR, Med Pol, ZH, 1978), but added potassium permanganate for background quenching

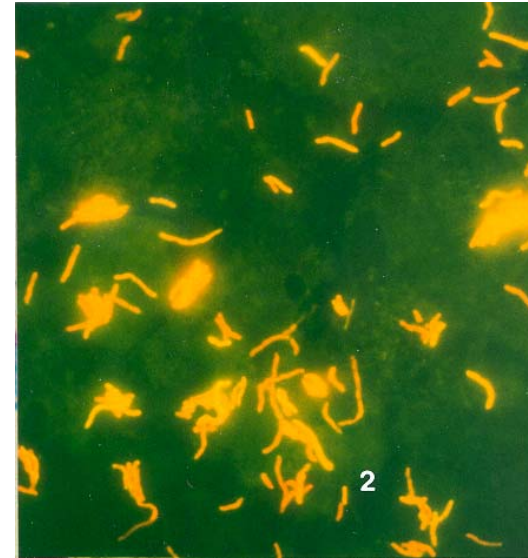
Magnification: 1,000x, oil immersion  
(Granulation disappears in photo due to long exposure time)

# Appearance of AFB in bright-field and fluorescence microscopy

Ziehl-Neelsen



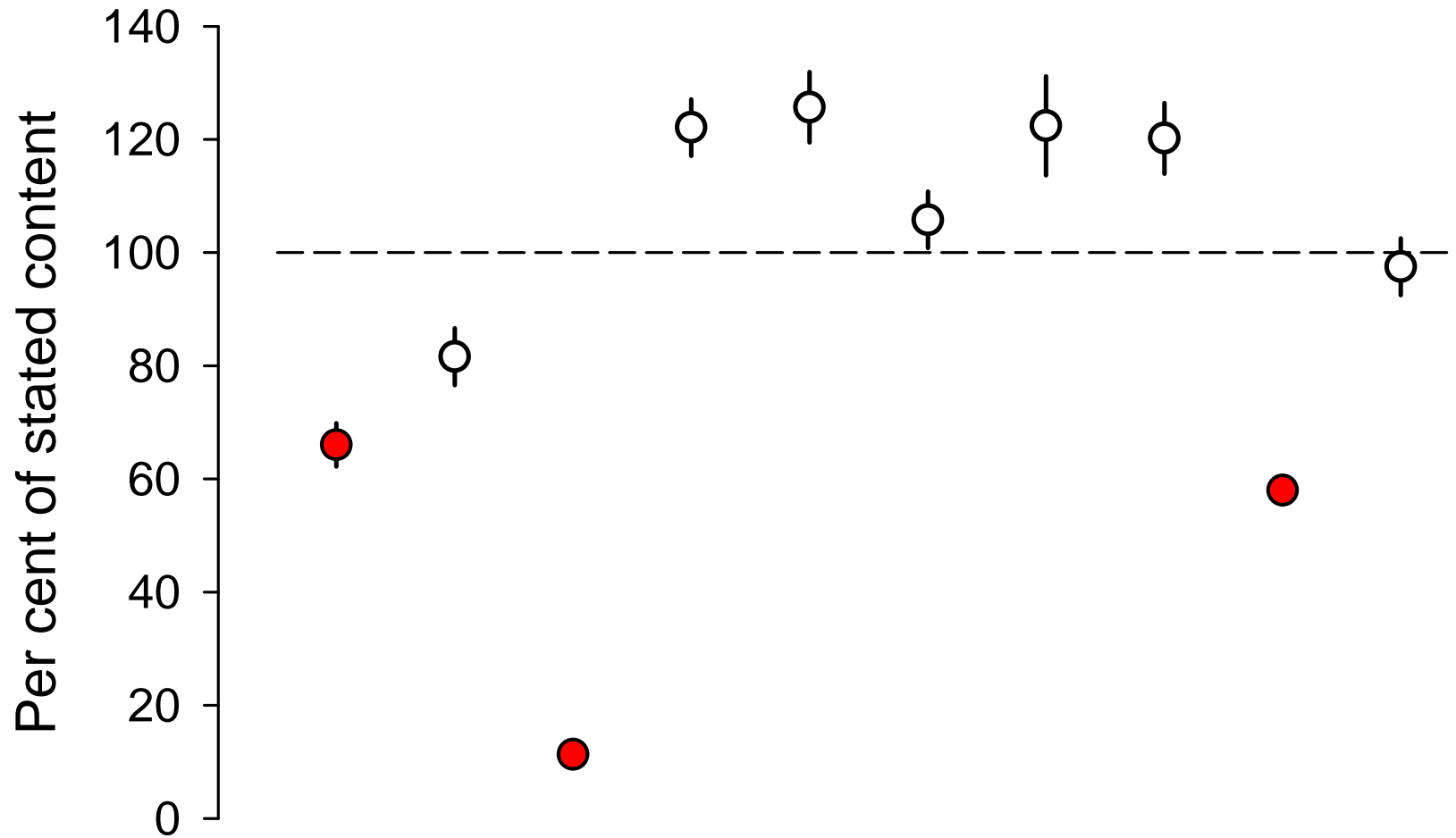
Fluorescence



*Picture courtesy:*

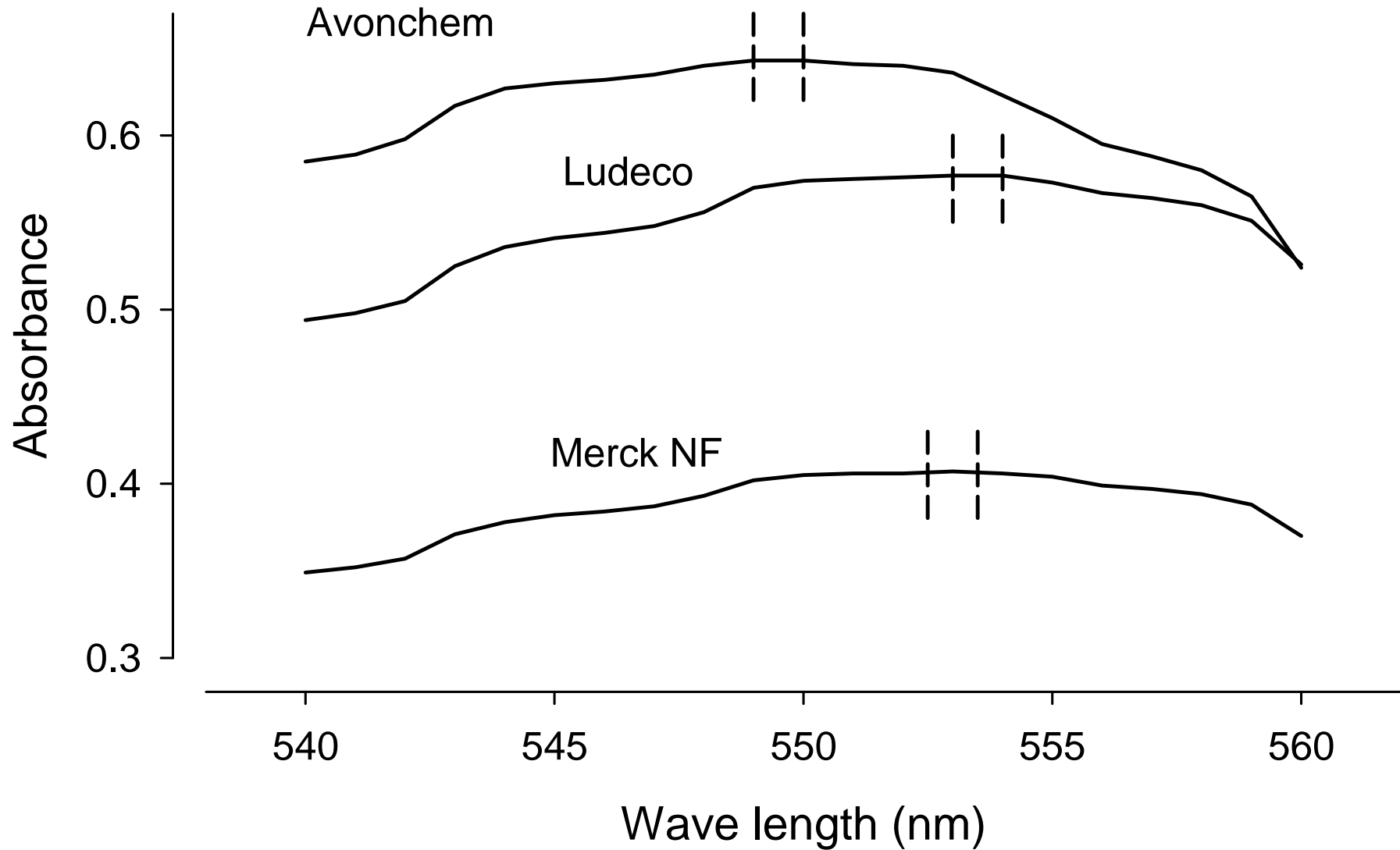
*Kim SJ. Unpublished lecture notes, Union Hanoi course, 4 September 2008*

# Variation in fuchsin content in 10 samples collected during routine field visits, China, 2005-2006



*Zhao YL, et al. Int J Tuberc Lung Dis 2009;13:126-9*

# Fuchsin Absorbance by Wave Length and Manufacturer



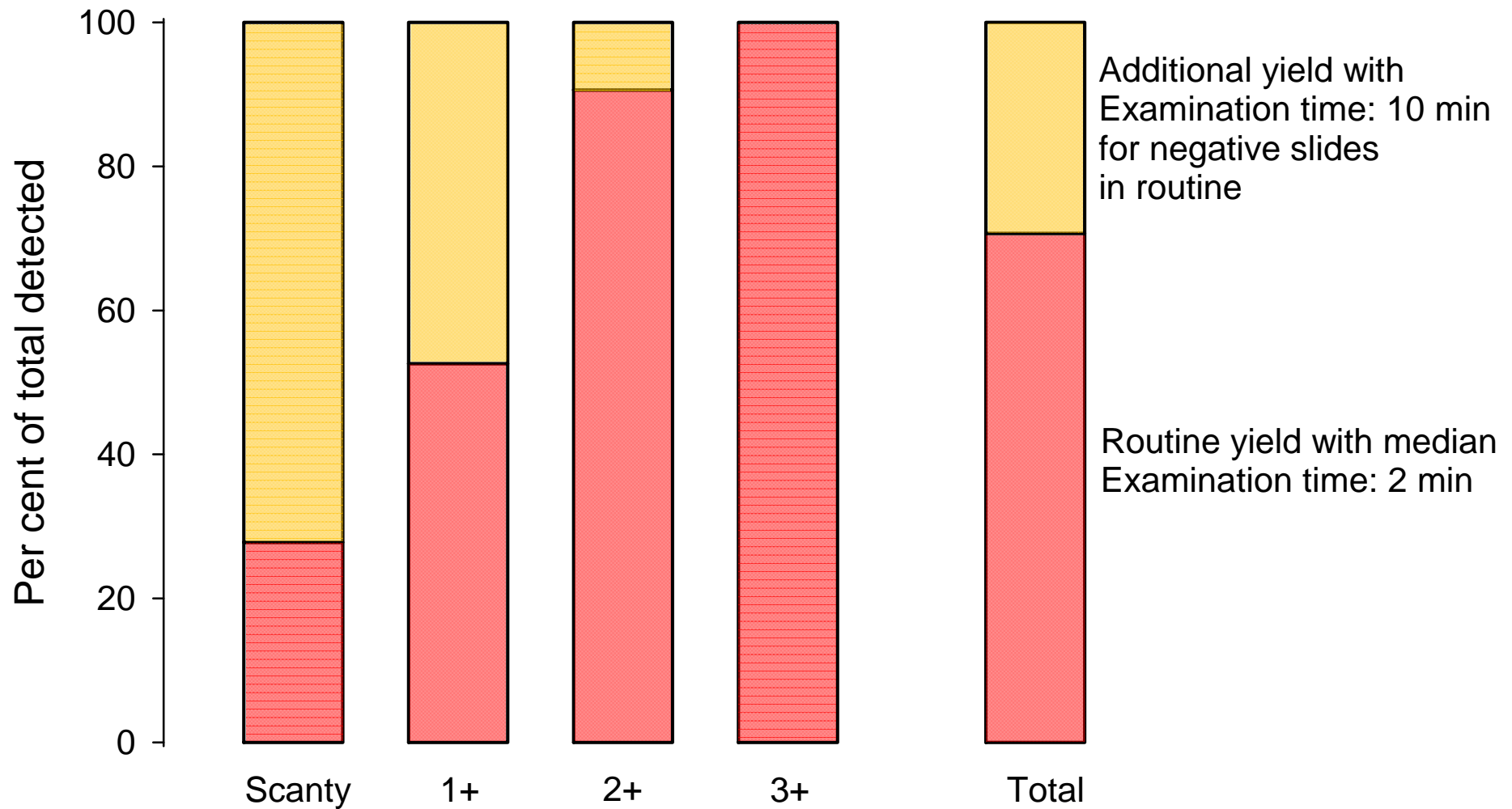
*Data courtesy: Van Deun A, unpublished experiments, 2008*

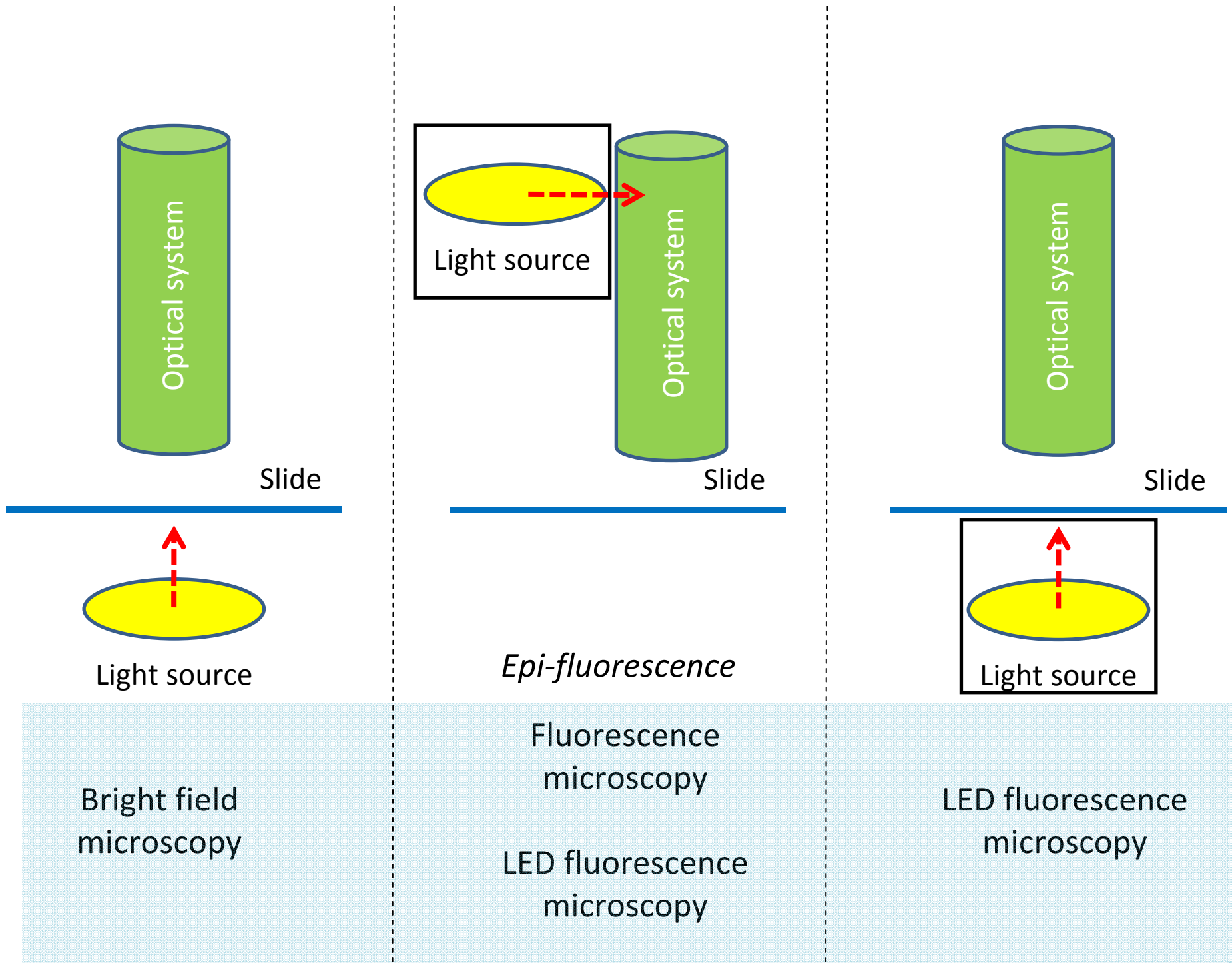
# Visualizing the fuchsin content of different stains by dilution



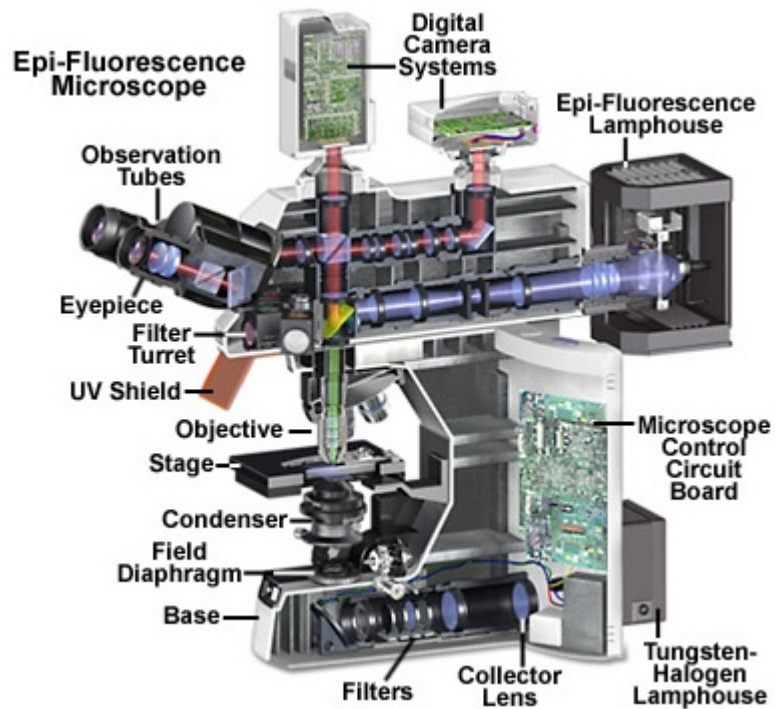
*Slide courtesy: Kam KM. Unpublished lecture notes, Union International Tuberculosis Course Hanoi, Viet Name, September 2008*

# Investing sufficient time in sputum smear examination, Cameroon





Principle of classical epi-fluorescence  
(Example: Nikon system)



Principle of “add-on” LED module  
in transmission mode  
(Example: Fraen system)



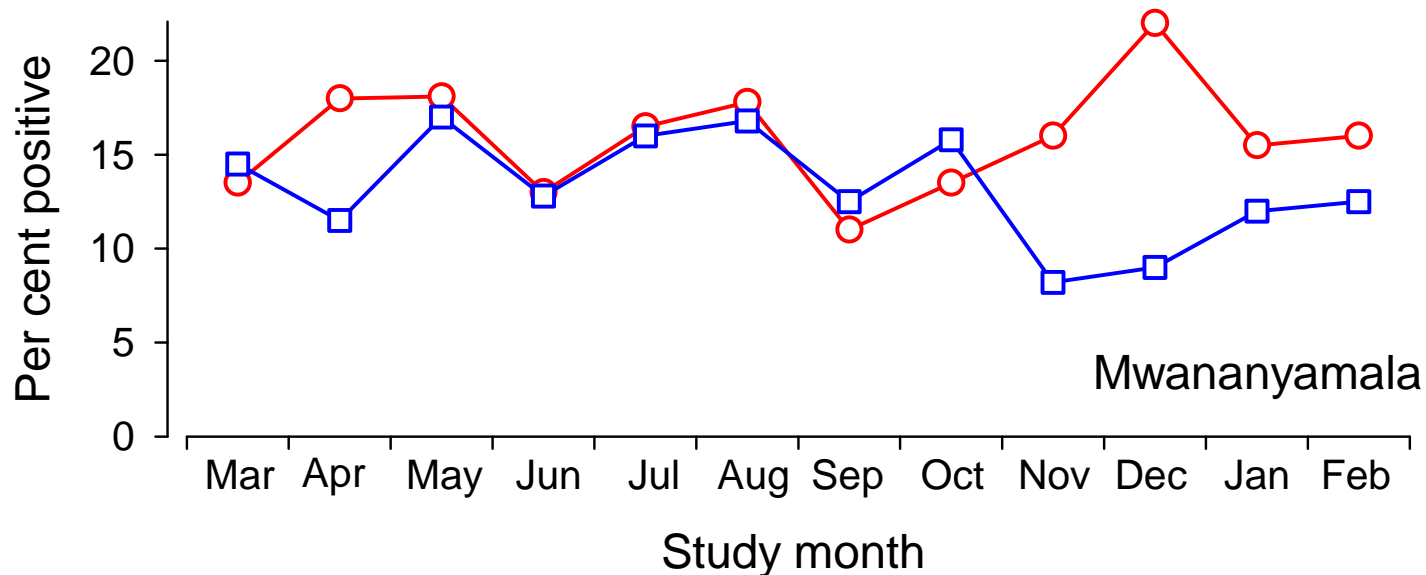
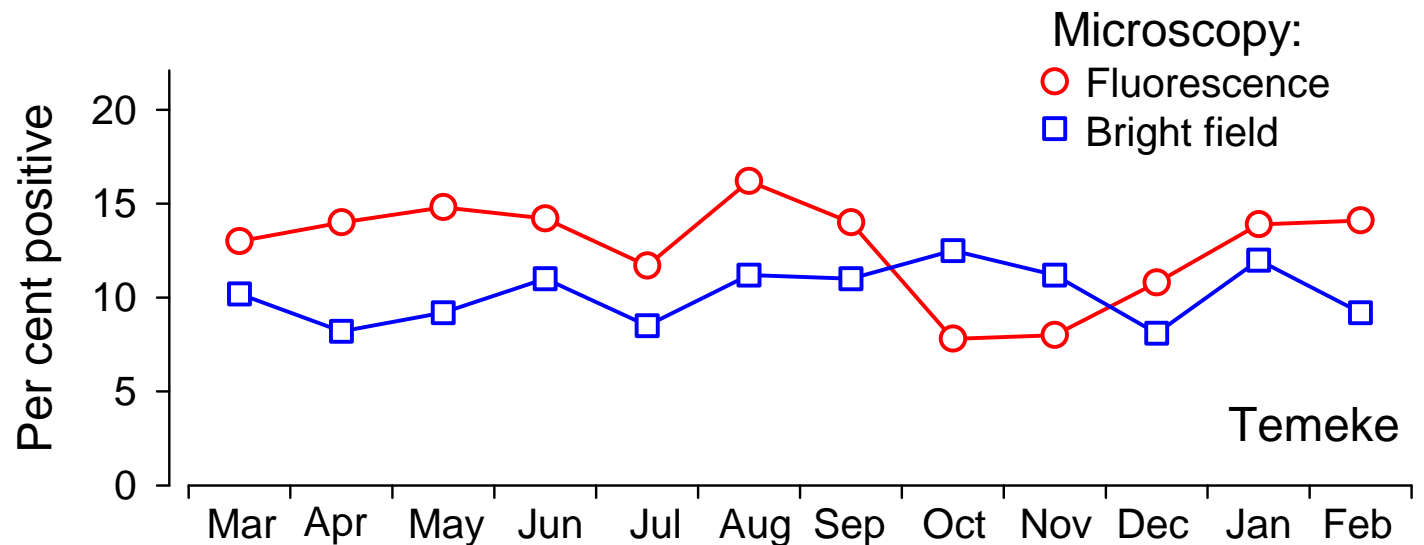
Added to an existing bright-field microscope without interference with the existing white-light function

## Classical vapor lamp versus light emitting diode fluorescence microscopy

Readings using LED	Readings using mercury vapor lamp					
	Negative	Scanty	1+	2+	3+	Total
Negative	396	6	2	0	0	404
Scanty	1	7	4	0	0	12
1+	1	1	23	2	0	27
2+	0	0	2	15	4	21
3+	0	0	0	4	24	28
Total	398	14	31	21	28	492

*Hung NV, et al. Lancet Infect Dis 2007;7:238-9*

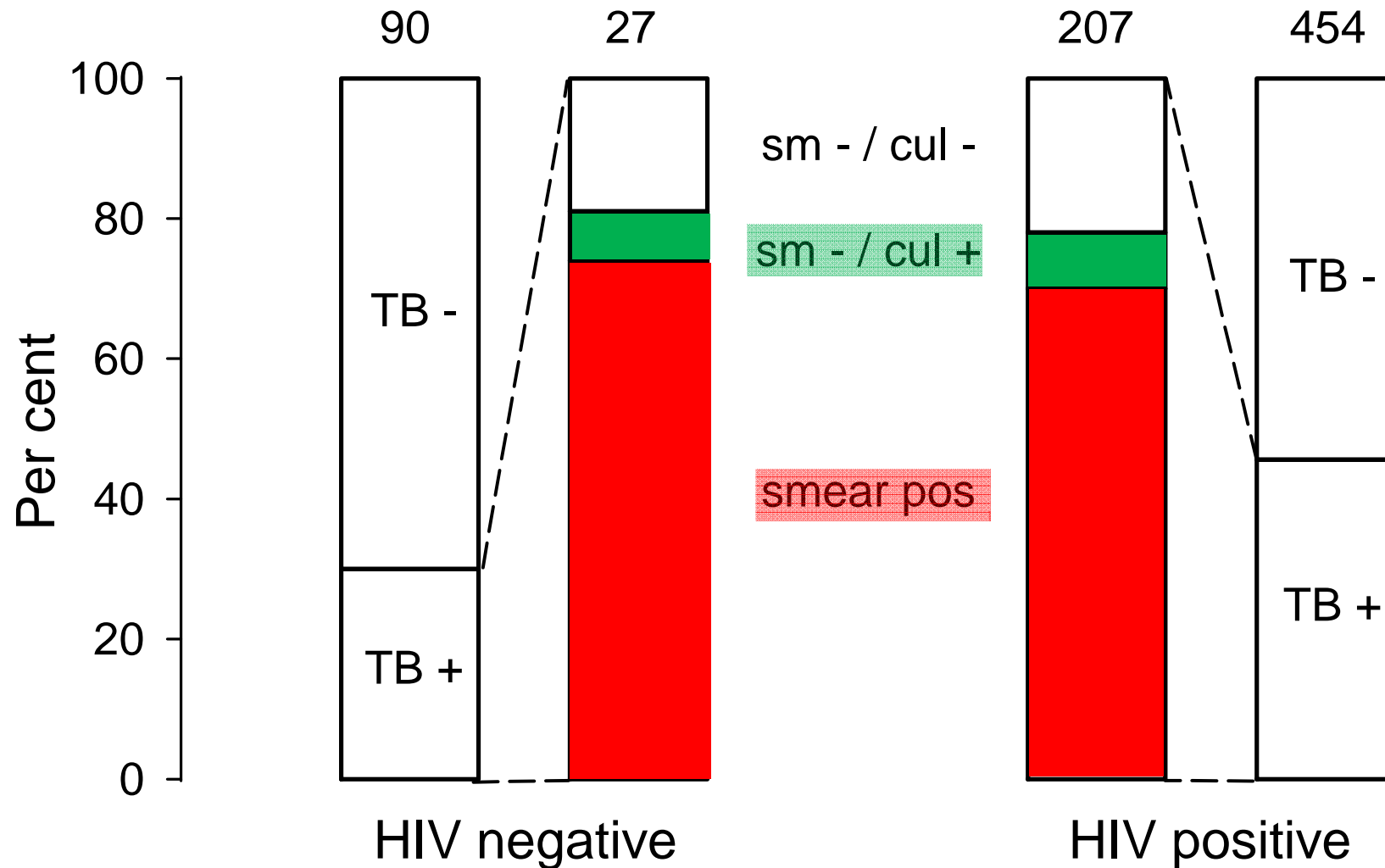
# Yield from bright field versus fluorescence (LED) microscopy in two districts, Tanzania, 2007-2008



## Some differences between microscopy techniques

	Bright-field	Fluorescence (classic)	Fluorescence (LED)
Stain	Fuchsin	Auramine	Auramine
Time for proper examination	5 to 10 min	2 min	2 min
Microscope cost (€)	1,000	8,000	1,000
Module cost (€)	--	--	1,000
Lamp life (hr)	10,000	200	100,000
Maintenance cost	Low	Very high	Low
Acceptability	High	Often very low	High

# Tuberculosis Among Primary Health Care Attendees with Prolonged Cough, Harare, Zimbabwe, 2003 (?)



Munyati SS, et al. Clin Infect Dis 2005;40:1818-27

## Main issue with fluorescence microscopy: acceptability, not test operating characteristics!

- o Fluorescence microscopy not accepted in Zurich until 1980 (Policy change introduced by Pr Dr A von Graevenitz, technique by Dr M Salfinger )!
- o Fluorescence microscopy break-through acceptance in the US only after 1962 (Truant, et al), “selling” that combining auramine plus rhodamine was the solution to earlier non-acceptance – everybody forgot that quirky piece of history – there is “evidence” and “evidence”!
- o “Classical” fluorescence microscopy not accepted in Dar es Salaam in the 1990s
- o LED fluorescence microscopy in Dar es Salaam 2007-2008:  
***microscopists refuse to return to Ziehl-Neelsen***