BRIEF REPORT

Light wave filtration by colored cellophane to optimize microscopic contrast ratio in pulmonary tuberculosis sputum observation [version 1; peer review: 1 not approved]

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V1 First published: 01 Mar 2022, 11:254
<https://doi.org/10.12688/f1000research.109146.1>

Latest published: 01 Mar 2022, 11:254
<https://doi.org/10.12688/f1000research.109146.1>

Abstract

Introduction: Indonesia has the second highest number of Tuberculosis (TB) in the world and tuberculosis still pose as a global health priority problem. For the diagnostics, microscopy is still one of important modalities in TB diagnosis especially in peripheral areas for screening, case-finding, and treatment evaluation. The use of microscopy is limited to operator visual burden and specimen load. This study aims to modify the use of the microscope by using everyday item to increase microscope operator workload.

Method: This is an analytic study of the use of colored cellophane in microscopic examination of sputum smear from 24 pulmonary TB patients from Jakarta and Surabaya. The sputum samples were confirmed positive by standardized ZN-AFB microscopy and/or Xpert MTB/RIF. Each sputum were made into two slides, for each slide, 10 oil immersion fields were captured using Optilab microscope camera mounted on microscope. A total of 480 data pairs were analysed by comparing color characteristic of acid-fast bacilli pixels and its adjacent background pixels and by counting Acid fast Bacilli for each observation field. IUATLD scale were also observed by 3 operators.

Results: Contrast ratio for each AFB and its adjacent pixels were significantly higher in groups using cellophane filter than without using filter ($p = 0.001$). AFB identification per image acquired using filter were not significantly higher ($p=0.815$).

Conclusion: Colored cellophane increases contrast ratio and eases microscopic examination, thus increases sputum smear microscopic observation capability. Also it might increase identification capability with digital image processing.

Open Peer Review

Approval Status

1

version 1

01 Mar 2022

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Acid-fast staining, Microscopy, Tuberculosis, Sputum

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Author roles: **Edbert D:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Visualization, Writing – Original Draft Preparation; **Mertaniasih NM:** Conceptualization, Data Curation, Methodology, Supervision, Validation, Writing – Review & Editing; **Endraswari PD:** Conceptualization, Methodology, Project Administration, Visualization, Writing – Review & Editing; **Koendhori EB:** Conceptualization, Data Curation, Methodology, Supervision, Validation

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

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How to cite this article: Edbert D, Mertaniasih NM, Endraswari PD and Koendhori EB. **Light wave filtration by colored cellophane to optimize microscopic contrast ratio in pulmonary tuberculosis sputum observation [version 1; peer review: 1 not approved]** F1000Research 2022, 11:254 <https://doi.org/10.12688/f1000research.109146.1>

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Introduction

Microscopy is one of the most important diagnostic modalities in Tuberculosis (TB) diagnosis and bacteriological follow-up especially in peripheral areas, sub district and community levels as a method for screening, case-finding, and treatment evaluation.¹ High-end modalities and trained staff to use nucleic acid amplification tests or fluorescent microscopy might not be available in limited resource settings and peripheral areas. While the need for large-scale screening and medical professional shortage are imminent.² This emphasizes the need to optimize currently available diagnostic methods and the use of digital image processing to help identification of pathogens.³

In 2019, approximately 5.6 million male, 3.2 million female, and 1.2 million children suffered TB. Primary TB and Multidrug resistant TB pose as one of 10 causes of death by infection. Between 2000 and 2019, 60 million lives had been saved by accurate diagnosis and treatment.⁴ In Indonesia, in 2019, 1 020 000 people suffered TB, with an incidence rate as high as 391 per 100 000 population. As of 2020, TB prevalence in Indonesia is still the second highest in the world after India^{1,4,5}

Acid-fast staining is the most versatile and the cheapest method in TB diagnosis. This staining can also be used in the diagnosis of non-pulmonary tuberculosis, lepra, parasitic diarrhea, and histopathological staining. Various methods have been proposed to increase the effectiveness of acid-fast staining in microscopy, such as fluorescence microscopy and additional pre-staining treatment. The use of fluorescence microscopy has topped the sensitivity of common hot Ziehl Neelsen (ZN) stains and cold Kinyoun-Gabbett-Tan Thiam Hok stains. Despite its performance, fluorescence microscopy needs a special observation room, trained staff, and its own hazards.⁶ Certain chemicals such as oxidator agents might be added to ZN stain or its alternative methods to increase sensitivity while eases the microscope operator observation skills, but generalized use of new reagents might need diagnostic study.⁷⁻⁹

Light wave modifications have been used to increase contrast in microscopy. Rheinberg's illumination has been used to increase microscopic contrast, while giving hue to the supposedly grayscale nature of microscopic slides. It is a variation of dark-field microscopy, but instead of using opaque (black) annular filters, this type of method uses translucent color filters. A layer of common colored cellophane placed under a microscope condenser can be used to increase contrast.¹⁰⁻¹³ A combination of blue and yellow using Rheinberg's Illumination is proven to increase microscope contrast in 10-40× magnification¹⁴ but its use in oil immersion field has not been evaluated, especially in stained preparation

This study aims to increase contrast ratio in observation of ZN stained sputum to ease the observation of acid-fast bacteria in sputum smear; in hope of increasing microscopic performance in limited resource settings and can be used to increase capability in digital image processing of acid-fast staining microscopy.

Methods

This experiment compares the use colored cellophane and standardized microscopy examination of 24 sputum of pulmonary TB patients: 8 sputums were from dr Soetomo Academic Hospital, Surabaya, and 16 sputums were from Atma Jaya Catholic University of Indonesia Microbiology Laboratory, Jakarta. These are stored reference sputum samples for quality control which were confirmed positive by standardized ZN-AFB microscopy and/or Xpert MTB/RIF. Each sputum was made into two microscopic slides, each shaped into a 2 cm × 3 cm oval. Each slide was heat-fixed.

Ethical statement

This research has been reviewed under dr. Soetomo Academic Hospital ethical committee and was granted approval (ethical certificate 0201/KEPK/V/2021). Written consent for biological material was acquired and patient related data are kept in confidentiality.

Staining

Standard Ziehl-Neelsen using heated 0.3% Carbol-fuchsin, 3% HCl-alcohol, and Levine's Methylene Blue were treated to all slides.

Microscope observation and image acquisition

LED microscope Nikon Eclipse E-100 and bulb microscope Olympus CX-21 was used to observe ZN stained slides. Blue cellophane and yellow cellophane were used to filter incoming light. This is to produce the highest possible contrast ratio by subtracting red and blue light waves.¹⁵ The blue light filters were placed on top of the light source, while the yellow filters were placed inside of the microscope camera or on one of the ocular lenses. The total of microscope magnification was 1000×. For each slide, ten oil immersion fields were captured using Optilab Advanced + microscope camera integrated with ocular lens and mounted onto ocular lens fitting until a total of 480 images were obtained. Images were saved in 2592×1944 JPEG format.

Figure 1. A) Adjacent Pixel (100× Objective, Maximum zoom in photo editing software). Red square: Acid fast bacilli pixel; Blue square: Adjacent background square. B) Example of a same observation field without filter (Left) and with filter (Right). The background is more homogenous in the filter use. Notice higher resolution and sharper margin of acid-fast bacilli when observed using filter (inset). C) Same sputum stained with Kinyoun-Gabbett-Tan Thiam Hok method.

Contrast ratio analysis

One pair of acid fast bacilli pixels and its adjacent background was noted from each of the 480 images to get a total of 960 contrast ratio data. RGB for acid fast bacilli and its adjacent background (Figure 1) were converted into sR/sG/sB values. The contrast ratio was then calculated according to contrast ratio formula.¹⁶

Results

Contrast Ratio and AFB count data were not normally distributed (Saphiro-Wilk value 0.000). Contrast ratio for each AFB and its adjacent pixel were significantly higher in filter group $p = 0.000$; Wilcoxon; CI 95%). AFB identification and enumeration per image using filter is not significantly higher ($p = 0.157$; Wilcoxon; CI 95%), reducing the possibility of false positives. IUATLD scales were analysed based on interobserver agreement from three microscope operators. IUATLD scales were slightly higher in four lower count slides that were observed using filters, especially in low IUATLD scores ($p = 1.000$; Wilcoxon; CI 95%) but the clinical relevance is still maintained (Table 1).

Digitally analyzed contrast ratio were higher in the use of light filter ($p = 0.000$). A slightly higher acid fast bacilli cell count was obtained in the use of cellophane filter use. The numbers of AFB count and IUATLD count do not differ significantly from standard Ziehl-Neelsen observation, which means no significant false positives were found (Table 1).

Table 1. General characteristics of measurements.

	Contrast ratio		AFB/OIF*		IUATLD scale	
	Filter	No filter	Filter	No filter	Filter	No filter
n**	480	480	480	480	48	48
Saphiro-Wilk	0.000	0.000	0.000	0.000	0.000	0.000
$\bar{X} \pm SD$	2.99 ± 0.18	2.38 ± 0.44	22.6 ± 1.02	22.47 ± 1.03	N/A	N/A
Median	2.6	2.2	11	9	3+	3+
Min	1	1	0	0	Scanty	Scanty
Max	8.5	6	50***	50	3+	3+
Wilcoxon (p value)	0.000		0.157		1.000	
Kappa (IUATLD Scale)					0.868 (Strong agreement)	

*Acid Fast Bacilli count per oil immersion field.

**Oil immersion field images for contrast ratio and AFB, Slides for IUATLD scale.

***Maximum count for AFB/OIF is 5 times the highest clinically relevant number (10 AFB/OIF).

Table 2. Crosstabulation of IUATLD scale based on observer agreement.

		No filter				Total
		Scanty	1+	2+	3+	
Filter	Scanty	5	0	0	0	5
	1+	2	8	0	0	10
	2+	0	2	5	0	7
	3+	0	0	0	26	26
Total		7	10	5	26	48

Each sputum was duplicated and observed separately.
Percent of similarity between group 91.67%.

Filter use increases the capability of finding scanty and low number of acid fast bacteria (1+ and 2+), the Kappa analyses showed strong agreement (Table 2).

Filter use limits the red wave spectrum almost entirely, creating black objects with light green backgrounds. The variance in filter group is smaller whether in RGB scale or HSL scale (Table 3); this can improve digital identification of AFB using image processing or artificial intelligence.

Discussion

During our preliminary trials, Malachite green counterstaining produces better contrast compared with Methylene Blue. This method is also applicable to Sulphuric acid modification staining and Kinyoun-Gabbet-Tan Thiam Hok cold staining method (Figure 1). This method also eases the finding of AFB in Reitz's serum observation for *Mycobacterium leprae* in our preliminary tests.

Light is a complex phenomenon that is classically explained with a simple model based on rays and wave fronts. Stained sputum slides act as a light wave filter as light passes through the translucent body of a cell. Various chemical reactions are repurposed to accommodate differentiation methods towards different cells. In acid-fast staining, Carbol fuchsin dye bends light waves with its unique refractive index, allowing only red light waves to pass. Methylene-blue stained structures allow only blue and green light waves to pass.^{17–19}

In a study in Teheran, approximately 2.42 ± 1.12 hours of microscopic work in a day is strongly correlated with visual fatigue. Dry eyes, burning eyes, headache, tearing of the eye and drowsiness are the five top complaints in high-burden microscope operators.²⁰ Therefore, most laboratories are trying to replace microscopic observation with image acquisition and digitization of images. WHO also try to replace microscopy with molecular approach, which is not feasible for most of healthcare facilities.¹

Table 3. Color characteristics of AFB and background pixels.

		Filter		No filter	
		AFB	Background	AFB	Background
R	$\bar{X} \pm SD$	0 ± 2	0	106 ± 50	91 ± 65
	Median (Min-Max)	0 (0-35)		112 (0-217)	112 (0-219)
G	$\bar{X} \pm SD$	89 ± 43	166 ± 29	81 ± 48	163 ± 35
	Median (Min-Max)	88 (8-203)	167 (59-253)	78 (0-209)	166 (22-248)
B	$\bar{X} \pm SD$	66 ± 43	80 ± 45	166 ± 44	177 ± 40
	Median (Min-Max)	60 (0-189)	78 (0-203)	172 (14-254)	183 (36-255)
H (°)	$\bar{X} \pm SD$	165 ± 24	150 ± 17	258 ± 45	177 ± 49
	Median (Min-Max)	166 (85-221)	150 (120-195)	266 (0-354)	183 (30-352)
S (%)	$\bar{X} \pm SD$	100	100 ± 3	51 ± 34	52 ± 29
	Median (Min-Max)	100	100 (94-100)	39 (0,2-100)	5 (0-100)
L (%)	$\bar{X} \pm SD$	$19 \pm 0,8$	33 ± 6	46 ± 13	54 ± 12
	Median (Min-Max)	19 (2-40)	33 (12-50)	44 (10-82)	55 (23-84)

Digital photomicrography opens more windows towards automation and in this case the use of image processing to identify acid-fast bacilli. Although photomicrography has been used for various microscopic methods, its use in the oil immersion field has not been explored.^{11,12} Color characteristics for background and the acid-fast “objects” are described in Table 3. this data can be used in the development of better image processing-based digital microscopic identification.

Digital image processing is versatile. One of the uses is to convert 24 bit true color image (RGB) to binary format (black and white) through some steps character extraction, noise filtering, grayscale, and threshold. By using representation of RGB (Red, Green, Blue) value, a true color image is converted to white color and gradation of black color that is usually called by grayscale image.²¹ If the observed RGB image is available in monochrome or in higher contrast, digital conversion of RGB to binary image is faster and shortens the image processing algorithm to identify Mycobacterial cell. The use of acid-fast stained sputum is in coherence that the black cells are acid-fast. For Red acid fast bacilli cells, the image processing algorithm can easily be modified to ‘learn’ stain characteristics by a color calibration step. That is, by comparison of color characteristics to the training database and flagged for difference in color histograms.²² However, staining errors are likely in laboratories that might use different stainings and might perform slight deviations of the quality control procedures recommended by the WHO. This disparity is reduced in this research by using black color as the object with homogenous green background, instead of red with blue or non-homogenous background.

In this study authors use a microscope using a light-emitting diode (LED) and a light bulb microscope. The LED microscope has a more homogenous light wave spectrum, compared to common light bulb lights which emit more orange to yellow light waves.¹⁹ Both LED and bulb microscopes are used in observation to reduce light wave bias in microscopic observation.

Conclusion

The use of cellophane as color filter in Ziehl-Neelsen reading increases contrast ratio and eases Acid-fast bacilli observation and is potential for the development of more accurate image processing software. No clinical differences were found in the use of colored cellophanes.

Data availability

All data underlying the results are available as part of the article and no additional source data are required.

Acknowledgement

We thank Dr. Soetomo Academic Hospital and Microbiology Laboratory of Faculty of Medicine and Health Sciences, Catholic University of Indonesia Atma Jaya for providing all necessary support in this research.

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Current Peer Review Status:

Version 1

Reviewer Report 07 November 2022

<https://doi.org/10.5256/f1000research.120611.r148881>

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Very interesting and simple innovation. With some additional improvements, this paper can be useful.

- Writing can be improved for better reading and clarity.
- The optical system description is inadequate to understand the logic. How are the filters used in the system? In combination what illumination? Which color filter?
- Improving contrast can help reduce the operator time per slide. There is no documentation on operator performance with microscope modification.
- Most of the TB microscopy is done manually and not with digital image acquisition. Does the filter use improve manual annotation versus digital image annotation?
- We need some more evidence to understand, how color selection can enable machine learning algorithm better.

Best wishes

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microscopy, Tuberculosis diagnostics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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