

DEVELOPMENT OF AN OPTICAL MODULE FOR NANO-ANALYSIS AND RAPID DIAGNOSIS OF INFECTIOUS DISEASES

VIKAS PANDEY



**DEPARTMENT OF CHEMICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

OCTOBER 2020

©Indian Institute of Technology Delhi (IITD), New Delhi, 2020

DEVELOPMENT OF AN OPTICAL MODULE FOR NANO-ANALYSIS AND RAPID DIAGNOSIS OF INFECTIOUS DISEASES

by

VIKAS PANDEY

Department of Chemical Engineering

Submitted

**In fulfillment of the requirements of the degree of Doctor of Philosophy
to the**



Indian Institute of Technology Delhi

OCTOBER 2020

Dedicated to
My Loved Ones

Certificate

This is to certify that the thesis entitled “**Development of an optical module for nano-analysis and rapid diagnosis of infectious diseases**”, being submitted by **Mr. Vikas Pandey** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy**, is a record of bonafide research work carried out by him, which has been prepared under our supervision and guidance of conformity with the rules and regulations of “Indian Institute of Technology Delhi”.

The research reports and the results presented in this thesis have not been submitted in part or full to any other University/ Institute for the award of any degree or diploma.

We certify that he has pursued the prescribed course of research.

Dr. Shalini Gupta
Associate Professor,
Department of Chemical Engineering
Indian Institute of Technology Delhi
New Delhi - 110016

Dr. Ravikrishnan Elangovan
Associate Professor,
Department of Biochemical Engineering &
Biotechnology
Indian Institute of Technology Delhi
New Delhi – 110016

Acknowledgments

The whole journey of my Ph.D. was a great learning experience. The completion of the thesis wouldn't have been possible without the encouragement and support of various individuals whom I would like to acknowledge here.

Firstly, I intend to express my deep sense of gratitude and indebtedness to Prof. Shalini Gupta who gave me wings to explore my scientific curiosity in her laboratory. Her faith and confidence in me, helped me work on this interdisciplinary research subject and a successful culmination of my thesis. As a true mentor, she helped me develop my research ideas and nurtured my investigative abilities that serve an important part in a research career. Her scientific approach and immense knowledge of the subject instilled great confidence in me to overcome the difficulties during my research work. I am hugely indebted and thankful to her for all the time and support she has provided me during my research.

I would like to thank Prof. Ravikrishnan Elangovan, for all the help and support right from the beginning of the research idea to making of the products out of the technologies developed during research work. He played a pivotal role in shaping my career into research and technology translation in the market. The late-night project works in the laboratories, long discussions about the politics along with science and tea breaks are something worth keeping in my sweet memories. It was a pleasure working with him in his laboratory.

From the bottom of my heart, I would like to thanks Prof. Seyed E Hasnain, Prof. Nasreen for their trust in me. They supported my ideas, technologies, and encouraged in all possible way to translate it into a product. They held my hand and poured their life experience and wisdom to

make me a different person. I would also want to thank all of my colleagues in Valetude Primus Healthcare, Manu Kadyan, Shruti Ahuja, Amir Khan, Avishke Dev, Sambit Dam, and Tanish Garg for their relentless support.

I am grateful to my SRC committee members: Prof. Anupam Shukla, Prof. Gaurav Goel, and Prof. Amartyasen Gupta for their valuable feedbacks to enhance my knowledge. I especially want to thank prof. Gaurav Goel for his keen and analytical suggestions about the subject. I am thankful to all of them for taking out time and reviewing my work annually.

I want to express my deep gratitude to my closest friends Saurabh Singh and Sumit Yadav who have helped me, supported me in each phase of my Ph.D. journey. We shared our happy, sad, emotional, and frustrating moments in campus canteens and hostel rooms. Our connection grew stronger and stronger with time, be it the professional venture together or friendship. I am thankful to God for their kind presence in my life.

I would like to thank Prof. Jesús Liñares Beiras, Prof. Vicente Moreno, Prof. Xesús Prieto Blanco, Dr. Carlos Montero from Universidade Santiago de Compostela for their keen insight and knowledge in optics which helped me refine my cTIRF & SeeTB devices further. I would also like to extend my thanks to Prof. Eva Acosta and Ana for all the help and logistic support they provided for crucial optics experiments in Spain.

Special thanks to Almighty. I dedicate all the success, achievements to Him. The invisible positive power of His presence in my life made me go on and on. I am grateful to my parents, siblings Mr. Vivek Kumar Pandey and Mrs. Amita Pandey, and my wife Mrs. Arunakshi Mishra for their unconditional love and relentless support towards me. Their solidarity in my decisions filled me with positivity. I can't thank them enough in this life.

Last but not least, I would like to thank all the colleagues in the lab Pooja Singh, Jyoti Sharma, Neha Khaware, Augustine Cleetus, Rohini Singh, Shruti Khanna, Surabhi, Puja Prashad, Promod Jagtap, Vidhu S Pillai, and Jagat Narayan for making the research duration enjoyable and fulfilled. I would like to especially mention Mr. Gaurav Jhakhda for his round the clock support and helpful gestures.

I acknowledge the Department of Biotechnology, Government of India, BIRAC, SRISTI, Honeybee network, FITT-IIT Delhi, and MHRD for all the financial support for our projects. I am very thankful to the Indian Institute of Technology (IIT) Delhi for transforming my life by providing me the knowledge, platform of opportunities and shaping me as a person to serve the society and mankind.

Vikas Pandey

Abstract

In the past few decades, different illumination methods in microscopy have shown significant enhancement in imaging resolution limit to capture even single-molecule. In this context, the total internal reflection (TIR) method stands on top as it generates exponential decaying electromagnetic disturbances also known as evanescent waves at the interface. This phenomenon is now widely exploited in various technologies such as total internal reflection fluorescence (TIRF) microscopy, surface plasmon resonance (SPR) spectroscopy, optical sensors, etc. In TIRF, the evanescent waves generated at the interface selectively excite the fluorophores and yield high z-resolution images. Despite resolution advantage, traditional TIRF microscopy has some shortcomings such as (i) its dependence on high numerical aperture (NA) objectives (> 1.4), (ii) the requirement of optical filters, (iii) low photon intensity illumination, and (iv) it's high cost.

During my Ph.D. thesis, I have addressed the above-mentioned challenges in TIRF microscopy by building a simple, portable, and large-area waveguide-based illumination device called the 'compact total internal reflection fluorescence illumination device' (cTIRF). cTIRF provides TIRF imaging capability in a simple brightfield microscope as well. cTIRF works on the principle of the planar optical waveguide. The optical filters are generally omitted in this setup as the direction of illumination and signal collection optics remain perpendicular to each other. This device is flexible to use with low magnification objective TIRF imaging as well. The device is also used to observe the scattering signals from the non-fluorescently labeled object.

We tested the cTIRF device for large-area illumination, high signal to noise ratio (SNR) imaging of mammalian cells, labeled and non-labeled nanoparticles (NPs), and for estimation of the diffusion coefficient of gold NPs in brownian motion through signal auto-correlation statistics. Moreover, we have also shown the implementation of TIRF imaging through cTIRF

device into infectious disease diagnosis by observing sputum smears prepared in the glass slides for *Mycobacterium tuberculosis* (MTB) diagnostics. A proprietary chemical composition called “CLR” has also been developed to optimize the sputum processing process. The combination of cTIRF in brightfield microscope and the CLR liquefaction is termed as SeeTB. The SeeTB has been shown a drastic improvement in TB diagnosis when tested over 500 clinical sputum samples. The sensitivity enhanced from 64% in the ZN test to 90 - 95% in SeeTB keeping GeneXpert and liquid culture as the gold standard.

सारांश

पिछले कुछ दशकों में सूक्ष्मदर्शी की विभिन्न दीप्ति विधियों के माध्यम से प्रतिबिंब विभेदन की क्षमता में कई महत्वपूर्ण उन्नति हुई है, जिसमें एकल अणु को प्रतिबिंबित करना भी शामिल है। इस संदर्भ में, पूर्ण आंतरिक परावर्तन विधि शीर्ष पर है क्योंकि इसमें अंतराफलक पर घातांकी क्षय होती हुई विद्युत चुंबकीय तरंगे उत्पन्न होती हैं, जिसे इवनएसेंट तरंगों के रूप में भी जाना जाता है। इस विधि को अब विभिन्न तकनीकों में जैसे कि पूर्ण आंतरिक परावर्तन प्रतिदीप्ति (टीआईआरएफ) सूक्ष्मदर्शी, सरफेस प्लास्मोन प्रतिध्वनि (एसपीआर) स्पेक्ट्रोस्कोपी में, ऑप्टिकल सेंसर इत्यादि में व्यापक रूप से इस्तेमाल किया जाता है। टीआईआरएफ में, अंतराफलक पर उत्पन्न होने वाली तरंगें चुनिंदा रूप से फ्लूरोफर्स को उत्तेजित करती हैं और उच्च अक्षीय विभेदन प्रतिबिंब बनाती है। उच्च अक्षीय विभेदन के लाभ के बावजूद, पारंपरिक TIRF सूक्ष्मदर्शी में कुछ कमियां हैं जैसे (i) इसकी उच्च न्यूमेरिकल एपर्चर (NA) लेंस (> 1.4) पर निर्भरता, (ii) प्रकाशीय फिल्टर की आवश्यकता, (iii) प्रतिदीप्ति फोटॉन तीव्रता की कमी, और (iv) इसकी उच्च लागत।

मेरे पीएचडी थीसिस के दौरान, मैंने TIRF सूक्ष्मदर्शी की उपर्युक्त चुनौतियों को एक सरल, व्यावहारिक और बड़े क्षेत्र में तरंग-निर्देश उत्पन्न करने वाले यंत्र द्वारा संबोधित किया है, जिसका नाम 'कॉम्पैक्ट पूर्ण आंतरिक परावर्तन प्रतिदीप्ति रोशनी यंत्र' (cTIRF) रखा गया है। cTIRF यंत्र TIRF इमेजिंग क्षमता को एक साधारण ब्राइटफील्ड सूक्ष्मदर्शी में भी प्रदान कर सकता है। cTIRF प्रकाशीय तरंग-निर्देश के सिद्धांत पर काम करता है। इस यंत्र में प्रकाशीय फिल्टर की आमतौर पर आवश्यकता नहीं पड़ती क्योंकि रोशनी और सिग्नल संग्रह प्रकाशिकी की दिशा एक-दूसरे के लंबवत रहती हैं। यह उपकरण कम आवर्धन में भी TIRF इमेजिंग उपयोग के लिहाज से लचीला है। डिवाइस का उपयोग गैर-फ्लोरोसेंटली लेबल वाली अति छोटे कणों से प्रकीर्णन वाले संकेतों का निरीक्षण करने के लिए भी किया जा सकता है।

हमने cTIRF यंत्र का परीक्षण बड़े क्षेत्र को प्रकाशित करने में, उच्च संकेत अनुपात (एसएनआर) में ममेलियन कोशिकाओं को प्रतिबिंबित करने में, लेबल और गैर-लेबल वाली नैनोकणों (एनपी) के प्रकीर्णन और ब्राउनिंग गति में स्वर्ण कणों के प्रसार गुणांक के आकलन के लिए किया है। इसके अलावा, cTIRF यंत्र द्वारा TIRF इमेजिंग की उपयोगिता संक्रामक रोगों के रोग-निर्णय के लिए छय रोगी Mycobacterium tuberculosis (MTB) की ग्लास स्लाइड में तैयार थूक स्मीयरो के अवलोकन के माध्यम से दर्शाया गया है। थूक प्रसंस्करण प्रक्रिया का अनुकूलन करने के लिए "सीएलआर" नामक एक सांपातिक रसायन भी विकसित किया गया है। ब्राइटफील्ड सूक्ष्मदर्शी में cTIRF और सीएलआर द्रवीकरण के संयोजित उपयोग को SeeTB (छयदृष्टि) का नाम दिया है। SeeTB का उपयोग 500 से अधिक नैदानिक थूक के नमूनों का निदान करने के लिए किया गया था और ZN परीक्षण में 64% से संवेदनशीलता में भारी सुधार देखा गया, जो GeneXpert और तरल culture को स्वर्ण मानक के रूप में रखते हुए 90 - 95% है।

Contents

Certificate.....	IV
Acknowledgments.....	V
Abstract.....	VIII
सारांश	X
List of Figures.....	XVI
Abbreviations.....	XX
Chapter 1 Introduction.....	1
1.1 Thesis objectives	4
1.2 Thesis layout	4
Chapter 2 Theory and background	5
2.1 Fluorescence microscopy	5
2.1.1 Fluorescence	5
2.1.2 Excitation and emission	6
2.1.3 Use of fluorescence in microscopy methods	7
2.1.4 Limitations of fluorescence microscopy	8
2.2 Total internal reflection fluorescence microscopy	9
2.2.1 TIRFM: A historical perspective	9
2.2.2 Evanescent waves	9
2.2.3 Different methods for TIRFM	11
2.2.4 TIRFM using optical waveguide	12
2.2.5 Theory of waveguide	12
2.2.6 Use of optical waveguides in microscopy	15
2.2.7 Methods for large area evanescent wave illumination.....	16
2.2.8 Evanescent scattering method.....	17
2.3 TIRFM in single-molecule imaging.....	18
2.3.1 Fluorescence correlation spectroscopy	18
2.4 Fluorescence microscopy in healthcare.....	19
2.4.1 The problem of TB diagnostics.....	19
2.4.2 Healthcare ecosystem in India	19
2.4.3 TB diagnosis scenario in India.....	20
2.4.4 Fluorescence microscopy in TB diagnosis	21

2.4.5	TB diagnosis improvement in India & other developing nations	22
Chapter 3	cTIRF device standardization	23
3.1	Characterization of cTIRF device	27
3.2	Materials and methods	27
3.2.1	Sample preparation	27
3.2.2	Fluorescence imaging of sample with cTIRF in a brightfield microscope	28
3.3	Results and discussion.....	29
3.4	Imaging of non-fluorescently labeled nanoparticles using cTIRF.....	38
3.5	Conclusions	38
Chapter 4	cTIRF for diffusion coefficient estimation	40
4.1	Introduction	40
4.2	Background	40
4.3	Theory	41
4.3.1	Diffusion coefficient	41
4.3.2	Fluorescence fluctuations.....	42
4.3.3	Correlation analysis	44
4.3.4	Determination of physical quantity.....	45
4.4	Experimental section and data analysis.....	47
4.4.1	Materials and methods	47
4.4.2	Gold nanoparticles	47
4.4.3	The thin-film CYTOP coating on glass coverslips	48
4.4.4	Experimental setup.....	50
4.5	Results	51
4.5.1	Detection volume	51
4.5.2	Fluctuation and autocorrelation functions	52
4.5.3	Discussion.....	54
Chapter 5	cTIRF system for TB diagnosis: SeeTB & FACEIT-TB.....	56
5.1	Materials and methods	56
5.1.1	Ethics approval and study setting	56
5.1.2	<i>Mycobacterium tuberculosis</i> culture on liquid medium	57
5.1.3	GeneXpert MTB/RIF (GeneXpert) assay	57
5.1.4	Fluorescence microscopy	58

5.1.5	Composition of CLR reagent	58
5.2	Clinical protocol.....	58
5.3	Results	59
5.4	Discussion	65
5.4.1	Advantages of SeeTB	65
5.4.2	Advantages of CLR reagent.....	65
5.5	Conclusion: SeeTB.....	67
5.6	cTIRF and artificial intelligence (AI) for efficient TB diagnosis: FACEIT-TB.....	68
5.6.1	Background	68
5.7	Fluorescence and artificial intelligence-based cell enumeration and imaging technology for tuberculosis (FACEIT-TB).....	73
5.7.1	Electronic circuit.....	73
5.7.2	Schematic of FACEIT-TB device.....	75
5.7.3	Image identification algorithm.....	76
5.7.4	Image annotation.....	77
5.7.5	Process flow for FACEIT- TB	80
5.8	Result and discussion: FACEIT-TB.....	81
Chapter 6	Summary and prospects	85
Chapter 7	Research outcomes.....	88
7.1	Patents	88
7.2	Publications	88
7.3	Conferences	88
7.4	Awards	88
References	89
Appendix-1	98

List of Figures

Figure 2-1: Jablonski diagram. The non-radiative energies are represented by the curved lines between the different energy states (S_0, S_1, S_2, S_3). The straight arrows up and down show the absorption and emission of the photon. Vibrational energy states are shown in $V_0, V_1, V_2, V_3, V_4, V_5$.	7
Figure 2-2: Phenomenon of TIR: In the area where the incident light angle is higher than the critical angle, the light gets TIR and generates evanescent waves in the low RI side of the plane. Exponentially decaying electromagnetic disturbance also known as evanescent waves of the same wavelength penetrates maximum depth (δ) of ~ 300 nm	10
Figure 2-3: It shows the two different TIRFM methods. The left side schematics show the prism-based TIRF illumination. The fluorescence signal from the specimen is captured using a 60x oil objective lens from the bottom side of the substrate while the specimen is sandwiched between prism and glass substrate. The right-hand schematic shows the propagation of light through the objective lens to generate TIR at the interface of the glass substrate and specimen. It is evident from these schematics that specimen is accessible in objective-based illumination method (shown in right). In both cases, a small spot of 0.5 to 1 mm^2 is illuminated using an objective lens with $\text{NA} > 1.4$. (Reference: MicroscopyU)	11
Figure 2-4: Glass substrate RI (n_1) sandwiched with lower RI (n_2), a light ray (in yellow) making angle (θ) in y-z plane moves inside the high RI region. The geometric representation of the waveguide is shown in the figure.	13
Figure 2-5: The electrical field distribution of the various modes in the above rectangular waveguide. (Figure courtesy: book “Fundamental of Photonics”) ³⁶	15
Figure 2-6: Schematic of a thick circular glass substrate sandwiched between light barriers and six high-power LEDs orderly arranged to illuminate from six different sides. The light rays above the critical angles enter into the glass substrate and generate evanescent waves in a large area. The sample is placed on top of the glass substrate and observed from the bottom (Ramachandran et al., 2013).	16
Figure 2-7: Left: High power laser-generated waveguide aligned with capture optics. This method generated fluorescence images with the use of optical filters and additional information of the non-labeled biological objects without optical filters (Bjorn Agnarsson et. al). Right: Schematic of a high-intensity laser light coupled into a beveled angle glass slide (Xiao-Hong, Z. et al.).	17
Figure 2-8: Healthcare diagnostics ecosystem in India along with the advantages of a portable and rapid POC test system over traditional central laboratory-based units.	20
Figure 2-9: TB diagnostics infrastructure in India. The majority of cases are first detected at the designated microscopy centers. In the second step, the sample is sent to the specialized intermediate laboratories.	21
Figure 3-1: A ray diagram was constructed on the geometric calculations through geometric optics. The ray diagram shows the incident light ray inserts at the glass substrate at an angle higher angle than the critical angle. The RIs of air, prism, glass, and sample are denoted as n_1, n_2 . EFL stands for the effective focal length of the cylindrical lens, and k is the distance between the prism and the cylindrical lens.	24

Figure 3-2: (A) The schematic of housing (COU) where all the optical components were fixed. (B) the schematics of metallic structure SCU where COU is fixed and glass slide can be inserted for light coupling. (C) the actual picture of COU. (D) 3D printed picture of SCU.....	25
Figure 3-3: The systematic improvements in cTIRF device with many designs and manufacturing iterations. These iterations incorporating operational challenges along with the user feedbacks.	26
Figure 3-4: cTIRF was used with an Olympus IX71 microscope to observe TIRF images.	29
Figure 3-5: Demonstration of cTIRF's large FOV excitation using 1 μm fluorescent latex beads at (A) $10\times$ & (B) $50\times$ magnification. (C) Enlarged image via digital magnification of a callout in B.....	29
Figure 3-6: Comparison between cTIRF and cTIRF + scattering mode (A) 1 μm fluorescent latex beads. (B) Intensity map along the lines shown in images in A. (C) Corresponding 3D images of each bead acquired in two different modes. All images were acquired using 50 ms exposure....	31
Figure 3-7: Micrographs of 2, 1, 0.5, 0.1 μm sized particles under brightfield, epi-, cTIRF, and cTIRF + scattering mode illumination. With small size particles, the cTIRF + scattering produced images with a substantial-high SNR in comparison to FM.	32
Figure 3-8: Epi- and cTIRF illumination of TRITC-phalloidin labeled HeLa cell in left and right respectively represents its attachment at maturation; the imaging of value for the study of the cell motion is possible through TIRF microscopy.....	33
Figure 3-9: Lateral decay of evanescent wave intensity from the light coupling point. (A) Representative cTIRF optical micrographs of 1 μm beads acquired at different distances along with the sample glass slide. The scale bar represents 10 μm . (B) The integrated fluorescence intensity values from a single bead as a function of the corresponding distance shown above. Each point is a signal-averaged from a minimum of 30 beads and error shown as standard deviation.....	34
Figure 3-10: (A) A schematic representation of evanescent wave excitation of particles of various sizes and their excitation depth showed as 2D illumination projection. The blue color represents 2D projected area in epifluorescence and the yellow color is the projected area for the same particle in cTIRF mode. (B) Images of 2 and 0.5 μm beads acquired using epi- and TIRF-illumination and their projections to calculate the penetration depth of the evanescent wave generated. The circles represent the calculated diameter in epi-illumination. (C) The study with the various dimension fluorescent beads showed that penetration depth is independent of the bead size was calculated to be 152 ± 32 nm.	35
Figure 3-11: The penetration depth of evanescent waves calculated using different-sized fluorescent particles. A geometrical relation between a partially illuminated particle and the penetration depth.....	36
Figure 3-12: Non-labelled NPs imaging via c-TIRF scattering. 100 nm non-fluorescent MNPs imaged under (A) epi-illumination and (B) cTIRF system. The scale bar represents 1 μm . Particles were not visible under epi-illumination and brightfield imaging. With evanescent scattering single, 100 nm particles can be visualized clearly with respect to the background. (C) The intensity surface plot of each particle denotes an approx. SNR of 5. Scattering signals from single-particle was Gaussian in nature and can be used for single-particle tracking experiments in biological samples. (D) A histogram of scattering signals from single NPs.	37

Figure 4-1: (A) Fluorescence fluctuation of the free-moving particles (1 & 2) crossing the focus of the laser beam in a confocal microscope. Two random trajectories of the freely moving particles move in an arbitrary path within the focused laser beam. The excitation and the detection probability of these particle particles are higher if they reside in the narrowest band area. The narrowest band area is the high-intensity illumination zone that fades away as we move away from the center. Since the particle moves randomly, there are chances that the particle crosses the focal volume repeatedly. (B) A time-dependent burst of photon counts recorded using a photodiode as the particles move in and out of the focal volume. Typically avalanche photodiodes are used for this purpose (figure courtesy: Jung et al)⁸⁴ 43

Figure 4-2: (A) Time-resolved photon burst Ft recorded using a photosensor. (B) Intensities plotted after subtracting the mean value from the actual ones. The positive value shows the presence of particles inside the detection volume. (C & D) The graph in B replotted after a small time-shift of τ_1 (C) and τ_2 (D) where, $\tau_1 < \tau_2$. The correlation with the original curve ($\tau = 0$) decreases as we move farther in time (figure courtesy: Jung et al)⁸⁴ 45

Figure 4-3: The hydrodynamic particle size distribution in the two batches of GNP suspensions (A) 15.6 ± 3.6 nm and (B) 17.4 ± 4.2 nm. 48

Figure 4-4: (Left) Optical waveguide generated in a glass coverslip coated with CYTOP (RI ~ 1.33) and propagating unhindered through an oil droplet put in its path. (Right) On the other hand, the optical waveguide is disrupted at the oil drop in an uncoated coverslip. Both panels are of the same orientation however, the right photo is a bit magnified as taken from closer. 49

Figure 4-5: (A) Thickness and (B) mean squared deviation vs position for coverslip 1. (C) Thickness and (D) mean squared deviation vs position for coverslip 2. 49

Figure 5-1: Schematic of clinical sputum sample processing, sputum smear preparation, and observation in the SeeTB system. 59

Figure 5-2: (A) Left to right: (i) ZN stained sputum slide imaged using BM. (ii) Auramine-O stained slide imaged using FM. (iii) Image acquired using cTIRF setup. (B) Comparison of the number of bacilli counted in FM and cTIRF setup 60

Figure 5-3: (A) *Mycobacterium tuberculosis* H37Ra cells stained using auramine-O. Cells were serially diluted and images were acquired randomly in 30 frames. (B) The average number of bacilli visualized using the SeeTB setup. 61

Figure 5-4: (A) Comparison of optical clarity of sputum samples with and without CLR treatment. All different grades of sputum samples are rapidly homogenized with CLR treatment. (B) Comparison of background staining of auramine-O with and without treatment of CLR reagent. (C) An average number of bacilli enumerated with (red bar) and without CLR treatment (blue bar). The error bars here show the standard deviation of the number of bacilli counted per frame. 62

Figure 5-5: Flow of participants in the study 63

Figure 5-6: The schematic of electronic circuit GPIO connections of stepper motors and their drivers are shown in the diagram 74

Figure 5-7: Complete Internal Mechanism of FACEIT-TB (Front View) 75

Figure 5-8: An example of 32 x 32 pixels is shown in the figure. The blue boxes correspond to the multi-channel features in the low resolution. The channel number is written on top of each box. The size of the pixel is written on the left side of the box. The type of arrow represents

different operations as mentioned in the figure. The white boxes show the copied feature maps.

.....	76
Figure 5-9: Classification into Negative, Positive or Clusters of <i>Mycobacterium tuberculosis</i> bacilli.....	77
Figure 5-10: Comparison of the original image (A) and the positive bacilli masked image (B) .	78
Figure 5-11: Summary of the model with the total number of trainable parameters and output..	80
Figure 5-12: Proposed Process Flow for TB Diagnosis using FACEIT-TB	81
Figure 5-13: Determination of the number of bacilli (output displayed as ‘Number of Positive Bacilli’) using input image (A1, and B1 respectively). The output can be seen in (A2, A3, B2, and B3 respectively)	82

Abbreviations

AFM	Atomic force microscopy
AFB	Acid-fast bacilli
AI	Artificial intelligence
BSC	Biosafety cabinet
BFM	Brightfield microscopy
CBNAAT	Cartridge-based nucleic acid amplification test
COU	Central optical unit
CCD	Charge-couple device
CLR	Clearing reagent
CEF	Collection efficiency function
cTIRF	Compact total internal reflection fluorescence
CMOS	Complementary metal-oxide semiconductor
CM	Confocal microscopy
CNN	Convolutional neural network
CVSVM	Cross-validation support vector machine
DNA	Deoxyribonucleic acid
DMC	Designated microscopy center
DIC	Differential interference contrast
DOT	Direct observation therapy
DMEM	Dulbecco's modified eagle media
DLS	Dynamic light scattering
EFL	Effective focal length
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
XRD-TB	Extreme drug resistance
FOV	Field of view
FITC	Fluorescein isothiocyanate
FACEIT-TB	Fluorescence and artificial intelligence-based cell enumeration and imaging technology for tuberculosis

FCS	Fluorescence correlation spectroscopy
FM	Fluorescence microscopy
fps	Frames per second
GFNN	Gaussian-fuzzy-neural network
GPIO	General-purpose input/output
GNP	Gold nanoparticles
GFP	Green fluorescent protein
HIV	Human immune virus
IKVSM	Intersection kernel support vector machine
kNN	k-nearest neighbor
LED	Light-emitting diode
LEDFM	Light-emitting diode fluorescence microscope
LOD	Limit of detection
LinSVM	Linear support vector machine
MNP	Magnetic nanoparticles
MDR-TB	Multi-drug resistance tuberculosis
MTBC	<i>Mycobacterium tuberculosis</i> complex
MTB	<i>Mycobacterium tuberculosis</i>
NALC	n-acetyl-l-cysteine
NTM	Non-tuberculosis <i>Mycobacterium tuberculosis</i>
NA	Numerical aperture
PDE	Particle detection efficiency
PC	Phase-contrast
PBS	Phosphate-buffered saline
PMT	Photon multiplier tube
PCR	Polymerase chain reaction
QY	Quantum yield
RF	Random-forest
RPi	Raspberry pi

ReLU	Rectified linear unit
RI	Refractive index
R-CNN	Region-based convolutional neural network
RNTCP	Revised national TB control programme
SCS	Scattering correlation spectroscopy
sCMOS	Scientific complementary metal-oxide semiconductor
SNR	Signal-to-noise ratio
SMD	Single-molecule detection
SCU	Slide coupling unit
SSM	Sputum smear microscopy
SPR	Surface plasmon resonance
TRITC	Tetramethylrhodamine
TIR	Total internal reflection
TIRFM	Total internal reflection fluorescence microscopy
TB	Tuberculosis
TB-AI	Tuberculosis artificial intelligence
UV	Ultraviolet
UTI	Urinary tract infection
VIA	VGG image annotator
WEFS	Waveguide evanescent field scattering
WExFM	Waveguide excitation fluorescence microscopy
WHO	World health organization
ZN	Ziehl-Neelsen