Investigations into high resolution optical fiber probe for targeted illumination and imaging around opaque obstacles

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**INVESTIGATIONS INTO HIGH RESOLUTION
OPTICAL FIBER PROBE FOR TARGETED
ILLUMINATION AND IMAGING AROUND
OPAQUE OBSTACLES**

ANANT SHINDE

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

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Abstract

Controlled illumination at targetted locations (targeted illumination) and imaging at high switching speed have found significant applications in different frontiers of science and technology such as optogenetics, photodynamic therapy (PDT), neurophotonics, and medical imaging. Although there are numerous optical systems reported with targeted illumination ability, most of these are modified standard (bench top) microscopes, hence not suitable for *in vivo* applications. Therefore, there is a critical need to develop optical imaging systems with targeted illumination suitable for *in vivo* studies.

The small diameter, user-defined length and high mechanical flexibility of fiber optic imaging bundle (FOIB) facilitates its positioning at remote and difficult-to-access *in vivo* sites. Recently, few FOIB based studies are reported, which have shown adaptation of targeted illumination. However, image resolution and switching speed achieved with these methods are found to be limited to 10 μm and 20 Hz, respectively. In order to investigate biological or chemical processes, the methodology adopted or equipment used should be able to provide higher resolution, faster-switching speed and an option for targeted illumination and imaging.

Moreover, images obtained with FOIB probes are always affected by pixelation noise which deteriorates the image resolution and contrast. Though there are many algorithms to remove pixelation noise, objective comparison of these algorithms are not possible due to the non-availability of a common test image database. Also,

there are no theoretical models available currently to simulate the image guidance through FOIB.

From this perspective, one of the objectives of this thesis is aimed at developing a high-speed imaging probe using a combination of FOIB and Digital Micromirror Device (DMD) with targeted illumination and imaging feature for potential *in vivo* applications. Compared to the earlier FOIB based probes, the newly developed probe has shown improved lateral and axial image resolution of $2.7\ \mu\text{m}$ and $5.5\ \mu\text{m}$, respectively. This imaging system also provides a larger field of view ($200\ \mu\text{m} \times 200\ \mu\text{m}$) at high resolution compared to earlier reported targeted FOIB probes. The developed FOIB probe's illumination switching speed is defined by the DMD, which is 10,000 Hz. The multiline digitally controlled laser source allows the FOIB probe to illuminate the targeted regions with different wavelengths.

Further, an objective comparison of different depixelation methods for FOIB imaging is also performed as part of the investigation. A theoretical model for FOIB based imaging is developed and used to generate images of simulated fiber pixelated images. The parameters such as packing fraction, fiberlet to fiberlet distance, fiberlet core diameter, core-cladding properties and light guiding properties of fiberlet are considered in the theoretical formulations. These studies have led to the development of a Fiber Pixelated Image Database (FPID), which now serves as a free open source common test image sample database for researchers working on the development of novel depixelation methods.

The effect of variable pinhole size on the imaging properties is also studied using the proposed probe. An illustrative demonstration is carried out for four different

imaging approaches using this targeted FOIB probe. These approaches are ‘targeted confocal imaging’, ‘multi-directional scanning in targeted sample regions’, ‘targeted time averaged imaging for contrast enhancement’, and ‘single shot multi-target multispectral imaging’, which were found to improve resolution, contrast, and imaging speed.

The potential of the FOIB probe is demonstrated by synchronous multispectral spatiotemporal illumination of targeted mouse kidney cells. Additionally, the efficiency of this FOIB probe for tracking and targeted illumination of dynamic (moving) particles is demonstrated as a proof of concept. This developed FOIB probe has also been demonstrated as a portable targeted illumination source for the standard bench-top microscope.

The second major objective of this thesis is aimed at imaging around opaque obstacles. Since there are numerous injuries (cuts) reported due to blind injection or improper position of the surgical tool, caused due to the blocking of the field of view of the sample by opaque surgical tools during surgery, an imaging system capable of imaging around obstacle would be helpful in avoiding such injuries. Hence in this thesis, a detailed research is carried out to come up with an optical system to image around obstacles.

In this thesis, use of an axicon lens is explored for imaging around opaque obstacles. The simulation of axicon lens to perform imaging around an opaque obstacle is demonstrated using Zemax software. This is further validated experimentally by imaging around different thick opaque obstacles of different shape and thickness, such as Allen key, syringe needle, metallic pin, hair, and

thread. Finally, the proof of concept of this method is demonstrated by imaging around a surgical needle during needle injection procedure.

It is envisaged that the invaluable advantages provided by the targeted probe along with its specialty features can make a great impact in the research and developmental arena such as optogenetics to selectively activate neuronal cells or cell organelles. Further the work done on the imaging round opaque obstacles are expected to contribute significantly during surgical procedures in the near future.

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Anant Shinde

Table of Contents

Abstract I

Acknowledgement.....V

Table of Contents VII

List of Figures..... XII

List of TablesXX

List of Symbols XXI

AbbreviationsXXV

Chapter 1: Introduction - 1 -

1.1 Background and motivation..... - 3 -

 1.1.1 FOIB for high resolution targeted illumination and imaging..... - 3 -

 1.1.2 Optical probe for imaging around obstacle (IAO) - 4 -

1.2 Research objectives - 4 -

1.3 Scope of the research - 5 -

1.4 Organization of thesis - 6 -

Chapter 2: Literature review - 10 -

2.1 FOIB based targeted illumination systems - 10 -

 2.1.1 Fiber optic imaging techniques..... - 11 -

 2.1.1.1 Wide-field imaging using coherent fiber optic bundle..... - 12 -

 2.1.1.2 Fiber bundle confocal microscope - 14 -

2.1.2	Structured illumination and imaging methods	- 18 -
2.1.2.1	Patterned array microscope	- 19 -
2.1.2.2	Structured illumination microscopy (SIM)	- 20 -
2.1.2.3	HiLo microscopy	- 21 -
2.1.2.4	SIM and HiLo based microendoscopy	- 22 -
2.1.3	Targeted illumination	- 23 -
2.1.3.1	In vitro studies	- 23 -
2.1.3.2	In vivo studies	- 26 -
2.2	Imaging around obstacles (IAO)	- 28 -
2.3	Summary	- 36 -
Chapter 3:	High resolution imaging fiber probe	- 41 -
3.1	Investigations into depixelation methods	- 41 -
3.1.1	Spatial averaging filters	- 43 -
3.1.2	Spectral filter	- 45 -
3.1.3	Interpolation method	- 49 -
3.1.4	Analysis of the depixelation methods	- 54 -
3.2	Theoretical model for FOIB imaging	- 63 -
3.3	Fiber Pixelated Image Database (FPID)	- 70 -
3.3.1	Simulation of pixelated images	- 70 -
3.3.2	Image Acquisition for experimental FPID	- 72 -
3.4	High resolution FOIB imaging with variable pinhole size	- 76 -
3.4.1	Wide-field imaging	- 76 -
3.4.1.1	Transillumination imaging	- 77 -
3.4.1.2	Reflective fluorescence imaging	- 78 -
3.4.2	Confocal imaging	- 79 -
3.4.3	Quasi-confocal or variable pinhole imaging	- 85 -

3.5	Summary	- 90 -
Chapter 4:	Targeted illumination and imaging fiber probe	- 91 -
4.1	Need for spatiotemporally targeted illumination probe	- 91 -
4.2	Experimental methodology	- 94 -
4.2.1	Optical configuration	- 94 -
4.2.2	DMD and control software for selective illumination	- 96 -
4.3	Methods for targeted illumination and imaging	- 98 -
4.3.1	Targeted illumination.....	- 98 -
4.3.2	Multispectral spatiotemporal switching.....	- 102 -
4.3.3	Multidirectional, multi-region scanning and Targeted confocal imaging.....	- 104 -
4.3.4	Contrast enhancement by targeted imaging	- 107 -
4.3.5	Selective wavelength illumination of targeted cell and time averaged imaging.....	- 110 -
4.4	Adaptable spatiotemporal probe for targeted illumination microscopy	- 112 -
4.4.1	Optical configuration	- 114 -
4.4.2	Combination of trans-illumination and reflection mode imaging with targeted illumination.....	- 115 -
4.5	Targeted illumination of dynamically moving particles.....	- 118 -
4.5.1	Algorithm for tracking and illumination	- 119 -
4.5.2	Tracking the glass bubble with targeted illumination	- 122 -
4.6	Summary	- 124 -
Chapter 5:	Optical probe for imaging around opaque obstacles	- 126 -
5.1	Introduction	- 126 -
5.2	Optical design for imaging around obstacle	- 127 -

5.3 **Optical setup for IAO - 130 -**

5.4 **Results of IAO - 133 -**

 5.4.1 White light reflection imaging behind thick opaque obstacle - 133 -

 5.4.2 White light transillumination imaging behind thick opaque obstacle - 134 -

 5.4.3 Fluorescence imaging behind thick opaque obstacle - 135 -

5.5 **Real-time IAO..... - 137 -**

5.6 **Microscopy around opaque obstacle - 142 -**

5.7 **Summary - 145 -**

Chapter 6: Conclusion and future work..... - 146 -

 6.1 **Conclusion..... - 146 -**

 6.2 **Major contributions - 151 -**

 6.3 **Future work - 152 -**

 6.3.1 Application of the developed system in optogenetics - 153 -

 6.3.2 Applications in ocular imaging - 154 -

 6.3.3 Miniaturization of IAO system - 154 -

Appendix A - 156 -

Fiber Pixelated Image Database - 156 -

Appendix B - 165 -

Sample preparation and materials - 165 -

Appendix C - 171 -

Axicon assisted gonioscopy for high resolution iridocorneal angle imaging- 171

-

List of publications.....- 174 -

References - 176 -

List of Figures

Figure 1.1 Hierarchical research roadmap.	- 9 -
Figure 2.1 Structure of the fiberlet showing total internal reflection.	- 11 -
Figure 2.2 End face Image of fiber optic imaging bundle. (a) Cross-Sectional image of the end face. (b) An enlarged version of inset area.	- 12 -
Figure 2.3 Optical schematic showing FOIB imaging with an external light source for sample illumination [51].	- 13 -
Figure 2.4 Wide-field FOIB imaging with inherent illumination [54]. O- objective, DM-dichroic mirror, EX-F -excitation filter, EM-F emission filter and CCD-charge coupled device camera.	- 14 -
Figure 2.5 Confocal microscope with coherent fiber optic bundle [58].	- 15 -
Figure 2.6 Experimental setup for a fiber bundle confocal system with Zeiss LM 310 Confocal scanning microscope [59].	- 16 -
Figure 2.7 Fiber confocal reflectance microscope [60].	- 16 -
Figure 2.8 Representative Galvanometric mirror assembly [9].	- 17 -
Figure 2.9 DMD based FOIB confocal system [66].	- 18 -
Figure 2.10 A simplified optical schematic for PAM imaging [67].	- 19 -
Figure 2.11 (a) Simplified diagram of 3D structured illumination apparatus. (b) Finite axial illumination pattern [1].	- 20 -
Figure 2.12 Optical configuration for SIM and HiLo micro-endoscopy [29]. ...	- 22 -
Figure 2.13 Targeted illumination of <i>C. elegans</i> with LCSLM based targeted multispectral microscope [20]. (a) Image of <i>C. elegans</i> obtained with wide-field illumination. (b-c) Processed image to select the target regions from FOV. (d) Multispectral targeted illumination of three selected regions.	- 23 -
Figure 2.14 Targeted illumination method to control neuronal cells [86].	- 24 -
Figure 2.15 Three LCSLM for multispectral selective illumination [20].	- 25 -
Figure 2.16 LED array with selective illumination. Inset showing a 3X3 array of LEDs with a central row of LEDs in ON state [27].	- 25 -
Figure 2.17 (a) A selective illumination probe with FOIB and electrodes. (b) Image showing three FOIB from the probe illuminated with uniform illumination. (c)	

Two sites (A and B) identified for selective illumination. (d) Direction for raster scanning with Galvo mirrors [12].	- 27 -
Figure 2.18 Optical schematic for targeted illumination using CGH. The figure beside DMD shows the pattern generated on DMD. The figure beside sCMOS shows the collected image [13].	- 28 -
Figure 2.19 Cloaked sphere by the technique of conformal mapping [116].	- 30 -
Figure 2.20 Schematic diagram of a fabricated carpet cloak showing the different regions, where C1 is the gradient index cloak and C2 is a uniform index background [118].	- 31 -
Figure 2.21 Illustration of paraxial cloaking. (a) Optical setup (b) Cloaking of hand demonstrated [111].	- 32 -
Figure 2.22 Axicon lens showing Non-diffraction zone.	- 33 -
Figure 2.23 (a) Optical setup for Bessel beam reconstruction around small rectangular objects. (b-c) Measured intensity reconstruction of Bessel-like beams at two different distances from the rectangular obstacle [124].	- 34 -
Figure 2.24 Propagation of Gaussian (a-d) and Bessel beam (e-h) in fluorescent gel with two large glass spheres of 8 μm diameter arranged consecutively at various distances from the beam axis b [125].	- 34 -
Figure 2.25 Fluorescence volume imaging with Bessel beam. (a) Optical schematic. (b-d) The intensity distribution of simulated fluorescence at the medium surface, axicon surface and at the detector respectively [126].	- 35 -
Figure 2.26 Optical schematic for variable depth imaging using multiple axicon lenses [127].	- 36 -
Figure 3.1 Spatial averaging filters, (a) Rectangular averaging (b) Disc averaging and (c) Gaussian smoothening.	- 45 -
Figure 3.2 Frequency domain image of Figure 3.1a showing a circular pattern of high-frequency components.	- 47 -
Figure 3.3 Filter masks (a) Suppressing high-frequency components by different suppression levels (b) Suppressing all high-frequency components (c) and (d) Two types of filter masks proposed by Winter <i>et al</i> [145].	- 49 -
Figure 3.4 Optical setup to obtain a calibration image.	- 50 -

Figure 3.5 (a) Primary candidates for center pixels (b) Score of Gaussian template matching (c) Delaunay triangulation of fiberlet center location and (c) Voronoi diagram for fiberlet pixel center.	51 -
Figure 3.6 Natural neighbor interpolation in Voronoi diagram, point p takes the gray area from neighboring points.	53 -
Figure 3.7 (a) USAF 1957 test chart image captured through imaging fiber bundle. It shows line l , and rectangular boxes showing bright and dark regions. Depixelated images reconstructed by (b) Gaussian smoothening (c) Spectral filter and (d) Spatial interpolation.	54 -
Figure 3.8 (a) Neuroblastoma cells captured through imaging fiber bundle and depixelated images reconstructed by (b) Gaussian smoothening (c) Spectral filter and (d) Spatial interpolation.	55 -
Figure 3.9 Variation of intensities in original and filtered images.	56 -
Figure 3.10 Histograms of original and filtered images.	58 -
Figure 3.11 Schematic diagram for single fiber confocal imaging. F- filter, BS- beam splitter, MO-microscope objective.	63 -
Figure 3.12 Cross-section of single fiberlet. (a) Continuous signal representation (b) Discrete signal representation.	66 -
Figure 3.13 Graph showing output amplitude vs input illumination power of FOIBs.	67 -
Figure 3.14 Representation of fiberlet assembly inside FOIB.	68 -
Figure 3.15 Synthetically generated fiber pixelated images. (a-d) and (i-l) are ground truth images while (e-h) and (m-p) are corresponding fiber pixelated images. (e-f) are the grating vertical patterns and (g-h) are inclined grating patterns. (m-n) are Siemens star pattern. (k) is pixelated color map image. (p) Shows how pixelation affects variations in individual colors such as red, green and blue.	71 -
Figure 3.16 Optical schematic for trans-illumination imaging through fiber bundle.	73 -
Figure 3.17 Optical schematic for reflective fluorescence imaging.	73 -

- Figure 3.18 Experimental fiber pixelated images of artificial patterns prepared on glass mask. (a) Grating pattern, (b) Siemens star, (c) Checkerboard pattern and (d) Smiley emoticon. - 75 -
- Figure 3.19 Experimental fiber pixelated images of natural samples. (a) Mouse kidney section imaged with 488 nm wavelength, (b) Glass bubbles in Rhodamine sample imaged with 532 nm wavelength, (c) Cross section of human eye imaged with 405 nm superluminescent LED. Image (a) and (b) were captured with the optical configuration shown in Figure 3.18 while optical setup shown in Figure 3.17 was used for imaging image (c). - 75 -
- Figure 3.20 Trans-illumination images of different samples captured with multiple light sources. Images (a) and (b) were captured with the white light source. Images (c) and (d) were captured with red and blue LED light, respectively. Samples used for imaging were (a) Glass bubble, (b) Cross-section of the human eye, (c-d) 1951 USAF test chart. - 77 -
- Figure 3.21 Images acquired through fiber optic probe in reflective fluorescence mode. (a) Fluorescent micro bubble 25, (b) mouse kidney section tagged with Alexa Fluor 488 and (c) Neuroblastoma cells tagged with Alexa Fluor 568. - 78 -
- Figure 3.22 Optical schematic for confocal imaging through FOIB. - 79 -
- Figure 3.23 Images of FOIB for DMD to fiberlet mapping. (a) with uniform illumination and (b) with spatial pattern illumination. - 82 -
- Figure 3.24 Flowchart for DMD to fiberlet mapping. - 82 -
- Figure 3.25 The FWHM for fiber confocal imaging. - 84 -
- Figure 3.26 Comparison of FOIB confocal with conventional confocal imaging. - 85 -
- Figure 3.27 Illustration of glass bubble images with different pinhole sizes. - 87 -
- Figure 3.28 Axial resolution variation against effective pinhole size. - 89 -
- Figure 4.1 Optical schematic for spatiotemporally targeted illumination and imaging fiber probe. - 94 -
- Figure 4.2 Graphical representation of spatial light modulation with DMD. The inset shows the pattern on DMD mirror pane with white and black part representing ON and OFF state mirrors respectively. - 96 -

- Figure 4.3 Procedure to illuminate selected region from the sample with targeted illumination probe..... - 97 -
- Figure 4.4 Selective illumination of fiber bundle end face. (a) Image fiber bundle with fiberlets clearly visible (with whole field illumination.) (b) Representative spatial pattern illuminating the fiber bundle end face. (c) Illumination of one fiberlet of the fiber bundle having 50000 fiberlets. - 99 -
- Figure 4.5 Comparison between non-targeted and targeted illumination and imaging of cells. (a) Image of mouse kidney section imaged in non-targeted illumination. (b) Illumination and imaging four targeted cells from the same section. Individual cells are clearly aimed through targeted illumination. .. - 99 -
- Figure 4.6 Real-time representation of pattern on DMD mirrors and the corresponding illumination pattern. The image on the left represents DMD mirror state selection, white color represent ON state mirror and the dark yellow color represents the OFF state mirrors. (a) A group of mirrors forming a rectangular shape are switched to ON state with a strip of OFF state mirrors surrounding them. (b) Small square blocks of ON state mirrors. - 100 -
- Figure 4.7 Targeted illumination of mouse kidney cells. (a) White light image of mouse kidney cells. (b) Image of cells collected with targeted illumination. (c-d) Images of cells collected with targeted illumination of cells and cell boundaries. - 101 -
- Figure 4.8 (a) Image of the mouse kidney section having multiple cells tagged with different fluorescent dyes. (b) Alternate illumination of two targeted cells, one with 488 nm and other with 561 nm. (c) The pulsed dual wavelength light source with the different pulse frequencies of 0.5 Hz for cell 1 (488 nm) and 1 Hz for cell 2 (561 nm) is used..... - 103 -
- Figure 4.9 Scanning of multiple targeted regions with single illumination beam spot in each region. - 105 -
- Figure 4.10 Stepwise increase in illumination beam size. (a-h) In each image number of fiberlets illuminating the sample are increased by 4 (2 from each side), eventually illuminating complete region..... - 106 -
- Figure 4.11 Images of the mouse kidney section in wide-field, targeted wide-field and targeted fiber confocal imaging. (a), (b), (c) and (d) show images captured

in a wide-field mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively. (e), (f), (g) and (h) show images captured in targeted wide-field mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively. (i), (j), (k), and (l) show images captured in a targeted fiber confocal mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively.....- 109 -

Figure 4.12 Intensity variation over the line marked in images Figure 4.11 (d), (h), and (l).- 110 -

Figure 4.13 Multiple regions of mouse kidney section captured with different exposure times in a single frame. Region 1 was illuminated with 488 nm light for 0.2 s. Region 2 and 3 were illuminated with 561 nm light for 0.4 s and 0.1 s, respectively.....- 112 -

Figure 4.14 An adaptable spatiotemporal probe (a) Optical schematic (b) Fiber probe adaptation into standard benchtop microscope (IX87, Olympus). ..- 115 -

Figure 4.15 Selective illumination and imaging of cells from mouse kidney section. (a) A microscope image of the mouse kidney section illuminated with 488 nm light. Images (b-e) and (f-h) are the microscope images of selectively illuminated glomeruli and convoluted tubules. Images (b) and (f) represent fluorescent images through fiber for targeted illumination while images (c) and (g) are the microscope images counterpart for (b) and (f) respectively.....- 116 -

Figure 4.16 Targeted imaging of mouse kidney section with spatiotemporal control of illumination. Images (a-d) are captured with targeted illumination of two cells for a period of 0.04 s, 0.1 s, 0.2 s and 0.3 s respectively. Images (e-h) are captured by using wide-field illumination for a period of 0.04 s, 0.1 s, 0.2 s and 0.3 s respectively.....- 117 -

Figure 4.17 Algorithm for calibration and particle tracking [181].- 121 -

Figure 4.18 Steps of calibration and selected particles. (a) Transillumination image of particles. (b) Selected rectangular region of interest (ROI). (c) Highlighted particles from selected ROI and (d-f) Illumination of the tracked particles.- 123 -

Figure 5.1 Illustration of image formation (point source) around an obstacle for the regular lens at different focus depth. (a) Ray diagram and detector view for biconvex lens placed at 120 mm from an obstacle (rectangular absorbing

medium, 2). (b) Ray diagram and detector view for biconvex lens placed at 57.5 mm from the obstacle. (c) Ray diagram and detector view for biconvex lens placed at 3 mm from the obstacle. Note: 1 is point source illumination, 2 is a rectangular obstacle (10 mm X 0.05 mm, absorbing medium), 3 is a biconvex lens (125 mm focal length, 25 mm diameter) and 4 is the detector. The distance between point source and obstacle is fixed at 5 mm for all the measurements. The detector is also placed at the same position during all measurements.....- 128 -

Figure 5.2 Illustration of image formation (point source) around an obstacle for different axicon focus depth. (a) Ray diagram and detector view for axicon lens placed at 120 mm from an obstacle (rectangular absorbing medium, 2). (b) Ray diagram and detector view for axicon lens placed at 57.5 mm from the obstacle. (c) Ray diagram and detector view for axicon lens placed at 3 mm from the obstacle. Note: 1 is point source illumination, 2 is a rectangular obstacle (10 mm X 0.05 mm, absorbing medium), 3 is an axicon lens (25 mm diameter) and 4 is the detector. The distance between point source and obstacle is fixed at 5 for all the measurements. The detector is also placed at the same position during all measurements.- 129 -

Figure 5.3 Schematic diagram of imaging around an obstacle.....- 131 -

Figure 5.4 Illustration of reflection and trans-illumination imaging set up. (a) Optical configuration for white light reflection imaging . (b) Optical configuration for trans-illumination.....- 132 -

Figure 5.5 Imaging behind thick obstacles using white light reflection method. (a) Image of USAF chart elements behind Allen key of thickness 0.7 mm, placed at a distance of 0.5 mm from the USAF chart. (b) Image of USAF chart elements behind surgical needle (eye) of thickness 0.35 mm, placed at a distance of 0.5 mm from the USAF chart. (c) Image of USAF chart elements behind pin head of 3.5 mm thickness placed at a distance of 3 cm from the USAF chart. (d) Image of USAF chart elements behind pin 0.5 mm thickness placed at a distance of 0.5 mm and 3 cm from the USAF chart.- 133 -

Figure 5.6 Imaging behind thick obstacles using trans-illumination method. (a) Image of USAF chart elements behind a stitching needle of thickness 0.6 mm.

(b) Image of the USAF chart elements behind a paper pin head of thickness 3.5 mm. The inset shows pin head region processed to enhance the contrast to see reconstruction with better clarity. (c) Image of USAF chart elements behind a syringe needle of 0.35 mm thickness.	- 136 -
Figure 5.7 Fluorescence imaging of mouse kidney section around the 0.7 mm thick Allen key	- 137 -
Figure 5.8 Experimental setup for real-time IAO. (a) Needle at 5 mm from the object to be imaged. The red arrow shows a location of the invisible needle. (b) Needle at 3 cm from object to be imaged.	- 138 -
Figure 5.9 Imaging arm of IAO with the images of sample movement around the obstacle. (a) Sample placed at 5 cm from axicon and 2 cm from the needle. (b) Sample placed at 5 cm from the axicon and 5 mm from the needle. (c) Sample touching the needle tip.	- 140 -
Figure 5.10 Illustration of image formation behind surgical needle during insertion. (a-c), Shows the movement of syringe needle kept in front of the biological sample. (d-e), Shows the insertion of the needle into the sample. (f-g), Fluid (stained) is injected into the sample. (h-i), Illustrate the retrace path of the needle after injection. The arrow in images shows the tip of the surgical needle.	- 141 -
Figure 5.11 Schematic diagram of imaging setup using a combination of the axicon and the objective lens.	- 143 -
Figure 5.12 Illustration of extended focus depth by ray diagram using Zemax simulations. (a) A normal plano-convex lens with shallow focus depth (focal length 125 mm). (b) Axicon lens (170°). (c) Combined axicon (170°) and a plano-convex lens.	- 144 -
Figure 5.13 Sequence of mouse kidney cells (microscopic) raw image formed behind hair (a-c). Each image has an inset attached to it showing specifically the imaged region behind the hair obstacle with improved contrast.	- 145 -
Figure 6.1 Schematic for proposed optical fiber probe-based IAO system.	- 155 -

List of Tables

Table 2.1 FOIB based optical illumination and imaging methods.- 37 -

Table 3.1 Calculation of contrast parameter for images shown in Figure 3.7- 59 -

Table 3.2 Smoothness(S), Detail preservation (R) and Performance measure for
depixelation images (*PD*).....- 61 -

Table 3.3 Reconstruction time for depixelation of images of different sizes- 62 -

Table 3.4 Contrast variation against pinhole diameter.- 88 -

Table 4.1 Comparison with state of the art fiber optic probes- 124 -

List of Symbols

mm	: millimeter
μm	: micrometer
Hz	: hertz
ms	: millisecond
fps	: Frames per second
α	: Apex angle
$o(x, y)$	: Pixel at location (x, y) in output image
$i(x, y)$	: Pixel at location (x, y) in input image
$h_{avg}(s, t)$	: Averaging filter coefficient at location (s,t) in the kernel
R	: Radius of the fiberlet core
$g(s, t)$	: Kernel for Gaussian filter
σ	: Standard deviation of the Gaussian filter mask
$1/2d_0$	: Nyquist limit on information retrieval in spectral domain
$I(u, v)$	: Image components at point (u, v) in frequency domain image
$M(k, l)$	: Mask components at point (k, l)
$F(k, l)$	: Filtered frequency domain image at point (k, l)

$i(x, y)$	: Spatial domain pixel value from inverse FFT image at point (x, y)
P	: Point p in spatial domain
$D(p)$	: Delaunay triangulation
$\lambda_{q_i}(p)$	: Natural neighbor coordinates of point p for Voronoi cells q_i
$Y(t, w)$	: Reflectivity of the sample
I_{max}	: Maximum brightness level
I_{min}	: Minimum brightness level
C	: Image contrast
I_D	: Pixel brightness from dark region
I_B	: Pixel brightness from bright region
A_R	: Area with alternating bright and dark pattern
S_{ls}	: Line separation value
R	: Rayleigh's parameter
γ	: Balancing factor
P_D	: Depixelation performance parameter
h	: Point Spread Function of the lens
M	: Magnification factor of the lens
U	: Spatial amplitude profile of the single mode fiber

f	: Fluorescent strength distribution function in the object plane
I	: Intensity distribution function
P_i	: Pupil function
h_e	: Effective ASF of lens
h_{eff}	: Effective PSF of lens
S	: Signal strength at a point p in fiberlet core
r	: Euclidian distance from the center of the fiberlet to point p
A	: Amplitude of the beam
s	: Discrete signal strength at a point p in fiberlet core
A_D	: Discrete amplitude of the beam
R_D	: Discrete value (number of pixels) fiberlet core radius
P_B	: Input beam power
h	: Distance between adjacent fiberlet rows
D	: Fiberlet to fiberlet distance
(x_o, y_o)	: Center coordinates for odd row fiberlets
(x_e, y_e)	: Center coordinates for even row fiberlets
f_{loc}	: 2D function that define the fiberlet placement in spatial domain

d_0	: Diffraction limit
I_{HiLo}	: HiLo image
η	: Scaling factor
I_{lp}	: Low resolution component of an image
I_{hp}	: High resolution component of an image
r	: Distance from the center of the fiberlet
A	: Amplitude of the beam
w_0	: Radius of the fiberlet
g	: Discrete Gaussian function
σ	: Variance
q_i	: Voronoi cell
$\lambda_{q_i}(p)$	: Natural neighbor co-ordinate for point p
Pr	: Probability
S_k	: Cumulative distribution function
n	: Number of pixels
C	: Contrast
Q	: performance measure for depixelation

Abbreviations

STED	: Stimulated Emission Depletion
STORM	: Stochastic Optical Reconstruction Microscopy
LCSM	: Laser Confocal Scanning Microscope
PAM	: Patterned Array Microscope
SIM	: Structured Illumination Microscopy
IAO	: Imaging Around Obstacle
SLM	: Spatial Light Modulator
DMD	: Digital Micromirror Device
FOIB	: Fiber Optic Imaging Bundle
TIR	: Total Internal Reflection
FCRM	: Fiber Confocal Reflectance Microscope
GRIN	: Gradient Index
LC-SLM	: Liquid Crystal Spatial Light Modulator
LCOS	: Liquid Crystal On Silicon
EMCCD	: Electron Magnified Charge Coupled Device
FOV	: Field Of View
LED	: Light Emitting Diode

CGH	: Computer Generated Holography
MO	: Microscope Objective
sCMOS	: Scientific Complementary Metal Oxide Semiconductor
CCD	: Charge Coupled Device
2D	: Two-dimensional
PSF	: Point Spread Function
SNR	: Signal to Noise Ratio
ROI	: Region of Interest
NNI	: Natural Neighbor Interpolation
CDF	: Cumulative Distribution Function
FPID	: Fiber Pixelated Image Database

Chapter 1: Introduction

This chapter begins with the background and motivation for undertaking this challenging research thesis. This will be followed by a brief review of the targeted imaging approaches adopted in clinical practices and some of the reported art followed by the significance of imaging around opaque obstacles. The chapter then discusses the objectives of this doctoral thesis followed by its scope and the research roadmap. The chapter concludes with the organization of the thesis.

Advancements in science and technology have contributed to the detailed understanding of the biological systems at the microscopic level. In particular, optical imaging methods have opened the way for detecting the morphological changes, specific events occurring in tissues, organs or environment [1-3]. Several microscopic imaging techniques have been developed for high resolution imaging such as Stimulated Emission Depletion (STED) microscopy, Stochastic Optical Reconstruction microscopy (STORM) and Confocal Laser Scanning Microscope (CLSM) [1, 3-5]. Although these techniques have achieved high spatial and axial resolutions of the order of sub-micrometers, they are not appropriate for targeted illumination studies. The Patterned Array Microscope (PAM) and Structured Illumination Microscopy (SIM) based commercial microscopic systems can be adapted for targeted illumination, however, they are limited to the *in vitro* applications.

Generally, endoscopes are widely used for *in vivo* imaging applications. Endoscopes are available in a variety of different sizes, shapes, and flexibility (maximum bending angle). Based on the working principle, they can be divided

broadly into three types: rod lens endoscope, tip chip endoscope, and fiberscope. Rod lens endoscope and tip chip endoscope are limited in their reach and size respectively [6, 7]. The imaging resolution achieved by these endoscopes are in the range of 0.1 mm. There are also different types of FOIB based *in vivo* imaging systems. These FOIB systems are capable of imaging with a higher resolution [8-11]. Further, these FOIB based optical methods have demonstrated their use for *in vivo* structured illumination imaging.

Recent developments in science and technology have observed significant growth in the targeted illumination and imaging methods for applications involving photo-activation. It is important to note that most of these techniques are suitable for *in vitro* applications. Also, in the controlled parametric studies which involve FOIB based targeted illumination of a single cell or cell organelle *in vivo*, only very few optical schemes have been reported [12, 13]. In fact, there is a greater need of the FOIB based targeted illumination and imaging system for niche bio-imaging applications.

Moreover, despite the advancements in optical concepts and optics-based imaging schemes, imaging around opaque obstacle has been hitherto impossible. Such an imaging method have potential applications in medical therapeutics. For instance, most frequently used medical procedure is injection, with an estimated 20 billion injections administered each year worldwide [14]. There are needle stick injuries (cuts) reported widely due to blind injection or improper position of the needle, caused due to the blocking of the field of view of the sample by the needle during insertion. There are also reports where the surgical tools obstruct the field of view, especially when dealing with extremely small structures [15]. Therefore there is a

critical need for designing imaging systems which can image around opaque obstacles for such applications.

Based on the identified research gaps, this thesis focuses mainly on the design and development of FOIB based optical imaging systems for targeted illumination. Additionally, it also investigates novel concepts for imaging around obstacles.

1.1 Background and motivation

1.1.1 FOIB for high resolution targeted illumination and imaging

In the last decade, there has been an exponential growth in the use of the *in vitro* targeted optical illumination for applications, such as optogenetics, selective filtering, microscopy, photodynamic therapy and particle sorting [16-22]. It is also a well-known fact that targeted illumination systems are of a greater demand in the field of neuronal studies [18, 20, 22-24]. A comprehensive study of the neuronal signaling demands the smallest pulse duration of the order of 10-20 ms (for dendrite activation) and the spatial resolution of 5- 10 μm (sub-cellular regions) [16, 23]. Based on the reported literature, the maximum achieved axial resolution with FOIB imaging system is 8.6 μm .

Furthermore, FOIB imaging systems have the inherent noise known as comb structure noise or pixelation noise. The pixelation noise removal assists in perceiving the information captured through FOIB by improving the image contrast [25]. In earlier studies on pixelation removal, researchers have often used their own set of images to perform depixelation which subdued their usage and adaptability

universally [26]. A database of pixelated images is the current requirement to meet the growing diagnostic needs in the healthcare arena.

The state of the art targeted illumination systems are the adaptations of the conventional bench top light microscopes [20, 23, 27]. These are limited to *in vitro* studies. It is important to note that the targeted light illumination based imaging at a micro-scale resolution for the *in situ* applications in the complex experimental environments remains unexplored due to the lack of high-performance probes [5, 9, 28, 29]. Further, the pattern switching speed of these FOIB probes are in the range of 2-20 Hz (low), which limits their use in applications such as optogenetics.

1.1.2 Optical probe for imaging around obstacle (IAO)

“The idea of invisibility has captured the imagination of humanity since ancient times” [30]. Cloaking techniques are used today to see behind obstacles by employing specialized materials and/or special optical configurations [30-32]. Though efficient, these methods are inappropriate for specific applications such as surgery and machine inspections. There is a definite need for an optical method to image the samples placed behind opaque obstacles.

1.2 Research objectives

The observed lack of suitable techniques to tackle the imaging problems related to targeted illumination and imaging around opaque obstacles have helped in defining the research objectives for this thesis work. They are -

- Research and Investigation into the design and development of a high resolution targeted illumination and imaging probe for *in vivo* diagnostic bio-imaging applications. The probe should be capable of targeted illumination with a small beam spot diameter (less than 5 μm) as well as high temporal switching speed (10 ms).
- Research and development of imaging biosamples around obstacles for diagnostic imaging. The optical system should be capable real time (25 fps) imaging at macroscopic (0.1 mm) and microscopic (1 μm) resolution.

1.3 Scope of the research

As discussed in the previous sections, the state of the art literature survey indicated that there is still a lack of targeted illumination probes with high spatial and temporal resolutions for specific bio-imaging applications. To address this void, a novel, fiber optic probe, which can illuminate the targeted region in the sample at micro-scale resolution, has been proposed and developed.

The scope of the thesis is summarized under three research challenges as detailed below:-

- Lateral and axial resolution enhancement -
 - Theoretical formulations for the imaging through the FOIB and MATLAB simulations to generate simulated images.
 - Investigations into suitable algorithms for the depixelation of images obtained with the FOIB. Development of a Fiber Pixelated Image Database (FPID).
 - Research and development of FOIB confocal system using a Digital Micro-mirror Device (DMD) based scheme for coupling the light into a single fiberlet for high resolution imaging.

- Investigations into the effect of variable pinhole diameter on the image resolution and contrast for FOIB imaging.
- FOIB based targeted illumination and imaging
 - The design and implementation of the FOIB based targeted illumination and imaging.
 - Design, analysis and custom fabrication of a multispectral targeted illumination probe for optical switching.
 - Investigations into specialty optical probe for particle tracking and targeted illumination.
- Imaging around obstruction
 - The Zemax simulation and design of a real-time IAO.
 - Development of IAO system for the macro and micro scale resolution imaging and its demonstration with obstacles of different shapes and sizes.
 - Development of real-time IAO with a demonstration of syringe needle insertion.

A detailed flowchart for the planned research is presented in Figure 1.1.

1.4 Organization of thesis

The motivation, objectives, and scope of the research work are discussed in Chapter 1. It also gives a detailed research roadmap with identified scope.

Chapter 2 provides an overview of the literature survey in the related research fields. It describes the standard FOIB based wide field, confocal scanning, and

structured illumination micro-endoscopy. The possibility of using these systems in *in vivo* and *in vitro* imaging has been evaluated with their advantages and disadvantages. However, it was evident from the literature survey that the FOIB based high resolution targeted illumination and imaging methods have remained relatively unexplored to their full potential till date.

The prominent noise in the images obtained with the FOIB probe is a honeycomb structured noise, also known as pixelation. The reason behind the occurrence of pixelation is elaborated and the reported depixelation algorithms are analyzed.

Chapter 2 also provides detailed literature review for optical methods for imaging around obstacles. Several cloaking techniques which make the object disappear are discussed in detail. The cloaking methods are observed to be not suitable for IAO applications. Further, in this chapter, special properties of the axicon lens and their applications are discussed.

The theoretical derivations for modeling of FOIB imaging, optical configurations for the high resolution imaging and the investigations into the depixelation techniques are discussed in Chapter 3. A mathematical model is developed for the imaging through FOIB. Based on this model, a MATLAB program is developed, which generates the fiber pixelated images from the reference images. The experimental setup for the confocal microscope using DMD is described. Different depixelation techniques are compared considering various performance measures. The two techniques, namely ‘Physically motivated interpolation’ and ‘Frequency domain depixelation’, stand out based on the quality of depixelated image and the time required for depixelation, respectively. Furthermore, a Fiber Pixelated Image

Database (FPID) is proposed and developed. The database serves as the input test sample space for research works associated to novel depixelation algorithms.

The design and experimental results for the FOIB based targeted illumination probe are described in Chapter 4. It describes the proposed optical configuration for the high resolution targeted illumination probe in detail. Specifically, a targeted imaging capability of the probe is demonstrated with the help of different samples. The illumination and imaging the developed FOIB probe is comparable to or better than the established *in vitro* illumination systems. The chapter also proposes novel imaging schemes, which are possible due to special features of the probe. The targeted FOIB probe is modified with particle tracking algorithms to perform targeted tracking and illumination. Moreover, developed FOIB probe is adapted to the standard bench-top microscope to be used as a portable targeted illumination source.

Chapter 5 describes investigations into design and development of IAO by using axicon lens. Zemax simulations compared the axicon lens and plano-convex lens in the perspective of IAO system design. Optical configuration for the proposed IAO technique using axicon lens is detailed. Further, real-time macroscopic and microscopic IAO is demonstrated with the obstacles of different shape, size and reflectivity. An axicon-objective unit is proposed to perform IAO at microscopic imaging resolutions. The proof of concept of this proposed IAO method is illustrated with imaging the sample placed behind a surgical needle during needle injection procedure.

Chapter 6 concludes this thesis with guidelines for further studies. Significant and original contributions made in this thesis are summarized followed by recommendations for future research directions.

Figure 1.1 Hierarchical research roadmap.

Chapter 2: Literature review

This literature review chapter is divided into two main sections based on the previous work done in the related area, which is relevant to this thesis. Section A focuses on the literature review related to the design aspects of high resolution FOIB based targeted illumination systems. This is further divided into three subsections. After a brief description of the structure of the FOIB, wide-field, and confocal imaging techniques are discussed in the first subsection. The second subsection gives a detailed overview of structured illumination techniques. The third subsection elaborates on the methods used for in vitro and in vivo targeted illumination systems. Section B briefly reviews the state of the art works reported with respect to imaging around obstacles and optical engineering challenges. An outcome of the literature review conducted which led to the identifying the research gaps and the directions for further research work are given at the end of the chapter.

SECTION A

2.1 FOIB based targeted illumination systems

FOIB probes are extensively used for illumination, sensing and imaging applications in science and technology [9, 33-43]. The small diameter, user-defined length and high mechanical flexibility of FOIB facilitates its positioning at remote or otherwise difficult-to-access sites [37, 44]. These features constitute the basis for the development of probes that fit into the instrument channel of standard medical endoscopes as demonstrated by Komachi *et al* [45]. The ability of FOIB probes to reach inside the complex structures and their insensitivity to electromagnetic fields

enable their use in various applications [46]. This chapter reviews the state of the art literature related to the proposed research.

2.1.1 Fiber optic imaging techniques

Figure 2.1 Structure of the fiberlet showing total internal reflection.

It is necessary to understand the structure of FOIB in order to comprehend the advantages of using fiber bundle in this study. Additionally, it lays down the groundwork for a discussion of optical configurations proposed in this thesis. FOIB consists of a large number (3000 to 100000) of single mode fibers, known as fiberlets. Each fiberlet has a core (usually circular in dimension) and a cladding surrounding it. Figure 2.1 depicts the structure of the single fiberlet. The refractive index of the core is slightly higher than that of the cladding. This difference in the refractive index causes light to reflect inside the core at the core-cladding interface as shown in Figure 2.1. This phenomenon is known as the Total Internal Reflection (TIR). Due to the TIR, the core of the fiberlet acts as a light guide and cladding behaves as an insulator blocking light.

These large number of fiberlets are densely packed to form a fiber optic imaging bundle. The fiberlet cores are arranged so as to keep minimum core to core distance. Instead of using separate cladding for each fiberlet core a single cohesive cladding is used for all the FOIB fiberlets, this reduces the overall FOIB diameter. Another important characteristic of the FOIB is that the fiberlets in FOIB are arranged coherently. The coherent arrangement in FOIB means that placement of a particular fiberlet with respect to the other fiberlets remains unchanged over the length of the fiber, this allows the FOIB to be used for imaging applications. Figure 2.2 shows the end face image of a fiber bundle.

Figure 2.2 End face Image of fiber optic imaging bundle. (a) Cross-Sectional image of the end face. (b) An enlarged version of inset area.

2.1.1.1 Wide-field imaging using coherent fiber optic bundle

Wide-field imaging methods using FOIB can be generally divided into two main categories based on the mode of illumination [9, 42, 47-49]. In the first type, FOIB is only used for imaging and the illumination is done separately by an external light

source. An optical schematic for FOIB imaging using external illumination source is shown in Figure 2.3.

In 1985, Pogrel *et al* have described one of the first basic FOIB based imaging configuration with external light source [42]. There are recent FOIB based studies which have used the wide-field imaging method. In 2011, Krishnan *et al* have described the use of external light source for FOIB imaging in trans-illumination mode [48, 50]. Phase gradient microscopy illustrated by Ford *et al* has utilized two external light sources with FOIB [51]. In 2014, Liu *et al* have demonstrated the use of two FOIB collection channels with an extra illumination source for Raman scattering and second harmonic generation imaging [49]. The external light source in these imaging methods increases their size, reducing their reach.

Figure 2.3 Optical schematic showing FOIB imaging with an external light source for sample illumination [51].

The second method of wide-field FOIB imaging techniques uses the same fiber probe for both the illumination as well as imaging. Basic optical schematic of this FOIB imaging schemes is shown in Figure 2.4. There are many studies which have reported the use of wide-field FOIB method [52-57].

Figure 2.4 Wide-field FOIB imaging with inherent illumination [54]. O- objective, DM-dichroic mirror, EX-F -excitation filter, EM-F emission filter and CCD-charge coupled device camera.

Though the wide-field imaging method for FOIB has been reported to be used in many different optical configurations, the images acquired with this method show poor imaging resolution. This has forced scientific community to focus on improving resolution.

2.1.1.2 Fiber bundle confocal microscope

To improve image resolution using FOIB, Gmitro *et al* have demonstrated the use of fiber bundle in confocal configuration [34, 58]. Figure 2.5 shows the schematic diagram of a confocal microscope using FOIB. In this system, a single fiberlet in fiber bundle acts as a pinhole. To generate an image, each fiberlet is illuminated individually over the entire FOV by implementing a beam scanning technique.

Later in the year 1994, Gmitro *et al* further modified the optical system by integrating their design with a commercially available Zeiss LM310 confocal microscope [58]. In this configuration, one end of the fiber bundle is fixed onto the stage of LM310 and the other end placed in proximity of the sample. Experimental setup for confocal scanning FOIB system using Zeiss LM 310 is shown in Figure 2.6. However, the lens unit used at the tip of the fiber bundle limits the FOIB systems use in *in vivo* applications [58].

Figure 2.5 Confocal microscope with coherent fiber optic bundle [58].

Figure 2.6 Experimental setup for a fiber bundle confocal system with Zeiss LM 310 Confocal scanning microscope [59].

Figure 2.7 Fiber confocal reflectance microscope [60].

In 2002, Sung, *et al* introduced a new approach named Fiber Confocal Reflectance Microscope (FCRM) [61, 62]. Figure 2.7 shows the optical configuration of FCRM. In this approach, two-dimensional scanning is achieved with a resonant scanner and galvanometric scan mirror or with a pair of galvanometric scan mirrors. Schematic diagram for a pair of galvanometric scan mirrors is shown in Figure 2.8, commonly

known as galvo mirrors. Movement of scan mirrors is controlled by a computer program. When compared with earlier FOIB studies, the design of FCRM with microscope objective at the fiber tip is also not suitable for *in vivo* application [62].

Figure 2.8 Representative Galvanometric mirror assembly [9].

The introduction of Gradient Index (GRIN) lens and miniature lens have replaced the use conventional microscopic objective in FOIB imaging schemes. This allowed the use of FOIB based imaging systems for *in vivo* applications. In 2001, Knittel *et al* used GRIN lens at the tip of the imaging fiber bundle. This design has demonstrated the use of FOIB-GRIN lens probe for *in vivo* imaging [59]. FCRM systems were also modified by replacing microscopic objectives with miniature lenses to perform *in vivo* experiments [61, 63, 64].

In 2000, Lane *et al* demonstrated FOIB optical setup with a DMD. Figure 2.9 shows the optical setup for the DMD integrated FOIB systems [65]. By using DMD, Lane *et al* have improved imaging speed and resolution (7 fps, axial resolution 16 μm) of the system as compared to the earlier studies which used galvo scanners.

Figure 2.9 DMD based FOIB confocal system [66].

2.1.2 Structured illumination and imaging methods

The previous section has described the conventional FOIB imaging techniques including different wide-field and confocal methods. In this section, basics of patterned array microscope, structured illumination microscope and HiLo microscope are discussed.

2.1.2.1 Patterned array microscope

Figure 2.10 A simplified optical schematic for PAM imaging [67].

Patterned array microscope commonly known as PAM is the first application of the use of structured illumination [67, 68]. The PAM uses the spatial illumination pattern modulated by Spatial Light Modulator (SLM). The PAM principle is based on collecting the conjugate information in two images and fusing them to generate a better-resolved image. One of the two images collects the information from the focal plane while the other image collects the conjugate information from defocused planes. Figure 2.10 shows a simplified schematic of PAM microscope. These two images are combined by simple image arithmetic to get the better-resolved image.

2.1.2.2 Structured illumination microscopy (SIM)

Figure 2.11 (a) Simplified diagram of 3D structured illumination apparatus. (b) Finite axial illumination pattern [1].

A vast number of examples of SIM technique are reported in the literature [1, 4, 5, 29, 69-72]. SIM technique enhances spatial resolution by collecting information from high-frequency space outside the observable region [73, 74]. Experimental implementation for SIM is achieved by multiple light patterns which when overlapped on one another forms Moiré fringes. (Moiré fringes are generated when two identical patterns are superimposed with slight displacement of rotation from one another.) SIM employs one of the following procedures to generate the grating: casting a mask onto the sample, placing a mask on the image of the sample,

coherent wave interference and the use of SLM like DMD, Liquid Crystal Spatial Light Modulator (LCSLM) or Liquid Crystal on Silicon (LCOS) [4, 73, 75-80].

The most commonly used illumination pattern for SIM is the rectangular grating pattern. Figure 2.11 represents a typical optical setup of SIM using a fixed grating. The sample is illuminated with three or more phase shifted patterns and the images are acquired. The images acquired with these illumination patterns are processed to extract the high-frequency components which provide better-resolved images. The optimal grating thickness and the optimal number of phase shifted patterns are evaluated for an individual optical setup. There are also commercially available SIM systems such as Elyra S.1 from Zeiss and FV1200 from Olympus. Typical resolution of SIM system is 130 nm lateral, 350 nm axial (Elyra S.1 from Zeiss).

Though SIM achieves high resolution it has to be noted that it requires minimum three images for the better-resolved image. Furthermore, to image finer details from the samples, illumination patterns with different orientations (0, 120, and 240) have to be used. This results in longer image processing time, which is the primary drawback of any SIM system.

2.1.2.3 HiLo microscopy

HiLo microscopy is another high resolution imaging method which uses the concept of conjugate image components[81]. In the case of HiLo microscopy, two different illumination patterns, uniform wide field illumination, and speckle are used to get the high resolution images. It can be implemented with the optical setup similar to that of the SIM microscopes. Lim *et al* have demonstrated that HiLo imaging can

achieve axial resolutions comparable to confocal [81]. Moreover, fast processing algorithm of HiLo imaging help to achieve high-speed acquisition at the rate of 7 fps.

2.1.2.4 SIM and HiLo based microendoscopy

Figure 2.12 Optical configuration for SIM and HiLo micro-endoscopy [29].

Jerome Mertz *et al* have extended SIM and HiLo techniques to micro-endoscope [29, 82, 83]. The FOIB with micro-objective is used for developing these micro-endoscopes, the optical schematic is shown in Figure 2.12.

All the above mentioned standard microscopes or endoscopes modified as PAM, SIM and HiLo systems are developed with a primary focus on improving the imaging resolution. However, it is important to note that illuminating only specific regions on the sample instead of the illuminating entire field of view (FOV) has many applications in science and technology [18, 20, 21, 84].

2.1.3 Targeted illumination

2.1.3.1 In vitro studies

Over the years, a large number of microscopic and FOIB systems such as PAM, SIM, and HiLo are reported with illumination arm modified using different spatial light modulators such as DMD, LC-SLM, and LCOS for performing structured illumination [16, 23, 27, 85-95]. However, as discussed in the earlier section 2.1.2, the targeted illumination for specific regions on the sample is not performed by these techniques.

Figure 2.13 Targeted illumination of *C. elegans* with LCSLM based targeted multispectral microscope [20]. (a) Image of *C. elegans* obtained with wide-field illumination. (b-c) Processed image to select the target regions from FOV. (d) Multispectral targeted illumination of three selected regions.

To achieve the targeted illumination, selected region of the sample should be precisely illuminated. Standard gratings as in SIM microscopes cannot be utilized

for targeted illumination of samples which dynamically changes its size. In contrast using SLM the targeted illumination is possible for moving samples (dynamically changing) [20, 23].

Figure 2.14 Targeted illumination method to control neuronal cells [86].

Figure 2.13 shows an example of targeted illumination and imaging with a microscope modified using SLM [20]. In 2007 Wang *et al*, presented a modified microscope system to achieve targeted illumination [86]. In this study, the author has modified a standard microscope by adapting DMD device in the illumination arm to achieve targeted illumination. Figure 2.14 shows the targeted illumination system with DMD [16, 86]. In recent years, there are many more *in vitro* studies reported using DMD for targeted illumination [16, 23, 85, 87]. The spatial

resolution of DMD-based approaches can reach the theoretical limit given by the optics (usually a microscope) [23].

Figure 2.15 Three LCSLM for multispectral selective illumination [20].

Figure 2.16 LED array with selective illumination. Inset showing a 3X3 array of LEDs with a central row of LEDs in ON state [27].

Liquid crystal spatial light modulator (LCSLM) is an alternative option for DMD's. LCSLM is transmissive SLM which can be added in the illumination path to achieve targeted illumination. In 2012, Stirman *et al* have demonstrated the multispectral targeted illumination microscope by using three LCSLM [20]. Figure 2.15 shows the optical configuration used by Stirman *et al*. Using LCSLM, selected regions of *C. elegans* are illuminated in the study (Figure 2.13). Many more studies have also used LCSLM to achieve targeted illumination. [96-98]. It is to be noted that LCSLM based system could achieve an approximate illumination resolution of 10 μm with a frame rate of 60 Hz [20].

Recently Grossman *et al* have demonstrated selective illumination microscopic method without any external beam shaping devices such as SLM, DMD or LCOS. In this study, selective illumination is achieved by an array of high power micro light emitting diodes (LED) [27]. Each LED is of 20 μm diameter and is placed at 50 μm center to center distance. Figure 2.16 shows the LED array with certain LEDs switched ON to display 'Opto Neuro' and the inset shows the enlarged view of 3X3 LED array. The temporal switching capacity for the LED array is reported to be 20 Hz.

2.1.3.2 *In vivo* studies

Though there are many targeted illumination systems using standard microscopes, designing of systems for *in vivo* applications are hitherto challenging. Till date, only two notable studies have demonstrated targeted illumination using FOIB.

In 2012, Hayashi *et al* have described a targeted illumination method with a fluorescence microendoscopy probe using FOIB and electrodes [12]. The probe described by Hayashi *et al* uses three FOIB as shown in Figure 2.17. The Galvo scanner is used to guide the light into FOIB. As shown in Figure 2.17, the selective illumination in this system could only achieve rectangular shaped illumination with pointwise raster scanning. In this study by Hayashi *et al*, the use of Galvo scanner limits the system’s ability to illuminate small regions (spots).

Figure 2.17 (a) A selective illumination probe with FOIB and electrodes. (b) Image showing three FOIB from the probe illuminated with uniform illumination. (c) Two sites (A and B) identified for selective illumination. (d) Direction for raster scanning with Galvo mirrors [12].

In 2014, Szabo *et al* have described a targeted illumination method with FOIB using computer-generated holography (CGH) [13]. Figure 2.18 shows the optical method

for targeted illumination and recording of neuronal signals using an FOIB coupled with the micro objective lens. Each fiberlet from the FOIB is capable of carrying the single pixel information of CGH. The smallest illumination beam spot diameter FOIB targeted illumination method presented by Szabo *et al* is 5 μm .

Figure 2.18 Optical schematic for targeted illumination using CGH. The figure beside DMD shows the pattern generated on DMD. The figure beside sCMOS shows the collected image [13].

SECTION B

2.2 Imaging around obstacles (IAO)

“The idea of invisibility has captured the imagination of humanity since ancient times” [30]. The invisibility science is about making the obstacles disappear to see (or image) the object placed behind. In any imaging system, imaging area is defined by the FOV of the system. However, any obstacle in the imaging path can hinder

the imaging capability of the system. Imaging behind obstacles placed in imaging path is always challenging.

In surgery, multiple accidents have been reported due to operating site being blocked by the operating tools [15, 99, 100]. For instance, WHO estimates 12 billion injections are given in health care settings around the world each year. The needle stick injuries are common due to multiple reasons. One of the most common types of needle injury is caused when the location of injection point is obstructed by the needle, resulting in insertion at wrong location. Other than needle stick injury, there are multiple instances in open surgery tools obstruct the view causing non-intended implications. These non- intended incisions are common in surgeries which lead to further complications [101, 102]. Imaging around the needle/ operating tool during medical procedure will be helpful to avoid such injuries.

In the field of engineering there are also instances where obstacle has blocked the similar problems have occurred due to obstructed view by obstacles in engineering disciplines [100, 103-105]. In instances of electronic soldering or mounting of electronic components on the PCB, small placement errors occur due to the blocking of the field of view by the tool [104, 105]. This is a common problem which can result in an inefficacious PCB. System for imaging around the soldering tools will allow correct placement of the components minimizing error in manufacturing of electronics circuit boards.

Similarly, there can be more instances of potential applications of imaging behind obstacles in different fields such as medical, biological sciences and engineering where different obstacles (tools) block the FOV [15, 103, 106-110].

The imaging behind obstacle is often associated with cloaking techniques [111, 112]. The cloaking of an object can be attained with different principles such as conformal mapping, carpet cloaking and optical cloaking [108, 111-117]. Leonhardt *et al* have developed a method for the design of media that create perfect invisibility [115]. The method is proposed based on the principle of angle preservation to make the object invisible, also known as conformal mapping. The conformal mapping is realized mostly using artificial electric and magnetic materials (metamaterials) [116]. Figure 2.19 shows the cloaked sphere object with the conformal mapping.

Figure 2.19 Cloaked sphere by the technique of conformal mapping [116].

Recently, cloaking theories are proposed which prove the schemes with transformational optics and conformal mapping [117, 118]. Valentine *et al* have given the first demonstration of optical cloaking at a range of wavelength frequencies using a carpet cloak [118]. This method has used an isotropic dielectric material which conceals an object that is placed under a curved reflecting surface by

imitating the reflection of a flat surface. Figure 2.20 shows a carpet cloak design that transforms a mirror with a bump into a virtually flat mirror.

Figure 2.20 Schematic diagram of a fabricated carpet cloak showing the different regions, where C1 is the gradient index cloak and C2 is a uniform index background [118].

The geometrical optical methods are also have been used to achieve invisibility cloaking [111, 119]. Recently, Choi *et al* demonstrated a geometrical cloaking method using paraxial ray optics [111]. The optical schematic of the paraxial ray cloaking method is shown in Figure 2.21. It involves the paraxial arrangement of multiple lenses such that the obstacle kept in between becomes completely invisible. Paraxial ray cloak does not work if the obstacle is placed in the illumination axis.

However, fundamentally all these methods bend and stretch the light. This also involves changing the environment of the object to be cloaked. The important limitation of the cloaking methods is the need for this special environment which is created to make the obstacle invisible. These environments involve placement of one or more optical components in between the obstacle and the object to be

imaged. For paraxial cloaking major limitation is that it needs multiple optical components to be placed behind the obstacle, creating cloaking. This limits paraxial cloaking concepts adaptation into standard microscopic or endoscopic systems.

Figure 2.21 Illustration of paraxial cloaking. (a) Optical setup (b) Cloaking of hand demonstrated [111].

In recent years, axicon lens has been utilized in many imaging configurations due to its interesting properties namely larger depth of focus and its ability to generate non-diffracting (or self-reconstruction) beams. Axicon lens was introduced by Macleod in 1954 [120]. An Axicon is a conical prism defined by its apex angle ($\pi - 2\alpha$), as shown in Figure 2.22. Unlike a converging lens (e.g. a plano-

convex, double-convex, or aspheric lens), which is designed to focus a light source along the optical axis, the design of an Axicon focuses a light source to a line consisting of multiple points along the optical axis.

Figure 2.22 Axicon lens showing Non-diffraction zone.

Self-reconstruction beams: The self-reconstruction or self-healing phenomenon of light beams has shown a great potential in imaging. Self-reconstruction beams have the property to reconstruct by the interfering beams after being obstructed by an obstacle. Bessel beam is one such beam type which has the remarkable characteristics of diffraction-free propagation with the self-reconstruction ability [121], which made it an excellent candidate in a wide range of applications such as optical imaging, atom tweezing, information extraction, metrology [122, 123]. In 1998, Bouchal *et al* using an axicon lens have demonstrated the self-reconstruction

of Bessel beam around small objects under free space propagation [124]. This is shown in Figure 2.23.

Figure 2.23 (a) Optical setup for Bessel beam reconstruction around small rectangular objects. (b-c) Measured intensity reconstruction of Bessel-like beams at two different distances from the rectangular obstacle [124].

Figure 2.24 Propagation of Gaussian (a-d) and Bessel beam (e-h) in fluorescent gel with two large glass spheres of 8 μm diameter arranged consecutively at various distances from the beam axis b [125].

Figure 2.25 Fluorescence volume imaging with Bessel beam. (a) Optical schematic. (b-d) The intensity distribution of simulated fluorescence at the medium surface, axicon surface and at the detector respectively [126].

Fahrbach *et al*, using an axicon lens have also demonstrated the self-reconstruction property in light sheet microscopic configuration [125]. Figure 2.24 shows the comparison of beam spreading of normal Gaussian and self-reconstructing beam when interacting with different scatterers (glass spheres).

A larger depth of focus: In addition to generation of non-diffracting Bessel-like beams, axicon lens is used specifically for increased depth of field [125-129]. A recent article by Zhang *et al* indicates, through simulations, that axicon lens can collect the fluorescence from the extended focal depth for volume imaging [126].

Figure 2.25 shows the optical schematic and the simulated results for fluorescence imaging. In 2014, Ong *et al* have demonstrated a variable focal depth imaging system using multiple axicon lenses [127]. Figure 2.26 shows the optical schematic for this system.

Figure 2.26 Optical schematic for variable depth imaging using multiple axicon lenses [127].

Surprisingly the features of axicon lens, namely focal depth and the collimating property have not been relatively explored to their potential in the context of IAO. There is no IAO system available which is free from special optical components placed in between the obstacle and the object to be imaged.

2.3 Summary

Section A: High resolution FOIB based targeted illumination systems

Table 2.1 FOIB based optical illumination and imaging methods.

	Imaging Modalities				Targeted Illumination			Illumination scanning		Imaging Resolution (μm)		Smallest Illumination beam spot diameter (μm)
Method	Wide-field	SIM	Confocal	Multispectral time averaged	Spatial	Temporal	Multispectral Switching	Raster scanning	Targeted scanning	Spatial	Axial	
Miniaturized fluorescence microscope [130]	✓											Widefield
SIM, HiLo Microendoscopy [82, 131]	✓	✓								2.6	17	30
Pixelation-free and diffraction-limited imaging [40]	✓		✓					✓		1.09	8.99	-
Spatially Selective Holographic Photoactivation And Functional Fluorescence Imaging [13]	✓	✓	✓		✓	✓		✓	✓	3.1	8.6	5

Table 2.1 summarizes all the FOIB based optical illumination and imaging systems by considering parameters namely spot diameter (Spatial resolution), temporal resolution, imaging resolution *etc.* The table also summarizes the imaging modalities demonstrated by different FOIB based optical systems. Spatially Selective Holographic Photoactivation And Functional Fluorescence imaging technique achieved the highest axial resolution, 8.6 μm [13]. It is evident from Table 2.1 that there is only one FOIB based imaging system which is capable of both targeted illumination and scanning. However, this system lacks the ability to perform multispectral illumination. Furthermore, targeted time averaged imaging

and controlled multispectral switching is not reported till date. Considering the significance and potential applications for targeted illumination and the available solutions, there is still a growing need for researching and developing these methods.

Limitations of earlier studies:

- 1 There is a greater demand for systems which can perform targeted illumination *in vivo* that can find potential applications optogenetics, neurophotonics, plasmonics and sensing. Till date, there are only two notable studies which demonstrated their potential for targeted illumination *in vivo* [12, 13].
- 2 Though there are number of FOIB based systems, best reported axial resolution is 8.6 μm (Refer Table 2.1). Therefore there is a need to improve the resolution.
- 3 Simultaneous multispectral targeted illumination and imaging is not demonstrated with the currently available targeted illumination systems. Furthermore, targeted systems having temporal and spectral illumination control have not explored the applications in time averaged targeted multispectral fluorescence imaging.
- 4 The FOIB targeted illumination systems have demonstrated smallest pulse durations of 5 ms with 20 Hz frequency. High speed switching is necessary needed for studies such as optogenetics. There is a need to improve the scanning speed of FOIB based imaging systems.

- 5 High resolution tracking and targeted illumination of dynamically moving particles is not yet demonstrated.

Section B: Imaging around obstacles (IAO)

Despite the advancements in optical technology and optics based imaging schemes, imaging around obstacle is still a challenge. There are needle stick injuries (cuts) reported due to blind injection due to the blocking of field of view by the needle during insertion. Further, there are also reports where the surgical tools obstructing the field of view, especially when dealing with extremely small structures [132]. Therefore there is a critical need for designing imaging systems which can image behind opaque obstacles for many such applications.

Presently cloaking methods are used for imaging around obstacles. The optical cloaking methods either involves changing the environment or use of optical components behind the obstacle [30]. Due to this reason, these optical cloaking methods cannot be easily adapted into microscopes and endoscopes. Axicon lens has special optical properties namely, extended depth of focus and ability to generate self-reconstruction beam. The axicon lenses have been used to illuminate the samples placed around the small obstacles.

To summarize, researchers have been studying various methods to develop a fiber probe for high resolution imaging. Though a number of targeted illumination methods have been reported for *in vitro* applications, there are hardly any reported studies on employing FOIB for targeted illumination and imaging for *in vivo* applications. It is evident from the literature survey that there is still a scope for improvement in the FOIB's imaging resolution. Accordingly, investigations in this

research focuses on design and development of high resolution fiber probe with targeted illumination and imaging feature. A novel targeted time averaged approach will be studied for improved resolution and contrast. In this study possibility of multispectral controlled temporal switching and particle tracking will be explored.

In the FOIB imaging research, the simulation of imaging is not possible due to unavailability of the theoretical model to represent image transfer through FOIB. Furthermore, the pixelation noise in the images captured through FOIB impedes the perceived information. The pixelation noise removal methods cannot be compared objectively as different researchers use different set of pixelated images to demonstrate the depixelation. In this regard, the research work also focuses on the development of a theoretical model for imaging through FOIB. Moreover this model is used to simulate the pixelated images. Further, standard test image database of pixelated images is created.

Presently, there is hardly any methods which demonstrate such capability to see around an obstacle. In this study axicon lens features such as large DOF and generation of non diffracting beams has been explored in the imaging context of viewing around opaque obstacles.

This chapter has provided a survey and analysis of relevant research to the proposed research objectives. The next three chapters, describe the details of research carried out towards development of novel FOIB based imaging systems, investigations for improvement of current FOIB systems and investigations into use of axicon lens for imaging around obstacles.

Chapter 3: High resolution imaging fiber probe

Since inherent pixelation noise affects the resolution of FOIB imaging, investigations into the state of the art methods for pixelation noise removal have been carried out in this chapter. A theoretical model is developed to study the effects of different parameters including pixelation on images guided through FOIB and to simulate imaging through FOIB to generate pixelated images. The “Fiber Pixelated Image Database” is developed which provides test images for researchers working on fiber pixelation noise removal methods. Moreover, the effect of pinhole size variation is investigated for the confocal, quasi-confocal and widefield configurations, in this chapter.

3.1 Investigations into depixelation methods

One of the objectives of this research is to come up with a new high resolution FOIB probe for imaging. FOIB based imaging system shows fiber pixelation effects due to their inherent honeycomb-like structure. This impedes the observer from perceiving the information from an image captured and hinders the direct use of image processing and machine intelligence techniques on the recorded signal. Removal of this pattern is an important image processing task in the development of FOIB probe imaging system [40, 133, 134].

Pixelation noise blocks the important information to be imaged; it hampers the quality of the captured image. At times, it is proved that human perception changes with each individual thus prone to errors. The same image can be perceived differently by two people. Computer vision methods such as object detection

automate the process of classification reducing the possible human errors. Furthermore, pixelation noise restricts the use of these computer vision techniques for object recognition and tracking.

The light inside each fiber core is in Gaussian distributed form; transmitting single intensity value from distal end to proximal end [43]. Each fiber generates pixels with discrete Gaussian distribution. Interfiber gap corresponds to dark pixels. This interlacing of bright and dark pixels creates a pattern known as a pixelation noise.

Several methods have claimed to be able to remove pixelation from the images obtained with a fiberscope. Aslan *et al* evaluated depixelation filters and several other filters used in commercially available fiberscopes but without mentioning underlying algorithm [135]. Pixelation noise makes it difficult to study a morphological and physiochemical characteristic of tissue as image information is lost. Several image processing approaches have been applied to remove pixelation noise from the images obtained with FOIB. These image processing approaches include static and dynamic approaches. Static approach refers to the processing of a single image to obtain depixelated image. In dynamic approach depixelated image is obtained from many images which are temporally or spatially related to each other [136]. The dynamic approach requires images to be captured in particular settings and these images are analyzed to extract parameters which are then used to remove the pixelation.

Static approaches mainly involve two types – spatial averaging filter and spectral filter. Dynamic approaches are interpolation methods where the image captured with special settings is used to identify the set of pixel locations used for

interpolation. In the following section, the spatial averaging filters used for depixelation are described.

3.1.1 Spatial averaging filters

Fiberlet core corresponds to brighter pixel areas in the image while fiber cladding corresponds to darker pixels surrounding brighter pixels. Bright circular areas surrounded by dark pixels form a pixelation pattern. Increasing intensity at dark pixel areas in accordance with nearby bright pixels can remove pixelation. The local averaging kernel can be used for reconstruction. Two dimensional (2D) convolution of an image and the averaging kernel is performed to get a depixelated image. It is implemented as [137],

$$o(x, y) = \sum_{s=-a}^a \sum_{t=-b}^b h_{avg}(s, t) i(x + s, y + t) \quad 3.1$$

Where, $o(x, y)$ is the pixel at location (x, y) in the output image, $i(x, y)$ is the pixel from input image and $h_{avg}(s, t)$ is averaging filter coefficient. For averaging filter mask of size $m \times n$, $a = (m-1)/2$ and $b = (n-1)/2$. Several averaging filters are available to choose from. Typical averaging filter used in image processing is rectangular neighborhood averaging filter. It is most widely used and easiest to implement. Eq. 3.2 shows the rectangular averaging filter kernel which has all the matrix values to be uniform.

$$h_{avg}(s, t) = \frac{1}{m \times n}, \quad s \in [-a, a] \text{ and } t \in [-b, b] \quad 3.2$$

The rectangular filter does not match the circularity of fiber cross section. Disc filter is proposed for accommodating this circularity [138]. Disc filter is similar to the

rectangular averaging filter but its kernel has non-zero values only in the circular region of radius R (radius of fiberlet core) beyond which kernel has zero-valued elements. The size of the disc filter is controlled by the radius r which is selected according pixel coverage of fiber in digital image. Disc filter provides slight improvement in depixelation compared to rectangular averaging filter but processed images are blurred with reduced contrast. Oh *et al* employed uniform circular averaging disc filter which reduces mean intensity by smoothening the pixelated image leading it to be darker [138]. Kernel function for disc filter is given as,

$$h_{avg}(s, t) = \frac{1}{R^2}, \quad \text{for } s^2 + t^2 \leq R^2$$

$$= 0, \quad \text{otherwise} \quad 3.3$$

Uniform averaging disc filter reduces visibility and increases blurring, by reducing contrast. The intensity profile of the light coming out of the fiber core is not uniform but Gaussian. Göbel *et al* improved the disc filter with non-uniform Gaussian averaging filter [139]. The contrast of the images processed with Gaussian filter is observed to be reduced than that of an original image. Han *et al* showed that the output of Gaussian filtering can be improved by pre-processing the images with histogram equalization [134]. Kernel for Gaussian filter can be written as,

$$g(s, t) = e^{-\frac{s^2+t^2}{2\sigma^2}} \quad s \in [-a, a] \text{ and } t \in [-b, b] \quad 3.4$$

Where, σ is the standard deviation of the Gaussian filter mask. Gaussian filter reconciles circularity of disc filter with weighted averaging. Gaussian filter is circularly symmetric around the center pixel and forms a bell shape with a peak at the center and diminishing with the Euclidean distance from the center.

Figure 3.1 shows kernels for different spatial averaging filters. Spatial averaging filter represents low pass filter, using this high-frequency information of the image is lost. Spectral filter overcomes this with the ability to suppress only those frequency components corresponding to pixelation pattern.

Figure 3.1 Spatial averaging filters, (a) Rectangular averaging (b) Disc averaging and (c) Gaussian smoothing.

3.1.2 Spectral filter

The important parameters detrimental for image guidance through FOIBs are the size of the fiber core, inter-fiber distance (i.e. the distance between two adjacent fiber centers), the total number of fiberlets, and the numerical aperture of individual fiber elements, fiberlets. As expected distance between two adjacent fibers is same the fibers in the bundle form a regular structure. FOIB, when used for imaging, creates a spatially repetitive pixelation pattern in captured images. Pixelation pattern corresponds to high-frequency components in frequency domain while all natural objects (which are to be imaged) correspond to low frequency components [140]. Also, it should be noted that a fiberlet can transmit only one intensity value [43], in other words, fiberlets in fiber bundle work as a sensor sampling at each

fiber location. According to Nyquist criteria in sampling theory signal information can be completely reconstructed if sampled with the sensor having sampling frequency at least twice of that of the maximum frequency component of the signal. Frequency transform of the image of fiber bundle shows a diffused ring corresponding to the pixelation pattern. Ideal frequency domain equivalent of the image should show a perfect ring of components with radius $1/d_0$ inverse of the radius of the fiber core at origin of frequency space. Thus frequency components corresponding to the circle of radius $1/2d_0$ can only be retrieved. But as the regularity in shape of fiber and distance between fibers is not perfect diffused rings of frequency components are observed. In order to generate clear image and eliminate pixelation pattern two approaches are in use one based on Nyquist criteria discarding all the frequency components beyond the half of the sampling frequency and second based on human intuition removing the frequency components only corresponding to the pixelation pattern. The second approach partially suppresses other high frequency components.

Dickens *et al* introduced spectral filtering techniques for depixelation [133, 141, 142]. Suter *et al* showed the use of spectral low pass filter in bronchoscopic imaging [143]. Lee implemented notch reject filter [144]. This filter removed pixelation pattern from the endoscopic images. Winter *et al* automated the process of spectral filtering of images with pixelation [145]. Winter *et al* also proposed a spectral filtering method for removal of pixelation from color images [146].

Figure 3.2 Frequency domain image of Figure 3.1a showing a circular pattern of high-frequency components.

Spectral filtering is a filtering scheme on images in reciprocal space or frequency domain. Two-dimensional Fourier transform converts image from the spatial domain to frequency domain [137]. Figure 3.2 shows the spectral representation of an image obtained with FOIB. Two-dimensional Fourier transform is given as,

$$I(u, v) = \sum_{x=0}^{M-1} \sum_{y=0}^{N-1} i(x, y) e^{-j2\pi(\frac{ux}{M} + \frac{vy}{N})} \quad 3.5$$

Where, $i(x, y)$ is an intensity at pixel location (x, y) , u and v are frequency domain variables. Fourier transform has to be calculated for $u = 0, 1, 2, \dots, M - 1$ and $v = 0, 1, 2, \dots, N - 1$. Suppressing specific frequency component is achieved with a mask. Mask is two-dimensional matrix of the same size as that of two-dimensional Fourier transform. Values in the mask are decided by the method adopted for filtering; which decide the level of suppression of particular frequency component. Multiplication of the mask with frequency domain image is element by element multiplication rather than conventional matrix multiplication. Figure 3.3 depicts

four mask designs for suppressing different frequency components. If mask is M and frequency domain image is I then frequency filtered image is given as,

$$F(k, l) = I(k, l)XM(k, l) \quad 3.6$$

Filtered frequency domain image is converted back to spatial domain by inverse Fourier transform. Inverse Fourier transform is given as,

$$i(x, y) = \sum_{u=0}^{M-1} \sum_{v=0}^{N-1} F(u, v) e^{j2\pi(\frac{ux}{M} + \frac{vy}{N})} \quad 3.7$$

Frequency spectrum corresponding to pixelation pattern has several high-frequency components. Instead of removing the high-frequency pattern, all high-frequency components can be eliminated to get pixelation free images [143]. Winter *et al* observed that pattern of frequency components corresponding to pixelation pattern; they are usually of two types [145]; ring of frequency components or six high-intensity frequency components depicting the corners of the hexagon. Thus a number of possible filters masks gets down to two, star-shaped mask and circular mask. Automation of filtering procedure becomes possible with a limited number of masks. Automated spectral filtering is assumed to require one prior image to the design of filter mask thus it can be considered as a partially dynamic depixelation approach. But it can be understood from the careful investigation that a prior image may not be necessary if detail information of system design and specifications are known, providing cues for filter mask design.

Spectral filtering notably improves the image quality compared to spatial smoothening filters. Automation of spectral filtering may not be always possible; filter may be required to be manually designed for the first time. Inappropriately designed spectral filter shows undesirable effects such as ringing in filtered image.

Object to be imaged might have some high-frequency content which might be suppressed by spectral filtering. Interpolation method explained below combats some of the above mentioned problems.

Figure 3.3 Filter masks (a) Suppressing high-frequency components by different suppression levels (b) Suppressing all high-frequency components (c) and (d) Two types of filter masks proposed by Winter *et al* [145].

3.1.3 Interpolation method

Elter *et al* devised a method for reconstruction of flexible endoscopic images, considering the physical behavior of the light inside fiber bundle [147]. Each fiberlet carries only one intensity value and amplitude profile of the end face follows 2D Gaussian representation with its center coinciding with the center of the

fiberlet core. It is obvious that the center of each fiberlet represents the value transferred by that fiberlet. The pixel locations for all the fiberlet centers' provide the information carried by the FOIB. Interpolation of intensity values carried by each fiberlet would provide depixelated images [146, 147].

Reconstruction requires two images: calibration image and a sample image. Calibration image is an image of illuminated FOIB. This image can be obtained either by capturing uniformly illuminated white surface with FOIB or illuminating FOIB at the proximal end with mirror orthogonal to the optic axis of FOIB at the distal end as shown in Figure 3.4. The sample image is obtained without altering anything but replacing a mirror with the sample.

Figure 3.4 Optical setup to obtain a calibration image.

Depixelation is a two-step process, the first step is to localize fiberlet centers from the calibration image. Fiberlet core corresponds to bright areas, i.e. center of fiberlet cores must reside in brighter areas of an image. Brightness variation in local

neighborhood provides primary candidates for the fiberlet centers. Subsequently, two-dimensional Gaussian template matching provides single center pixel per fiberlet. Template matching function is given as,

$$m = \sum_{x=-a}^a \sum_{y=-b}^b [I(x, y) - T(x, y)]^2 \quad 3.8$$

Maximum matching value with each local neighborhood is found out. It is made sure that two center locations are located apart from each other by some threshold distance. Figure 3.5 shows the intermediate steps in localizing the center pixels for fiberlets.

Figure 3.5 (a) Primary candidates for center pixels (b) Score of Gaussian template matching (c) Delaunay triangulation of fiberlet center location and (d) Voronoi diagram for fiberlet pixel center.

FOIB's one end is fixed with respect to the camera, and thus fiberlet core to camera pixel mapping is required to be done only once. It implies that fiberlet center locations don't change if the arrangement of FOIB relevant to the camera is not changed. Amplitude corresponding to each fiberlet changes with a change in the object as well as with the relative variation in the distal end position. All the fiberlet center points P are inserted into Delaunay triangulation $D(P)$. Delaunay triangulation has the max-min property which is known to be suitable for interpolation. Amongst important interpolation techniques, Natural Neighbor Interpolation (NNI) is the most suitable for depixelation [148]. NNI uses Voronoi diagrams to calculate natural neighbor coordinates for every point in an image except fiber center pixels. Insertion of a point in Voronoi diagram chops off areas from its neighboring Voronoi cells. Figure 3.6 shows a representative Voronoi diagram. If a point p is inserted into the Voronoi diagram it chops off small areas from its neighboring cells to create one of its own. As shown in Figure 3.6, a point p inserted into Voronoi diagram steals some area from cells q_3, q_5, q_6 and q_7 to create its own cell represented by grey color. Natural neighbor coordinates of point p with neighboring Voronoi cells of q_i are given as,

$$\lambda_{q_i}(p) = \frac{A(q_i)}{\sum_i A(q_i)} \quad 3.9$$

Natural neighbor coordinates represent the area of cell q_i taken by the inserted point p . These coordinates are required to be calculated for every point in image to be reconstructed.

Similar to fiberlet centers from the digital image, natural neighbor coordinates also remain constant if the proximal end of the FOIB is fixed relative to the camera. It

implies that all above mentioned procedures need to be done only once and look up table can be prepared for using NN coordinates multiple times. Natural neighbor coordinates and intensities of the fiber center pixels are used to generate depixelated image. Image interpolated with NNI is,

$$I(p) = \sum_i \lambda_{q_i}(p) \times I(q_i) \quad 3.10$$

Figure 3.6 Natural neighbor interpolation in Voronoi diagram, point p takes the gray area from neighboring points.

Output image is continuous function of intensities $I(q_i)$ except at the fiberlet center pixels. Localizing the fiberlet center pixels, finding natural neighbors for every pixel and calculating natural neighbor coordinates is a time consuming task. Ideally it needs to be done only once, but in practice there are changes in position of proximal end of FOIB relative to camera; stressing the need for recalibration. In practice, calibration needs to be done after every few frames resulting in delayed signal display, which also hampers the frame rate of the flexible endoscopic system.

Interpolation method uses calibration image or a priori information to retrieve physical information about the FOIB. It exploits the physical properties of the FOIB to remove pixelation artifact, and thus is more specific to the FOIB used in the optical setup. The approach can be considered as a more customized approach for FOIB used compared to other filtering approaches.

3.1.4 Analysis of the depixelation methods

Figure 3.7 (a) USAF 1957 test chart image captured through imaging fiber bundle. It shows line 1, and rectangular boxes showing bright and dark regions. Depixelated images reconstructed by (b) Gaussian smoothing (c) Spectral filter and (d) Spatial interpolation.

The response of the algorithm at the edge is an important parameter for comparing image processing methods; sharp edges or lines with contrast background are the most suitable test patterns. USAF test chart has sharp edges and edge separating black-white boundary, which make it a desirable test object. Original sample image and its depixelated images are shown in Figure 3.7 for USAF 1951 test chart and in Figure 3.8 for human neuroblastoma cell.

Figure 3.8 (a) Neuroblastoma cells captured through imaging fiber bundle and depixelated images reconstructed by (b) Gaussian smoothing (c) Spectral filter and (d) Spatial interpolation.

USAF test chart has alternating bright and dark bars of uniform thickness. Ideally, intensities at a pixel line orthogonal to this bar pattern should generate square wave signal. The arrangement of fiberlet in FOIB and inherent properties of fiberlet does not capture the underlying signal as a square wave but as an alternating pattern with

varying amplitude. In Figure 3.7a, bright line l is a set of pixels orthogonal to bar pattern of group 6 element 3 from USAF test chart.

Figure 3.9 Variation of intensities in original and filtered images.

Figure 3.9 shows a variation of pixel intensities in the original image and in depixelated images with three depixelation methods. Pixel intensities for original image form a rapidly alternating signal with an average period equal to the average of fiberlet-to-fiberlet distance. Dark pixels represent the cladding region, large variation in intensities is observed in pixels corresponding to bright bar pattern while small variation is observed in pixels corresponding to dark bar pattern. Gaussian averaging eradicates the rapid alterations from the signal, revealing smoothened underlying pattern.

Rapid alterations are considerably reduced in the spectrally filtered image but the local variation in signal is more than that of the Gaussian filtered image. Pixels

from interpolated image show almost no local variation in intensities. Brightness spreading is observed in Gaussian smoothing and interpolation method; thus observed size of the bright line is more compared to dark line. It is observed that at some pixel locations in the reconstructed images, intensity values are greater than that of the original image. This is because line l may not go through center pixel of fiberlet (max intensity for fiberlet) but reconstruction algorithm considers surrounding pixels of fiberlets' center pixels for depixelation.

The histogram represents distribution of number of pixels against pixel intensities. Depixelation method alters the gray level of pixels corresponding to the cladding. As there are changes in pixel intensities, a histogram of original and depixelated images would give better insights into the effectiveness of depixelation algorithms. Ideally, enhanced image should have a uniform histogram that means all the intensity levels have the same number of pixels. Figure 3.10 shows the histograms of images shown in Figure 3.7. Histogram of Gaussian filtered image is similar to that of the original images showing no significant improvement. Images enhanced with spectral filtering and interpolation method have well spread out histogram which is similar to a uniform distribution. It should be noted that for better perception only part of the histogram is shown; it has large number of pixels (14×10^4) with intensity value zero where displayed histogram's number of pixels ranges up to 3.5×10^4 and maximum possible intensity value is 255 but as none of these images have any pixels with intensity beyond 165, intensity range is shortened to 200.

Figure 3.10 Histograms of original and filtered images.

Dynamic range of an image is the difference between maximum (I_{max}) and minimum (I_{min}) brightness level. Smaller dynamic range suggest image having less contrast but the converse is not true; Image might have very few pixels with extreme intensity values increasing the dynamic range [149]. This asks for parameter other than dynamic range. The contrast ratio can be used as a parameter for comparison of images [48],

$$C = \frac{(I_B - I_D)}{(I_B + I_D)} \quad 3.11$$

where, I_B and I_D are the average intensity values of bright and dark regions of the image respectively. Table 3.1 enlists the contrast values for the images from Figure 3.7.

Table 3.1 Calculation of contrast parameter for images shown in Figure 3.7

<i>Images</i>	I_B	I_D	C
<i>Original image</i>	90.64	22.17	0.6069
<i>Gaussian Smoothing</i>	51.79	9.74	0.6832
<i>Spectral Filtering</i>	101.70	18.58	0.6911
<i>Interpolation method</i>	146.37	55.30	0.4516

Rupp *et al* proposed a quantitative performance measure for objective evaluation which takes into account the ability of depixelation and impact of blurring [150]. Depixelation method should be capable of removing honeycomb pattern without losing the underlying features from the image. Spatial regions from an image can be classified as smoothly varying or quickly varying areas. The output of depixelation over a smoothly varying region of an image shows the efficacy of depixelation. It can be measured with smoothness parameter S [150].

$$s = 1 - \frac{\sigma_s}{\sigma_0} = 1 - \sqrt{\frac{\sum_{i,j \in A_s} (i_s(i,j) - \mu_s)^2}{\sum_{i,j \in A_s} (i(i,j) - \mu_0)^2}}$$

3.12

Here, σ_0 and μ_0 are standard deviation and mean in smoothly varying region of unfiltered image; σ_s and μ_s are standard deviation and mean of smoothly varying region of filtered image.

It is important to check whether details underlying the pixelation noise are reflected in the depixelated image. The detail-preserving ability of the method is defined by Rayleigh's line based separation criteria. Line separation is calculated over an area of USAF 1951 test chart image having alternating bright and dark bar pattern. Let's say A_R is an area of image having alternating bright and dark lines of varying thickness. Here, the width of the smallest bar pattern should not be less than that of the diameter of a fiberlet. Line separation value for area A_R is given as [150],

$$S_{ls}(k) = \frac{1}{M} \sum_{i \in A_{R,M}} i_{line}(k, i) \quad \text{for } k \in A_{R,N} \quad 3.13$$

Where, $A_{R,M}$ and $A_{R,N}$ containing row and column pixels coordinates respectively.

Rayleigh defined normalized line separation parameter as [150],

$$R = \frac{S_{ls,max} - S_{ls,min}}{S_{ls,max}} \quad 3.14$$

As smoothness parameter over smoothly varying region of an image and detail preservation criteria over an area of image having alternating pattern, these two parameters can be combined to form a single performance measure for depixelation method [150],

$$P_D = \gamma S + (1 - \gamma) R \quad 3.15$$

Table 3.2 Smoothness(*S*), Detail preservation (*R*) and Performance measure for depixelation images (*P_D*).

Depixelation method	<i>S</i>	<i>R</i>	<i>P_D</i>	
			$\gamma=0.5$	$\gamma=0.8$
Original image	0	0.9577	0.4788	0.1915
Gaussian filter	0.2713	0.9268	0.6138	0.4043
Spectral filter	0.5144	0.9563	0.7216	0.5985
Interpolation	0.7725	0.8971	0.8348	0.7984

Selected value of balancing factor γ for depixelation technique signifies the importance of depixelation ability over the detail preservation. Higher value of P_D means the method provides better combination of S and R for γ . Table 3.2 shows calculations of S , R and P for USAF chart images with two values of balancing factor (0.5 and 0.8). According to Eq. 3.15, smoothness is calculated by subtracting ratio of variance of filtered image to that of original image from unity and that leads to the S value of original image to zero. Opposed to smoothness, detail preservation in original image bears maximum value. Smoothing is improved from Gaussian averaging towards interpolation method, as represented by parameter S . Detail preservation parameter is affected due to variations in the intensities i.e. by pixelation endured in the reconstructed images. Performance value for both the γ values affirms superiority of interpolation method. Value of P_D for spectral filtering is not far behind if both smoothness and detail preservation are given equal importance.

Probe imaging system requires images to be displayed at the rate of 25 fps to provide the user with experience of non-jerky continuous observation of the sample; Speed of reconstruction is crucial when probe system is used for real-time imaging.

We evaluated the reconstruction speed for the three depixelation methods mentioned in the paper. MATLAB with image processing toolbox is used on computing machine with the i7-3930K processor at 3.2 GHz and having 16 GB RAM. The time required reconstructing images of 3 different sizes (100x100 pixels, 300x300 pixels and 500x500 pixels) using previously discussed depixelation methods are clocked and listed in Table 3.3. It is clear that interpolation method requires long time as compared to other methods, time complexity increases with increase in the size of the image. The time required for spectral filtering is least of the three methods under consideration.

Table 3.3 Reconstruction time for depixelation of images of different sizes

<i>Depixelation method</i>	<i>100x100 pixels</i>	<i>300x300 pixels</i>	<i>500x500 pixels</i>
<i>Gaussian Smoothing</i>	<i>0.0320s</i>	<i>0.039s</i>	<i>0.0520s</i>
<i>Spectral Filtering</i>	<i>0.0055s</i>	<i>0.011s</i>	<i>0.0154s</i>
<i>Interpolation Method</i>	<i>0.0940s</i>	<i>0.288s</i>	<i>0.7190s</i>

This section investigated the static depixelation methods and other image processing algorithms for FOIB imaging [25]. Gaussian averaging, spectral filtering and interpolation method for depixelation are reviewed. The advantages and limitations of each methodology are compared by taking standard resolution charts and neuroblastoma cells as test samples in the proposed fiber probe imaging configurations. It is observed that different depixelation methods use different test images which mean the output of the two methods cannot be compared based on the reported results. Since researchers have often used their own set of images without

making source data available which subdued their usage and adaptability universally. A database of pixelated images is the current requirement to meet the growing diagnostic needs in the healthcare arena. Such a database can consist of simulated images and experimental data. To generate a simulated pixelated data, a mathematical model is required which defines the image transfer through imaging fiber bundle.

3.2 Theoretical model for FOIB imaging

To develop a theoretical model for defining imaging through FOIB, it is necessary to understand how the image is formed through individual fiberlet. Let us consider initially the case of confocal imaging using a single fiberlet as shown in Figure 3.11. In this system, both the illumination and collection is performed through the same single mode fiberlet. A monochromatic light source is used for illumination. A confocal lens unit is used to focus light onto and collect from the object. The object in this configuration is characterized by the spatial fluorescent strength distribution.

Figure 3.11 Schematic diagram for single fiber confocal imaging. F- filter, BS- beam splitter, MO- microscope objective.

According to the imaging theory [151], intensity (I) distribution at point l_0 is,

$$I(l_0, l_s) = \left| \int h\left(l_1 + \frac{l_0}{M}\right) U(l_1) dl_1 \right|^2 f(l_s - l_0) \quad 3.16$$

Where,

h and M are Point Spread Function (PSF) of the lens and the magnification factor of the lens, respectively.

U is the spatial amplitude profile of the single mode fiber. $f(l_s - l_0)$ is the fluorescent strength distribution function in the object plane.

Let us begin by assuming that illumination and reflection beam has the same wavelength. The light reflected from a point (O) in the object plane can be expressed as a delta function. The amplitude distribution at point (O_1) after the collector lens is

$$U'(l_o; l_1) = h\left(l_o + \frac{l_1}{M}\right) \quad 3.17$$

Further, the Amplitude Spread Function (ASF) of the lens [152].

$$h_e(l_o) = \int h\left(l_o + \frac{l_1}{M}\right) U^*(l_1) dl_1 \quad 3.18$$

$h_e(l_o)$ is the effective ASF of lens. U^* is a complex conjugate of spatial amplitude profile of the single mode fiber.

Thus the effective PSF for a lens at point O is given by,

$$h_{eff}(l_o) = \left| \int h\left(l_o + \frac{l_1}{M}\right) U^*(l_1) dl_1 \right|^2 \quad 3.19$$

Combining Eq. 3.19 with Eq. 3.16 shows the intensity collected by the detector as a function of scanning coordinates.

$$I(l_s) = \int \left| \int h \left(l_o + \frac{l_1}{M} \right) U^* (l_1) dl_1 \right|^2 \times \int h \left(l_1 + \frac{l_o}{M} \right) U (l_1) dl_1 \int f(l_s - l_o) dl_o \quad 3.20$$

The Eq. 3.20 represents image intensity collected by the detector through the single mode fiberlet. To calculate the image intensity collected through FOIB (having many fiberlets), an ASF for single mode fiberlet (U) has to be replaced with the ASF of FOIB (U_F) in Eq. 3.20.

In order to calculate ASF for FOIB, ASF of single mode fiberlet and the 2D spatial arrangement of fiberlets in FOIB have to be considered. ASF of single mode fiberlet has a two-dimensional (2D) Gaussian-shaped intensity profile, independent of the properties of the input beam [43]. It implies that if the fiberlet core is transmitting the monochromatic light, at a particular instant of time, a cross section at any point in the fiberlet core depicts a single information value with amplitude A and 2D Gaussian spread. Thus the signal strength function (S) at any point in the cross section of single fiberlet (Figure 3.12) is given as,

$$S(r) = Ae^{-\frac{2r^2}{R^2}} \quad 3.21$$

Where,

r is the distance of the point p from the center of the fiberlet, R is the radius of the fiberlet, and A is the amplitude of the beam.

Figure 3.12 Cross-section of single fiberlet. (a) Continuous signal representation (b) Discrete signal representation.

When this light is captured by a digital sensor such as CCD, oversampling of signal occurs as single intensity value is being imaged onto an array of sensor elements (pixels). The discrete signal at any point in this 2D array is given by,

$$s(m, n) = A_d e^{-\frac{m^2 + n^2}{R_d^2}} \quad 3.22$$

m and n represent the coordinates of point p in the digital image. The center of the fiberlet is considered as the origin of the coordinate system. A_d and R_d represent the discrete values for amplitude and radius, respectively.

The amplitude of the collected signal depends on the input beam power. To understand this relationship between amplitude (A_d) and input beam power, experiments were performed on three FOIB with different fiberlet core diameter. The FOIBs used for this experiment were FIGH 15-600 (Fujikura, 15000 fiberlets with core diameter 3 μm), FIGH 15-600 with GRIN lens (Fujikura, 15000 fiberlets with core diameter 2.8 μm) and IGN 11/50 (Sumitomo electric, 50000 fiberlets with

core diameter 2.7 μm) with GRIN lens. The input power was measured with the power meter (Nova display and 12 AV1, Ophir). The images were collected using a Charge Coupled Device (CCD) camera which identified the output amplitude (brightness value) corresponding to the input beam power. The experiment was repeated with the five beam power levels and with the three different FOIB. Amplitude values collected through the fiberlets were averaged to get output amplitude value for FOIB. Figure 3.14 shows the relationship of the output amplitude collected against the input illumination beam power. It was observed that irrespective of the different types of FOIB, ratio of output amplitude with respect to the input illumination power remains the same.

Figure 3.13 Graph showing output amplitude vs input illumination power of FOIBs.

Based on the graph shown in Figure 3.14,

discrete amplitude (A_d) is given by,

$$A_d = CXP_B \quad 3.23$$

Where,

C is the proportionality constant and P_B is input beam Power

To calculate the 2D spatial arrangement of fiberlets in FOIB, it is important to analyze the structural details of FOIB. Figure 3.15 shows the typical arrangement of fiberlets in FOIB. It is evident from the figure that these fiberlets are placed equidistantly to form a hexagonal comb structure. The smallest arrangement unit is an equilateral triangle (D is the length of one side which also represents the center to center distance between fiberlets.); the fiberlets are arranged with its center matching with the vertices of the triangle.

Figure 3.14 Representation of fiberlet assembly inside FOIB.

In Figure 3.15, R is the fiberlet radius and h is the distance between adjacent rows.

For odd rows, fiberlets center coordinates are

(x_o, y_o) , where $x_o \in X_odd \cap y_o \in Y_odd$.

X_odd and Y_odd are the set of x and y coordinates.

$$X_{odd} = [R+h, R+h+D, R+h+2D, R+h+3D...] \text{ and}$$

$$Y_{odd} = [R, R+2h, R+4h...].$$

For even rows, fiberlets center coordinates are

$$(x_e, y_e), \text{ where } x_e \in X_{even} \cap y_e \in Y_{even}.$$

X_{even} and Y_{even} are the set of x and y coordinates corresponding to the centers of fiberlets placed in even rows.

$$X_{even} = [R, R+D, R+2D, ...] \text{ and}$$

$$Y_{even} = [R+h, R+h+D, R+h+2D, R+h+3D...].$$

After the fiberlets center locations are identified, 2D ASF for a single fiberlet (Eq. 3.9) is applied at each fiberlets location to obtain ASF for FOIB,

$$U_F = \int f_{loc}(r_p - r_o) S(r) dr_p \quad 3.24$$

f_{loc} is the 2D function which defines the fiberlet placement in spatial domain. S represents the 2D ASF for a single mode fiberlet.

Using Eq. 3.20 and 3.24, the image intensity collected by the detector through FOIB is given by

$$I(l_s) = \int \left| \int \int h\left(l_o + \frac{l_1}{M}\right) f_{loc}(r_p - r_o) S(r) dr_p dl_1 \right|^2 \left| \int \int h\left(l_1 + \frac{l_o}{M}\right) f_{loc}^*(r_p - r_o) S(r) dr_p dl_1 \right|^2 X f(l_s - l_o) dl_o \quad 3.25$$

This section has described a theoretical model for imaging through FOIB. The model helps to calculate output image $I(l_s)$ for an object with fluorescence strength

distribution $f(l_s - l_0)$ when the object is captured through the FOIB. The model described here has been used to simulate pixelated database, which is described in the below section.

3.3 Fiber Pixelated Image Database (FPID)

There is a lack of open source Fiber Pixelated Image Database (FPID) resource for depixelation studies, to improve image resolution of FOIB imaging. Availability of FPID allows the researchers to perform quantitative evaluation of depixelation algorithms against a reference data. There are two main aspects of fiber pixelated image database - simulated and experimental.

3.3.1 *Simulation of pixelated images*

To synthetically generate a pixelated image, it is necessary to simulate the FOIB first. MATLAB program is written to simulate the FOIB, based on the theoretical model presented in Section 3.2. When this model is applied to the reference image from the input sample space, corresponding fiber pixelated output image is obtained. A set of conditions for natural samples are applied for generating a reference image [26]. One of the most important natural condition to identify from images acquired is edge detection.

There are different types of edges that occur in natural sample space. In order to replicate possible natural phenomenon through simulation, grating pattern images with several frequency components and different orientation angles are designed. For simplicity of the implementation, only 50% duty cycle grating patterns are used. Figure 3.15 (a-d) shows the pixelated grating pattern images and pixelated grating

pattern images with a 5-degree angle of orientation with their corresponding ground truth (reference) images. For each reference samples, their corresponding pixelated images are also simulated.

Figure 3.15 Synthetically generated fiber pixelated images. (a-d) and (i-l) are ground truth images while (e-h) and (m-p) are corresponding fiber pixelated images. (e-f) are the grating vertical patterns and (g-h) are inclined grating patterns. (m-n) are Siemens star pattern. (k) is pixelated color map image. (p) Shows how pixelation affects variations in individual colors such as red, green and blue.

Edge identification is a significant feature of any imaging system. It is equally important to identify two intersecting edges with a minimum angle of intersection between those two. Siemens star of different frequencies is used to replicate most of

the intersection angles and orientations occurring in natural samples. Siemens star with increasing frequencies and their corresponding pixelated images are shown in Figure 3.15 (i-j).

Prior to the evaluation of grating patterns and Siemens star, the response of a system to the minimum and maximum intensity values of camera's dynamic range has to be identified. Two large patterns with constant intensity value are used as input samples. These patterns serve as smoothly varying intensity patterns or constant intensity patterns for pixelated fiber configuration. Further, test images were simulated with different intensity values which covered the desired dynamic range. The input sample space also has two images which represent different colors from RGB color map. Figure 3.15 shows 8 different patterns from the synthetically generated FPID and their ground truth images.

Finally, simulated FPID consists of 28 pairs of reference images (ground truth images) and their corresponding simulated pixelated images.

3.3.2 Image Acquisition for experimental FPID

In addition to simulations, FOIB based experiments were performed to generate pixelated images. There are many possible optical configurations which can be used to capture an image through imaging fiber bundle [48, 81, 131, 153-156]. In this study, two optical configurations for the acquisition of fiber pixelated images are used namely trans-illumination wide-field imaging configuration (Figure 3.16) and epifluorescence imaging configuration (Figure 3.17).

Figure 3.16 Optical schematic for trans-illumination imaging through fiber bundle.

The optical schematic for the trans-illumination imaging is shown in Figure 3.16. An LED light source is placed on one side of the sample. The light source can be chosen based on the sample specification. A white light fiber illuminator can be used in most of the cases, while an LED emitting specific wavelength can be used for fluorescence imaging.

The imaging system is placed on the other side of the sample. FOIB collects an image of the sample through the distal end and projects it at the proximal end. The microscope objective collects the image projected at the proximal end of the FOIB and transfers it to the camera lens. The CCD camera is used to digitally record the image. An optical filter is used to achieve wavelength selective imaging.

Figure 3.17 Optical schematic for reflective fluorescence imaging.

It is not always possible to place an illumination source on the other side of the sample. Moreover, object to be imaged may not always be thin and transparent. Thick opaque objects have to be imaged in the reflective imaging mode. Further, to image the target that is situated in complex cavities, i.e. *in situ* environment, placing light source on the other side of the sample to provide the uniform illumination is not possible. The other solution would be to add an extra light source which may increase the size of the catheter, restricting its reach. FOIB has the ability to illuminate and collect through the same conduit. This allows it to be used as both illumination and reflective imaging conduit. Figure 3.17 shows the optical schematic for reflective fluorescence imaging.

For generating pixelated images using FOIB configurations, two sets of test samples are prepared, the first set consists of a glass mask marked with different patterns and the second set consists of standard test samples such as mouse kidney section, the cross section of the human eye and glass bubbles in fluorescent solution. A glass mask is marked with different patterns such as grating patterns of different period and 50% duty cycle, star-shaped patterns with several frequencies, checkerboard patterns with increasing box sizes and some miscellaneous patterns. These patterns are marked onto the coated glass mask, thus trans-illumination imaging setup described in Figure 3.16 is used to capture images with this mask. Selected images from the mask are shown in Figure 3.18.

Figure 3.18 Experimental fiber pixelated images of artificial patterns prepared on glass mask. (a) Grating pattern, (b) Siemens star, (c) Checkerboard pattern and (d) Smiley emoticon.

Figure 3.19 Experimental fiber pixelated images of natural samples. (a) Mouse kidney section imaged with 488 nm wavelength, (b) Glass bubbles in Rhodamine sample imaged with 532 nm wavelength, (c) Cross section of human eye imaged with 405 nm superluminescent LED. Image (a) and (b) were captured with the optical configuration shown in Figure 3.18 while optical setup shown in Figure 3.17 was used for imaging image (c).

The second set of samples involves biological, fluorescent and non-fluorescent samples. Instead of mimicking scenarios in bio-imaging, real biological samples were used for capturing images through FOIB (Figure 3.19). Fluorescently tagged mouse kidney section from Life Technologies was used for fluorescence imaging in

reflective mode. Fluorescent dye used in this sample was Alexa Fluor 488 wheat germ agglutinin (W-11261). Cross-sectional slice of human eye cut through the iris is imaged. Further, glass microbubbles (10-25 μm) in Rhodamine 6G are used.

With the above mentioned two configurations, a dataset of 54 experimental FPID images is prepared. Both the simulated and experimentally obtained images are provided in appendix A [157].

In addition to depixelation methods using simulated and experimental data, the effect of variable pinhole size is explored for improving image resolution in the following section.

3.4 High resolution FOIB imaging with variable pinhole size

Currently available FOIB imaging systems demonstrate axial resolution of 8.6 μm . There is a need of resolution improvement for FOIB imaging. In this context, the change in the image contrast and resolution is explored by varying the pinhole size (number of illuminated fiberlets) of FOIB. The imaging modalities can be classified into three categories based on the pinhole size, viz wide-field, confocal and quasi-confocal imaging configuration.

3.4.1 Wide-field imaging

For FOIB based optical methods, a wide-field imaging method is the most commonly used image acquisition technique. It is easy to implement and achieves a high speed of image acquisition. The imaging speed in wide-field imaging technique is limited only by the speed of the camera device.

Two methods in which wide-field imaging can be performed with fiber bundle are described below.

3.4.1.1 Transillumination imaging

Figure 3.20 Trans-illumination images of different samples captured with multiple light sources. Images (a) and (b) were captured with the white light source. Images (c) and (d) were captured with red and blue LED light, respectively. Samples used for imaging were (a) Glass bubble, (b) Cross-section of the human eye, (c-d) 1951 USAF test chart.

Fiber optic transillumination configuration is suitable for imaging transparent or thin samples in a wide field imaging perspective. The optical configuration is explained in Section 3.3.2 (Figure 3.16). The technique generally demands the use of very thin partially transparent sample such as cells fixed on glass slides, thin slices of the animal organ etc. Figure 3.20 shows images captured with the trans-illumination configuration.

3.4.1.2 Reflective fluorescence imaging

The optical configuration for reflective fluorescence imaging is described in Section 3.3.2 (Figure 3.17). Figure 3.21 demonstrates the different sample images acquired with the reflective fluorescence FOIB configuration. The reflective imaging configuration uses the same FOIB as illumination and the imaging conduit which in turn reduces the size of the imaging system.

Figure 3.21 Images acquired through fiber optic probe in reflective fluorescence mode. (a) Fluorescent micro bubble 25, (b) mouse kidney section tagged with Alexa Fluor 488 and (c) Neuroblastoma cells tagged with Alexa Fluor 568.

Wide-field reflective and trans-illumination imaging configurations are simpler to design, their image resolution is poor. Compared to wide-field FOIB imaging, it is difficult to implement confocal FOIB system. However, earlier reports suggest that the confocal imaging provides better axial resolution. The following section 3.4.2 details of the proposed confocal FOIB imaging system that was developed to achieve high resolution imaging.

3.4.2 Confocal imaging

The standard table top laser scanning confocal system achieves the best possible image resolution in any of the continuous light source based imaging techniques. The confocal imaging relies on the ‘removal of out of focus light’ to improve the imaging resolution. The pinhole helps in avoiding the out of focus light. In the literature review, it is shown that the image captured with smaller pinhole have better sectioning ability compared to larger pinholes. In the FOIB imaging, smallest possible pinhole diameter is same as that of the size of single fiberlet. As discussed in Chapter 2, the pure fiber confocal imaging involves the collection of the signal through one fiberlet at a time. Figure 3.22 shows the optical schematic used for confocal imaging through FOIB.

Figure 3.22 Optical schematic for confocal imaging through FOIB.

In this configuration, 30mW, 532nm diode pumped solid state (DPSS) laser is used as the illumination light source. The laser beam is further expanded by a beam

expander (BE06R, Thorlabs). Expanded laser beam impinges on and is reflected from DMD (0.7 XGA DDR DMD, Texas Instruments) with selected spatial patterns towards the dichroic mirror. The dichroic mirror then reflects this spatially controlled laser light through a microscope objective to collimate into thin FOIB of 1 mm diameter (Sumitomo Electric, Japan). FOIB has 50,000 individual fiberlets, each fiberlet has an average core diameter of $2.7\text{ }\mu\text{m}$ and the average center to center distance between two neighboring fiberlets is $4.4\text{ }\mu\text{m}$. The appropriate optical filter is selected to block the light reflected from the proximal end of FOIB and to allow fluorescence from sample towards the camera. Fluorescence signal from the sample is collected by Electron Magnified Charge Coupled Device (EMCCD) camera (Andor iXon 887). Additionally, a custom GRIN lens is designed for the FOIB to focus the light and collect emission from the same point of the sample.

In this proposed probe embodiment, effective implementation of single fiber illumination is realized by properly configuring the optics as well as by mounting the DMD at the desired angle with the optic axis. The DMD is able to precisely control the illumination beam so as to illuminate one fiberlet from the proximal enface of FOIB by switching on the selected micromirrors. The DMD is able to illuminate a single fiberlet because of the information gained through the DMD-fiberlet mapping.

It is necessary to perform the mapping to find out when certain DMD mirrors are switched to ON state, which fiberlets are illuminated. The DMD to fiberlet mapping involves three steps. Mapping of the fiberlets from the FOIB onto the camera pixels; recording which camera pixels capture information from each of the fiberlet in the field of view (FOV). Mapping of fiberlets onto the camera pixels is achieved

with the sample image captured through the fiber optic imaging bundle. Secondly, the DMD mirrors are mapped onto the camera pixels. The DMD to camera mapping is achieved by imaging the proximal end of the sample illuminated with spatially selective light. The mapping information of 'DMD mirrors to camera pixels' and 'fiberlets to camera pixels' is used to map 'DMD mirrors onto the fiberlets.'

The sample image through fiber bundle is captured by illuminating the sample with the uniform illumination. The wavelength different than that of the sample fluorophores' excitation wavelength is used for the uniform illumination beam (Figure 3.23a). The reflected sample image is transferred through the GRIN lens to the distal end of the FOIB. Fiber conduit passes the sample image to the proximal end of the FOIB. The sample is imaged at the proximal end of the fiber with the EMCCD camera. The microscope objective is used to magnify the sample image at the proximal end of the fiber and transfer it towards the camera. The captured image represents the X and Y locations of the image pixels that show the core of each fiberlet.

The DMD mirrors to the camera pixels mapping is obtained from the image captured with selective illumination (Figure 3.23b). A spatial rectangular pattern is created onto the DMD mirror pane which modifies and reflects the laser beam onto the FOIB. The image of the spatial pattern reflected from the proximal end of the fiber bundle is captured with the EMCCD camera. The excitation filter is removed to capture the reflected image. The light reflected from the proximal end of the fiber is of the shape of the DMD pattern. A DMD pattern (rectangle) is matched with the image captured by the EMCCD camera, to get the mapping of the 'DMD mirrors to camera pixels'.

The ‘DMD mirrors – fiberlet mapping’ means that for each of the fiberlet from fiber bundle a group of DMD mirrors has to be located which when switched to ON state reflect the illuminated light beam towards the core of that selected fiberlet. The two mappings the ‘fiberlet to camera pixels’ and the ‘DMD mirrors to camera pixels’, can be combine to deduce the ‘fiberlet to DMD mirrors’ mapping’. This process can be summarized into flowchart as shown in Figure 3.24.

Figure 3.23 Images of FOIB for DMD to fiberlet mapping. (a) with uniform illumination and (b) with spatial pattern illumination.

Figure 3.24 Flowchart for DMD to fiberlet mapping.

The DMD to fiberlet map described above is used to identify the DMD pixels which would illuminate individual fiberlet from the Region of Interest (ROI). The illumination light is coupled through one fiberlet at a time and the reflected light is collected through the same. The collected light holds a single information value corresponding to a spatial location of the fiberlet. Collecting the information through all the fiberlet provides pixel intensity values for a number of spatial locations which is equal to the number of fiberlets. The natural neighbor interpolation is used to reconstruct an image from the spatial locations and intensity values.

The fluorescent sample was used to image with the FOIB confocal probe. The sample used was glass bubble mixed with Rhodamine 6G. The PSF for the confocal imaging probe is shown in Figure 3.25. The PSF was measured with the help of 4 μm fluorescent beads having an excitation wavelength of 540 nm and emission wavelength of 560 nm. The details of all the samples used are provided in the Appendix B. It is clear from the PSF that full width half maximum (FWHM) for FOIB confocal system presented here is 5.5 μm . Here, the spatial resolution is 2.7 μm and axial resolution is 5.5 μm . The resolutions achieved with this probe are better than that of the probe systems presented in the reported literature.

The main reasons for the resolution improvement of the proposed probe are spatial selectivity of the illumination system and the size of the individual fiberlet. The developed probe is capable of illuminating individual fiberlet without illuminating adjacent ones which creates perfect fiber confocality which was not achieved

earlier. The other reason for the improved resolution is the diameter of the individual fiberlet. Compared to previously reported works in this area, diameter of the fiberlet used in the developed probe was smaller ($2.7\mu\text{m}$). As a future work direction, the target will be on further reducing the fiberlet diameter and its associated improvement in the resolution.

The FOIB confocal system is compared with the conventional Zeiss confocal laser scanning microscope (LSM 510 META). A glass bubble of $25\mu\text{m}$ was imaged with both the confocal imaging systems and results are shown in Figure 3.26. It is observed that the images of $25\mu\text{m}$ glass bubble using FOIB confocal is compared to that of Zeiss laser scanning the confocal system. However, it should be noted that the Zeiss laser scanning confocal can achieve better axial resolution (less than $1\mu\text{m}$) compared to that of the developed probe.

Figure 3.25 The FWHM for fiber confocal imaging.

Figure 3.26 Comparison of FOIB confocal with conventional confocal imaging.

3.4.3 *Quasi-confocal or variable pinhole imaging*

In previous sections, the wide-field and confocal FOIB imaging techniques are described. Wide-field imaging method illuminates the sample; and the reflected intensities from the FOV of the sample surface are collected through the FOIB. This results in poor image contrast. Wide-field imaging has better image acquisition speed compared to any other technique, which is only limited by that of the camera's frame capture rate. On the contrary, the FOIB confocal imaging speed is directly proportional to the number of fiberlets in the ROI i.e. the time required to capture an image increases with the size of the image.

Wide-field and confocal are two extreme ends of the imaging modalities with the image quality from worst to best and imaging speed from high to low. Using the pinhole larger than that of single fiberlet can help improve the speed of confocal imaging. Moreover, the imaging quality (contrast and resolution) would be better

than that of the wide-field imaging. This imaging method which uses more than one fiberlet as a pinhole can be classified as the quasi-confocal.

The selection of optimal pinhole size is the key problem in the quasi-confocal imaging. Image quality and speed of imaging are the two parameters to be considered for selection of optimal pinhole size. This is possible after studying the images captured with a variable pinhole.

Figure 3.27 a, c and e show proximal end image of FOIB illuminated through pinhole diameter size of one fiberlet, two fiberlets, and four fiberlet respectively. Images b, d and f show the entire reconstructed glass bubble images corresponding to different pinhole sizes of one fiberlet, two fiberlets and four fiberlets respectively. Images g and h show the wide-field illumination and image reconstruction of the glass bubble.

Figure 3.27 Illustration of glass bubble images with different pinhole sizes.

The effect of variation of pinhole size on the image contrast area was investigated. Figure 3.27 shows images of a glass bubble collected with imaging modalities having four different pinhole sizes ($2.7\mu\text{m}$, $7.1\mu\text{m}$, $16\mu\text{m}$ and ROI size). These varying pinhole sizes classify imaging resolution that can be achievable by fully confocal, quasi-confocal and wide-field imaging. It is evident that improved contrast is observed with smaller pinhole size. With the increase in pinhole diameter the intensity difference of the pixels showing the bright region and dark region

reduces. Intensity difference of bright and dark region reduces with increase in pinhole diameter and remains constant for $d > 20 \mu\text{m}$, where d is effective pinhole diameter. It also signifies that in the optical setup described, the imaging system is not confocal or quasi-confocal but it is wide-field imaging system for $d > 20 \mu\text{m}$.

A quantitative evaluation of contrast value for imaging modalities with four different pinhole sizes was performed. To calculate contrast, two different regions of the image were selected. The first region was chosen inside the region of glass bubble (non-fluorescing) I_{glass} and the second region was outside the bubble (fluorescing area) I_{fluoro} . Contrast value is calculated as,

$$C = \frac{I_{\text{glass}} - I_{\text{fluoro}}}{I_{\text{glass}} + I_{\text{fluoro}}} \tag{3.26}$$

Calculated contrast values and respective pinhole diameter are listed in Table 3.4. The results show that the image contrast decreases with increase in the pinhole size, validating the contrast dependency on pinhole diameter.

Table 3.4 Contrast variation against pinhole diameter.

Pinhole diameter	Contrast
2.7 μm ,	0.312
7.1 μm	0.284
16 μm	0.259
Wide-field	0.055

Figure 3.28 Axial resolution variation against effective pinhole size.

Furthermore, the axial sectioning capability of the quasi-confocal imaging fiber probe is investigated by using 4 μm fluorescent beads. The effective pinholes of size 1 fiberlet to 8 fiberlets are used for this study. The Figure 3.28 shows the axial resolution with respect to the effective pinhole size. It is observed that after the pinhole size of 4 fiberlets the axial resolution reduces exponentially. Pinhole size of 7 fiberlets and 8 fiberlets could not achieve any axial sectioning.

Different FOIB based optical imaging techniques are described in this section. Furthermore, the quantitative parametric comparison of these methods is presented which will help in the selection of a suitable method for different imaging application.

3.5 Summary

Pixelation noise which is inherent in the images captured with FOIB based systems hampers the contrast and image resolution. The analysis of the state of the art depixelation methods is performed with multiple quantitative parameters such as intensity variation, image histogram and time required for depixelation. The need for a standard test image database for depixelation research was identified. In this context, an open source “Fiber Pixelated Image Database” is proposed and developed. The database consisted of two subsets simulated images and experimentally acquired images. The developed database is made available to the researchers on the university web page. To generate a simulated pixelated image, a theoretical formulation defining the image transfer through FOIB is needed. In this chapter, a theoretical model for imaging through FOIB is developed by incorporating the FOIB parameters viz placement of fiberlets in FOIB (controlled by number of fiberlets, fiberlet to fiberlet distance, fiberlet core diameter and packing fraction) core-cladding properties, and light guiding properties of the single mode fiberlet. This model is implemented in MATLAB to generate pixelated images stored as the simulated images in the database.

The FOIB confocal probe system presented in this chapter has demonstrated an axial resolution of 5.5 μm which is better than the reported earlier methods [13]. It can be mainly attributed to improved illumination scheme used. Further, the effect of a change in the effective pinhole size on the imaging contrast and resolution is investigated and a detailed analysis is given as well.

The next chapter discusses the design and development of novel methods for targeted illumination and imaging.

Chapter 4: Targeted illumination and imaging fiber probe

This chapter focuses on design and development of FOIB based targeted illumination and imaging probe. To demonstrate the targeted illumination and imaging capability of the developed FOIB probe, multiple scanning, switching and imaging modalities are described and demonstrated. The targeted illumination is adapted into conventional bench-top microscope to illustrate the ability of the probe to be used as portable targeted illumination source. Additionally, FOIB targeted illumination probe functionality is enhanced by using the object tracking algorithms. The chapter ends with the brief summary of the work presented.

4.1 Need for spatiotemporally targeted illumination probe

The optical systems with a targeted spatial illumination and the ability to change the spatial pattern at a high speed have applications in the different frontiers of science and technology such as optogenetics, selective filtering, microscopy and particle sorting [16-18, 20, 21, 84, 158]. A comprehensive study of neuronal signaling demands the temporal precision of the order of milliseconds and illumination beam spot of diameter less than equal to 5 μm [16, 159].

In recent years, the targeted illumination has served as an important aid in scientific advancement. However, these tools are capable of illuminating only one selected region at a time with the standard bounding box of circular or rectangular shape.

Zhang *et al* showed the *Drosophila* behavior can be controlled and Stirman *et al* demonstrated the *C. Elegans* movements can be guided with the targeted light [20,

160]. The spatial and temporal resolution of the illumination system demonstrated in these experiments was 100 μm and 111 ms respectively [20, 160]. Grossman *et al* used an array of the micro-LEDs placed in a close proximity of the sample for illumination with a spatial resolution of 50 μm [27]. The multiphoton excitation shows the ability of a targeted illumination with a spatial resolution of 1 μm , but this improvement in the resolution comes with a high installation and maintenance costs for the system [161, 162]. DMD-based targeted illumination systems have achieved the spatial resolution of 10 μm and 2 Hz [16, 23]. These systems allow the improved localization of the light and are capable of illuminating multiple irregularly shaped regions in the sample. Most of these techniques use reflection mode imaging for the recording of the photo-activated processes or the effects of photonic stimulation. Furthermore, the use of the non-coherent or monochromatic light source prohibits them from using the samples having multiple photosensitive materials or reagents [18, 19]. The state of the art targeted illumination methods use microscope objective for illumination which limits their use to the samples that are only suitable for benchtop microscope.

Another limitation common to these methods is the large size of the microscope and the bulky microscope objective which restricts their usability in *in situ* studies for the samples placed in the miniaturized complex structures with restricted access such as microfluidic channel [163-166]. Such miniaturized nature devices provide a rapid analysis platform. The characteristics such as small size, minimized use of materials, flow control and rapid analysis time have triggered wide acceptance for miniaturized devices in optofluidics, biomedical engineering and other interdisciplinary fields [165, 166]. The complex and miniature size of these devices

restricts the use of the targeted illumination technology. In this context, there is a growing demand for the specialized targeted illumination and imaging system for such devices. It is important to note that this system should be capable of illuminating the sample placed in different fluidic mediums in complex miniaturized structures. Furthermore, imaging samples from behind the thick transparent materials can be more helpful in many experiments which use petri dish, capillary tube, cuvette and similar setup as the sample holders. In such cases, imaging from both the sides is found to be more beneficial to gather information.

Moreover, other than the spatial selectivity, a temporal switching is also important for the study of living organisms. The continuous illumination of the selected particles reduces the longevity of the experiment. For these reasons, particle tracking and illumination methods with a spatial and temporal selectivity feature are critical for the life science and molecular studies.

As discussed, the targeted illumination, imaging and recording for the different applications remains unexplored due to the lack of the high performance targeted probes [5, 9, 21, 51, 82, 131, 167]. To address this void, a novel, high-speed probe is developed that can illuminate and manipulate targeted region in the sample space at a microscale resolution.

Following section details the optical schematic for the targeted illumination and imaging probe.

4.2 Experimental methodology

4.2.1 Optical configuration

Figure 4.1 Optical schematic for spatiotemporally targeted illumination and imaging fiber probe.

A schematic diagram representing the optical configuration of the developed targeted illumination and imaging fiber probe is shown in Figure 4.1. A multiline laser combiner with the diode lasers of wavelengths 488 nm, 532 nm and 561 nm serves as a digitally controlled multispectral illumination source. The laser light beam (3 mm diameter) is further expanded with a beam expander [BE06R, Thorlabs] to cover the complete pane of the DMD mirrors (14 mm X 10 mm). The DMD [0.7 XGA DDR, Texas Instruments] is a reflective SLM with 1024 X 768 micro-mirrors.

An array of the DMD mirrors acts as a reflective grating creating the higher ordered diffraction beams. The zeroth order beam reflected from the DMD is allowed to

pass through a condenser lens assembly of the plano-convex lenses (focal length 35 mm and 75 mm) and a microscope objective lens (Magnification 20x; NA 0.4 air objective) reducing the beam size to 200 μm X 200 μm .

A FOIB [Sumitomo Electric, IGN-11/50] of 1 mm diameter is used as the probe arm of the developed system. A custom gradient index (GRIN) lens [GT-IFRL-100-cus-50-NC, Grintech GmbH] having a focal length of 0.3 mm is attached to the distal end face of the fiber probe. The FOIB-GRIN lens assembly is secured in a steel sheath (1.1 mm inner diameter and 1.2 mm outer diameter). A beam condenser assembly made up of the two plano-convex lenses is used along with the microscope objective to couple the light beam into the fiber bundle through the proximal end. An EMCCD [iXon 887, Andor] camera is used to capture the images from the proximal end of the fiber bundle. The bright field image collected through the fiber bundle is used as a reference input to select the regions in the sample to be illuminated. Using a commercial software [Pixel precision, 3DIcon], specific DMD mirrors are activated to perform targeted illumination.

4.2.2 DMD and control software for selective illumination

Figure 4.2 Graphical representation of spatial light modulation with DMD. The inset shows the pattern on DMD mirror pane with white and black part representing ON and OFF state mirrors respectively.

DMD is an array of micromirrors with an associated electronics in the embedded chip. This DMD chip is incapable of receiving signals directly from the computer. Therefore, Texas Instruments' DMD Discovery 1100 controller board is used, which provides interface between Texas instruments' 0.7 XGA DDR chip DMD and the computer. The DMD used in this experiment has a full frame switching frequency of 10,000 Hz and a spectral sensitivity range of 400 nm to 700 nm. The DMD has 1024 X 768 micromirrors, each of size 13.7 μm X 13.7 μm .

Figure 4.2 shows a graphical representation of the spatial light modulation with the DMD device. As shown in the Figure, the DMD is illuminated with a monochromatic light. The spatial pattern is prepared on the DMD micromirror array (shown in inset) with the help of the pixel precision software. The spatial pattern reflects selective light into the optical system and discards the unwanted light beam. In the previous chapter, the DMD to fiberlet mapping is elaborated. Such a DMD to fiberlet map is used for the targeted illumination.

Figure 4.3 Procedure to illuminate selected region from the sample with targeted illumination probe.

The image captured with an EMCCD camera is used as a reference image to select the region to be illuminated. This image shows which region of the sample is

illuminated by a specific fiberlet. This information is used to identify the fiberlets through which light has to be coupled for illuminating a target cell or region in the sample. A spatial pattern on DMD is designed based on the 'DMD to fiberlet map' to couple the light through the identified fiberlets. The process is depicted in Figure 4.3.

4.3 Methods for targeted illumination and imaging

This section demonstrates the targeted multispectral spatiotemporal illumination capability of the probe with some of the possible illumination techniques followed by an experimental validation by using the different test samples.

4.3.1 Targeted illumination

For the targeted illumination, it is important to couple the light through the selected fiberlets so as to illuminate the targeted regions of the sample. Figure 4.4a shows the image of the fiber bundle endface which clearly shows the individual fiberlets being illuminated. The capability of coupling the light into the selected individual fiberlet is shown in Chapter 3, the confocal imaging section (Section 3.2.2). Each fiberlet in the fiber bundle acts as a separate light conduit with the negligible cross talks between the adjacent fibers. This preserves the spatial shape of the light beam while it propagates through the fiber bundle. Figure 4.4b illustrates the probe illuminated with the light beam of the shape of smiley emoticon. This also signifies that sample can be illuminated with any non-regular shaped light beam. Further,

Figure 4.4c illustrates the single fibrelet being illuminated, which is the smallest possible illumination spot which can be achieved with this probe (2.7 μm). As discussed in chapter 3, the property of single fiber illumination enables the fiber confocal imaging.

Figure 4.4 Selective illumination of fiber bundle end face. (a) Image fiber bundle with fiberlets clearly visible (with whole field illumination.) (b) Representative spatial pattern illuminating the fiber bundle end face. (c) Illumination of one fiberlet of the fiber bundle having 50000 fiberlets.

Figure 4.5 Comparison between non-targeted and targeted illumination and imaging of cells. (a) Image of mouse kidney section imaged in non-targeted illumination. (b) Illumination and imaging four targeted cells from the same section. Individual cells are clearly aimed through targeted illumination.

Using the probe, the targeted illumination and fluorescence imaging of the cells are achieved. Figure 4.5a shows an image of the mouse kidney section, which is used as a test sample, imaged in the non-targeted illumination. Figure 4.5b shows the potential of the probe in illuminating and imaging the four targeted cells from the same kidney section. It demonstrates the targeted illumination capability of the probe at the cellular level resolution

Figure 4.6 Real-time representation of pattern on DMD mirrors and the corresponding illumination pattern. The image on the left represents DMD mirror state selection, white color represent ON state mirror and the dark yellow color represents the OFF state mirrors. (a) A group of mirrors forming a rectangular shape are switched to ON state with a strip of OFF state mirrors surrounding them. (b) Small square blocks of ON state mirrors.

Figure 4.6 shows the two representative picture frames which demonstrate the pattern on DMD mirrors and the corresponding spatial optical patterns illuminated on the sample using the developed probe.

The screen on the left-hand side of the image is a laptop screen. The pixel precision software window can be observed in the laptop. The screen on the right represents a computer system which is connected to the camera device which shows the corresponding image through a camera. As the DMD pattern is changed in the pixel precision software, the light pattern illuminating the fiber changes accordingly.

Figure 4.7 Targeted illumination of mouse kidney cells. (a) White light image of mouse kidney cells. (b) Image of cells collected with targeted illumination. (c-d) Images of cells collected with targeted illumination of cells and cell boundaries.

The mouse kidney cells selected in Figure 4.3 are illuminated with a sequence of targeted patterns and the real time images are recorded as shown in Figure 4.7. Figure 4.7a shows the white light image of the mouse kidney cells. Figure 4.7(b-d) show the images captured with different illumination patterns which include illumination of targeted cells or cell boundaries. The optical configuration described in Figure 4.1 and process described in Figure 4.3 are used to achieve this targeted illumination.

This section has described the spatial selectivity for illumination and imaging of the sample through fiber probe. The following section describes the spatiotemporal switching of illumination with controlled multispectral light.

4.3.2 Multispectral spatiotemporal switching

The organelles in cells can respond differently to the individual wavelengths. It is reported that the different organelles in the cells may require light pulses for the different wavelengths and different exposure times in certain scenarios [168-170]. In addition, there are the reagents and dyes which get activated by the illumination light beam. Different dyes respond to the different wavelengths of light.

Figure 4.8 demonstrates the ability of the probe to illuminate the targeted cells of the mouse kidney section using two different wavelengths (488 nm and 561 nm) and their subsequent imaging. Cell 1 and cell 2 are tagged with the fluorescent dyes, Alexa Fluor 488 wheat germ agglutinin ($\lambda_{Ex\ Peak} = 490\ nm, \lambda_{Em\ Peak} = 525\ nm$)

and Alexa Fluor 568 phalloidin($\lambda_{Ex\ Peak} = 578\text{ nm}$, $\lambda_{Em\ Peak} = 603\text{ nm}$), respectively.

Figure 4.8 (a) Image of the mouse kidney section having multiple cells tagged with different fluorescent dyes. (b) Alternate illumination of two targeted cells, one with 488 nm and other with 561 nm. (c) The pulsed dual wavelength light source with the different pulse frequencies of 0.5 Hz for cell 1 (488 nm) and 1 Hz for cell 2 (561 nm) is used.

The continuous exposure of the biological sample to the laser light illumination for a long period of time can cause adverse effects such as overheating and cell death. In order to subdue the adverse effects of illuminating organelles for longer exposure times, this probe can perform a temporal control on the illumination. Moreover, certain studies involving the photo-activation need the sample regions to be targeted with a train of light pulses with independently controllable ON and OFF periods. In Figure 4.8, alternate illumination of the two cells with two different wavelengths of light is demonstrated. Furthermore, to demonstrate the potential of the probe to independently control the ON and OFF periods of illumination, a timing diagram of targeted wavelength specific illumination is also provided. The potential of the probe is demonstrated by synchronous multispectral spatiotemporal illumination of multiple regions.

4.3.3 Multidirectional, multi-region scanning and Targeted confocal imaging

As discussed in Section 4.1, the conventional confocal scanning microscopes incorporate the scanning mechanisms to scan the illumination beam spot. The small light beam spot is scanned across a sample and the light reflected from the sample is collected pointwise. The image is stitched together to get a high resolution image. The scanning mechanism for such a microscope is fixed i.e. it does not vary from sample to sample. Furthermore, in most of these methods, a beam spot is scanned to collect information from each point. The limitations of these methods can be summarized as,

- These methods cannot be adjusted to scan only the targeted region such as a cell shape because the scan region is fixed to regular shape e.g. a rectangle and cannot be changed. Furthermore, the scanning direction is also fixed such as raster scanning.
- A small regular shaped (circular or rectangular) region can be selected but selection of the multiple regions is not possible
- The time taken to capture one image is equal to the number of scan points.
- These methods only support single scan point i.e. only one-point information is acquired at a given time.

Figure 4.9 Scanning of multiple targeted regions with single illumination beam spot in each region.

Unlike these methods, the probe proposed in this thesis allows the multiple regions to be scanned either simultaneously or at sequentially in any direction with a greater

precision. Figure 4.9 demonstrates the region being scanned by two light spots. The two light spots that represent the light beam emanating from two fiberlets of the fiber bundle. It demonstrates the probes' ability to scan an irregularly shaped region with multiple scan beams. The object imaged in the Figure 4.9 is a broken glass bubble in a Rhodamine 6G –PVA mixture on a glass slide, illuminated with a 532 nm laser. As observed from the Figure 4.9, the region to be illuminated is divided into two small regions and the two regions are scanned simultaneously.

Figure 4.10 Stepwise increase in illumination beam size. (a-h) In each image number of fiberlets illuminating the sample are increased by 4 (2 from each side), eventually illuminating complete region.

Additionally, two regions shown in Figure 4.10 are illuminated with a stepwise increase in the size of the illuminated region. The illumination starts with illuminating only one spot from each region through the two different fiberlets. In

each step the number of illumination spots, and hence the fiberlets are increased by two for each region, eventually illuminating the complete region.

It is evident from these results that simultaneous scanning of the multiple regions for any arbitrary directions can be performed by the proposed probe. Illuminating the sample region through a single fiberlet and imaging through the same is the principle of the fiber confocal microscopy. As described above, this can be achieved with the developed probe for any targeted regions. This fiber scanning confocal imaging in the targeted region is termed as a targeted fiber confocal imaging. Figure 4.11 shows the comparison of the other imaging techniques with the targeted confocal imaging. (More details are provided in the next section.)

4.3.4 Contrast enhancement by targeted imaging

There are instances when only a few cells or subcellular regions are of interest for imaging. However, the wide-field illumination generally reduces the image contrast. Additionally, wide-field imaging involves exposure to light over the entire FOV, which causes unintentional decay in the fluorescence signal in the non-targeted sample region. A targeted wide-field imaging captures information from the target cells by illuminating only the selected cells. This technique improves the image contrast compared to wide-field imaging as it avoids the light reflected from non-targeted parts of the sample.

To demonstrate the improved contrast by targeted imaging using this probe, a comparison of wide-field imaging and targeted illumination of three mouse kidney

cells is done (Figure 4.11). The targeted region of the kidney sample is illuminated for the controlled time period and the fluorescence images are acquired. (The targeted regions (three cells) are marked in Figure 4.11b.) The targeted illumination and imaging provides a better contrast compared to the wide-field imaging. As compared to the conventional wide-field imaging and the targeted imaging, this proposed probe demonstrates that it can also selectively image using the individual fiberlet with a single fiber confocal resolution which leads to better image quality and contrast. Figure 4.11 shows four images captured with each imaging method having exposure times 0.04 s, 0.1 s, 0.2 s and 0.3 s respectively. Figure 4.12 depicts the intensity variation across a line shown in the images acquired in wide-field (Figure 4.11d), targeted wide-field (Figure 4.11h) and targeted confocal mode (Figure 4.11l). As the pinhole size increases the contrast decreases. Similar effect is observed in Figure 4.12 with intensity at its minimum in the wide-field imaging, at an increased value in the targeted wide-field imaging and at its peak in the targeted confocal imaging. In Figure 4.12, intensity variation in normalized arbitrary unit (a.u.) is plotted against the pixel number.

Figure 4.11 Images of the mouse kidney section in wide-field, targeted wide-field and targeted fiber confocal imaging. (a), (b), (c) and (d) show images captured in a wide-field mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively. (e), (f), (g) and (h) show images captured in targeted wide-field mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively. (i), (j), (k), and (l) show images captured in a targeted fiber confocal mode with exposure times of 0.02 s, 0.1 s, 0.2 s and 0.3 s, respectively.

Figure 4.12 Intensity variation over the line marked in images Figure 4.11 (d), (h), and (l).

4.3.5 Selective wavelength illumination of targeted cell and time averaged imaging

In fluorescence imaging experiments, the sample can have an uneven concentration of fluorescent dyes. The uneven concentration of fluorescence in the sample always poses a hurdle in imaging with optimal quality. The exposure time of illumination system and the collection time of camera are controlled to improve image quality. However, the full FOV has to be illuminated for the longer time duration to capture a good quality image of the sample region with a low fluorescence concentration. This can cause either the pixel saturation in some parts of the image, bleach or even damage to the whole sample structure. On the contrary, for imaging the samples with high fluorescence concentration, the exposure time has to be reduced. It

means, if the sample has multiple cells tagged with the different concentrations of the fluorescent dye then the multiple images have to be captured and stitched together for an optimal image. In another scenario, if the sample has multiple cells tagged with the fluorescent dyes which have different excitation wavelengths, then multiple images need to be captured separately at different times in order to prevent unnecessary exposure to cells. In both the cases, multiple images have to be captured and stitched together which takes a longer image capture time and overexposes the samples to the irrelevant wavelengths of light. Also, it requires extra image processing to combine the images captured with different exposure times.

The developed probe enables a targeted illumination of the multiple regions from FOV with the freedom to select each region with different exposure times (Figure 4.13). The potential advantage of the proposed probe comes from this perspective. The probe embodiment can illuminate multiple cells or cell organelles from the sample with the controlled exposure times. Figure 4.13 shows an image captured by illuminating three cellular regions from the test sample with two different wavelengths and different exposure times. The region 1 and 2 as shown in Figure 4.13 are two cells from mouse kidney section while the region 3 is the remaining part of the FOV. These three sample regions are illuminated for different time durations with a region specific monochromatic light and a single image is captured with long camera exposure time.

Figure 4.13 Multiple regions of mouse kidney section captured with different exposure times in a single frame. Region 1 was illuminated with 488 nm light for 0.2 s. Region 2 and 3 were illuminated with 561 nm light for 0.4 s and 0.1 s, respectively.

4.4 Adaptable spatiotemporal probe for targeted illumination microscopy

The probe embodiment described in Section 4.2 and 4.3 represent a targeted fiber probe for a high resolution illumination and imaging. In this section, the use of the fiber probe to modify the conventional microscope is described. The modified microscope is capable of a multimodal spatiotemporally selective illumination and imaging.

Most of the high resolution spatiotemporally selective illumination systems are modified conventional microscopes. But, all these systems are suitable only for the *in vitro* applications. Moreover, these methods are designed to illuminate and image

the sample placed behind the thin coverslips. It means that for the wet or fluidic samples that are prepared in a petri dish or a cell culture flask, these methods are not suitable. Even the glass slide based samples can be imaged only from the coverslip side of the sample.

In this context, an adaptable fiber probe to modify the conventional microscope into a fully reversible targeted illumination microscope is presented. The probe allows a spatially selective illumination and reflective fluorescence imaging through the fiber probe. Further, the modified microscope allows the imaging in trans-illumination mode from behind the thick transparent medium. The use of fiber optic imaging bundle in the illumination and imaging arm allows the spatiotemporally selective *in situ* illumination and imaging for different sample holders such as flow cell and cell culture flask, microfluidic channel. Further, the fiber probe with a proper modification GRIN lens can be inserted into the cell culture fluid which may not be possible with the microscope objective. Advantages of this modified microscope are its high resolution spatiotemporal illumination capability in *in situ* environment and its ability to use the multiple light beams and imaging multi-fluorescent samples simultaneously. Imaging samples from the two sides provides multiple perspectives to the sample under observation. Also, the two illumination beams can be used to image with different spectral characteristics.

As discussed in Section 4.3, the spatially modulated light beam is allowed to pass through the imaging fiber bundle to illuminate the selected parts of the sample. Microscope camera is used to image the sample in trans-illumination mode. The reflectance image is captured through the imaging fiber bundle. The ability of this probe to modify the conventional microscope for targeted illumination and imaging

is elucidated with the images of mouse kidney section. Optical configuration for this adaptive probe is a modification of spatiotemporally selective illumination probe.

4.4.1 Optical configuration

Adaptable targeted illumination probe consists of an optical setup for the selective spatiotemporal illumination and a miniature fiber probe adapted to a microscope. This optical setup is described in detail in the Section 4.2.1.

Figure 4.14 shows the schematic diagram of the adaptable spatiotemporal probe for targeted illumination microscopy. As evident from the schematic, the spatiotemporally selective illumination probe is adapted into the microscopic setup. In this case, microscope setup is developed with the microscope objective, optical filter, and a camera system. Thorlabs 20X, 0.4 NA microscope objective is used to image the sample illuminated with the targeted illumination. Optical filters are used to discard the excitation wavelength from the collected image. Andor sCMOS Neo camera is used to image the samples. The microscope setup is used to image the sample in trans-illumination mode, while fiber probe is used to image in reflection mode. The setup is same as the microscope's imaging arm. This allows the probe to be added to the microscope with a suitable fiber holder. This means there is no physical modification in the microscope structure. In Figure 4.14, a modified dual modality microscope with fiber probe adaptation is shown.

The bright field image collected through fiber bundle is used as a reference input to select the regions in the sample to be illuminated. The optical configuration is

designed to illuminate the targeted region keeping the non-targeted illumination to a minimum. Results obtained with this adaptive probe are discussed in the next section.

Figure 4.14 An adaptable spatiotemporal probe (a) Optical schematic (b) Fiber probe adaptation into standard benchtop microscope (IX87, Olympus).

4.4.2 Combination of trans-illumination and reflection mode imaging with targeted illumination

Figure 4.15 shows the targeted illumination microscopy images. Figure 4.15 (b-h) shows the images of the sample by restricting the illumination onto Glomeruli and the convoluted tubules of the mouse kidney section. Figure 4.15a depicts the image of the sample illuminated with a wide field illumination. Figure 4.15b and Figure 4.15c are the images captured by illuminating the same region from the sample in the reflection and trans-illumination mode respectively. Similar is the case with Figure 4.15f and Figure 4.15g, where the four small sub-regions are illuminated and

imaged in the reflection and trans-illumination modes respectively. Figure 4.15d, Figure 4.15e, and Figure 4.15h represent the ability of adaptive probe for selective illumination and imaging. As discussed in the previous section, the fiber probe is used to image the sample in the reflection mode by placing it close to the sample. Microscope setup images the sample in trans-illumination mode with the samples kept behind the thick transparent surfaces. The targeted selective illumination strand of Glomeruli and convoluted tubules cells shown in Figure 4.15. A transition from the illumination of a continuous strand of cells to the illumination of selected parts of the strand of cells is depicted (Figure 4.15).

Figure 4.15 Selective illumination and imaging of cells from mouse kidney section. (a) A microscope image of the mouse kidney section illuminated with 488 nm light. Images (b-e) and (f-h) are the microscope images of selectively illuminated glomeruli and convoluted tubules. Images (b) and (f) represent fluorescent images through fiber for targeted illumination while images (c) and (g) are the microscope images counterpart for (b) and (f) respectively.

In Figure 4.16, the ability of the adaptive spatiotemporal probe to illuminate and capture spatially as well as temporally selective image is demonstrated. The two cells from the mouse kidney section were selected for the targeted spatiotemporal

illumination and imaging. The spatial light beam was modulated to illuminate only the selected two cells. Further, these cells were illuminated for controlled time periods elucidating both the spatial and temporal selectivity. These images were compared with the wide-field illumination for similar time periods. Figure 4.16a-d represents the microscopic images with a spatially selective illumination. Figure 4.16 e-h represents the microscopic images with a wide-field illumination. The controlled illumination time of 0.04 s, 0.1 s, 0.2 s and 0.3 s is used for targeted and wide-field imaging.

Figure 4.16 Targeted imaging of mouse kidney section with spatiotemporal control of illumination. Images (a-d) are captured with targeted illumination of two cells for a period of 0.04 s, 0.1 s, 0.2 s and 0.3 s respectively. Images (e-h) are captured by using wide-field illumination for a period of 0.04 s, 0.1 s, 0.2 s and 0.3 s respectively.

It is evident from Figure 4.16 that images captured with a targeted illumination microscopy hold more information than that of the wide-field illumination microscopy. The information content of the datasets can be compared with the help of a quantitative value, standard deviation. It is observed that the targeted illumination microscopy images contain more information compared to that of

wide-field illumination microscopy. In Section 4.2 - 4.4, the two optical configurations for a fiber probe based targeted illumination and imaging are discussed. Furthermore, multiple imaging modalities suitable for specific applications are proposed and demonstrated with static samples. In the following section, an imaging modality suitable for dynamically moving particles is described.

4.5 Targeted illumination of dynamically moving particles

In the biological experiments, the photosensitive reagents are usually tagged to a particular organelle from the selected group of cells. Other than biological studies, the photoactive materials are also used in other engineering disciplines. Photoactive materials have a special property of changing their physical characteristics after illumination with the specific wavelength of light.

Study of the dynamic particles or the living cell requires their spatial and temporal information [160, 171-174]. In such studies, researchers rely on the techniques such as time-lapse imaging and longitudinal examinations to acquire the relevant images [171, 175-177]. Massive amounts of the data produced from imaging experiments need rigorous analysis to retract the information of the cell locomotive principles and motility [178-180]. Apart from the imaging techniques, an important technique in the quest for quantitative analysis of the animate entities is the particle tracking [171-173, 175, 176, 178, 179]. In recent years, there are many studies in the imaging and tracking of the targeted cells or organelles for a variety of biomedical and lab-on-chip applications that include particle movement. Multiple optical techniques have been proposed for the targeted illumination of the selected particle.

However, there is no targeted illumination technique available to illuminate dynamically moving particles. In this context, a fiber optic probe embodiment for the tracking and controlled spatiotemporal illumination of the multiple particles is described in this section.

A fiber optics based probe system described in Section 4.2 (Figure 4.1) incorporating an SLM and an imaging fiber bundle is used for the selective illumination of the targeted moving particles. The selective illumination of the particle under conversation is possible with the developed fiber probe. Every time the particle changes its position the illumination pattern needs to be changed in order to illuminate the selected particle.

4.5.1 Algorithm for tracking and illumination

A flowchart representing the detailed algorithm developed for the calibration and tracking of particles is described in Figure 4.17. The first part of the algorithm describes a calibration which involves acquiring the reference images and using them for DMD-fiber mapping. DMD fiber map is then used to achieve tracking of particles.

The tracking and illumination of the particles that are moved with the sample require initial calibration which involve series of operations - calibration involves a series of operations - First, the image of the sample is collected with the red LED light in trans-illumination mode of operation. This image (Figure 4.18a) is used to map the fiberlets from the fiber bundle onto the camera pixels. Following this step, a group of DMD mirrors covering a rectangular region is switched to ON state. This

allowed only the top hat portion of the laser beam to pass onto the fiber bundle. Image of fiber bundle illuminated with a rectangular shape light beam is captured by EMCCD camera, as shown in Figure 4.18b. Information of the DMD mirrors (like X and Y coordinates) which are in the ON state and an image of the illuminated fiber bundle (Figure 4.18b) are used as inputs for mapping of DMD mirrors onto the camera pixels. ‘Fiberlets to camera pixels mapping’ and ‘DMD mirrors to camera pixels mapping’ are used to perform mapping of DMD mirrors onto the fiberlets. The ‘DMD to fiberlet mapping’ identifies which DMD mirrors to be switched to ON state for coupling light into selected fiberlets.

After mapping, the final step for the calibration of an optical system for particle tracking is to identify which particles are to be tracked. All the identified particles from the sample are localized by the thresholding operation, which separates the particles from the background. Figure 4.18c shows two particles in the ROI highlighted by the rectangular box which are selected for illumination and tracking. For each selected particle’s centroid and the bounding box region surrounding it are illuminated by coupling light through the corresponding fiberlets. This is followed by moving the sample in X and Y direction. With the help of ‘DMD –fiberlet mapping’, the DMD pattern is changed manually to illuminate the selected particles. This process is repeated for further tracking of the selected particles. Particle tracking algorithm is described in the flow chart shown in Figure 4.17.

The spatial illumination pattern has to be modified for every instance of the sample movement. In the following subsection, the modified algorithm for real-time illumination and tracking is described. This method is not suitable for the real-time targeted illumination of the dynamically moving particles. In order to achieve real-

time targeted illumination, the particle location, and the size has to be identified in real time. This can be achieved with an object identification and particle tracking algorithm used in computer vision. The selective fiber optics probe used in combination with the computer vision algorithm can be used as a real-time illumination probe for dynamically moving particles.

Figure 4.17 Algorithm for calibration and particle tracking [181].

4.5.2 Tracking the glass bubble with targeted illumination

In Figure 4.18, different calibration steps and particles selected for tracking are shown. As discussed in Section 4.5.1, Figure 4.18a and Figure 4.18b are used to get the fiberlet to DMD mapping. Figure 4.18c shows all particles from the ROI box illuminated, as explained in the previous section. This has demonstrated the ability of the system to target and illuminate multiple particles simultaneously.

Figure 4.18d shows the two particles which were selected for tracking and illumination. Illuminated rectangular ROI boxes were used as markers to locate the particles in the image. The centroid of the particles was illuminated by the laser light. This demonstrated the ability of targeted illumination system to illuminate regions from the sample even at the micron scale.

Figure 4.18 shows the multi-particle tracking and illumination. In this experiment, a static sample of glass bubbles in fluorescent dye placed on a glass slide is used. It is moved to simulate the movement of the particle. Micrometer resolution X-Y translation stages [T-LS28E, Zaber Technologies] were used to move the sample in X-Y plane. Every time the sample was moved along the X-Y plane, the DMD pattern was adjusted to illuminate the selected particles. The ability of the targeted illumination is demonstrated with bounding box illumination for each particle. Figure 4.18 (d-f) shows targeted illumination of the two dynamic particles.

Figure 4.18 Steps of calibration and selected particles. (a) Transillumination image of particles. (b) Selected rectangular region of interest (ROI). (c) Highlighted particles from selected ROI and (d-f) Illumination of the tracked particles.

In recent years, the different automated object tracking techniques have been proposed for machine vision applications [173, 178, 179]. These techniques require manual intervention only to mark the object to be tracked. These methods can track the object movement in the FOV of the camera. Adoption of object tracking technique in our system would allow real-time particle tracking and illumination. Further, use of multiline lasers, temporally selective illumination capability and automated object tracking algorithm expected to make this an indispensable optical technique for living organism research.

4.6 Summary

Table 4.1 Comparison with state of the art fiber optic probes

Method	Imaging Modalities			Targeted Illumination and imaging		Optical switching		Illumination scanning		Imaging Resolution (μm)	
	Widefield	SIM	Confocal	Spatial-Temporal Multi-target	Multispectral time averaged	Non-Targeted	Multi-targeted	Raster scanning	Region specific Scanning patterns	Spatial	Axial
Miniaturized fluorescence microscope [182]	✓									2.8	NA
SIM, HiLo Microendoscopy [82, 131]	✓	✓								2.6	17
Pixelation-free and diffraction-limited imaging[40, 183]	✓		✓					✓		1.09	8.99
Spatially Selective Holographic Photoactivation And Functional Fluorescence Imaging [13]	✓	✓	✓	✓		✓		✓		3.1	8.6
Probe method presented in this article	✓		✓	✓	✓	✓	✓	✓	✓	2.7	5.5

In this chapter, the targeted illumination and imaging fiber probe methodology is described in detail. Further, the probe's ability for performing multispectral illumination is also described by taking multi-fluorescent samples for imaging. The Potential of the fiber probe for several possible functionalities is demonstrated. These include 'spatially selective targeted illumination and imaging' (Resolution $2.7\text{ }\mu\text{m}$ and the FOV $200\mu\text{m} \times 200\mu\text{m}$), 'Temporally selective illumination' (minimum illumination, where beam pulse duration is 0.1 ms), 'Multidirectional scanning', 'Targeted confocal', 'Contrast enhancement with time averaging' and 'Wavelength selective targeted illumination and switching'. The probe has demonstrated improved speed of imaging as well as improvement in the image quality. Additionally, the adaptation of the probe as an illumination source for the conventional microscope is proposed and developed. Further, the design and algorithm for targeted illumination of dynamic (moving) particles in real time are described. Additionally, this probe can be used to illuminate any targeted region with the precise shape selectivity. The comparison of the feature of the developed probe with fiber optic probes demonstrated earlier is summarized in the table 4.1. The table provides clear idea about the improvements over earlier reported works.

In this chapter, a multimodal fiber probe for targeted illumination and imaging is proposed. Following chapter focuses on the investigations carried out into the use of an axicon lens for imaging around obstacles. It details the research and development of a novel real time method for imaging around obstacles at macroscopic and microscopic resolutions.

Chapter 5: Optical probe for imaging around opaque obstacles

This chapter describes the design and implementation of an axicon based IAO method. The design concept is illustrated with the help of Zemax simulations for imaging through an axicon lens. Real-time imaging around various obstacles having different shapes, sizes and textures are carried out using white light (incoherent) and laser beam (coherent) sources. The method is further improved to achieve high resolution IAO microscope incorporating an integrated axicon-objective lens combination. The chapter ends with the brief summary of the work presented.

5.1 Introduction

The imaging around opaque objects is found to be useful in wide areas including medical, biological and engineering. Though optical concepts and optics-based imaging schemes have shown tremendous potential in the recent past for live cell imaging as well as therapeutic arena [184-188]. These methods are limited in their potential for applications which involve imaging sample around opaque obstacle.

As discussed in the literature review chapter, axicon lens may be used for designing an IAO system because of its properties such as generation of self-reconstruction beam and extended focal depth. In this chapter, the use of axicon lens based imaging systems to image around obstacles is explored.

5.2 Optical design for imaging around obstacle

In this section, with the assistance of Zemax simulations, the suitability of the axicon lens for performing IAO is explained.

The conventional lens has a fixed focal length. Here, the bi-convex or convex lens is used to explain the principle but same can be applied to other types of conventional lenses such as a plano-convex lens or concave lens. The convex lens is used for imaging of a point source; it collects the part of light diverging from the point source which incidents on the lens. If the point source is placed at the focal point of the lens and image plane is placed on the focal spot on the other side of the lens, the rays incident on the lens converge to form an image of the point source at the image plane. This is the ideal imaging setup for a convex lens. When the lens is moved closer to the point source the rays incident onto the lens diverge and no image is formed. On the contrary, when the lens is moved away from the point source the image is formed at a plane different than that of ideal imaging setup. Furthermore, in this case, the number of rays incident on the lens reduce the distance from a point source which reduces the intensity of the image formed. It means that, for a fixed-point source and the image plane, the convex lens can only be placed at a single location (focus spot) to create an image of a point source at an imaging plane.

Figure 5.1 shows the Zemax simulation for imaging of a point source around the obstacle with a bi-convex lens. The simulation results show that a normal lens can only image around an obstacle when the sample to be imaged is kept at the focus spot or near to the focus depending on the Depth of Focus (DOF) of the lens. It is

evident from Figure 5.1b and 5.1c when the sample is out of focus the detector view is clearly blocked by the obstacle.

Figure 5.1 Illustration of image formation (point source) around an obstacle for the regular lens at different focus depth. (a) Ray diagram and detector view for biconvex lens placed at 120 mm from an obstacle (rectangular absorbing medium, 2). (b) Ray diagram and detector view for biconvex lens placed at 57.5 mm from the obstacle. (c) Ray diagram and detector view for biconvex lens placed at 3 mm from the obstacle. Note: 1 is point source illumination, 2 is a rectangular obstacle (10 mm X 0.05 mm, absorbing medium), 3 is a biconvex lens (125 mm focal length, 25 mm diameter) and 4 is the detector. The distance between point source and obstacle is fixed at 5 mm for all the measurements. The detector is also placed at the same position during all measurements.

Figure 5.2 Illustration of image formation (point source) around an obstacle for different axicon focus depth. (a) Ray diagram and detector view for axicon lens placed at 120 mm from an obstacle (rectangular absorbing medium, 2). (b) Ray diagram and detector view for axicon lens placed at 57.5 mm from the obstacle. (c) Ray diagram and detector view for axicon lens placed at 3 mm from the obstacle. Note: 1 is point source illumination, 2 is a rectangular obstacle (10 mm X 0.05 mm, absorbing medium), 3 is an axicon lens (25 mm diameter) and 4 is the detector. The distance between point source and obstacle is fixed at 5 for all the measurements. The detector is also placed at the same position during all measurements.

This can be further elaborated with the Zemax simulations. In Figure 5.2, a Zemax simulation for IAO of point source through axicon lens and a small rectangular object is provided. The point source and the imaging plane are fixed in the positions with axicon lens placed in between. The obstacle is placed 5 mm away from the

source. Axicon lens is moved at three positions and the imaging process is simulated with Zemax. Figure 5.2 shows that an axicon lens with an extended focal depth is able to image around such opaque obstacle for samples placed at different imaging plane, even when the axicon lens is kept close to the obstacle or imaging object.

Through simulations, it has been proved that axicon lens can collect the fluorescence from the optical axis and concentrate into a single spot. The advantage with the axicon lens compared to the conventional lens, in this case, is that the lens can be moved with respect to the image plane and still the single spot will be formed. Further, the Zemax simulations made it clear that the imaging with a convex lens the obstacle creates a shadow as the lens was moved closer to the obstacle. While the axicon lens imaged the point source under the identical conditions. This helped to conclude that axicon lens can be used to image around the obstacle.

5.3 Optical setup for IAO

In this study, three different working optical configurations using an axicon lens are used to demonstrate imaging capability behind an obstacle in free space: white light based, laser based (both in reflection imaging mode, Figure 5.3), and trans-illumination imaging.

In all these experimental configurations, Plano-Convex Axicon lens (Conical lens, Apex angle, 170° , Material: UVFS, Thorlabs) have been used. A 488 nm continuous wave laser (Coherent Inc) is used as the source light for the laser based system

configuration. A tube lens (ITL200, Thorlabs) is used before the camera for effective light collection. The tube lens consists of two doublets that are corrected for lateral and axial aberration across the entire field of view (FOV). Therefore, geometrically correct images are produced, and image distortion is minimized. The fluorescence emission is filtered by an emission filter and imaged by the camera. All the images were captured by Andor iXon3 EMCCD camera.

Figure 5.3 Schematic diagram of imaging around an obstacle.

Both white light reflection and trans-illumination configurations can be applied for image acquisition behind opaque obstacles. Figure 5.4a and 5.4b show the optical setups for performing reflection and trans-illumination, respectively. It was made sure that the sample is always positioned in the focal plane. A typical plano-convex axicon (Conical lens, Apex angle, 170° , Material: UVFS, Thorlabs) was used. The axicon lens and tube lens were separated by 8 cm (fixed position). The images were

acquired by a standard CCD camera (Lumenera Corporation) through a tube lens.

The camera was positioned at the focus position of the tube lens (14.8 cm).

Figure 5.4 Illustration of reflection and trans-illumination imaging set up. (a) Optical configuration for white light reflection imaging . (b) Optical configuration for trans-illumination.

Results with the above three optical configurations are discussed in the following section.

5.4 Results of IAO

5.4.1 White light reflection imaging behind thick opaque obstacle

Figure 5.5 Imaging behind thick obstacles using white light reflection method. (a) Image of USAF chart elements behind Allen key of thickness 0.7 mm, placed at a distance of 0.5 mm from the USAF chart. (b) Image of USAF chart elements behind surgical needle (eye) of thickness 0.35 mm, placed at a distance of 0.5 mm from the USAF chart. (c) Image of USAF chart elements behind pin head of 3.5 mm thickness placed at a distance of 3 cm from the USAF chart. (d) Image of USAF chart elements behind pin 0.5 mm thickness placed at a distance of 0.5 mm and 3 cm from the USAF chart.

This section demonstrates the ability of the proposed method to image samples behind various thick opaque obstacles having different shapes, sizes, and texture with white light reflection imaging. Images captured with the optical configuration shown in Figure 5.4a are shown in Figure 5.5.

Figure 5.5a and 5.5b show the USAF resolution chart behind an Allen key (steel) of thickness 0.7 mm and behind a surgical needle (stainless steel) of thickness 0.35 mm respectively. These two images show IAO capability of the optical system with different obstacles placed at the same position. Image formation of USAF chart elements behind pin head (plastic coated with acrylic paint) having a thickness of 3.5 mm is illustrated in Figure 5.5c. USAF chart imaged behind a stainless steel pin of size 0.5 mm is depicted in Figure 5.5d, placed at 0.5 mm and 3 cm from the object to be imaged. It should be mentioned that the axicon position need to be optimized with respect to the sample in order to get the image reconstructed without the obstacle masking it. This optimal position depends on the size of the obstacle. Images of the sample behind thick obstacles as big as 0.7 mm are achieved even when obstacles are placed as close as 0.5 mm from the sample. Further, it is observed that when the obstacle is moved closer to the object to be imaged, the brightness of the captured image reduces.

5.4.2 White light transillumination imaging behind thick opaque obstacle

The results obtained with the proposed scheme for imaging behind obstacles using trans-illumination modality are presented in Figure 5.6. (The schematic diagram of the simple trans-illumination setup is shown in Figure 5.4b). Figure 5.6a and 5.6b

show the obtained imaging results using USAF resolution chart as the test samples kept behind a stitching needle of thickness 0.6 mm and a metal pin head of thickness 3.5 mm, respectively. Further, the processed image of the reconstructed region is shown as an inset with higher contrast in Figure 5.6b. Image formation of USAF chart elements behind the surgical needle having a thickness of 0.35 mm is illustrated in Figure 5.6c. These results confirm that axicon lens can be used to image behind a thick obstacle in white light using simple trans-illumination configuration.

An earlier study by Bouchal *et al* showed that an ideal non-diffracting beam can exactly reconstruct its initial intensity profile behind an obstacle of arbitrary form and size [22]. However, it is important to note that, in the approach presented here, the illumination and imaging is performed through the same axicon lens. This means either the reflected or fluorescence light from the object is imaged by the camera through the axicon lens. The reflected or fluorescence light from the object does not have self-healing properties as required for the case in [22]. Only the coherent excitation beam when passed through axicon can generate a non-diffracting beam. However, in the presented approach the imaging is demonstrated using white light trans-illumination setup, which clearly show that the focusing plane of the axicon lens is the key contributor.

5.4.3 Fluorescence imaging behind thick opaque obstacle

The schematic diagram of the laser-based fluorescence imaging system is illustrated in Figure 5.3. A Bessel-like beam was generated by propagating a Gaussian beam

through an axicon lens, and was used to illuminate the sample. Both the illumination and imaging are carried out through the same axicon lens. A high pass filter (above 500 nm) is used in the fluorescence imaging mode to discard the excitation beam where 488 nm laser source is used for excitation. The sample is positioned within or at the end of reconstruction plane of the Bessel beam as shown in Figure 5.3.

Figure 5.6 Imaging behind thick obstacles using trans-illumination method. (a) Image of USAF chart elements behind a stitching needle of thickness 0.6 mm. (b) Image of the USAF chart elements behind a paper pin head of thickness 3.5 mm. The inset shows pin head region processed to enhance the contrast to see reconstruction with better clarity. (c) Image of USAF chart elements behind a syringe needle of 0.35 mm thickness.

The feasibility of imaging around a thick opaque obstacle is demonstrated by imaging mouse kidney cell clusters by using an Allen key of thickness 0.7 mm (Figure 5.7). Inset image, which is the processed image of the region marked, is an image with enhanced contrast. This validates the proposed concept and methodology that such structures can be imaged even when there are opaque obstacles between the sample and imaging unit. It should be noted that in the above experiments the obstacles were placed between the axicon and the sample, at a plane as close as possible to the sample.

Figure 5.7 Fluorescence imaging of mouse kidney section around the 0.7 mm thick Allen key

5.5 Real-time IAO

The previous section illustrated the images obtained with several imaging modalities using axicon-based IAO. These results confirm that axicon lens can be used to image behind thick obstacles in fluorescence imaging, white light using simple reflection or trans-illumination configuration.

As discussed in Section 5.1 there is a need for imaging around an obstacle in different fields. Specifically, in the case of surgery, there is a severe need for real time IAO method. This would help in reduction in the injuries due to blocked view. In this section, the ability of the proposed method to perform IAO in real time is demonstrated.

Figure 5.8 Experimental setup for real-time IAO. (a) Needle at 5 mm from the object to be imaged. The red arrow shows a location of the invisible needle. (b) Needle at 3 cm from object to be imaged.

The ability of the imaging system to image around an obstacle in real time with white light illumination is demonstrated in Figure 5.8. Images from Figure 5.8 show the imaging end of the system on the left and real-time image collected through the CCD camera on the right side. A stainless steel syringe needle of diameter 0.35 mm was used as an opaque obstacle. The sample used in the setup was a styrofoam board. The distance between the sample and the axicon lens was 7.5 cm. Tube lens was used in between the camera and the axicon lens. The syringe needle was moved from the sample towards the axicon lens. Figure 5.8 shows that the imaging around the syringe needle is possible irrespective of the needle movement.

This was followed by the movement of the object to be imaged towards the needle (Figure 5.9). Syringe needle remains completely invisible until it touches the sample. The presence of the needle in the imaging path is confirmed with the plastic syringe visible and marked in Figure 5.9b and 5.9c. When the sample and syringe are close by the syringe outline can be identified which looks like a transparent glass rod and the sample can be clearly seen through that. The needle becomes visible when it touches the sample.

The robustness and versatility of the conceived idea are demonstrated by simulating a surgical injection ambiance. The axicon-based fluorescence imaging system is further used to image biological specimen kept behind a surgical needle (0.35 mm) and the image of the sample, kept behind the needle, was found to be formed in real time. The raw sequence of needle insertion is shown in Figure 5.10.

Figure 5.9 Imaging arm of IAO with the images of sample movement around the obstacle. (a) Sample placed at 5 cm from axicon and 2 cm from the needle. (b) Sample placed at 5 cm from the axicon and 5 mm from the needle. (c) Sample touching the needle tip.

Figure 5.10 Illustration of image formation behind surgical needle during insertion. (a-c), Shows the movement of syringe needle kept in front of the biological sample. (d-e), Shows the insertion of the needle into the sample. (f-g), Fluid (stained) is injected into the sample. (h-i), Illustrate the retrace path of the needle after injection. The arrow in images shows the tip of the surgical needle.

Based on both white light and fluorescence imaging, it can be inferred, that the slow insertion of the syringe needle cannot be seen due to the image formation around the obstacle, which is the basic principle of the proposed method. This section described the examples of IAO in the macroscopic setting for imaging of large objects around obstacles of few mm thickness. The IAO can be very useful in the

microscopic environment such as cell level or tissue level manipulations and soldering of microchips in embedded circuit. In the following section, the IAO in microscopy is discussed which can be used for above mentioned applications.

5.6 Microscopy around opaque obstacle

As discussed in previous sections, with axicon lens in the imaging arm it is possible to image behind opaque obstacles. However, it is evident from the USAF chart imaged through axicon lens that elements from group 4 and 5 cannot be resolved (Figure 5.6 and 5.7). This means using only an axicon at the imaging arm, it could not perform an IAO of microscopic objects. As an extended embodiment of the previously explained configuration, a simple microscope is designed using a combination of axicon and objective lenses. Figure 5.11 shows the optical setup for the same. In the figure, the normal lens is shown as a representative for an objective lens.

In IAO with an axicon, the object to be imaged can be placed at any point in the depth of field, shown by multiple reconstruction planes in Figure 5.10. For axicon-objective combination the depth of field is very narrow outside the objective and it reduces with the depth of field of the axicon. Axicon lens with smaller apex angle will be difficult to employ in such configuration due to a smaller depth of field. Axicon lenses with different apex angles such as 110° , 140° , 160° and 170° are studied. As the system requires a large depth of field, it is found that the apex angle with 170° is more suitable. Moreover, an axicon lens is placed close to the objective lens so as to maximize the possible DOF outside objective lens. Compared to a fixed focus point for a normal objective lens, axicons with apex angle above 170°

have larger DOF. The imaging plane can be from the surface of the axicon lens to different planes along very long DOF (Figure 5.11). This is not possible for a normal lens to image an object at all points from the lens surface to its focal position. Figure 5.12 shows an optical ray diagram illustrating the increased overall DOF of the combined axicon and normal lens unit as compared to the single normal lens. To illustrate the effect of combined lens system, first, the individual ray diagrams are also provided. As mentioned above the distance between the lenses should be minimum, which was 10 mm in the simulation presented.

Figure 5.11 Schematic diagram of imaging setup using a combination of the axicon and the objective lens.

Further to validate the combination of axicon-objective for microscopic IAO, a real time sequence of mouse kidney cells image reconstructed behind 60 μm hair using this microscope is presented in Figure 5.13. It is evident from these results that the resolution of the system is improved (imaged kidney cells, spatial resolution less than 3 μm).

Figure 5.12 Illustration of extended focus depth by ray diagram using Zemax simulations. (a) A normal plano-convex lens with shallow focus depth (focal length 125 mm). (b) Axicon lens (170°). (c) Combined axicon (170°) and a plano-convex lens.

Figure 5.13 Sequence of mouse kidney cells (microscopic) raw image formed behind hair (a-c). Each image has an inset attached to it showing specifically the imaged region behind the hair obstacle with improved contrast.

5.7 Summary

Zemax simulations have proved the potential of axicon lens for IAO method. A new imaging concept using axicon lens to image samples behind obstacles is demonstrated with samples of different material types, shapes, sizes, and reflectivity. Using white light and as well as laser light as the illuminating source, the phenomenon of image formation behind obstacle is demonstrated in both the reflection and trans-illumination imaging mode. The real-time IAO for fluorescence and non-fluorescence sample is demonstrated. In order to enable microscopic IAO, an axicon-objective combination is used and demonstrated.

To further improve the performance parameters, we need to do simulations and designs to optimize the object distance from the surface and the diameter size. In the current study, we have used a standard axicon lens of diameter 25 mm. Using customized larger lens diameter axicon lens, imaging behind large obstacle can be performed.

Chapter 6: Conclusion and future work

This chapter begins with the major conclusions derived out of this research thesis. Major contributions made during the course of research are stated in sequence followed by suggestions for future work directions.

6.1 Conclusion

Targeted *in vivo* illumination and imaging systems have numerous significant applications in diagnostics and therapeutics apart from other branches of science and technology. An extensive literature survey of targeted illumination and imaging systems has been carried out to understand this niche research domain. Despite the overwhelming need of targeted illumination in *in vivo* systems, there are very few studies which have addressed this problem. In this regard, this primarily focuses on the design and development of fiber optic imaging bundle (FOIB) based optical imaging systems for targeted illumination.

In this research, FOIB was chosen to design targeted illumination probe as it has small diameter, user-defined length and high mechanical flexibility to access *in vivo* sites. The system uses FOIB in combination with DMD mirrors (Texas instruments) to achieve the targeted illumination. Real time switching of DMD mirrors is employed to generate the desired spatial optical illumination pattern. Further, the ability of FOIB probe to scan multiple regions either simultaneously or sequentially by using multiple light spots has been demonstrated for confocal imaging. The multiline digitally controlled laser source is integrated into the probe to perform targeted illumination with different wavelengths.

Though there are a few notable studies reported which use the FOIB probe for imaging, they could claim the highest possible axial resolution of only around 10 μm [12, 13]. The image resolution of the proposed and developed FOIB probe, as part of this thesis, has demonstrated an improved axial resolution of 5.5 μm , which is the highest compared to the reported methodologies. The developed FOIB probe uses GRIN lens with magnification factor of 1, which implies that the smallest illumination beam spot demonstrated with the developed probe is 2.7 μm . It is important to note that earlier reported targeted FOIB systems have shown the smallest illumination beam spot of size 5 μm diameter. Compared to earlier reported works, this probe also provides a larger field of view (200 μm X 200 μm) at a higher resolution.

There are many thrust niche areas such as optogenetics, neurophotronics, and Photodynamic therapy (PDT) which requires a method such as targeted illumination with high switching speed. In neurophotronics research, illuminating the parts of the brain with light for few tens of milliseconds generates heat at the non-targeted parts of the brain [84, 189]. The heat generated may trigger an unplanned neuronal process sabotaging the correctness of the study. This elucidates the importance of targeted illumination with improved spatial and temporal resolution. Current available targeted illumination FOIB probes have demonstrated the optical switching speed in the range of 20 Hz. For the developed probe, the optical switching speed is defined by the DMD switching frequency, which is 10,000 Hz. This high-speed switching makes it suitable for studies which need high illumination switching speed.

There is a lack of a proper theoretical model to analyze the image transfer through FOIB which has become a bottleneck for researchers to accurately simulate image transmission through such FOIB based systems. Pixelation noise is inherent to all the images acquired with FOIB. It hinders the important information in full to be imaged and it affects the quality of the captured image. In this context, a detailed quantitative and subjective comparison of the depixelation methods is provided. There are many depixelation algorithms reported earlier to remove the pixelation noise such as Gaussian smoothing, Spectral filtering, and interpolation methods. After a careful study of these methods these depixelation methods, it was found that there were no common set of the test images available which can be used to demonstrate the depixelation performance. Moreover, presently there was no free open source database of pixelated images available.

These observation has led to the development of a Fiber Pixelated Image Database (FPID), which now serves as a common test image sample space for researchers working on the development of novel depixelation methods. The FPID database consists of two subsets simulated pixelated images and experimentally acquired images through FOIB. To generate simulated fiber pixelated images, a theoretical model for FOIB based imaging is developed. Packing fraction, fiberlet to fiberlet distance, fiberlet core diameter, core-cladding properties and light guiding properties of fiberlet were the parameters considered in the theoretical formulations.

The effect of variable pinhole size has been studied in the standard bench-top microscopes. However, in the case of FOIB based imaging systems, effects of the variable pinhole sizes on image resolution have not been studied so far. Therefore, in this thesis, using the targeted FOIB probe, the effect of using a variable pinhole

sizes, (i.e. variable number of fiberlets) is investigated for parameters such as resolution and contrast.

Based on the point-wise scanning by FOIB probe in the targeted regions, a special type of confocal imaging method is also demonstrated, termed as the targeted confocal imaging. In FOIB confocal method sequential illumination and scanning are performed through a single fiberlet in raster scanning approach. There are instances where the entire sample need not be imaged, and targeted confocal imaging is a promising approach in such scenario. In targeted confocal method only selected regions (non-sequential approach) of the sample are imaged, thereby improving the image capture time while maintaining the same image resolution as that of the FOIB confocal. This has been demonstrated by contrast enhancement imaging performed on the mouse kidney fixed cells.

To perform targeted illumination in live samples, tracking feature is needed. The targeted illumination probe can find application in illuminating the samples such as cells, microorganisms, and active materials which have moving particles. In this context, a proof of concept of tracking and targeted illumination by FOIB probe is demonstrated for glass microspheres.

The second main objective of the thesis is to design and develop an optical method for imaging around an opaque obstacle (IAO). It is noticed that there are no available optical methods for imaging around the opaque obstacles. There are many cases of injuries reported every year, which are caused because of improper positioning of the surgical tool, due to the blocking of the FOV of the sample by opaque surgical tools during surgery. From this perspective, an imaging system is

designed incorporating an axicon lens to enable imaging around such opaque obstacles.

Using this imaging system, imaging around different thick opaque obstacles of different shapes and thickness, such as Allen key, syringe needle, metallic pin, hair, and thread has been demonstrated. Using the axicon lens based method presented in this thesis, it is possible to image the sample at a different DOF, irrespective of the position of the obstacle or the sample position. In this approach, the obstacle can also be positioned in the axis of the imaging plane. Further, the obstacle can be placed very close to the sample to be imaged. IAO method is demonstrated with the obstacle as close to the object to be imaged as 1 mm. Due to the low numerical aperture of axicon lens, generally, it is not suitable for microscopic imaging. Hence to improve the imaging resolution, a combined axicon-objective unit is proposed and demonstrated by performing high resolution imaging of mouse kidney cells.

The significance of this developed method for diagnostic medical imaging and therapeutics is tremendous and has great relevance in clinical imaging. The FOV of imaging systems used during microsurgery is generally restricted to the FOV of the microscope. It is possible to cause damage from surgical instruments unless the surgeon is always aware of the position of the targeted structures in the surgical site. Therefore, in this study, the proof of concept of seeing the structures around the needle during surgical injection is demonstrated.

The major research contributions from this thesis are listed in the following section.

6.2 Major contributions

1. High resolution FOIB confocal imaging probe was proposed, developed and experimentally validated. Effects of variable pinhole size on the imaging quality were investigated for fiber optic imaging bundle. The effective pinhole sizes considered in this study were as low as that of a single fiberlet diameter and as high as the diameter of a group of eight fiberlets.
2. Fiber pixelation noise removal techniques were investigated for different test samples. A database for fiber pixelated images was proposed and developed, which serves as input test sample space for the researchers developing new depixelation techniques.
3. An *in vivo* fiber optic probe for targeted illumination and imaging was proposed and demonstrated with the different samples. The probe is capable of spatially targeted illumination, temporal switching and multispectral illumination. These features were demonstrated by capturing images and videos of mouse kidney samples in different imaging modes.
4. The fiber optic probe was developed that was capable of simultaneous scanning of multiple illumination spots in the targeted regions from the sample. The targeted confocal imaging of multiple regions was demonstrated using this feature of the probe. Additionally, a selective illumination and tracking probe for dynamically moving particles was developed and demonstrated.
5. Optical technique for imaging around opaque obstacle was simulated with Zemax software. Based on the simulation results an optical system for IAO is designed, developed and demonstrated.

6. Experimental results for the real time IAO are presented with macroscopic and microscopic imaging resolution. The real time IAO is further demonstrated with the needle injection experiment.

The following section discusses future research directions which are identified to improve the current limitation of the developed probe for its applications in specific areas. A brief account these initial investigations that are carried out and which are proposed are detailed below.

6.3 Future work

Research carried out as part of this thesis has the potential for further studies to be carried out in the future. The following are the recommendations for some of the suggested future work directions. The future work will be concentrating towards employing the developed targeted illumination FOIB probe concepts for optogenetics applications. Similarly, for IAO system research- the future work will be concentrating on the development of a fully functional probe based IAO method. Further, the study of axicon lens based system for imaging applications reported of achieving imaging resolutions beyond the diffraction limit will be further explored. Use of such a high resolution imaging for eye imaging will also be explored as part of the future work directions. Such investigations require modifications to the current probe design, which will be discussed below.

6.3.1 Application of the developed system in optogenetics

Optogenetic research has highlighted the need for targeted illumination schemes [18, 20, 21, 84, 159, 190]. The FOIB probe designed as part of this thesis has potential for *in vivo* optogenetic applications. The studies will be conducted to analyze the possible issues, which will be associated with the adaptation of the probe for *in vivo* applications. Although no major changes need to be done in the optical configuration, possible variations for application specific requirements need to be considered. Two main challenges to be addressed are-

- The fiber probe has to be designed to be suitable for water immersion applications as the optogenetic studies need an optical probe to be inserted into different fluidic channels.
- The probe embodiment has to be modified and suitable cleaning procedure has to be developed so as to use the same probe multiple times without contamination.

Furthermore, the FOIB probe has been demonstrated to perform particle tracking and illumination for fixed FOV. In different biological studies, continuous monitoring of the targeted cells is required with the capability to stimulate (illumination with a light beam of a particular wavelength) the targeted cells intermittently. If targeted cell moves out of the fixed FOV, the existing particle illumination and tracking FOIB probe method won't be able to provide such continuous monitoring. In this context, FOIB probe has to be modified to provide extended FOV. This can be achieved by adding mobility to the FOIB probes distal end and controlling its movement based on the movement of the targeted cell.

6.3.2 Applications in ocular imaging

The trabecular meshwork (TM) in the iridocorneal angle (ICA) of the eye, plays a vital role in aqueous outflow. Direct visualization and assessment of the iridocorneal angle (ICA) region with high resolution is important for the clinical evaluation of glaucoma. However, the current clinical imaging systems for ICA do not provide sufficient structural details due to their poor resolution.

To address this void, an initial study has been conducted using axicon lens assisted Bessel Beam Microscopy (BBM). This work was able to achieve imaging resolutions beyond the diffraction limit. Gonio lenses are the standard solution for imaging of ICA regions, however, the image resolution through Gonio lenses is very poor. In this regard, use of Gonioscope in combination with axicon lens-based imaging system would be able to image ICA at high imaging resolutions. The results obtained during technical investigations are detailed in Appendix C.

6.3.3 Miniaturization of IAO system

The IAO imaging method demonstrated in this thesis is suitable for the applications where the samples are in different examples such as an open surgery. In the case of samples which are not readily accessible, a probe based IAO method is needed and hence one of the potential future research should be along this direction. An initial investigation was carried out and a probe embodiment is conceived as shown in Figure 6.1

Figure 6.1 Schematic for proposed optical fiber probe-based IAO system.

This optical configuration is a modification of the IAO system described in Section 5.3. The initial configuration consists of a plano-convex axicon lens (Conical lens, Apex angle, 170° , Material: UVFS, Thorlabs) and a flexible fiber probe of 1 mm diameter and 15000 fiberlets which collect the light from the axicon lens. The wide acceptance angle (70°) GRIN lens is attached to the fiber probe. A custom lens system is designed to collect the image through the fiber onto the camera sensor. A CCD camera (Lumenera Inc.) is used to record the image. A poly-light source is used to illuminate the sample with light of particular wavelength.

The proposed probe based IAO can be improved for true in vivo imaging around the obstacle by incorporating micro-axicon and fiber probe. This research expected to has potential bioimaging applications in the near future.

Appendix A

Fiber Pixelated Image Database

In this thesis, Fiber Pixelated Image Database (FPID) is developed as common sample test image database with a free open access. This will facilitates the researchers working in the area of FOIB depixelation research. The details of the open source FPID which is developed is also reported in journal publication (Refer [2] from the List of publications).

In this appendix, more examples of images from Fiber Pixelated Image Database (FPID) are given. Generation of both simulated and experimental pixelated images are described in Chapter 3. Figure A.1- A.4 illustrate the simulated FPID images. Both original and its corresponding pixelated image (in the adjacent row below) is given for all the simulated image data.

Simulated images of FPID

Reference image patterns are created first with the MATLAB program. And its corresponding pixelated image is created by using following equation.

$$I(l_s) = \left| \int \left| \int \int h\left(l_o + \frac{l_1}{M}\right) f_{loc}(r_p - r_o) S(r) dr_p dl_1 \right|^2 \right. \\ \left. \int \int h\left(l_1 + \frac{l_o}{M}\right) f_{loc}^*(r_p - r_o) S(r) dr_p dl_1 \right|^2 \\ \times f(l_s - l_o) dl_o \quad \text{A.1}$$

Relevant theoretical details are provided in Section 3.2 of Chapter 3. The examples of simulated images of FPID are given below.

Figure A.1 (a-c) Original image, 5-degree inclined grating pattern with frequency 16, 32 and 64 (e-h) simulated pixelated images of reference patterns in images (a-c).

Figure A.2 (a-b) Colormap pattern, (c-d) Grayscale variation (e-h) simulated pixelated images of reference patterns in images a-d. (i) Uniform bright image (j) uniform Grayscale pixel value image (k) uniform dark image (l) Siemens star with frequency 2. (m-p) simulated pixelated images of reference patterns in images (i-l).

Figure A.3 (a-d) Siemens star pattern with frequency 4,8,16 and 32 (e-h) simulated pixelated images of reference patterns in images (a-d). (i-l) Grating patterns with frequency 1,2,4 and 8. (m-p) simulated pixelated images of reference patterns in images (i-l).

Figure A.4 (a-d) Grating patterns with frequency 16, 32, 64 and 128. (e-h) simulated pixelated images of reference patterns in images (a-d). (i-l) 5-degree inclined rating patterns with frequency 1, 2, 4 and 8. (m-p) simulated pixelated images of reference patterns in images (i-l).

Experimental images of FPID

In addition to simulated images, pixelated images of various samples are acquired performing FOIB based imaging experiments. The experimental configurations used are explained in Chapter 3. In this database, pixelated images of different types of samples are provided. This include images of mouse kidney cells, glass bubble, thin slice of human eye cross section, and artificial patterns marked on a glass substrate.

Figure A.5 (a-c) Images of glass bubble sample (e-g) Images of a cross section of the human eye. (d and h) images of mouse kidney section.

Figure A.6 Images of checkerboard marked on a glass substrate. A box size varying from 30 μm to 1 μm .

Figure A.7 Images of grating pattern marked on a glass substrate. A line thickness varying from 50 μm to 1 μm .

Figure A.8 Images of Siemens star marked on glass substrate. Star pattern frequency varying from 2 to 36. (n) Miscellaneous smiley emoticon marked on the glass substrate and imaged through FOIB.

Appendix B

Sample preparation and materials

Glass mask with patterns for generating FPID images

In order to generate images for fiber pixelated image database, a photomask is prepared on quartz glass substrate of 5 inch X 5 inch X 0.09 inch with different patterns (Figure B.1). The patterns were designed using L-Edit software in a 10 cm diameter circle. The patterns were made on the quartz by laser lithography. Based on the considerations discussed in Section 3.3 different shapes and sizes were selected for the patterns. Smallest pattern in this photomask was of size 1 μm and the largest feature size was 500 μm .

Figure B.1 Glass mask with patterns for generating FPID images.

Fiber Optic Imaging Bundles

Research work carried out in this thesis have utilized three different fiber optic imaging bundles. Detailed specifications for these three FOIBs are given in the Table B.1. The IGN 11/50 is used for the development of Targeted FOIB probe. All the FOIBs were used for investigation into relationship of input beam power and output amplitude. Further, for proposed future work FOIB with 15000 fiberlets will be more suitable due to their increased flexibility.

Table B.1: The details of the three FOIBs used in this thesis

Parameters	Sumitomo Electric IGN-11/50	Fujikura FIWL 15-600N	Fujikura FIGH 15-600N
Number of fiberlets	50000	15000	15000
Numerical Aperture	0.35	0.41	0.40
Fiberlet core diameter	2.7 μm	2.8 μm	3 μm
Fiberlet to Fiberlet distance	4.4 μm	4.3 μm	4.46 μm
Picture diameter	0.99 mm	0.695 mm	0.7 mm
Length of FOIB	50 cm	1 m	2 m

GRIN lens

Light emanating from the distal end of the FOIB would tend to spread (diverge). To collimate the output light of the fiber, custom Gradient Index (GRIN) lenses are used. The GRIN lens was attached to FOIB distal end by using epoxy glue.

Appendix B

For the targeted illumination FOIB probe, GRIN lens of 1 mm diameter was used. The GRIN lens FOIB assembly was then put inside the metal casing of diameter 1.1 mm.

Specifications for this GRIN lens

Outer diameter	: 1 mm
Working distance	: 0.3 mm @ 540 nm
Magnification factor	: 1:1
Length	: 20 mm

Figure B.2 Illustration of FOIB probe with GRIN lens

Mouse kidney section

Thin slice (~15 μm) of mouse kidney section fixed onto glass slide was used in this study. It was procured from Life technologies (Currently Thermo Fisher Scientific). Mouse kidney section was tagged with Alexa Fluor® 488 WGA, Alexa Fluor® 568 Phalloidin, and DAPI. The optical configurations described in this thesis used three

Appendix B

excitation wavelengths (488, 534 and 561 nm). Hence, only two types of signatures from the mouse kidney section are demonstrated in this thesis.

Sample preparation for PSF experiments

Pure water is mixed with PVA powder. It is heated and stirred until PVA is dissolved completely and solution is transparent.

Glass bubbles of the desired sizes are added to this mixture.

Rhodamine 6G is added to the solution and it is stirred well until the solution has a uniform pink colour.

Finally, the prepared solution is transferred onto a glass slide surface and allowed it to solidify and form thin films.

USAF test chart

Quantitative evaluation of optical imaging system requires the imaging resolution values. 1951 USAF test chart provides such standard to test the image resolution. It is a glass mask marked with dark and bright grating lines of different thickness. In USAF test chart, each grating line thickness has 3 pairs of dark and bright lines. The smallest line thickness inscribed in the USAF test chart used for this study was 0.78 μm .

Figure B.3. 1951 USAF test chart image.

Digital Micromirror Device

Figure B.4 Texas instruments Discovery DMD 1100 controller board and starter kit.

Appendix B

Digital micro mirror (DMD) device from Texas instruments is used as a spatial light modulator. DMD has an array of large number of very small rectangular mirrors arranged in matrix form. Area of each of the mirror varies with the model. For DMD1100P, area of single mirror is $179.56 \mu\text{m}^2$. Mirrors are arranged on to the rectangular pane. Each mirror can have three states of operation- ON, OFF and FLAT. All mirrors are in FLAT state after DMD is powered on until they are activated for particular pattern. Pattern is a design over a pane of mirrors created by individually switching ON some of the mirrors; all other mirrors remain in OFF state. Change of mirror state from OFF state to ON state is reflected physically by change in the angle of mirror axis by 12 degrees. As specific patterns can be generated on to the DMD, it can be used as very high speed controlled spatial beam reflector. Figure B.4 shows the DMD system used for the study (Texas instruments, DDR1100). Further, the ability of DMD mirror to illuminate any shape is shown in Figure B.5.

Figure B.5 Tajmahal image pattern by switching DMD mirrors.

Appendix C

Axicon assisted gonioscopy for high resolution iridocorneal angle imaging

In this appendix, preliminary results of investigations into the use of axicon assisted gonioscopy for high resolution iridocorneal angle (ICA) imaging are described.

Optical setup

In the gonioscopic setup shown in Figure C.1, a white light source (Correct Shimadzu FA-150EN Fiber Illuminator, Japan) was used for illumination. The imaging head is a gonioscopic lens (Hoskins-Barkan Goniotomy adult 11mm lens; Ocular Instruments, WA, USA). Through the gonioscopic lens, ICA region of the eye was imaged by a microscopic objective lens (LSM03 - 4.6X, VIS OCT scan lens, Thorlabs, USA). The long working distance of the objective lens (25mm) and its large field of view facilitated the effective adaption of the microscopic lens with a gonioscopic lens. The light from the microscopic objective was then collected via a tube lens (ITL 200, Thorlabs). A convex lens (LB-1844-A, Thorlabs) was placed at a focal length distance from the focal plane of the tube lens. An axicon lens was placed in close proximity of convex lens. The combination of convex lens and axicon lens was known to transform the wavefront of a point source into a Bessel beam, which has the special property to propagate without diffracting. A beam passing through axicon lens was captured by the CCD camera (EXi Aqua Bio-Imaging Microscopy Camera, QImaging, Canada). Lateral resolution of the imaging system is around 3 μ m.

Figure C.1 Optical setup of developed indirect gonioscopic imaging system. a, Optical setup of the developed imaging system. b, Inset, illustrating the front view of our system. [M: Eye model; G: Hoskins-Barkan Goniotomy lens; L1: objective lens; L2: tube lens; L3: convex lens; L4: axicon lens; L5: zoom lens; C: CCD; 9] [185].

The optical setup described above have demonstrated the ability to perform imaging at high resolution ICA imaging. The ICA of the porcine eyes imaged with the described system is shown in Figure C.2. Figure C.2a is the raw image, it has structures slightly false colored since the light source used to illuminate has yellow tint and not perfectly white. The processed image is shown in Figure C.2b. It is evident that the different regions in the ICA of the porcine eye are distinctly visible in the image.

The trabecular meshwork (TM) region is very distinct in the image (Figure C.2b). However, TM meshwork structures are further highlighted by performing edge detection on the image. This result is shown in Figure C.2c.

Figure C.2 Acquired image of iridocorneal angle of porcine eye imaged by the developed system. a, Raw TM region image of porcine eye. b, Processed TM region image of porcine eye. c, Edge detected image allowing structures inside TM region to be visualized [185].

Results shown above demonstrate the ability of axicon assisted gonioscopy to perform high resolution ICA imaging. For further details, please refer to [3] from list of publication.

List of publications

Journals

1. A. Shinde and V.M. Murukeshan, "Pixelate removal in an image fiber probe endoscope incorporating comb structure removal methods," *Journal of Medical Imaging and Health Informatics* **4**(2), 203-211 (2014).
2. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Fiber Pixelated Image Database," *Optical Engineering* **55**(8), 083105 (2016).
3. S.M. Perinchery, A. Shinde and V.M. Murukeshan, "High resolution iridocorneal angle imaging system by axicon lens assisted gonioscopy," *Scientific Reports* **6**, 30844 (2016).
4. S.M. Perinchery, A. Shinde and V.M. Murukeshan, "Imaging behind opaque obstacle: a potential method for guided in vitro needle placement" *Biomedical Optics Express* **7**(8), 5308-5324 (2016).
5. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Simultaneous shape selective imaging and stimulation probe for optogenetics" *Scientific Reports* (Minor revision submitted, 2017).
6. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Adaptable spatiotemporal probe for selective illumination microscopy" (*To be communicated*) (2017).
7. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Investigations into variable pinhole" (*To be communicated*) (2017)

International conferences

8. A. Shinde and V.M. Murukeshan, "Synthetically generated fiber pixilated image database," in *SPIE Photonics Europe*, 91280K-91280K-11 (2014).
9. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Variable resolution imaging fiber probe using digital spatial light modulator," in *International Conference on Optical and Photonic Engineering (icOPEN2015)*, 95242F-95242F-7 (2015).
10. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Targeted illumination and tracking using optical fiber probe for optogenetics application," in *SPIE BiOS*, 969028-969028-7 (2016).
11. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Fiber optic probe for region of interest (ROI) selective time averaged multi-fluorescence imaging," in *International Conference on Optical and Photonics Engineering* (2017).
12. A. Shinde, S.M. Perinchery and V.M. Murukeshan, "Random patterned illumination for high resolution imaging," in *International Conference on Optical and Photonics Engineering* (2017).

List of Publications

13. S.M. Perinchery, A. Shinde and V.M. Murukeshan, "Imaging objects behind small obstacles using axicon lens," in *International Conference on Optical and Photonics Engineering* (2017).
14. S.M. Perinchery, A. Shinde and V.M. Murukeshan, "Microscopy using grid pattern with randomized speckle illumination," in *International Conference on Optical and Photonics Engineering* (2017).

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