

**TAILORING EXCITATION STRATEGIES FOR  
STRUCTURED ILLUMINATION  
MICROSCOPY**

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by

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Department of Physics

Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the



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*Dedicated to my loving parents and grandparents*



# Certificate

This is to certify that the thesis entitled “**Tailoring Excitation Strategies for Structured Illumination Microscopy**”, submitted by **Krishnendu Samanta** to the Indian Institute of Technology Delhi, for the award of the degree of **Doctor of Philosophy** in Physics, is a record of the bonafide research work carried out by him under my supervision and guidance. The thesis has reached the standards fulfilling the requirements of the regulations related to the award of the degree.

The results contained in this thesis have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma to the best of my knowledge.

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# *Abstract*

The thesis primarily highlights the super-resolution optical microscopic imaging in the transmission architecture. At the heart of the thesis, there is structured illumination microscopy (SIM) that has been explored with different perspectives. The conventional SIM deals with a single objective lens for both illumination as well as detection purposes. Hence, both the excitation and fluorescent signal collection are restricted by the diffraction limit of the objective lens under consideration. In the race of achieving best possible resolution, high numerical aperture (NA) objective lenses are typically employed in the standard SIM systems. Thus, the super-resolution image using such objective lens appears by compromising with the imaging field of view. However, the entire study is performed employing low NA objective lens that confirms a large field of view imaging at the cost of poor resolution. Implementing sinusoidal structured illumination generated by tilt-mirror based illumination module, the resolution is enhanced afterwards. Two oppositely faced mirror facets are functional at once to produce the sinusoidal interference pattern and the hexa assembly of the mirror facets serves the purpose of isotropic resolution in three orientations. It is also demonstrated that by tuning the angle of the tilt-mirror, high frequency illumination pattern is created which further improve the image resolution while maintaining the same field of view. The technique is experimentally demonstrated for both the fluorescence nano particles and biological samples.

Next, the tilt-mirror based illumination configuration is exploited for the high space-bandwidth product SIM imaging. By incorporating a sequence of illumination patterns with gradually increasing spatial frequencies generated by the tilt-mirror assembly covers up the frequency spectra in a cumulative manner and thus delivers high space-bandwidth product SIM imaging. However, it is observed that the existing reconstruction methods fail to deliver the appropriate reconstruction for the illumination frequencies beyond the detection passband support. A reconstruction strategy is presented that is capable to provide appropriate SIM reconstructed images even if the excitation peak lies very far outside the passband limit. It is verified that the proposed scheme can be extended up to infinity in ideal situations and the quality is degraded with the presence of noise in realistic scenario. The existing reconstruction algorithms developed for the SIM technique suffers from several

artifacts e.g. honeycomb, ghosting, hatching and so on, these artifacts sometimes provide misinterpretations about the sample. In order to get rid these issues, a blind reconstruction algorithm is proposed which does not require any prior knowledge of the illumination. It brings the spatial fluctuation and temporal photo-switching based imaging techniques together under a general category. The only criteria required to be satisfied for the algorithm is that the average over the illumination patterns is uniform. The super-resolution capability of the proposed algorithm is verified for both the sinusoidal and multi-periodic lattice illumination microscopy.

The trans-illumination type SIM architecture is further exploited for the nonlinear SIM imaging as well. A broader range of illumination frequencies acquired by the detection system result in an improved achievable spatial resolution, which represents that a high-frequency excitation pattern is potentially beneficial to provide superior resolution. Following this fundamental concept, a novel super-resolution technique called saturable absorption assisted nonlinear structured illumination microscopy (SAN-SIM) is proposed by exploring the saturable absorption property of a material. The higher intensity regions of the sinusoidal illumination possess higher transmittance through the saturable absorber as compared to the low-intensity region. As a result, the effective illumination becomes sharper in comparison to the input sinusoidal pattern and contains broader spectral support. The technique is capable of providing a resolution similar to the conventional nonlinear SIM without the requirement of highly photo-stable fluorescence tagging molecules which work at high power illumination or specific dyes that exhibit nonlinear response at low intensity level. The effective nonlinear illumination possesses higher harmonics which multiply fold high frequency components within the passband and hence offers more than two-fold resolution improvement over the diffraction limit. The theoretical background of the technique is presented supported by the numerical results. The simulation is performed for both symmetric as well as random samples where the raw moiré frames are processed through a blind reconstruction approach. The results demonstrate the super-resolution capability of the proposed technique.

In conclusion, some current state-of-the-art technical developments are discussed in a nutshell. Finally, some future outlooks are presented which might develop fruitfully and pave the way for new discoveries in the near future.

## सार

यह शोध प्रबंध मुख्य रूप से प्रतिसरण बनावट में उत्तम-विश्लेषण प्रकाशिक सूक्ष्मदर्शिकी प्रतिबिंब पर प्रकाश डालती है। शोध प्रबंध के केंद्र में, संरचित प्रदीपन सूक्ष्मदर्शन (सिम) पद्धति है जिसे विभिन्न दृष्टिकोणों के साथ समन्वेषण किया गया है। पारंपरिक सिम में प्रदीपन तथा संग्रहण के लिए एक ही वस्तुनिष्ठ लेंस का प्रयोग किया जाता है। इसलिए, प्रदीपन और फ्लोरोसेंट उत्सर्जन प्रकाश संग्रह दोनों विचाराधीन वस्तुनिष्ठ लेंस की विवर्तन सीमा द्वारा प्रतिबंधित हैं। सर्वोत्तम संभव विश्लेषण प्राप्त करने की दौड़ में, उच्च संख्यात्मक द्वारक (एनए) वस्तुनिष्ठ लेंस आमतौर पर मानक सिम पद्धति में नियोजित होते हैं। इसलिये, इस तरह के वस्तुनिष्ठ लेंस का उपयोग करने वाली उत्तम-विश्लेषण प्रतिबिंब, अपना द्रश्य-क्षेत्र के साथ समझौता करती दिखाई देती है। हालांकि, पूरा अध्ययन कम एनए वस्तुनिष्ठ लेंस को नियोजित करके किया जाता है जो खराब विश्लेषण की कीमत पर प्रतिबिम्बन के एक बड़े द्रश्य-क्षेत्र की पुष्टि करता है। झुकाव-दर्पण आधारित बनावट द्वारा उत्पन्न साइनसॉइडल संरचित प्रदीपन के प्रयोग द्वारा विश्लेषण को बाद में बढ़ाया जाता है। दो विपरीत स्थापित किए गए दर्पण मुख साइनसॉइडल संरचित पैटर्न का उत्पादन करने के लिए एक बार में कार्यात्मक होते हैं और दर्पण मुखों की षट्कोणीय श्रंखला तीन अभिविन्यासों में समदैशिक विश्लेषण के उद्देश्य को पूरा करती है। यह भी प्रदर्शित किया जाता है कि झुकाव-दर्पण के कोण को परिवर्तित करके, उच्च आवृत्ति प्रदीपन पैटर्न बनाया जाता है जो समान द्रश्य-क्षेत्र को बनाए रखते हुए प्रतिबिंब विश्लेषण को और बेहतर बनाता है। यह प्रविधि को फ्लोरोसेंस नैनो कणों और जैविक नमूनों दोनों के लिए प्रयोगात्मक रूप से प्रदर्शित किया गया है।

इसके बाद, उच्च स्पेस-बैंडविड्थ गुणनफल सिम प्रतिबिंब के लिए झुकाव-दर्पण आधारित प्रदीपन विन्यास का प्रयोग किया जाता है। झुकाव-दर्पण बनावट द्वारा उत्पन्न धीरे-धीरे बढ़ती स्थानिक आवृत्तियों के साथ प्रदीपन पैटर्न के अनुक्रम को शामिल करके आवृत्ति स्पेक्ट्रा को संचयी तरीके से विस्तारण किया जाता है और इस प्रकार उच्च स्पेस-बैंडविड्थ गुणनफल सिम प्रदान करता है। हालांकि, यह देखा गया है कि मौजूदा पुनर्निर्माण विधियां डिटेक्शन पास पारण बैंड समर्थन से परे प्रदीपन आवृत्तियों के लिए उपयुक्त पुनर्निर्माण देने में विफल रहती हैं। एक पुनर्निर्माण रणनीति प्रस्तुत की जाती है जो उपयुक्त सिम पुनर्निर्मित चित्र प्रदान करने में सक्षम है, भले ही उत्तेजन शीर्ष पारण बैंड सीमा से बहुत दूर हो। यह सत्यापित किया जाता है कि प्रस्तावित योजना को आदर्श स्थितियों में अनंत तक बढ़ाया जा सकता है और यथार्थवादी परिदृश्य में शोर की उपस्थिति के साथ गुणवत्ता खराब हो जाती है। सिम तकनीक के लिए विकसित मौजूदा पुनर्निर्माण एल्गोरिदम कई कलाकृतियों से ग्रस्त हैं जैसे हनीकॉम्ब, घोस्टिंग, हैचिंग आदि, ये कलाकृतियाँ कभी-कभी नमूने के बारे में गलत व्याख्याएँ प्रदान करती हैं। इन मुद्दों से छुटकारा पाने के लिए, एक अंधा-पुनर्निर्माण एल्गोरिथ्म प्रस्तावित किया गया है जिसमें प्रदीपन के किसी भी पूर्व ज्ञान की आवश्यकता नहीं है। यह एक सामान्य श्रेणी के तहत स्थानिक उतार-चढ़ाव और सामयिक फोटो-

स्विचिंग आधारित प्रतिबिंबन तकनीकों को एक साथ लाता है। कलनविधि सफल होने के लिए आवश्यक एकमात्र मानदंड यह है कि प्रदीपन पैटर्न का औसत एक समान हो। प्रस्तावित कलनविधि की उत्तम-विश्लेषण क्षमता को साइनसाँइडल और बहु-आवधिक जाली प्रदीपन सूक्ष्मदर्शन दोनों के लिए सत्यापित किया गया है।

गैर-रेखीय सिम प्रतिबिम्बन के लिए भी संचारित-प्रदीपन प्रकार के सिम संरचना का उपयोग किया जाता है। डिटेक्शन प्रणाली द्वारा प्राप्त प्रदीपन आवृत्तियों की एक विस्तृत श्रृंखला के परिणामस्वरूप एक बेहतर स्थानिक विश्लेषण प्राप्त होता है, जो दर्शाता है कि बेहतर विश्लेषण प्रदान करने के लिए एक उच्च आवृत्ति उत्तेजन पैटर्न संभावित रूप से फायदेमंद है। इस मौलिक अवधारणा के बाद, एक पदार्थ की संतृप्त अवशोषण विशेषता का प्रयोग कर एक नवीन सुपर- विश्लेषण तकनीक जिसे सैचुरेबल एब्जॉर्प्शन असिस्टेड गैर-रेखीय संरचित प्रदीपन सूक्ष्मदर्शन (सैन-सिम) पद्धति कहा जाता है, प्रस्तावित है। साइनसाँइडल प्रदीपन के उच्च तीव्रता वाले क्षेत्रों में निम्न-तीव्रता वाले क्षेत्र की तुलना में संतृप्त अवशोषक के माध्यम से उच्च संप्रेषण होता है। नतीजतन, प्रभावी प्रदीपन इनपुट साइनसाँइडल पैटर्न की तुलना में तीक्ष्ण हो जाता है और इसमें वर्णक्रमीय विस्तार व्यापक होता है। तकनीक अत्यधिक फोटो-स्थिर प्रतिदीप्ति टैगिंग अणुओं की आवश्यकता के बिना पारंपरिक नॉनलाइनियर सिम के समान एक विश्लेषण प्रदान करने में सक्षम है जो उच्च शक्ति प्रदीपन या विशिष्ट रंजकों पर काम करते हैं जो कम तीव्रता पर गैर-रेखीय प्रतिक्रिया प्रदर्शित करते हैं। प्रभावी गैर-रेखीय प्रदीपन में उच्च हार्मोनिक्स होते हैं जो पारण बैंड के भीतर उच्च आवृत्ति घटकों को गुणा करते हैं और इसलिए विवर्तन सीमा पर दो गुना से अधिक विश्लेषण सुधार प्रदान करते हैं। तकनीक की सैद्धांतिक पृष्ठभूमि संख्यात्मक परिणामों द्वारा समर्थित प्रस्तुत की जाती है। सिमुलेशन दोनों सममित और यादृच्छिक नमूनों के लिए किया जाता है जहां रॉ मॉडरे फ्रेम को अंधा पुनर्निर्माण दृष्टिकोण के माध्यम से संसाधित किया जाता है। परिणाम प्रस्तावित तकनीक की उत्तम-विश्लेषण क्षमता को प्रदर्शित करते हैं। उपसंहार के रूप में कुछ वर्तमान अत्याधुनिक तकनीकी विकासों पर संक्षेप में चर्चा की गई है।

अंत में, कुछ भविष्य के दृष्टिकोण प्रस्तुत किए जाते हैं जो फलदायी रूप से विकसित हो सकते हैं और निकट भविष्य में नई खोजों का मार्ग प्रशस्त कर सकते हैं।

## Organization of the thesis

Chapter 1 provides the preface and essential outlines of Structured Illumination Microscopy (SIM) as a widefield super-resolution imaging technique. At first, the history of microscopy, basics of the fluorescence microscopy, diffraction limited resolution are discussed in a nutshell. Then a few significant techniques are mentioned which are primarily developed to overcome the diffraction limited resolution of the fluorescent microscopes and influenced the bio-medical, bio-engineering studies and research to visualize the sub-cellular features with fine details. These ground-breaking revolutionary super-resolution approaches are capable to push the resolution limit of microscopes towards the nanoscale dimension and hence a new field of research called ‘optical nanoscopy’ is emerged in the last two decades. Although all the super-resolution techniques are launched in order to serve a common purpose, they are distinguished in their origin. Each of the techniques appears with its unique working principle and their characteristics relative to each other are described briefly. Next, the SIM is highlighted with a short discussion on its advantages and disadvantages with respect to other techniques. Then, the details of SIM technique is described including the fundamental theory as well as the reconstruction approach existing so far in the literature.

Chapter 2 presents tilt-mirror assisted transmission structured illumination microscopy. In the conventional linear SIM technique, a single objective lens is employed for illuminating the sample as well as for collecting the fluorescence signal. Consequently, both the illumination and the detection optics are diffraction limited by the same objective lens and the system is strictly restricted to offer  $\leq 2$ -fold resolution improvement over the diffraction limit. However, in the race of achieving the best possible resolution, very high numerical aperture objective lenses are utilized in general for the SIM imaging. The super-resolution image in SIM employing the high NA objective lens is obtained by compromising with the field of view (FOV). This limitation appears because of the inherent trade-off between the resolution and the FOV of the standard imaging system. So, the next level super-resolution techniques deal with large FOV imaging while improving the resolution. Nonetheless, this typical trade-off can be tricked if the illumination path is decoupled from the

collection path using a trans-illumination kind of optical configuration. Thus, making the illumination optics free from the diffraction limit of the collection objective lens facilitates the full flexibility to play with the illumination configuration. Herein, we demonstrate the experimental proof-of-concept of the tilt-mirror assisted transmission type SIM technique which possesses potential to exploit the high throughput SIM utilizing the free space optics in a cost-effective way. This is also the first experimental proof that describes it is possible to design the illumination for large FOV SIM without guiding the light through any condenser lens, waveguide chip or advanced materials.

In chapter 3, the potential of transmission SIM approach is presented which can offer multiple-fold improvement of the lateral resolution that requires sequential higher frequency sinusoidal illumination patterns. Employing low-magnification/low-NA objective lens in the detection system, the super-resolution imaging is performed with a large FOV. In addition, employing sequential illumination patterns with gradually increasing spatial frequencies in tSIM covers up the frequency spectra without compromising with the FOV i.e. it delivers high space-bandwidth product SIM imaging. However, the existing reconstruction algorithms delivers wrong estimation of the pattern frequency when the illumination frequency lies very far from the passband limit and fail to provide appropriate reconstruction. In this chapter, a correlation based frequency estimation approach is described for sequential high frequency illumination patterns with different illumination frequencies in gradually increasing orders; the results are shown for three different sets of illuminations. Additionally, the effect of different noise-levels in the reconstruction is also demonstrated; which reveals that noise affects image contrast as well as resolution. However, the proposed reconstruction approach is capable to robustly estimate illumination frequency under different noise-levels. It can be extended further for more sets of illuminations and can be employed to obtain theoretically unlimited resolution. This technique is considered to be an efficient approach of SIM reconstruction which can be implemented in variety of custom-designed systems where illumination frequency lies far outside the passband limit (e.g. non-linear SIM, plasmonic SIM and so on).

In chapter 4, the utility of optical lattice illumination is envisaged and a super-resolution image reconstruction approach is presented without assuming the knowledge of the illumination patterns. Sinusoidal and multi-periodic patterns are considered with three different orientations that are generated by the phase engineered interference of coplanar beams. In both cases, the raw moiré frames are acquired using a test object with multiple phase-shifted lattice illuminations. The phase-shifting is executed in consecutive steps such that the average of all the recorded frames over the entire acquisition remains uniform. The change in the illumination patterns results in the variation of fluorescence emission at a given point in the sample over time. Therefore, methods that use temporal variations in fluorescence intensity are the candidates for reconstruction algorithms. However, most of these methods are based on the assumption that each fluorescent emitter behaves independent to other emitters in terms of photo-emissions. This condition is violated in the case of optical lattice illuminations since photo-emissions from different emitters are a function of the spatial distribution of illumination patterns and are, therefore, not independent. The raw frames over the entire acquisition in such cases are processed via multiple signal classification algorithm generate a super-resolved image, where it exploits the systematic variation in illumination distribution for super-resolution. In this work, the reconstruction approach creates a bridge that connects temporal fluctuation and spatial fluctuation based imaging, together. The use of a periodic pattern in this blind reconstruction requires only as many raw images as needed for diffraction-limited isotropic optical lattice microscopy images. This implies super-resolution at no extra trade-off in temporal resolution, having a direct advantage against a limited photon budget. It also implies that a special arrangement for speckle-like or pseudo-random illumination can be excluded by the current approach of performing SIM without the knowledge of the illumination patterns. The efficiency of reconstruction approach is explained through the eigen-image analysis. Additionally, the multi-periodic illumination pattern generated by the interference of four coplanar beams containing both lateral as well as axial periodicity offers potential new approaches in three-dimensional super-resolution imaging and light-sheet microscopy which can be used for the future explorations.

In chapter 5, a novel nonlinear SIM approach called the saturation absorption assisted nonlinear structured illumination microscopy is presented by exploiting the saturable absorption property of the absorbing material. The proposed method is

capable to provide resolution similar to the traditional nonlinear-SIM without the requirement of a highly photo-stable fluorescence tagging molecules which work at high power illumination. Moreover, depending upon the nature of the absorbing layer, the intensity of the illumination pattern is scalable and an appropriate selection of material can offer to configure the system suitable to operate in different wavelength regime. The proposed nonlinear illumination scheme is designed for the transmission SIM architecture where the detection is decoupled from the illumination. Thus, the incident sinusoidal pattern is converted into an effective illumination with nonlinear characteristics instigated by the multiple harmonics present in the pattern after propagating through the SA medium. The effective nonlinear illumination containing higher harmonics multiply folds the high frequency components within the detection passband and hence offers more than 2-fold resolution improvement over the diffraction limit. The theoretical background of the technique is presented supported by the numerical results. The simulation is performed for both symmetric as well as random samples where the raw moiré frames are processed through a blind reconstruction approach developed for the nonlinear SIM. The results demonstrate the super-resolution capability of the proposed technique.

Chapter 6 sums up the research works reported in the previous chapters in a common thread and provides the important conclusions of the thesis. The studies performed so far deals only with the 2D SIM cases, which can be extended towards 3D SIM. Besides, multi-colour SIM, live-cell imaging, fluorescent dipole mapping, digital holographic microscopy, coherent imaging, expansion microscopy and so on might be the possible future explorations into the tSIM architecture. Moreover, the implementations of the SIM technique in various other imaging modalities is described in order to enhance their imaging performances with the augmented capabilities. Nonetheless, some future outlooks in the research area of SIM are mentioned which might develop fruitfully and pave the way for new discoveries in the near future.

# Contents

Certificate

Acknowledgements

Abstract

Organization of the thesis

Contents

List of Figures

List of Tables

Abbreviations

Symbols

<b>1</b>	<b>Introduction</b>	<b>1</b>
1.1	Brief history of microscopy . . . . .	1
1.1.1	Fluorescence microscopy . . . . .	2
1.1.2	Diffraction limited resolution . . . . .	4
1.2	Super-resolution microscopy (optical nanoscopy) . . . . .	5
1.3	Structured Illumination Microscopy (SIM) . . . . .	8
1.3.1	Theoretical background . . . . .	8
1.3.2	Image reconstruction . . . . .	11
1.3.2.1	fairSIM . . . . .	14
1.3.2.2	OpenSIM . . . . .	15
1.3.2.3	SIMcheck . . . . .	15

1.3.2.4	SIMToolbox . . . . .	15
1.3.2.5	ML-SIM . . . . .	16
1.3.2.6	HiFi-SIM . . . . .	17
1.4	Various SIM techniques . . . . .	18
1.4.1	TIRF SIM . . . . .	18
1.4.2	Non-linear SIM . . . . .	19
1.4.3	Plasmonic SIM . . . . .	21
1.4.4	Speckle SIM . . . . .	23
1.4.5	Spot-scanning SIM . . . . .	25
1.4.6	Lattice SIM . . . . .	27
1.5	Objectives of the thesis . . . . .	30
<b>2</b>	<b>Tilt-mirror assisted transmission SIM</b>	<b>31</b>
2.1	Introduction . . . . .	31
2.2	Theory and optical system . . . . .	33
2.3	Results . . . . .	38
2.3.1	tSIM imaging with conventional illumination . . . . .	38
2.3.2	tSIM imaging with high frequency illumination . . . . .	42
2.3.3	Scalable tSIM imaging . . . . .	44
2.4	Conclusion . . . . .	45
<b>3</b>	<b>Image reconstruction of high space-bandwidth product SIM</b>	<b>49</b>
3.1	Introduction . . . . .	49
3.2	High space-bandwidth product SIM imaging . . . . .	51
3.3	Reconstruction strategy for high SBP SIM . . . . .	54
3.3.1	Phase estimation of the illumination pattern . . . . .	54
3.3.2	Illumination spatial frequency estimation . . . . .	56
3.4	Results . . . . .	60
3.4.1	Results for isotropic resolution enhancement . . . . .	66
3.4.2	Effect of different photon-levels in the reconstruction . . . . .	67
3.5	Conclusion . . . . .	69
<b>4</b>	<b>Blind reconstruction technique for SIM</b>	<b>71</b>
4.1	Introduction . . . . .	71
4.2	Lattice illumination microscopy with the blind reconstruction . . . . .	73
4.2.1	MUSICAL in sinusoidal lattice illumination . . . . .	75
4.2.2	MUSICAL in multi-periodic lattice illumination . . . . .	84
4.3	Conclusion . . . . .	88
<b>5</b>	<b>Saturation absorption assisted nonlinear SIM</b>	<b>89</b>
5.1	Introduction . . . . .	89
5.2	Theory . . . . .	90

5.3	Results . . . . .	95
5.4	Conclusion . . . . .	100
<b>6</b>	<b>Conclusion</b>	<b>101</b>
6.1	Important conclusions of the thesis . . . . .	101
6.2	Current state-of-the-art . . . . .	103
6.3	Scope for future study . . . . .	105
<b>A</b>	<b>Tilt-mirror assisted transmission SIM</b>	<b>109</b>
A.1	Comparison between conventional SIM and transmission SIM . . . . .	109
A.2	Preparation for fluorescent-beads sample . . . . .	110
A.3	Cell culture and labeling . . . . .	111
A.4	Experimental setup and data acquisition . . . . .	111
A.5	Image reconstruction . . . . .	112
A.6	Tilt-mirror angle-tuning and volumetric interference . . . . .	112
A.7	Nitty-gritty details of the experimental setup . . . . .	114
A.8	Analysis of tSIM result for fluorescent beads . . . . .	115
A.9	Power spectral support for the beads data . . . . .	116
A.10	Raw moiré frames and fringe contrast . . . . .	117
A.11	Analysis of the SIM result for the actin . . . . .	118
<b>B</b>	<b>High space-bandwidth product SIM algorithm</b>	<b>121</b>
B.1	Algorithm pseudo-code . . . . .	121
<b>C</b>	<b>Moiré frames for high space-bandwidth product SIM</b>	<b>123</b>
C.1	Raw moiré frames used in the reconstruction process . . . . .	123
<b>D</b>	<b>Blind reconstruction technique for SIM</b>	<b>127</b>
D.1	Summary of the MUSICAL algorithm . . . . .	127
D.2	Eigen-images and eigen-spectra . . . . .	128
D.3	Performance of MUSICAL . . . . .	130
D.4	Scheme of phase-modulated multi-periodic lattices . . . . .	132
D.5	Experimental work . . . . .	134
D.6	Frequency support of MUSICAL . . . . .	135
<b>E</b>	<b>Saturable absorption assisted nonlinear SIM</b>	<b>137</b>
E.1	Saturable absorbing materials . . . . .	137
E.2	Flowchart of the reconstruction . . . . .	139
E.3	Result for isotropic resolution improvement . . . . .	140
E.4	Effect of noise for the Siemen's star image . . . . .	142
E.5	Analysis for different sample regions . . . . .	142

E.6	Power spectral support of different techniques . . . . .	143
E.7	Effect of noise to the random sample . . . . .	144
E.8	Effect of number of frames to final image . . . . .	145
E.9	Graphics of the SAN-SIM setup . . . . .	146
<b>F</b>	<b>Code Resources</b>	<b>147</b>
	<b>Bibliography</b>	<b>149</b>
	<b>List of Publications</b>	<b>163</b>

# List of Figures

1.1	Scenario of the scale of resolution in terms of commonly known objects	2
1.2	(a) Jablonski diagram represents the fluorescence and phosphorescence mechanism; (b) fluorescence excitation-emission spectra of a commercial dye (Alexa Fluor 532) plotted using the spectraviewer of Thermo Fisher Scientific. . . . .	3
1.3	(a) Point spread function of a microscope; (b) two points emitters separated at a distance below diffraction limit; (c-e) two points emitters separated according to Sparrow, Abbe and Rayleigh criteria respectively. . . . .	5
1.4	Visualization of the moiré patterns: (a) the superposition of two 1D grating patterns twisted by $8^\circ$ and $-6^\circ$ (clockwise) w.r.t. the central grating; (b) overlap of two 2D hexagonal patterns rotated by $18^\circ$ and $6^\circ$ (clockwise) with respect to the middlemost pattern . . . . .	9
1.5	Basic concept of SIM: (a) frequency content of sinusoidal illumination pattern (for $\theta = 0^\circ$ ); (b) passband support of imaging system (OTF); (c) spectral content of structured illuminated sample (for $\theta = 0^\circ$ ) composed of linear combination of three different spectral regions; (d) isotropic frequency extension in three orientations; (e) hypothetical sample spectra (for explanation); (f) diffraction limited spectra; (g) spectra of moiré image; (h) separated frequency components (pseudo-coloured components represent different region of sample spectra); (i) stitched spectra combining the frequency components. . . . .	10
1.6	(a) TIRF SIM illumination scheme; (b) standing evanescent wave generation at the interface of the two medium . . . . .	19
1.7	(a) Diffraction limited spectral support (OTF); (b) frequency of the equivalent non-linear structured illumination pattern; (c) SIM frequency extension for non-linear SIM; (d) isotropic filling of frequency space combining 6 different orientations. . . . .	21
1.8	(a) Dispersion relation between temporal frequency( $\omega$ ) vs. wavevector( $k$ ); (b) frequency support of linear SIM; (c) surface plasmon polariton and localized plasmon assisted SIM frequency support. . . . .	22
1.9	Typical system setup of (a) multifocal structured illumination microscopy (MSIM) and (b) optical photon reassignment microscopy (OPRA). . . . .	26

2.1	<p>Concept of tSIM: (a) scheme of the experimental setup for one orientation; (b) tunable frequency illumination patterns are generated by a pair of mirror facets corresponding to low and high frequency illumination respectively; (c) scheme of polarization conversion of the interfering beams where top, middle and below panel corresponds to the state of polarization above, on and below the vortex HWP respectively; (d) hexagonal arrangement of mirror-facets for three orientation angles required for isotropic resolution enhancement; (e) resolution limit represented by the optical transfer function (solid circle), maximal shift (dotted circle) in conventional SIM offers 2-fold resolution enhancement. (f) maximum spectral support of tSIM governed by with periodicity <math>\frac{\lambda}{2\sin\theta_1}</math>, tunable under different interfering angles <math>\theta</math>; (g,h) frequency support of a low-NA detection objective lens under similar illumination configurations. . . . .</p>	34
2.2	<p>Experimental results of fluorescent beads: (a) single FOV tSIM reconstruction and widefield image of 200 nm fluorescent beads; (b) diffraction limited spectra; (c) reconstructed tSIM spectra; (d,f) magnified widefield images of white box in <math>R_1</math> and <math>R_2</math> regions, respectively; (e,g) closeup of the tSIM reconstruction in <math>R_1</math> and <math>R_2</math> regions, respectively; (h) intensity line-profiles of (d,e); (i) intensity line-profiles of (f,g). . . . .</p>	39
2.3	<p>tSIM imaging of actin filaments in U2OS cells: (a) single FOV tSIM reconstruction vs. diffraction limited image with the excitation/emission wavelengths of 532 nm/560 nm and inset shows spectra of the reconstructed image; (b,d) diffraction limited images of regions <math>R_1</math> and <math>R_2</math>; (c,e) reconstructed tSIM image of regions <math>R_1</math> and <math>R_2</math> respectively; (f,g) intensity profile of a line marked between the arrowheads in (c.e). . . . .</p>	41
2.4	<p>High-frequency tSIM imaging of actin-stained U2OS cell: (a) single FOV tSIM reconstruction and diffraction limited image with the excitation/emission wavelengths of 532 nm/560 nm and inset shows spectra of the reconstructed image; (b,d) widefield image of <math>R_1</math> and <math>R_2</math> regions respectively; (c,e) reconstructed image of <math>R_1</math> and <math>R_2</math> regions respectively; (f,g) intensity line profiles between the magenta and blue arrowheads. . . . .</p>	43
2.5	<p>Actin of U2OS cell stained with alexa 532: (a) widefield image; (b) SIM reconstruction image; (c,d) magnified widefield images of white box in <math>R_1</math> and <math>R_2</math> regions respectively; (e,f) closeup of the SIM images in <math>R_1</math> and <math>R_2</math> regions respectively; (g) intensity line-profiles of (c,e) shown by magenta colour; (h) intensity line-profiles of (d,f) shown by blue colour. . . . .</p>	44

3.1	Different sets of illuminations: (a) set $j=1$ corresponds to conventional SIM case, (b) set $j=2$ and (c) set $j=3$ represent cases where illumination frequencies lie outside the detection passband limit (OTF) in a sequence. . . . .	52
3.2	(a) Sample spectra, (b) SIM spectra (log scale) for illumination frequency inside OTF support (inset: spectral correlation shows sufficient overlap), (c) SIM spectra (log scale) for illumination frequency outside OTF (inset: less spectral correlation), (A) Ground-truth test object, (B) SIM reconstructed image for illumination frequency within OTF, (C) SIM image artifacts for illumination frequency outside OTF. . . . .	55
3.3	Reconstruction strategy for illumination frequencies $j=1, j=2, j=3$ . Nine raw moiré frames for each set (three $\theta$ s and three $\varphi$ s) are considered as the input and the Fourier transformed raw frames are shown by the image stack. In each set ( $j$ ), phase has been optimized first and then three frequency lobes are separated by solving the matrix equations. For set $j=1$ , correlation between central lobe and one of the side frequency lobe efficiently estimates the pattern frequency (for $\theta = 0$ ). Then frequency hepta-lobes obtained from the three orientations ( $\theta$ s) are merged together to obtain $\tilde{C}_{j=1}^{SIM}(\mathbf{k})$ . In the next set ( $j=2$ ), the SIM frequency has been utilized; correlation is evaluated between $\tilde{C}_{j=1}^{SIM}(\mathbf{k})$ and one of the side component of second set. Isotropic spectral stitching leads to synthesize broad spectra $\tilde{C}_{j=2}^{SIM}(\mathbf{k})$ which is used further for the third set ( $j=3$ ) and so on. In the similar way, the scheme can be extended for $j= 4, 5 \dots$ to $\infty$ . . . . .	58
3.4	(a-c) Frequency support of synthesized SIM spectra for the set of illumination patterns $j=1, j=2, j=3$ considered individually; (A-C) reconstructed SIM images obtained from the inverse Fourier transforms of the frequency spectra (a-c) respectively. . . . .	59
3.5	(a) Sample spectra; (b) diffraction limited spectra; (c) SIM spectra considering upto three sets of illumination patterns $j=1, j=2, j=3$ together; (A) ground-truth sample; (B) diffraction limited image; (C) high SBP SIM reconstructed image for collective contribution from three sets. . . . .	61
3.6	(a,b,c) SIM image considering 10%, 20% and 30% additive Gaussian noise for the set $j=1$ ; (a <sub>1</sub> -c <sub>1</sub> ), (a <sub>2</sub> -c <sub>2</sub> ) magnified views of the selected box in region 1 and region 2 of (a-c) respectively; (d <sub>1</sub> -d <sub>2</sub> ) corresponding intensity profiles along a line in region 1 and region 2, green, cyan and magenta curves correspond to the 10%, 20% and 30% noise-level. . . . .	62

3.7	(a-c) SIM spectra for the set of illumination patterns $j=1, j=2, j=3$ considered individually for $\theta_1 = 30^\circ, \theta_2 = 150^\circ, \theta_3 = 270^\circ$ , (d) SIM spectra considering upto three set of illumination patterns $j=1, j=2, j=3$ together for six orientations collectively (no gap in the spatial frequency space), (A-D) reconstructed SIM images obtained from the inverse Fourier transforms of the frequency spectra (a-d) respectively.	66
3.8	(a-d) reconstructed images for different average photon levels of $10^0, 10^1, 10^2, 10^3$ considering three sets of illuminations $j=1, j=2, j=3$ together; (A-D) magnified versions of a small regions of (a-c) shown by green, blue, magenta and yellow colour respectively.	67
3.9	Plot of average phase errors with different photon-levels for three sets of illuminations $j=1, j=2, j=3$ shown by blue, red and magenta colours respectively.	68
4.1	Example structure and simulation results of acquired data using sinusoidal lattice illumination and processing with MUSICAL: (a) Ground-truth sample; (b) widefield image for the emission ( $\lambda_{em}$ ) = 450 nm and NA =0.3; (c) reconstructed image obtained using MUSICAL; (d) magnified view of a small portion of (c); (e) radial line-scan of ground-truth, widefield and reconstructed image; (f) spatial frequency spectra of the imaging configuration for the lattice illumination in three different orientations $R_1, R_2$ and $R_3$ .	76
4.2	(a1-a7) Projection of different orders or eigen-images displayed in increasing order, scalebar = $2\mu m$ ; (b1-b7) Fourier spectra of the eigen-images (a1-a7) displayed in the $\log_{10}$ scale, scalebar = $0.8\mu m^{-1}$ ; (c-e) spectra of ground-truth sample, widefield image and MUSICAL image; (f) plot of the normalized power spectral spread.	78
4.3	Experimental results with 100 nm (diameter) fluorescent beads sample: (a) widefield image; (b) MUSICAL reconstruction; (A-B) the magnified version of a selected region of (a-b) with scalebar $1\mu m$ ; (c) intensity scan profile across a line within the selected box.	80
4.4	(a-b) MUSICAL analysis on SLM-SIM fluorescent bead dataset: (a) Widefield image, (b) MUSICAL reconstruction. B1 and B2 corresponds to the MUSICALy reconstructed versions of the magnified sections A1 and A2 in the widefield image. (c-e) Similar analysis on TIRF-SIM tubulin dataset: (c) Widefield image, (d) MUSICAL reconstruction. D corresponds to the MUSICALy reconstructed version of the section C in the widefield image (e) intensity scan profile along a line segment shown in image C and D.	82

4.5	Further analytical insight into the microtubulin example in figure 4.4 (c,d) where MUSICAL does not apparently provide significant resolution enhancement. (a) shows the average image, (b) shows the standard deviation image, (c) shows the ratio of standard deviation to the average intensity computed for each pixel. The colorbar in (a-b) shows intensity units, (c) shows ratio (unit-less). In (c), two regions are identified, where region A is already analyzed figure 4.4 and region B is a region where the ratio of standard deviation to the average intensity is quite high. Region B indicates a region in which illumination contributed significant fluctuations. (d) shows the MUSICAL reconstruction reproduced from figure 4.4. (e,f) show the zoom-ins of regions A and B (both widefield and MUSICAL), and also the comparison at a relevant cross-section. . . . .	83
4.6	Scheme of multi-periodic lattice illumination and simulation results: (a) Graphic of illumination scheme for multi-periodic lattice through interference of four coplanar beams; (b) magnified view of the volumetric interference pattern in the sample (S) region; (c) frequency spectra of the multi-periodic lattice illumination; there is a mixture of four different frequencies $\frac{(f_2-f_1)}{2} = 0.3\lambda^{-1}$ , $f_1 = 0.68\lambda^{-1}$ , $\frac{(f_2+f_1)}{2} = 0.98\lambda^{-1}$ and $f_2 = 1.28\lambda^{-1}$ in increasing order respectively; (d) 2D spectral spread in multi-periodic lattice illumination imaging with isotropic coverage of the higher frequency space in three orientations for numerical aperture NA = 0.3 and excitation wavelength = 400 nm; (e) Diffraction limited widefield image of the sample; (f) MUSICAL reconstructed image for multi-periodic pattern; (g) magnified view of a small portion of (f); (h) radial line-scan of ground-truth, widefield and MUSICAL image. . . . .	86
5.1	(a) Graphical representation of the illumination configuration; (b) magnified view of the conversion scheme of the illumination pattern; (c) efficiency of selected SA material; (d) frequency spectra of the nonlinear and sinusoidal illumination shows the strengths of different harmonics; (e) line profiles of the effective nonlinear and the sinusoidal illumination pattern. . . . .	92
5.2	(a) Synthetic ground-truth sample; (b) wide-field image of the sample; reconstructed images with (c) sinusoidal pattern (conventional SIM) and (d) SAN-SIM approach. . . . .	96
5.3	(a) Random ground-truth sample; (b) wide-field image; (c) conventional SIM image; (d) reconstructed image for SAN-SIM; (e-h) magnified view of a region indicated by the white box in (a-d) respectively, scale bar = 0.5 $\mu\text{m}$ ; (i) intensity profiles across a line between the arrows in (e-h). . . . .	98

A.1	Schematic of the experimental setup of (a) conventional SIM and (b) tilt-mirror assisted transmission SIM. . . . .	110
A.2	(a) Side-view of the graphical experimental setup; (b) volumetric interference and span of illumination region. . . . .	113
A.3	(a) Photograph of the real experimental setup; (b) metallic hexa mirror arrangement with the micrometer assisted angle tuning; (c) magnified view of a single tilt-mirror unit; (d) perspective view of single mirror unit; (e-f) angle tuning scheme of a single mirror facet; (g) coupled translation and rotation stage for the change of orientation and phase-shifting of the physical grating. . . . .	114
A.4	Experimental results of fluorescent beads: (a) diffraction limited image; (b) SIM reconstructed image; (a <sub>1</sub> -a <sub>6</sub> ) magnified views of (a) in different regions; (b <sub>1</sub> -b <sub>6</sub> ) magnified views of (b) in different regions. . . . .	115
A.5	2D spatial frequency spectra of SIM data for fluorescent beads: (a-c) three separated spectral bands used for the reconstruction process where peaks corresponding to the illumination frequency and orientation are marked with the circles; (d) diffraction limited spectra; (e) reconstructed SIM spectra. . . . .	116
A.6	Raw moiré frames for the actins in three different set of illumination patterns (a1-c1,a2-c2,a3-c3). . . . .	117
A.7	Intensity line profiles of the contrast of the fringes for three phase-shifted patterns. . . . .	118
A.8	Experimental results of actin filaments of U2OS cell stained with alexa-fluor 532 fluorescent tagging: (a) diffraction limited image; (b) SIM reconstructed image; (a <sub>1</sub> -a <sub>4</sub> ) magnified views of (a) in different regions; (b <sub>1</sub> -b <sub>4</sub> ) magnified views of (b) in different regions. . . . .	119
C.1	Moiré frames for 3 orientations and 3 phases in illumination set $j=1$ , scalebar = 3 $\mu\text{m}$ . . . . .	124
C.2	Moiré frames for 3 orientations and 3 phases in illumination set $j=2$ , scalebar = 3 $\mu\text{m}$ . . . . .	125
C.3	Moiré frames for 3 orientations and 3 phases in illumination set $j=3$ , scalebar = 3 $\mu\text{m}$ . . . . .	126
D.1	Summary of the MUSICAL reconstruction used for sinusoidal optical lattice raw data. The green panel below represents flowchart of the MUSICAL. . . . .	127
D.2	Projection of the set of eigen-images of the circle-sample for sinusoidal illumination, scalebar corresponds to 2 $\mu\text{m}$ . . . . .	129
D.3	Frequency spectra of the projection of eigen-images of the circle-sample, plotted in $\log_{10}$ scale shown by hot color-map, scalebar corresponds to 0.8 $\mu\text{m}^{-1}$ . . . . .	130

D.4	Comparison of the MUSICAL reconstruction with fairSIM reconstruction with 100 nm fluorescent beads sample: (a) widefield image, (b) Wiener filtered widefield image, (c) fairSIM reconstruction, (d) MUSICAL reconstruction; (A-D) are the magnified view of (a-d) with scalebar 1 $\mu\text{m}$ , (e) intensity profiles along a line in the selected box. . . . .	131
D.5	Multi-periodic lattice illumination scheme: (a,b,c) stack of lattice illumination patterns generated by four beam interference with phase step $2\pi/N$ corresponding to $R_1$ , $R_2$ , and $R_3$ configuration respectively; (d) schematic of $R_1$ configuration showing geometry for four coplanar beams interference, $\theta_1 = 20^\circ$ , $\theta_2 = 40^\circ$ are the polar angles of first and second order beam pairs respectively w.r.t. the $z$ axis; (e) average of the lattice illumination patterns for the stack of lattices in the $R_1$ configuration. . . . .	133
D.6	Top row: (a-f) average and bottom row: (g-l) standard deviation of the multi-periodic lattice illumination patterns in $R_1$ configuration for different number of phase-steps $N = 4$ to 9, respectively. . . . .	134
D.7	Graphical representation of the frequency support. . . . .	136
E.1	The efficiency of saturable absorbing material for wavelength $\lambda = 532$ nm. . . . .	138
E.2	The summary of the reconstruction in the form of flowchart. . . . .	140
E.3	(a) Ground-truth sample; (b) widefield image; (c) reconstructed image for conventional SIM; (d-f) reconstructed image for SAN-SIM considering 3, 6 and 9 orientations respectively; (g-i) power spectra represents the frequency support corresponding to the reconstructed image shown in (d-f) respectively. . . . .	141
E.4	(a-d) SAN-SIM results corresponding to the average light-level of $10^0$ , $10^1$ , $10^2$ , $10^3$ photons per pixel respectively; (e-h) magnified view of the white box in (a-d) respectively. . . . .	142
E.5	(a) Ground-truth fluorescent sample; (b) widefield image; (c) reconstructed image for conventional SIM; (d) reconstructed image for SAN-SIM; (a1-d1), (a2-d2) enlarged views of two selected regions of (a-d) shown by yellow and blue coloured boxes respectively, scale bar = 0.5 $\mu\text{m}$ ; (e-f) corresponding intensity profile across a line indicated by the arrows shown by red, green, magenta and blue colour respectively. . . . .	143
E.6	(a-d) Frequency spectra of ground-truth fluorescent bead sample, widefield image, conventional SIM image and SAN-SIM image respectively, scalebar = 4 $\mu\text{m}^{-1}$ ; (e) line profiles of normalized power spectra of (a-d) plotted in $\log_{10}$ scale. . . . .	144
E.7	(a-d) SAN-SIM results for beads sample corresponding to the average light-level of $10^0$ , $10^1$ , $10^2$ , $10^3$ photons per pixel respectively; (e-h) magnified view of the white box in (a-d) respectively. . . . .	144

E.8	(a-f) Reconstructed images for SAN-SIM by considering different number $N = 3, 5, 8, 10, 12, 20$ of frames respectively. . . . .	145
E.9	Graphical representation of the experimental setup of SAN-SIM. SF: spatial filter, CL: collimating lens, BS: beam splitter, SLM: spatial light modulator, L: lens, PH: pinhole, M: mirror, S: sample, MO: microscope objective, FF: fluorescent filter, TL: tube lens. . . . .	146

# List of Tables

1.1	Comparison among various super-resolution techniques . . . . .	7
1.2	Features of different SIM reconstruction tools . . . . .	16
2.1	Super-resolution achieved by tSIM under different illumination-detection conditions . . . . .	45
3.1	Different sets of illumination patterns employed for the study with $\lambda_{ex} = 532$ nm, $\lambda_{em} = 570$ nm, objective lens = 20X/0.4 . . . . .	57
4.1	Resolution obtained using various lattice illumination for $\lambda_{em} = 450$ nm and $NA = 0.3$ , and with and without MUSICAL. The maximum frequency support and resolution gain for MUSICAL is explained in Appendix D6 . . . . .	87
5.1	Parameters used in the simulation study . . . . .	95
C.1	Different set of illumination patterns employed for the simulation . . . . .	123
E.1	Material parameters related to the saturable absorption . . . . .	139



# Abbreviations

<b>PSF</b>	<b>P</b> oint <b>S</b> pread <b>F</b> unction
<b>OTF</b>	<b>O</b> ptical <b>T</b> ransfer <b>F</b> unction
<b>FOV</b>	<b>F</b> ield <b>O</b> f <b>V</b> iew
<b>NA</b>	<b>N</b> umerical <b>A</b> perture
<b>SIM</b>	<b>S</b> tructured <b>I</b> llumination <b>M</b> icroscopy
<b>TIRF</b>	<b>T</b> otal <b>I</b> nternal <b>R</b> eflection <b>F</b> luorescence
<b>FWHM</b>	<b>F</b> ull <b>W</b> idth <b>H</b> alf <b>M</b> axima
<b>CW</b>	<b>C</b> ontinuous <b>W</b> ave
<b>SP</b>	<b>S</b> urface <b>P</b> lasmon
<b>LP</b>	<b>L</b> ocalized <b>P</b> lasmon
<b>SPP</b>	<b>S</b> urface <b>P</b> lasmon <b>P</b> olariton
<b>HWP</b>	<b>H</b> alf <b>W</b> ave <b>P</b> late
<b>SBP</b>	<b>S</b> pace <b>B</b> andwidth <b>P</b> roduct
<b>MUSICAL</b>	<b>M</b> Ultiple <b>S</b> Ignal <b>C</b> lassification <b>A</b> Lgorithm
<b>SA</b>	<b>S</b> aturable <b>A</b> bsorber
<b>UV</b>	<b>U</b> ltra <b>V</b> iolet
<b>SNR</b>	<b>S</b> ignal to <b>N</b> oise <b>R</b> atio



# Symbols

$\mathbf{r}$	position vector
$\mathbf{k}$	spatial frequency/wavevector
$\omega$	angular frequency
$n$	refractive index of the medium
$\lambda_{ex}$	excitation wavelength
$\lambda_{em}$	emission wavelength
$\theta$	polar angle
$\varphi$	phase value
$S$	sample distribution
$I$	intensity of illumination
$h$	point spread function
$H$	optical transfer function
$\mathbf{E}$	electric field vector
$c$	speed of light (free space)
$\varepsilon$	electric permittivity
$N$	number of frames