

**MULTI-MODAL AND HIGH RESOLUTION
QUANTITATIVE PHASE AND FLUORESCENCE
IMAGING SYSTEMS FOR BIOLOGICAL
APPLICATIONS**

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**DEPARTMENT OF PHYSICS
INDIAN INSTITUTE OF TECHNOLOGY DELHI, INDIA
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QUANTITATIVE PHASE AND FLUORESCENCE
IMAGING SYSTEMS FOR BIOLOGICAL
APPLICATIONS**

by

SHILPA TAYAL

DEPARTMENT OF PHYSICS

Submitted

in fulfilment of the requirement for the degree of Doctor of Philosophy

to the



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Dedicated to,

My parents and husband

CERTIFICATE

This is to certify that the thesis entitled, “**MULTI-MODAL AND HIGH RESOLUTION QUANTITATIVE PHASE AND FLUORESCENCE IMAGING SYSTEMS FOR BIOLOGICAL APPLICATIONS**” submitted by **Ms. SHILPA TAYAL** to the Department of Physics, Indian Institute of Technology Delhi for the award of the degree **DOCTOR OF PHILOSOPHY**. This thesis is a record of bonafide research work carried out by her. She has worked under my guidance and supervision and has fulfilled the requirements, which to our knowledge have reached the requisite standard for the submission of this thesis.

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

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Shilpa Tayal

ABSTRACT

The biomedicine industry is rapidly growing and diagnosis requirements have also been increased from last few decades. Most of the biological specimens like HeLa, MG63, human MSc cell line, RBCs etc. are transparent in nature. A traditional optical microscope is well-known technique to visualize micron size particles, but it fails to provide good contrast images of the transparent specimen. In abundant cases, the functional as well as quantitative information is required to analyze the effect of the diseases. Quantitative phase microscopy (QPI) works on the principle of wave-front distortion, where collimated beam illuminates the sample and depending upon the structure of the specimen; the wave-front got distorted which interferes with the reference beam wave-fronts to form the interference pattern on the CCD camera. This technique has been utilized to obtain various quantitative parameters like refractive index, cell thickness, phase etc. of the biological specimen. On the other hand, in fluorescence technique, the specimen is tagged with the specific dye which provides molecular specific information about the biological specimen.

We have developed multi-modal systems that can be utilized to obtain multi-fold information at the same location of the specimen. These multi-modal bio-microscopic systems could be utilized for the diagnosis of various types of diseases. The improvement in spatial phase sensitivity is obtained and multi-fold information about the specimen is estimated with high accuracy and precision. By incorporating speckle-free light source, the spatial phase sensitivity is improved by an order of magnitude compared to the fully coherent light source. The resolution of the system also improves from $1.5\mu\text{m}$ to $1.1\mu\text{m}$ (Using 10x MO, NA=0.25).

Abstract

Further, in the next work, we have performed two step phase improvements including experimental as well as computational approach. The location of the carrier peak is in sub-pixel, but the off-axis interferograms are analyzed using the Fourier fringe analysis method which limits the accuracy of the phase maps by pixel size. For experimental part, we have used spatially partially coherent light source and in computational approach, the hamming window is used for accurate estimation of the original signal frequency information and HR discrete Fourier transform (DFT) is used for sub-pixel accuracy in the estimation of the carrier peak location is obtained.

Next work is conducted to improve the stability of the system. The common-path sheared interferometer is developed with speckle-free illumination and the temporal phase stability of the system is estimated to be 0.0066 rad, which is much better compared to the off-axis systems. In another work, the multi-modal compact system is developed using sheared interferometer. The overall reduction in form factor of the system is achieved by employing shear plate additionally as a beam splitter for the fluorescence imaging part of the system. A compact total internal reflection fluorescence (TIRF) unit is fabricated with 3D printing and LED circuit assembly which is attached compactly with the interferometer to obtain multi-fold information of the biological specimen with high spatial phase sensitivity and high temporal stability.

Then, we have developed a high-resolution WLQPN system that can be utilized to visualize nanoparticles and sub-cellular features of the biological specimens. The 5-phase shifting technique, along with deconvolution, is adopted to obtain super-resolution in phase imaging. The phase shifting technique can provide full detector resolution, making it beneficial as compared to the well-known Fourier analysis method. The Fourier transform method requires minimum angle of $\sin^{-1}(3f_x\lambda)$, where f_x is maximum achievable spatial frequency. It limits the

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highest achievable resolution to much below the actual diffraction limit of the system. Thus, to obtain a high-resolution phase map of the biological specimen, a two-step process is adopted. Firstly, the phase map is recovered using the 5-phase shifting algorithm, with full detector spatial resolution. Secondly, the complex field is obtained from the recovered phase map and further processed using the Richardson Lucy total variation deconvolution algorithm to obtain super-resolution phase images. The present technique was tested on 1951 USAF resolution chart, 200nm polystyrene beads and E-coli bacteria using a 50X, 0.55NA objective lens. The 200nm polystyrene beads are visually resolvable and sub-cellular features of the E-coli bacteria are also observed, suggesting a significant improvement in the resolution

Finally, we have developed an adaptive optimization approach to reconstruct low fringe density interferogram in single shot with high-resolution. The simulation and experimental results obtained using the constraint optimization approach is compared with the Fourier transform and 5-phase shifting results, and found that the optimization approach is much better as compared to the single-shot Fourier transform which provides significant improvement in the phase map.

The present thesis will discuss improvements, advantages, challenges and developments in the system designing as well as in the numerical analysis methods for the betterment of the field.

सार

बायोमेडिसिन उद्योग तेजी से बढ़ रहा है और पिछले कुछ दशकों से निदान आवश्यकताओं में भी वृद्धि हुई है। हेला, एमजी63, मानव एमएससी सेल लाइन, आरबीसी आदि जैसे अधिकांश जैविक नमूने प्रकृति में पारदर्शी हैं। एक पारंपरिक ऑप्टिकल माइक्रोस्कोप माइक्रोन आकार के कणों की कल्पना करने के लिए प्रसिद्ध तकनीक है, लेकिन यह पारदर्शी नमूने के अच्छे विपरीत चित्र प्रदान करने में विफल रहता है। प्रचुर मात्रा में मामलों में, बीमारियों के प्रभाव का विश्लेषण करने के लिए कार्यात्मक और साथ ही मात्रात्मक जानकारी की आवश्यकता होती है। मात्रात्मक चरण माइक्रोस्कोपी (क्यूपीआई) तरंग-सामने विरूपण के सिद्धांत पर काम करता है, जहां समांतरित बीम नमूना रोशन और नमूना की संरचना पर निर्भर करता है, वेव-फ्रंट विकृत हो गया जो सीसीडी कैमरे पर हस्तक्षेप पैटर्न बनाने के लिए संदर्भ बीम वेव-फ्रंट में हस्तक्षेप करता है। इस तकनीक का उपयोग जैविक नमूने के अपवर्तक सूचकांक, सेल मोटाई, चरण आदि जैसे विभिन्न मात्रात्मक मापदंडों को प्राप्त करने के लिए किया गया है। दूसरी ओर, प्रतिदीप्ति तकनीक में, नमूने को विशिष्ट डार्क के साथ टैग किया जाता है जो जैविक नमूने के बारे में आणविक विशिष्ट जानकारी प्रदान करता है।

हमने मल्टी-मोडल सिस्टम विकसित किया है जिसका उपयोग नमूने के एक ही स्थान पर बहु-गुना जानकारी प्राप्त करने के लिए किया जा सकता है। इन मल्टी-मोडल बायो-माइक्रोस्कोपिक प्रणालियों का उपयोग विभिन्न प्रकार की बीमारियों के निदान के लिए किया जा सकता है। स्थानिक चरण संवेदनशीलता में सुधार प्राप्त किया जाता है और नमूने के बारे में बहु-गुना जानकारी उच्च सटीकता और सटीकता के साथ अनुमानित है। यहां, प्रयोगात्मक प्रणाली में धब्बेदार मुक्त रोशनी को शामिल करने से पूरी तरह से सुसंगत प्रकाश स्रोत की तुलना में परिमाण के आदेश से स्थानिक चरण संवेदनशीलता में सुधार होता है। सिस्टम का रिज़ॉल्यूशन 1.5 मिमी से 1.1 मिमी (10x एमओ, एनए = 0.25 का उपयोग करके) में भी सुधार करता है।

इसके अलावा, अगले काम में, हमने प्रयोगात्मक और कम्प्यूटेशनल दृष्टिकोण सहित दो चरण चरण सुधार किए हैं। वाहक चोटी का स्थान उप-पिक्सेल में है, लेकिन ऑफ-एक्सिस इंटरफेरोग्राम्स का विश्लेषण फूरियर फ्रिंज विश्लेषण

पद्धति का उपयोग करके किया जाता है जो पिक्सेल आकार द्वारा चरण मानचित्रों की सटीकता को सीमित करता है। प्रायोगिक भाग के लिए, हमने स्थानिक रूप से आंशिक रूप से सुसंगत प्रकाश स्रोत का उपयोग किया है और कम्प्यूटेशनल दृष्टिकोण में, हैमिंग विंडो का उपयोग मूल संकेत आवृत्ति जानकारी के सटीक अनुमान के लिए किया जाता है और एचआर असतत फूरियर ट्रांसफॉर्म (डीएफटी) का उपयोग अनुमान में उप-पिक्सेल सटीकता के लिए किया जाता है। वाहक शिखर स्थान प्राप्त किया जाता है।

प्रणाली की स्थिरता में सुधार के लिए अगला काम किया जाता है। कॉमन-पाथ शीयर्ड इंटरफेरोमीटर को स्पेकल-फ्री रोशनी के साथ विकसित किया गया है और सिस्टम की अस्थायी चरण स्थिरता 0.0066 रेड होने का अनुमान है, जो ऑफ-अक्ष सिस्टम की तुलना में बेहतर है। एक अन्य कार्य में, मल्टी-मोडल कॉम्पैक्ट सिस्टम को कतरनी इंटरफेरोमीटर का उपयोग करके विकसित किया गया है। सिस्टम के फॉर्म फैक्टर में समग्र कमी प्रणाली के प्रतिदीप्ति इमेजिंग भाग के लिए एक बीम फाड़नेवाला के रूप में अतिरिक्त कतरनी प्लेट को नियोजित करके प्राप्त की जाती है। एक कॉम्पैक्ट कुल आंतरिक प्रतिबिंब प्रतिदीप्ति (टीआईआरएफ) इकाई 3D प्रिंटिंग और एलईडी सर्किट असेंबली के साथ निर्मित है जो उच्च स्थानिक चरण संवेदनशीलता और उच्च अस्थायी स्थिरता के साथ जैविक नमूने की बहु-गुना जानकारी प्राप्त करने के लिए इंटरफेरोमीटर के साथ कॉम्पैक्ट रूप से जुड़ी हुई है।

फिर, हमने एक उच्च-रिज़ॉल्यूशन WLQPN प्रणाली विकसित की है जिसका उपयोग जैविक नमूनों के नैनोकणों और उप-कोशिकीय विशेषताओं की कल्पना करने के लिए किया जा सकता है। चरण इमेजिंग में सुपर-रिज़ॉल्यूशन प्राप्त करने के लिए 5 चरण स्थानांतरण तकनीक, विक्षेपण के साथ-साथ अपनाया जाता है। चरण स्थानांतरण तकनीक पूर्ण डिटेक्टर संकल्प प्रदान कर सकती है, जिससे यह प्रसिद्ध फूरियर विश्लेषण विधि की तुलना में फायदेमंद हो सकती है। फोरियर परिवर्तन विधि के लिए न्यूनतम कोण $\sin^{-1}(3f_x\lambda)$, की आवश्यकता होती है, जहां f_x अधिकतम प्राप्त करने योग्य स्थानिक आवृत्ति है। यह उच्चतम प्राप्त करने योग्य संकल्प को सिस्टम की वास्तविक विवर्तन सीमा से बहुत नीचे तक सीमित करता है। इस प्रकार, जैविक नमूने का एक उच्च-रिज़ॉल्यूशन चरण मानचित्र प्राप्त करने के लिए, एक दो-चरण प्रक्रिया अपनाई जाती है। सबसे पहले, चरण मानचित्र को 5-चरण

शिफ्टिंग एल्गोरिदम का उपयोग करके पुनर्प्राप्त किया जाता है, जिसमें पूर्ण डिटेक्टर स्थानिक रिज़ॉल्यूशन होता है। दूसरा, जटिल क्षेत्र बरामद चरण मानचित्र से प्राप्त किया जाता है और आगे रिचर्डसन लुसी कुल भिन्नता deconvolution एल्गोरिथ्म का उपयोग कर सुपर-रिज़ॉल्यूशन चरण छवियों को प्राप्त करने के लिए संसाधित किया जाता है। वर्तमान तकनीक 1951 USAF संकल्प चार्ट, 200nm पॉलीस्टीरीन मोती और ई-कोली बैक्टीरिया एक 50X, 0.55NA उद्देश्य लेंस का उपयोग कर परीक्षण किया गया था। 200nm पॉलीस्टायर्न मोती दृष्टि से हल करने योग्य हैं और ई-कोली बैक्टीरिया की उप-कोशिकीय विशेषताएं भी देखी जाती हैं, जो संकल्प में महत्वपूर्ण सुधार का सुझाव देती हैं

अंत में, हमने उच्च-रिज़ॉल्यूशन के साथ एकल शॉट में कम फ्रिज घनत्व इंटरफेरोग्राम के पुनर्निर्माण के लिए एक अनुकूली अनुकूलन दृष्टिकोण विकसित किया है, बाधा अनुकूलन दृष्टिकोण का उपयोग करके प्राप्त सिमुलेशन और प्रयोगात्मक परिणामों की तुलना फुरियर परिवर्तन और 5-चरण स्थानांतरण परिणामों के साथ की जाती है, और पाया कि एकल-शॉट फ्रूरियर ट्रांसफॉर्म की तुलना में ऑप्टिमाइज़ेशन दृष्टिकोण बहुत बेहतर है और चरण मानचित्र में महत्वपूर्ण सुधार प्रदान करता है।

वर्तमान थीसिस सिस्टम डिजाइनिंग के साथ-साथ क्षेत्र की बेहतरी के लिए संख्यात्मक विश्लेषण विधियों में सुधार, फायदे, चुनौतियों और विकास पर चर्चा करेगी।

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LIST OF ABBREVIATIONS

CCD	Charge Couple Device
CNNs	Convolutional Neural Networks
DFT	Discrete Fourier Transform
DHM	Digital holography Microscopy
DIC	Differential Interference Contrast
DPM	Diffraction phase Microscopy
dSTORM	direct Stochastic Optical Reconstruction Microscopy
FFT	Fast Fourier Transform
FT	Fourier Transform
FOV	Field of View
FBS	Fetal Bovine Serum
HR	High resolution
hMSCs	human Mesenchymal Stem Cells
He-Ne	Helium-Neon
MO	Microscope Objective
MMFB	Multi-mode fiber bundle
NA	Numerical Aperture
o-TIRF	Objective Based TIRF
OPD	Optical Path Difference
p-TIRF	Prism Based TIRF
PCM	Phase Contrast Microscopy
PZT	Piezo Electric Transducer

List of Abbreviations

PALM	Photo-Activated Localization Microscopy
PBS	Phosphate-Buffered Saline
QPM	Quantitative Phase Microscopy
QPI	Quantitative Phase Imaging
RBCs	Red Blood Cells
RL-TV	Richardson Lucy total variation
SNR	Signal to Noise Ratio
STED	Stimulated Emission Depletion
STORM	Stochastic Optical Reconstruction Microscopy
SI-QPI	Structured Illumination Quantitative Phase Imaging
SLD	Super-luminescent Diode
SLIM	Spatial light-interference microscopy
TIR	Total Internal Reflection
TIRF	Total Internal Reflection Fluorescence
WLQPN	White light quantitative phase nanoscopy
wTIRF	waveguide Total Internal Reflection Fluorescence

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