DEVELOPMENT OF HIGHLY STABLE, WIDE-FIELD AND HIGH-RESOLUTION QUANTITATIVE PHASE MICROSCOPIC SYSTEM FOR BIOLOGICAL APPLICATIONS

VEENA SINGH

DEPARTMENT OF PHYSICS

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

VEENA SINGH

DEPARTMENT OF PHYSICS, IIT DELHI

Submitted

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Dedicated to,

My husband

&

My parents

Certificate

This is to certify that this thesis entitled **DEVELOPMENT OF HIGHLY STABLE, WIDE-FIELD AND HIGH-RESOLUTION QUANTITATIVE PHASE MICROSCOPIC SYSTEM FOR BIOLOGICAL APPLICATIONS**, being submitted by Mrs. Veena Singh to the Indian Institute of Technology Delhi for the award of the degree Doctor of Philosophy 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.

October, 2020

Date:

Place:

Dr. Dalip Singh Mehta
Professor
Department of Physics
Indian Institute of Technology Delhi
Hauz Khas, New Delhi -110016
INDIA

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Abstract

Most of the biological specimens are transparent in nature, making their imaging and measurement difficult under a bright field microscope. Various techniques have been developed in order to make them visible and provide qualitative as well as quantitative information about the specimen. Quantitative phase microscopy (QPM) is a non-contact, and label-free technique that provides quantitative information about the sample and finds its application in biological as well as industrial applications such as life sciences, material science, electronics, photonics, etc. Most of the QPM systems demonstrated so far are based on Mach-Zehnder and Michelson configurations and have been highly successful, but both of these systems are highly unstable. The aim of the present thesis is the development of highly stable, wide field of view and high-resolution quantitative phase microscopy, which can be utilized for industrial and biological applications.

QPM of various biological cells and tissues aids in their visualization as well as in the determination of different biophysical parameters such as morphology, refractive index, cell dry mass, etc. In general, non-common path QPM systems are employed for the experimentation in which the light travels through reference and object paths separately and through different sets of optical elements used in the system. But they suffer from low temporal stability compared to the common path systems. Common path interferometers have higher temporal stability and thus useful in dynamic applications. Here, we have developed a common path interferometer using Fresnel biprism, which is simple, easy to align, and nevertheless highly stable. Fresnel Biprism divides the incoming beam into reference and object beam without any power loss. The system has been implemented in spatial filtering as well as self-referencing mode with the temporal stability of 6 mrad without any vibration isolation.

Due to high stability, the developed highly stable common path system can be easily employed in dynamic applications. Most of the biological cell/tissue properties such as refractive index, cell dry mass, protein concentration are birefringent and strongly dependent on the wavelength of light such that their spectral response may alter in normal and disease state. Thus, their multispectral response can be utilized in disease detection. Here, in this thesis, we have studied the spectral response of human RBCs at different wavelengths ranging from 510nm to 650 nm

with a gap of 10nm. The highly stable interferometer helps in the accurate measurement of phase in this dynamic application at multiple wavelengths. The refractive index has been determined at each of the wavelengths to provide us with the multispectral response of RBC in the visible range of 510 to 650 nm.

In addition to the temporal stability, the accuracy in the phase measurement is also dependent on the spatial phase sensitivity of the system. Common path systems usually employ lasers as the light source, thus suffers from spurious fringes and speckle artifacts. These degrade the phase measurement accuracy and image quality. Here, we have implemented the above developed highly stable common path QPM system using partially spatially coherent multi-spectral light source. The spatial coherence of red and green lasers was reduced with the help of a rotating diffuser, and QPM was implemented using Fresnel biprism in self-referencing mode. The system gives the advantage of high spatial phase sensitivity in addition to easy to implement, no optical power loss, and high temporal stability. Further, the system completely replaces the need for spatial filtering at the source end as well as for the reference beam generation.

In addition to QPM using Fresnel biprism, the same can be utilized for 3D shape measurement of reflecting industrial objects. Here, we have extended the use of Fresnel biprism from QPM system to a single shot fringe projection system as it provides us with the benefit of tunable frequency and region of interference. Fourier transform profilometry being a single-shot technique necessitates the use of sufficient fringe density in order to distance out the first-order term from the DC term in the Fourier domain. The tunable fringe density obtained by the use of biprism helps in getting appropriate fringe density for single-shot measurement. The system is low cost, offers no optical power loss, and provides high-stability in fringe generation. Further, it gives the advantage of tunability in the region of interference. The tunability in fringe density along with region of interference helps in the profilometry of large range of sample sizes that are microscopic as well as macroscopic samples.

The above developed system provides us with qualitative as well as quantitative information about the specimen, but the information is still confined by the diffraction limit of the system. In modern healthcare, there is an urgent need for a system that can provide us with high-resolution data along with wide field of view. Thus, a system that utilizes high NA (numerical aperture) objectives in order to resolve finer details of the specimen but consecutively, there is a decrease

in the field of view of the system. Here, we have implemented simultaneous high resolution and wide field of view using Fourier Ptychography. Fourier Ptychography is a non-interferometric technique which involves illuminating the samples from various angles and computationally combining all the low resolution angularly illuminated images into a high resolution and wide field of view image. Fourier Ptychography usually employs LED array as the light source, but it offers disadvantage of low light illumination, which leads to dark field images having very low SNR due to camera noise. Here, we have used lasers as the partially spatially coherent light source as it provides a solution to low light illumination and non-uniform intensity. It offers the advantage of higher sharpness and higher brightness compared to an LED array. Experimental results of biological samples with high resolution as well as wide field of view are reported.

Multi-modality in biomedical imaging combined with machine learning is one of the pioneer technique which helps in achieving fast and early detection of disease. Multi-modality helps in achieving a large number of parameters and thus aid in increasing the accuracy of the system. Here, we have developed a multimodal system using white light interference microscopy, micro-spectrocolorimetry, and machine learning for the automatic screening of RBCs. White light interference microscopy helps in determining the morphological parameters such as area, perimeter, phase, etc. and micro-spectrocolorimetry gives us color coordinates at various spatial locations of RBCs. The features extracted from this multimodal technique combined with machine learning achieved average specificity, sensitivity, and accuracy of 95.52%, 95.58%, and 95.55%, respectively, with testing data in classification of healthy and unhealthy RBCs. A better result is obtained by using synergies amongst data as compared to QPI based features only.

सार

अधिकांश जैविक नमूने प्रकृति पारदर्शी होती है, और उज्ज्वल क्षेत्र सूछमदर्शी के अधीन उनका छायांकन तथा मापन कठिन होता है। न्यादर्शो को देखने के साथ ही साथ उनकी गुणात्मक तथा संख्यातमक सूचनाओं को मुहैया कराने हेतु बहुत सी वैज्ञानिक तकनीके विकसित की जा चुकी हैं। मात्रात्मक कला सूक्ष्मदर्शी (मा० क० सू०) एक संपर्क रहित तथा लेबल मुख्त तकनीक है जो की न्यादर्श के सम्बंद में मात्रात्मक सूचनाएँ प्रदान करती है और इसका अनुप्रयोग जैव - वैज्ञानिक साथ ही साथ औद्यागिक छेत्रों जैसे कि जीव विज्ञान , पदार्थ वियान , इलेकट्रॉनिक्स तथा फोटोनिक्स आदि में होता है। अभी तक प्रदर्शित की जा चुकी मा० क० सू० प्रणालियों मैक जेंडर तथा माईकल्सन विन्यासो पर आधारित है तथा अत्यधिक सफल हैं , किन्तु यह दोनों ही प्रणालियाँ अत्यदिक अस्थाई हैं। इस शोध ग्रन्थ का लक्ष्य अत्यधिक स्टीर, व्यापक दृश्य क्षेत्र तथा उच्च रेजोलुशन मात्रात्मक कला सूक्ष्मदर्शी प्रणाली विकसित करना है , जिसको औद्यागिक तथा जैव- वैज्ञानिक अनुप्रयोगों हेतु उपयोग किया जा सके।

जैविक कोशिकाओं तथा ऊतकों का मा० क० सू० उनके प्रत्योंक्षकरण साथ ही साथ विभिन्न जैव भौतिक प्रचालों जैसे की आकृति अपवर्तकनांक तथा कोशिका शुष्कद्रव्यमान आदि का निर्धारण करती है। सामान्यतः अनुभयनिष्ठ मार्ग मा० क० सू० प्रणालियों को उन प्रेक्षणों हेतु आरोपित किया जाता है , जिनमें प्रकाश संदर्भ तथा वास्तु पथों पर अलग अलग गमन करता है तथा तंत्र में प्रयुक्त किये गए विभिन्न प्रकाशिए अवयवों के समुच्यों से होकर गुजरता है। किन्तु उनमें उभयनिष्ठ मार्ग प्रणाली की तुलना में काम सामयिक स्थिरता की समस्या है। सामान्य पथ इंटरफेरोमीटर में उच्च सामयिक स्थिरता होती है और इस प्रकार यह गतिशील अनुप्रयोगों में उपयोगी होता है। यहाँ, हमने फ्रेस्नेल बिप्रिस्म का उपयोग करते हुए एक सामान्य पथ इंटरफेरोमीटर विकसित किया है जो सरल, सरेखित करना आसान है और फिर भी अत्यधिक स्थिर है। फ्रेस्नेल बिप्रिस्म, आने वाली बीम को संदर्भ और ऑब्जेक्ट बीम में बिना किसी प्रकाशीय ऊर्जा को हानि पहुँचाये विभाजित करता है। प्रणाली को स्थानिक फ़िल्टरिंग के साथ-साथ स्व-संदर्भ मोड में लागू किया गया है, जिसमें किसी भी कंपन अलगाव के बिना 6 mrad की सामयिक स्थिरता है।

उच्च स्थिरता के कारण, गतिशील अनुप्रयोगों में विकसित अत्यधिक स्थिर सामान्य पथ प्रणाली को आसानी से नियोजित किया जा सकता है। अधिकांश जैविक कोशिका / ऊतक गुण जैसे कि अपवर्तक सूचकांक, कोशिका शुष्क द्रव्यमान, प्रोटीन सांद्रता, द्विगुणित और दृढ़ता से प्रकाश की तरंग दैर्ध्य पर निर्भर होती है, ताकि उनकी वर्णक्रमीय प्रतिक्रिया सामान्य और रोग की स्थिति में बदल सकती है। इस प्रकार, बीमारी का पता लगाने में उनकी बहुउद्देशीय प्रतिक्रिया का उपयोग किया जा सकता है। यहां, इस थीसिस में, हमने 10nm के अंतराल के साथ 510nm से 650 nm तक विभिन्न तरंग दैर्ध्य पर मानव RBCs की वर्णक्रमीय प्रतिक्रिया का अध्ययन किया है। अत्यधिक स्थिर इंटरफेरोमीटर कई तरंग दैर्ध्य में इस गतिशील अनुप्रयोग में चरण के सटीक माप में मदद करता है। अपवर्तक सूचकांक 510 से 650 nm के दृश्यमान रेंज में आरबीसी की बहुउद्देशीय प्रतिक्रिया के साथ हमें प्रदान करने के लिए प्रत्येक तरंग दैर्ध्य पर निर्धारित किया गया है।

सामयिक स्थिरता के अलावा, कला माप में सटीकता भी प्रणाली की स्थानिक कला संवेदनशीलता पर निर्भर है। सामान्य पथ प्रणालियां आमतौर पर लेज़रों को नियोजित करती हैं क्योंकि प्रकाश स्रोत इस प्रकार से भ्रामक भित्तिचित्रों और धब्बेदार कलाकृतियों से ग्रस्त है। ये कला माप सटीकता और छवि गुणवत्ता को ख़राब करते हैं। यहाँ, हमने आंशिक तथा स्थानिक रूप से सुसंगत बहु-स्पेक्ट्रल प्रकाश स्रोत का उपयोग करके ऊपर विकसित अत्यधिक स्थिर सामान्य पथ मा० क० सू० प्रणाली को लागू किया है। लाल और हरे रंग की लेज़रों के स्थानिक सुसंगतता को एक घूर्णन विसारक की सहायता से कम किया गया और मा० क० सू० को सेल्फ-रेफ़रेंसिंग मोड में फ्रेस्नेल बिप्रिस्म का उपयोग करके लागू किया गया। प्रणाली लागू करने में आसान, कोई प्रकाशीय शक्ति की हानि

और उच्च सामयिक स्थिरता के अलावा उच्च स्थानिक कला संवेदनशीलता का लाभ देती है। इसके अलावा, सिस्टम पूरी तरह से स्रोत के अंत में और साथ ही संदर्भ बीम पीढ़ी के लिए स्थानिक फ़िल्टरिंग की आवश्यकता को प्रतिस्थापित करता है।

फ्रेसेल बिप्रिस्म का उपयोग करते हुए मा० क० सू० के अलावा, प्रतिबिंबित औद्योगिक वस्तुओं के लिए तीन आयामी आकार माप प्राप्त करने के लिए उपयोग किया जा सकता है। यहां, हमने मा० क० सू० प्रणाली से फ्रेसेल बाइप्रिज्म के उपयोग को एकल शॉट फ्रिंज प्रोजेक्शन सिस्टम तक विस्तारित किया है क्योंकि यह हमें आवृत्ति अनुकूलन और व्यतिकरण क्षेत्र के लाभ प्रदान करता है। फूरियर ट्रांसफ़ॉर्म प्रोफिलोमेट्री एक एकल-शॉट तकनीक है, फ़्यूरियर डोमेन में डीसी से प्रथम-ऑर्डर को बाहर करने के लिए पर्याप्त फ्रिंज घनत्व का उपयोग आवश्यक है। बिप्रिस्म के उपयोग से प्राप्त अनुकूलन करने योग्य फ्रिंज घनत्व एकल-शॉट माप के लिए उपयुक्त फ्रिंज घनत्व प्राप्त करने में मदद करता है। सिस्टम कम लागत वाला है, कोई प्रकाशीय शक्ति नुकसान नहीं करता है और फ्रिंज पीढ़ी में उच्च स्थिरता प्रदान करता है। इसके अलावा, यह व्यतिकरण क्षेत्र में सामर्थ्य का लाभ देता है। व्यतिकरण क्षेत्र के साथ-साथ फ्रिंज घनत्व में अनुकूलन नमूना आकार की बड़ी रेंज के प्रोफिलोमेट्री में मदद करती है जो सूक्ष्म और साथ ही स्थूल नमूने हैं।

उपर्युक्त विकसित प्रणाली हमें गुणात्मक के साथ-साथ नमूना के बारे में मात्रात्मक जानकारी प्रदान करती है लेकिन यह जानकारी अभी भी सिस्टम की विवर्तन सीमा तक ही सीमित है। आधुनिक स्वास्थ्य सेवा में, एक ऐसी प्रणाली की तत्काल आवश्यकता है जो हमें व्यापक क्षेत्र के साथ-साथ उच्च-रिज़ॉल्यूशन डेटा प्रदान कर सके। इस प्रकार, एक प्रणाली जो नमूना के बारीक विवरण को हल करने के लिए उच्च एन० ए० (न्यूमेरिकल अपर्चर) ऑब्जेक्टिव का उपयोग करती है, लेकिन लगातार, सिस्टम के दृष्टिकोण के क्षेत्र में कमी होती है। यहां, हमने फ्यूरियर टाईकोग्राफी का उपयोग करके एक साथ उच्च रिज़ॉल्यूशन और व्यापक क्षेत्र को लागू किया है। फूरियर टाईकोग्राफी एक गैर-इंटरफेरोमेट्रिक तकनीक है जिसमें विभिन्न कोणों से नमूने को रोशन करना और कम्प्यूटेशनल रूप से सभी कम रिज़ॉल्यूशन को कोणीय रूप से प्रकाशित छवियों को एक उच्च रिज़ॉल्यूशन और दृश्य छवि के विस्तृत क्षेत्र में संयोजित करना शामिल है। फूरियर टाईकोग्राफी आमतौर पर एलईडी सरणी को प्रकाश स्रोत के रूप में नियुक्त करता है लेकिन यह कम रोशनी की प्रदीप्ति का नुकसान प्रदान करता है जो कि कैमरा शोर के कारण बहुत कम संकेत-रव अनुपात वाले अंधेरे क्षेत्र की छवियों की ओर जाता है। यहां, हमने लेज़रों का आंशिक तथा स्थानिक रूप से सुसंगत प्रकाश स्रोत के रूप में उपयोग किया है क्योंकि यह कम रोशनी और गैर-समान तीव्रता का समाधान प्रदान करता है। यह एक एलईडी सरणी की तुलना में उच्च तीक्ष्णता और उच्च चमक का लाभ प्रदान करता है। उच्च संकल्प के साथ-साथ दृश्य के व्यापक क्षेत्र के साथ जैविक नमूनों के प्रायोगिक परिणामों की सूचना दी जाती है।

मशीन लर्निंग के साथ संयुक्त बायोमेडिकल इमेजिंग में मल्टी-मॉडेलिटी अग्रणी तकनीक में से एक है जो रोग का तेजी से और जल्दी पता लगाने में मदद करती है। मल्टी-मॉड्युलिटी बड़ी संख्या में मापदंडों को प्राप्त करने में मदद करती है और इस प्रकार सिस्टम की सटीकता को बढ़ाने में सहायता करती है। यहां, हमने आरबीसी की स्वचालित स्क्रीनिंग के लिए श्वेत प्रकाश व्यतिकरण माइक्रोस्कोपी, माइक्रो-स्पेक्ट्रोकोरोनोमेट्री और मशीन लर्निंग का उपयोग करके एक मल्टीमॉडल प्रणाली विकसित की है। श्वेत प्रकाश हस्तक्षेप माइक्रोस्कोपी क्षेत्र, परिधि, चरण आदि जैसे रूपात्मक मापदंडों को निर्धारित करने में मदद करता है और माइक्रो-स्पेक्ट्रोकार्टोमीरी हमें आरबीसी के विभिन्न स्थानिक स्थानों पर रंग निर्देशांक देता है। मशीन लर्निंग के साथ संयुक्त इस मल्टीमॉडल तकनीक से निकाली गई विशेषताओं ने स्वस्थ और अस्वस्थ आरबीसी के वर्गीकरण में डेटा का परीक्षण करने के साथ क्रमशः 95.52%, 95.58% और 95.55% की औसत विशिष्टता, संवेदनशीलता और सटीकता प्राप्त की। केवल मा० क० सू० आधारित सुविधाओं की तुलना में डेटा के बीच तालमेल का उपयोग करके एक बेहतर परिणाम प्राप्त किया जाता है।

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List of Abbreviations

3D	3 Dimensional
CCD	Charged coupled device
CMOS	Complementary metal oxide semiconductor
DHM	Digital holography microscopy
DIC	Differential Interference contrast
DOF	Depth of field
DRS	Diffused reflectance spectroscopy
EM	Electromagnetic
EPRY	Embedded pupil function recovery
ESF	Edge spread function
FOV	Field of View
MF	Magnification
FP	Fourier Ptychography
FPM	Fourier Ptychography Microscopy
FWHM	Full width half maxima
Hb	Hemoglobin
LASER	Light amplification by stimulated emission of radiation
LED	Light emitting diode
LSF	Line spread function
MCHb	Molecular hemoglobin concentration
MO	Microscopic objective
MTF	Modulation transfer function

NA	Numerical aperture
OBJ	Objective
PIE	Ptychographic iterative engine
PSF	Point spread function
PSI	Phase shifting interferometry
QPI	Quantitative phase imaging
QPM	Quantitative phase microscopy
RBC	Red blood cell
SBP	Space bandwidth product
SLD	Super luminescent diode
SR	Super resolution
SVM	Support vector machine
TIRF	Total internal reflection fluorescence
WLIM	White light interference microscopy