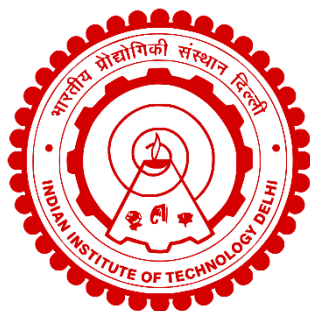


**HIGH CONTRAST LABEL-FREE SINGLE-SHOT QUANTITATIVE
PHASE IMAGING AND SPECTROSCOPIC TECHNIQUES FOR
BIOLOGICAL APPLICATIONS**

SUNIL BHATT



DEPARTMENT OF PHYSICS

INDIAN INSTITUTE OF TECHNOLOGY DELHI

FEBRUARY 2024

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by

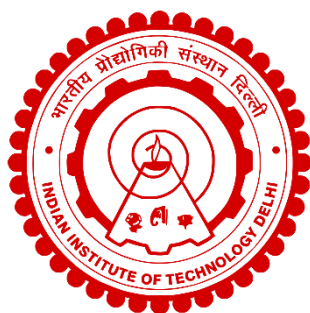
SUNIL BHATT

DEPARTMENT OF PHYSICS

Submitted

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

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

FEBRUARY 2024

Dedicated to,

My parents

CERTIFICATE

This is to certify that the thesis entitled “**HIGH CONTRAST LABEL-FREE SINGLE-SHOT QUANTITATIVE PHASE IMAGING AND SPECTROSCOPIC TECHNIQUES FOR BIOLOGICAL APPLICATIONS**” submitted by **Mr. SUNIL BHATT** to the Department of Physics, Indian Institute of Technology Delhi for the award of the degree of **DOCTOR OF PHILOSOPHY**. This thesis is a bonafide record of the research carried out by him under my guidance and supervision. In my opinion, the thesis has reached the standards fulfilling the requirements for the submission relating to the degree.

The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Date:

Place: New Delhi, India

Prof. Dalip Singh Mehta

Department of Physics,

Indian Institute of Technology Delhi

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Sunil Bhatt

ABSTRACT

Quantitative phase imaging (QPI) is the most widely used label-free optical imaging technique to visualize the cellular and subcellular structures of a sample with high contrast and quantitative information. In the past, advanced QPI techniques were developed to improve the contrast, acquisition speed, resolution, sensitivity, and specificity. In addition, label-free spectroscopic techniques are essential to investigate molecular fingerprint information about the sample. Therefore, label-free optical imaging and spectroscopic techniques are introduced to extract the microscopic, nanoscopic, and molecular fingerprint information about the sample and thus gained popularity in various applications. The aim of the present work is to develop label-free optical imaging and spectroscopic techniques for industrial and biological applications.

First, we present a single-shot phase-shifting interferometric technique for accurate phase measurement using white light phase-shifting interference microscopy (WL-PSIM) and deep neural network (DNN). WL-PSIM is a prominent technique for high-resolution QPI of industrial and biological samples. However, multiple interferograms with accurate phase shifts are essentially required in WL-PSIM for measuring the accurate phase of the object, which is time-consuming. Thus, we trained a DNN for the generation of intermediate multiple equal phase-shifted interferograms that are finally used in a traditional phase-shifting interferometry (PSI) algorithm for phase reconstruction. In a similar manner, the network is further trained on the same dataset for the direct phase map from a single low fringe density interferogram without any intermediate multiple phase-shifted interferograms in PSI. The network was trained and validated on two different samples: the optical waveguide and MG63 osteosarcoma cells. The phase map comparison and structure similarity index measure (SSIM) value between the experimental and network-generated data results validates the proposed approach for high-resolution single-shot phase reconstruction in WL-PSIM.

In QPI, the phase (basically refractive index) of cells and tissues are wavelength-dependent properties and accurate measurement with spectral dependence is required to increase the accuracy of diagnostic applications. However, multispectral quantitative phase imaging (MS-QPI) is an imaging platform used for the quantitative analysis of phase maps with spectral dependence. But, to perform MS-QPI, we require a multiwavelength light source with multiple optical components to avoid dispersion and a multi-chip camera or multiple single-chip camera, which makes the system bulky and slow. In addition, the color cross-talk from a multichip camera detector is another limitation in MS-QPI. Therefore, we aim to extract spectral-dependent quantitative phase information in a single shot using a deep learning (DL)-based QPI system. The network was trained and validated on two different samples: the optical waveguide and MG63 osteosarcoma cells. For the training of the DNN, we acquire a series of multispectral datasets by scanning the multiwavelength light source and their corresponding phase maps. After successful training, the trained DL-based model is used to generate multispectral

quantitative phase map generation corresponding to a single wavelength interferogram. The phase map comparison and image quality assessment between the experimentally reconstructed and network-generated MS-QPI images provide the validation for the proposed study. The proposed DL-based MS-QPI system overcomes the limitations of traditional MS-QPI approaches and provides significant time, performance, resources, and cost reduction management.

So far, we performed the study to ease the complexity of the QPI technique in less time. Now, we showed the application of QPI for nanoscale motion tracing of moving samples. Filtration of male semen samples and selection of healthy spermatozoa (sperm cells) is an important step in assisted reproduction technologies. The motion of the spermatozoa indicates their motility and is therefore used as a visual cue for selecting high-quality sperms during intracytoplasmic sperm injection and in-vitro fertilization. For this, we combine an optical imaging platform with an intensity fluctuation-based optical nanoscopy method (primarily used in a labelled technique for super-resolution) for the motion trace analysis of the specimen. We used a multiple signal classification algorithm (MUSICAL) with QPI sequences to perform nanoscale motion trace analysis; refer to this technique as MusiQ, where ‘Musi’ stands for MUSICAL, and ‘Q’ stands for QPI. For the proof of concept (POC) of the proposed approach, a 1- μm polystyrene bead particle is trapped and propelled in the evanescent field of the waveguide and traced its motion using MusiQ with finer details, although the object was diffraction limited in each frame. Further, we trace the motion of human sperm cells in a straight path with helical motion trace with finer features. We achieved motion precision in the range of 340 nm using a 10 $\times$, 0.25 NA lens, whereas the diffraction-limited resolution at this setting was 1320 nm. Our technique presents nanoscale motion features beyond the conventional features used for motility and progressive motility analysis, allowing further distinction among the progressively motile sperms in terms of the regularity of their motion patterns.

Further, the application of QPI is combined with multimodal imaging and spectroscopic techniques to investigate the morphological and molecular fingerprints of the Penguin brain sample. The aim of the current work is to study the Penguin brain and provide different parameters in order to ultimately create a comparative digital brain atlas. Here, we used multiple label-free optical techniques, such as QPI, autofluorescence spectroscopy, and Raman spectroscopy, along with cresyl violet (CV)-staining to extract the optical parameters of the Penguin brain for their morphological and molecular study. All these techniques provide the microscopic and molecular fingerprints of the Penguin brain, which can be useful for understanding the anatomical, physiological, and social behaviour of the Penguins.

Although the QPI technique provides the morphological information of the thin biological samples with high resolution and contrast, it still has limitations when we apply it to thick biological samples. In the case of thick biological samples, the scattered light has the sample information from the multiple layers or out-of-focus planes, which results in a noisy image. So, we introduced the concept of longitudinal

spatial coherence (LSC) gated full-field optical coherence microscopy (FF-OCM) for the cellular and subcellular imaging of thick multilayered biological samples. We synthesized a partially spatially coherent (PSC) light from a monochromatic light source and utilized its LSC property for the high-resolution sectioning of multilayered thick biological specimens. First, we determined the LSC length of the light source and performed topography of a standard gauge block with a height difference of $20\mu\text{m}$. Further, we performed the tomography of a multilayered biological and an oral cancer tissue sample. The PSC-FFOCM system provides ± 20 mrad spatial phase sensitivity and ± 2 mrad temporal phase sensitivity with a high axial resolution of $6\mu\text{m} - 8\mu\text{m}$.

The multimodal and multispectral techniques discussed in this thesis provide morphological and molecular fingerprints of biological samples. The present thesis discusses motivation, challenges, advantages, results, and opportunities associated with the methods utilized during various biological studies.

सार

मात्रात्मक चरण इमेजिंग (क्यूपीआई), व्यापक रूप से इस्तेमाल की जाने वाली लेबल-मुक्त ऑप्टिकल इमेजिंग तकनीक है जिसका उपयोग सैम्पल से उच्च कंट्रास्ट और मात्रात्मक जानकारी के साथ सेलुलर और उपसेलुलर संरचनाओं को दिखाने के लिए किया जाता है। अतीत में, उन्नत क्यूपीआई तकनीकों का विकास कंट्रास्ट, अधिग्रहण गति, रिजॉल्यूशन, संवेदनशीलता और विशिष्टता में सुधार के लिए किया गया है। इसके अलावा, लेबल-मुक्त स्पेक्ट्रोस्कोपी तकनीक सैम्पल के बारे में आणविक फिंगरप्रिंट की मौलिक जानकारी की जांच करने के लिए एक आवश्यक है। इसलिए, लेबल-मुक्त ऑप्टिकल इमेजिंग और स्पेक्ट्रोस्कोपी को सैम्पल के सूक्ष्म, नैनोस्कोपिक और आणविक फिंगरप्रिंट की जानकारी निकालने के लिए पेश किया गया है। इस प्रकार विभिन्न अनुप्रयोगों में लोकप्रियता हासिल हुई। वर्तमान कार्य का उद्देश्य औद्योगिक और जैविक अनुप्रयोगों के लिए लेबल-मुक्त ऑप्टिकल इमेजिंग और स्पेक्ट्रोस्कोपी तकनीकों को विकसित करना है।

सबसे पहले, हम सफेद प्रकाश चरण-स्थानांतरण विकिरण माइक्रोस्कोपी (डब्ल्यूएल-पीएसआईएम) और गहरे न्यूरोल नेटवर्क (डीएनएन) का उपयोग करके सटीक चरण माप के लिए एकल-शॉट चरण-शिफ्टिंग इंटरफेरोमेट्रिक तकनीक प्रस्तुत करते हैं। डब्ल्यूएल-पीएसआईएम औद्योगिक और जैविक नमूनों के उच्च-रिजॉल्यूशन क्यूपीआई के लिए एक प्रमुख तकनीक है। हालाँकि, वस्तु के सटीक चरण को मापने के लिए डब्ल्यूएल-पीएसआईएम में सटीक चरण बदलाव वाले कई इंटरफेरोग्राम की अनिवार्य रूप से आवश्यकता होती है, जिसमें समय लगता है। इसलिए, हमने एक डीएनएन को प्रशिक्षित किया ताकि पारंपरिक चरण-शिफ्टिंग इंटरफेरोमेट्री (पीएसआई) एंजोथ्रिथम के लिए मध्यवर्ती एकाधिक समान चरण-स्थानांतरित इंटरफेरोग्राम की पीढ़ीया उत्पन्न की जा सके, जो अंततः चरण पुनर्निर्माण के लिए उपयोग हो। इसी तरह से, नेटवर्क को पीएसआई में किसी मध्यवर्ती एकाधिक चरण-स्थानांतरित इंटरफेरोग्राम के बिना एकल कम फ्रिंज घनत्व इंटरफेरोग्राम से सीधे चरण माप के लिए समान डेटासेट पर प्रशिक्षित किया जाता है। नेटवर्क को दो अलग-अलग नमूनों पर प्रशिक्षित और मान्य किया गया था: ऑप्टिकल वेवगाइड और एमजी63 ओस्टियोसारकोमा कोशिकाएं। प्रयोगात्मक और नेटवर्क जनित डेटा परिणामों के बीच चरण माप तुलना और संरचना समानता सूचकांक माप (एसएसआईएम) मान डब्ल्यूएल-पीएसआईएम में उच्च रिजॉल्यूशन एकल-शॉट चरण पुनर्निर्माण के लिए प्रस्तावित दृष्टिकोण को मान्य करता है।

क्यूपीआई में, कोशिकाओं और ऊतकों के चरण (मूल रूप से अपवर्तक सूचकांक) तरंग दैर्ध्य पर निर्भर गुण होते हैं और नैदानिक अनुप्रयोगों की सटीकता बढ़ाने के लिए वर्णक्रमीय निर्भरता के साथ सटीक माप की आवश्यकता होती है। हालाँकि, मल्टीस्पेक्ट्रल मात्रात्मक चरण इमेजिंग (एमएस-क्यूपीआई) एक इमेजिंग प्लेटफॉर्म है, जिसका उपयोग वर्णक्रमीय निर्भरता के साथ चरण माप के मात्रात्मक विश्लेषण के लिए किया जाता है। लेकिन, एमएस-क्यूपीआई को करने के लिए, हमें बहुविकिरण प्रकाश स्रोत की आवश्यकता होती है जिसमें प्रसारण से बचने के लिए अनेक ऑप्टिकल घटक होते हैं और बहु-चिप कैमरा या कई एकल चिप कैमरा की जरूरत होती है, जो प्रणाली को भारी और समय लेने वाला बना देता है। इसके अलावा, बहु-चिप कैमरा डिटेक्टर से कलर क्रॉस टॉक एमएस-क्यूपीआई में एक और सीमा है। इसलिए, हमारा लक्ष्य डीप लर्निंग (डीएल) आधारित क्यूपीआई प्रणाली का उपयोग करके एकल शॉट में स्पेक्ट्रल आश्रित मात्रात्मक चरण की जानकारी निकालना है। नेटवर्क को दो अलग-अलग नमूनों पर प्रशिक्षित और मान्य किया गया था: ऑप्टिकल वेवगाइड और एमजी63 ओस्टियोसारकोमा कोशिकाएं। डीएनएन के प्रशिक्षण के लिए हम बहुविकिरण प्रकाश स्रोत को स्कैन करके और उनके संवर्धन चरण मानचित्रों के साथ एक श्रृंखला मल्टीस्पेक्ट्रल डेटासेट प्राप्त करते हैं। सफल प्रशिक्षण के बाद, प्रशिक्षित डीएल-आधारित मॉडल का उपयोग एकल तरंग दैर्ध्य इंटरफेरोग्राम के अनुरूप मल्टीस्पेक्ट्रल मात्रात्मक चरण मानचित्र पीढ़ी उत्पन्न करने के लिए किया जाता है। प्रयोगात्मक रूप से पुनर्निर्मित और नेटवर्क जनित एमएस-क्यूपीआई छवियों के बीच चरण मानचित्र तुलना और छवि गुणवत्ता मूल्यांकन प्रस्तावित अध्ययन के लिए सत्यापन प्रदान करता है। प्रस्तावित डीएल-आधारित एमएस-क्यूपीआई प्रणाली पारंपरिक एमएस-क्यूपीआई दृष्टिकोण की सीमाओं को पार करती है और और महत्वपूर्ण प्रदर्शन, संसाधन और लागत की कमी के प्रबंधन के साथ-साथ 31X महत्वपूर्ण समय कटौती प्रदान करती है।

अभी तक, हमने कम समय में क्यूपीआई तकनीक की जटिलता को कम करने के लिए अध्ययन किया। अब, हमने गतिमान नमूनों की नैनोस्केल गति का पता लगाने के लिए क्यूपीआई का अनुप्रयोग दिखाया है। पुरुष वीर्य के नमूने का निस्पंदन और स्वस्थ शुक्राणु (शुक्राणु कोशिकाओं) का चयन सहायक प्रजनन प्रौद्योगिकियों में एक महत्वपूर्ण कदम है। शुक्राणु की गति उनकी गतिशीलता को इंगित करती है और इसलिए इसका उपयोग इंट्रासाइटोप्लाज्मिक शुक्राणु इंजेक्शन और इन-विट्रो निषेचन के दौरान उच्च गुणवत्ता वाले शुक्राणुओं के चयन के लिए एक दृष्टिगत संकेत के रूप में किया जाता है। इसके लिए, हम नमूने के मोशन ट्रेस विश्लेषण के लिए एक ऑप्टिकल इमेजिंग प्लेटफॉर्म को तीव्रता के उतार-चढ़ाव आधारित ऑप्टिकल नैनोस्कोपी विधि (मुख्य रूप से लेबल तकनीक में सुपर रिजॉल्यूशन के लिए उपयोग किया जाता है) के साथ जोड़ते हैं। हमने नैनोस्केल मोशन ट्रेस विश्लेषण करने के लिए क्यूपीआई अनुक्रमों के साथ मल्टीपल सिग्नल वर्गीकरण एल्गोरिदम (म्यूजिकल) का उपयोग किया, इस तकनीक को म्यूसीक्यू के रूप में संदर्भित किया, जहां 'म्यूसी' का मतलब म्यूजिकल है, और 'क्यू' का मतलब क्यूपीआई है। प्रस्तावित दृष्टिकोण की अवधारणा (POC) के प्रमाण के लिए, एक 1-माइक्रोन पॉलीस्टीरिन बीड कण को वेवगाइड के अप्रचलित दायर में फंसाया जाता है और महीन विवरण के साथ म्यूसीक्यू का उपयोग करके इसकी गति का पता लगाया जाता है, हालांकि वस्तु प्रत्येक फ्रेम में विवर्तन सीमित थी। इसके अलावा, हमने सीधी पथ में हेलिकल गति करते मानव शुक्राणु कोशिकाओं की गति का पता बेहतर विशेषताओं के साथ लगाया। हम $10\times, 0.25$ एनए लेंस का उपयोग करके 340 नैनोमीटर की सीमा में गति परिशुद्धता प्राप्त करते हैं जबकि इस सेटिंग में विवर्तन सीमित रिजॉल्यूशन 1320 नैनोमीटर था। हमारी तकनीक प्रगतिशीलता और प्रगतिशीलता विश्लेषण के लिए उपयोग की जाने वाली परंपरागत विशेषताओं से परे नैनोस्केल गति विशेषताएँ प्रस्तुत करती है, जिससे प्रगतिशील गतिशील शुक्राणुओं को उनकी गति नियमितता और गति पैटर्न के संदर्भ में और भी विशेषता से भिन्न किया जा सकता है।

इसके अलावा, पेंगुइन मस्तिष्क नमूने के रूपात्मक और आणविक फिंगरप्रिंट की जांच के लिए क्यूपीआई के अनुप्रयोग को मल्टीमॉडल इमेजिंग और स्पेक्ट्रोस्कोपी तकनीक के साथ जोड़ा गया है। वर्तमान कार्य का उद्देश्य पेंगुइन मस्तिष्क का अध्ययन करना और अंततः एक तुलनात्मक डिजिटल मस्तिष्क एटलस बनाने के लिए विभिन्न पैरामीटर प्रदान करना है। यहां, हमने उनके संरचनात्मक और आणविक अध्ययन के लिए पेंगुइन मस्तिष्क के ऑप्टिकल मापदंडों को निकालने के लिए क्रिसिल वायलेट (सीवी)-स्टेनिंग के साथ-साथ क्यूपीआई, ऑटोफ्लोरोसेंस स्पेक्ट्रोस्कोपी और रमन स्पेक्ट्रोस्कोपी जैसी कई लेबल-मुक्त ऑप्टिकल तकनीकों का उपयोग किया। ये सभी तकनीकें पेंगुइन के मस्तिष्क के बारे में सूक्ष्म और आणविक फिंगरप्रिंट प्रदान करती हैं जो पेंगुइन के विश्लेषणात्मक, शारीरिक और सामाजिक व्यवहार को समझने के लिए उपयोगी हो सकती हैं।

हालांकि, क्यूपीआई तकनीक पतले जैविक नमूनों की संरचनात्मक जानकारी उच्च रिजॉल्यूशन और कंट्रास्ट के साथ प्रदान करती है, फिर भी जब इसे मोटे जैविक नमूनों के विश्लेषण उपयोग किया जाता है तो इसकी सीमाएँ हैं। मोटे जैविक नमूनों के मामले में, प्रकीर्णन हुई प्रकाश में सैपल की जानकारी एकाधिक परतों या फोकस क्षेत्रों से आती है, जिसके परिणामस्वरूप शोर वाली छवि बनती है। इसलिए, हमने मोटे बहुस्तरीय जैविक नमूनों की सेलुलर और उपसेलुलर इमेजिंग के लिए अनुदैर्ध्य स्थानिक सुसंगतता (एलएससी) गेटेड पूर्ण-क्षेत्र ऑप्टिकल सुसंगत माइक्रोस्कोपी (एफएफ-ओसीएम) की अवधारणा पेश की। हमने एक मोनोक्रोमैटिक प्रकाश स्रोत से आंशिक रूप से स्थानिक रूप से सुसंगत (पीएससी) प्रकाश को संश्लेषित किया और बहुस्तरीय मोटे जैविक नमूनों के उच्च-रिजॉल्यूशन सेक्शनिंग के लिए इसकी एलएससी संपत्ति का उपयोग किया। सबसे पहले, हमने प्रकाश स्रोत की एलएससी लंबाई निर्धारित की और 20-माइक्रोन की ऊंचाई के अंतर के साथ मानक गेज ब्लॉक की स्थलाकृति प्रदर्शित की। इसके अलावा, हमने एक बहुस्तरीय जैविक और एक मौखिक कैंसर ऊतक के नमूने की टोमोग्राफी की। पीएससी - एफएफ-ओसीएम प्रणाली ± 20 मिलीरेडियन स्थानिक चरण संवेदनशीलता, ± 2 मिलीरेडियन कालिक चरण संवेदनशीलता 6-माइक्रोन - 8-माइक्रोन की उच्च अक्षीय रिजॉल्यूशन संकल्पना प्रदान करती है।

इस थीसिस में चर्चा की गई मल्टीमॉडल और मल्टीस्पेक्ट्रल तकनीकें जैविक नमूनों के रूपात्मक और आणविक फिंगरप्रिंट को प्रदान करती हैं। वर्तमान थीसिस विभिन्न जैविक अध्ययनों के दौरान उपयोग की जाने वाली विधियों से जुड़ी प्रेरणा, चुनौतियों, लाभों, परिणामों और अवसरों पर चर्चा करती है।

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ABBREVIATION

BF- Bright field
DF- Dark field
PCM- Phase contrast microscope
DIC - Differential interference contrast
FM- Fluorescence microscopy
QPI- Quantitative phase imaging
QPM - Quantitative phase microscopy
OPD- Optical path difference
FT- Fourier transform
DFT- Discrete Fourier transform
FOV- Field-of-view
DHM- Digital holographic microscope
PSI- Phase-shifting interferometry
PZT- Piezoelectric transducer
WL-PSIM- White light phase-shifting interference microscopy
F-WL-PSIM- Filtered white light phase-shifting interference microscopy
CMOS- Complementary metal-oxide semiconductor
CCD- Charge-coupled device
TIE- Transport of intensity equation
PSC- Partially spatially coherent
MS-QPI- Multispectral quantitative phase imaging
MMFB- Multi-multimode fiber bund
MFB- Multimode fiber bundle
RD- Rotating diffuser
MO- Microscope objective
NIR- Near infrared
WT- Waveguide trapping
IFON- Intensity fluctuation-based optical nanoscopy
MUSICAL- Multiple signal classification algorithm
PALM- Photoactivated light microscopy
SOFI- Super-resolution optical fluctuation imaging
SRRF- Super-Resolution Radial Fluctuations
SACD- Super-resolution with autocorrelation two-step deconvolution
PSF- Point spread function
OCT- Optical coherence tomography
TD-OCT- Time-domain optical coherence tomography
FD-OCT- Frequency-domain optical coherence tomography
SD-OCT- Spectral-domain optical coherence tomography
SS-OCT- Swept-source optical coherence tomography
FF-OCM- Full-field optical coherence microscopy
NA- Numerical aperture
DOF- Depth-of-field
LSC- Longitudinal spatial coherence
NADH- Nicotinamide adenine dinucleotide
FAD- Flavin adenine dinucleotide

FWHM- Full-width half maximum
RR- Resonance Raman
SERS- Surface-enhanced Raman spectroscopy
AgNDs- Silver nano-dendrites
ML- Machine learning
DL- Deep learning
ANN- Artificial neural networks
DNN- deep neural network
GAN- Generative adversarial network
Leaky ReLU- Leaky rectifier linear unit
GT- Ground truth
SSIM- Structure similarity index measure
FSIM- Feature similarity index measure
MSE- Mean square error
RMSE- Root mean square error
SNR- Signal-to-noise ratio
PSNR- Peak signal-to-noise ratio
POC- Proof of concept
MusiQ- MUSICAL+QPI
ART- Assisted reproductive technology
IUI- Intrauterine insemination
IVF- In-vitro fertilization
ICSI- Intracytoplasmic sperm injection
CASA- Computer-aided semen analysis
CV- Cresyl violet
DMEM- Dulbecco's modified eagle medium
FBS- Fetal bovine serum
PBS- Phosphate buffered saline
PDMS- Polydimethylsiloxane
BV- Blood vessels BC- Blood cells
GC- Glial cells
Ne- Neurons
CM- Cerebellum medulla
ML- Molecular layer
GL- Granular layer
NS- Nissl substance