

HOLOGRAPHIC IMAGING FLOW CYTOMETRY USING THREE-DIMENSIONAL MICROFLUIDIC HYDRODYNAMIC FOCUSING

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Holographic imaging flow cytometry using three-dimensional microfluidic hydrodynamic focusing

by

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Certificate

This is to certify that the thesis entitled “**Holographic imaging flow cytometry using three-dimensional microfluidic hydrodynamic focusing**”, being submitted by, **Mr. Yogesh Maheshbhai Patel** to the Indian Institute of Technology, Delhi, for the award of the degree of **Doctor of Philosophy**, is a bonafide record of original research work carried out by him under my supervision in conformity with the rules and regulations of the institute. 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.

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Abstract

Holographic flow cytometry (HFC) is a cell-analysis technique that employs digital holographic microscopy (DHM) to image and classify cells flowing in a microfluidic device. The current HFC techniques are based on phase reconstruction followed by numerical refocusing as pre-processing steps. The numerical refocusing method to retrieve the phase images is computationally expensive and, hence, limits the speed of current HFC techniques. Moreover, while applying numerical refocusing algorithms, if an individual object is far from the nominal plane that is in focus while recording the digital hologram, the corresponding object field may be highly blurred, and there is a possibility of missing such objects completely. As a result, the accuracy of classification and quantification of cells is affected. The accurate and rapid classification and quantification in HFC require the randomly suspended cells in the inlet stream to be three-dimensionally focused into a single file arrangement. Current three-dimensional (3D) sheathing devices focus the cells at the midplane of microchannels, necessitating the fabrication of devices with smaller depths through precise methods to keep cells within the working distance of the microscope objective. Furthermore, existing approaches for 3D hydrodynamic focusing using sheath flows require microfluidic devices with multi-layer construction, introducing complexity to the fabrication process and escalating device costs. As microfluidic devices for HFC are discarded after a single use to prevent contamination, it is imperative to lower the cost of 3D sheathing devices through uncomplicated designs and low-cost fabrication techniques without affecting functionality.

Firstly, we demonstrate the design, fabrication, characterization, and testing of a sheath-flow based microfluidic device for 3D hydrodynamic focusing of cells. Our device hydrodynamically focuses the cells in a single-file near the bottom wall of the microchan-

nel which allows imaging cells with high magnification and low working distance objectives, without the need for small device dimensions. The relatively large dimensions of the microchannels enable easy fabrication using less-precise fabrication techniques, and the simplicity of device design avoids the need for tedious alignment of various layers. We have characterized the performance of the device with 3D numerical simulations and validated these simulations with experiments of hydrodynamic focusing of a fluorescently dyed sample fluid. The simulations show that the width and the height of the 3D focused sample stream can be controlled independently by varying the heights of the main and side channels of the device and the flow rates of sample and sheath fluids. Based on simulations, we provide useful guidelines for choosing the device dimensions and flow rates for focusing cells of a particular size. Thereafter, we demonstrate the applicability of our device for imaging a large number of red blood cells using brightfield microscopy.

Next, we demonstrate an HFC technique using 3D hydrodynamic focusing for accurate visualization, classification, and quantification of the cells from a mixture. Our HFC approach uses high-resolution, single-shot digital holographic microscopy to image flowing cells in a microfluidic device that orders the cells in a single file close to the bottom wall of the channel. The integration of a 3D focusing device with DHM allows high-magnification holographic imaging without the need for numerical refocusing. Moreover, the specially designed 3D sheathing device prevents the clustering of cells. To demonstrate the efficacy of our HFC method, we consider a challenging case of classification from a mixture of unstained red blood cells and polystyrene particles, which are otherwise indistinguishable in brightfield and phase-contrast microscopy. Through experiments with cell-particle mixtures with varying proportions, we show that our HFC technique can precisely count and classify the cells and particles based on their reconstructed phase values while avoiding computationally expensive and time-consuming numerical refocusing. We also discuss the choice of the region of interest and camera frame rate so as to image each cell and particle individually in our device.

Lastly, we demonstrate the application of our HFC approach for specific biological studies. We commence with presenting our HFC method for the classification of un-

stained trypsinized flowing MDA-MB-231 and ZR-75-1 breast cancer cells in suspension. The classification of MDA-MB-231 and ZR-75-1 cells serves as a challenging problem to showcase the capability of our HFC approach because these cells are indistinguishable under brightfield and phase-contrast microscopy without staining. Furthermore, we demonstrate the capability of our HFC approach for drug-susceptibility testing of cancer cells while avoiding numerical refocusing and staining. This is in contrast to conventional approaches for drug susceptibility assessment, wherein numerical refocusing and staining of cancer cells is necessary to visualize and estimate the proportion of live and dead cells in suspension. The findings of the work presented in this thesis can address a major limitation of HFC to compete with other high-throughput cell imaging and classification techniques. Our HFC method of cell classification opens the arena for its usage in various disciplines, including intelligent healthcare, medicine, and biophysics.

सार

होलोग्राफिक फ्लो साइटोमेट्री (एचएफसी) एक सेल-विश्लेषण तकनीक है जो माइक्रोफ्लुइडिक डिवाइस में बहने वाली कोशिकाओं की छवि और वर्गीकरण के लिए डिजिटल होलोग्राफिक माइक्रोस्कोपी (डीएचएम) का उपयोग करती है। वर्तमान एचएफसी तकनीक चरण पुनर्निर्माण पर आधारित है जिसके बाद पूर्व-प्रसंस्करण चरणों के रूप में संख्यात्मक रीफोकसिंग होती है। चरण छवियों को पुनः प्राप्त करने के लिए संख्यात्मक रीफोकसिंग विधि कम्प्यूटेशनल रूप से महंगी है और इसलिए, वर्तमान एचएफसी तकनीकों की गति को सीमित करती है। इसके अलावा, संख्यात्मक रीफोकसिंग एल्गोरिदम लागू करते समय, यदि कोई व्यक्तिगत वस्तु डिजिटल होलोग्राम रिकॉर्ड करते समय फोकस में मौजूद नाममात्र विमान से दूर है, तो संबंधित ऑब्जेक्ट फ्रील्ड अत्यधिक धुंधला हो सकता है, और ऐसी वस्तुओं के पूरी तरह से गायब होने की संभावना है। परिणामस्वरूप, कोशिकाओं के वर्गीकरण और परिमाणीकरण की सटीकता प्रभावित होती है। एचएफसी में सटीक और तीव्र वर्गीकरण और परिमाणीकरण के लिए इनलेट स्ट्रीम में बेतरतीब ढंग से निलंबित कोशिकाओं को एक फ़ाइल व्यवस्था में त्रि-आयामी रूप से केंद्रित करने की आवश्यकता होती है। वर्तमान त्रि-आयामी (3डी) शीथिंग उपकरण कोशिकाओं को माइक्रोचैनल के मध्य तल पर केंद्रित करते हैं, जिससे कोशिकाओं को माइक्रोस्कोप उद्देश्य की कार्यशील दूरी के भीतर रखने के लिए सटीक तरीकों के माध्यम से छोटी गहराई वाले उपकरणों के निर्माण की आवश्यकता होती है। इसके अलावा, शीथ प्रवाह का उपयोग करके 3डी हाइड्रोडायनामिक फ़ोकसिंग के लिए मौजूदा तरीकों के लिए मल्टी-लेयर निर्माण के साथ माइक्रोफ्लुइडिक उपकरणों की आवश्यकता होती है, जिससे निर्माण प्रक्रिया में जटिलता आती है और डिवाइस की लागत बढ़ जाती है। चूंकि संदूषण को रोकने के लिए एचएफसी के लिए माइक्रोफ्लुइडिक उपकरणों को एक बार उपयोग के बाद त्याग दिया जाता है, इसलिए कार्यक्षमता को प्रभावित किए बिना सरल डिजाइन और कम लागत वाली निर्माण तकनीकों के माध्यम से 3डी शीथिंग उपकरणों की लागत को कम करना जरूरी है।

सबसे पहले, हम कोशिकाओं के 3डी हाइड्रोडायनामिक फ़ोकसिंग के लिए एक शीथ-फ्लो आधारित माइक्रोफ्लुइडिक डिवाइस के डिजाइन, निर्माण, लक्षण वर्णन और परीक्षण का प्रदर्शन करते हैं। हमारा उपकरण हाइड्रोडायनामिक रूप से कोशिकाओं को माइक्रोचैनल की निचली दीवार के पास एक एकल-फ़ाइल में केंद्रित करता है जो छोटे उपकरण आयामों की आवश्यकता के बिना, उच्च आवर्धन और कम कार्य दूरी के उद्देश्यों के साथ कोशिकाओं की इमेजिंग की अनुमति देता है। माइक्रोचैनल के अपेक्षाकृत बड़े आयाम कम-सटीक निर्माण तकनीकों का उपयोग करके आसान निर्माण को सक्षम करते हैं, और

डिवाइस डिज़ाइन की सादगी विभिन्न परतों के कठिन संरेखण की आवश्यकता से बचाती है। हमने 3डी संख्यात्मक सिमुलेशन के साथ डिवाइस के प्रदर्शन की विशेषता बताई है और फ्लोरोसेंटली रंगे नमूना तरल पदार्थ के हाइड्रोडायनामिक फोकसिंग के प्रयोगों के साथ इन सिमुलेशन को मान्य किया है। सिमुलेशन से पता चलता है कि 3डी केंद्रित नमूना धारा की चौड़ाई और ऊंचाई को डिवाइस के मुख्य और साइड चैनलों की ऊंचाई और नमूना और शीथ तरल पदार्थों की प्रवाह दरों को अलग-अलग करके स्वतंत्र रूप से नियंत्रित किया जा सकता है। सिमुलेशन के आधार पर, हम किसी विशेष आकार की कोशिकाओं पर ध्यान केंद्रित करने के लिए डिवाइस आयाम और प्रवाह दर चुनने के लिए उपयोगी दिशानिर्देश प्रदान करते हैं। इसके बाद, हम ब्राइटफील्ड माइक्रोस्कोपी का उपयोग करके बड़ी संख्या में लाल रक्त कोशिकाओं की इमेजिंग के लिए अपने डिवाइस की प्रयोज्यता प्रदर्शित करते हैं।

इसके बाद, हम मिश्रण से कोशिकाओं के सटीक दृश्य, वर्गीकरण और मात्रा निर्धारण के लिए 3डी हाइड्रोडायनामिक फ़ोकसिंग का उपयोग करके एक एचएफसी तकनीक का प्रदर्शन करते हैं। हमारा एचएफसी दृष्टिकोण एक माइक्रोफ्लुइडिक डिवाइस में बहने वाली कोशिकाओं की छवि के लिए उच्च-रिज़ॉल्यूशन, एकल-शॉट डिजिटल होलोग्राफिक माइक्रोस्कोपी का उपयोग करता है जो चैनल की निचली दीवार के करीब एक फ़ाइल में कोशिकाओं को ऑर्डर करता है। डीएचएम के साथ 3डी फोकसिंग डिवाइस का एकीकरण संख्यात्मक रीफोकसिंग की आवश्यकता के बिना उच्च-आवर्धन होलोग्राफिक इमेजिंग की अनुमति देता है। इसके अलावा, विशेष रूप से डिज़ाइन किया गया 3डी शीथिंग उपकरण कोशिकाओं के समूहन को रोकता है। हमारी एचएफसी पद्धति की प्रभावकारिता को प्रदर्शित करने के लिए, हम बिना दाग वाली लाल रक्त कोशिकाओं और पॉलीस्टायरीन कणों के मिश्रण से वर्गीकरण के एक चुनौतीपूर्ण मामले पर विचार करते हैं, जो अन्यथा ब्राइटफील्ड और चरण-कंट्रास्ट माइक्रोस्कोपी में अप्रभेद्य हैं। अलग-अलग अनुपात वाले सेल-कण मिश्रण के प्रयोगों के माध्यम से, हम दिखाते हैं कि हमारी एचएफसी तकनीक कम्प्यूटेशनल रूप से महंगी और समय लेने वाली संख्यात्मक रीफोकसिंग से बचते हुए कोशिकाओं और कणों को उनके पुनर्निर्मित चरण मूल्यों के आधार पर सटीक रूप से गिन और वर्गीकृत कर सकती है। हम रुचि के क्षेत्र और कैमरा फ्रेम दर की पसंद पर भी चर्चा करते हैं ताकि हमारे डिवाइस में प्रत्येक कोशिका और कण की व्यक्तिगत रूप से छवि ली जा सके।

अंत में, हम विशिष्ट जैविक अध्ययनों के लिए अपने एचएफसी दृष्टिकोण के अनुप्रयोग को प्रदर्शित करते हैं। हम निलंबन में बिना दाग वाले ट्रिप्सिनाइज्ड प्रवाहित एमडीए-एमबी-231 और जेडआर-75-1 स्तन कैंसर कोशिकाओं के वर्गीकरण के लिए अपनी एचएफसी विधि प्रस्तुत करने के साथ शुरुआत करते हैं। एमडीए-एमबी-231 और जेडआर-75-1 कोशिकाओं का वर्गीकरण हमारे एचएफसी दृष्टिकोण की

क्षमता को प्रदर्शित करने के लिए एक चुनौतीपूर्ण समस्या के रूप में कार्य करता है क्योंकि ये कोशिकाएं बिना दाग के ब्राइटफील्ड और चरण-कंट्रास्ट माइक्रोस्कोपी के तहत अप्रभेद्य हैं। इसके अलावा, हम संख्यात्मक रीफोकसिंग और स्टेनिंग से बचते हुए कैंसर कोशिकाओं की दवा-संवेदनशीलता परीक्षण के लिए अपने एचएफसी दृष्टिकोण की क्षमता प्रदर्शित करते हैं। यह दवा की संवेदनशीलता के मूल्यांकन के लिए पारंपरिक दृष्टिकोण के विपरीत है, जिसमें निलंबन में जीवित और मृत कोशिकाओं के अनुपात की कल्पना और अनुमान लगाने के लिए कैंसर कोशिकाओं की संख्यात्मक रीफोकसिंग और धुंधलापन आवश्यक है। इस थीसिस में प्रस्तुत कार्य के निष्कर्ष अन्य उच्च-थ्रूपुट सेल इमेजिंग और वर्गीकरण तकनीकों के साथ प्रतिस्पर्धा करने में एचएफसी की एक प्रमुख सीमा को संबोधित कर सकते हैं। सेल वर्गीकरण की हमारी एचएफसी पद्धति बुद्धिमान स्वास्थ्य देखभाल, चिकित्सा और बायोफिज़िक्स सहित विभिन्न विषयों में इसके उपयोग के लिए क्षेत्र खोलती है।

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FC Flow cytometry	1
MFC microfluidic flow cytometry	4
IFC Imaging flow cytometry	4
DHM Digital holographic microscopy	6
HFC holographic flow cytometry	7
ROI region of interest	8
3D three-dimensional	10
2D two-dimensional	16
PDMS polydimethylsiloxane	16
RBCs red blood cells	19
DI deionized	19
CFD computational fluid dynamics	22
CMOS complementary metal–oxide–semiconductor	27

CCD charge-coupled device	27
PMT photomultiplier tubes	27
APD avalanche photodiodes	27
PMMA polymethyl methacrylate	48
CNC computer numerical control	48
IPA isopropyl alcohol	48
PBS phosphate buffer saline	56