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(please insert ref. above)

The Research Grants Council of Hong Kong
NSFC/RGC Joint Research Scheme
Joint Completion Report

(Please attach a copy of the completion report submitted to the NSFC by the Mainland researcher)

Part A: The Project and Investigator(s)

1. Project Title

Investigation of technology and mechanism of in/ex vivo stem-cell differentiation by femtosecond laser

2. Investigator(s) and Academic Department/Units Involved

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Prof. Ho Ho Pui (New PI)	Prof . He Hao
Post	Professor	Professor
Unit / Department / Institution	Department of Biomedical Engineering, CUHK	School of Biomedical Engineering, Shanghai Jiao Tong University (SJTU)
Contact Information	aaron.ho@cuhk.edu.hk	haohe@sjtu.edu.cn
Co-investigator(s) *(with title and institution)*	Prof. Kong Siu Kai School of Life Sciences, CUHK Prof. Kwan Yiu Wa School of Biomedical Sciences, CUHK	Prof. Wei Xunbin School of Biomedical Engineering, SJTU

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted)*
Project Start date	1 January 2017	N/A	
Project Completion date	31 December 2020	N/A	
Duration *(in month)*	48 months		
Deadline for Submission of Completion Report	31 December 2021		

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1) To develop an all-optical method to induce stem cell differentiation by femtosecond laser in in vivo and ex vivo model.
2) To study the role of mitochondria in the laser-induced differentiation.
3) To develop a droplets-in-oil micro-device for the laser-induced stem cell differentiation.

5.2 Revised Objectives

Date of approval from the RGC: _____

Reasons for the change: _____

_____

N.A.

1.
2.
3.

5.3 Realisation of the objectives

1) To develop an all-optical method to induce stem cell differentiation by femtosecond laser in in vivo and ex vivo model.

In this study, we focused our femtosecond laser in cells cytosol within 2 μm2. Optimized laser stimulation parameters such as energy density, repetition rate, pulse duration and exposure time for osteogenic differentiation were subsequently identified to avoid lethality. After confirming 140 mW/μm2 , 200fs, 80Hz was the best stimulation parameter through the real-time response similar to those triggered by growth factor stimulation, osteoblastic gene RunX2, Osterix and SOD2 were found to be activated in the hMSCs 3 days after with the pulsed laser illumination alone, without any biochemical reagents and physical contact. After 21 days, the laser-illuminated hMSCs could form extracellular mineralized nodules.

To demonstrate that this photo-stimulation is able to induce differentiation in vivo, hMSCs and femtosecond laser (fsL) stimulated hMSCs were subcutaneously inoculated to the left and right nude mice ears, respectively, with the animal research ethics approval. After 14 days, histological sectioning with Von Kossa staining was performed and more calcium deposits were found in the laser treated group when compared to that of control. To conclude, the laser stimulated cells showed the ability to differentiate into osteoblasts and finally developed into bone tissue in vitro and in vivo.

2) To study the role of mitochondria in the laser-induced differentiation.

The mitochondrial trans-membrane potential (MMP) in the stimulated area showed a moderate depolarization after 140 mW/μm2 fsL stimulation and 350 mW/μm2 nanosecond laser (nsL) stimulation, which could direct osteogenic differentiation. This suggested that both a high peak power generated non-linear effect and total energy stimulation generated thermal effect, could generate MMP depolarization. However, the rise of reactive oxygen species (ROS) could hardly be obtained after laser perturbation when compared to other cell lines. To further illustrate this phenomenon, we found that the mRNA and protein expression of antioxidant enzymes manganese SOD2 could be activated in Day-3 and -7 after fsL stimulation for 1 s, which was able to clear mitochondrial ROS. Therefore, our laser stimulation might activate the antioxidant system in the hMSCs cells mitochondria similar to that in the conventional chemical osteogenic differentiation method.

3) To develop a droplets-in-oil micro-device for the laser-induced stem cell differentiation.

We used droplet-based microreactors to improve cell transfection efficiency. However, due to the different phases occurred in droplet-in-oil technique that may introduce optical aberration, we decided to use single phase microfluidic technique instead. Here, we developed the optical platform in both lab-on-a-chip and centrifugal microfluidic

techniques. For cells stimulated on a lab-on-a-chip platform, a 72.5% stimulation efficiency could be achieved. After 3 weeks, extracellular mineralized nodules were formed. For cells stimulating on a centrifugal microfluidic disc under a 100 rpm, 40 mins rotation, more RunX2 and SOD2 could expressed when compared to control groups after 2 weeks, which holds promising prospects to induce osteogenic differentiation.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	Addressed *(please tick)*	Percentage achieved *(please estimate)*
1. To develop an all-optical method to induce stem cell differentiation by femtosecond laser in in vivo and ex vivo model.	√	100%
2.To study the role of mitochondria in the laser-induced differentiation.	√	80%
3. To develop a droplets-in-oil micro-device for the laser-induced stem cell differentiation.	√	90%

6. Research Outcome

Major findings and research outcome
(maximum 1 page; please make reference to Part C where necessary)

Here, we present a new strategy to induce osteogenic differentiation in the hMSCs by a highly focused pulsed laser without any chemical or exogenous materials interference. Through modulating the $[Ca^{2+}]i$, ROS, MMP in the bone marrow derived hMSCs, we found 140 $mW/\mu m^2$, 200fs, 80MHz fsL with 1s illumination was able to promote osteogenic differentiation. An early expressed RunX2, SOD2, Osterix, ALP was found in the light-induced stimulation in 7 days when compared to the conventional chemical induction, which demonstrates an early osteogenic differentiation initiation. After 21 days, the laser stimulation group with 1 s light dosing for single cycle illumination could achieve a 2.1-fold increase in the red precipitants when compared to the control group without exogenous chemical perturbation 21 days after, in the final mineralization stage. Based on our single point laser stimulation technique, the major hurdle in regenerative medicine caused by leveraging *ex vivo* culture and exogenous materials could be overcome. A high spatial resolution and clean *in situ* manipulation is possible to be achieved by our pure use of laser induced osteogenic differentiation.

To satisfy the requirements of 1 s precise sequential dosing of fsL and automation in individual cells, we programmed the sample stage to an automatic zig-zag stimulation pattern and combined it with a microfluidic trapping device for fsL stimulation. A 72.5% stimulation efficiency could be achieved by this method. The expression of RunX2 and Osterix was found to increase 7 days after. Moreover, the laser-illuminated hMSCs could

form extracellular mineralized nodules after 21 days. Consequently, an accurate stimulation in individual cells is developed for multiphoton-led differentiation.

To improve cell-cell communications, we further designed the photo-stimulation on a centrifugal microfluidic disc. Working with centrifugal force, loaded cells in the inner arc of the disc were prone to flow to the outer edge of the disc. A focused laser spot was addressed in any point of the outer edge and every cells stay in the same radius are capable to receive light dosing during the disc spinning. A $[Ca^{2+}]i$ upregulation was obtained after 10 mins under 100 rpm rotation. Followed by an increased RunX2, SOD2 expressions in Day-7 and -14 were found to be upregulated for cells rotated for 40 mins under 100 rpm. Through this fully automatic lab-on-a-disc platform, an evaluated through-put and repeatable platform has been developed.

Potential for further development of the research and the proposed course of action *(maximum half a page)*

In this study, we provide a minimally-invasive and clean method for osteogenic differentiation and can be potentially applied to tissue regeneration. But there are still some bottlenecks to increase the utility of this photo-stimulation platform. Paying the price for a high spatiotemporal-specific manipulation, the harsh requirements for a successful multiphoton excitation which mostly happens in the focal point that limits the throughput and platform selectivity of this method. Nevertheless, tissue regeneration needs a specific microenvironment and enough cell population for cell-cell interactions. Microfluidic is one of the possible solutions to solve this dilemma. However, different phases between the lens and cells will generate light abbreviation and influence the stimulation result. And even a slightly floating of cells in the channel could lead an off-target of the laser focus. Therefore, the microfluidic channel dimension and device balancing should be carefully designed. Ongoing advances in photonics technology are possible to lead to future simplification and convenience of this study including scanning and parallel photo-stimulation techniques. At the same time, holographic optical tweezers are currently the most advanced optical manipulation technique. This technique can facilitate a multitude of laser stimulation simultaneously. By combining these techniques with a high N.A. soft-glass multimode fibre can realize a 3D manipulation *in vivo*. Consequently, a hybrid platform linking photonics technique with microfluidic system may serve as a practical system to address tissue regeneration *in situ* and a full understanding of cell and light interaction.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

A variety of physical and chemical methods have been developed in research laboratories for the induction of stem cell differentiation. However, the non-specific interference from these methods is one of the major hurdles to their widespread utility. To develop a clean and precise differentiation induction approach with minimal invasion, a modulation method purely employing pulsed laser without other external perturbation was established in this study. We optimized the optical parameters and found that the 140 $mW/\mu m^2$ femtosecond laser (fsL) (200 fs) stimulation for 1 second could induce stem cells to differentiate into bone cells by screening the bone cell related markers at different differentiation stages. However, the conventional approach using optical stimulation by a focused beam for cell activation is limited by the low throughput, small-scale production.

To satisfy the requirements of sequential dosing of individual cells and automation, we developed a fully automatic zig-zag pattern stimulation in a lab-on-a-chip device, finally to an optical stimulation integrated lab-on-a-disc. The microfluidic approach coupled with our ultrashort-pulsed laser could eventually harvest a successful differentiation. Collectively, we described a novel and automated fsL stimulation induced bone cell differentiation method in a clean, labor free and controllable manner.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project

(Please attach a copy of each publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s)	Title and Journal/ Book	Submitted to RGC	Attached to this report	Acknowledged the support of this Joint Research Scheme	Accessible from the institutional repository
Year of publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*	***(bold** the authors belonging to the project teams and denote the corresponding author with an asterisk*)*	*(with the volume, pages and other necessary publishing details specified)*	*(indicate the year ending of the relevant progress report)*	*(Yes or No)*	*(Yes or No)*	*(Yes or No)*
2018				Shaoyang Wang, Yaohui Liu, Dapeng Zhang, Shih-chi Chen, **Siu-Kai Kong,** Minglie Hu,* Youjia Cao, and **Hao He***	Photoactivation of Extracellular-Signal-Regulated Kinase Signaling in Target Cells by Femtosecond Laser. Laser Photonics Rev. 2018, 12, 1700137 (8 pages)	2018-12-31	Yes	Yes	Yes
2019				Dongping Wang , Jacky Fong Chuen Loo , Jiajie Chen, Yeung Yam, Shih-Chi Chen, **Hao He**, **Siu Kai Kong** and **Ho Pui Ho***	Recent Advances in Surface Plasmon Resonance Imaging Sensors. Sensors. 2019,19(6),1266.	2018-12-31	Yes	Yes	Yes
2019				Yuye Wang, **Shiyue Liu,** Tiankai Zhang, Hengji Cong, Yuanyuan Wei, Jianbin Xu, Yi-Ping Ho, S**iu-Kai Kong** and **Ho-Pui Ho***	A centrifugal microfluidic pressure regulator scheme for continuous concentration control in droplet-based microreactors Lab Chip, 2019,19, 3870-3879.		Yes	Yes	Yes

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered *(Please attach a copy of each delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)*

Month/Year/ Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
2-7 Feb 2019 California, United States	Laser-induced plasmonic heating for the detection of genetic markers	Photonics West 2019	2018-12-31	Yes	Yes	No
27 Jan - 1 Feb 2018 California, United States	In situ cell lysing and DNA amplification with localized heating induced by plasmonic absorption	Photonics West 2019	2018-12-31	Yes	Yes	No
9-12 Oct 2018 Xi'an, China	Plasmonic Absorption Induced Heating for Single Cell Manipulation and Detection of Genetic Markers	The 7th Conference on Advances in Optoelectronics and Micro/nano-optics (AOM 2018), Xi'an, China	2018-12-31	Yes	No	No
23/Oct/2017/ Institute for Advanced Study, HKUST	Using Light to Induce and Detect Stem Cell Differentiation in Microfluidic Device	AoE On-site Visit Conference - Novel wave functional materials for manipulating light and sound	2018-12-31	Yes	No	No
April/2021/on line	Ultrafast Laser Stimulation in Stem-cells and Its Potential for Cell Differentiation Induction	Biophotonics Congress: Optics in the Life Sciences 2021		Yes	Yes	Yes

10. Student(s) trained *(Please attach a copy of the title page of the thesis.)*

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Liu Shiyue	Ph.D.	01/Aug/2017	Jan/2022
Wang Yuye	Ph.D.	01/Aug/2017	June/2021

Osteogenic Differentiation of Mesenchymal Stem Cells by Near-infrared Pulsed Laser Induction

Liu, Shiyue

A Thesis Submitted in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy in Biochemistry

The Chinese University of Hong Kong

January 2022

Novel Strategies for Sample Manipulation and Biodetection in Centrifugal and Optical Microfluidic Systems

WANG, Yuye

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biomedical Engineering

The Chinese University of Hong Kong

June 2021

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

Certificate of Awards “Champion and special award”, awarded by “Professor Charles K. Kao Student Creativity Awards 2021”.

香港中文大學
The Chinese University of Hong Kong

Professor Charles K. Kao Student Creativity Awards 2021

Certificate of Award

This is to certify that the following student(s) participated in the Professor Charles K. Kao Student Creativity Awards 2021 and won in the competition.

Award:	Champion, Special Award in Life Sciences
Entry Category:	Postgraduate Individual
Name of Student:	LIU Shiyue
Project:	Ultrafast Laser Stimulation in Stem-cells and Its Potential for Cell Differentiation Induction

Professor Rocky S. TUAN
Vice-Chancellor and President
The Chinese University of Hong Kong

28 May 2021

12. Statistics on Research Outputs *(Please ensure the summary statistics below are consistent with the information presented in other parts of this report.)*

	Peer-reviewed journal publications	Conference papers	Scholarly books, monographs and chapters	Patents awarded	Other research outputs (Please specify)
No. of outputs arising directly from this research project [or conference]	3	5	N.A.	N.A.	N.A.