A multiplexed intracellular probing (IP) nano-chip for interrogation of myo-fibroblasts and cardiomyocytes gene in cardiac fibrosis

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Heart disease remains global leading cause of mortality. In organ, most cardiac diseases are accompanied with fibrosis, a procedure that cardiac fibroblasts accumulate and over-express extracellular matrix (ECM) proteins, eventually leading to dysfunctions of cardiomyocytes. At cellular level, however; there is a lack of intracellular biomarkers of the activated myo-fibroblasts primarily due to its phenotypic heterogeneity[1]. Furthermore, comprehensive understanding of the mechanism of fibroblast-secreted extracellular matrix (ECM) proteins in regulating the gene expression of cardiomyocytes have been hampered due to the challenges of intracellular analysis in single living cell, which leads to inconsistent conclusions between previous reports[2]. Current tools remain challenging to address these two specific issues at single cell level.

We herein report an advanced intracellular probing (IP) nano-chip for precise detection of gene expression within myo-fibroblasts and cardiomyocytes in cardiac fibrosis model (**Fig. 1**). The silicon chip was fabricated using cleanroom techniques, mainly including projection photolithography and deep reactive ion etching (DRIE) (The inset of **Fig. 1a**)[3]. Cells were positioned on IP nano-chip where each single cell is aligned with a Z-directional nanochannel (**Fig. 1b**). The buffers of IPs (i.e. molecular beacons (MBs)) are filled in a bottom chamber under the chip. A pulsed electric field is applied over IP nano-chip and cells between a pair of electrodes (**Fig. 1c**). The nanochannel focuses the electric field within a narrow area on the cell membrane, enabling safe and localized electroporation. MBs are electrophoretically driven into the cell under previse dose control (**Fig. 1b&c**).

The nanochannel-assisted electroporation and electrophoretic IP delivery require that the single cell tightly connects with a nanochannel. To achieve this aim, we developed a novel on-chip microfluidics approach for moving and trapping cardio-cells on the nanochannel array, where > 10,000 cells can be massively parallel probed over a 1 cm^2 single chip (**Fig. 2a**). Micro-well array (SU-8 photoresist), designed to capture individual cells, were photolithographically patterned around each nanochannel on the chip (**Fig. 2b**). For flowing cell to micro-well, microfluidic channels were fabricated on a PDMS membrane by soft-lithography. Controlling flow rate achieved precise positioning each single cell into microwell.

We have designed and synthesized MBs to detect the expression of mRNAs which are hypothesized to indicate the biomarkers of myo-fibroblasts (e.g. Tcf-21, Pdgfr-α, α-SMA)[2] (**Fig. 2c**). The expression of specific mRNAs allows to identifying the biomarkers those can exclusively 'label' the activated myo-fibroblasts rather than their counterparts (e.g. epithelial fibroblasts). MBs were also applied to investigate GATA4 and HDAC2, two critical genes in cardiomyocytes in cardiac fibrosis. At single cell level, the novel IP nanochip characterized the activation of 'myo-fibroblasts' and provided comprehensive analysis of the biomarkers for acthe gene expression in cardiomyocytes in response to the activated myo-fibroblasts in cardiac fibrosis.

[1] Krenning, G. *et al.* 2010. ***Journal of Cellular Physiology***, 225, 631.
[2] Tallquist, M.D. *et al.* 2017, ***Nature Reviews Cardiology***, 14, 484.
[3] Chang L. et al. 2016, ***Small***, 12, 5971.

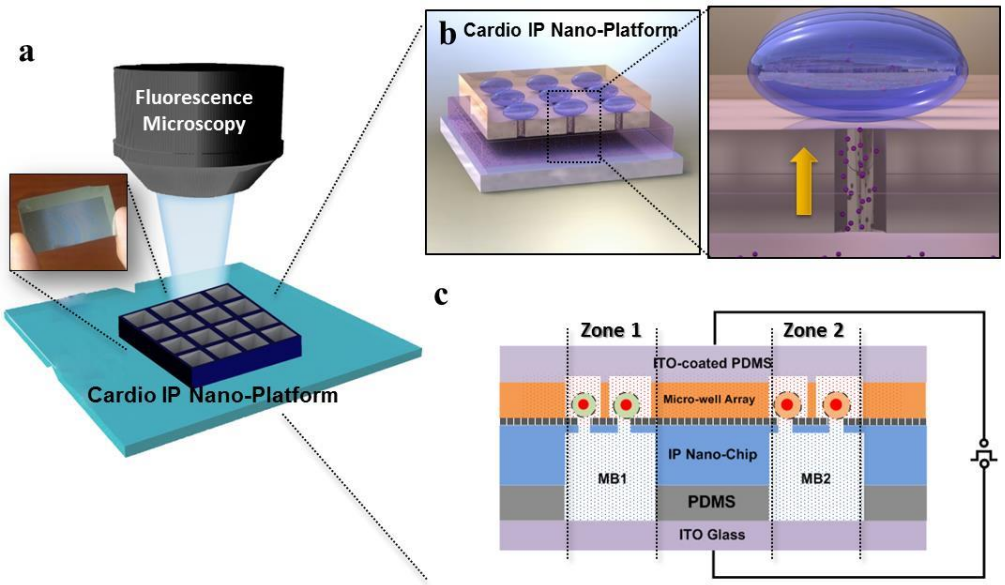

Fig. 1. The overall description of the novel Cardio IP nano-platform for identifying intracellular biomarkers of activated-myofibroblasts and interrogating gene expression in cardiomyocytes in response to activated myo-fibroblasts in cardiac fibrosis. (a) The schematic and photograph of the fabricated silicon IP nano-chip. (b) Intracellular probes (e.g. molecular beacons) are delivered into loaded cells through Z-directional nanochannel under electrophoresis. (c) The cross-section schematic of the cell nano-electroporation on IP nano-chip, which achieved intracellular delivery at single cell level.

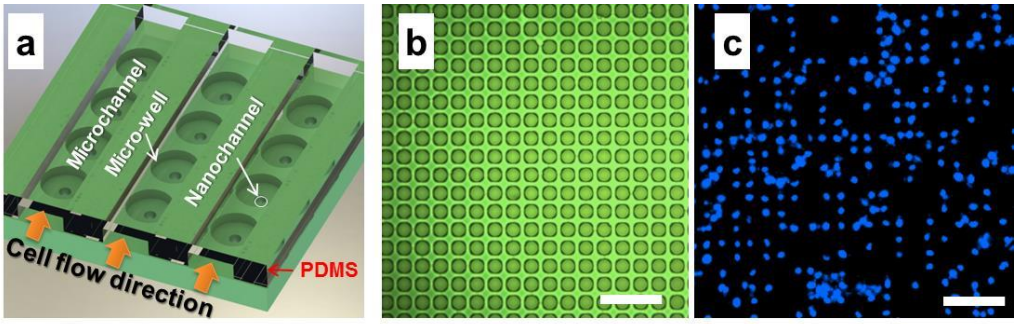

Fig.2 Micro-well and microchannel on IP nano-chip for microfluidic-assisted cardio cell trapping and single cell biomarker detection. (a) The schematics of array of micro-well array patterned around nanochannel for cell seeding. Cells flow within in microchannel manufactured on PDMS mold. (b) High density micro-well array (10 μm in diameter) has been patterned on the nanochannel array chip using photolithography. (c) Molecular beacons recognize the expression of mRNAs intracellularly in single cell. Scale bar: (b) &(c) 20 μm.