Electronic Supplementary Material (ESI) for RSC Advances.

Metabolic variation of HeLa cells migrating on microfabricated cytophilic channels studied by fluorescence lifetime of NADH

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Materials and methods

Cell culture substrate:

Cytophobic substrates, for patterning cytophilic domains, were fabricated by coating a borosilicate glass substrate (D263, Schott AG, Germany, 0.17 mm thickness) with gold layer and cytophobic polymer layer sequentially. Prior to the coating, the glass substrate was sequentially cleaned by ethanol (98%), and deionized water. The cytophobic top surface of the substrate is formed by coating a layer of 2-methacryloxyethylphosphorylcholine (MPC) polymer. MPC coating was done by soaking the substrate in a 0.2% (w/v) solution of polymer in ethanol. The substrate was dried under clean air and stored at room temperature. MPC polymer is transparent to 1064 nm wavelength of light from Nd:YVO_4 laser, although the gold layer absorbs the same light. Thus 30 nm gold layer was used in between the polymer and glass surface as thermal transducer for photothermal ablation, which was formed by physical vapor deposition using an electron beam evaporation system. A 7 nm thin platinum layer was coated over the glass plate prior to gold coating to achieve proper attachment of the gold layer.

Laser fabrication setup:

Overview of the setup is shown in supplementary Fig. 1. Q-switched Nd:YVO_4 laser is the ablation light source lasing at 1064 nm. Galvanometer mirrors (ScanMate, Laser Solution

Technologies Co., Ltd., New Taipei Taiwan) scan the laser over the substrate to draw patterns on it. These Galvanometer mirrors are coupled with laser control unit to control laser irradiation. The laser scanning was controlled by Marking Mate software (Eastern Logic,l Taipei, Taiwan) to fabricate patterns. The maximum scanning speed was 800 mm/s. The patterns were designed on computer as graphics and laser drew those patterns on the substrate. A piloting diode laser (653 nm) helps to focus the ablation laser, which were coaxially synchronized by a dichroic mirror. The laser was focused by a 10× water immersion objective (Olympus, Japan) of numerical aperture (NA) 0.3 coupled in a modified Olympus BX51microscope (Olympus, Japan). Maximum laser power at the focal plane was detected to be 120 mW.

Cell culture:

Cervical carcinoma HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, low glucose, GlutaMAX^{tm}, Life Technologies) supplemented with streptomycin (100 μg/ml), penicillin (100 uits/ml) and 10% fetal bovine serum (FBS). Cells were seeded on a substrate at density of 3000 cells/cm^2. Seeded cells were incubated in humidified atmosphere at 37^{0}C with 5% CO_2.

Multiphoton excitation for measuring NADH fluorescence lifeime:

A mode-locked Ti:sapphire Mira F-900 laser (Coherent, Santa Clara, USA) was used to excite NADH, which is capable of producing femtosecond laser pulse train at 76 MHz, pumped by a solid-state frequency-doubled 532 nm Verdi laser (Coherent). The spectral range of Ti:sapphire is 700 to 1000 nm. The maximum wavelength for two-photon excitation of cellular NADH is 740 nm. The excitation beam was coupled to the FV300 (Olympus, Japan) scanning unit with scanning speed set by a function generator (AFG310, NJ 07740, USA) to optimize the photon acquisition. Measurements were conducted on a modified inverted Olympus microscope IX 71

(Olympus, Japan). Supplementary Figure 2 schematically depicts the FLIM microscopy setup used in this experiment. The laser beam was focused on the cells with a Plan Apochromat oil immersion objective (60×, NA 1.3, Olympus, Japan). An average laser power of ~ 3.5 mW was used to avoid photo damage of the cells under investigation.

Photon acquisition:

To match the spectral characteristics, band-pass filter of 447±30 nm was used for collecting NADH autofluorescence emission (Semrock, USA). An additional 633 nm short-pass filter was used to avoid the excitation light at 740 nm. Finally, the photons of autofluorescence signals were detected by a cooled photon counting photomultiplier tube (PMT; H7422-P40, Hamamatsu Photonics, Hamamatsu, Japan). The FLIM data were acquired by a time-correlated single photon counting system (TCSPC) (SPC-830, Becker & Hickl, GmbH, Germany). All FLIM images were taken at 256 x 256 pixels resolution with the accumulation time of 600 sec to have sufficient photon counts for statistical data analysis.

Data analysis

Data were analyzed using SPCImage (v. 2.8) software (Becker & Hickl, GmbH, Germany). As the cellular system has both free and protein-bound NADH, lifetime calculation from the complex fluorescence decay was done by fitting to a double exponential model function. Lifetimes from the composite decays of NADH were derived by convolution of an instrument response function (IRF), I_{instr}, with a function, defined in Eq. (1), with offset correction for the ambient light and/or dark noise $I_{0,}$ to obtain calculated lifetime decay function $I_c(t)$ in Eq. (2)

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \quad \ldots\ldots\ldots\ldots (1),$$

$$I_c(t) = \int_{-\infty}^{\infty} I_{instru}(t)\{I_o + F(t)\}dt \quad \text{.... (2).}$$

Where τ_1 and τ_2 denote short and long lifetime constants of NADH free and bound species respectively; a_1 and a_2 refer to the corresponding relative amplitudes; $a_1 e^{-t/\tau_1}$ and $a_2 e^{-t/\tau_2}$ represent the contributed fluorescence decays from free and protein bound components of NADH, respectively. The, I_{instr} was measured experimentally with the periodically poled lithium niobate (PPLN) crystal at 370 nm (i.e. the second harmonic of 740 nm from the Ti:Sapphire laser). The instrument response is the inherent lifetime of the instruments without any sample or the lifetime with a fast decay time. Usually, second harmonic is a scattering process with a short decay time in comparison to any fluorescence decay. Second harmonic generated by PPLN was used to measure the instrument response. The average lifetime was calculated as an amplitude-weighted parameter of the two lifetime components:

$$\tau_m \equiv \frac{a_1\tau_1 + a_2\tau_2}{a_1 + a_2} \quad \text{..... (3).}$$

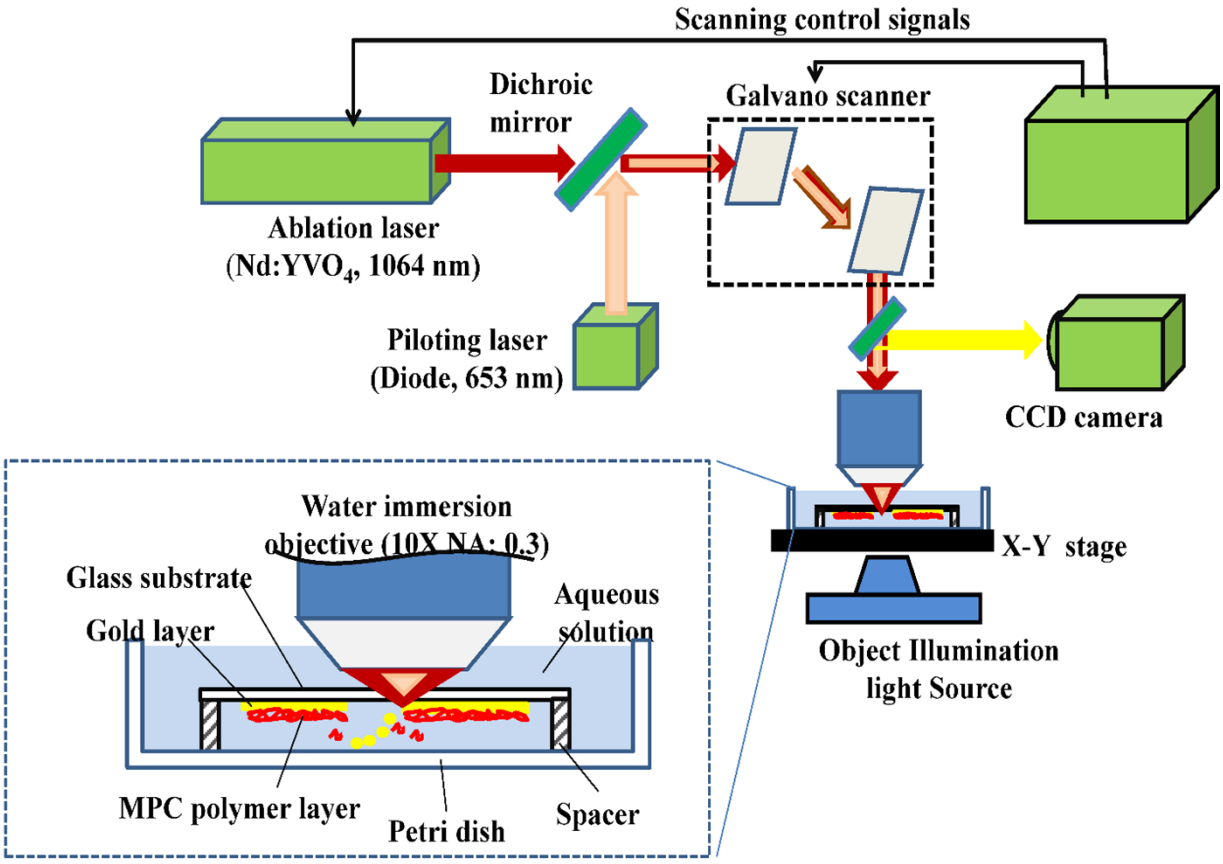
The model parameters (i.e. a_i and τ_i) were derived by fitting the decay $I_c(t)$, from Eq 2, to the actual data $I_a(t)$ by minimizing the goodness-of-fit function defined in Eq. (3), using the Levenberg-Marquardt search algorithm,

$$\chi_R^2 = [\sum_{k=0}^{n} [I_a(t) - I_c(t)]^2 / I_a(t)]/(n-p) \quad \text{.....3(a),}$$

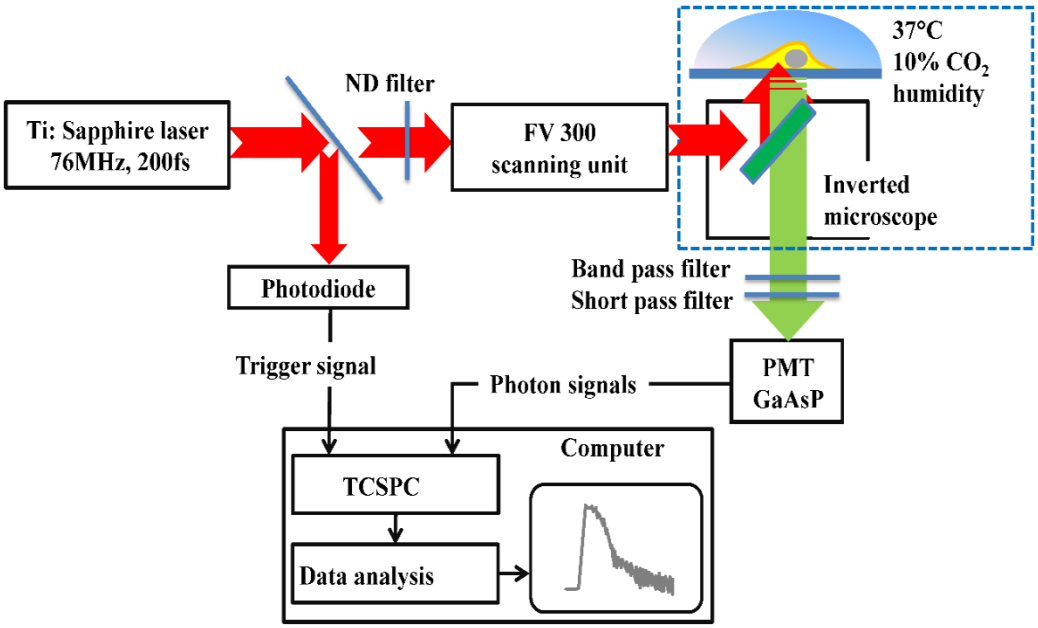
where n denotes the number of data (time) points (equal to 256), and p represents the number of model parameters. The ratio of a_1 and a_2 is the best indicator of free and protein-bound states of

NADH, which can be used to depict the status and changes in cellular metabolism. Note that higher value of a_2 represents higher fraction of protein bound NADH. Thus, the value of a_1/a_2 ratio is inversely proportion to the metabolic activity. These values also can represent shift in metabolic pathways from oxidative phosphorylation to glycolysis in direct relation for cancer growth.

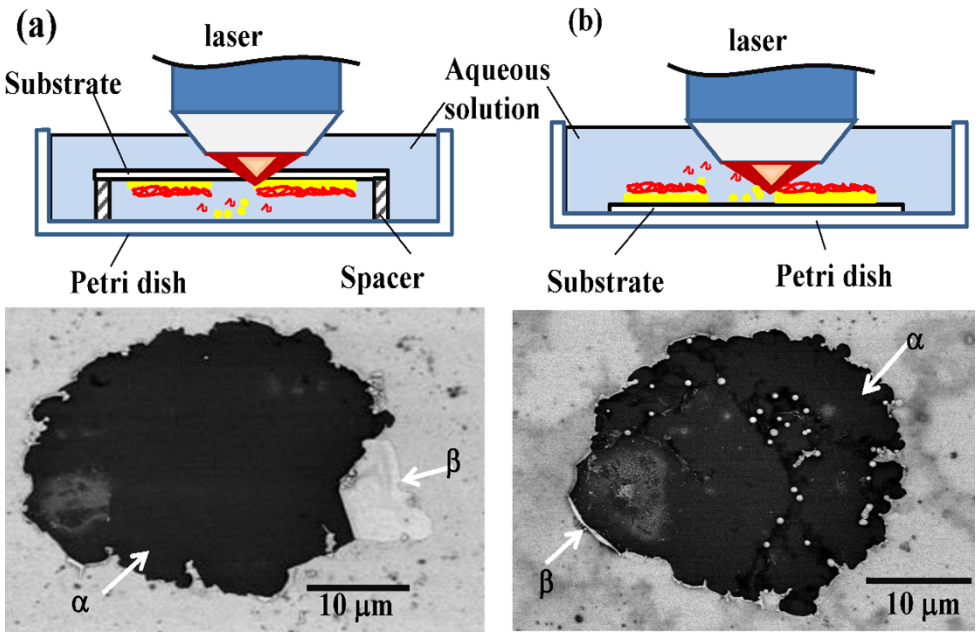
Supplementary Figures



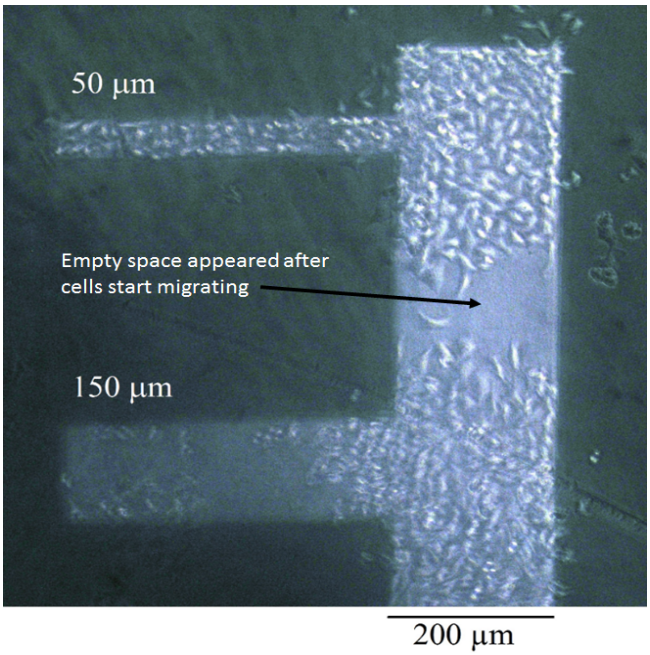
Supplementary Fig. 1 Schematic diagram of laser fabrication setup. The system is designed to ablated the upper cytophobic layer on the substrate under physiologic aqueous conditions with high scanning rate.



Supplementary Fig. 2 Schematic view of FLIM setup. The Inverted microscope was installed inside an incubation chamber (fitted with 10% CO2, 37°C and approximate 90% humidity controller) for live cell imaging under physiological conditions,.



Supplementary Fig. 3 Two different ablation conditions. Laser was focused to the substrates (a) gold and MPC polymer layer facing down ward, with laser irradiation from above through the glass surface and (b) gold and MPC polymer layer held upward facing the laser from top. In the figure the first row depicts diagrammatic representation of laser ablation process and the second row is the representative SEM images of the corresponding ablated areas. Arrows α and β indicate the ablated domains and the folded edges of gold layer by laser ablation.



Supplementary Fig. 4: Migration of cells in two different channels. The channel widths are 50 μm and 150 μm, respectively.