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Versatile on-Stage Microfluidic System for Long Term Cell Culture, Micromanipulation and Time Lapse Assays

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Abstract
We report here a versatile on-stage microfluidic cell culture and assay system which is compatible with different microscopes and sensors, can simultaneously perform steps of long term cell culture, high throughput time lapse cell assays/imaging, and cell micromanipulations. With the system, we cultured a variety of cells for different periods of time and monitored their cell morphology, migration and division. We also performed a series non-invasive real time in situ time lapse assays and micromanipulations on different cells. They include: the first time lapse imaging and measurements on the instantaneous variations of morphology, biomechanical properties and the intracellular protein of human red blood cells in responding to pH fluctuation, drug action and electromagnetic radiation; the first continuous time lapse Raman micro-spectroscopy on a CHO cell in different phases of its entire life cycles; the micro-transfection of GFP into B16 cells and the follow up observation of the cell’s morphology and expressed GFP fluorescence varying with incubation time and cell generations. The performance of these experiments not only demonstrated the capability of the system, but also proposed a variety of novel methods for obtaining time- and spatially-resolved information about the cellular and molecular heterogeneity and transformation during development or stimulations.

Keywords
Microfluidic cell culture and assay system; Long term cell culture; Time lapse cell assay; Cell micromanipulation

1. Introduction
To explore cellular processes and dynamic events, long term continuous monitoring of living cells on a microscope stage is strongly desired nowadays. Meanwhile, to study the cell responses to different physical and chemical stimulations at single-cell level, on stage cell assay and manipulation are also required so that the steps of cell culture and assay/manipulation can be performed simultaneously. However, it poses a number of significant biotechnological and bioengineering challenges in such experiments. For they need to have an onstage cell culture system to provide a clean, sterile,
endotoxin-free environment with a CO₂ atmosphere and suitable temperature for mammalian cell to maintain living cells in a healthy state for long time. The culture system should be also equipped with perfusion/microfluidic devices to create and vary the cell culture conditions for modeling physiological functions of cells. Furthermore, to be able to perform various assays and micromanipulations on the culturing cells, the system should be compatible with different microscopes and sensors, and have the capability to couple with various techniques such as time-lapse fluorescence imaging, optical trapping and dissection, microinjection, and Raman microspectroscopy.

Actually, to meet the requirements of live-cell research, a wide variety of on-stage living cell culturing and assay chambers have developed since the early twentieth century after mammalian cell culture techniques were developed(Dailey et al. 2016). In the first generation, the chambers were just either simply sandwiching of two coverslips or a Petri Dish without the capability to exchange culture medium. The second generation was characterized by different kind of perfusion chambers which enabled the continuous addition of fresh culture medium, though most of them were lack of satisfactory laminar flow characteristics. They mainly fall into two basic functional categories: closed and open systems. The open systems allow quick access to the culturing cells, thus permitting microinjection, changing of the culture medium, addition of drugs, or other manipulations to the cells. While closed chambers provide better insulation from the external environment but have ports that permit the addition of fresh medium and drugs during the experiment. As the development of microfluidic technique in the last ten years, the chambers have coupled with microfluidic devices and become microfluidic culture and assay systems. Microfluidic cell culture systems have several advantages over the previous perfusion chambers. They can allow exquisite control over experimental conditions to more closely mimic a cell's natural microenvironment, either by continuous laminar flow perfusion and waste removal or periodic perfusion with chemical gradients. They also offer convenience to couple with various downstream biochemical analytical or on-chip analysis platforms to study on low numbers of cells or single cells in high temporal / spatial resolution and with efficient high throughput. Nowadays, microfluidic cell culture and assay systems have a variety of models from two-dimensional(Halldorsson et al. 2015) to three-dimensional cell culture and assay(Edmondson et al. 2014), and even microfluidic organs-on-chips to synthesize minimal functional units that recapitulate tissue- and organ-level functions(Bhatia and Ingber 2014); or from a mini-bioreactor with just several hundred femtoliters in volume for one cell culturing and used optical force for precise amount of additive feeding(Huang and Huang 2011), to microwell array which could simultaneously culture 1500 clones for tracking the cells’ lineage development for cell life events(Chen et al. 2011). Meanwhile, some specific models such as portable platform(P.Walzik et al. 2015) and systems which were able to perform simultaneous and uninterrupted extracellular electrophysiology and time-lapse imaging(Saalfrank et al. 2015) were also developed. However, none of them has integrated the functions of long term mammalian cells culturing, time lapse assay and cell micromanipulation in one system, can satisfy the requirements of simultaneously performing steps of long term cell culture, high throughput time lapse
cell assays/ imaging, and micromanipulations for cell screen, transfection and recovery. Therefore, we developed a versatile on-stage microfluidic cell culture and assay system (abbreviated as CCA system) which can simultaneously satisfy the aforementioned requirements. In here we describe the construction of the system and the applications of the system with various techniques for long term cell culture, time lapse cell assays, and cell micromanipulations. With the system, we cultured a variety of adherent and suspended cells for different periods of time from several hours to several days and monitored their cell morphology, migration and division. We also performed a series high throughput time lapse assays and micromanipulations on different kinds of cells, not only to demonstrate the capability of the system, but also to develop novel and challenging methods for the information of the cells’ cellular and molecular heterogeneity and transformations during development or stimulations. They include: (1) The first time lapse measurements on the instantaneous variations of morphology, biomechanical properties and the intracellular protein concentration of human red blood cells in responding to pH fluctuation, drug action and electromagnetic radiation. (2) The first continuous time lapse Raman micro-spectroscopy on a CHO cell in different phases of its entire life cycles. (3) The micro-transfection of GFP into B16 cells and the follow up observation on the cells’ morphology and expressed GFP fluorescence varying with incubation time and cell generations post microinjection.

2. Materials and Methods

2.1. Cell lines and reagents
B16 cell line was kindly provided from Prof. LX Wang, South East University of China. CHO cell line was purchased from the Procell Biological Science and technology Company (Wu Han, China). GFP plasmid and the TurboFect Transfection Reagents were kindly provided from Prof. Ming Hu, Texas University, USA. RBCs were obtained from the Foshan blood center, China. Drug hydroxyxafflor yellow A was purchased from the Shang Hai YuanYe Biotechnology Company, China. In the experiment, it was added into 50 μl blood samples with a concentration of 0.4 mg/ml to incubate with the cells for 4 hours at 37°C. The gold nanoparticle solution for surface-enhanced Raman micro-spectroscopy was prepared with 0.01% HauCl₄•4H₂O (Sinopharm Chemical Reagent Co., Shang Hai, China) and 1% C₆H₅Na₃O₇•2H₂O (DaMao Chemical Reagent Co., Tian Jin, China).

Study on blood of volunteers (providing informed written consent) was approved by Ji Nan University Animal Care and Use Committee conforming to the Chinese Public Health Service Police on Human Care and Use of Laboratory Animals.

2.2. Cell culture media and stimulations
The culture media for RBC was PBS-BSA solution. For the adherent cells, RPMI-1640 culture medium (Gibco) or DMEM culture medium (Gibco) was used
accompanied with fetal bovine serum (Gibco), penicillin (Gibco) and streptomycin (Gibco).

The 900 MHz mobile phone electromagnetic field was emitted by a VS401A RF RFEMF emitter (Shenzhen Weikete Technology Company, Ltd. China) mounted beside the CCA system. The specific absorption rate of the cultured cells from the radiation was 0.43 W/kg so as to mimic the situation that people usually may absorb in an environment within a distance of 20 meters from mobile phone relay stations.

2.3. Long term imaging and time-lapse assays
The imaging camera was a PCO camera (Germany). The time lapse imaging for RBC’s bending modulus $K_c$ and shear elasticity $\mu_c$ measurements was performed with a Nikon TE 300 inverted microscope using our technique of dynamic image analysis (J Li and Huang 2006). Based on the thermal fluctuation (“flickering”) effect of living cell membrane, this technique detects the cells’ elastic properties ($K_c$ and $\mu_c$) by measuring the minute fluctuation $\delta n_m$ of the cell membrane. In the measurements, the video of the RBCs in flickering was recorded at the rate of 30 fps for 2 minutes, and then the fluctuation of the cell edge in four azimuths (or eight azimuths) was tracked to obtain the $K_c$ and $\mu_c$ values as described previously (J Li and Huang 2006) (see the Supplementary Movie 1 and SFig.2). For the time-lapse assay using our fast multi-channel micro-spectrophotometry (Huang 2005), the absorption spectra of RBCs under different stimulations were recorded at a rate of 10 spectra per second. Then the variation of the peak intensity and peak shift with time were deduced from the spectra. The two means were coupled with the microscope via a digital camera and a spectrum sensor respectively. They were quite simple and easy, and just needed the ordinary illumination of the microscope for video and absorption spectrum recording during the MPEF radiation but without disturbance on the cells. Since both the video and spectrum recording can be performed simultaneously on all the cells in the field of view of the microscope (usually several tens to hundreds cells), the measurements were multi-parameters and efficient high throughput experimentation, but at the same time, they could be performed at single cell resolution.

2.4. Raman micro-spectroscopy
The Raman micro-spectroscopy was performed using a Horiba JY RAM INV Raman scattering system with an inverted Olympus optical microscope (see Supplementary SFig.1). The exciting laser was a He-Ne laser with a wavelength of 633 nm and output power of 1 mW. Before the Raman measurement, the CHO cells were cultured in the CCA system for 24 hours to let them completely adhered to the culture slide. Then the solution of gold nanoparticle was added in with a volume ratio of 20%. The gold nano-particle was proved previously by MTT assay to be successfully delivered into all CHO cell differentiation stages without affecting the cell’s viability or proliferation. After 24 hours incubation with the gold nanoparticle, the cells were measured by the Raman scattering system with an acquisition range of 300~3600 cm$^{-1}$ and a spectrum resolution of 1 cm$^{-1}$. The exposure time was 10 s and the grating was 600 lines/mm. Such a short time exposure was proved to be safe to the cells by a trial
experiment taken before the measurement. In the SERS measurements on the nucleus of a CHO cell at different cell cycles, each Raman spectra was averaged from the spectra taken at five points in the nucleus of the cell, and at each point, ten spectra were taken for average.

2.5. Micromanipulation on cultured cells
The micromanipulations were performed using a micromanipulator (Narishige, NAI-3N, Japan) mounted on an Axiovert 200 inverted microscope of Microlaser Combisystem (Zeiss, Germany). The microcapillaries used for microinjection were with an outer diameter of 0.8 μm and an inner diameter of 0.5 μm, while those used for negative-pressure inspiration were with an inner diameter of 6 μm. They accessed the culture cells at a 45° angle through the top window of the CCA system which was opened for the micromanipulation but closed after the micromanipulation. The cells were injected with GFP plasmid (83μg/L in concentration) into the nucleus, and the average injection volume was 180 fL. A square frame on the culture slide shown in Fig. 5 was sculptured by the violet laser beam of the Microlaser Combisystem to indicate the position of the selected cell.

3. Results
3.1. Microfluidic cell culture and assay system
As shown in Fig.1, our versatile on-stage microfluidic cell culture and assay system consisted with a cell culture chamber which was made of PMMA, a microfluidic perfusion system, a pH-CO₂ control system and a temperature control system. The cell culture chamber had a top cover with glass window and an observing window on its bottom. The top cover was generally closed to provide insulation from the external environment, but it could be removed to offer free access to the cultured cells for micromanipulation. The chamber also has two side windows for pH sensor and temperature sensor respectively. The cell culture slide was mounted on the observing window with a fixed tray and secured with sealants to provide a watertight seal. To be compatible with high numerical aperture optics for better quality optical observation and for Raman spectroscopy, round coverslips or quartz round coverslips were usually used as the cell culture slide. Since all the parts were dismountable, the chamber and the cell culture slides can be easily cleaned, sterilized and carried out surface treatment and coating.

The microfluidic perfusion system was used to pattern the cell culture substrates and vary the composition of culture medium in either continuous or periodic perfusion manner. With a slow linear perfusion rate and by the damping effect of the fixed tray, the perfusion media could be directed gently across the specimen on the culture slide as shown in Fig.1(B) so that there was no disturbance on the specimen lateral position or axial focal plane. It was also demonstrated by the concentration distribution test with our technique of microsphere imaging(Yue et al. 2011) that there was no concentration gradient across the cell culture slide after a new solution was perfused.
into the chamber for several ten seconds. However, flow stress or concentration gradient can be generated when required. At the same time, the culture pH was monitored and controlled by a pH regulator (Harvard Apparatus, HI931700) with CO₂ super fusion so that the deviation of pH away from the set value was less than ±0.3 during a cell culture period of several days. While the temperature of the system was monitored and controlled with an accuracy of ± 0.2 °C by a temperature controller with its temperature regulated clamps (Harvard Apparatus, TC-202A).

3.2. Long term on-stage observation of single cell morphology, migration, division and differentiation
We used our CCA system to culture various adherent and suspended cells such as CHO, 3T3 cells, HUVEC cells, mesenchymal stem cells and red blood cells for several hours to several weeks until the cells had exceeded their useful passage limit. At the same time, we performed on stage continuous observation on the cell morphology and transmigration, division and differentiation at single cell level. Fig.2(A) shows the HUVEC cells cultured in the CCA system. We can see that the cells grew well with normal division and differentiation, and showed no difference with those cultured in a Petri dish inside a CO₂ incubator(see Fig.2(B)), indicating that our CCA system was competent in long term cell culture. Fig.2(C) shows the shape, size and position changes of six CHO cells during the culture period from the 18th to 21st hour. From which we can obtain quantitative temporal information about the cell migration; extension or retraction of cell processes and cell-to-cell variability that cannot be otherwise resolved in bulk assays.

3.3. Time lapse assays
To monitor the cell responses to various stimulations, we performed time lapse assays on human red blood cells (RBC) incubated in the CCA system. The stimulations included drug action (with drug Hydroxysafflor yellow A), pH fluctuation of the culture medium, and the radiation of 900 MHz mobile phone electromagnetic field. We used several techniques which can perform non-disturbance and real-time in situ time lapse measurements to monitor the morphological, biomechanical and molecular changes during the stimulations. They included the technique of dynamic image analysis(J Li and Huang 2006) which can monitor the bending modulus $K_c$ and the shear modulus $\mu_c$ of RBC to obtain information about the cell flexibility and adhesiveness(Huang et al. 2011). And the fast multi-channel micro-spectrophotometry(Huang 2005) which can record 100 absorption spectra every second on the time course of the absorption spectra of RBC to monitor the variations of the molecular structure & concentration of the intracellular protein during the stimulations. Since both kinds of the measurements can be performed at the same time, the information about the instant responses of the cells to the stimulations can be obtained simultaneously at cellular and molecular levels.

Fig.3(A) illustrates the typical morphological changes of human RBCs when the pH of the culture medium in the CCA system varied from 5.0 to 9.7. The cell in the upper row of Fig.3(A) was incubated with the solution of Hydroxysafflor yellow A for
four hours before the assay. After the incubation, it was perfused with a solution of PBS+BSA at different pH values. Compared with the RBC which had not been incubated with the solution of Hydroxysafflor yellow A before the assay, the RBC with drug action could keep its normal shape of discocyte during the variation of the pH value. In contrast, the RBC without drug action became echinocyte in acidic conditions but spheroechinocyte in alkaline environment. The $K_c$ and $\mu_c$ obtained from the time lapse dynamic image analysis of the cell (see the Supplementary Movie 1) shown in Fig.3(C and D) respectively also suggested that the RBC under drug action could keep its membrane flexibility and adhesiveness during the pH variation, whereas the cell without drug action lost its membrane flexibility and adhesiveness a lot when pH was away from normal value. The results suggest that Hydroxysafflor yellow A has the ability to protect human RBC from pH variation, and it will be helpful in blood diseases treatment and blood storage.

Fig.3 also shows the results of the time lapse assays on the RBCs under the radiation of a 900 MHz mobile phone electromagnetic field (MPEF). We can see that a 20-30 minutes exposure of the radiation could induce the cells to transfer from discocyte to echinocyte (see Fig.3(B)). The continuous on-site real time dynamic image analysis of the cells indicated that the $K_c$ of the cells under exposure increased with time during the exposure, but decreased as soon as the exposure was turned off and almost recovered to normal value 100 minutes post radiation(Fig.3(E)). Repeating the exposure three times with an interval of 30 minutes between each one, we found that the RBC under the exposure showed similar response each time to increase its $K_c$ when the radiation was on, and decrease its $K_c$ when the radiation was off. Although the effect of the radiation seems to have accumulated so that the $K_c$ was higher and higher each time, it would still recover to almost the normal value when the time was long enough after the last exposure (see Fig.3(F)). The fast multi-channel micro-spectrophotometry performed at the same time indicated that the density of the hemoglobin in the cells decreased with time during the exposure and couldn’t recover to its original value after the exposure (Fig.3(G and F)). Moreover, both the $\alpha$ peak and $\beta$ peak of the hemoglobin were found to be blue shifted for about 1.5 nm after the exposure, suggesting that the molecular structure of the protein had also changed. These results provide us the first experimental evidences for the real time direct effects of 900 MHz mobile phone electromagnetic field on human cells.

3.4. Long term continuous on-site Raman micro-spectroscopy on live cells

By the aid of the CCA system, we performed long term continuous in situ Raman micro-spectroscopy on living CHO cells. Considering that CHO cells are adherent cells and their Raman signal is very week in general(Kang et al. 2008), we used gold nanoparticle mediated surface-enhanced Raman micro-spectroscopy (SERS) for the Raman detection of CHO to enhance the signal and shorten the acquisition time of detection.

We firstly performed time lapse SERS at different locations of the cells to see the signal temporal variance of each point. Fig.4(A) shows the result of a typical time course SERS measurement in which ten Raman spectra were taken every ten seconds.
(the signal accumulated time for each spectrum was also 10 seconds) at a point of a cell. We can see that although there was slight variance in the Raman bands due to the Brownian motion of molecules in the cell, or the fluctuations of nanoparticle attachment/positions in the vicinity of measurements, the general features of the spectra were similar. This indicated that the average of the ten spectra represented the general information about the molecular composition and density at the point of the cell. On this basis, we performed SERS on the nucleus of a CHO cell at different cell cycles to see its biochemical changes due to cell growth and proliferation. The reason we just focused on the cell’s nucleus was that we mainly concerned about the DNA content variation in different cell cycles. Fig.4(B) illustrates the SERS spectra of a CHO cell in different phases of its entire cell cycle accompanied with its morphological variations during the process. We can see that as the cell changed its shape from sphere to ellipsoid, and then adhered to the bottom of the culture chamber to become spindle shape and grew to larger size, the Raman shift and peak intensity also changed accordingly. At the early stage (0-5 h), the major peaks were those assigned to the S-S and C-S vibration and the Amide III of proteins (505 cm\(^{-1}\), 657 cm\(^{-1}\) and 1308 cm\(^{-1}\)). Most of them and those assigned to the phosphodiester bond stretching vibration of nucleic acid (830 cm\(^{-1}\), 1028 cm\(^{-1}\), 1316 cm\(^{-1}\), 1502 cm\(^{-1}\) and 1581 cm\(^{-1}\)) were of highest intensities among the spectra taken for 24 hours. This suggested that at the phase (especially at the 5\(^{th}\) h), the contents of protein and nucleic acid of the cell were higher than other phases. By the characteristics of the Raman spectra and the morphology of the cell in this phase, we could judge that the cell was in G\(_1\) or G\(_1\)/S phase from 0 to the 5\(^{th}\) h. For in the phases, the cell synthesized a number of proteins and enzymes that were involved in DNA synthesis and replication, so the contents of protein and nucleic acid were highest at the period. However, at the 11\(^{th}\) h, the contents of both the protein and amino acid lowered down, indicating that the cell was in S/G2 phase. Form the 16\(^{th}\) to 24\(^{th}\) h, the peaks at 595 cm\(^{-1}\),1235 cm\(^{-1}\),1244 cm\(^{-1}\),1265 cm\(^{-1}\) and 1284 cm\(^{-1}\) were those of amide bonds correlated with the \(\alpha\) helix, \(\beta\)-strands and random-coil of proteins, they should be the signal of tubulin so that the cell would be at the G\(_2\) phase.

Based on our knowledge, this was the first time to perform a continuous Raman spectrum measurement on a single cell in its entire cell cycle. These experiments indicated that the time course analysis of the observed Raman DNA and protein peaks allowed us to observe and monitor cell processes in vitro and identify the different phases of the cell cycle. This technique could be used for cell sorting, disease diagnostics and improve our understanding of the molecular mechanisms of cellular functions such as division, death, signaling, and drug action.

3.5. Micromanipulation on cultured cells and the follow-up long term analysis
Since it can retain the cells under micromanipulation on the culture chamber for the follow-up long term observation and analysis, our CCA system has several advantages over conventional incubating systems for the gene transfection of cells with microinjection technique: (1) Each cell’s transgene expression post microinjection can be directly correlated with the injection quantity and subcellular location as well as its
cell cycle in injection, so that the optimal microinjection method and parameters for a stable recombinant cell line can be simply determined. (2) Each microinjected cell can be followed specifically and analyzed for gene expression and cell growth, so that the growing colony is guaranteed from a single cell. (3) It can be easier to study the dynamic movement of a given protein or subcellular structure, and evaluate the efficiency of recovery of recombinant cell lines and the stability of reporter protein expression over time.

Fig. 5 shows the results of our experiment in which B16 cells were capillary microinjected with GFP plasmid. After 24 hours incubation post injection, we just retained one of the cells with GFP expression but took off all the other cells by a negative pressure micro-inspirator. By this way the entire process in which how a cell grew and divided to create a colony with stable GFP expression and the efficiency of the recovery of recombinant cell line can be followed up. We can see that the cell grew much slowly at the initial four days post cell screening than the later 7 days(Fig.5(B), it probably due to the fact that the cell lacked of intercellular contact to stimulate cell growth during the initial lag phase(Frame and Hu 1988; Lee 2002). The mean fluorescent intensity of the cells decreased in the first four days post transfection and then increased (see Fig. 5(C)). And in the early stage, GFP just expressed in the nucleus of the cell as the cell was under nucleus-injection, however after cell division, GFP expressed in the whole cell of all the daughter cells though their nuclei showed higher GFP fluorescence(Fig.5(B)). When the cells needed to be passaged, we transferred some of them to a Petri dish and incubated in a CO\textsubscript{2} incubator. After one month incubation, the cells still grew normally and stably expressed GFP fluorescence, indicating that it’s a stable recombinant cell line.

4. Discussions

We have reported a versatile on-stage microfluidic cell culture and assay system, and demonstrated that the system can be used to perform a variety of challenging and interesting experiments. From the performances of the system in cell culture, real time in situ time-lapse cell assay and manipulation, we can see that the system have several advantages over other existing systems. First, the CCA system is not only able to provide a suitable environment for long term cell culturing, but also compatible with various microscopes and sensors thus integrating multifold functions of different cell culture, manipulations and assays in one system. As a result, it is able to perform steps of cell culture and multi-parameters high throughput assay as well as manipulation simultaneously. Based on our knowledge, no other previously reported cell culture system has such capability (Bhatia and Ingber 2014; Dailey et al. 2016; Halldorsson et al. 2015). On the other hand, it can vary the culturing conditions for different temperature, pH value, and perfusion modes such as continuous or periodic, with or without flow stress and concentration gradient, to meet the requirements of different cell culturing and assays. Furthermore, all the parts in the culture chamber of the system are dismountable for cleaning, sterilization and surface treatment/coating, so
the system can be repeatedly used for multifold functions.

With the capability of simultaneously performing long term cell culture, cell assay and manipulation, the CCA system enable us to on-stage culture a variety of adherent or suspended cells for different long periods of time from several hours to several days or even weeks. And at the same time, it allows investigators to obtain quantitative information about single-cell processes such as the cell’s migration, extension or retraction, cell-to-cell variability as illustrated in Fig.2 that cannot be resolved in bulk assays, provides unique mechanistic insights into cellular spatial and temporal dynamics. More importantly, our CCA system can carry out some experiments that cannot be achieved by other microfluidic systems, such as the transfection experiment on B16 cell mentioned above. In the experiment, we could perform microinjection and micro-inspiration on the cells in the CCA system and then on stage follow up each cell’s gene expression, cell growth and division, and its process to become a colony with stable GFP expression. Since the whole process from microinjection to the colony generation was taken in the same culture chamber, investigators would have several privileges for their study. First, they can exactly identify the transfection effect for each way of microinjection including its injected quantity and location, and the cell’s phase, etc., so that they can simply determine the suitable microinjection way for a certain cell line. Second, as the cells were not needed to transfer to other culture chamber after the microinjection, it greatly reduced the disturbance and damage on the cells, so that the natural state of the cell growth after microinjection can be obtained. Third, the investigator cannot only ensure that the growing colony was from a single cell, but also easily monitor the dynamic movement of a given protein during the cell growth, and evaluate the efficiency of recovery of recombinant cell lines. The whole continuous on-stage experiment, from the pre-transfection cell culture to microinjection of GFP and then the post-transfection observation on the process of the cell growing to become a colony with stable GFP expression, lasted for two weeks. This period was much longer than the time of on-stage cell culture and observation reported by previous microfluidic systems (Chen et al. 2011; Etzrodt et al. 2014; Spiller et al. 2010; Stevenson et al. 2008). Even more significant is that, the experiment created a precedent that performed steps of on-stage long term cell culture, cell assay and micromanipulation simultaneously in one system in one time.

Since the CCA system is easy to couple with different sensors and assay techniques, and compatible to various microscopes, it offers convenience to perform real time in situ high throughput measurement on the culture cells to study the immediate response of the cells to different stimulations. Our time lapse experiments on the responses of RBCs to mobile phone radiation and environment pH were good examples of such measurements.

As we know that although there were already huge amount of studies on the effects of MPEF on human health, a strong debate over the issue still exists(Hardell and Sage 2008; Khurana et al. 2009; N et al. 2010). The major reason for the confusion on the issue is that it is extremely difficult to detect the instant effect on living cells to determine what kind of responses directly resulted from the radiation,
so most of the previous studies were unable to obtain the true and direct evidence of the effect. On the other hand, up to now little is known about how the intracellular/intraneuronal concentrations of proteins correlate with the physiological state of a cell and whether they can significantly change along the cell development and under external stimulation, although such information would have a fundamental significance. Accordingly, we designed and performed the experiments as mentioned in section 3.3. In the experiment, during the MPEF radiation, the culturing RBCs were under time lapse measurements by the technique of dynamic imaging for biomechanical parameters and by the fast multi-channel micro-spectrophotometry for the information about intracellular molecule composition and density. As mentioned in section 2.3 that the measurements were just simply making video and absorption spectrum recordings while without disturbance on the cells. Therefore, it helped us to get the instant effects purely from the MPEF radiation on the cells from cellular to molecular levels.

It is interesting to note that both the biomechanical parameters \( K_e \) and \( \mu_e \) increased with time during the exposure, but decreased as soon as the exposure was turned off. At the same time, the cells transferred from discocyte to echinocyte, and the \( \alpha \) peak and \( \beta \) peak of the intracellular hemoglobin were shifted. The increase of \( K_e \) and \( \mu_e \) indicated a reduction in the cells’ deformability(Huang et al. 2016), while the \( \alpha \) peak and \( \beta \) peak shift suggested that the hemoglobin became deoxygenated or partly deoxygenated(Sugita 1975). Both these changes would influence the blood circulation and effective oxygen transport and delivery. According to our previous research(Lu et al. 2012), they were most probably induced by the oxidative stress of the MPEF radiation. When Hb is partially deoxygenated, it would result in an increased rate of autoxidation and increased aggregation and affinity for the RBC membrane, so the cell’s membrane became less flexible(Huang et al. 2011). When the MPEF was removed, as soon as the release of oxidative stress, most deoxygenated Hbs would return to oxygenated state and the deformability of the cells recovered, though some of the Hbs might have undergone slow oxidation to methemoglobin (metHb)(Ideguchi 1999), so the density of Hb couldn’t recover to its original value after the exposure.

Based on our knowledge, our time lapse assay on the response of RBCs to the mobile phone radiation was the first real time in situ measurement on living human blood cells under the radiation stimulation. It provided the first direct evidence for the instant effect of the mobile phone radiation on human cells in their morphology, biomechanical properties and intracellular proteins. The significance of this finding is that, it not only tell us that the MPEF would instantly influence RBC’s deformability and the oxygenate state of hemoglobin, but also reveals for the first time that the response of human RBCs to the stimulation is instantaneous, and the cells have the ability to heal/restore themselves after the exposure. Therefore, if we were unable to perform the real time in situ time lapse measurement on the cells, we would have missed such a response. It may also help to explain why there were so many discrepancies in the results of previous research on the biological effect of mobile phone radiation. Since the intensity of mobile phone radiation is weak, as a non-ionizing radiation, its induced biological effect would be also weak and retain for
short time in case of low dose exposure. However, most of the previous researches were unable to perform measurement during the radiation. Depending on the time interval from the end of radiation to detection, their obtained results might just partially reflect the effect of the radiation in different extents because of the self-recovery ability of the cells so they were contradictory. Therefore, whether the measurement is performed during the radiation is crucial for the research of the effect of MPEF radiation. For this reason, having a CCA system with the coupled techniques for such measurements is significant as it is much more accurate and comprehensive for investigating cell instant responses to various stimulations via a series high throughput multi-parameter assays. Our experiment on the response of the RBCs with drug action to pH fluctuation was another good example to explain it.

Our time lapse SERS measurements on the CHO cells at different cell cycles was also a pioneering experiment for it was the first time-course Raman measurement on a single biological cell during various phases of its entire cell cycle. Although numerous attempts have been taken to perform Raman measurements on living cells such as multidimensional scanning(Jin et al. 2017; Kang et al. 2008), detecting the chemical changes due to proliferation(Short et al. 2005), and characterizing phenotype(Smith et al. 2016), no one was able to continuously perform Raman detection on a cell at various phases of its entire cell cycle. The most probable reason is that without a versatile on-stage long term cell culturing system like our CCA system to maintain the cell in a healthy state for long time and with the capability to couple with a Raman microscope to perform cell culture and cell assay simultaneously, it is incredible to perform such an experiment. Continuously tracking the molecular changes in the same cell due to cell growth and proliferation should give more accurate information about the intranuclear molecular composition and concentration of the cell varying with cell cycle, than that obtained from different groups of cells(Short et al. 2005). Therefore, our experiment opens up a new way to perform both qualitative and quantitative cell biochemical analysis for revealing cellular dynamics events and function at the single-cell level. It is of particular importance to regenerative medicine and tissue engineering applications such as stem cell therapy, for the Raman measurements can provide time- and spatially-resolved information regarding the molecular properties of stem cells during differentiation and the cell quality and phenotype heterogeneity of stem cell progeny. From this point of view, the significance of our CCA system for this kind of experiment is far beyond that. Since on one hand it is a microfluidic sterilized cell culture system enabling long term real-time Raman monitoring of cellular processes and phenotypic identification of stem cell progeny. But on the other hand, it is a versatile system which allows micromanipulations on the cells, so that one can take off the undifferentiated cells but retain the fully committed mature cell for further proliferation to guarantee a stable cell line is from a single cell as the way we carried out on the B16 cells described above.
Conclusion

The CCA system we developed is a versatile on-stage microfluidic cell culture and assay system which is compatible with various microscopes and sensors, and integrates the functions of long term cell culture, time lapse assay and cell micromanipulation in one system. The main feature and advantages of the system are that, it can satisfy the requirements of simultaneously performing steps of on-stage long term cell culture, multi-parameters efficient high throughput time lapse cell assays, and micromanipulations for cell screen, transfection and recovery at single cell resolution. It is convertible between close and open configurations, dismountable for cleaning, sterilization and surface treatment/coating, and can be repeatedly used for multifold functions. A variety of novel experiments performed with the CCA system demonstrated that, the system can provide a suitable environment for long term on-stage culturing different adherent or suspended cells for several days or even weeks. It can perform various challenging experiments which were unable or difficult to achieve with previous microfluidic systems. Including the multi-parameter high throughput time lapse assays on the instantaneous responses of human red blood cells to pH fluctuation, drug action and electromagnetic radiation during the stimulations to obtain the first direct evidence for the instant effects of the stimulations on human cells at both cellular and molecular levels. The first continuous time lapse Raman micro-spectroscopy on cells in different phases of their entire life cycles for the information about the composition and concentrations of intranuclear proteins correlate with the physiological state of the cells. And the microinjection of GFP into cells and the follow up observation of the cells’ morphology and expressed GFP fluorescence varying with incubation time and cell generations. This multi-procedures experiment, integrated on-stage long term cell culture, cell assay and manipulation in one system and in one time, was a good proof for the advantages of the CCA system. We believe that our technique of versatile on-stage microfluidic cell culture and assay system will promote more and more interesting experiments for time- and spatially-resolved information about the cellular and molecular heterogeneity and transformation during development or stimulations in the future.

Author contributions:
YXH conceived and designed the experiments. CLH, YTP, PW, WWT, CCY and YLY performed the experiments. YXH, CLH and YTP analyzed the data. YXH wrote the paper.

Conflict of interest:
The authors declare that they have no conflict of interest.
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Figure legends

Fig. 1. The versatile on-stage microfluidic cell culture and assay system. (A) The construction of the CCA system; (B) The fluid flow patterns in the culture chamber of CCA system.

Fig. 2. Long term on-stage observation of single cell morphology, migration, division and differentiation. (A) The HUVEC cells cultured in the CCA system; (B) The HUVEC cells cultured in a Petri dish inside a CO2 incubator; (C) The morphological and position changes of six CHO cells during the culture period from the 18th to 21st hour, in the right one, the color curves are the migration tracks of the cells, a18 means the size of the cell at the 18th h, a21 is the size of the cell at the 21st h.
Fig. 3. The responses of living cells to various stimulations. (A) The response of human red blood cells to pH fluctuation, upper row, the RBCs with drug action, lower row, the RBCs without drug action; (B) The morphology of RBCs before (upper one) and after (low one) a 20-30 minutes exposure of the electromagnetic field radiation; (C) and (D) The $K_c$ and $\mu_c$ of RBCs as functions of pH. ■: the cells with drug action, ●: the cells without drug action; (E) and (F) the $K_c$ of RBCs changed with time during and after the exposure of MPEF radiation; (G) and (H) the relative absorbance of $\alpha$ peak and $\beta$ peak of the hemoglobin respectively changed with time during and after the exposure of MPEF radiation.

Fig. 4. The time course SERS spectra of CHO cells. (A) The morphology of a CHO cell and ten Raman spectra taken every ten seconds at the same point of the cell; (B) a CHO cell in different phases of its entire cell cycle and the corresponding SERS spectra.

Fig. 5. The microinjection of a B16 cell with GFP plasmid and the follow up observation on its growth and GFP expression post microinjection. (A) The microinjection of a B16 cell with GFP plasmid; (B) The growth and GFP expression post microinjection of a selected B16 cell changed with time, upper row, bright field images, lower row, fluorescent images; (C) The relative intensity of the expressed GFP fluorescence in the cell as a function of time.

Supplement materials:

SFig. 1. The Horiba JY RAM INV Raman spectrometer accompanied with the CCA system for long term continuous on-site Raman micro-spectroscopy on living cells.

SFig. 2. The result panel of $K_c$ and $\mu_c$ measurements. (A) The position variation data of the cell edge in four azimuths obtained from the video of the time laps imaging for RBC’s $K_c$ and $\mu_c$ measurements; and (B) The corresponding position variation curves; (C) The calculated $K_c$ and $\mu_c$ from the position variation data.

S Video 1: The time lapse imaging for RBC’s $K_c$ and $\mu_c$ measurements.
Figures:

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Highlights

1. A versatile microfluidic cell culture and assay system which is compatible with different microscopes and sensors.
2. It has the capability to couple with various techniques such as time-lapse fluorescence imaging, optical trapping and dissection, microinjection, and Raman microspectroscopy.
3. It can perform steps of long term cell culture, time lapse assays and micromanipulations simultaneously in one system.
4. Can achieve a variety of novel methods for obtaining time- and spatially-resolved information about the cellular and molecular heterogeneity and transformation during development or stimulations.