DESIGN AND PREPARATION OF EIGHT Cr-Au ELECTRODES FOR THE MICROFLUIDIC DETECTION OF APOPTOTIC CELLS

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Abstract

A microchip with eight Cr-Au electrodes was elaborated, designed, and fabricated for the microfluidic detection of apoptotic cells. The eight Cr-Au electrodes consist of four pairs of electrodes located opposite to each other and form a ring. The inner diameter of the ring section for microfluidic droplet placement was 400μm. Then a rotating electric field is produced by the eight-electrode structure. The electric field at the center of the microchip circle was nearly consistent and stable enough to ensure that almost all the tested biological particles will have a consistent rotation speed. The proposed microfluidic biochip with eight Cr-Au electrodes is recommended for the cell screening and rapid clinical detection of damaged cells at the middle and late stages of apoptosis.

Introduction

Apoptosis is a process of programmed cell death that occurs in multicellular organisms (Jin *et al.* 2011) under the gene regulation to maintain the body normal development and homeostasis. In contrast to necrosis, which is a traumatic cell death induced by the acute cellular injury, apoptosis is a highly regulated and controlled process, which occurs regularly during each organism's lifecycle under the gene regulation to maintain the body normal development and homeostasis. However, any disorder of the apoptosis mechanism can directly or indirectly cause cancer, myocardial infarction (Kitsis and Mann 2005), neurodegenerative and other diseases (Horvitz 1999, Ghavami *et al.* 2014). The apoptosis activation mechanisms are conventionally subdivided into extrinsic and intrinsic pathways. The former is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex (DISC). The intrinsic pathway is activated by intracellular signals generated when cells are stressed and depends either on the release of proteins from the intermembrane space of mitochondria, the so-called mitochondrial-cytochrome C pathway or involves the endoplasmic reticulum (ER) signal pathway (Hotz *et al.*1994, Gogvadze *et al.* 2006 and Kadowaki and Nishitoh 2013). There are many external biochemical stimuli, which may trigger apoptosis-inducing signals. When cells are poisoned by toxic substances or infected by pathogens, they take the initiative to produce apoptosis mechanisms to protect the stability of the environment (Norbury and Hickson 2001). Considering the importance of basic research and clinical application value of apoptosis, it has become one of the research hotspots in life sciences and medicine field in recent years.

The apoptosis detection is a necessary step in the study of apoptosis. Cells in the event of apoptosis will produce special morphological and biochemical changes. The production of apoptotic bodies is the main morphological feature of apoptosis (Hacker 2000), and its most significant biochemical property is chromatin degradation initiated by the Caspase protein family, wherein the chromatin DNA is gradually decomposed into many 180 ~ 200 bp fragments.

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Additional biochemical features include the expression of cell surface markers (Bratton *et al.* 1997). At present, the commonly used apoptotic detection methods are based on the morphological and biochemical characteristics of apoptotic cells. With the deepening and cognitive increase in the field of research, the demands for apoptosis detection technology and the portability of apoptosis detection equipment are also increasing. The most available detection methods are quite expensive and cumbersome, with the exception of the microfluidic detection technology (MDT), which is recognized as one of the most promising analytical tools, due to such advantages as miniaturized components, microliter level of demanded sample volume, easy operation, adaptability to automation, low cost, etc (Yamada *et al.* 2015). The microfluidic technology is a new inter disciplinary technology based on microelectronics, micromechanics, biotechnology and so on. The microfluidic chip concept was introduced in the 1990s (Manz *et al.* 1990), while microfluidic systems are widely used in chemistry and biotechnology fields (Yamaguchi and Miyazaki 2015). Through the comprehensive utilization of hydrodynamics, electrical and optical techniques at the microscopic scale, the MDT can easily and quickly achieve the test material preparation, separation, testing and other functions, especially when the state-of-the-art microfluidic chip technologies are combined with the dielectrophoresis.

Dielectrophoresis (DEP) is a phenomenon, in which a force is exerted on dielectric/neutral polarizable particles (such as cells) when they are subjected to a non-uniform electric field. DEP induces particle motion by forces arising from the difference in polarizability between the particles and the fluid. The most prevalent biological applications envisioned for DEP are trapping or separation of individual cells or particles, which often rely on the frequency-dependent, dielectric responses of particles. By manipulating the particles in the electric field without any external marking allows one to achieve the cell capture, transport, separation, and other purposes (Cen *et al.* 2004).

By combining the dielectrophoresis and microfluidic chip technologies, an eight-electrode microchip with Cr-Au materials, which exhibits some advantages over the existing four-electrode ones were elaborated, designed, and produced. Here HeLa cells were used to verify the electrorotation microchip feasibility. HeLa cells, like other cell lines, are termed "immortal" in that they can divide an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met (i.e., being maintained and sustained in a suitable environment).

Materials and Methods

Currently, microfluidic chips are produced from silicon, polydimethylsiloxane (PDMS), and other polymer materials, which have certain drawbacks. The silicon materials are brittle and fragile, fail to withstand high voltage, and provide insufficient electrical insulation.

After the comprehensive consideration, at the initial test stage in this study, a traditional low-cost printed circuit board (PCB) structure, which has the advantages of mature processing technology and convenient production, was used for placement of microelectrodes and microchip operation simulation, but at the later stage this material was replaced by the improved glass-based transparent material. The composition of a PCB generally consists of four layers, which are heat laminated together into a single layer. The material used in PCB includes the following layers from top to bottom: silkscreen, solder mask, copper, and substrate. The substrate is made of fiberglass and is also known as FR4, with the FR letters standing for "fire retardant."

At the initial stages of this study, the conventional PCB material was used to design the electric rotating chip. However, despite the ease of processing of PCB materials, the micro-scale process accuracy is not adequate, due to the excessive electrode metal sheet volume and high probability of

occurrence of voids and pores. Moreover, since PCB is a non-transparent material, only metallographic microscopy can be used for the observation of cells in the droplet placed in the ring electrode cell of the microchip, while the observation effect will be affected by the droplet height. On the other hand, the state-of-the-art glass chip micro-processing technology is very lucrative, since glass surface properties are relatively stable and have good biocompatibility, while glass transparency makes it possible to use a transmission microscope for observing the cell motion and migration. So the experimental design was optimized and produced microfluidic chips using glass materials and applying the coating process for depositing the chromium-golden microelectrodes.

The model of microfluidic chip with eight electrodes was elaborated using the computer-aided design (CAD). The chip geometry is shown in Fig. 1. Here the conductor length is 30 mm, conductive module length is 5mm, its width is 2.5 mm, and the distance between the conductive modules is 0.5 mm (Fig. 1A), while the ring section for microfluidic droplet placement has the inner diameter of 400 μm (Fig. 1B).
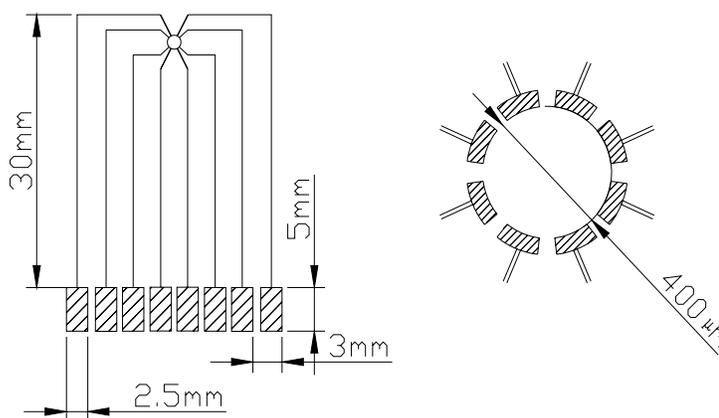

Fig. 1. The design of the microfluidic chip with eight Cr-Au electrodes. A. General design of the chip, B. Detail of the ring electrode cell.

The designed chip is intended to provide a periodic rotation of the electric field in the two-dimensional planes of the ring electrode, in order to manipulate particles in the droplet placed into the ring electrode cell, wherein the electric field direction can be changed by controlling the voltage of each electrode. To ensure this function, an external signal controller was designed to achieve the control voltage and AC frequency signal processing. The control circuit generates the corresponding electrode-driving signal according to the input signal source, and the amplitude controller ensures that the electric field drives the circuit module. The voltage range of this signal controller is 0 - 15 v, and the frequency range is 0 - 1 MHz.

A simulation experiment was done on the electrode. Fig. 2 depicts the operation of the 8-electrodes' chip, which consists of four pairs of electrodes, which are located opposite to each other, when the same phase voltage is applied to the two (upper) adjacent electrodes and a voltage of the opposite phase is applied to their opposite (lower) two electrodes, the remaining four electrodes are in a high impedance state, the spatial potential of the chip being shown in Fig. 2A, while the spatial electric field distribution is illustrated in Fig. 2B. Due to the periodicity of the voltage applied to each electrode, the spatial potential of the chip and the spatial electric field are rotated by 45° in turn. As compared to the four and six electrodes' schemes with the rotation angles

of 90° and 60°, respectively, the eight-electrodes' rotation angle is smaller, and the electric field variation is more continuous, which is beneficial for the rotation of cells.
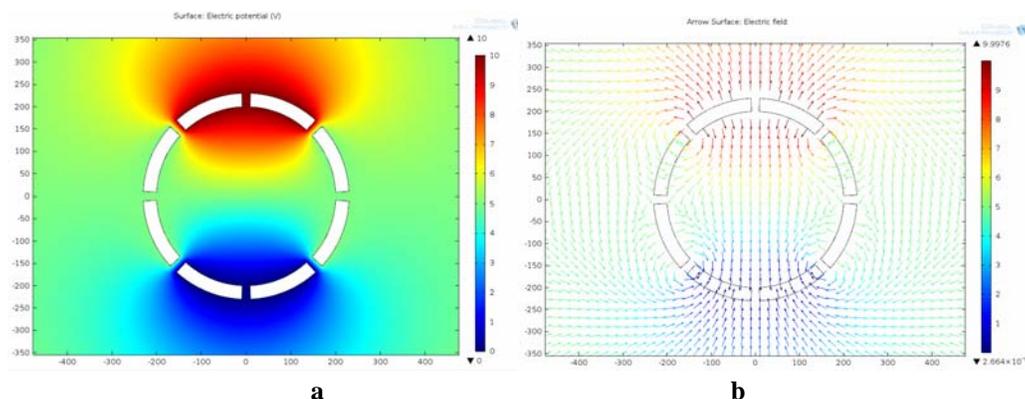

Fig. 2. Simulation diagram of the eight-electrodes' chip operation.

The following describes the preparation process. Firstly, the mask production was performed, since the chip production process requires the mask to transfer the design to the chip for lithography. Secondly, the glass substrate was processed by the ultrasonic wave method, and rinsed with acetone and deionized water to ensure that the material is clean enough to provide the enhanced adhesion with the metal film. Thirdly, the glass surface was coated with a 3μm-thick layer of photoresist high-temperature hard film, which procedure included its ultraviolet exposure and development. Finally, the glass chip was placed into the magnetron sputtering system for chromium-gold coating. The photoresist film was later removed by acetone and, thus, the chip preparation was completed. The produced microchip was used to test the rotational rate of apoptotic cells at different stages of growth.

Results and Discussion

The physical map of the produced microchip with eight Cr-Au electrodes is presented in Fig. 3. An enlarged view of the Cr-Au ring electrode was obtained through the dark field positive microscope (LEICA DM4000 M).

As seen from Fig. 4, the final chip was embedded into glass and its electrodes were made from Cr-Au materials. The eight conductive electrodes formed the ring, where a droplet was placed. The conductive electrodes were connected to an external signal controller. Then an alternating current signal was generated, which induced an alternating electric field in the chip ring. During the experiment, the cell suspension was put in the middle of the rotating electrode, adjusted the voltage and frequency, and then recorded the rotation speed of particles (cells).

It was experimentally proved that the ring electrode is suitable for exerting a relatively stable cell motion in the central part of the ring and then tested the cell rotation effect. A droplet of normal 5 μl cell suspension was carefully placed in the middle of the rotating electrode, the rotary switch was turned on, the voltage and frequency were set to 8V and the range of 0 - 1 MHz, respectively. Then the cell rotation speed was measured and recorded. Through the experiments, it was found that the response of cells occurred in the frequency range of 10 - 300 Hz and resulted in their clockwise rotation. The cell rotation speed is faster in the higher frequency range and vice versa.

By comparing the rotation of normal HeLa cells with that of apoptotic ones, it was observed that the former was faster and its speed was more consistent, compared to the latter. The apoptosis-induced cells showed a relatively slow rotation speed and a large difference in rotational speed. The analysis of the experimental results made it possible to conclude that degrees of cell apoptosis corresponded to rotation speeds of the respective apoptotic cells was different: at the early stage of apoptosis, the damaged cells' rotation speed exhibited a small difference from that of the normal cells, while the rotation speeds of cells at the middle and late stages of the apoptosis were increasingly different from that of the normal ones.
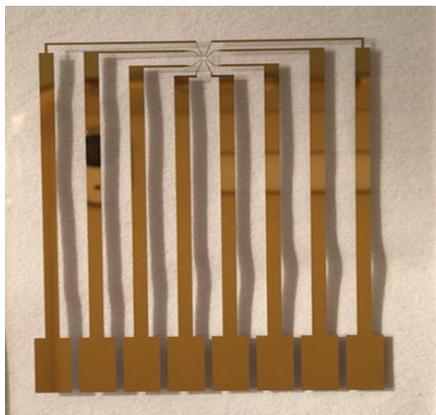

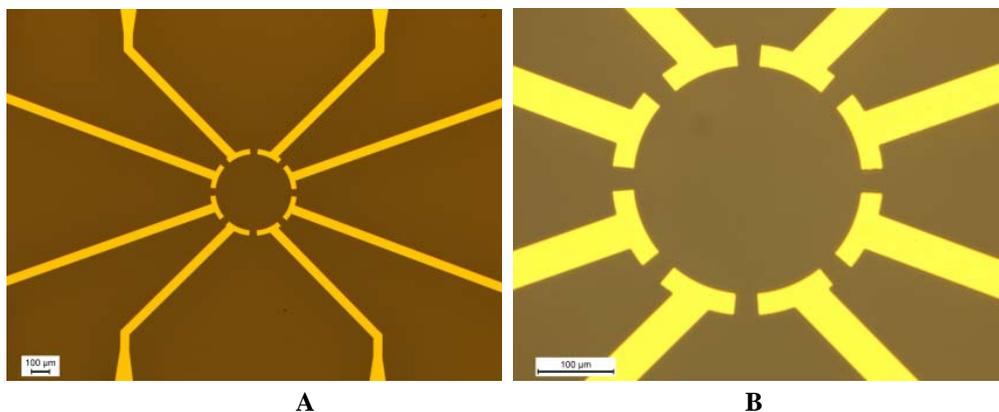Fig. 3. Physical map of the micro- chip with eight Cr-Au electrodes.

| A | B |

Fig. 4. The ring Cr-Au electrode with five-fold (A) and twenty-fold, (B) amplification.

The rotating electric field was produced by the eight-electrode structure, which consisted of four pairs of electrodes located opposite to each other. When the same phase voltage was applied to the two (e.g. upper) adjacent electrodes and a voltage of the opposite phase was applied to their opposite (e.g. lower) two electrodes, the remaining four electrodes are in a high impedance state, providing the hyperbolic electric field distribution. The electric field at the center of the microchip circle was nearly consistent and stable enough to ensure that the tested biological particles, in general, will have a consistent rotation speed. In addition, in contrast to the six-electrode scheme, the design of eight-electrode microchip will reduce the rotational acceleration and make the rotation of the cell more smooth with a nearly constant angular velocity of the electric field rotation, thereby

reducing the effect of Brownian motion and fluid flow on the rotation of cells. The rotating electric field produced by the six-electrode scheme proposed by Shan in 2015 failed to produce smooth enough cell rotation (Shan 2015). The eight-electrode scheme proposed in this case was added to solve this problem and produced a smooth rotation of cells.

Therefore, the microfluidic chip designed in this study can detect apoptotic cells at the middle and late stages. The proposed technique is recommended for cell screening and rapid clinical detection of apoptotic cells.

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