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CYTOSOLIC CALCIUM MEASUREMENT FOR SINGLE-CELL DRUG EFFICACY AND CARDIOTOXICITY EVALUATIONS USING MICROFLUIDIC BIOCHIPS

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ABSTRACT

Intracellular calcium ($[Ca^{2+}]_i$) regulates a diverse range of cellular functions and signaling pathways. This review article aims to highlight applications of microfluidic single-cell analysis in drug discovery including drug efficacy test and drug side-effect test, based on intracellular calcium measurement.

Keywords: Cytosolic calcium, microfluidic chip, cytotoxicity, drug efficacy, drug cardiotoxicity, single-cell analysis, herbal ingredients, licorice.

INTRODUCTION

Although medicinal herbs have been used in folk medicine with a long history, only recently has the screening of natural anticancer drugs from herbs gained much interest (Lee *et al*., 2007; Kanazawa *et al*., 2003; Srivastava and Gupta, 2007). Colorimetric cytotoxicity assays, such as the one using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), are widely used for drug screening. However, these conventional assays usually require substantial amounts of herbal ingredients which are often expensive and limited in amount. In addition, these assays are time-consuming, usually taking ~ 4 days for experiments (Dell'Erba *et al*., 2005). Moreover, when screening for herbal compounds, the reliability and sensitivity of this assay are sometimes affected by the presence of antioxidants and colored substances that may lead to chemical and color interferences, respectively (Wang *et al*., 2006). Therefore, a new cell-based technique is needed to test the drug efficacy of herbal compounds.

It is well-known that intracellular calcium acts as a universal second messenger to regulate a diverse range of cellular functions (e.g., cell death and myocyte contraction) (Dorsam and Gutkind, 2007). In addition, the elevation of cytosolic Ca^{2+} concentration or $[Ca^{2+}]_i$ is associated with the activation of cell membrane-bound G-protein-coupled receptors (GPCRs), which represent the drug targets of 50-60% of current therapeutic agents (Dorsam and Gutkind, 2007). Therefore, the cytosolic Ca^{2+} measurement is now one of the most important cell-based assays in screening for new drug candidates (Monteith and Bird, 2005; Worley and Main, 2002). For example, it is found that various stimuli (e.g., anti-cancer drugs) can cause sustained $[Ca^{2+}]_i$ elevations, which disrupt the Ca^{2+} homeostasis and result in cytotoxicity, and even cell death (Orrenius *et al*., 2003). Moreover, this disruption is believed to be an early event of cytotoxicity (Orrenius *et al*., 2003; Gurfinkel *et al*., 2006). Therefore, the effect of anticancer herbal compounds on cancer cells can be rapidly evaluated by $[Ca^{2+}]_i$ measurement.

During the process of drug discovery, drug biosafety (e.g. cardiac effect) must also be evaluated. It is known that any disturbance to cytosolic Ca^{2+} may cause adverse effects on the contractility of myocytes, leading to cardiotoxicity (Missiaen *et al*., 2000). For instance, many chemicals or drugs (e.g., caffeine and cocaine) have undesirable side-effects on the heart as measured by their effects on the $[Ca^{2+}]_i$ mobilization (Sardao *et al*., 2002). A high plasma caffeine concentration (> 1-2 mM) is known to be lethal for adult humans, in which a large increase in cytosolic calcium occurs by emptying the internal store sarcoplasmic reticulum (SR) of heart muscle cells (Sardao *et al*., 2002). In addition, many anticancer drugs (e.g., daunorubicin, or DNR) have serious toxic effects on the heart (Olson and Mushlin, 1990). Therefore, the $[Ca^{2+}]_i$ measurements can provide useful information for cardiotoxicity of drug candidates before their animal tests and human trials.

Miniaturized microfluidic devices have been used for cellular analysis (see Fig. 1). They require much less reagent consumption when compared with the traditional microtiter plate-based assays. In addition, the small dimensions of microfluidic devices, which are compatible with the sizes of biological cells, allow for the study of a

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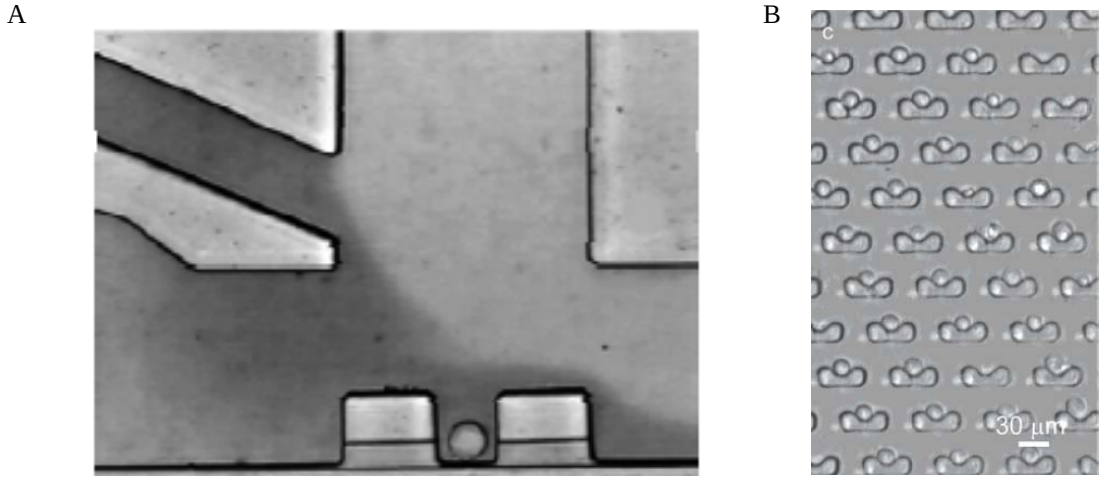

Fig. 1. Different microstructures for single-cell capture (A) from (Wheeler *et al*., 2003) and multiple single-cell capture (B) from (Di Carlo *et al*., 2006).

small number of cells, e.g., rare cells (Li *et al*., 2008). This has made the cell-based assay one of the most popular micro total analysis system (μTAS) applications (Dittrich *et al*., 2006; Li *et al*., 2012; Li *et al*., 2011; Kovarik *et al*., 2012). Among the microfluidics-based cellular applications, much emphasis has been placed on single-cell analysis (Sims and Allbritton, 2007; Di Carlo and Lee, 2006), which helps observe cellular heterogeneity and can provide information about cell-to-cell variations (Teruel and Meyer, 2002; Wheeler *et al*., 2003). For instance, Wheeler *et al*. (2003), Peng and Li (2004, 2005), Li *et al*. (2009), Yin *et al*. (2008), Yang *et al*. (2002), and Zhang *et al*. (2006) have measured calcium changes of spherical cells due to chemical stimuli using fluorescence detection. Furthermore, single cylindrically shaped cardiomyocytes (heart muscle cells) have been measured for calcium changes (Li and Li, 2005; Li *et al*., 2007; Li and Li, 2006; Kaji *et al*., 2003; Klauke *et al*., 2003; Cheng *et al*., 2006; Klauke *et al*., 2007; Klauke *et al*., 2006).

Among these microfluidic measurements on calcium, Li *et al*. quantified $[Ca^{2+}]_i$ of single cells (Li and Li, 2005), showing that only quantified $[Ca^{2+}]_i$, but not fluorescence intensity, can accurately represent the $[Ca^{2+}]_i$ changes of cells in response to drug stimulation. This quantitative method was applied to single cancer cells using a microfluidic biochip for drug efficacy test (see Fig. 2) (Li *et al*., 2009). This method quickly detected the sustained $[Ca^{2+}]_i$ increase, an early event of cytotoxicity, caused by different reagents on leukemia cancer cells. Meanwhile, by using a microfluidic chip with improved cell retention of cardiomyocytes (see Fig. 3), drug cardiotoxicity test on calcium mobilization of single cardiomyocytes from various chemicals (e.g. caffeine), the chemotherapeutic drugDNR, and anti-cancer drug candidate isoliquiritigenin (IQ) were studied (Li *et al*., 2007).

Drug efficacy evaluations on cancer cells

Daunorubicin, a highly effective chemotherapy drug of the anthracycline family, has been reported to cause apoptosis in leukemic cell lines, such as U937 and HL60 (Vial *et al*., 1997; Durrieu *et al*., 1998). Since $[Ca^{2+}]_i$ mobilization precedes the caspase activation in the apoptosis pathway (Durrieu *et al*., 1998), the $[Ca^{2+}]_i$ measurement method can reveal the early-stage information of cell death (Li *et al*., 2009).

The schematic diagram of the chip, the cell retention structure and single RAW cell retained within the structure are shown in figure 2A, 2B and 2C, respectively. Figure 2D shows the $[Ca^{2+}]_i$ mobilization of a single RAW cell (cell 1) by the DNR treatment. After DNR was added, $[Ca^{2+}]_i$ did not increase very much during the first 1200 s (see curve 1). Then a sustained $[Ca^{2+}]_i$ increase up to ~420 nM was observed after ~3800 s of the DNR treatment. A control experiment on another individual cell (cell 2) without Fluo-4 loading was performed, showing no obvious cellular fluorescence increase observed due to the DNR accumulation into the cell (see curve 2). This confirmed that DNR accumulation did not interfere with the fluorescent measurement of $[Ca^{2+}]_i$ because of the different emission wavelengths of DNR (585 nm) and Fluo 4-Ca^{2+} (525 nm) (Li *et al*., 2008; Li and Li, 2005). At a higher concentration of DNR (35 μM), the $[Ca^{2+}]_i$ increase was even faster, and it was observed that some RAW cells died within ~4500 s.

Isoliquiritigenin (IQ), a flavonoid ingredient from licorice, was found to exhibit cytotoxic effects on human prostate

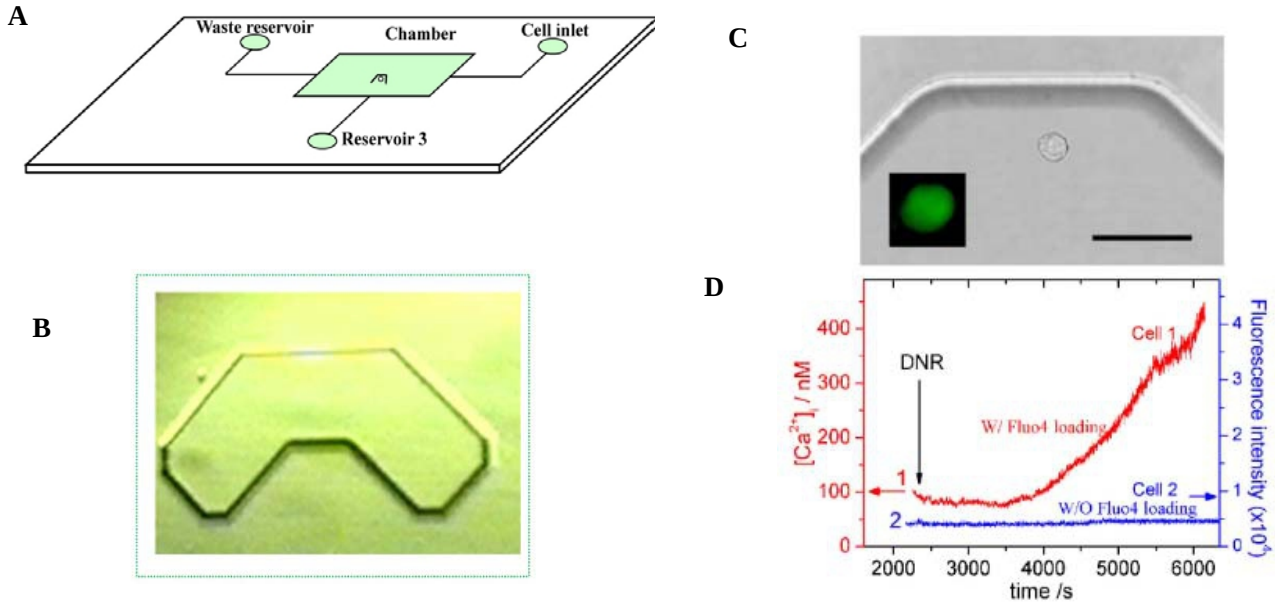

Fig. 2. Drug efficacy evaluations on cancer cells. (A) Microchip layout. (B) Single-cell capture structure. (C) A captured RAW 264.7 cell in the microstructure. (D) DNR-induced $[Ca^{2+}]_i$ mobilization on single leukemia RAW cells. The figure is adapted from Ref (Li *et al*., 2009).

(Kanazawa *et al*., 2003), gastric (Ma *et al*., 2001), heptoma (Hsu *et al*., 2005) and breast cancer cells (Maggiolini *et al*., 2002), and also on mouse renal (Yamazaki *et al*., 2002) and melanoma cells (Iwashita *et al*., 2000). As it was reported that the cytotoxic effect of IQ on gastric cancer cells may involve a calcium-dependent pathway (Ma *et al*., 2001), the anticancer effect of IQ on leukemia cells was investigated by monitoring $[Ca^{2+}]_i$ mobilization (Li *et al*., 2009).

It was shown that 50 μM IQ has caused a sustained $[Ca^{2+}]_i$ increase on a single cell reaching a level of 342±39 nM (n=3) after 1.5 h (Li *et al*., 2009). Glycyrrhizin (GL), which is a major ingredient of licorice (Pompei *et al*., 1979; Sung and Li, 2004), has various desirable pharmacological properties such as anti-viral (Cinatl *et al*., 2003), anti-inflammatory (Tanaka *et al*., 1987), has also been studied to see whether this ingredient has anticancer or cytotoxic effects on leukemia cells. But no sustained increase of $[Ca^{2+}]_i$ on single RAW cells from GL (up to 100 μM) was observed (Li *et al*., 2009).

Drug cardiotoxicity evaluations on cardiomyocytes

In drug development, after efficacy test of drug candidates, it is important to evaluate their cardiovascular safety. Figure 3C shows a single cardiomyocyte retained in the cell retention chamber of a chip with improved cell retention (Fig. 3A and B) for cylindrical cardiomyocytes as described elsewhere (Li *et al*., 2007).

The known cardiac effect of caffeine was first verified by the real-time monitoring of the $[Ca^{2+}]_i$ change on the contraction of a single cardiomyocyte (Li *et al*., 2007). After 40 mM caffeine was added, a rapid $[Ca^{2+}]_i$ increase of 580 ± 65 nM was observed, which was due to the transient release of Ca^{2+} from SR (Bers, 1987). This $[Ca^{2+}]_i$ increase induced the cardiomyocyte to quickly contract by ~40% afterward. These $[Ca^{2+}]_i$ changes in cardiomyocytes indicate the early event of cardiotoxicity, as compared to the previous observations of cytotoxicity in RAW cells.

The effect of IQ on $[Ca^{2+}]_i$ of a single cardiomyocyte is shown in figure 3D (curve 1). Before drug treatment, the resting $[Ca^{2+}]_i$ was ~118 nM. After 100 μM IQ was introduced, $[Ca^{2+}]_i$ showed a slow increase after ~20 min (at ~4400 s), until it flattened off at ~279 nM after ~80 min (at ~8000 s). The results showed that IQ did increase the $[Ca^{2+}]_i$ of cardiomyocyte in a time-dependent manner, but the change was not dramatic, which indicated that IQ might have a low toxic effect on cardiomyocytes. For comparison, the cellular response of 3.5 μM of DNR was also measured, as shown in figure 3D as curve 2. It can be seen that the $[Ca^{2+}]_i$ increase due to DNR is much higher

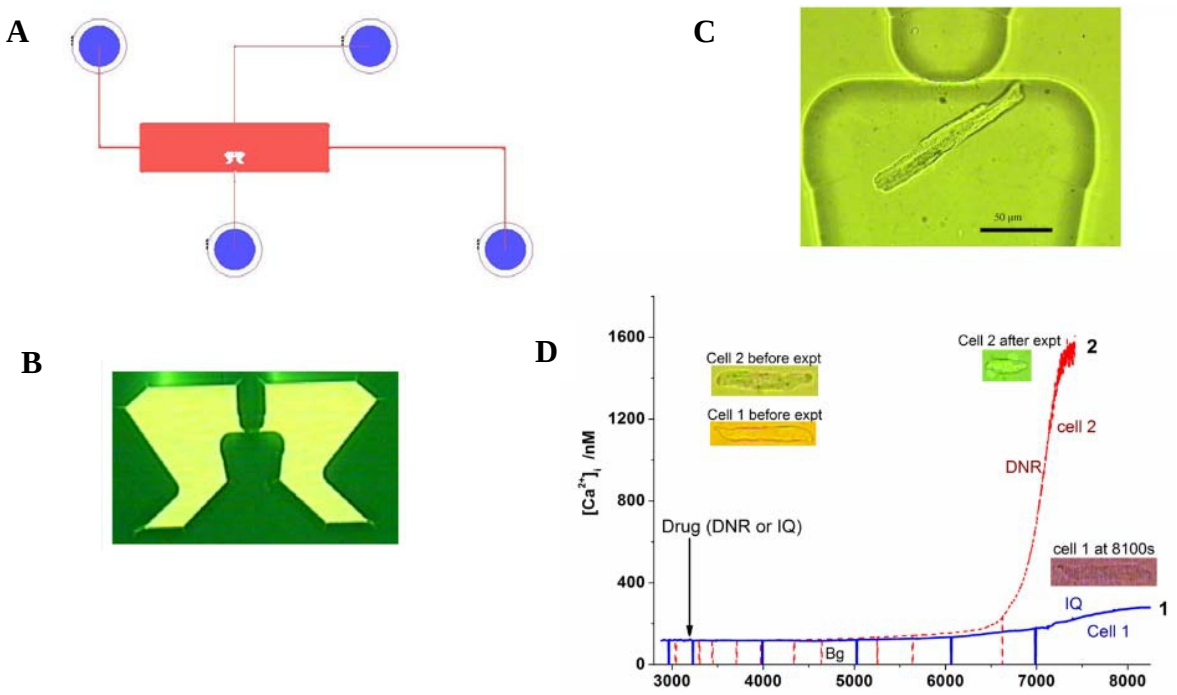

Fig. 3. Microfluidic single cell analysis for drug cardiotoxicity evaluation. (A) Chip layout. (B) Single-cell capture structure. (C) A single cardiomyocyte retained in the capture structure. (D) Cardiotoxicity evaluations on cardiomyocytes as depicted by the $[Ca^{2+}]_i$ responses after drug treatment of DNR and IQ. The figure is adapted from Li *et al.* (2007).

than that resulted from IQ. For instance, DNR caused $[Ca^{2+}]_i$ to increase up to 1563 nM, even when a much lower DNR concentration (3.5 μM) was used, while 100 μM IQ increased $[Ca^{2+}]_i$ only up to 279 nM. The very high $[Ca^{2+}]_i$ concentration of 1563 nM is detrimental to the cells (Olson and Mushlin, 1990), as it has been reported that $[Ca^{2+}]_i$ above ~10^{-6} M or 1000 nM is lethal to cells (Hesketh *et al.*, 1983). More experiments show that the maximum $[Ca^{2+}]_i$ due to DNR and IQ within 90 min are 1507±80 nM and 255±35 nM (n=3), respectively. These data suggest that DNR has a much greater cytotoxic effect than IQ on cardiomyocytes. In addition to the Ca^{2+} data, the microfluidic single-cell method provided the data of the cell morphology changes before and after the drug treatment (Fig. 3D). For instance, after DNR treatment, cell 2 showed cell shortening and membrane blebbing, which is indicative of an unhealthy or dying cell, while cell 1 still showed healthy status after the IQ treatment.

Although DNR is widely used in treatment of leukemia, the use of DNR is unfortunately limited by its potentially fatal cardiotoxicity (Olson and Mushlin, 1990; Olson *et al.*, 2000). As compared with DNR, IQ has less effect on $[Ca^{2+}]_i$ of heart muscle cells, and hence less cardiotoxicity. In conjunction with the finding from the previous section that 50 μM IQ has a cytotoxic effect on leukemia cells, IQ can be a potential drug candidate with less cardiotoxicity. But the findings of these cell-based in vitro tests are subject to the confirmation by in vivo assays.

SUMMARY

Intracellular calcium measurement using microfluidic devices provides a versatile platform for drug discovery from drug efficacy test to side-effect test. Such an approach, together with the effort to develop high throughput capability and automation, will be useful to evaluate drug efficacy and cardiosafety, without the 4-day wait when using the conventional assays.

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