Microsyst Technol
DOI 10.1007/s00542-015-2565-9

TECHNICAL PAPER

Effect of a microwave warming of cell culture media on cell viability and confluence rate

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Received: 15 April 2015 / Accepted: 28 April 2015

Abstract Here we present a method for rapidly and stably warming up a small volume of cell culture media that can maintain cell viability and confluence rate. This method uses microwave radiation for warming without any direct contact with water, preventing the potential issue of contamination induced by the use of a water bath. To demonstrate the proof of concept validation, we used a conventional microwave oven for warming cell culture media. In our experiments, it took only 10 s to warm a 50 mL-media tube (mostly proper volume for the use of microfluidic cell culture experiments) up to 37 °C. Multiple tubes can also be used to increase the volume of cell culture media by placing them in a plastic support within the oven at the same time in a scalable manner. The results show that there was no jump discontinuity to a higher temperature than 37 °C within 10 s. Both apoptosis and necrosis were monitored and examined to confirm whether the new method can affect cell viability and metabolism. The proposed method is fast, easy and user-friendly in conventional cell culture process, even scalable for the use of large media volume, and free of biological contamination due to water contact occurred by use of conventional water baths. We furthermore believe that this approach can be potentially helpful for advancing on-chip cell culture process that may require a small volume of cell culture media often used in microfluidic devices.

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1 Introduction

Conventional cell cultures and tissue engineering applications use thermal conduction in water bath for warming cell culture media in laboratory and cell culture room (Ventura et al. 2007; Bang et al. 2008; Lee et al. 2010). Mostly it takes more than 10 min to warm refrigerated media bottle or freezed pre-aliquoted tube using water bath with a constant temperature (37 °C), even if the sample volume is small for cell manipulation in microfluidics (Park et al. 2012; Yun et al. 2013, 2014). Although water bath can warm up cell culture media mildly, it involves the potential issue of biological contamination by water mold or bacteria. Even though people usually clean the water bath and replace water with fresh, distilled water at regular intervals, it is often occurs from careless handling and insufficient cleaning of bottle or tube with 70 % EtOH after contacting the water. Besides, it takes a longer time to increase the media's temperature to a required level due to the heat transfer mechanism of thermal conduction (Yen et al. 2014). Therefore, a non-contact heating method with fast heating speed is preferred for stably heating cell culture media, which would be another option to advance cell culture process.

There are two major equipments to warm up and cook food at home, i.e., gas stove and microwave oven. Gas stove uses natural gas or other flammable gas as a fuel source to heat the air or container around for heating food. Microwave oven uses short wavelength (2.4–2.5 GHz)

Published online: 17 May 2015

(Smith et al. 2014), which can penetrate inside food and generate heat by molecular vibrations allowing more uniform and deeper heating of food in a shorter time (Mishra et al. 2012). Although a microwave oven uses a magnetron to create short wavelengths which results in high electricity consumption (approximately 2 kW of electricity for creating high-frequency wavelengths of 1.2 kW) (Metcalf and Codd 2000), it uses less energy than a gas stove when heating up a plate of food according to the US Environmental Protection Agency (EPA). Microwave oven is usually installed with a cooking timer as it generally requires short heating time due to the heat transfer mechanism of radiation. Besides, accumulating evidences has also shown that microwave oven does not have enough energy that may impact the cell nutrients' molecular bonds of food (Baqai and Hafiz 1992). Interestingly, the microwave approach has been used for rapidly and selectively detecting mycobacteria in biochip applications in the range of 10–26 GHz (Jing et al. 2004). In addition, the use of microwave has been a fascinating tool for assisting the synthesis polymeric microstructures of carbon nanodots, since it can shorten the reaction time (Wang et al. 2012; Lee et al. 2009). These facts inspire us to think over a new microwave approach that can be useful for warming up a small volume of cell culture media rapidly and stably, leading to a low possibility of contamination due to the use of water bath.

In this study, we presented a microwave approach for overcoming the problems: (1) unwanted relatively longer heating time of preparing for cell culture media, even a small volume of media to the ready-to-feed state in conventional water bath methods, and (2) high chance of contamination due to the use of water contact and. Our attempt is also to explain the biological stability of new cell media heating method in these experiments. To determine the biological stability of microwave media-warming, we measured the cell number and viability, and observed the healthy condition of the cultured cells through the fluorescence imaging and time-lapse image analyses. When using a conventional water bath method, the media temperature of 50 mL-volume Falcon tube could reach 37 °C after 1 h immerged into the bath. As stated earlier, a water bath requires longer heating time to distribute the heat through water's heat conductivity than a microwave oven (Millin 2001). Through the microwave, it took 10 s or so as the same condition of conventional. Here we aimed to demonstrate the proof of concept validation of the microwave method as an effective method for warming up the culture media, especially for a small volume of cell culture media in a rapid and stable manner.

2 Materials and methods

2.1 Media warming

Water bath (Fig. 1a) to heat the cell culture media uses heat conductivity of water and transfer the heat from outside to inside. In contrast, microwave oven uses heat radiation and heats uniformly by penetrating and vibrating water molecule inside the target. Microwave oven is an appliance that sets the heating time, different from a conventional heating device which sets and controls the

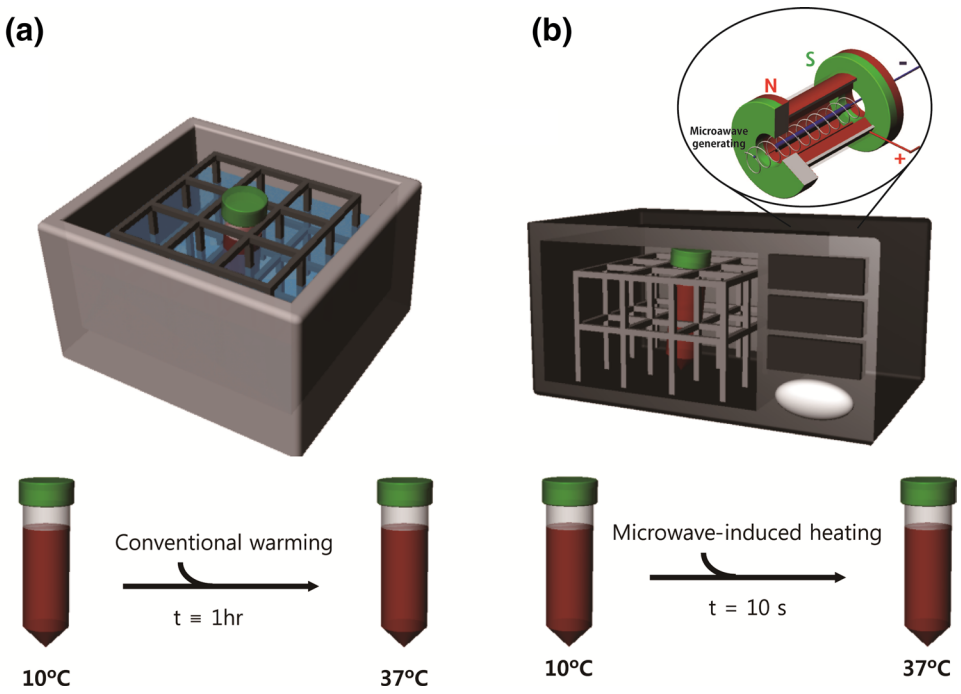


Fig. 1 **a** Schematic image of conventional water bath for media heating. **b** The schematic image of the microwave oven applied for media heating

target temperature. To measure temperature variation of media during microwave warming, we use an infrared thermometer that measures the temperature by detecting infrared radiation emitted by the surface energy. The media temperature varied from 20 to 50 °C, depending on the heating time around 10 s. We have measured the temperature change depending on the heating time and the media's volume. The water bath used in this study is Gaon SW-90WP, with rate voltage in 220 V/60 Hz. The microwave oven used in this experiment is Samsung RE-C21AB, with rated voltage in 220 V/60 Hz and the oscillation frequency is 2450 Hz.

2.2 Cell culture

The cell culture media used in the biological stability test consists of Dulbecco's Modified Eagle Media (DMEM), Fetal Bovine Serum (FBS), and Penicillin streptomycin (PS). There are various media for animal cell cultures, and we chose the most appropriate media for HeLa cell. Generally, DMEM and Minimum Essential Media (MEM) which are added four times more amino acid and ferric nitrate based on Basal Media Eagle (BME) are widely used for adhesive animal cell culture. Composition of DMEM used in this experiment is 4.5 g/L D-Glucose, L-Glutamine and 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. FBS provides hormone, protein that need for growth and functionality of the cell. PS causes lysis by suppressing the growth of cell wall that kills bacteria in the media. The cell culture media can be made by adding 5 mL FBS and 500 μL PS on 45 mL DMEM.

To find any effect in cell growth and viability in two different media-warming methods, cell culture performed using two different media, which heated by water bath and microwave oven individually. If there are some changes in media acts as a cell fuel, cell growth and viability would be different between two groups. To see the cell viability, the cell was cultured for 7 days in the incubator under the condition of 37 °C, 5 % CO_2 without adding additional materials and observed condition at day 4 and day 7.

2.3 Cell viability test

The total cell number and cell viability were measured to confirm the biological stability of two different media-warming methods. The total cell number was counted by cell counter (Eve™, NanoEnTek Inc., Korea). The cell viability was measured after staining the cell with Live/Dead kit, consisting of Calcein AM and EthD-1. The cell viability in different groups was measured using a real time imaging system (JuLI™ Br, NanoEnTek Inc., Korea) installed in the cell culture incubator.

2.4 Time lapse imaging

Real time imaging system installed in incubator observes changes of cells and measures the confluence in the cell culture flask in real time. Since this system uses the dual camera system, it is able to compare cell growth and confluence in real time or with user fixed interval time lapse.

2.5 Cell image analysis

The total cell number and viability were measured and analyzed using an automatic cell counter image-based analysis after staining cells with trypan blue. Trypan blue has a strong affinity to protein. If cell membrane was cracked or destroyed after cell death, non-viable cells will be stained with blue dye in core part. To monitor and identify cell status (live, dead, and necrosis), we use apoptosis assay kit (TaLI™ apoptosis kit, life technologies) containing Annexin binding buffer (ABB), Annexin V Alexa Fluor 488 (AF488), and Propidium Iodide (PI). Annexin V is a 35-36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidyl serine (PS). Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS. PI is a cell-impermeant to live cells, but easily enters dead cells where it binds to nucleic acids and becomes fluorescent (red). After staining using the apoptosis assay kit, stained cells were measured and analyzed by an image-based cytometer. Apoptotic cells show green fluorescence, dead cells shows red and green fluorescence (observed as yellow), and live cells show little or no fluorescence.

3 Results and discussion

3.1 Ten seconds are enough to warm up a small volume of cell media in a ready-to-feed state

Cell culture media contains many proteins and nutrients, which are necessary for cell survival. Due to the enzyme activity, cellular metabolism is sensitively influenced by the temperature of environment. For the cells from human tissue or body, the optimal temperature for maximizing enzyme reactions is around 37 °C. At lower temperature, the reaction rate will decrease with limited collisions between enzyme and substrate. At higher temperature, enzymes and proteins will be denaturated and the reaction rate will decrease rapidly. Therefore, it is important that media-warmer should have the ability to reach and maintain the target temperature precisely. Figure 2a shows temperature changes with time in different warming methods. Microwave warming increased the temperature of cell culture media to the target temperature (37 °C) within 10 s, while

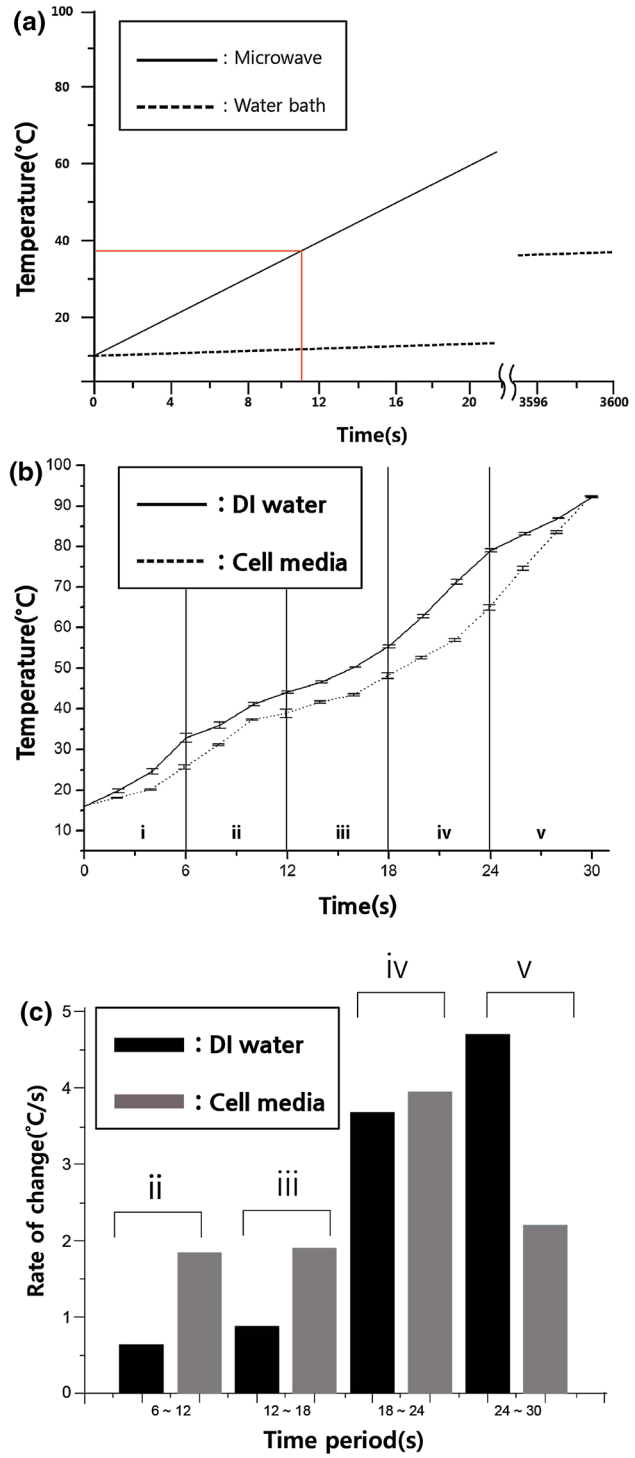


Fig. 2 **a** Temperature changes by times in different media-warming methods, **b** enlarged graph of temperature changes of media and water warmed up by microwave oven. **c** Mean rate of change of media and water

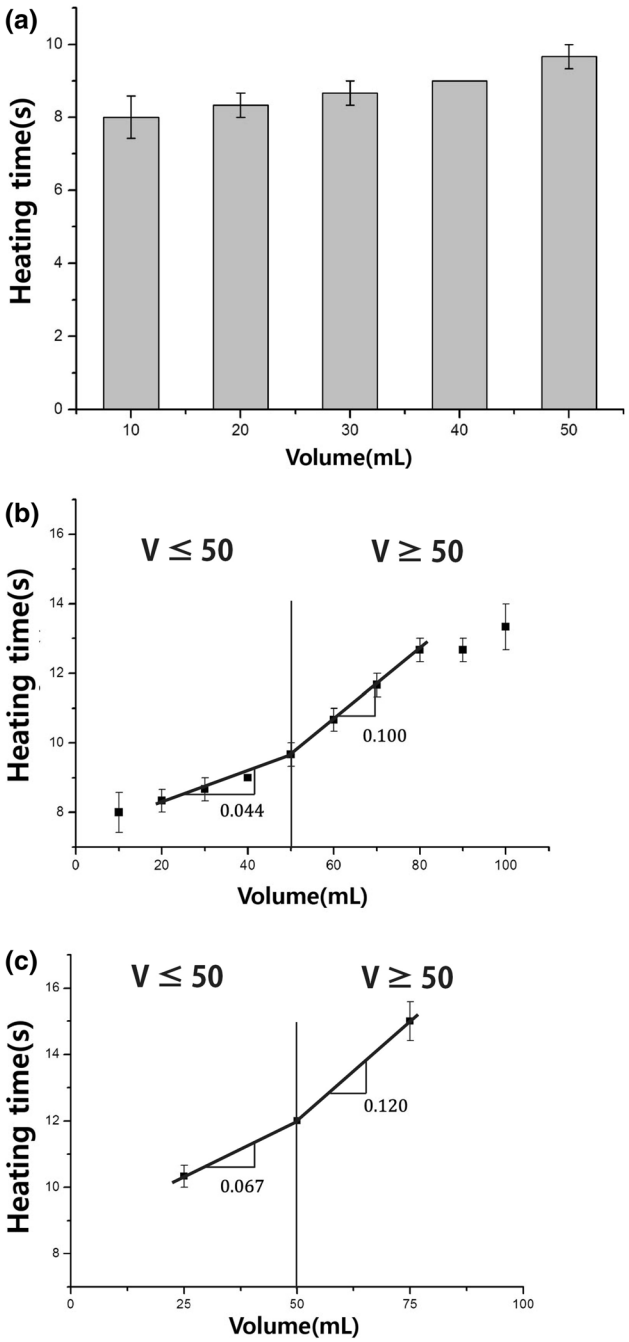


Fig. 3 **a** Required heating time to warm up to 37 °C by microwave oven depend on media volumes. **b** Graph between heating time to 37 °C and media volume of 20–50, 50–80 mL. **c** Heating time to 37 °C and media volume of 25–50, 50–75 mL

the conventional media heating method by water bath took 1 h (i.e., 3600 s). Comparing the temperature rising slope of two methods, microwave and water bath in media-warming is 2.7 and 0.0075 °C/s, respectively. The media-warming speed using the microwave oven is 360 times faster than that using the conventional water bath (see Table 1). The microwave electromagnetic radiation penetrated through media, causing dielectric warming primarily by absorption of the energy in water. Due to the reason, the results show that the microwave method can warm up the cell culture media very rapidly. Figure 2b shows the temperature changes of

Table 1 Summary of a microwave-induced method in comparison to conventional methods

	Conventional method (water bath)	Microwave-induced method
Heat transfer	Indirect (conduction)	Direct (absorption of radiation)
Heating method	Electric heating (using resistance coil)	Electromagnetic radiation (using magnetron)
Heating time	Continuous heating	Prompt heating

cell culture media and deionized distilled water warmed up by microwave oven. The little temperature gap between cell culture media and D. I. water would be from the difference in the percentage of water content. The pure water is easier to heat in initial period when same energy is applied (Fig. 2b, c). The inclination of the temperature changes in media is larger than that of water in period (1) and (2), similar in (3) and smaller in (4). This difference may occur because of characteristics between the mixture and the pure substance. Figure 3 shows difference of required heating time of media with various volumes, warmed up to 37 °C by microwave oven. The time gap between different media volumes occurs because of penetrating volume. The slope of required heating time shows in Fig. 3b and c. It presents the large volume of media required longer time and energy to reach the target temperature. The slope of required time in the region under 50 mL-media would have small value compared to the upper region (50–80 mL).

3.2 Cells survive and even confluence well after being supplied with microwave-warmed media

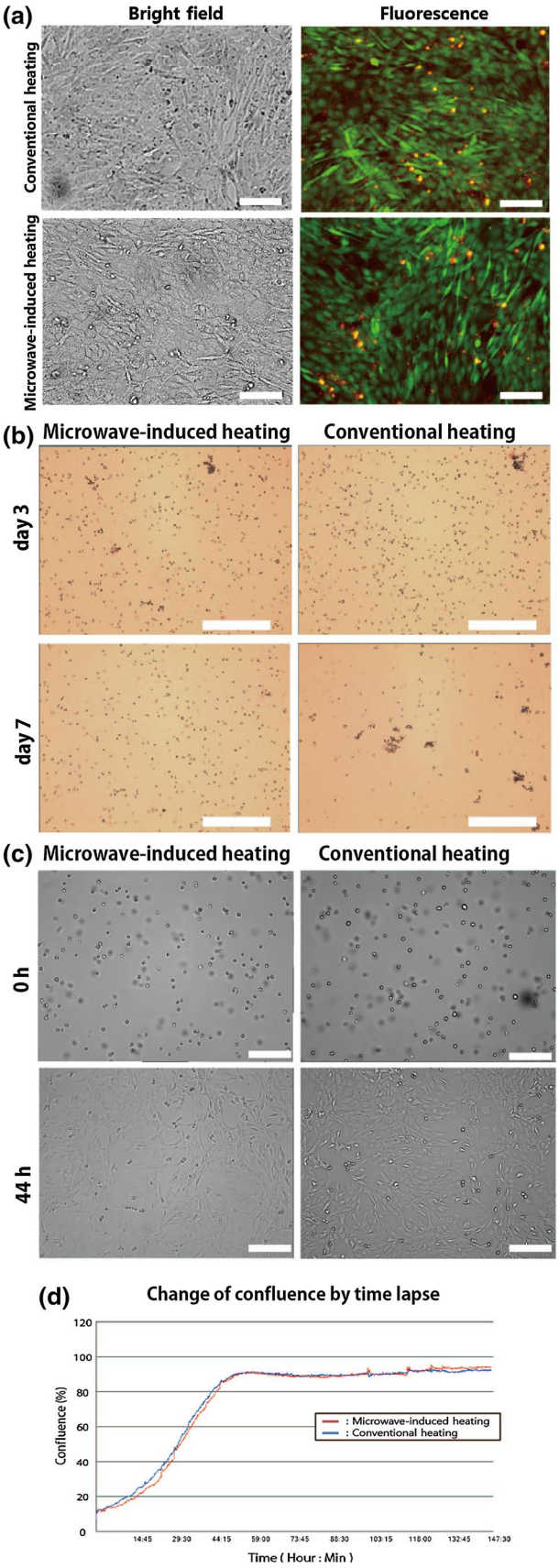
To confirm the biological stability of two different media-warming methods, the total cell number and cell viability were measured. The viability per cell's unit volume is shown in Table 2. The results show that there was no significant difference on day 3 showing each 90 and 93 % viability rate, and there was less than 5 % of the differences of 87 and 82 % on day 7. Based on the results, it can be recommended to further examine not only the cell's viability rate, but also the functional changes in cells. For the long-term culture, in particular, the cell viability in the group with microwave warmed media was 5 % higher than the other group. The results show that there was no significant difference between two groups cultured with different media, warmed up by water bath and by a microwave oven, respectively. Based on the result, we also believe that the protocol for warming culture media with microwave might not denature and broke the protein and enzyme to be harmful for cells. The results of live/dead cell analysis are shown in Fig. 4a. The fluorescence images of stained cells were taken on the day 7 cultured with two different media. The cell viability after Trypan blue staining was measured using a cell imaging and counting system that can quantify the result on the day 3 and the day 7, respectively. Figure 4b is a fluorescence image of cells stained with Trypan blue to be used for the cell counter. The real-time cellular images, especially showing the change in confluence rate, are acquired and analysed by a real-time live cell movie analyser as shown in Fig. 4d. In particular, Fig. 4c shows the time lapse images of sub-cultured cells after 0 and 44 h, respectively. The results of Table 2 were compared to the confluence change in Fig. 4d, but there was no significant difference. From the results of bright field and fluorescence images, there was no significant difference found in the numbers of total cells and cell viability. This may be because the ingredients of microwaved media are hardly modified within the short time and modestly warmed at the media temperature. In contrast, a direct exposure of the microwave to the cultured cells can obviously cause severe damages to them (Esmekaya et al. 2013). In our experiments, however, it was a way of warming cell culture media, not directly to the cells, even not more than 10 s at conventional media temperature with no jump continuity.

Table 2 Cell viability cultured with different media warming

		Water bath	Microwave oven
Day 3	3.9×10^6	Total cell (cells/mL)	3.5×10^6
	93 %	Viability	90 %
Day 7	2.0×10^6	Total cell (cells/mL)	2.7×10^6
	82 %	Viability	87 %

3.3 Possibility of being contaminated by microwave warming can also be decreased

The possibility of media contamination can be reduced using microwave ovens, in which microwave is carried through air, in contrary the conventional water bath is exposed in direct contact with water. As the result indicates 10 s heating time in a microwave oven versus 1 h in a water bath, it was confirmed the cell heating time can be greatly reduced using a microwave appliance. The contamination by water mold or bacteria may occur in a water bath, which uses the water temperature conduction, can be effectively reduced by using a microwave oven which uses thermal radiation in the air. Recently, it was reported that the use of microwave can kill pathogenic microorganisms that often contaminate food (Rasooly et al. 2014).



◀**Fig. 4** **a** Images of bright field and fluorescence after staining with EthD-1 and Calcein AM (*red* dead cells, *green* cells live cells). **b** Images of stained HeLa cells by Trypan blue. **c** Images of sub-cultured cell after 0 and 44 h. **d** Graph of cell confluence in culture flask (*Scale bar* 100 μm) (color figure online)

In addition to microwave-induced method by heat radiation, radiation heat transfer method, infrared heater and ultraviolet heater with sterilizing can be compared with microwave-induced method and water bath or convection oven by raising temperature also can be compared with conventional water bath heating method.

4 Conclusions

Here we demonstrated feasibility of the method by performing 7-day cell cultures with high cell viability and confluence rate. In particular, we found that a microwave approach is easily accessible and very convenient to warm up cell culture media in a fast and controlled manner. In addition, this approach is mainly made by thermal energy radiation in air, rather than thermal conduction by water contact which can cause unwanted fungal contamination. In our experiments, the microwave warming was performed within a protected safety chamber at a fast heating rate, especially for a 50-mL media volume, mostly appropriate for the use of microfluidic cell applications. We furthermore believe that this approach could be a fascinating method to heat up cell culture media with several advantages; (1) much less time of warming to be required (in particular, very useful in the case of using a small volume of media, at least 50 mL), (2) lower chance to be contaminated (due to water fungi), and (3) more convenient and easier use of warming by a clear time-setup within a controlled safety chamber. We expect that this approach can be potentially helpful for those who need a quick warming of cell media with small volume in on-chip microfluidic cell culture applications, where urgent media supply is often required at every injection event of experiments.

Acknowledgments This research was supported by the National Research Foundation of Korea (NRF-2012029193) and Basic Science Research Program through the NRF, funded by the Ministry of Science, ICT and Future Planning (2013010861). This research was supported by a grant of the Korea Health technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea. (A111345). F. X. acknowledged the financial support from the Key (Key grant) Project of Chinese Ministry of Education (313045) and International Science and Technology Cooperation Program of China (2013DFG02930).

Conflict of interest The authors have no conflict of interest to declare.

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