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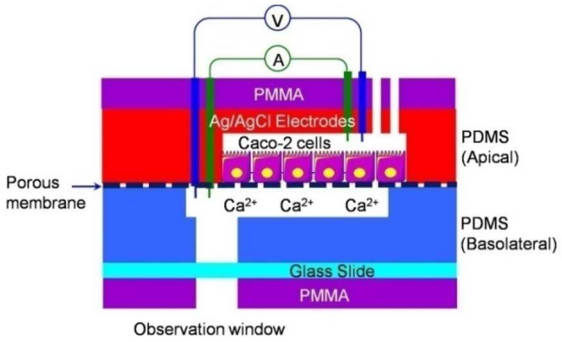

Microfluidic-based Caco-2 culture model provides a useful tool for investigating of metabolites transport and nutrikinetics studies.

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Microfluidic chip for monitoring Ca^{2+} transport through a confluent layer of intestinal cells

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Abstract. The absorption of dietary calcium through the intestinal barrier is essential for maintaining health in general and especially of the bone system. We propose a microfluidic model that studies free calcium (Ca^{2+}) transport through a confluent monolayer of caco-2 cells. The latter were cultured on a porous membrane that was positioned in between a top and bottom microfluidic chamber. Fresh cell culture medium was continuously supplied into the device at a flow rate of 5 nL/s and the culture progress of the cell monolayer was continuously monitored using integrated Transepithelial Electrical Resistance (TEER) electrodes. The electrical measurements showed that the caco-2 monolayer formed a dense and tight barrier in 5 days. The transported free Ca^{2+} from the top microfluidic chamber to the basolateral side of the cell monolayer was measured using the calcium-sensitive dye fura-2. This is a ratiometric dye which exhibits an excitation spectrum shift from 340 nm to 380 nm, when it binds to Ca^{2+} with an emission peak at 510 nm. Therefore, the concentration of free Ca^{2+} is proportional to the ratio of fluorescence emissions obtained by exciting at 340 nm and 380 nm. The barrier function of the cell monolayer was evaluated by a measured rate of Ca^{2+} transport through the monolayer that was 5 times lower than that through the bare porous membrane. The continuous perfusion of cell nutrients and the resultant mechanical shear on the cell surface due to the fluid flow are two key factors that would narrow the gap between the in vivo and in vitro conditions. These conditions significantly enhance the caco-2 cell culture model for studying nutrients bioavailability.

Introduction

Dietary calcium intake has an important impact on human health and chronic calcium deficiency resulting from inadequate intake or poor intestinal absorption is a major cause of reduced bone mass and osteoporosis. Calcium is absorbed in the mammal along two routes: (i) a transcellular mechanism, predominant in the duodenum and regulated by vitamin D, and (ii) a paracellular, concentration-dependent diffusional process that takes place throughout the length of the intestine [1]. In normal conditions, calcium predominantly traverses the intestinal epithelium via the paracellular pathway [2], which is driven by the voltage gradient between the apical and basolateral side of the gut epithelium, and regulated by the tight junction proteins, which polymerize to form an array of channel-like paracellular pores [3]. *In vivo* methods, which provide direct data of bioavailability, have been used to measure the bioavailability of a great variety of metabolites [4,5]. Such studies imply the consumption of a certain dose of a nutrient by either humans or animals, and subsequently monitoring its concentration in the blood plasma over time and compare it to an equivalent nutrient dose found in a food source[6]. However, *in vivo* studies are technically difficult, costly, and limited by ethical constraints. Another major drawback of *in vivo* data is the variability in physiological state of individuals and the possible interaction of the nutrient with other components in the diet[7]. Therefore, alternative methods are needed.

In vitro models of the human gastrointestinal tract (GIT), that closely mimic the physiological processes of absorption, may provide efficient tools for the bioavailability measurements, and therefore can show more systematic human tissue response to nutrients and significantly reduce experimentation expenses. The standard *in vitro* model used for studying the bioavailability of nutrients in food is a confluent layer of epithelial cells [8, 9], with caco-2 cells the most popular choice for modeling the human GIT [10,11]. In this model, the cell monolayer is grown on a porous membrane, such as a Transwell insert (Millipore, USA), which is placed inside a culture well to form a double-compartment system. Such system allows accessing the cells from both the top/apical and bottom/basolateral side. When cultured on a micro-porous membrane, caco-2 cells form dense intercellular junctional complexes, resulting in a tight epithelium and presenting a unique paracellular and transcellular barrier [12], thus providing a useful physiological tool to identify metabolites transport originating from a variety of digested food types [13]. However, these *in vitro* methods require a large amount of cells, reagents and culture media, and, additionally, these tools are static and less suited to provide a dynamically controlled flow of cell nutrients and stimuli[14].

Microfluidics-based cell culture systems have the potential to create dynamic *in vivo*-like cell microenvironments [15, 16] and therefore can be useful tools for screening of the physiological properties and Ca imaging [17, 18]. This paper introduces a technique to measure Ca^{2+} transport through a confluent monolayer of epithelial cells cultured in a double-layer microfluidic chip by using a ratio imaging method. Two pairs of Ag/AgCl TEER microelectrodes are integrated into the chip to continuously monitor the quality of the cell monolayer during the cell incubation, as well as throughout the Ca^{2+} transport experiments. Fig. 1 shows a conceptual schematic view of the Ca^{2+} transport microfluidic chip, which comprises a confluent monolayer of caco-2 cells cultured on a porous membrane sandwiched in between two microfluidic chambers made in polydimethylsiloxane (PDMS) and clamped by two polymethylmetacrylate (PMMA) plates.

The chip was fabricated of two individually molded/micro patterned PDMS layers, which are termed here as the 'apical' and the 'basolateral' layers, respectively. These two layers sandwich a polyethylene terephthalate (PET) membrane with pore size of 0.4 µm. The assembled chip was sandwiched in between two PMMA layers and firmly mechanically clamped to prevent fluidic leakage. The PMMA fitting also facilitates inserting the TEER probes, as well as connecting the chip to the external fluidic apparatus for cell loading and continuous infusion of culture media. To maintain the optical path length, the base of the basolateral chamber was made of a glass cover slip with thickness of 170 µm. Our device allowed leakage-free operation during the experiments, as tested by injecting colored solutions in the microfluidic chip. For epithelial cell layer culture, 0.1 mL of a caco-2 cells suspension at a density of 2×10^6 cells/cm^2 was loaded into the chip using a syringe pump at a flow rate of 10 nL/s. After cell loading, the chip was inserted into the incubator at 37 °C with 5% CO_2 for cell sedimentation and proliferation. Fresh cell medium flow was continuously supplied at5nL/s during incubation. The cells formed a fully confluent monolayer after 5-6 days of culture. Caco-2 cells were also cultured in parallel in Transwell devices for comparison.

Several fluorescent probes show a spectral response upon binding to Ca^{2+}, which in principle enables investigating changes in transport and absorption of Ca^{2+} using fluorescent microscopy. Fura-2 is the most popular dye for ratio-imaging microscopy: it is a ultraviolet (UV)-excitable Ca^{2+}indicator that undergoes a shift in absorption upon binding to Ca^{2+} [13]. The dye is excited at a wavelength of 340 nm and 380 nm and the ratio of the emissions monitored at ~510 nm corresponding to those wavelengths can be correlated to the amount of calcium in solution. The use of the ratio allows canceling out confounding variables such as variable dye concentration and liquid volumes. The setup used for detecting the calcium transport includes a dedicated imaging system which relies on the ratio imaging technique. Fig. 2a shows a schematic of the ratio imaging apparatus (Visitron, Germany), which was integrated onto an inverted microscope (Axio Observer, Zeiss). An illumination system (Lambda DG4, Sutter instruments, USA) with a 300 W Xenon lamp and rapid wavelength switching function was used to excite the fura-2 dye. The dual galvanometer design of this system allowed switching between the two excitation filters within 2 milliseconds, which facilitates the ability to follow fast changes in Ca^{2+} concentrations. The Ca^{2+} images were captured using an integrated CCD camera (ORCA-D2, Hamamatsu, Japan) and the Ca^{2+} ratio was calculated using VisiView Premier Image acquisition Software (Visitron, Germany).

Results and Discussion

Prior to running the ratio measurements, the ratio imaging apparatus had to be calibrated. Samples with different concentrations of Ca^{2+}, ranging from 0 to 40 µM, diluted in a calcium calibration buffer #1 (prepared from 50 mL of 10 mM K_2EGTA and 50 mL of 10 mM CaEGTA stock solution, which can be blended to make buffers having free Ca^{2+} ranging from 0 µM to 39 µM), containing fura-2 were prepared and the ratio of the fluorescence emission intensity at the two excitation wavelengths were measured. Fig. 2b shows the calibration line of the free calcium containing 0-40 µM in 10 µM fura-2 on the chip. The emission intensities obtained at the two excitation wavelengths are defined as I_{340} and I_{380} for the excitation at 340 nm and 380 nm, respectively. The Ca^{2+} concentration has been obtained from the equation [19]:

$$[Ca^{2+}] = K_d \times \left[{(R - R_{min})}/{(R_{max} - R)} \right] \times S_{fb} \qquad (1)$$

Where K_d (for Ca^{2+} binding to fura-2 at 37°C) = 225 nM, R is defined as the ratio I_{340}/I_{380}, R_{max} is this ratio under Ca^{2+}-saturating conditions, R_{min} is this ratio under Ca^{2+}-free conditions, and S_{fb} is ratio of the fluorescent signal I_{380} at zero and saturated Ca^{2+}. The calibration line in Fig.2b, calculated from Eq.(1), fits well the standard calibration line.

For comparing the experiments on confluent layer cell culture in our microfluidic device, caco-2 cells with a concentration of 5.5×10^4 cells/cm^2 were also seeded into Transwell devices and incubated. In parallel, 0.1 mL of caco-2 cells suspension with a cell density of 2×10^6 cells/cm^2 were loaded onto the chip at a flow rate of 10 nL/s and continuously supplied with DMEM cell media （Dulbecco's Modified Eagle's Medium） at a flow rate of 10 nL/s for 6 days. Successful cell loading into the chip was enhanced by blocking the fluidic ports at the apical side (except the cell loading port) and allow fluid aspiration from the basolateral side. All the introduced cells were observed to aggregate in the vicinity of the PET membrane pores. Fully confluent monolayers of caco-2 cells were observed after 6 days in both the Transwell device and the microfluidic chip (see Fig. 3). The Ca^{2+} transport through the Caco-2 cell monolayer was carried out only after the monolayer reached 100% confluence, as confirmed by the TEER measurements. TEER measurements were normalized to account for the membrane surface. TEER increases during the cell incubation and reaches a maximum value after 6 days after cell seeding and then maintains a stable high value during further culture. However, the caco-2 monolayer on the chip displayed a 7-fold higher value of TEER compared to that of a monolayer on a Transwell device (Fig. 4).

For studying the Ca^{2+} transport through a confluent caco-2 cell layer, DMEM cell media with a free Ca^{2+} concentration of 33 µM was used as a source of Ca^{2+} and was perfused through the apical chamber at a flow rate of 10 nL/s for 10 h. At the same time, the lower compartment was fluidically isolated; therefore, the liquid from the apical to the basolateral compartment could be only transported through diffusion. As a control experiment, Ca^{2+} transport measurements through a cell-free PET membrane were also conducted (see Figure S1 of the Supporting Information). The emission intensities in the basolateral chamber obtained at the two excitation wavelengths, I_{340} and I_{380}, were measured at different time intervals (see Fig. 5(a)). A notable feature is the opposite trend of the two intensities: while I_{340} increases over time, I_{380} decreases with increasing transport of the Ca^{2+} through the membrane. This also is further reflected in the ratio curve of Fig. 5(b). We use this ratio to calculate the free Ca^{2+} concentration in the basolateral compartment (see Fig. 5(c)). Moreover, this figure shows also the obtained Ca^{2+} concentration for our control experiment, in which we only have the

membrane in the device and not the caco-2 cells. Ca^{2+} transport through the caco-2 monolayer was ~ 5 times slower than that through the bare membrane. While the latter show sharp increase of Ca^{2+} concentration after 8 hours of Ca^{2+} supplement injection, the Ca^{2+} concentration significantly increased only after 11 hours in the basolateral chamber beneath the cell monolayer, effectively demonstrated the barrier function of the epithelial cell layer. 4-5 μM of free Ca^{2+} originating from the DMEM cell media was transported through the caco-2 monolayer and was detected after 19 h in the basolateral chamber. This corresponds to 14% of the concentration of free Ca^{2+} in the cell culture medium in the apical chamber. The corresponding basolateral to apical ratio of the calcium concentration measured over 19 hours is shown in Fig. 5d.

Conclusions

Our microfluidic device enabled monitoring the Ca^{2+} transport through a confluent intestinal cell monolayer over prolonged periods of time, while the integrated TEER measurements provided a continuous feedback on the quality of the cell monolayer. Confluent cell monolayers were reached after 5 day of cell seeding, as indicated by TEER. Fura-2 was used as an indicator of the free Ca^{2+} and a ratio imaging technique was employed to measure the transported free Ca^{2+}. Significant Ca^{2+} transport was observed 11 hours after applying the Ca^{2+} supplement-containing cell culture media in the apical chamber and we could check the barrier function of the epithelial cell layer. From these results, we envision wider applicability of our device for measuring the bio-availability and transport of several nutrients. Also we could perform real-time measurements at the cellular level, by incorporating a live-cell imaging system, which comprises a cell incubation system that is directly integrated with the ratio imaging setup. Such system could also be used to differentiate the paracellular and the transcellular transport of calcium.

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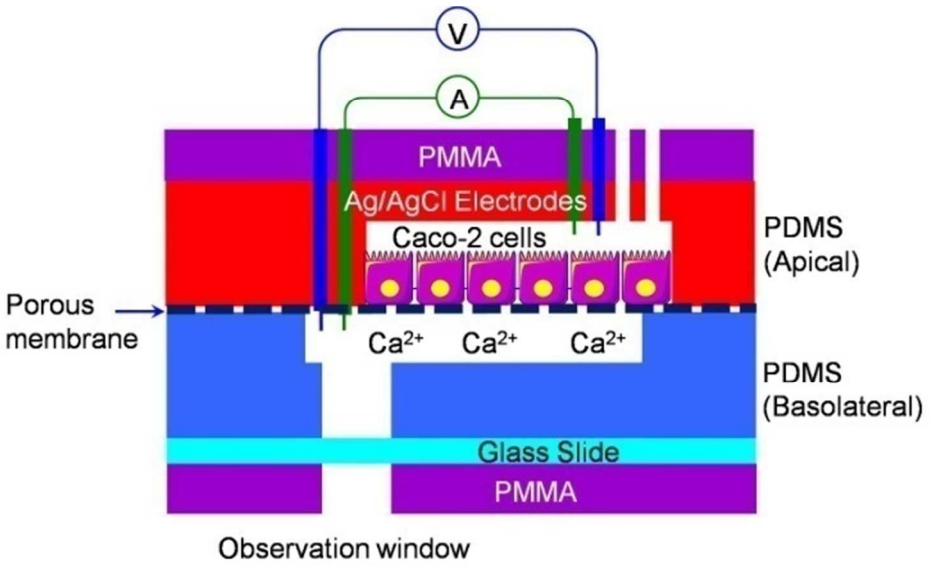
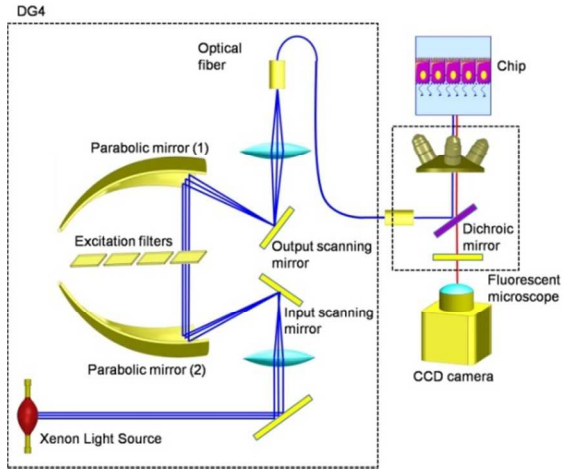
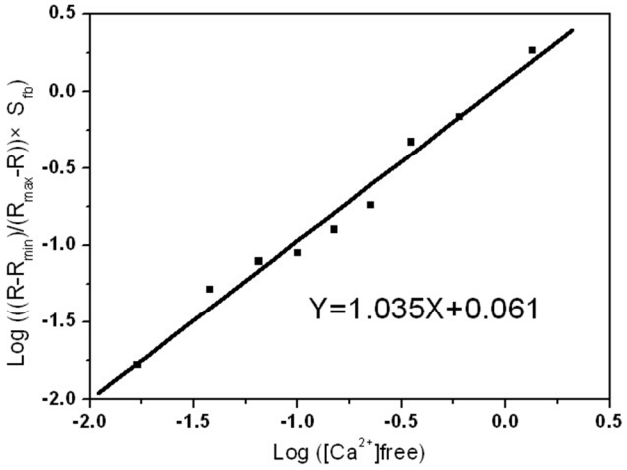


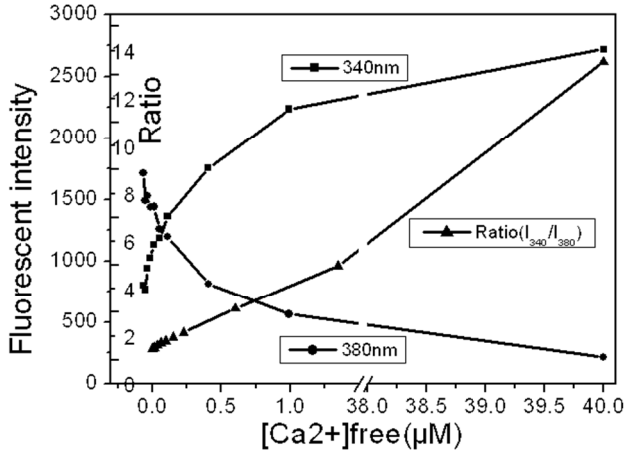
Figure 1: Cross-sectional schematic drawing of the microfluidicchip, showing the apical and basolateral PDMS chambers and the electrical connections (TEER electrodes). The PDMS structure is assembled in between two PMMA sheats, resulting in a tight leakage-free assembly.



(a)



(b)



(c)

Figure 2: (a) Schematic of the custom-built ratio imaging apparatus for measuring the free concentration of Ca^{2+} in the basolateral (lower) compartment of our microfluidic chip. (b) Calibration line of calcium buffer solution ranging from 0-40 μM which prepared from 50 mL of 10 mM K_2EGTA and 50 mL of 10 mM CaEGTA stock solution and using 10 μM fura-2 in the buffer. (c) Fluorescent intensity against the free calcium concentration within the range of 0-40 μM and the corresponding ratio (I_{340}/I_{380}).

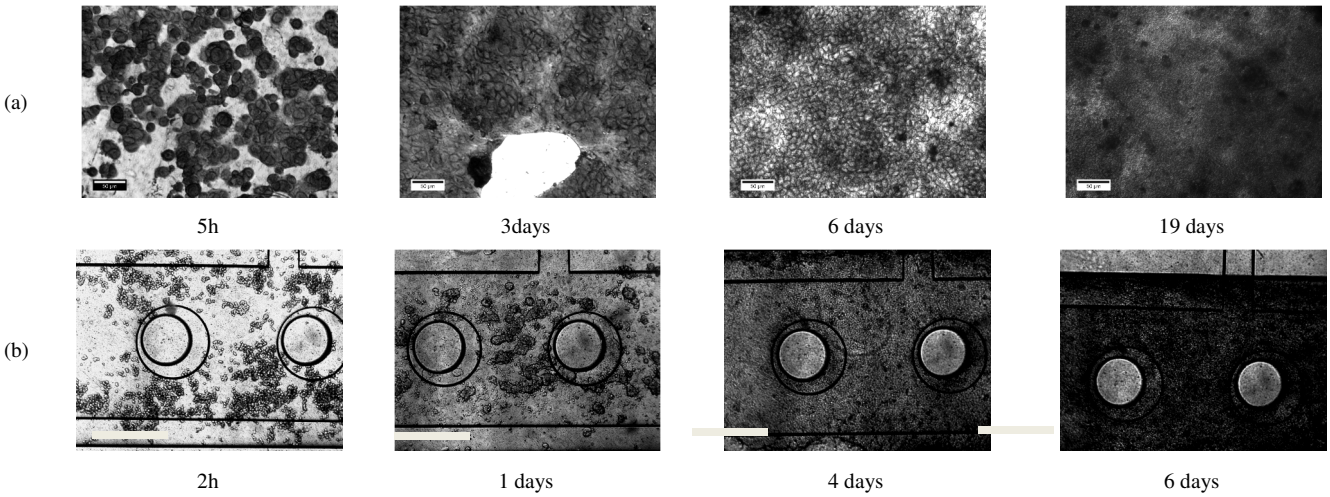
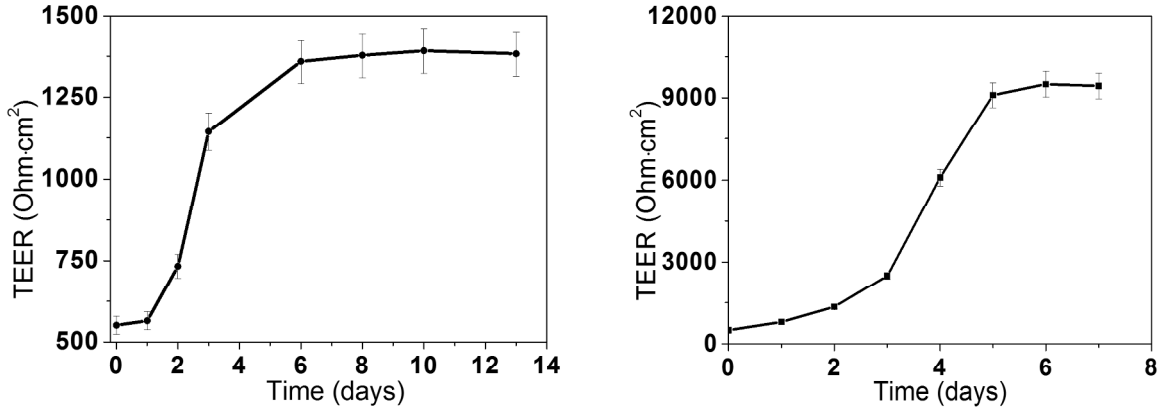
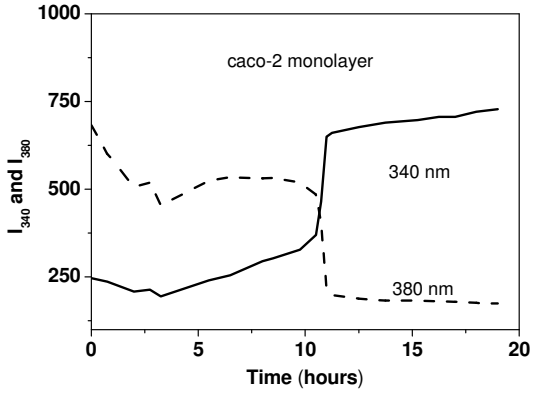


Figure 3: Optical microscope images of caco-2 cells forming a confluent layer over time in: (a) Transwell device and (b) our microfluidic chip. Scale bar is 50 μm. Posts (circles) in (b) are for membrane support.

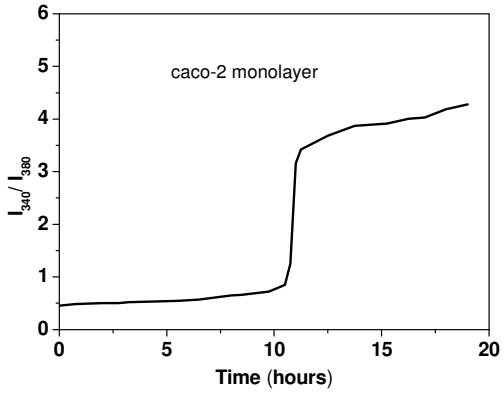


(a) (b)

Figure 4: Typical TEER values of a caco-2 cell monolayer cultured (a) in a Transwell device and (b) on our microfluidic chip. All experiments were performed three times.



(a)



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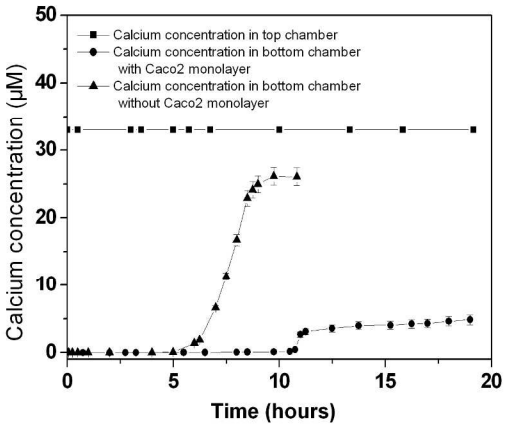
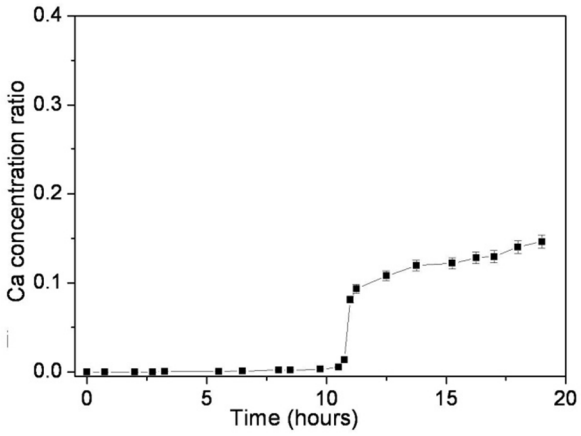


(c)



(d)

Figure 5: (a) Fluorescent intensities in the basolateral compartment of the microfluidic chip: I_{340} is increasing with time and I_{380} shows the opposite trend; (b) calculated ratio I_{340}/I_{380}; (c) calculated Ca^{2+} concentration from the data of (b) by using eq. (1) (circles); for comparison, the measurement of the concentration in the basolateral compartment of microfluidic chip is presented, when just using the porous membrane, but not the cultured cells (triangles). (d) The basolateral/apical ratio of the calcium concentration measured with a continuous supply of Ca at a concentration of 33μM.

