Chapter 10

Chemically Induced Cell Cycle Arrest in Perfusion Cell Culture

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Abstract

In contrast to most present methods, continuous imaging of live cells would require full automation in each processing step. As an integrated system that would meet all requirements does not exist, we have established a long-term scanning-perfusion platform that: (a) replaces old medium with fresh one, (b) bypasses physical contact with the cell culture during continuous cell growth, (c) provides uninterrupted photomicrography of single cells, and (d) secures near physiological conditions and sterility up to several weeks. The system was validated by synchronizing cells using serum starvation and butyrate-induced cell cycle arrest of HaCaT cells.

Key words Time-lapse imaging microscopy, Mammalian cell culture, Perfusion system, Cell cycle synchronization, Serum starvation, Sodium-butyrate synchronization

1 Introduction

Perfusion operations in cell culture have been widely used to improve the cell productivity of bioreactors. Perfusion bioreactors feed solutions and media constantly to reduce the waste of nutrients, remove by-products under relatively high flow rate without washing out cells from the bioreactor. Cells can be retained by filtration, sedimentation, or centrifugation. Filtration techniques require the replacement of filters or keeping filters free of clogging by tangential crossflow. Sedimentation has gained only limited commercial use, due to the adjustment to the specific settling requirements. Centrifugal devices face the difficulty of keeping them under commonly used sterile conditions required for cell cultures. Most of the perfusion systems pump the cells through the separation chamber and back to the bioreactor. Beside the difficulties of maintaining sterility, cells are damaged by shearing forces, oxygen depletion, declining productivity, and quality. Benchtop

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bioreactors have been used for fermentation to provide optimal growth conditions for different types of cell cultures [1]. Bioreactors in combination with innovative process technologies contributed to the advancement of antibody industry [2]. Perfusion systems improved the productivity achieved by high cell number in industrial, benchtop, and disposable bioreactors (*see* **Note 1**).

Perfusion chambers with live-cell imaging fulfill two important requirements by maintaining cells in healthy conditions and observing them under high resolution. Open chambers are easily accessed, approach equilibrium with the surrounding atmosphere, but exhibit restricted control. Closed chambers avoid the evaporation of medium, allow the control of temperature, pH and CO_2 concentration. The addition of fresh medium or drugs is favored in long-term experiments focusing on the design of microfluidic chips in 2D and 3D cell cultures. To obtain high optical quality the depth of the perfusion chamber is to be minimized. Large coverslip surface area is prone to leaks and physical damages. Unbalanced perfusion may cause hydrodynamic pulsation of liquid, cellular damages and produce pressure under the coverslip.

The continuous long-term operation of bioreactors could be exploited especially during time-lapse microscopy and image analysis of single cells grown in a cell population. Earlier studies have combined time-lapse fluorescence microscopy and automated image analysis to investigate the dynamic events at the single-cell level [3]. This was followed by the automated analysis of fluorescent foci in single cells [4]. Computer-automated platform combined time-lapse image analysis with a cell tracking software has assisted studies of early embryonal development and helped to improve embryo selection [5].

Time-lapse scanning (TLS) microscopy is an integral and critical branch of live-cell imaging that provides a wide spectrum of design during the microscopic observations of individual cells. Long-term scanning video-microscopy offers excellent optical conditions while allowing specimens to be maintained for varying amounts of time. The essence of LTS is that digital photos are taken every minute and the light microscopic observation of cells lasts during the whole period of examination. The digital pictures are converted to videos and/or analyzed along quantitative parameters. TLS allows the long-term observation of individual cells and cell cultures at population level.

Our computer-automated platform combines time-lapse image analysis that is tracking individual cells, with a perfusion system aided by a control system and software (Fig. 1). The perfusion subsystem is controlled and protected against contamination, mechanical manipulation and disturbance of growth and by avoiding any physical contact with the cell culture during the experiment. Perfusion contributes to the extended observation of specific effects that would be interrupted by the removal of tissue flasks from the incubator, and by the addition of fresh medium or chemicals. The in vitro

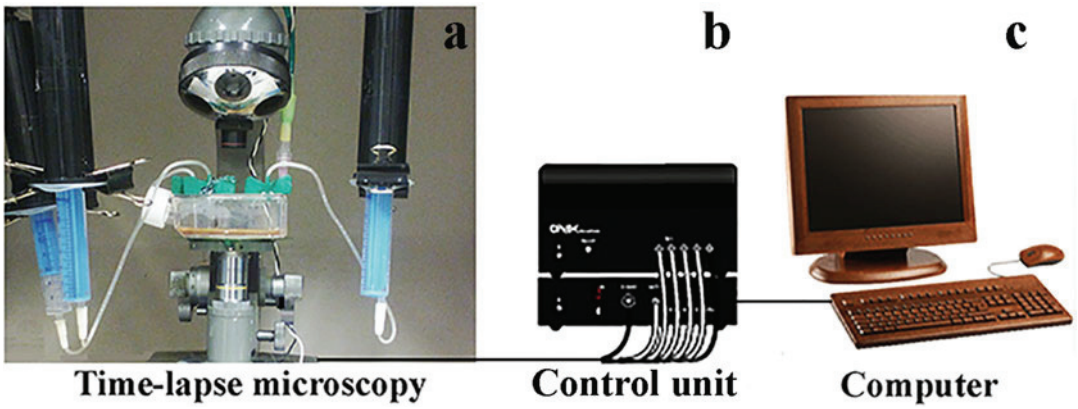

Fig. 1 Time-lapse scanning—perfusion platform. The system combines time-lapse microscopy with perfusion and control units. (**a**) Cell growth is taking place in the perfusion unit sitting in the carbon dioxide incubator. The perfusion unit serves the administration of fresh RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and addition of substances to the cell culture while the old medium is being removed. Parts of the perfusion unit are: (1) pneumatic cylinders, (2) sterile syringes, (3) tissue culture flask (T-flask), (4) charge coupled device (CCD camera), (5) inverse microscope. (**b**) Control panel responsible for the electronic steering and contains the main switch for the activation of the system and other switches responsible for the direction of four working cylinders and the vacuum. (**c**) Software

evolutionary perspective of the LTS-perfusion system (LTS-PS) lies in the observation of individual cells for several generations.

2 Materials

2.1 Disposables

1. T-25 flasks for cell culture.
2. 20 ml syringes.
3. 0.09 mm injection needles for outlet.
4. Butterfly infusion needles for inlet.

2.2 Media and Solutions

1. RPMI 1640 growth medium.
2. Fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS): 2.7 mM KCl, 4.3 mM sodium phosphate dibasic (Na_2HPO4), 1.8 mM potassium phosphate monobasic (KH_2PO_4), 137 mM NaCl, pH 7.2. Sterilize in an autoclave.
4. Sodium-butyrate solution for synchronization.

3 Methods

3.1 The TLS-PS System

The TLS-PS system consists of the time-lapse video microscopy (TLS), electric steering, and computer subsystems (Fig. 1).

The perfusion subsystem was designed to run the time-lapse imaging system (TLS) under physiological conditions and provide a simple and reliable means to test the effect of serum deprivation and sodium butyrate on individual cells without being in physical contact with the cell culture (*see* **Note 2**).

Time-lapse microscopy subsystem consists of:

1. *Inverse m\icroscope* sitting in CO_2 incubator (Fig. 1a).
2. High-sensitivity digital camera.
3. Illumination under minimized heat- and photo toxicity, operated in the near-infrared range (940 nm), with light emitting diodes synchronized by image-acquisition periods.
4. *Cell culture*. The growth of HaCaT cells in a 25 ml TL-flask starts at low (10–20%) confluency. Cell confluence is measured by the adaptation of the Image J program based on the occupation of the empty space by the cells in the visual field not in the entire growth chamber. The culture is placed under the inverse microscope and subjected to time-lapse videomicroscopy. Frames are recorded every minute and the video sequence converted to database form. The time of exposure is indicated in the filename of each frame. Exposures are converted to video films by speeding up the projection to 30 exposures/s. Proliferation of cells is measured by (a) the observation of cells undergoing cell division expressed on graphs as number of cells/frame and (b) as the surface area covered by cells (confluency) in square pixels [6–9].
5. *Control unit*. Electronic steering takes place through the control unit (Fig. 1b). This unit is located outside the CO_2 incubator and contains the activating contact stud, the activating buttons of the working chambers, and the vacuum/pressure switch. The control panel is working parallel with the relay-driven computer interface to provide full manual control during the experiment.
6. *Image acquisition computer system* (Fig. 1c). Open-source software Fiji is used for quantitative image analysis. Image sequences are converted to 8-bit grey scale. Resulting image stacks are deflickered to eliminate transient brightness changes. Background is homogenized by fast Fourier transformation and bandpass-filtering. Covered area (confluency) is determined by the dynamic thresholding of the image J progam of the National Institute of Healthy after background removal. This plugin is corrected for uneven illuminated background by using a rolling-ball algorithm. The number of dividing cells is determined by dynamic under/over thresholding and particle size analysis and circularity calculations of the segmented binary images frame by frame. Due to the large frame number providing fast results, operations are optimized for calculated speed.

3.2 Subsystems of TLS-PS

3.2.1 Perfusion Subsystem

The vacuum and the pneumatic pressure are measured by the vacuum gauge (VG) and manometer (MM), respectively. The vacuum is built up by the pneumatic pump and is directed toward the vacuum reservoir (VR). Leaky vacuum hoses and tubes could cause poor pump performance and in severe cases cylinder failures to function properly. To avoid such malfunction the vacuum reservoir was inserted to buffer and to protect the air vacuum pump that generates full vacuum within a minute after connecting it to the air line. The vacuum spreads from the vacuum reservoir through the three-way plastic valve (PV) to the distribution box that directs the low pressure either to the perfusion system or to the vacuum flask (VF). During the assembly the perfusion unit, including the vacuum flask is placed inside the laminar flow hood under sterile conditions. To secure sterility all instruments and materials placed under the hood are sprayed with 70% ethanol, the sterile air flow is turned on for at least 30 min to avoid the danger of setting on fire. The vacuum-side cleaner runs from the pump to the tissue culture (TC) and forces the debris into the vacuum flask. The overflow of the vacuum flask containing old medium, washing buffer, detached dividing or damaged cells can be disconnected from the system if necessary. The vacuum-side ends in the distribution box (DB) (Fig. 2a).

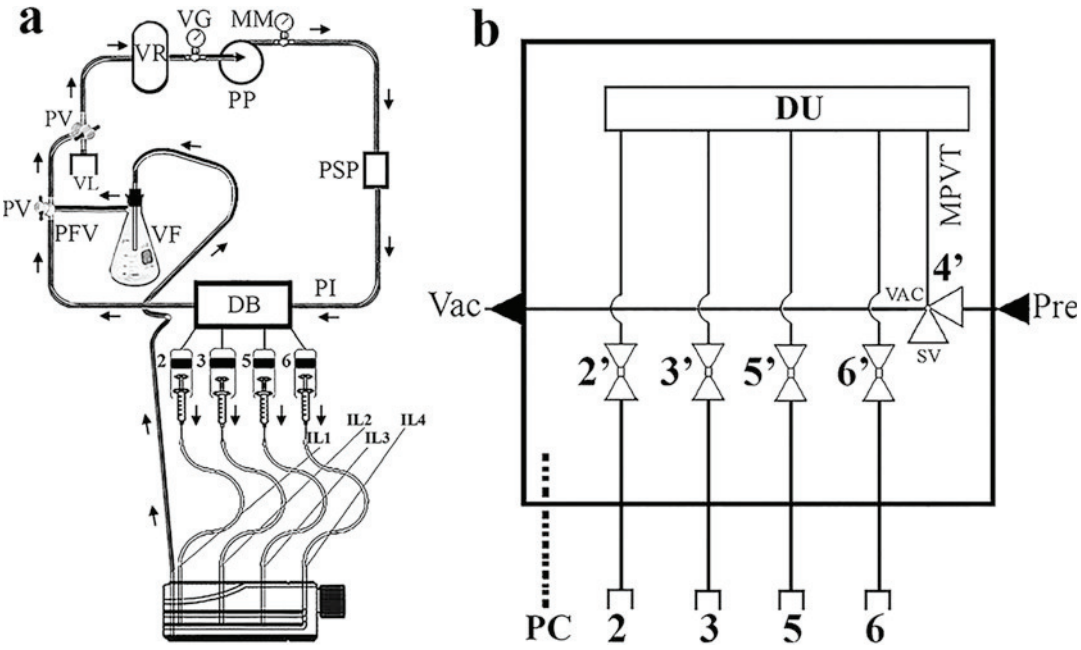

Fig. 2 Schematic representation of perfusion unit. (**a**) Parts of the perfusion circuits: *VR* vacuum reservoir, *VG* vacuum gauge, *PP* pneumatic pump, *MM* manometer, *PSP* pressure side port, *PI* pressure inlet, *DB* distribution box, *VF* vacuum flask, *PFV* perfusion vacuum, *PV* plastic valve, *VL* vacuum toward the laminar box. (**b**) Schematic view of the distribution box (DB) of perfusion system. *Vac* vacuum, *MPVT* major pressure-vacuum tube, *Pre* pressure, *DU* distributing unit, *PC* personal computer

3.2.2 Attachment of Liquid-Containing Syringes to the Perfusion Subsystem

All liquids to be added to the cell culture during the experiment are stored in syringes and incorporated in the perfusion system before starting the experiment. Each syringe is filled with the appropriate liquid under the sterile hood and two more ml-s of sterile air secure that the input tubing runs empty at the end of the injection steps. The piston is placed on the plunger and the syringe is fitted to the working chamber. By pressing the button belonging to one of the pistons of the control panel and at the same time pressing the vacuum knob, the plastic pads and the syringes are clamped to the pistons in standby position.

3.2.3 Vacuum-Side of Perfusion Unit

Safety considerations dictate the application of a pneumatic rather than a hydraulic perfusion unit. The hydraulic pressure could not only disrupt the plastic tubing system that is transporting the liquid, but the fluid could also cause short circuit.

The perfusion circuit is maintained by the pump that is compressing air at one end and generating vacuum at the other end. The vacuum-side of the system generates low pressure circuit through the inlet of the pump in the closed tubing system. The outlet-side of the pump, called the pressure circuit, works in compression mode.

The pressure-side of the perfusion unit is connected to the distribution box (Fig. 2a), starting at the outlet of the pump and containing a security pressure-side port. The function of the port is to prevent the sudden increase of pressure that can go up to 80–100 hPa during the installation of the system. The sudden pressure elevation may disrupt the tubing system at the connections. This is to be prevented at the pressure-side port by releasing the excess pressure. The circuit can be closed again and the normal working pressure restored.

The main function of the distribution box is to spread the pressure and vacuum evenly and in a regulated manner among the working chambers and to provide the uniform motion of the liquid contained in the syringes (lower part of Fig. 2a). The distribution box (DB) contains five electronic safety valves (2′, 3′, 5′, 6′, and 4′ in Fig. 2b). Four of them are connected to the working chambers (2, 3, 5, and 6 in Fig. 2b). The fifth three-way safety valve provides connection between the pressure and vacuum circuit branches, and is responsible for the electronic steering of the distribution box (valve 4′ in Fig. 2b). The principal function of this valve is to place the system under pressure or vacuum. Under electric current the valve introduces vacuum to the distribution box. The other four valves are turned off in the absence of electric signals. Upon activation the valves open toward the working chambers building up the vacuum/pressure in the cylinders.

3.3 Running the TLS-PS System

1. Activate the perfusion system by turning on the units in the following order:

- Pump.
- Interface electric power.
- Steering control panel.

2. After activating the system, let each working chamber work independently directed by its own valve.

3.3.1 Cell Growth in the TLS-Perfusion System

1. Perform all sterile operations under the laminar flow hood. Alcoholic disinfection at this point is not allowed due to the danger of inflammation while inserting the hot perfusion needles.
2. Heat the butterfly blood collection needle gradually to red-hot temperature and insert it into the T-flask (*see* **Note 3**).
3. Remove the first plug of the butterfly blood collection needle before the syringe is attached to it. This needle serves to introduce solutions to the flask.
4. Fix the needle to the flask with hot (200 °C) glue. The number of further needles inserted depends on the number of planned additions of solutions.
5. Add each solution through a separate sterile needle and syringe.
6. Cut the outlet needle blunt-ended, then heat, insert, and place near the bottom of the culture flask, then fix with hot glue (*see* **Note 4**).

3.3.2 Connecting the Cell Culture Flask to the TLS Unit

1. After the inserted needles cooled down to room temperature, but before starting the TLS experiment in the CO_2 incubator, spray and disinfect the outer surface of perfusion system with 70% ethanol.
2. Transfer the perfusion subsystem to the nearby CO_2 incubator and combine with the TLS subsystem. Place and fix the cells in the T flask supplied with the inlet and outlet needles, containing medium and cells to the object table of the inverted microscope sitting in the carbon dioxide incubator.
3. After fixing the flask to the object holder, focus the microscope to give sharp images of cells and start the TLS-PS experiment.

3.3.3 Running the TLS-PS Experiments

Replacement of the culture medium and other solutions during the TLS-perfusion experiments takes place in the closed sterile system without opening the carbon dioxide incubator. Carry out the experiments as follows:

1. Turn ON the TLS-perfusion system and the power unit by separate switches and by the “ON” button of the control panel.
2. To develop vacuum and pressure turn on the vacuum pump. Allow to develop proper vacuum pressure (45–50 hPa) and manometric overpressure (40 hPa) to run the perfusion system.

3. Make corrections by screw valves to run the system under optimal working conditions.
4. Add solution from the corresponding syringe to the culture flask by pressing the button of the control panel as long as needed. By pressing one of the 2′, 3′, 5′, or 6′ buttons the pressure increases in the corresponding 2, 3, 5, or 6 working camber and the pneumatic pressure forces the liquid out of the syringe into the T-flask (Fig. 2b).
5. By pressing simultaneously the VACUUM knob and one of the buttons (2′, 3′, 5′, 6′, Fig. 2b) of the control panel the opposite process takes place, namely the generated vacuum pulls back the liquid to the syringe. This operation is used when the suspended cells that were not attached to the monolayer were withdrawn without being discarded. The removal of the excess material from the T-flask takes place through the unit with its own switches directing the liquid through the outlet needle from the cell culture to the vacuum flask (*see* **Note 5**).

3.4 Cell Cycle Synchronization

To prove the applicability and reproducibility as well as to demonstrate the advantage of the LTS-perfusion system, two validation studies have been performed by synchronizing HaCaT cells. With respect to the most frequently used synchronization methods, bulk synchronization by physical separation, flow cytometry, cytofluorometric purification, and dielectrophoresis was not applicable to the LTS-perfusion system. The synchronization of cells by the LTS-perfusion system was limited to chemical blockade. Metabolic reactions of cells are most often blocked by nutritional deprivation and inhibition of DNA synthesis. Of the known classes of synchronization [10], nutrient deprivation and butyrate treatment belonging to the classes of chemical synchronization [11, 12] were used to test the TLS-PS system (*see* **Note 6**).

3.4.1 Synchronization by Serum Starvation

Nutritional serum starvation has been widely used for synchronization by arresting cells in the G_0/G_1 phase of the cell cycle, but it often reduced cell survival and increased DNA fragmentation [13–15]. In transformed cells, the proliferation turned out to be relatively independent of serum and growth factors, thus neoplastic cells do not necessarily cease proliferation immediately upon serum deprivation and subjection to stringent G_0 arrest [16]. HaCaT-transformed cells served to prove that serum deprivation could be used for their synchronization. Follow the protocol given below:

1. In the control experiment grow HaCaT cells and visualize growth by time-lapse microphotography in RPMI 1640 medium containing 10% FBS using the perfusion attachment as described.
2. After 24 h growth add fresh medium containing 10% FBS by the perfusion unit to maintain the logarithmic growth of cells.

Cell growth (Fig. 3a) and increase in confluency (Fig. 3b) show the continuous growth and 100% confluency attained after 66 h incubation.

3. Visualize cell growth by time-lapse microscopy with dividing cells being in the foreground, and monolayer cells in the background (Fig. 3c). Black numbers at the bottom of each panel

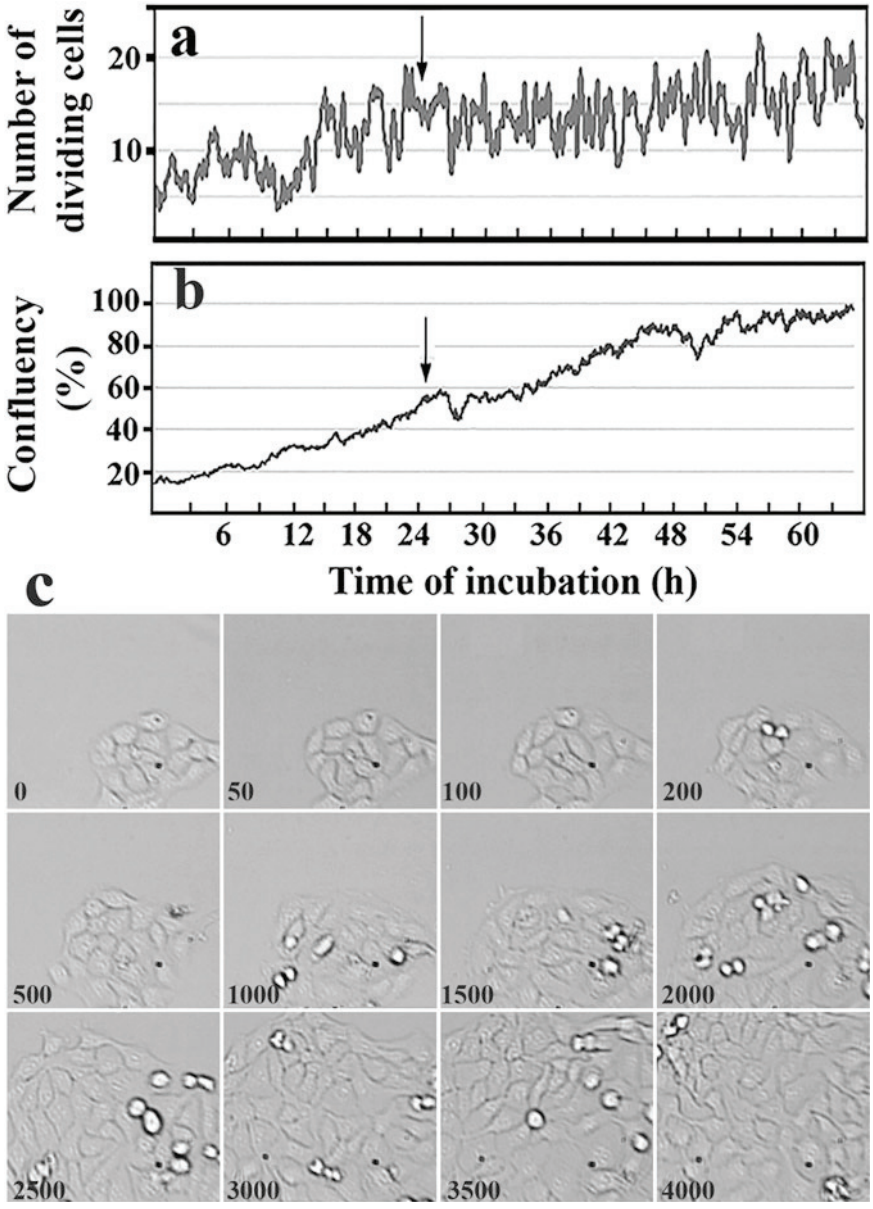

Fig. 3 The growth of control cells started in 10% serum containing RPMI medium. After 24 h additional 10% FBS was added and grown to 66 h. (**a**) Number of dividing cells and (**b**) increase in confluency were measured by time-lapse scanning microscopy. (**c**) Cell growth of a group of cells was revealed in the visual field of the microscope minute-by-minute

show the time in minutes passed from the recording. Images are to be brightness and contrast balanced, freed of interlacing and flickering, and gradient equalized, then subjected to histogram equation [6].

4. Start serum deprivation in RPMI 1640 medium in the absence of FBS causing a stagnation, then a drop, followed by a short increase in the mitotic cell number (Fig. 4a). Serum starvation increases temporarily the confluency as a consequence of expansion of cells binding to the attachment surface representing

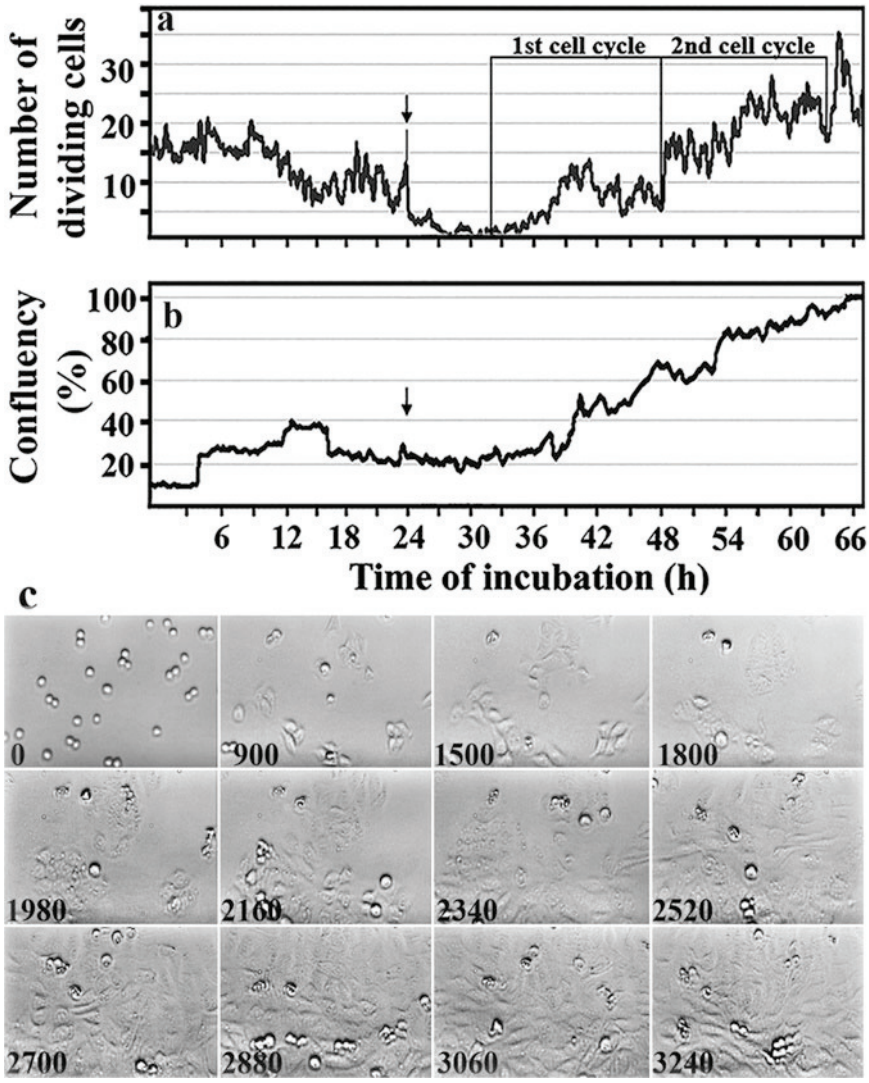

Fig. 4 Growth of HaCaT cells in serum-free RPMI medium. After 24 h serum deprivation was terminated and growth was continued in the presence of 10% FBS. (**a**) Number of dividing cells and (**b**) confluency changes were measured by time-lapse scanning microscopy. *Black arrow* indicates the time of serum addition. (**c**) Cell growth of a group of cells was revealed in the visual field of the microscope minute-by-minute

larger cell surface than rounded up mitotic cells that are not attached to the surface (Fig. 4b). The temporary increase of mitotic cell number after serum deprivation could be explained by the observation of others that HaCaT cell cultures could extend their proliferation in serum-free medium in contrast to normal human keratinocytes whose growth in vitro requires a feeder layer and/or the supplementation with hormones and growth factors [17]. The extension of the proliferation of HaCaT cells can be accounted for by the delayed attachment of cells to the monolayer that could be observed even after 10 h of incubation (Fig. 4c). In conformity with the autocrine activation of the epidermal growth factor (EGF) receptor on keratinocytes, extended proliferation has been recognized as an important growth regulatory mechanism involved in epithelial homeostasis [18]. These observations underline the importance of using cell cultures rather than individual cells in cell cycle studies.

5. After 24 h serum starvation, remove old medium and add fresh medium containing 10% FBS to the cell culture through the perfusion unit. The cell cycle from the lowest mitotic activity (32 h 50 min) to the next mitotic minimum (48 h 05 min) lasted for 15 h 15 min, corresponding to the length of the first synchronized cell cycle. In the next synchronized cell cycle with an initial logarithmic growth and mitotic drop after 63 h 25 min incubation, the length of the cell cycle corresponds to 15 h 20 min (Fig. 4a).

3.4.2 Synchronization with Butyrate

DNA synthesis can be blocked by butyrate as an inhibitor of DNA replication [19–21]. The major population of butyrate-treated cells is arrested at the very early G_1 phase, possibly immediately after mitosis, with some cells resting in G_0 [12].

1. Grow HaCaT cells in RPMI 1640 medium, 10% FBS and 10 mM sodium butyrate for 24 h.
2. Wash cells with PBS and replace by regular RPMI 1640 containing 10% FBS. After the removal of the cell cycle inhibitor fast growth and differentiation takes place that is manifested as expansion and motility changes of separate groups of morphological changes of clumping cells as well as individual round cells being in metaphase.
3. Take micrographs every minute during the differentiation process and collect data for image analysis showing changes in the cell number of dividing cells and confluency in the cell culture (Fig. 5). The cells treated with butyrate retain their initial growth characteristics, without significant changes in growth dynamics (Fig. 5a) and confluency increase (Fig. 5b) relative to the growth without butyrate. In the presence of butyrate the confluency level reach its maximum at ~12 h, followed by a

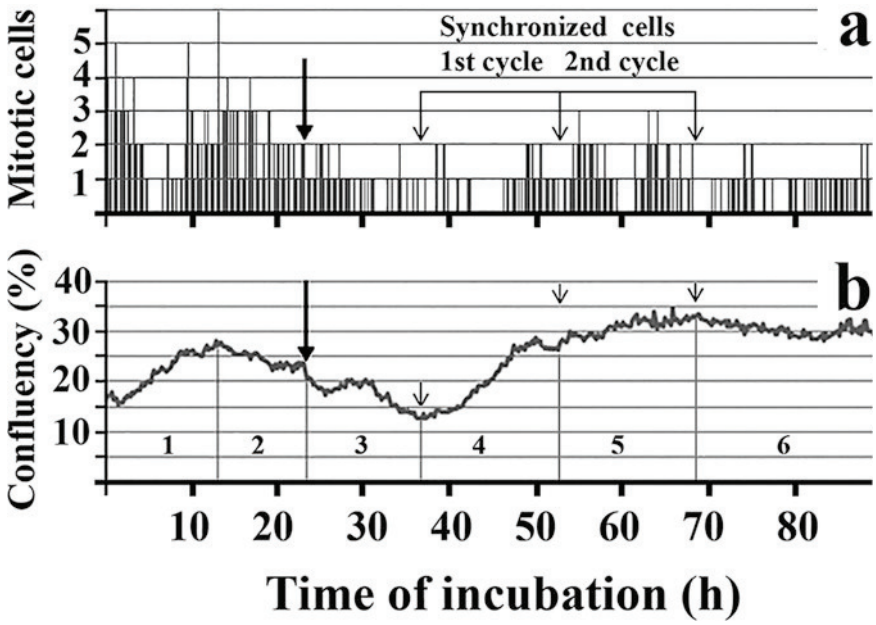

Fig. 5 Synchronization of HaCaT cells with sodium butyrate. HaCaT cells were grown in RPMI 1640 medium containing 10 % FBS and 10 mM sodium butyrate for 24 h. Then the medium was removed by the perfusion system and the cells were washed with PBS and fresh RPMI 1640 medium containing 10 % PBS culture was added. (**a**) Number of mitotic cells in the TLS-perfusion experiment. *Large black arrow* indicates the time of butyrate removal. *Small arrows* indicate the boundaries of the two synchronized cell cycles. (**b**) Confluency changes during synchronization with butyrate. Subphases of cell growth: (1) Residual growth in the presence of butyrate. (2) Reduction of mitotic cells and confluency. (3) Further reduction of mitotic cells and confluency changes after removal of butyrate. (4) First cell cycle of synchronized cells. (5) Second cell cycle after synchronization. (6) Loss of synchrony

gradual decrease, before the mitotic cell number culminates at ~14 h. This discrepancy could be explained by those dividing cells that detached from the monolayer, rounded up and caused a temporary confluency decrease. Further reduction in mitotic cell number and confluency points to apoptotic cell death. Butyrate treatment causes the clustering of apoptotic cells that could be distinguished and subtracted from the number of round metaphase cells (not shown).

4. After the removal of butyrate and replacement of medium, a significant reduction of confluency level became visible by TLS microscopy as a result of cellular shrinkage. The maximal value of shrinkage and minimal value of mitosis become detectable 38.3 h (2300 min) after the removal of butyrate. The regeneration of synchronized population could be divided into two consecutive cell cycles, the first one lasting for 15 h 34 min and the second cell cycle for 15 h 50 min indicated by the small arrows in Fig. 5a, b. These values of cell cycle length are somewhat higher, nevertheless comparable to those obtained by the synchronization of serum deprivation (15 h 15 min and 15 h 20 min).

One of the differences relative to the synchronization with serum deprivation was that 96 h after butyrate treatment the control population did not reach its initial confluency also pointing to the damaging effect of butyrate on the proliferative activity of cells and its toxicity causing death of some of the cells in the HaCaT population. In spite of these differences one can conclude that synchronization with serum deprivation and butyrate treatment can be performed by the TLS-perfusion system. The reproducibility is indicated by two consecutive cell cycles with the two different methods used for synchronization resulting in measurements with similar lengths of cell cycles:

Serum starvation:	15 h 15 min and 15 h 20 min (Fig. 4a)
Butyrate treatment:	15 h 34 min and 15 h 50 min (Fig. 5a)

The confluency during the second cell cycle was somewhat higher (Fig. 4b and Fig. 5b, respectively), while the cell cycle length was moderately extended in the second cell cycle, reflecting that confluency did not impact severely growth dynamics (*see* **Note** 7).

4 Notes

1. A wide range of commercially available perfusion and imaging systems are available, offering designs such as glass bottom dishes, multiwell chambers on microscope slides, heating stages with perfusion adapters, specialized chambers with conductive coatings for tight control of temperature. Typical packages include: chamber, temperature control, stage adapter, and accessories but no carbon dioxide incubator. The long-term use of these systems is limited due to the small volume that is another limiting factor to get a large and homogeneous monolayer. Cells in our system are growing in a 25 ml T-flask providing sufficient growth capacity against temporary fluctuations other systems are likely to be subjected.
2. Originally we started to add solutions manually to the cell culture through butterfly blood collection needles. Incandescent hot needles were used to penetrate the T-flask and fix them with hot (200 °C) glue. The inlet needle was directed toward and touching the wall of the T-flask to avoid the turbulence of the cell culture medium. The end of the outlet needle was cut blunt before its insertion, then placed as close to the bottom of the flask as possible. The T-flask was slightly tilted (3%) toward the outlet needle to secure complete removal of the old medium. Initially, the culture medium was removed manually through the outlet needle with another needle and syringe. Although the T-flask was fixed to the stage of the inverted

microscope, it occasionally changed slightly its position during the manipulation. Due to these technical hindrances, it was mandatory to improve and automate the perfusion system. As a result we have developed a pneumatic-driven force transduction method, to keep the system as simple and fail-safe as possible. In this setup during the experiment the working chambers push or pull the plunger of the syringe without touching the T-flask or the syringe.

3. Use another incandescent hot needle of 3 mm diameter to penetrate the T-flask before inserting the inlet and outlet needles. This way, the molten plastic in the inlet and outlet needles will not caulk the flow of solutions.
4. In the course of working, you could get some non-sterile tools in the laminar flow hood (e.g., the glue gun for fixing the needles)—be very careful not to contaminate the cell culture while using them.
5. Cleaning of the perfusion system: Parts of the perfusion system were autoclaved then connected under the laminar flow hood and rinsed with 70% ethanol. The ethanol was removed by pumping and drying the passages with sterile-filtered compressed air. The vacuum flask containing the discarded solution was removed and sterilized separately.
6. HaCaT cell synchronization in the perfusion system is based on the visibility of dividing cells. The number of dividing cells corresponded to the number of mitotic cells counted every minute by time-lapse photomicroscopy. Before division detached cells were rounded up and after division the two daughter cells separated and reattached to the monolayer. Detached cells through a thin plasma bridge did not lose contact with the monolayer [7]. The detachment of mitotic cells and their division was visualized by time-lapse photomicroscopy that turned out to be a reliable measure to follow synchronization without necessitating markers for synchronized and non-synchronized cells.
7. We confirm the existence of a significant lag period, before butyrate-induced G_1-synchronized cells entered S phase in agreement with the observation of others [12]. The length of the two consecutive cell cycles after serum starvation was 15 h 15 min and 15 h 20 min *versus* 15 h 34 min and 15 h 50 min measured after synchronization by butyrate treatment. Due to the undisturbed growth conditions in the TLS-perfusion system, the length of the cell cycle of HaCaT cells was significantly shorter and uniform relative to the consecutive cell cycles measured in single cells by the TLS system without perfusion [22]. TLS visualizes not only the cell growth of a group of cells in the visual field of the microscope, but also the fate of

individual cells can be followed minute-by-minute. Synchronization did not measurably affect growth. 100% confluency in control population was attained after 66 h. Similarly, after serum starvation 100% cell confluence was obtained after 66 h growth and after butyrate treatment at around 68 h.

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