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A whole-cell biosensor as *in vitro* alternative to skin irritation tests

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ABSTRACT

This study presents the time-resolved detection of chemically induced stress upon intracellular signaling cascades by using genetically modified sensor cells based on the human keratinocyte cell line HaCaT. The cells were stably transfected with a HSP72-GFP reporter gene construct to create an optical sensor cell line expressing a stress-inducible reporter protein. The time- and dose-dependent performance of the sensor cells is demonstrated and discussed in comparison to a label-free impedimetric monitoring approach (electric cell-substrate impedance sensing, ECIS). Moreover, a microfluidic platform was established based on μSlides[0.4]Luer to allow for a convenient, sterile and incubator-independent time-lapse microscopic observation of the sensor cells. Cell growth was successfully achieved in this microfluidic setup and the cellular response to a cytotoxic substance could be followed in real-time and in a non-invasive, sensitive manner.

This study paves the way for the development of micro-total analysis systems that combine optical and impedimetric readouts to enable an overall quantitative characterization of changes in cell metabolism and morphology as a response to toxin exposure. By recording multiple parameters, a detailed discrimination between competing stress- or growth-related mechanisms is possible, thereby presenting an entirely new *in vitro* alternative to skin irritation tests.

1. Introduction

Many chemicals can induce irritant contact dermatitis. Several irritation models ranging from single-cell assays, epidermal equivalents, skin equivalents to excised skin have been described (Netzlaff et al., 2005; Schafer-Korting et al., 2008). Keratinocytes, the predominating cell type of the human skin, are the first to encounter chemicals that come into contact with the skin; they are involved in the immune reaction, for example, by producing cytokines. Therefore, keratinocytes represent a valuable model cell type to screen for dermatological effects in response to skin-damaging agents. However, this does not mean that all dermatological effects of the human skin can be studied within this particular cell culture model, because the three-dimensional tissue architecture of the *in vivo* environment and some of its unique transport pathways, *e.g.* penetration of the *stratum corneum*, are missing. Nevertheless, we need to establish standard operating procedures for future *in vitro* assays whose relevance and reliability should be based on internationally recognized protocols.

Equally important to the type of model used to investigate irritancy is the type of biomarker used as readout parameter. Currently those approaches are in focus that aims to investigate the early irritation response, preferably via real-time monitoring before visible cell damage occurs, to provide an alternative to conventional endpoint measurements (Gibbs, 2009). Among these techniques *in vitro* cell culture in microfluidic devices has attracted considerable interest and has led to a substantial impact on cell biology research (Sung and Shuler, 2010; Yeo et al., 2011).

Different microfluidic platforms have been developed for on-chip cell culture (Zhang et al., 2009), allowing quantitative readouts of cell-based assays to study cellular responses to chemical gradients (Hung et al., 2005) or cell/reagent manipulation (Wang et al., 2008) even for incubator-independent conditions (Petronis et al., 2006).

Using genetically modified cells that carry reporter genes under the control of stress-sensitive promoters provide functional information about the impact of cytotoxic reagents on cell physiology (El-Ali et al., 2006). The use of reporter genes, including their individual merits and limitations, has been extensively reviewed (Ghim et al., 2010; Palmer et al., 2011).

It has been reported repeatedly that inducible heat shock protein (HSP) genes are up-regulated in mammalian cells by very different forms of cellular stress via binding of heat shock factor to the heat shock element (Morimoto, 1998). This strategy of cell

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survival is highly conserved in almost every mammalian cell type and has been successfully used in reporter assays (Ait-Aissa et al., 2000; Migita et al., 2010).

With respect to the detection of skin irritation and the use of keratinocytes it has been described that this cell type shows a significant basal HSP72 expression even without any acute cell stress involved. The HSP72 seems to be "superinduced" by underlying continuous environmental stress factors (Jonak et al., 2006). HSP27 has also been reported to be a key player in apoptosis and differentiation (Sur et al., 2007) and it has been suggested to be a useful sensitive marker for skin irritation (Boxman et al., 2002).

Among different irritation models the recently established KeratinoSens seems to be the most promising tool in toxicology to distinguish skin sensitizers from non-sensitizers. The sensor cell holds a luciferase reporter gene under control of a single copy of the antioxidant response element of the human AKR1C2 gene stably inserted into HaCaT keratinocytes (Emter et al., 2010). The major and crucial drawback of this system is the endpoint measurement due to the use of a luciferase reporter.

Besides monitoring cell stress by means of reporter gene expression, several label-free approaches have been described, among which *Electric Cell-Substrate Impedance Sensing* (ECIS) is the most versatile and established sensor device that uses low amplitude AC signals to measure the electrical impedance of small gold-film electrodes that are deposited on the bottom of the culture dish (Giaever and Keese, 1984). When cells attach and spread on the electrode surface, the impedance increases accordingly as the dielectric cell bodies force the current to flow around the cells. Thus, ECIS reports very sensitively on changes in cell shape and electrode coverage. As the three-dimensional cell shape responds to chemical, biological or physical stimuli, ECIS has become an enormously versatile monitoring device for adherent cells that works label-free and non-invasively with a time resolution down to seconds (Arndt et al., 2004).

In the present study we describe the time-resolved detection of chemically induced stress upon intracellular signaling cascades by using genetically modified sensor cells based on the human keratinocyte cell line HaCaT. The cells were stably transfected with a HSP72-GFP reporter plasmid to create an optical sensor cell line for prescreening applications in cosmetic and pharmaceutical industry. The time- and dose-dependent performance of the sensor cells is demonstrated and will be compared to real-time monitoring using electric cell-substrate impedance sensing (ECIS) measurements as well as endpoint measurements via MTT. Our studies will pave the way to establish combined optical and impedimetric readouts for skin irritation tests.

2. Material and methods

2.1. Cell culture

HaCaT cells (Boukamp et al., 1988) were grown in DMEM Low Glucose medium (Biowest) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 μg/ml) in a humidified atmosphere/cell culture incubator with 5% CO_2 at 37 °C. With the exception of cytotoxicity assay (MTT) cells were deprived of serum (1% FBS) for at least 24 h prior to treatment with $CdCl_2$ or heat shock (43 °C). For time-lapse microscopy of cells on μSlidesI^{0,4}Luer (Ibidi) DMEM Low Glucose was replaced by CO_2-independent medium.

2.2. Cytotoxicity testing

Cell viability was essentially determined using the MTT assay as described (Mosmann, 1983). 15,000 cells were seeded in each well of a 96-well plate. After 24 h the cells were treated with varying concentrations of $CdCl_2$ (0–1 mM) for 24 h in medium containing either 1% or 10% FBS. After the incubation time the assay was performed as described previously, but dissolving the blue crystals was achieved using DMSO and Soerensen's buffer. Cytotoxicity (in percent) was expressed by dividing the absorbance detected for treated cells by that for untreated controls.

2.3. RNA extraction and two step quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from HaCaT cells using peqGOLD TriFast (Peqlap) according to the manufacturer's protocol. One microgram of total RNA was reversely transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) applying oligo(dT) primers following the standard protocol. Resulting cDNAs were diluted to a total volume of 100 μl with water (PCR grade). The reaction mixture for quantitative RT-PCR consisted of qPCR GreenMaster (Jena Bioscience), cDNA and 0.2 μM of the specified primers (MWG operon). The protocol included an initial denaturation step at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Data obtained on a Corbett Rotor-Gene 6000 were analyzed with the delta-Cq quantification model (Hellemans et al., 2007) using GAPDH as reference gene. Primer sequences for quantitative RT-PCR were: HSPA1A fwd 5′-ACCACTTCGTGGAGGAGTTCAAGA-3′ and rev 5′-ACGTGTAGAAGTCGATGCCCTCAA-3′; HSPB1 fwd 5′-AACGAGATCACCATCCCAGTCACCTT-3′ and rev 5′-TAAGGCTTTACTTGGCGGCAGTCT-3′; GAPDH fwd 5′-TTCGACAGTCAGCCGCATCTTCTT-3′ and rev 5′-GCCCAATACGACCAAATCCGTTGA-3′. All RT-PCR expression values were determined at least three times from three independent experiments. Data was analyzed by the Student's *t*-test.

2.4. Engineering the sensor cell line

The DNA segment containing the region from nt −165 to +91 of the *HSPA1A* gene was amplified from the human genome by PCR using 5′-AAAAACTCGAGCACTCTGGCCTCTGATTGGT-3′ and 5′-AAAAACCCGGGACAG GTTCGCTCTGGGAAG-3′ as primers. The PCR amplicon was ligated into pJET1.2/blunt. After amplification the plasmid was digested with the restriction enzymes *XhoI* and *XmaI*. The fragment containing the functional promoter region of *HSPA1A* (Morgan et al., 1987) was then subcloned into the *XhoI* and *XmaI* sites of pAcGFP1-1 (Clontech) to create pHSP72$_p$-AcGFP. Stable transfection of the cells was achieved with TurboFect (Thermo Fisher Scientific) according to the manufacturer's protocol and subsequent selection in medium containing 1 mg/ml G418. Resistant cells were harvested and initially screened by fluorescence-activated cell sorting (FACSAria IIU, Becton Dickinson) to eliminate cells that had a constitutively high GFP expression without induction of the functional promoter (negative screening). Cells demonstrating low GFP expression were again maintained under normal culture conditions. Cells were cultured for 2 h at 43 °C in an incubator followed by 12–16 h recovery at 37 °C. Only cells responding to heat shock with a high level of GFP expression were collected for single clone selection (positive screening). Cells were diluted to 30 cells/ml and seeded into 6-well tissue culture plates using culture medium supplemented with 0.5 mg/ml G418. Well-separated, G418-resistant single colonies from each dish were harvested using trypsin-impregnated filter paper (5 mm diameter) and placed in 24-well plates with selective medium including 0.5 mg/ml G418. Inducibility of single cell lines was again checked by microscopy and FACS to select a cell line showing the strongest response to heat shock (2 h, 43 °C) and $CdCl_2$ (6 h, 25 μM).

2.5. Quantitative fluorescence microscopy

Fluorescence microscopy was used for the quantitative assessment of cellular expression levels in an incubation time- and dose-dependent manner. The sensor cell line was seeded on coverslips at a density of 50,000 cells/cm^2 in 24-well culture plates. Following stress induction with $CdCl_2$ the cells were kept under normal culture conditions up to 18 h. GFP expression levels were observed using an Axio Imager M2 (Zeiss). Cell culture images were digitally recorded using an AxioCam MRm camera attached to the microscope using the filter set 38 HE and a 20× objective (Zeiss). Fluorescence images showing a confluent cell layer were analyzed, corrected for medium and device fluorescence, and compared to an untreated control to obtain relative fluorescence values.

2.6. Impedance-based monitoring of $CdCl_2$ cytotoxicity (ECIS)

The cellular response of genetically-engineered HaCaT cells to increasing concentrations of $CdCl_2$ was analyzed by time-resolved ECIS recordings. Measurements were performed using commercial 8-well electrode arrays with gold-film electrodes deposited on the bottom of each well (Applied BioPhysics Inc.; type 8w1e). Each well contains a circular *working electrode* with 250 μm diameter and a coplanar *counter electrode*. The surface area of the *counter electrode* is approximately 1000 fold larger and it is also covered with the cells under study that form a continuous monolayer. Due to the difference in surface area between *working electrode* and *counter electrode* and the fact that the impedance scales with the inverse of the area the total impedance of the system is dominated by the impedance of the cell-covered *working electrode*. Counter electrode, bulk electrolyte and wiring do not contribute significantly to the overall impedance readout. This allows for a direct correlation between the impedance measurements and microscopic observations of the cells on the small *working electrode*. Once the gold-film electrodes are immersed in cell culture medium, a monomolecular film of the amino acid L-cysteine forms at the electrode surface in a self-assembly process within minutes. This cysteine layer stabilizes the interface impedance of the electrodes and makes gold-film electrodes hydrophilic and very cytocompatible as cysteine is in its zwitter ionic form at physiological pH. The gold-films are thin enough to allow for routine inspection of the cells on the surface by regular phase contrast microscopy.

The 8-well electrode array was placed in a humidified cell culture incubator at 37 °C with 5% CO_2. Electrodes were interfaced to the electronic equipment located outside the incubator by an electrode holder (Applied BioPhysics Inc.). A relay allowed switching between the different electrodes. Relay and impedance analyzer were connected to an ordinary PC. Further experimental details are described elsewhere (Stolwijk et al., 2011). To acquire ECIS data, the continuous wave impedance analyzer (Solartron Instruments, SI-1260) applied a non-invasive AC voltage of 70 mV amplitude (rms) to the electrodes. This amplitude provided a sufficiently high signal-to-noise ratio for the measured impedance without leaving the linear current–voltage regime (Supplementary material). Moreover, the signal generator was programmed to clamp the DC potential difference between the two electrodes to 0 mV. Measurements were performed at 61 designated frequencies (1 Hz–1 MHz) equally spaced on a logarithmic scale. The associated current was measured and provided the impedance of the system as a function of frequency.

When cells adhere and spread on the electrode surface, they behave essentially like insulating particles forcing the current to flow around the cell bodies as long as the AC frequency, which is used for the measurement, is below a certain threshold value. Under these 'low frequency conditions' the AC current probes the extracellular spaces around the cells (paracellular current pathway). Thus, the measured impedance reports sensitively on changes in cell shape as cell shape determines the dimensions of these extracellular current pathways underneath and between cells. The threshold frequency mainly depends on electrode size and cell type and it is on the order of 10 kHz for the experimental conditions applied here. Above this threshold frequency the AC current can capacitively couple through the cell membranes and get across the cell layer on transcellular pathways. Under these conditions the measured impedance reports most specifically on coverage of the working electrode with intact cells as only intact cells impede transcellular current flow. If the cells on the electrode round up or if they lose their membrane integrity as a result of a toxic response, dielectric coverage of the electrode decreases and the impedance decreases accordingly. Thus, the time course of the impedance, recorded above the threshold frequency, reports on electrode coverage with intact cells and is, thus, well suited to serve as a toxicity indicator. For the electrode sizes used here we selected a sampling frequency of 40 kHz to monitor the cytotoxic response of the cells under study. The impedance magnitude $|Z|$ at any time of the measurement was normalized to the last value of $|Z|$ recorded before $CdCl_2$ addition.

To monitor $CdCl_2$ cytotoxicity the sensor cells were seeded into the 8-well electrode array (360,000 cells/ml) and cultivated overnight in a humidified cell culture incubator followed by serum starvation for 24 h. After recording baseline impedance data of the untreated cell layer for about 1 h low serum medium (1% FBS) supplemented with 0–100 μM $CdCl_2$ was applied to the cells for 6 h while continuously monitoring the impedance. Thereafter, the $CdCl_2$ containing medium was exchanged by cadmium-free cell culture medium (10% FBS) and impedance monitoring was continued for another 18 h.

2.7. Cell culture in μSlides

To allow for a convenient, sterile, incubator-independent time-lapse observation of a cytotoxic response, a perfusion setup was established based on μSlides I^{0,4} Luer (Ibidi) as culture dishes. The setup consisted of a Thermal-Clear™ transparent heater equipped with a Heaterstat™ sensorless controller (Minco), a spacer to allow optimal temperature distribution and a chip clamp to enable precise positioning on the motorized microscope stage. A perfusion setup based on two neMESYS Syringe pumps (Cetoni) was used either to apply medium supplemented with $CdCl_2$ (0–50 μM) or to provide cells with fresh medium using a maximum flow rate of 10 μl/min. Optimal temperature distribution within the μSlide channel was checked prior to flow experiments by sidewise insertion of a thermal element.

Sensor cells were seeded into the μSlide with a density of 400,000 cells/ml and cultivated under static conditions overnight to allow for cell adhesion followed by serum starvation for 24 h. Thereafter, the μSlide I^{0,4} Luer was connected to the perfusion setup applying CO_2-independent medium supplemented with 0–50 μM $CdCl_2$ for varying time intervals. To allow cells to recover from cadmium exposure and providing optimal conditions for GFP expression the incubation was continued with regular cell culture medium. Phase contrast and fluorescence images were taken every 30 min and analyzed as described above.

3. Results and discussion

3.1. Comparison of biomarkers for early detection of cytotoxic response

To establish, optimize and validate an alternative *in vitro* skin irritation test, we used the response of the HaCaT sensor cells to $CdCl_2$ as proof-of-concept experiments. To start with, the effect of

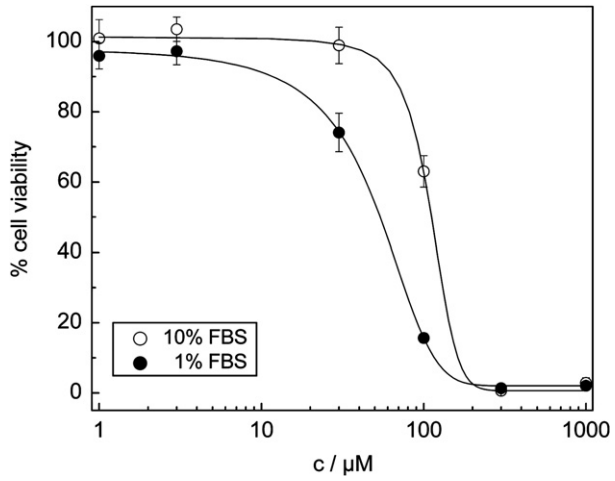

Fig. 1. Dose-response analysis of $CdCl_2$ ($t=24$ h) dissolved in culture medium containing 10% or 1% FBS as obtained from the MTT assay with HaCaT cells as sensors. Results are expressed as mean ± SE of three independent experiments. The EC_{50} values were obtained by fitting the dose response function to the experimental data using nonlinear regression analysis.

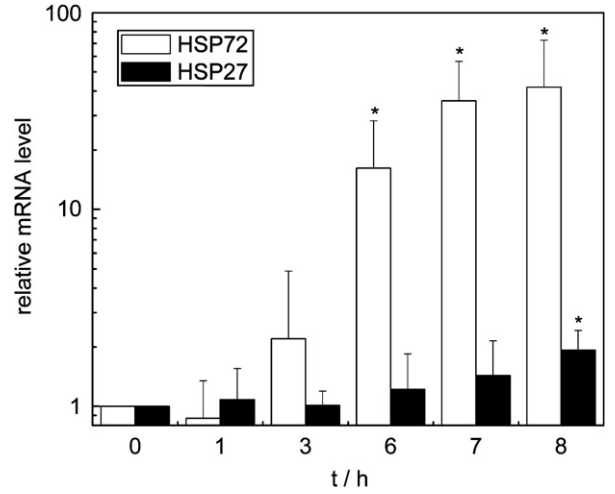

Fig. 2. Detection of the relative mRNA level of HSP72 (*HSPA1A*) and HSP27 (*HSPB1*) following incubation with $CdCl_2$ (25 µM; 0, 1, 3, 6, 7, 8 h). Relative expression relates to corresponding values for samples from untreated cells set as 1 [means ± SE; $n=3$; $^*P<0.05$].

$CdCl_2$ on cell viability ($t=24$ h) was measured under normal (10% FBS) and serum-deprived (1% FBS) conditions using the MTT endpoint cytotoxicity assay. Dose-response curves are presented in Fig. 1. The EC_{50} value determined under normal culture conditions (110 ± 10) µM was somewhat higher than under serum-deprived conditions (45 ± 7) µM. This may be due to the binding of cadmium to serum albumin and the associated reduction in cytotoxicity (DelRaso et al., 2003). To further investigate the impact of $CdCl_2$ on signaling cascades, HaCaT cells were exposed to sublethal concentrations of $CdCl_2$ and experiments were conducted under serum-deprived conditions.

In this study we focused on the genes coding for HSP72 (*HSPA1A*) and HSP27 (*HSPB1*) as potentially inducible stress indicators expressed in human keratinocytes. The suitability of these genes as biomarkers to be used in the HaCaT cell model of skin irritation was tested by the analysis of gene expression by quantitative reverse-transcription PCR (qRT-PCR) in cells exposed to 25 µM $CdCl_2$ as well as heat treatment (43 °C). The experiments revealed a significantly increased mRNA level of HSP72 after incubation of cells with 25 µM $CdCl_2$ for 6 h or longer (Fig. 2). Under these conditions expression of HSP72 increased approximately 20-fold. Likewise, an 8-fold and 20-fold induction of the biomarker was observed following heat treatment for 1 h and 2 h, respectively (data not shown). The relative mRNA level of HSP27 showed a considerably smaller but still significant change after exposure to 25 µM $CdCl_2$ for longer than 8 h.

We conclude that an approximately 2-fold induction of HSP27 may not provide sufficient sensitivity to be used in a reporter assay, whereas induction of HSP72 expression is sensitive enough to serve as a sensor for the detection of xenobiotic stress imposed on HaCaT cells.

3.2. Construction and evaluation of sensor cell line under normal culture conditions

According to the results described above a sensor cell line was established consisting of HaCaT cells stably transfected with plasmid $pHSP72_p$-AcGFP, which allows for the expression of green fluorescent protein (GFP) under the control of the HSP72 promoter. Reporter gene expression was tested after exposure of sensor cells to $CdCl_2$ and heat shock. For each sensor cell clone the absolute emission output and the dynamic range of GFP induction in different stress situations was evaluated using flow cytometry. One clone was selected for further investigations because it showed the best signal to noise ratio and the highest dynamic range if treated for short time periods. Fig. S1 (Supplementary material) shows phase contrast and fluorescence images of the selected clone, as well as typical histograms of fluorescence intensity distribution prior to and following stress exposure. The threshold point was determined from the histogram as the fraction including approximately 95% of the control cells that were not exposed (intrinsic fluorescence). Then, the number of GFP+ cells was determined. The fraction of GFP+ cells after exposure of the sensor cells to 25 µM $CdCl_2$ was 24.1% (4 h exposure) and 47.3% (6 h exposure), respectively. 44.9% of the cells were GFP+ after 2 h exposure of reporter cells to 43 °C.

In the next series of experiments, cells were seeded on coverslips for fluorescence microscopy to quantitatively investigate the expression levels in an incubation time- and dose-dependent manner under normal cell culture conditions. The fluorescence emission of the sensor cells increased with time after exposure of cells to $CdCl_2$, reaching a maximum level 22–24 h after exposure (data not shown). When the sensor cells were exposed to 15, 25 and 35 µM $CdCl_2$, a time-dependent increase in GFP fluorescence was observed, reaching a maximum for 35 µM $CdCl_2$ after 6 h of incubation (Fig. 3A). When exposing sensor cells to 45 µM $CdCl_2$, the cell population showed an unusual morphology and some cells died, which was associated with a decrease in fluorescence intensity.

Moreover, fluorescence intensity was recorded for different $CdCl_2$ concentrations after 6 h of incubation to determine the lowest detectable $CdCl_2$ concentration. The limit of detection (LOD) was defined as the concentration that induces fluorescence intensity equal or bigger than the mean fluorescence of untreated control cells plus three times the standard error of the measurement (Fig. S2, Supplementary material). Based on this definition the sensor cells displayed an LOD of 7 µM $CdCl_2$ for a 6 h exposure time. This data suggests that the sensor cells are suitable to quantitatively detect cytotoxicity under sublethal conditions and shorter incubation periods compared to endpoint measurements.

The cytotoxic response of the sensor cells to $CdCl_2$ (0–100 µM) was analyzed impedimetrically, following the normalized impedance at an AC frequency of 40 kHz over time. Cells were continuously monitored during pre-equilibration and exposure (arrow 1 in Fig. 3B). Fig. S3 (Supplementary material) provides an overlay of the frequency-dependent impedance and phase angle of eight electrodes covered with continuous layers of HaCaT

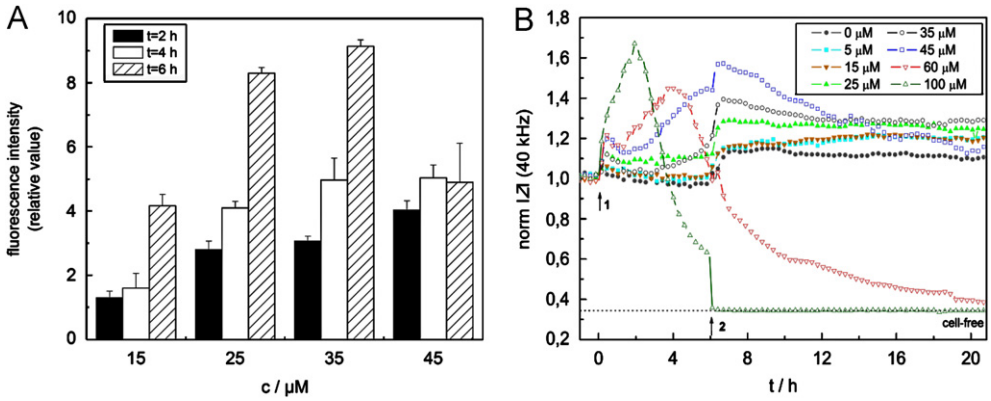

Fig. 3. $CdCl_2$ response of sensor cells analyzed by quantitative fluorescence microscopy (A) or ECIS measurements (B). (A) Fluorescence intensity of GFP expressed in sensor cells after exposure to 15, 25, 35, 45 µM $CdCl_2$ for 2, 4, 6 h [means ± SE; $n=3$]. (B) Time course of the normalized impedance $|Z|$ at a sampling frequency of 40 kHz before and after $CdCl_2$ addition (arrow 1). After 6 h of $CdCl_2$ exposure $CdCl_2$ containing medium was exchanged by cell culture medium (arrow 2). The impedance was normalized to the last value before $CdCl_2$ addition.

sensor cells prior to toxin exposure as an indicator for the reproducibility of the measurement.

Upon exposure of cells to 5 and 15 µM $CdCl_2$ the time course of the normalized impedance closely paralleled the $CdCl_2$-free control, indicating no impact on the cells. Addition of $CdCl_2$ in concentrations of 25 and 35 µM has led to a marginal increase of the normalized impedance. When the concentration was raised to 45, 60 and finally 100 µM, a distinct and dose-dependent cellular response was observed by ECIS recordings. Immediately after $CdCl_2$ addition beyond a threshold concentration, the impedance passed a transient maximum before it descreased in a dose-dependent manner. For a $CdCl_2$ concentration of 45 µM, the peak was followed by an impedance recovery to baseline values with no sign of cell retraction or lysis within the observation time. $CdCl_2$ added to the cell layer at a concentration of 60 µM and higher (100 µM) induced an immediate morphological change, which decreased the measured impedance to the values of a cell-free electrode, indicating complete loss of membrane integrity and, thus, cell death. The impedance decrease triggered by 60 µM of $CdCl_2$ was somewhat slower compared to 100 µM and approached cell-free electrode values only after 21 h. Exposure to 100 µM of $CdCl_2$ weakened cell-substrate interactions rather drastically, such that the exchange of culture medium after 6 h removed the remaining cells from the electrode surface. The slight impedance increase observed for all conditions after 6 h was due to this exchange of incubation fluid to cadmium-free culture medium.

Direct comparison of the reporter gene assay on the one hand, and label-free impedimetric monitoring on the other hand, revealed their individual strengths and limitations. Whereas the reporter gene reported more sensitively on small concentrations of cadmium in the extracellular buffer than ECIS recordings, it failed when cadmium concentrations were beyond the threshold concentration of acute cytotoxicity. Under these conditions cell physiology was compromised severely enough by the presence of toxins that protein biosynthesis was impaired and, therefore, the expression of the reporter gene was repressed. In contrast, ECIS is independent of active cellular processes. It reports passively to cell body contraction or membrane lysis induced by the toxin. Therefore, ECIS recordings correctly mirror the cell response until the cell was entirely permeabilized or detached from the electrode surface. Obviously, either assay alone has its limitations, but when both assays are performed in parallel, they provide complementary information over the entire concentration range and allow for a much more comprehensive and reliable analysis of the investigated irritant. In ongoing research we will combine both assays in one experimental setup in order to collect the available information from one and the same cell population and to describe the characteristic irritation profile with more than a single parameter.

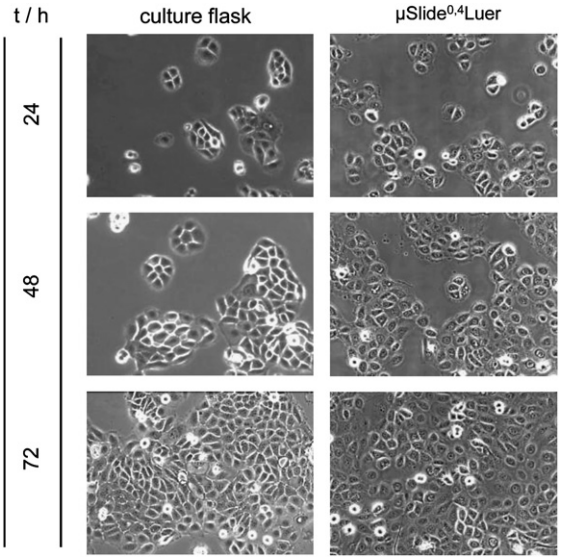

Fig. 4. Phase contrast micrographs of HaCaT cell growth under normal cell culture conditions using a culture flask (left panel) and under incubator-independent conditions using a µSlide^{0,4}Luer (right panel).

3.3. Incubator-independent time-lapse observation of cytotoxic response in µSlides

To allow for a convenient, sterile, and incubator-independent time-lapse observation of the cytotoxic response profile, the genetically modified sensor cells were grown in a microfluidic platform based on µSlidesI^{0,4}Luer (Ibidi) as culture substrate. First, we optimized the experimental setup to provide optimal culture conditions and similar proliferation rates as in conventional cell culture dishes. Cells were seeded at defined density in a µSlide and a culture flask at the same time. Images of nine randomly chosen areas of each culture were documented every 24 h to determine cell growth curves by counting cells within the defined areas. A set of images is exemplarily shown in Fig. 4. For both environments an increase in cell number by a factor of 1.9 was observed in 24 h. This in turn was important for monitoring reliable, high-quality time course changes in fluorescence of sensor cells without using a

large-scale incubator and for the ability to compare and relate these to conventional cell culture practice.

A representative set of time-lapse observations of sensor cells grown in μSlidesI$^{0.4}$Luer exposed to 35 μM $CdCl_2$ for 6 h is shown in Fig. 5. Images were taken 12–24 h after addition of the toxin, resulting in a maximum GFP expression after 22 h. This delay is obviously due to time required for transcription, translation, maturation, and accumulation of the AcGFP gene in the sensor cells.

Figs. 3 (normal cell culture) and 6 (microfluidic platform) provide a comparison of the cell response for both culture conditions. A comparable response profile to varying $CdCl_2$ dosages (t=6 h) was observed, and no increase in fluorescence was measured in untreated populations at any time point (Fig. 6). As expected, recording of fluorescence intensities in the microfluidic platform suffered from higher noise levels originating from the slide. This gave rise to a higher background signal, which has been subtracted in the results depicted in Fig. 6. Thus, the lower relative fluorescence intensity value presented in Fig. 6 was a result of background correction rather than a lower sensitivity obtained with the microfluidic platform.

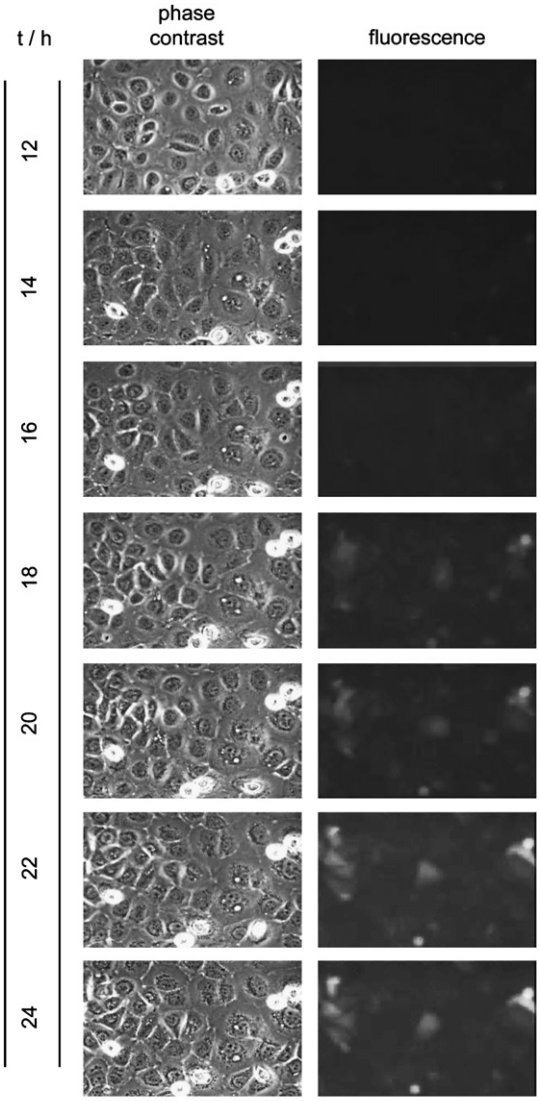

Fig. 5. Time-lapse observation of HaCaT sensor cells grown in μSlidesI$^{0.4}$Luer. Examplary response of sensor cells exposed to 35 μM $CdCl_2$ for 6 h. Phase contrast and fluorescence micrographs are shown 12–24 h after adding the toxin, reaching a maximum GFP expression after 20 h.

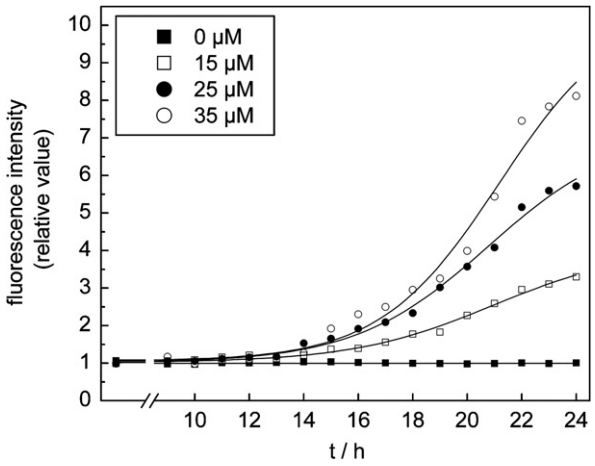

Fig. 6. Time course changes in GFP fluorescence of the sensor cells exposed to varying concentrations of $CdCl_2$ grown in μSlidesI$^{0.4}$Luer.

4. Conclusions

In this study we have demonstrated, by combining an optical readout based on genetically modified sensor cells and impedance measurements for quantification of cell morphology, the suitability of both approaches to quantify the early irritation response upon exposure to a certain test substance in a sensitive and real-time manner. The assay allows a detailed discrimination of competing growth and stress-related effects. Furthermore, we have shown that the sensor cell line works with the same efficiency/sensitivity in an incubator-independent environment under microfluidic control. The present study proves the reliability of the sensors based on genetically modified reporter cells and the significance of the results received by combining optical and impedimetric readouts and it may pave the way to the further developments and applications of micro-total analysis systems in dermatology, toxicology, pharmacology and drug screenings.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2012.07.075.

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