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Validation of in vitro assays in three-dimensional human dermal constructs / Idrees, Ayesha; Chiono, Valeria; Ciardelli, Gianluca; Shah, Siegfried; Viebahn, Richard; Zhang, Xiang; Salber, Jochen. - In: INTERNATIONAL JOURNAL OF ARTIFICIAL ORGANS. - ISSN 0391-3988. - 41:11(2018), pp. 779-788. [10.1177/0391398818775519]

Availability:

This version is available at: 11583/2710542 since: 2020-02-25T20:45:27Z

Publisher:

SAGE Publications

Published

DOI:10.1177/0391398818775519

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Validation of *in vitro* assays in three-dimensional human dermal constructs

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The International Journal of Artificial
Organs

2018, Vol. 41(11) 779–788

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DOI: 10.1177/0391398818775519

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Abstract

Three-dimensional cell culture systems are urgently needed for cytocompatibility testing of biomaterials. This work aimed at the development of three-dimensional *in vitro* dermal skin models and their optimization for cytocompatibility evaluation. Initially “murine *in vitro* dermal construct” based on L929 cells was generated, leading to the development of “human *in vitro* dermal construct” consisting of normal human dermal fibroblasts in rat tail tendon collagen type I. To assess the viability of the cells, different assays CellTiter-Blue[®], RealTime-Glo[™] MT, and CellTiter-Glo[®] (Promega) were evaluated to optimize the best-suited assay to the respective cell type and three-dimensional system. Z-stack imaging (Live/Dead and Phalloidin/DAPI-Promokine) was performed to visualize normal human dermal fibroblasts inside matrix revealing filopodia-like morphology and a uniform distribution of normal human dermal fibroblasts in matrix. CellTiter-Glo was found to be the optimal cell viability assay among those analyzed. CellTiter-Blue reagent affected the cell morphology of normal human dermal fibroblasts (unlike L929), suggesting an interference with cell biological activity, resulting in less reliable viability data. On the other hand, RealTime-Glo provided a linear signal only with a very low cell density, which made this assay unsuitable for this system. CellTiter-Glo adapted to three-dimensional dermal construct by optimizing the “shaking time” to enhance the reagent penetration and maximum adenosine triphosphate release, indicating 2.4 times higher viability value by shaking for 60 min than for 5 min. In addition, viability results showed that cells were viable inside the matrix. This model would be further advanced with more layers of skin to make a full thickness model.

Keywords

Three-dimensional cell culture, two-dimensional cell culture, cytocompatibility evaluation, human *in vitro* dermal construct, human dermal fibroblasts

Date received: 29 January 2018; accepted: 16 April 2018

Introduction

Before testing new drugs, theranostics or medical devices containing one or more new bioactive biomaterials in “First-in-Man Phase I Clinical Trials”, highly valid and reliable data are required. Lack of transferability of experimental data of *in vivo* animal trials and their inequivalency to human biology necessitate the use of human cell-derived model systems that can range from single cells via three-dimensional (3D) models to organs-on-chips.^{1–6} Taking this into consideration, human-tissue-related 3D cell cultures have the ability to recapitulate characteristics of tissue physiology and pathophysiology and are emerging as an attractive model system to provide more reliable preclinical outcomes.

Autologous skin or tissue-engineered skin grafts have been used as epidermal/dermal substitutes to treat burns and

wounds.^{7–10} Commercially available dermal constructs for clinical use include either chemically treated allografts

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(e.g. AlloDerm®)¹¹ or *in vitro* cultured human fibroblasts in a matrix (Dermagraft®).^{12,13} One recent application of grafts is in the field of *in vitro* testing systems.^{14,15} Skin substitutes have been used in pharmacological and basic research either for hazard assessment of chemical compounds (e.g. skin irritation/absorption/corrosion)^{14,16–19} or to understand fundamental processes (e.g. the effect of fibroblasts on microenvironment for epidermal regeneration).^{20,21} The development of *in vitro* tools for testing has also been stimulated by evolving regulations: the European Union (EU) 7th amendment of the “Cosmetics Directive” imposed to replace all animal experiments on cutaneous resorption with reliable *in vitro* tests by the year 2009.²² This led to the development of the 3Rs principle “replacement, reduction and refinement.”²³ In agreement with this principle, cell lines are used to assess general *in vitro* cytotoxicity based on DIN EN ISO 10993-5, while any specific cytotoxicity should be tested using specific primary cells.²⁴ In addition, *in vitro* bioevaluation is more accurate using 3D testing systems than two-dimensional (2D) cultures.^{25–27} Cell-based assays being routinely used for therapeutic screening are the methods based on a specific biomarker for detecting cell viability and cytotoxicity with an established 2D cell culture system. There have been precise adaptations of these assays to spheroids-based 3D systems;^{28–32} however, to evaluate the viability of primary cells-based 3D model, it is crucial to select the best suited assay for each cell type in 2D and then in 3D system.

In this work, a human dermal skin model was developed and aimed at the future preclinical testing of new biomaterials for wound healing, reducing the number of needed animal experiments. Human skin is composed of three layers: epidermis, dermis, and the underlying hypodermis, also called subcutaneous connective tissue.³³ A preliminary 3D dermal model using L929 cells (mouse fibroblast cell line) was constructed and named as “murine *in vitro* dermal construct.” Knowledge arising from this model allowed the subsequent development of human dermal skin model using primary normal human dermal fibroblasts (NHDF), named as “human *in vitro* dermal construct.” The models were characterized for cell viability and morphology as a function of time. Different viability assays were applied on the models under different conditions to select the best one as advanced testing system for preclinical evaluation. There is a need for more than one type of test methods to imply in the 3D cell culture system based on different parameters, for example, nicotinamide adenine dinucleotide phosphate (NADPH) enzyme activity, adenosine triphosphate (ATP) content of cells, and/or DNA content to reveal all the different aspects of nonphysiologic or pathophysiologic reactions occurring in this system.

Methods

Cell source and materials

L929 cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures). Primary cells

including NHDF and normal human epidermal keratinocytes (NHEK) were obtained from PromoCell. ISO standardized materials, that is, polyethylene (PE) and zinc diethyldithiocarbamate containing polyurethane (ZDEC-PU) were obtained from Goodfellow and Hatano Research Institute, respectively, and used as control materials for cytotoxicity testing. Collagen type I (col. I) from rat tail tendons was obtained from Ibbidi. Lysis solution of 9% Triton® X-100 in water from Promega was used to create “lysis control.”

Dermal construct fabrication

Gelation of col. I solution was performed in 10X media (M199-Sigma), in the presence of additives (L-glutamine) and sodium bicarbonate (NaHCO₃) resulting in a final col. I concentration of 1.5 mg/mL (containing a final salt concentration of 1X mixture with a pH of 7.2–7.4).

L929 cells maintained in cell culture media RPMI 1640 with stable glutamine (PAN Biotech) containing 10% fetal bovine serum (FBS; PAN Biotech) under physiological culture conditions (37°C, 5% CO₂), and subcultured using 0.25% Trypsin (Gibco).

NHDF were maintained in fibroblasts growth media 2 (FGM2, Promocell) under the physiological culture conditions (37°C, 5% CO₂), and subcultured using DetachKit2-Promocell HEPES BSS (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline solution); 0.04% trypsin/0.03% Ethylenediaminetetraacetic acid (EDTA); trypsin neutralizing solution (TNS) containing 0.05% trypsin inhibitor from soybean/0.1% bovine serum albumin).

Dermal constructs were prepared by fabricating acellular (200 µL) and cellular layers (400 µL) of col. I matrix on polyester membrane of 12 well insert (Corning) constituting a 5-mm thick dermal construct.

Cellular layers: Actively dividing mitotic cells (8 × 10⁵ L929 cells/mL to obtain the murine *in vitro* dermal construct; 8 × 10⁴ NHDF cells/mL to obtain the human *in vitro* dermal construct) were embedded in col. I solution and poured onto the top of the previously deposited acellular col. I layer. When the cellularized matrix underwent gelation, the system was fed with fresh cell culture medium. The system was incubated for 5–7 days to allow hydrogel remodeling by the embedded cells.

Morphological analysis

Morphological appearance of cells in 2D (cells grown on tissue culture polystyrene (TCPS)) and 3D col. I matrix was assessed using bright field and fluorescent microscopy (Olympus IX51). Fluorescent staining was performed using Live/Dead staining kit-Promokine containing calcein-AM and ethidium homodimer III (EthD-III) to see live and dead cells in 2D and/or 3D matrix. F-actin/nuclei staining was performed using Phalloidin/DAPI stain-Promokine.

Cell viability analysis

To assess the viability of the cells in 2D and 3D system quantitatively, different cell viability and cytotoxicity assays, for example, CellTiter-Blue® (CTB), CytoTox-ONE™ (CTO), RealTime-Glo™ MT (RTG MT), and CellTiter-Glo® (CTG)-Promega, were evaluated to monitor cell viability of the respective cell types according to standard protocols and for their 3D dermal constructs with modified standard protocols.

The CTG assay was used for matrix-based 3D cultured cells for the first time after optimizing the shaking time using the reagent at the same concentration as described in the standard protocol with monolayer culture. The parallel Z-stack microscopic observation showed that 60 mins of shaking time (10 min for signal stabilization) was enough to lyse cells and release the maximum ATP content. The effect on the decay of signal over time was confirmed by recording the luminescence over time.

The CTB assay was used for matrix-based 3D cultured cells for the first time after optimizing the test design using the reagent at the same concentration as described in the standard protocol with monolayer culture. The reagent was added only on the top of the construct, shaken for a few minutes, and incubated for 2 h at 37°C. The tiny seeping reagent was transferred every 40 min ($T_{40\text{min}}$, $T_{80\text{min}}$, $T_{120\text{min}}$) back to the insert during the incubation period. Compared to higher incubation times, 2 h was also good enough to reduce blue resazurin to pink resorufin in this 3D system.

Statistical analysis

Experiments were carried out in triplicates ($n=3$) and results were expressed as mean \pm standard deviation. For statistical analysis, GraphPad Prism 5.00.288 (Inc., San Diego, CA, USA) was used to evaluate the significance of the differences in cell viability data. *T*-test was used when the comparison involved two groups. Significance between groups was considered for $p < 0.05$.

Results

L929 cells in 2D and 3D models: cytocompatibility evaluation

Morphology of cells changed depending if they had been cultured on 2D flat surface or within a 3D col. I matrix: a well-connected network of L929 cells was found in 3D matrix (Figure 1(b)) while in 2D cultures, cells showed flat morphology (Figure 1(a)).

We intended to develop an in vitro human dermal skin model for bioevaluation of novel drug-free antibacterial hybrid biopolymers for medical applications. Therefore, apart from the company's recommended controls, we used according to the DIN EN ISO 10993-5 standardized positive (PE) and negative control materials (ZDEC-PU), since

we intended to analyze the novel biomaterials' interaction with our 3D system.

CTB assay measures cell viability and is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end-product (resorufin), while CTO assay evaluates the cytotoxicity by measuring the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. Both assays were performed in multiplexed format to get more information from the same sample. As a proof of concept, the performed experiment (Figure 2(a)) showed that CTB assay works well with L929 cells, indicating an increasing viability signal with an increase in cell number.

This was also confirmed by monitoring the cytocompatibility of ISO standardized positive (PE) and negative control materials (ZDEC-PU) with L929 cells, indicating cell viability and cytotoxicity signals, respectively (Figure 2(b)). The fluorescent micrographs using Live/Dead staining of L929 cells (Figure 2(c)) showed dead cells (red) as a result of exposure with ZDEC-PU and live cells (green) with well-preserved morphology when exposed to PE. These data completely correlated with the cytocompatibility data obtained by CTB assay shown in Figure 2(b).

The assays were optimized in murine in vitro dermal construct demonstrating that both assays can be adapted to this 3D system (Figure 3(a)). This experiment was performed to know if the cell viability can be assessed for the same sample at multiple time points. For this, the reagent exposure time was 2 h only, at every 2 days, leading to washing and feeding with fresh media until the following measurement. The results of repeated reagent exposure and as an end-point reagent exposure were compared. Cells were viable for at least 20 days in matrix, which was a prerequisite for in vitro experiments. Furthermore, effect of repeated CTB reagent exposure on viability of L929 cells in 3D matrix demonstrated that this reagent had no toxic effect for at least 18 days' exposure (Figure 3(b)). Therefore, this assay can be used as a method to monitor cytotoxic effect of the same sample in this 3D system over an extended period, at multiple time points.

NHDF cells in 2D and 3D models: cytocompatibility evaluation

CTB assay was performed with primary cells of skin, that is, NHDF and NHEK (Figure 4(a)). The experiment showed an increasing viability signal with an increase in cell number showing that CTB assay is suitable on these cell types. This concept was further demonstrated by monitoring the cytocompatibility of ISO standardized positive (PE) and negative control materials (ZDEC-PU) with NHDF (Figure 4(a)). However, microscopic observations (Figure 4(c)) showed that CTB reagent affected cell morphology (shrunken cells) of NHDF, suggesting reagent interference with cell normal biological activity and possibly resulting less reliable data.

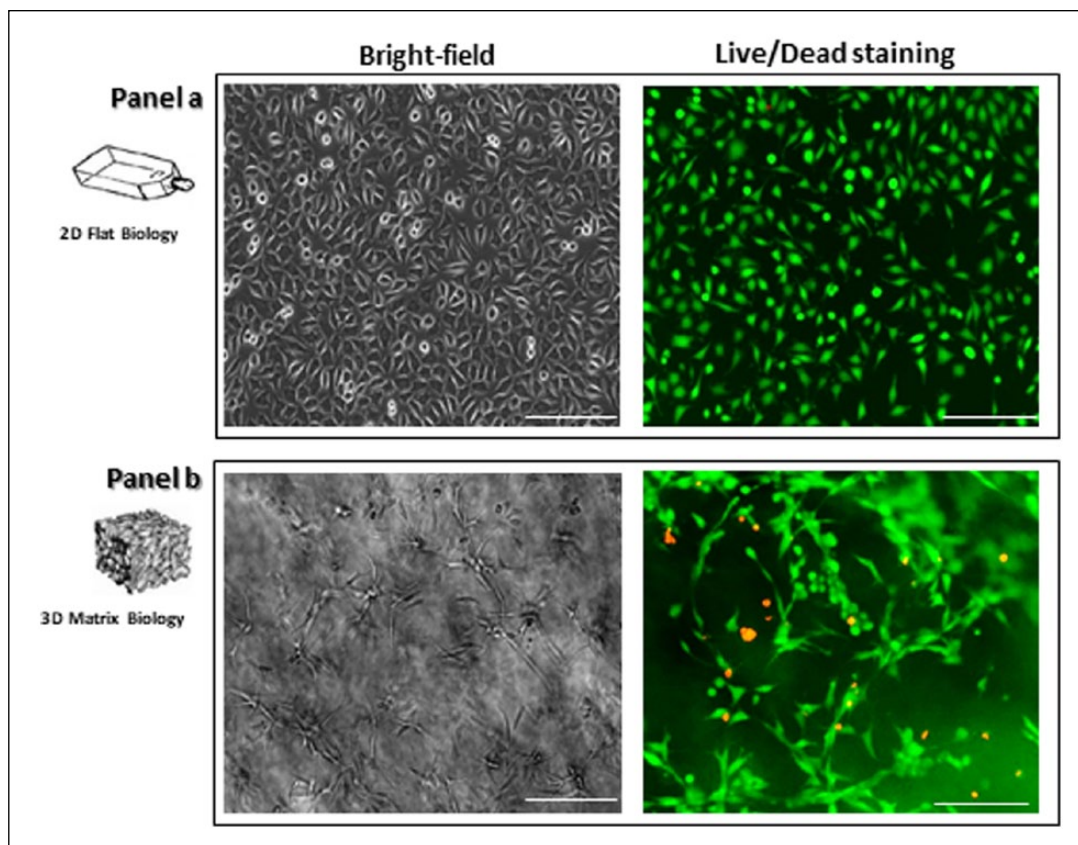


Figure 1. Morphology of L929 cells in 2D versus 3D. Bright field (left) and fluorescence micrographs (right) of L929 on 2D flat surface (Panel a) and inside 3D col. I matrix (Panel b). Fluorescent staining was performed using Live/Dead staining kit (Calcein-AM and EthD-III). Scale bar = 200 μm .

In an exploration of an appropriate viability assay for NHDF, RTG MT assay was selected because of its continuous-read format to analyze cell viability in real time. The assay is based on the ability of viable cells to reduce a substrate to produce a luminescent signal by NanoLuc[®] luciferase. This assay did not show linearity of luminescence signal with an increasing time (Figure 5). Similar results were obtained with L929 cells (data not shown), demonstrating that the linearity in the signal was only present when using a lower cell number than 1250, irrespective of cell type. For this reason, this assay was not suitable for 3D systems that involve higher cell number.

Finally, CTG assay was performed: it is based on the generation of luminescent signal, proportional to the amount of ATP present in cells. The assay showed an increasing viability signal with an increase in cell number (Figure 6(a)). Half-life of luminescent signal was assessed as >4h (data not shown) and CTG assay was found to be an optimal cell viability assay among those analyzed.

Human in vitro dermal construct showed the filopodia-like morphology with dendritic extensions in the matrix and a uniform distribution of NHDF in different planes inside the matrix (Figure 6(b)).

After selecting CTG assay as an appropriate assay for this cell type, the assay adapted to 3D system by changing the shaking time to optimize the cell lysing ability for ATP release. Increased shaking time resulted in higher cell lytic capacity for the maximum ATP release: 60 min shaking time for a cultured dermal construct on day 7 showed 2.4 times higher luminescent signal for ATP release than shaking for 5 min (Figure 6(c)). This demonstrated that, NHDFs were viable inside the matrix. If an increased ATP content with time was due to proliferating NHDF inside, the matrix needs further exploration using a proliferation assay (Figure 6(d)).

Discussion

Type I collagen is a fibrous protein and a major structural component of extracellular matrix (ECM) in skin and thus can simulate the 3D in vivo cell environment. Collagen type I from “rat tail tendons” well serves the purpose of ECM because of its irregular fibrils formation that resembles more in vivo-like reconstitution. Interactions between fibroblasts and ECM are the core of 3D cell cultures, which is different from the focal and fibrillar adhesions on

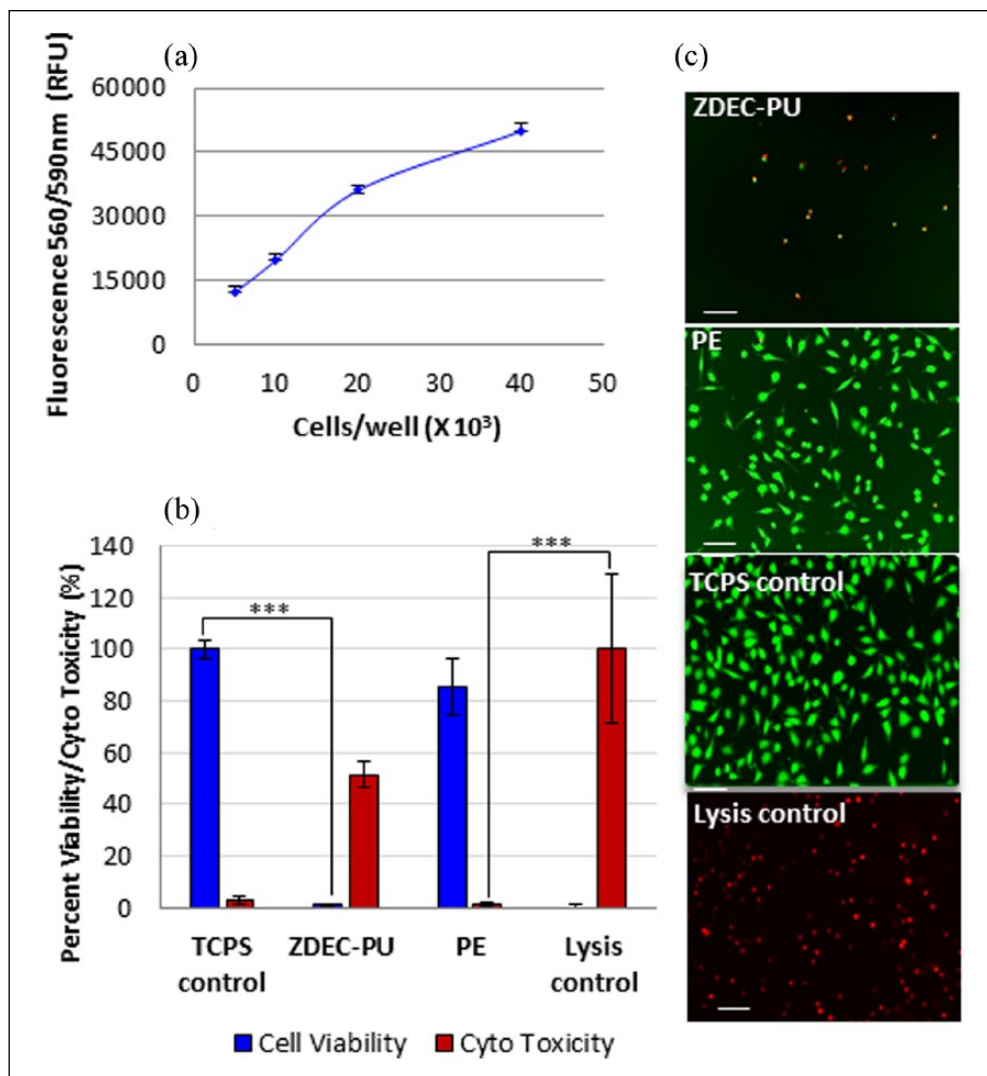


Figure 2. (a) CellTiter-Blue® (CTB) assay with L929 cells. The graph shows cell viability data of L929 cells analyzed using CTB assay. (b) Cytotoxicity evaluation of control materials. The cytocompatibility of ISO standardized positive and negative control materials with L929 cells monitored using CTB and CTO assays. PE indicates polyethylene and ZDEC-PU indicates zinc diethyldithiocarbamate containing polyurethane, while TCPS is tissue culture polystyrene. (c) Live/dead staining. The fluorescent micrographs show L929 cells after exposure with the control materials. Staining was performed using Live/Dead staining kit showing dead cells as red and live cells as green. Scale bar = 100 μ m. Significant difference between viability values of TCPS control and ZDEC-PU was $p=0.0001$, and between viability values of lysis control and PE it was $p=0.0009$.

2D surfaces.³⁴ Cells in 3D have enhanced cell biological activities and narrowed integrin usage,³⁴ displaying a distinctive morphology (as seen in Figures 1 and 6(b)).

Among the cytocompatibility assays, resazurin-based assay recognized as the AlamarBlue® or CTB is exceedingly used because it is sensitive as well as cost-effective.³⁵ This assay works well with an established 2D cell culture system; however, to assess the cytotoxic effect of therapeutics in the 3D cell culture system, it has to be re-evaluated to get more reliable results. The test has been previously used to screen cytocompatibility of cell spheroids after treatment with an agent, for example, staurosporine, able to disrupt the tight cell junctions for resazurin

uptake.³⁶ In this study, the test was successfully adapted to matrix-based 3D system of “murine *in vitro* dermal construct,” measuring the cell viability at different time intervals (Figure 3).

Resazurin is reported nontoxic to cells for short-term incubation times, but can affect cell survivability for extended exposure times (in terms of days) by interfering with the cell’s metabolic activity, DNA content, and glucose consumption depending on the cell line.³⁷ Changes in morphology is a cell’s stress response to an environmental insult that can result in fragmentation of Golgi complex, swelling of mitochondria, compromised integrity of nucleoli, or alterations in cytoskeleton especially in intermediate

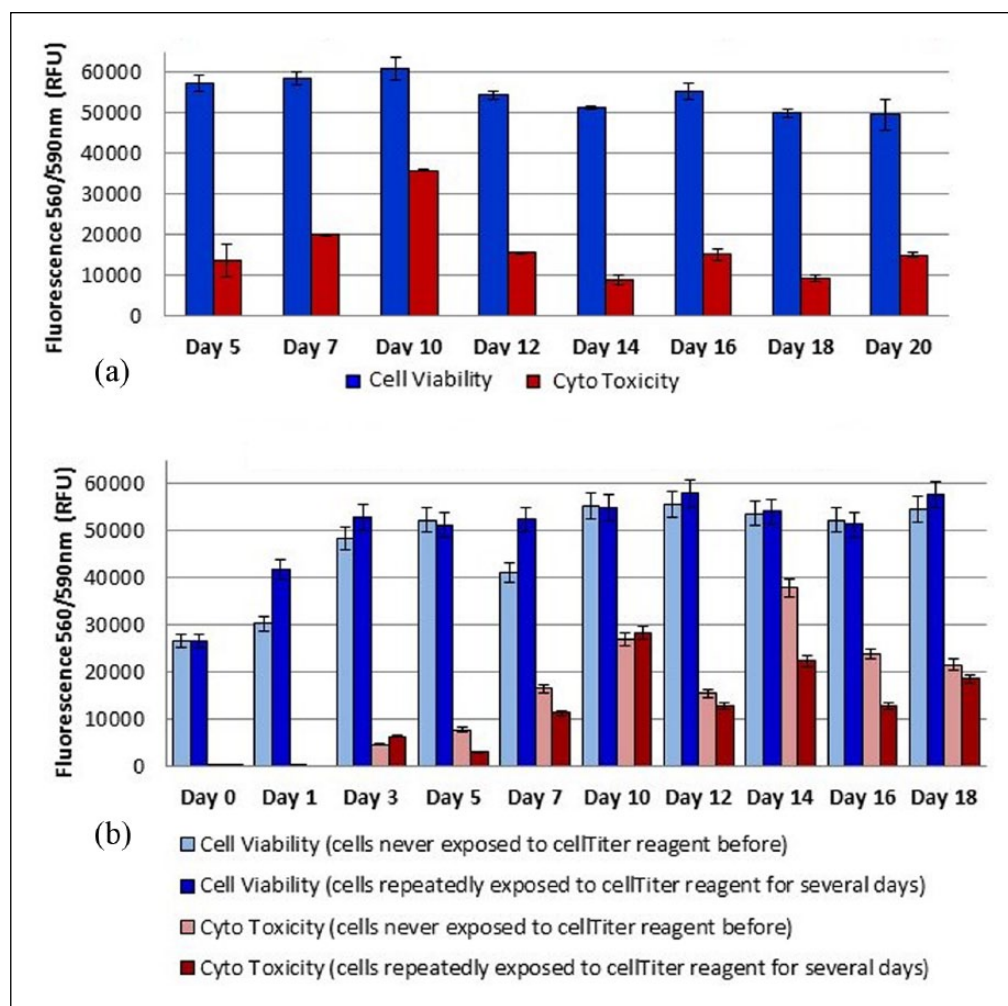


Figure 3. (a) Cell viability of murine *in vitro* dermal construct (based on L929 cells). The graph shows cell viability (blue bars) and cytotoxicity (red bars) assessment of murine 3D dermal construct (based on L929 cells) over time analyzed by CellTiter-Blue® (CTB) and CytoTox-ONE™ (CTO) assays. (b) Effect of repeated reagent's exposure on 3D L929 cells. The viability of constructs that were repeatedly exposed to CTB reagent was measured for 18 days.

filaments.³⁸⁻⁴⁰ In this study, an altered morphology of NHDF that might be resulting from a collapsed cytoskeleton was observed after a short-term exposure (≤ 1 h when the reduction of the blue resazurin to pink resorufin has not fully occurred yet) of CTB reagent (Figure 4(c)). Therefore, to provide definitive viability data in a clinically relevant 3D model based on human primary cells (that is more sensitive than a model based on cell lines), a panel of cytocompatibility assays was tested for NHDF. In this regard, RTG MT assay lost (Figure 5) its linearity at a very low cell seeding density. Here, the substrate concentration can be a limiting factor as well as the cell itself, based on the fact that the number of cells (and the subsequent metabolism) does not change any more. In this case, an increase in the substrate concentration would not solve that effect. As per company's recommendation, it was strongly needed to test the best cell number for each respective cell type for RTG MT assay. This nonlinearity effect was further tested with

L929 cell line. The effect stayed similar (results not shown) and, thus, was not suitable for our 3D system.

However, the most advanced and sensitive assay for testing viability of 3D systems has been the CTG assay, based on the quantification of intracellular ATP content.²⁸ This assay can be employed on 3D spheroids after optimizing the lysis conditions.^{29-32,41} For the "human *in vitro* dermal construct" developed in this work, CTG assay was selected and experimental parameters for reagent penetration were optimized. In 3D systems, there are strong cell-to-cell and cell-to-matrix interactions resulting in tight junctions that hinder the reagent uptake. In this study, CTG assay was used for "human *in vitro* dermal construct" (matrix-based) for the first time, by optimizing the "shaking time" to enhance reagent penetration for the maximum ATP release (Figure 6(c)) and thus cell viability of NHDF was measured inside the matrix (Figure 6(d)). However, shaking times varied for the measurement of cell viability

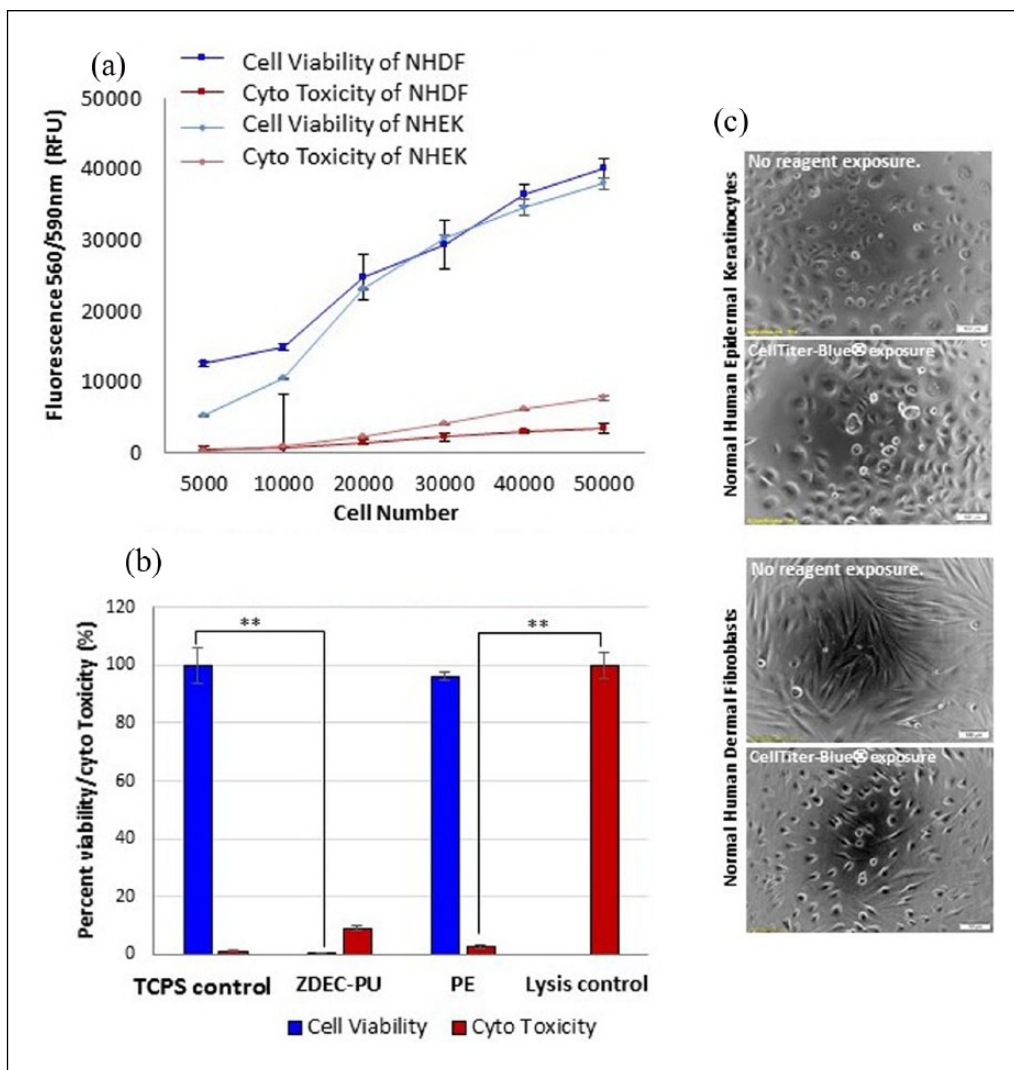


Figure 4. (a) CellTiter-Blue® (CTB) assay with human primary cells. The graph shows cell viability data of human skin primary cells, that is, normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) analyzed by CTB and CytoTox-ONE™ (CTO) assays. (b) Cytotoxicity evaluation of control materials with human dermal fibroblasts. The cytocompatibility of ISO standardized positive and negative control materials with NHDF monitored using CTB and CTO assays. PE indicates polyethylene, ZDEC-PU indicates zinc diethyldithiocarbamate containing polyurethane, while TCPS is tissue culture polystyrene. Significant difference between viability values of TCPS control and ZDEC-PU was $p=0.0022$; and between viability values of lysis control and PE it was $p=0.0016$. (c) Effect of CTB reagent on cell's morphology. Bright field micrographs show NHEK and NHDF with and without CTB reagent exposure. Scale bar = 100 μm .

of matrix-based 3D system at different time points, indicating the varying matrix–cell interaction with increasing culture time. CTG assay was found to be the optimal cell viability assay for 3D matrix-based dermal system among those studied in this work.

The modified protocol for 3D system used a longer shaking time of 60 min in our 3D system to improve reagent penetration in comparison with 5-min shaking time (as described by company for monolayer cell culture). Therefore, it was important that half-life showed the stability of the signal over time, thus providing the flexibility for measuring time.

The parameters that are important in optimizing an assay in a 3D system include type of cells, number of cells, period of cultivation, frequency of media exchange, culture conditions, type of assay, reagent interaction with cells, assay conditions, detection range of assay, and signal half-life. Depending on the intrinsic characteristics of a 3D system, for example, spheroids are different from cells embedded in a 3D matrix; extrinsic parameters have to be defined differently for each system. For this reason, a portfolio of assays is crucial to be tested for each cell type in 2D and then in 3D system.

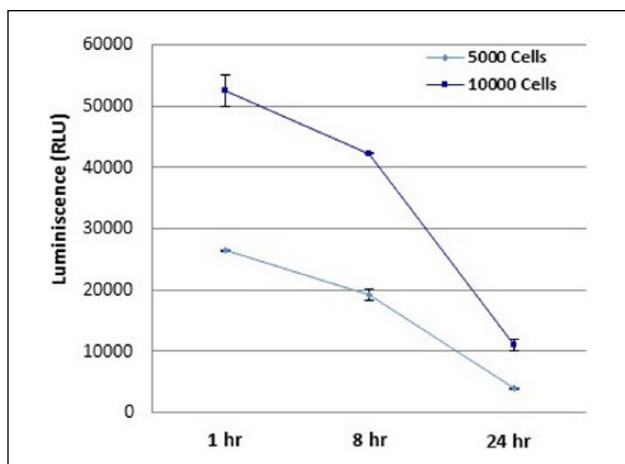


Figure 5. RealTime-Glo™ MT (RTG MT) assay with human dermal fibroblasts. The graph shows the cell viability data of normal human dermal fibroblasts (NHDF) analyzed by RTG MT assay at different time points of 0, 8, and 24 h (T_{0h} , T_{8h} , T_{24h}).

Conclusion

Development of human-based 3D in vitro systems will serve as an advanced and more complex system in the future, to perform more reliable in vitro preclinical studies for the bioevaluation of cytotoxicity of biomedical materials. Although a 3D model based on human primary cells is clinically more relevant, it is more sensitive. In this study, the differences in morphology were shown when cells were grown on a 2D surface and inside the 3D matrix. Moreover, different cell viability assays were tested to adapt to the matrix-based 3D systems. The CTB assay was adapted by optimizing the test design to the 3D system. It worked well with 3D murine in vitro dermal constructs (based on L929 cells), but affected the cell morphology of NHDF (unlike L929). In an exploration of the assays tested with NHDF, CTG was found to be the most optimal in this study. This assay was adapted to 3D human in vitro dermal constructs by optimizing the “shaking

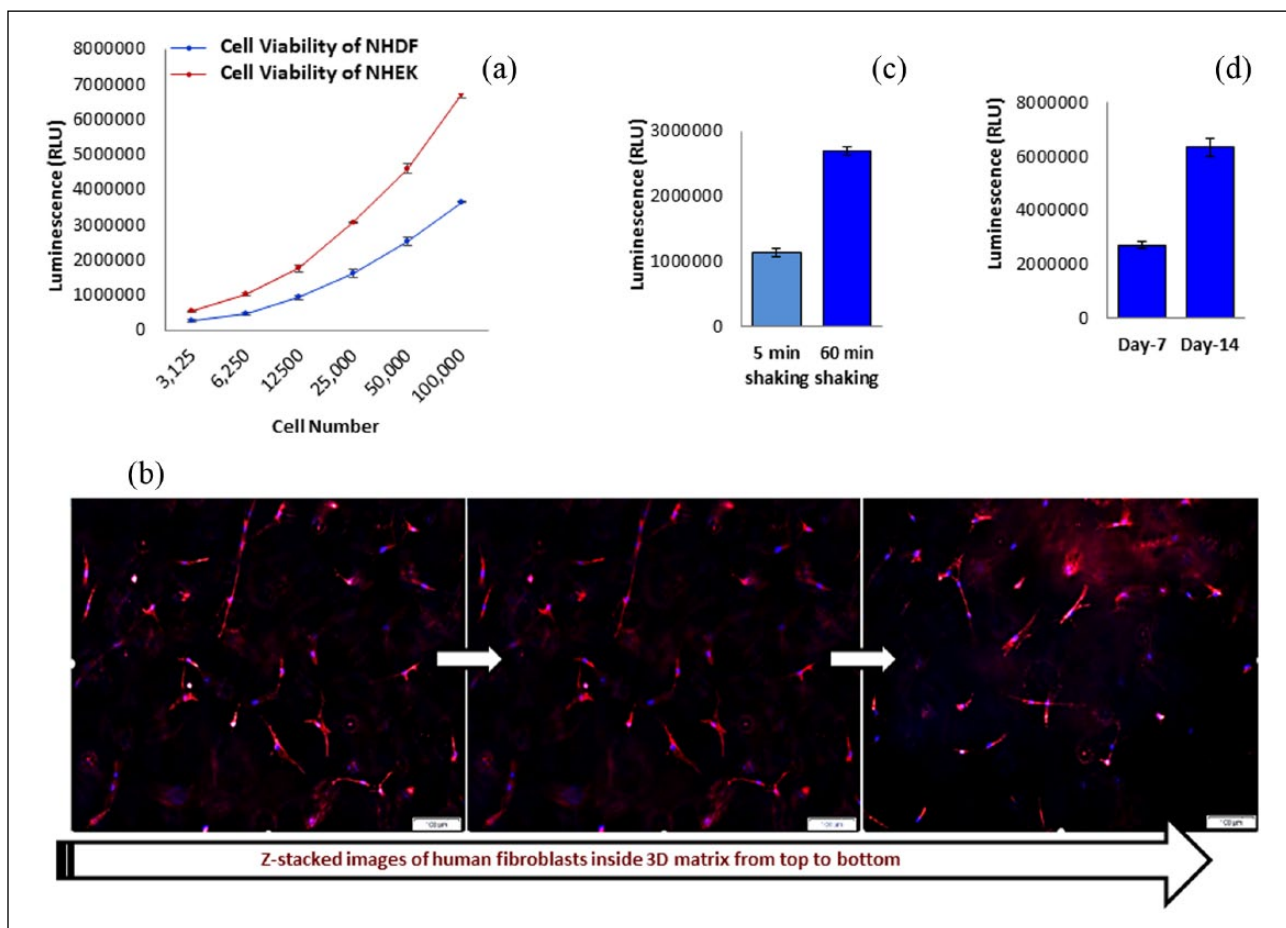


Figure 6. (a) CellTiter-Glo® (CTG) assay with human primary cells. The graph shows the cell viability data of human skin primary cells, that is, normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) analyzed by CTG assay. (b) Visualization of 3D NHDF inside human in vitro dermal construct. The fluorescent micrographs from top to bottom show the Z-stacked images of in vitro human dermal construct revealing the NHDF inside the matrix. Staining was performed using DAPI and Phalloidin. Scale bar = 100 μ m. (c) Optimization of CTG assay with human in vitro dermal construct. The graph shows the results of viability of NHDF in 3D matrix of dermal construct demonstrating the optimization of shaking time. (d) Cell viability of human in vitro dermal construct. The bar graph shows the cell viability of 3D dermal construct analyzed by optimized CTG assay at different time points of 7 and 14 days of culture.

time” to enhance the reagent penetration and thus, the maximum ATP release (assisted by parallel Z-stack microscopic observation for confirmation of cell lysis throughout the depth of human in vitro dermal construct), indicating 2.4 times higher viability value by shaking it for 60 min compared to 5 min, a time stated in the standard protocol of the respective assay for monolayer cell cultures. There is a need for more than one type of test methods to reveal all the different aspects of nonphysiological or pathophysiological reactions, respectively, occurring in this system. Measuring more than one parameter would offer more valid data.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant agreement No. 643050 (HyMedPoly Project).

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