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Fluorescein Diacetate for Determination of Cell Viability in Tissue-Engineered Skin

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ABSTRACT

Assurance of the quality of cultured skin substitutes (CSSs) currently relies on representative histology and determination of surface hydration, which provide limited sampling at selected points. To evaluate uniformity of cell density on the collagen matrices before clinical use, a field assessment of cell viability is advantageous. This study aimed to develop a field measure of cell viability in CSSs *in vitro* using fluorescein diacetate (FdA). CSSs were stained 3 days after keratinocyte inoculation using 0.04 mg/mL FdA followed by exposure to 366 nm of ultraviolet light. CSS fluorescence quantified using Metamorph image analysis was correlated with inoculation density, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) values and histology of corresponding biopsies. CSS fluorescence correlated significantly with inoculation density ($p < 0.001$) and MTT values ($p < 0.001$) of biopsies collected immediately after FdA staining. Fluorescence at day 3 also predicted day 10 MTT values. No toxicity was detected in CSSs, and normal *in vitro* and *in vivo* histology was demonstrated after FdA exposure. In conclusion, measurement of intracellular fluorescence with FdA allows for the early, comprehensive measurement of cellular distributions and viability in engineered skin and may therefore facilitate quality assurance.

INTRODUCTION

With the progression of tissue engineering research, the possibilities for clinical applications of 3-dimensional engineered tissues and organs are increasing. Rapid, accurate evaluation of cell distribution and viability is highly advantageous in tissue engineering to assure proper function of the tissue. Loss of cell viability in a natural or bioengineered tissue can lead to a severe reduction in efficacy once implanted. For example, engraftment of split-thickness allografts has been related to allograft tissue viability.[1] Thus the development of a rapid, non-destructive assay for the assessment of cell distribution and viability would provide a valuable step forward for assuring the quality of engineered tissues. Fluorescein diacetate (FdA) has been used in the laboratory to assess the viability of a wide variety of cells types and tissues, including human fetal cerebral cortical cells,[2] human keratinocytes,[3] and ovine articular cartilage,[4] and in the clinical setting to assess skin flap viability[5] and burn depth.[6] By virtue of its bipolar side chains, FdA easily penetrates the cell membrane. FdA remains colorless until esterases, which convert the non-fluorescent FdA to fluorescein, non-specifically cleave the acetate moieties in FdA.[7] Metabolically active cells with intact cell membranes can then be visualized under ultraviolet (UV) light;[8,9] the intensity of fluorescence seen under UV light is therefore directly proportional to the number of viable cells. With fluorescein and carbon dioxide as the only byproducts, this assay should not result in significant toxicity and therefore may be suitable for non-destructive, quantitative measurements of cellularity in engineered tissue and organs.

The treatment of full-thickness burns with cultured skin substitutes (CSSs) has been clinically successful in acute

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burn patient trials.[10] CSS use is associated with reduced mortality from massive burns using a minimum amount of available donor skin.[10,11] CSSs are fabricated by manually inoculating a collagen-based sponge with autologous dermal fibroblasts and epidermal keratinocytes.[12] It is desirable to confirm uniform cell distribution and viability within the sponge to evaluate the suitability of the initial bioengineered constructs for subsequent application to the patient. Currently, cell viability of CSSs is determined according to point assessments using punch biopsies, which are destructive, reduce the available surface area of CSSs for clinical use, and can only assay a small fraction of the total graft area. For these reasons, a non-destructive assay of cell viability in the entire engineered tissue would represent an important advancement in quality assurance for clinical use.

The aim of this project was to develop a non-destructive field assessment of cell viability in cultured skin *in vitro*, using fluorescein diacetate (FdA) staining and computer-assisted planimetry. Fluorescence was successfully demonstrated with CSSs; the assay accurately reflected the presence of viable cells at the time of staining and predicted viability at a later time points. The toxicity of the staining conditions was thoroughly tested on CSSs and monolayer cultures of fibroblasts and keratinocytes.

MATERIALS AND METHODS

Cultured skin substitute fabrication

Cultured skin substitutes were fabricated according to previously described protocols.[13,14] Briefly, human fibroblasts (HFs) and keratinocytes (HKs) were isolated from tissue discarded after surgery and grown in selective growth medium.[15] HFs and HKs were inoculated on collagen–glycosaminoglycan (GAG) scaffolds fabricated via freeze-drying.[16] HFs were inoculated onto the collagen–GAG sponges at a density of 0.5×10^6 cells/cm^2 and incubated at 37°C and 5% carbon dioxide (CO_2) in UCMC 160 medium.[15] After 1 day of incubation, the sponges were inoculated with HKs at a density of 1×10^6 cells/cm^2. The following day (CSS incubation day 1), the CSSs were placed onto perforated stainless steel platforms covered by a cotton pad to establish an air–liquid interface and incubated up to 21 days, with the CSS culture medium replaced daily.

FdA assay

The methodology for staining viable cells using fluorescein diacetate developed by Jones *et al.*[17] was modified for use with standard CSSs. A stock solution of fluorescein acetate salt in acetone (5 mg/mL; Sigma, St. Louis, MO) was made and stored at 4°C, protected from light. A fresh dilution of FdA in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffered saline (HBS) was prepared at the time of each assay. Various dilutions (0.01, 0.02, 0.04, and 0.1 mg/mL) of FdA–HBS were prepared to determine the concentration of FdA staining that would provide the best contrast between cell-dense and cell-free CSSs. The FdA–HBS solutions were vacuum filtered to ensure sterility, using a 0.2–μm-pore filter (Pall Corporation, East Hills, NY). The solutions were warmed to room temperature before use and protected from light. For all preliminary experiments, a 2-×2-cm square of CSSs was cut using sterile technique and transferred to a separate culture dish, along with a 2-×2-cm square of rehydrated collagen without cells. Freshly diluted FdA solution was poured into the culture dish until the CSS pieces floated in the dish. The lid was replaced and the dish shielded from light. After soaking in the FdA solutions for various time intervals (2, 5, 10, 20, and 40 min), the CSS samples were exposed to UV light for 10 s to evaluate the contrast in fluorescence between the control and cell-populated collagen sponges. Three replicate samples were used to test each FdA concentration at 5 time points each.

After the optimal exposure time and concentration were determined, it was necessary to establish the most advantageous time point in the culture period for using the FdA assay with CSSs. Samples of CSSs ($n=3$) were stained with 0.04 mg/mL FdA for 20 min at different time points (3, 5, and 7 days) after keratinocyte inoculation.

Correlation of FdA intensity with inoculation density and 3-(4,5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide assay

To determine whether FdA fluorescence accurately reflects inoculation density, rehydrated collagen–GAG scaffolds were inoculated with fibroblasts only. Collagen–GAG scaffolds were placed into 6-well-plate Snapwell inserts (1.13 cm^2 growth area; Corning, Inc., Corning, NY) that had their polycarbonate membranes removed. Scaffolds were inoculated with fibroblasts at different densities (0/cm^2, 5E3/cm^2, 1E4/cm^2, 5E4/cm^2, 1E5/cm^2, 5E5/cm^2, 1E6/cm^2, 5E6/cm^2; $n=8$ for 1E4–5E5/cm^2 and $n=4$ for 1E6–5E6/cm^2). Collagen–fibroblast constructs (CFCs) were exposed to FdA 1 day after inoculation to prevent normalization of inoculation density differences with time. After a 20-min exposure to 0.04 mg/mL of FdA, the CFCs were transferred to an empty 150-mm culture dish (Corning. Inc.) for photographs. The lid of the culture dish was removed, and a camera hood (Electrophoresis Systems, Fisher BioTech, Pittsburgh, PA) was placed in the dish to block out all other light. A 312-nm emission UV lightbox (Fisher BioTech) was lit, and the resultant CFC fluorescence was captured on Polaroid black-and-white film, type 667 (Sigma). A photo of each CFC was scanned, and the fluorescence was quantified using Metamorph software (Molecular Devices Corporation, Downington, PA). The average fluorescence intensity for the acellular grafts (0E5 cells/cm^2) or the background was subtracted from the cell-containing groups. For each CFC, the average fluorescence intensity of the circular construct was correlated with the inoculation density, using

the Pearson correlation coefficient. In this way, the variability in the regional cell density could be determined.

To determine whether this assay could reliably determine cell viability of the CSSs, which contain fibroblasts and keratinocytes, at the time of staining and 7 days after staining, collagen–GAG scaffolds were placed into 6-well-plate Snapwell inserts (Corning, Inc.) that had their polycarbonate membranes removed. HFs and HKs were inoculated onto the collagen at 0, 0.125, 0.25, 0.5, 1, and 2 times standard inoculation density (HFs = 5.0E5/cm^2, HKs = 1.0E6/cm^2, $n = 8$/group), with the medium replaced daily. The CSSs were exposed to FdA 3 days after HK inoculation, and an 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was performed immediately. Average fluorescence intensity (with the background substracted) for each CSS was then correlated with the MTT values. In separate experiments, CSSs ($n = 8$/inoculation density) were stained with FdA at day 3, photographed under sterile conditions, and returned to the 6-well plates with fresh medium. After an additional 7 days in culture, an MTT assay performed on the CSS and MTT values at day 10 were correlated with fluorescence intensity at day 3.

To perform the MTT assay, each Snapwell insert was incubated with 4 mL of MTT stock solution (0.5 mg MTT/mL PBS) for 3 h. After 3 h, the MTT solution was removed and 1 mL of ethylene glycol (Sigma) was added and agitated for 3 h. The amount of MTT–formazan product released was measured at 590 nm on a microplate reader (Spectracount, Packard Bioscience Corporation, Meriden, CT), with values reported as mean optical density ± standard error of the mean (SEM). To ensure that the initial inoculation density and MTT values accurately represented the number of cells within the CSSs, deoxyribonucleic acid (DNA) quantification was performed on grafts that were inoculated at 0, 0.125, 0.25, 0.5, 1, and 2 times standard inoculation density (HFs = 5.0E5/cm^2, HKs = 1.0E6/cm^2, $n = 5$/group). Each circular CSS was divided in half, and an MTT assay was performed on one half, and a 6-mm biopsy punch taken from the other half for DNA quantification. DNA was isolated from the biopsies using an Easy-DNA kit (Invitrogen, Carlsbad, CA) and quantified using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). DNA content was reported as total ng ± SEM.

Toxicity

The toxicity of the FdA staining procedure was first determined using monolayer cultures of fibroblasts or keratinocytes that were inoculated at 2 different densities in 6-well plates (Corning, Inc.). Fibroblasts or keratinocytes were inoculated into the wells at 1E5 or 5E5 cells/well (1.05E4 cells/cm^2 or 5.26E4 cells/cm^2). Medium was changed in the wells every day (UCMC 160 for HF, 153 for HK). One day after inoculation, the medium was aspirated in all the wells and replaced with freshly prepared FdA solution (0.04 mg/mL in HBS) for the experimental group, an acetone–HBS solution (1:125) to examine the delivery vehicle, and HBS for the control. After leaving the dishes at room temperature for 20 min, the solutions were aspirated from the wells, and the FdA exposed dishes were exposed to the UV light. Wells were then rinsed twice with the appropriate medium and returned to incubate for an additional 2 h. The metabolic activity of the cells was then assayed using AlamarBlue (Biosource International, Inc., Camarillo, CA) at culture days 1, 3, 5, and 7 ($n = 12$ at each time point). To perform the AlamarBlue assay, the medium was removed from the wells and replaced with a 1:10 dilution of alamarBlue in culture medium. The cells were incubated at 37°C and 5% CO_2 for 3 h, and the fluorescence was read at 590 nm emission (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA). Absorbance counts were collected for each well at each time point and reported as relative fluorescence units ± SEM. In addition, cell morphology was qualitatively assessed using bright-field microscopy. Images of cells in the 6-well plates were taken after 1 day in culture and at 1-day intervals after exposure to HBS, acetone–HBS or FdA–HBS and UV light.

The toxicity of the FdA to CSSs was determined using standard CSSs (5E5 HF/cm^2 and 1.0E6 HK/cm^2). Experimental CSSs were exposed to FdA and UV light at culture day 3, whereas control CSSs were exposed only to HBS. An MTT assay was performed on the CSSs 2 and 7 days after FdA exposure. MTT absorbance was reported (mean ± SEM). Biopsies for histological evaluation were collected before and after exposure to FdA to characterize any changes to the CSS anatomy as a result of the FdA staining procedure.

Animal studies

The University of Cincinnati Institutional Animal Care and Use Committee approved all procedures using animals in this study, which followed National Institutes of Health guidelines. At culture day 14, control CSSs and CSSs that had been exposed to FdA at culture day 6 were grafted onto 2-× 2-cm full-thickness wounds prepared surgically in athymic mice (nu/nu, Jackson Labs, Bar Harbor, ME; $n = 3$ per condition). An occlusive dressing with antibiotic ointment was applied to the graft as previously described.[18] Two weeks after surgery, dressings and stent sutures were removed, and animals were maintained without dressings for the remainder of the test period. Photographs of the wound were taken at weekly intervals from 2 to 4 weeks. The animals were euthanized at week 2, and graft biopsies were taken from each animal for paraffin embedding. The paraffin sections were hematoxylin and eosin stained to assess cellular organization.

Statistical analyses

All data was analyzed using SigmaStat 3.10 (Systat Software, Inc., San Jose, CA). Correlations were determined

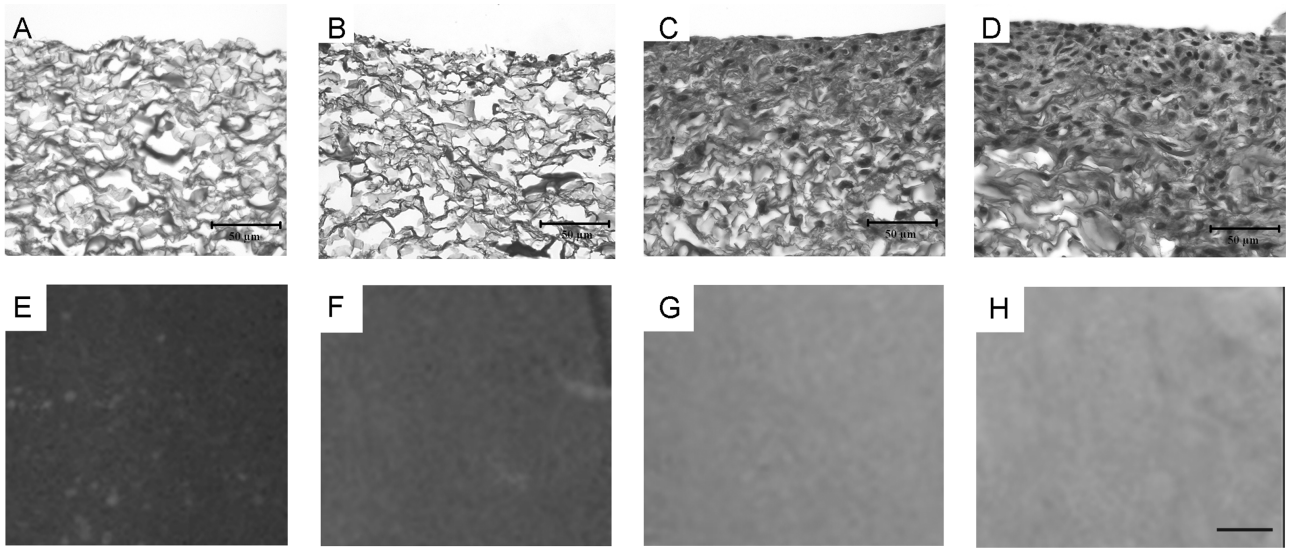

FIG. 1. Qualitative assessment of collagen–glycosaminoglycan (GAG) sponges inoculated with fibroblasts at variable densities; 0 cells/cm^2 (**A and E**), 5E4 cells/cm^2 (**B and F**), 5E5 cells/cm^2 (**C and G**), and 5E6 cells/cm^2 (**D and H**). Black-and-white photographs of fluorescein intensity via exposure to a fluorescein diacetate–N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffered saline solution (0.04 mg/mL) for 20 min (**E–H**) and hematoxylin and eosin–stained cross-sectional histology of collagen–GAG sponges embedded in paraffin (**A and D**). Scale bar = 200 μm (**A–D**) and 1 cm (**E–H**).

using the Pearson correlation coefficient. Differences between the FdA-exposed and control groups were analyzed using Student *t*-tests. *P* values of 0.05 or less were considered statistically significant.

RESULTS

CSSs were exposed to increasing dilutions (0.01, 0.02, 0.04, and 0.1 mg/mL) of FdA in HBS at time intervals spanning 2 to 40 minutes. At time points less than 20 min, no significant difference in fluorescence between CSSs and acellular controls was observed at concentrations less than 0.04 mg/mL (data not shown). A combination of 0.04 mg/mL and 20 min produced the most significant contrast between acellular scaffolds and CSSs with the least amount of exposure time and FdA and was therefore used for all subsequent experiments.

The accuracy of this staining procedure was demonstrated through correlation of CFC fluorescence with fibroblast inoculation density. A qualitative assessment of fluorescence of different fibroblast inoculation densities on collagen is depicted in Figure 1E–H, which was also histologically confirmed according to an observed increase in fibroblast density (Fig. 1A–D). As can been seen in Figure 1E, acellular collagen–GAG sponges weakly autofluoresce, and when this autofluorescence was quantified, the sponges had an average fluorescence intensity of 22.09 ± 5.26. The background autofluorescence was subtracted from each cell-containing group. A correlation coefficient of 0.853 was obtained after an analysis of MTT and fibroblast-inoculated collagen samples (Fig. 2).

CSSs, inoculated at increasing densities, were exposed to FdA, and fluorescence values correlated significantly with MTT values ($r = 0.857$; Fig. 3A). Fluorescence also correlated with initial inoculation density of the CSSs ($r = 0.847$, Fig. 3B). DNA content also strongly correlated with MTT (Fig. 3C; $r = 0.903$) and inoculation density (Fig. 3D; $r = 0.926$). The effectiveness of day 3 CSS fluorescence intensity at predicting MTT values of CSSs at incubation

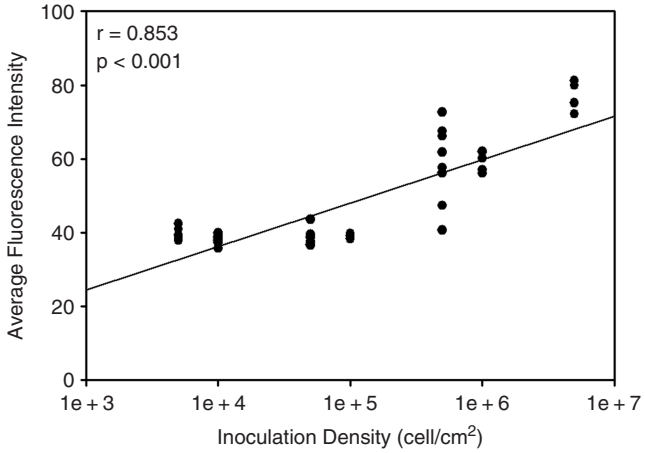

FIG. 2. Quantitative correlation between average fluorescein intensity measured with Metamorph software and fibroblast inoculation density 1 day after fibroblast inoculation.

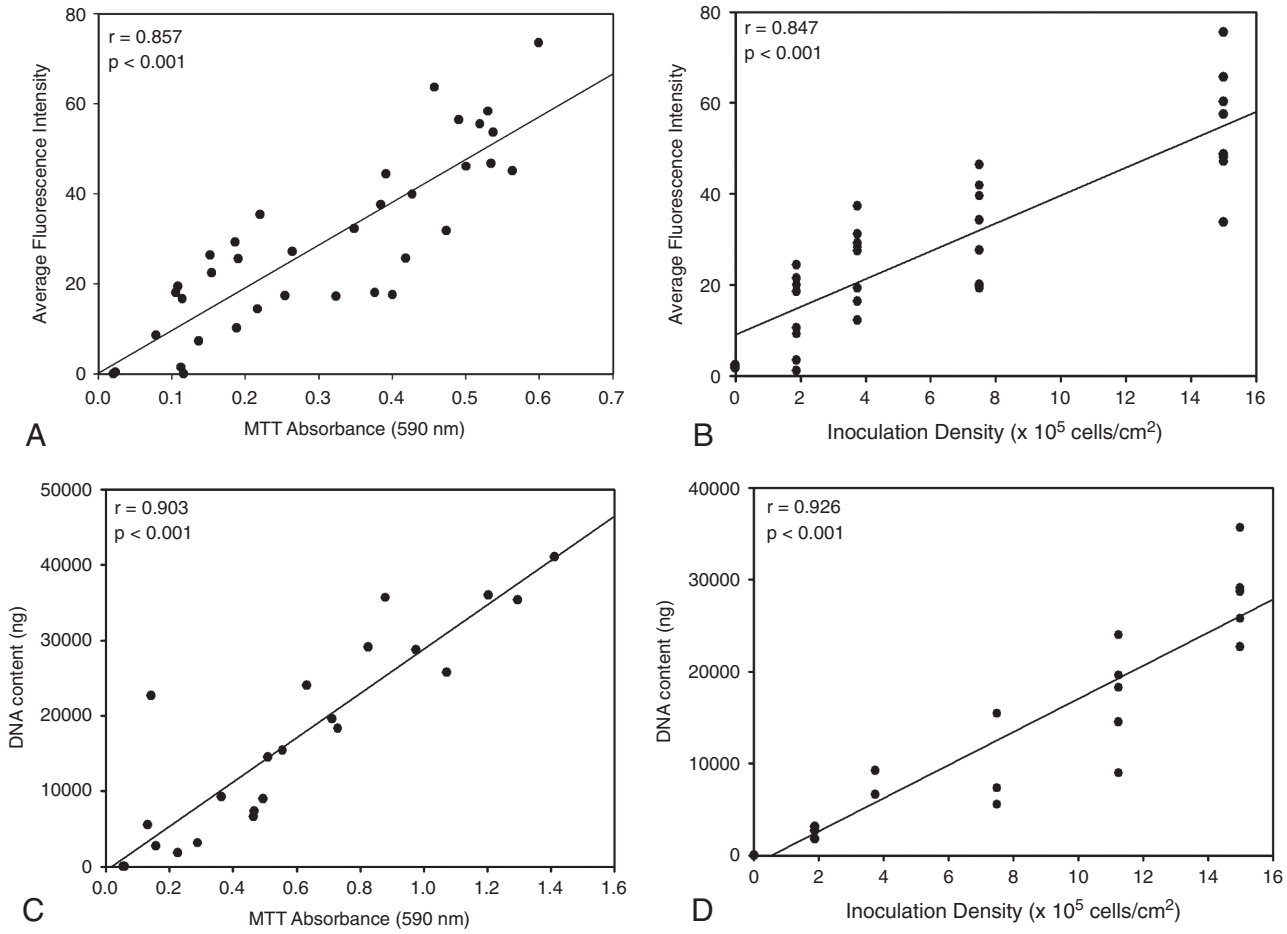

FIG. 3. Correlation between fluorescein intensity of cultured skin substitutes (CSSs) and immediate 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) absorbance (**A**) or inoculation density (**B**) at day 3. Correlation of total deoxyribonucleic acid content from 6-mm punch biopsies and immediate MTT absorbance (**C**) or inoculation density of the CSSs (**D**).

day 10 was also assessed. Early CSS fluorescence intensity positively correlated with late MTT values, with a correlation coefficient of 0.712 (Fig. 4).

Experiments were conducted to detect the toxicity of the assay components on the cells. Toxicity of the drug vehicle (acetone) and the combination of the vehicle and drug (FdA–HBS solutions, 1:125 acetone:HBS) was demonstrated in monolayer cultures of keratinocytes and fibroblasts. AlamarBlue values for monolayer cultures of HFs inoculated at 5E5 cells/well were significantly lower at 1 and 3 days after exposure to acetone or the FdA–HBS solution and UV light (Fig. 5A). At later time points, reduced viability was not seen in the fibroblasts (Fig. 6A, B). A similar phenomenon was seen in monolayer cultures of HKs at 5E5 cells/well that had reduced cell viability in FdA–UV and acetone-exposed wells at day 1 and FdA–UV treated wells at day 5 but no reduction in cell viability at day 7 (Fig. 5A, B, $n = 12$ per group). Additionally, there were no visible changes in cell morphology in either cell type (data not shown).

In contrast, no toxicity, either delayed or immediate, was seen in FdA-exposed CSS samples in terms of MTT values of 6-mm punch biopsies (Fig. 6). Two days after the FdA and UV light exposure, no significant toxicity was found ($p = 0.47$, $n = 18$). Punch biopsies were also obtained on CSS samples kept in culture for 1 week after FdA and UV light exposure. An analysis of 18 samples did not demonstrate any significant delayed toxicity to the CSS samples ($p = 0.84$). Histology of CSS samples exposed to FdA and UV light did not reveal any significant change in tissue morphology (Fig. 7A). CSSs grafted on full-thickness wounds in athymic mice ($n = 3$/group) healed well, with normal graft histology demonstrated 14 days post-operatively (Fig. 7B).

DISCUSSION

The purpose of this study was to develop a non-destructive assay that could reliably measure cell viability in a 3-dimensional skin substitute *in vitro*. A number of *in vitro* cell viability assays have been developed more specifically for use in 2-dimensional cell populations, but limitations may arise when these assays are applied to 3-dimensional

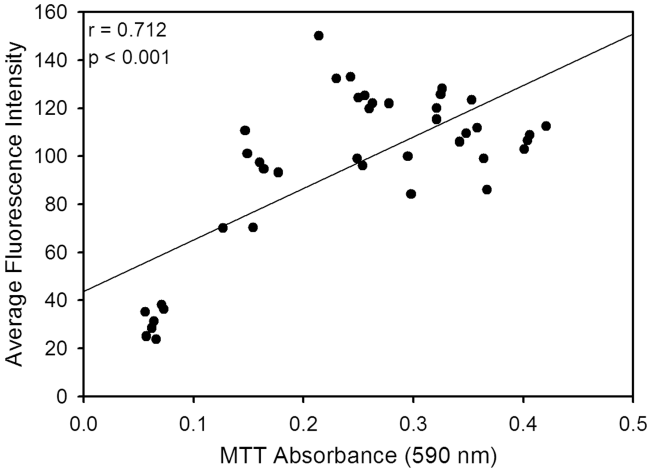

FIG. 4. Correlation between CSS fluorescein intensity at day 3 and 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide absorbance at day 10.

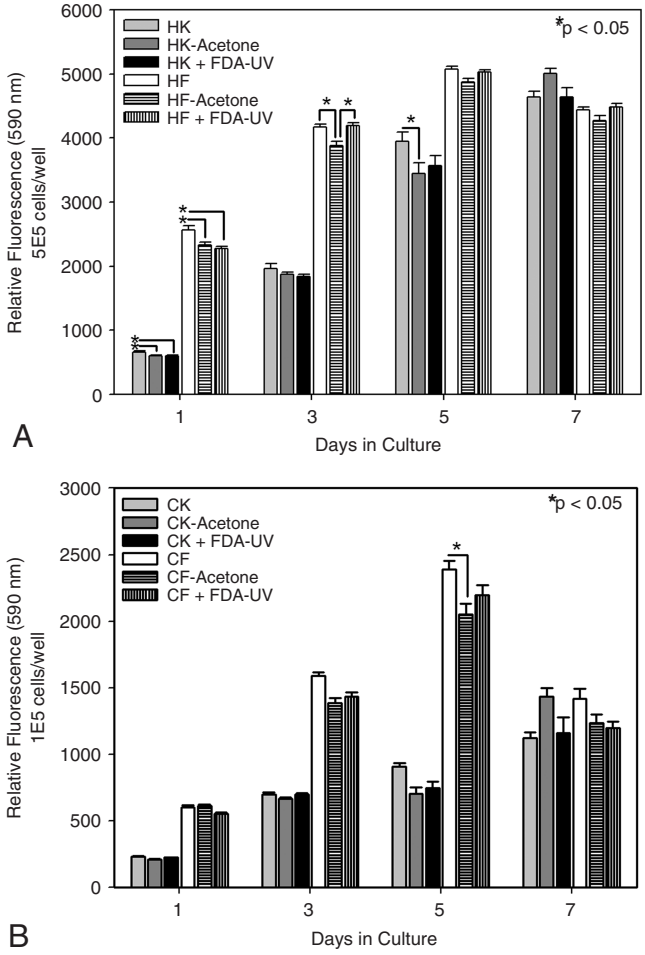

FIG. 5. AlamarBlue assay of monolayer cultures of keratinocytes or fibroblasts exposed for 20 min to N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffered saline (HBS) alone, the fluorescein diacetate (FdA) vehicle, acetone at a ratio of 1:125 with HBS, or FdA-HBS and ultraviolet irradiation for 10 s. Cultures were exposed to the stimuli 1 day post-inoculation, with the assay performed at culture days 1, 3, 5, and 7. (**A**) High- (5E5 cells/well) and (**B**) low- (1E5 cells/well) cell-density cultures.

cell-seeded scaffolds. Radioactive labeling of viable cells can be achieved through the addition of low concentrations of ^{3}H-glycine to the culture medium.[19] The inconvenience associated with the long half-life of radioactive isotopes such as ^{3}H (12.35 years) precludes their use with clinically implantable tissues.[19] Live/dead fluorescent labeling using 5-chloromethylfluorescein diacetate and ethidium homodimer-1 was evaluated as a means of assaying cell viability in cultured cancellous bone, but significant background staining of the hydroxyapatite matrix precluded any specific measurement of cell viability.[20] Pancreatic islet viability can be accurately determined with SYTO-13/ethidium bromide or calcein AM/ethidium homodimer stains, both of which are destructive.[21] Intervertebral disc explants were assayed for cell viability using cellular uptake of a fluorescent marker after a 4-day incubation with 50 µmol/L of dextran conjugated to tetramethylrhodamine.[22] However, cell quantification could only be performed using confocal microscopy on frozen cross-sections of the disc tissue; thus, cell viability was not preserved. Likewise, the commonly used MTT and the lesser known 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays also result in tissue destruction following exposure to the assay conditions.[23] These assays are essential for experimental tissue research but might be prohibitively costly in the context of clinical tissue production quality assurance.

Quality assurance measurements of CSSs before grafting are essential to ensuring proper engraftment and wound healing. Currently, point assessment of keratinocyte differentiation in conjunction with microscopic analysis of histological sections at days 6 and 9 are used to determine the quality of the CSSs before grafting. An assessment that could quantify the viability of the entire skin substitute and predict the viability of the CSSs earlier in the culture period could provide significant cost benefits (reductions in labor and media consumption) from the early elimination of suboptimal grafts. A significantly positive correlation between fluorescence intensity and immediate MTT values was established in CSSs at day 3 (Fig. 3A; $r = 0.857$, $p < 0.001$), and CSS fluorescence at day 3 positively correlated with MTT values at day 10 (Fig. 4; $r = 0.712$). These results suggest that the staining technique could be used as a valid, quantitative assessment of cell viability in engineered tissues.

Exposure of HFs and HKs to the FdA vehicle, acetone, and FdA-HBS and UV light induced a small amount of cytotoxicity in the high-density cultures (5E5/well) at days 1, 3, and 5 post-exposure (Fig. 6A) but was not apparent at 7 days post-exposure. The FdA vehicle and FdA-UV–exposed group had similar fluorescence values in the AlamarBlue assay, suggesting that the observed toxicity was largely due

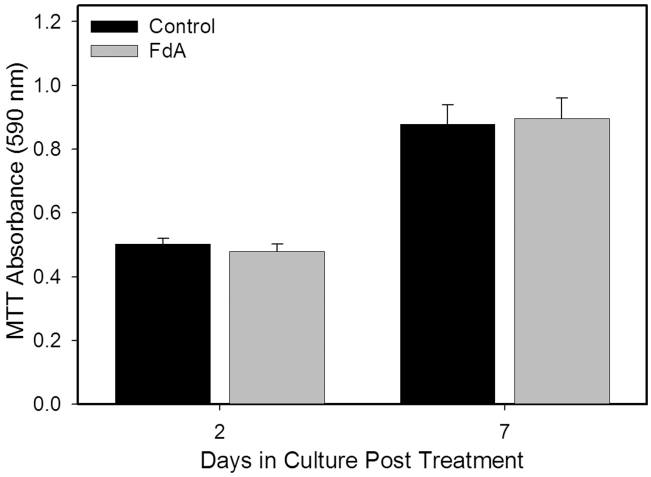

FIG. 6. 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay of control and fluorescein diacetate (FdA)-exposed cultured skin substitutes 2 and 7 days after exposure to FdA. No significant difference in MTT values was found between the control and experimental groups.

to the FdA vehicle and not the FdA itself or the brief exposure to UV light. Exposure to FdA and UV or acetone alone at any cell density or time point did not alter cellular morphology (data not shown). No immediate nor delayed toxicity was seen in the CSSs (Fig. 6), with no changes in cell organization of the CSSs in response to the FdA (Fig. 7A). Further experiments would be needed to determine whether a different vehicle could be used to reduce the cytotoxicity in monolayer culture, although because no toxicity was demonstrated in the CSS samples, the relevance of these additional tests would be secondary. Additionally, no alterations in engraftment of CSS treated with FdA were seen along with similar histological appearance of control and FdA-exposed grafts *in vivo* (Fig. 7B). Thus it is likely that the levels of FdA to which the CSSs are exposed present no clinically significant toxicity. Therefore, it is possible that the FdA assay could be used clinically for CSS quality assurance.

CONCLUSION

A quantitative assessment of cell viability in bioengineered tissues is an essential measurement for pre-clinical experiments or clinical use. Thus, the viability assay must be simple to perform, rapid, accurate. and cost-effective. Fluorescein was used to non-destructively determine the viability of cells in CSSs. The intensity of fluorescence correlated positively with inoculation density and was shown to predict the viability of CSSs 7 days after exposure to FdA. In addition, FdA produces no significant toxicity to the CSSs, and the assay can be performed under sterile conditions. The use of fluorescein as a measure of cell viability in bioengineered tissues represents a quality assurance method that could be used to quantitatively and non-destructively assess an entire engineered tissue before transplantation.

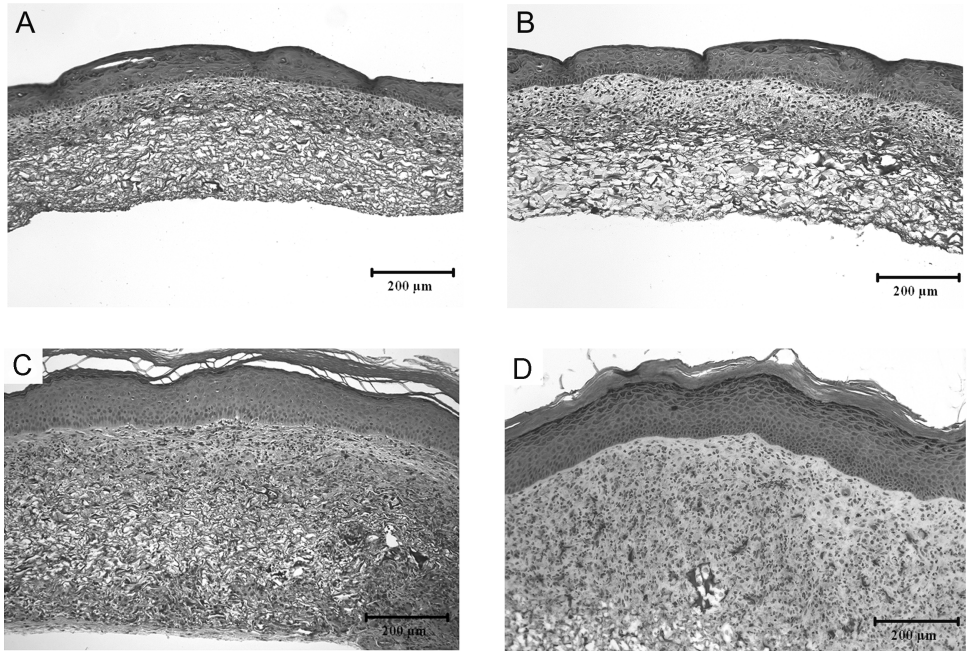

FIG. 7. Histological images of hematoxylin and eosin–stained biopsies from (**A**) control and (**B**) fluorescein diacetate (FdA)-exposed cultured skin substitutes (CSSs) at culture day 7 and healed skin 2 weeks after grafting with control (**C**) and FdA-exposed CSSs (**D**). Scale bar = 200 µm.

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