Chapter 13

Fluorescein Diacetate for Determination of Cell Viability in 3D Fibroblast–Collagen–GAG Constructs

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Abstract

Quantification of cell viability and distribution within engineered tissues currently relies on representative histology, phenotypic assays, and destructive assays of viability. To evaluate uniformity of cell density throughout 3D collagen scaffolds prior to in vivo use, a nondestructive, field assessment of cell viability is advantageous. Here, we describe a field measure of cell viability in lyophilized collagen–glycosaminoglycan (C–GAG) scaffolds in vitro using fluorescein diacetate (FdA). Fibroblast–C–GAG constructs are stained 1 day after cellular inoculation using 0.04 mg/ml FdA followed by exposure to 366 nm UV light. Construct fluorescence quantified using Metamorph image analysis is correlated with inoculation density, MTT values, and histology of corresponding biopsies. Construct fluorescence correlates significantly with inoculation density ($p < 0.001$) and MTT values ($p < 0.001$) of biopsies collected immediately after FdA staining. No toxicity is detected in the constructs, as measured by MTT assay before and after the FdA assay at different time points; normal in vitro histology is demonstrated for the FdA-exposed constructs. In conclusion, measurement of intracellular fluorescence with FdA allows for the early, comprehensive measurement of cellular distributions and viability in engineered tissue.

Key words: Quality assurance, Tissue engineering, 3D scaffold, Cell viability, Intracellular fluorescence

1. Introduction

Engineered tissues have the potential to replace or repair diseased or damaged tissues. Their outcome in vivo is highly correlated with their viability and function prior to implantation (1–3). A large number of engineered tissues are fabricated by the inoculation of single or multiple cell types onto three-dimensional (3D) tissue engineering scaffolds (4–7). For example, engineered skin is formed by serial inoculation of human autologous fibroblasts and keratinocytes on a 3D collagen scaffold (8–11). Engineered blood vessels can be made by inoculating vascular smooth muscle cells on

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3D polyurethane scaffolds (12). Autologous endothelial cell-seeded heart valves are designed to minimize thrombogenicity postimplantation (13). Bone tissue has been engineered by seeding human mesenchymal stem cells on decellularized trabecular bone (14). As cell distribution, density, and viability are intimately linked with tissue function, it is essential to evaluate uniformity of cell distribution and viability within a 3D scaffold to assess the suitability of engineered tissues for clinical application/implantation. Quality assurance of engineered tissues is commonly determined using histology, functional assays of differentiated phenotypes, such as surface hydration or transepidermal water loss for skin (15–17), contractility in response to endothelin-1 for vessels (18), platelet activation and adherence assays for heart valves (13), and metabolic viability assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (11, 14, 17, 19). These assessments are often destructive, thereby reducing the tissue available for clinical use, and/or can only assay a small fraction of the total tissue area. Thus, a nondestructive assay of cell viability in the entire engineered tissue would represent an important advancement in preclinical quality assurance.

Fluorescein has been used in the laboratory to assess the in vitro viability of a wide variety of cell types and tissues including human fetal cerebral cortical cells (20), human keratinocytes (21), and ovine articular cartilage (22), and to assess skin flap viability (23) and burn depth (24) in vivo. By virtue of its bipolar side chains, fluorescein diacetate (FdA) easily penetrates the cell membrane. FdA remains colorless until the acetate moieties in FdA are cleaved nonspecifically, by intracellular esterases which convert the nonfluorescent FdA to fluorescein (25). Metabolically active cells with intact cell membranes can then be visualized under ultraviolet (UV) light (26, 27); the intensity of fluorescence seen under UV light is therefore directly proportional to the number of viable cells.

FdA staining and computer-assisted planimetry allow direct visualization of cell distribution and viability in vitro. The FdA assay accurately reflects the presence of viable cells when compared to traditional destructive assays and can be used to predict viability at a later time points with little to no cytotoxicity. Care must be taken when performing the assay to properly quantify scaffold autofluorescence and to calibrate the FdA intensity to known cell densities.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum (FBS) and 1% antibiotic–antimycotic for dermal fibroblast culture.

2. HEPES-buffered saline (HBS): HEPES is dissolved in tissue culture water (see Note 1) at 30 mM, with 10 mM dextrose, 3 mM potassium chloride, 0.13 M sodium chloride, and 1 mM sodium phosphate heptahydrate, pH to 7.40, sterile filter, and stored at 4°C.
3. Solution of trypsin (0.025%) and ethylenediamine tetraacetic acid (EDTA, 1 mM) in HBS.
4. Water bath.
5. Fetal bovine serum.
6. Hemocytometer.
7. Inverted brightfield microscope (Nikon, Melville, NY).
8. Refrigerated tabletop centrifuge.
9. Sterile stereological and aspirating pipettes.

2.2. 3D Collagen–GAG Scaffolds

1. Fibrous collagen from bovine hide (Kensey Nash, Exton, PA) dissolved in 0.5 M acetic acid at 0.6 wt/vol%.
2. GAG–acetic acid solution: Chondroitin-6-sulfate dissolved in acetic acid at 0.35 wt/vol%.
3. Cold room (4°C) (see Note 2).
4. Digital stir plate and stir bar.
5. Syringe pump.
6. 60 ml syringe.
7. Casting frame: Rectangular metallic mold (aluminum plate and bar stock, Lyon Industries, South Elgin, IL) formed out of two sheets of metal separated by a 1-mm thick and 5-mm wide rubber spacer (Small Parts Inc., Miramar, FL) fitted around the edges of the metal. A cavity (20 cm × 10 cm × 1 mm) is created by the spacers.
8. –80°C Freezer.
9. 95% Ethanol (EtOH) bath. 2 l of ethanol poured into a 24 in. × 12 in. metal container.
10. Lyophilizer-Virtis Advantage XL (SP Industries, Gardiner, NY).
11. Vacuum oven.
12. Cryo-gloves.

2.3. Cell-Scaffold Construct Preparation

1. Cells from Subheading 2.1.
2. 150-mm cell culture dish.
3. Variable speed pipette-aid.
4. Merocel (Medtronic/XoMed, Minneapolis, MN).
5. N-terface (Winfield, TX).
6. Blunt edge forceps.

7. Bunsen burner.
8. Glass beaker containing 95% ethanol.

2.4. FdA Assay

1. FdA dissolved in acetone at 5 mg/ml and stored protected from light at 4°C.
2. UV lightbox (Fisher BioTech).
3. Camera hood (Electrophoresis Systems, Fisher).
4. Polaroid black and white film type 667.
5. Dell 1815 Scanner (see Note 3).
6. Image Analysis software (Metamorph) (see Note 4).
7. Snapwell cell culture inserts and tissue culture plate (Corning, NY).
8. Sterile surgical gloves.
9. Sterile 12 mm diameter biopsy punch (see Note 5).

2.5. Correlation of FdA Assay to MTT Assay and Total DNA Content

1. Thiazolyl blue tetrazolium bromide (MTT) dissolved in phosphate-buffered saline (pH 7.4) at 0.5 mg/ml, sterile filter, and stored at 4°C.
2. 2-Methoxy ethanol.
3. UV/Vis Spectrophotometer with 548-nm filter (Spectracount, Packard Bioscience Corporation, Meriden, CT).
4. Easy-DNA kit (Invitrogen).
5. ND-1000 spectrophotometer (Nanodrop, Wilmington, DE).
6. Statistical software (SigmaStat, San Jose, CA).

3. Methods

FdA rapidly penetrates the cellular plasma membrane and is cleaved to form fluorescein. The FdA solution at 0.04 mg/ml is sufficiently concentrated to allow detection of cells above any scaffold autofluorescence. Correlations between intensity of fluorescence can be made with cell number, punch biopsy histology, and viability. Assessment with FdA provides a global, nondestructive measure of cell distribution and viability within 3D scaffolds.

We developed a standardized, quantitative assay using FdA to evaluate fibroblast density and distribution across a collagen–glycosaminoglycan scaffold. This assay was necessary for us to be able to monitor cell inoculation uniformity when using larger surface area scaffolds or different scaffold preparations. The methods

described here outline the steps to prepare a standard collagen scaffold inoculated with fibroblasts, and to evaluate its cell density 1 day later using the FdA assay. The fibroblast-inoculated scaffolds are immersed in the FdA solution at a nontoxic concentration, photographed immediately under fluorescent conditions, and rinsed prior to being returned to the incubator in fresh culture media. We also outline the techniques for and results of correlating the nondestructive FdA assay with the destructive MTT assay and DNA content measurements with this construct. The toxicity of the FdA assay has been evaluated with respect to keratinocytes, fibroblasts, fibroblast-inoculated scaffolds, and keratinocyte–fibroblast-inoculated scaffolds (28). Fluorescence can be quantified according to (1) the average over a defined surface area (Fig. 2), (2) the degree of variability between the maximum and minimum fluorescence over a given surface, or (3) the percent surface area above a defined threshold fluorescence. By establishing standard curves for fluorescence by inoculation density, MTT assay, or total DNA content, a suitable threshold can be determined for specific cell-scaffold combinations.

3.1. Preparation of Collagen Scaffolds

1. The raw collagen from bovine hide is mixed with 0.5 M acetic acid at 300 rpm on a stir plate within a refrigeration room (see Note 6). Mix continuously for 24 h.
2. Calculate the amount of GAG–acetic acid solution required for scaffold fabrication (add 1 ml of GAG solution for every 6.5 ml of collagen solution).
3. Fill the syringe with the appropriate amount of solution. Using the syringe pump, slowly add (~12 ml/h) GAG–acetic acid mixture to collagen–acetic acid solution. Mix for a total of 6 h.
4. Pour solution into a casting frame and freeze in the ethanol bath at –80°C for 1 h.
5. Remove the top section from the casting mold (see Note 7) and place the bottom section and frozen collagen–GAG solution into prechilled lyophilizer.
6. Lyophilize the frozen solvent and place the resultant porous collagen–GAG scaffold into vacuum oven set to 140°C and 30 mmHg for 24 h.
7. Cut collagen–GAG scaffold to desired size and sterilize by placing scaffold into a sterile culture dish filled with 70% EtOH. Procedure performed in class II biological safety cabinet (BSC). Alternatively, the scaffolds may be packaged into peel packs, and sterilized by gamma-irradiation (25 kGy) for later use.
8. Rinse with HBS for 10 min, repeat four times followed by 2–30 min rinses in cell culture medium (see Note 8).

3.2. Cell Harvesting

1. Warm fibroblast medium, HBS, and trypsin to 37°C in water bath.
2. Supplemented fibroblast culture medium is used to neutralize trypsin in fibroblast cultures.
3. Once fibroblasts have reached approximately 80% confluence, remove from incubator, aspirate medium, and rinse once with HBS for 2 min (see Note 9).
4. Aspirate HBS, add trypsin to each flask, and reclose flask cap. Place flasks in incubator for approximately 2 min.
5. Tap side of flask with hand to detach cells from growth surface.
6. Add equivalent amount of supplemented medium to flasks, rinse growth surface of flask with medium three times, remove cell-containing medium, and place into sterile conical tube.
7. Centrifuge at 1,000 rpm ($200 \times g$) for 7 min.
8. Resuspend and count cells using hemocytometer.

3.3. Preparation of Fibroblast-Scaffold Constructs

1. Using flame sterilized forceps, place a sterile piece of Merocel into a 150-mm culture dish. Immerse in cell culture medium. Rinse twice.
2. Place an 8 cm × 8 cm square of sterile N-terface on the Merocel followed by the collagen scaffold (see Note 10).
3. Aspirate excess medium from Merocel containing dish.
4. Inoculate collagen scaffold with desired density of fibroblasts (e.g., $1 \times 10^4/\text{cm}^2$).
5. Add medium to dish until medium level reaches the top of the Merocel. Do not allow medium to flow over the top of the Merocel. Incubate at 37°C, 5% CO_2 for 3 h (see Note 11).
6. Lift cell-scaffold constructs on the N-terface from the Merocel and place into a 150-mm cell culture dish containing 50 ml medium.
7. Culture for up to 21 days with medium changed daily.

3.4. FdA Assay

1. Warm HBS and cell culture medium to 37°C.
2. Wipe UV lightbox with 70% EtOH and place into BSC (see Note 12).
3. Using stock solution of FdA in acetone, mix a fresh dilution of 0.04 mg/ml of FdA in HBS. Sterile filter into foil covered bottle (see Note 13).
4. Remove cell–collagen construct from incubator and aspirate medium (see Note 14).
5. Add FdA solution being careful to fully saturate all sides of construct. Allow construct to soak in solution for 20 min at room temperature.

6. Aspirate FdA solution and rinse cell–collagen construct thoroughly with HBS.
7. Transfer cell–collagen construct to empty sterile 150-mm cell culture dish and place dish on UV lightbox (see Note 15).
8. Turn lightbox on for 20 s to convert the nonfluorescent FdA to fluorescein.
9. Wipe edges of camera hood with 70% EtOH.
10. Place camera hood over the dish to block out all other light. Turn on UV lightbox and take picture (1/8 s shutter speed) after lightbox has been on for 5 s (see Note 16).
11. Allow photo to develop for 60 s.
12. While photos are drying, transfer cell–collagen constructs back into cell culture dish containing medium and incubate. After 3 h, change medium to ensure that there is no residual FdA solution within the construct.
13. Scan photographs at 300 dpi in tiff format (Fig. 1).
14. Measure average fluorescence intensity using Metamorph software (see Note 17).
15. In Metamorph, use the select area tool to select just the cell–collagen construct within the photograph. Measure grayscale intensity values including average, high, and low. The average fluorescence intensity for the acellular grafts (0 cells/cm^2) or the background was subtracted from the cell containing groups (Fig. 1).

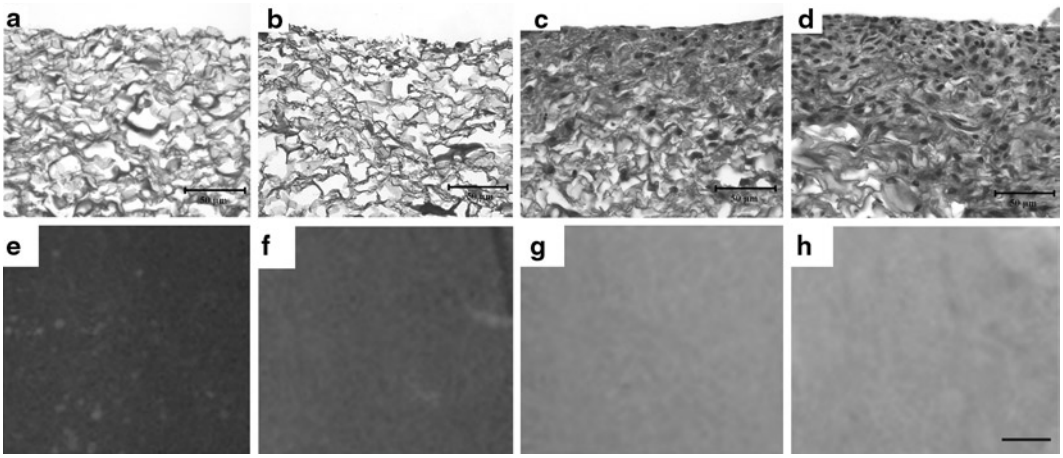

Fig. 1 Qualitative assessment of collagen–GAG scaffolds inoculated with fibroblasts at variable densities; 0 cells/cm^2 (**a**, **e**), 5E4 cells/cm^2 (**b**, **f**), 5E5 cells/cm^2 (**c**, **g**), and 5E6 cells/cm^2 (**d**, **h**). *Black* and *white* photographs of fluorescein intensity via exposure to FdA–HBS (5 mg/ml) for 20 min and H&E stained cross-sectional histology of collagen–GAG scaffolds embedded in paraffin (**a**, **e**). *Scale bar* = 200 μm (**a**–**d**) and 1 cm (**e**–**h**). (Reproduced from ref. 28, with permission from Mary Ann Liebert Inc.).

3.5. Standard Curves for FdA Intensity, Inoculation Density, DNA Content, and MTT Assay

1. Prepare collagen scaffolds as in Subheading 3.3.
2. Use sterile biopsy punch to cut 15 mm diameter circles in the rinsed collagen scaffold or use a sterile scalpel to cut small squares approximately 15 mm × 15 mm in size.
3. Place sterile Snapwell inserts and 6-well plate into BSC and remove lid.
4. Using sterile technique, remove lower ring from Snapwell insert (see Note 18).
5. Place lower ring with collagen scaffold back onto upper portion of insert affixing the collagen scaffold to the insert.
6. Harvest cells following Subheading 3.2. Inoculate cells onto collagen within Snapwell insert at 0, 5.0e3, 5.0e4, 5.0e5, and 5.0e6/cm^2. Adjust the total volume of inoculum to range between 250 and 500 µl.
7. Incubate for 1 day and perform FdA assay. Assay can be performed with scaffolds in the inserts or scaffolds can be removed prior to the assay.
8. After the assay has been performed, return Snapwell inserts to 6-well plate containing medium. Rinse twice with medium (30 min per rinse, return plate to incubator after each rinse).
9. Remove cell–collagen constructs from Snapwell insert and place into a new 6-well plate containing 4 ml of MTT–PBS solution.
10. Incubate for 3 h.
11. Remove MTT–PBS solution and add 4 ml of methoxy ethanol in a chemical fumehood. Place on a plate shaker for 3 h at room temperature.
12. Remove cell-scaffold constructs and read optical density on a spectrophotometer at 590 nm.
13. Perform steps 1–8 again with a new set of fibroblast-scaffold constructs.
14. After rinsing the constructs, remove the construct and perform the DNA quantification following the instructions provided by the Easy-DNA kit.
15. Use statistical software to correlate inoculation density, MTT absorbance values, and DNA content with FdA intensity values. For fibroblasts on collagen scaffolds the relationship is linear but this may not be true for all cell types on all types of scaffolds (Fig. 2) (see Note 19).
16. The standard curves can then be utilized to extract additional quantitative data from the FdA intensity values.

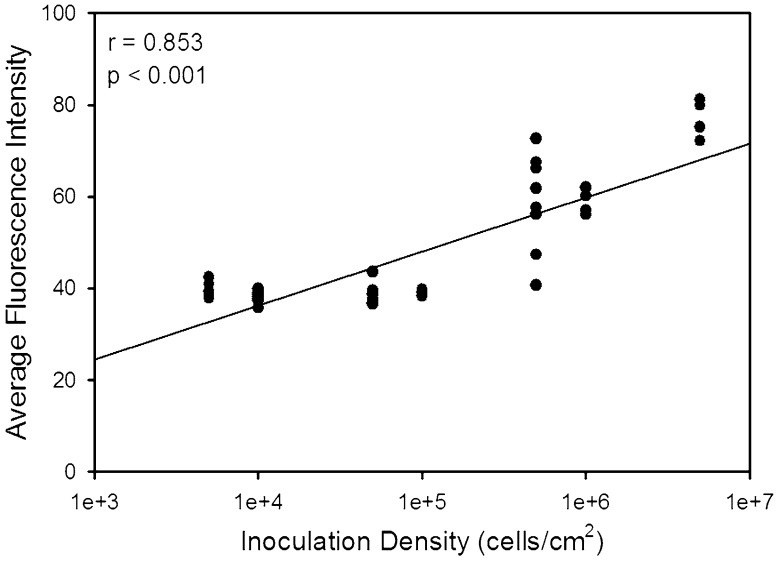

Fig. 2. Quantitative correlation between average fluorescein intensity measured with Metamorph software and fibroblast inoculation density 1 day after fibroblast inoculation. (Reproduced from ref. 28 with permission from Mary Ann Liebert Inc.).

4. Notes

1. All solutions should be prepared in water with a resistance of 18.2 MΩ cm. This is referred to as "water" in the text.
2. Cold room can be substituted by a stir plate within a 4°C refrigerator.
3. Most general office scanners will suffice. Caution is taken to avoid scanning the images at resolutions less than 300 dpi as significant pixilation can occur.
4. Other image software such as ImageJ may be used to calculate average grayscale intensity.
5. A biopsy punch is not essential. Collagen scaffolds can be cut into small square pieces using a sterile scalpel and prep blade or sterile scissors.
6. The quantity of collagen–GAG solution required is proportional to the area of collagen scaffold needed. 30 ml will form approximately 15 cm^3 ($15 \times 10 \times 0.1$ cm), or 150 cm^2.
7. When removing frozen collagen–GAG mold from casting frame, caution is required to open frames quickly and gently to avoid cracking.
8. Any commercially available scaffold can also be utilized for this assay. Be sure to quantify the autofluorescence for each scaffold type used.

9. All procedures involving cells and/or sterile scaffolds are performed within a BSC using aseptic technique.
10. Flame sterilized instruments (forceps, scalpel, prep blade) are used to manipulate sterile materials (collagen–GAG scaffolds, Snapwell inserts, etc.). Sterile technique is assumed for all procedures unless otherwise noted.
11. Cells will be incubated at 37°C, 5% CO_2. Unless otherwise noted these will be the incubation parameters.
12. To keep the cell-scaffold construct sterile, the entire assay will be performed within the BSC. All instruments brought into the safety cabinet will be wiped down with 70% ethanol prior to set up.
13. FdA is very light sensitive. Both the stock solution and working solution are formed without laboratory lights on.
14. Once items are removed from the incubator they are placed in the BSC. All procedures are performed in a BSC from this point forward.
15. Fluorescein can be excited at a wide range of wavelengths, with a maximal excitation at 494 nm (29). The FdA assay may be used either qualitatively or quantitatively. Following cell inoculation, cell adherence and localization on the matrix can be visualized topographically and evaluated for uniformity for quality control of the inoculation and collagen scaffold production methods. Such a qualitative analysis would require only a handheld Wood's lamp to visualize the fluorescence, as early as day 3 after inoculation. Alternatively, cell density and distribution on the matrix can also be quantified, in order to compare results between the groups.
16. Personal protective equipment (UV goggles or face shield) is required to avoid physical injury due to the UV light.
17. Quantification requires strict adherence to sterile cell culture techniques, including the use of a fluorescent UV light box and black and white camera in the laminar flow hood. The surface area which can be analyzed in this manner depends on the size of the camera hood available to capture fluorescent images. Once the photographic data is obtained, the fluorescence can be quantified using an image software program. The localization of the fluorescence can also be quantified in terms of the percent of the total surface area fluorescing over a defined threshold.
18. Sterile gloves can be donned to remove the lower rig from the Snapwell insert and for the insertion of the collagen–GAG scaffold.
19. The FdA assay can also be correlated to other biological properties such as total protein content.

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