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Detection of Viable and Non-Viable Cells in Connective Tissue Explants Using the Fixable Fluoroprobes 5-Chloromethylfluorescein Diacetate and Ethidium Homodimer-1

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The efficacy of connective tissue explants is difficult to determine, particularly where autopsy material is required for research or clinical applications. We report here an optimised protocol using 5-chloromethylfluorescein diacetate (CMFDA) and ethidium homodimer-1 to distinguish viable and non-viable cells in a range of connective tissue explants. Biopsies and explants of corneae, arteries, cartilage and skin were loaded with fluoroprobes for extended periods (≤ 24h) at low temperatures (4°C), fixed in paraformaldehyde, and processed using a variety of embedding, sectioning, autoradiographic, and immunohistochemical procedures. Detection of fluorescent green CMFDA and red ethidium homodimer was achieved using epi-illuminated light or dual channel confocal microscopy, and clearly differentiated live from dead cells throughout the explants. Furthermore, the intracellular distribution of CMFDA provided superior images of cell shape and morphology not previously available using conventional histochemical techniques. Adaptations of this protocol could prove valuable in a variety of research and clinical applications.

Key Words: *5-chloromethylfluorescein diacetate, ethidium homodimer-1, connective tissues, cell viability, confocal microscopy*

INTRODUCTION

The low ratio of cells to matrix in connective tissues poses particular problems for researchers investigating the metabolism of tissue explants and surgical biopsies maintained in organ culture. The reliability of data derived from such studies is dependant on the assumption that the majority of the relatively small numbers of cells in the sample remain viable. Where tissue samples are obtained at biopsy or immediately after death, there is usually little doubt expressed as to the efficacy of the preparation although viability is seldom assessed. In studies on human tissues however, autopsy specimens are frequently the only option, raising questions as to the viability of cells at the time of sampling. This is particularly important when tissue explants are collected for medium to long storage, as occurs with corneae and heart valves for transplantation.

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As an essential prerequisite to our studies on a range of connective tissues it became necessary to devise a protocol which would allow us to differentiate between viable and non-viable cells in small connective tissue explants. Four criteria were essential to the development of a protocol. (i) The size of the explant had to be of sufficient dimensions to allow for tissue dissection, dye diffusion and downstream processing. (ii) Fluorescent dyes were required which could diffuse into and differentially load viable and non-viable cells throughout the depth of the explant with no disruption of normal cellular metabolism. (iii) Loaded cells had to be aldehyde-fixable to allow long term preservation and storage with minimal tissue distortion or deterioration, and subsequent assessment of dye retention in morphologically intact specimens processed through a variety of embedding procedures. (iv) Fluorescent dyes had to be compatible with conventional epi-illuminated light microscopy and the specialised requirements of confocal laser scanning microscopy.

In this report we detail an optimised protocol for the simultaneous detection of viable and non-viable cells in a variety of connective tissue explants cultures loaded with

the fluorochromes 5-chloromethylfluorescein diacetate (CMFDA) and ethidium homodimer-1. CMFDA is a glutathione-reactive dye which freely diffuses into the cytoplasm of viable cells where it is catalysed by the ubiquitous enzyme glutathione S-transferase to produce a cell-impermenant, non-fluorescing product conjugated to glutathione and other intracellular thiols.[1,2] In a separate reaction, intracellular esterases, common to most viable cells, cleave the acetate group from the conjugated dye molecule to produce a 'glutathione-fluorescent dye adduct',[2] spread throughout the cell cytoplasm and concentrated within specifically reactive organelles. Hence CMFDA can only be loaded into living cells where the plasma membrane is intact and intracellular processes maintained.

In contrast to CMFDA, ethidium homodimer-1 labels the nuclei of non-viable cells in which plasma membrane integrity and cellular metabolism have been compromised, and has demonstrated advantages over other nuclear stains such as propidium iodide or ethidium bromide.[2,3] In particular, ethidium homodimer has a very low membrane permeability and is thus excluded from viable cells, but has an extremely high affinity for nuclear DNA,[3] accessible only when membrane integrity and function have been compromised.

The results presented show that both dyes are compatible with metabolic studies, both can be fixed with formaldehyde after loading, and both survive the numerous processing steps required for wax and resin embedding, cryo- and vibratome sectioning, immunohistochemistry, autoradiography, and confocal microscopy. Taylored adaptations of this protocol for specific connective tissues, and other tissue types, could prove valuable to researchers and clinicians wishing to identify simultaneously, viable and non-viable cells in tissue explants.

MATERIALS AND METHODS

Tissue Penetration and Dye Loading

The penetration characteristics and loading conditions of the viable cell probe 5-chloromethylfluorescein diacetate (CMFDA), and the dead cell probe ethidium homodimer-1, were first determined empirically using a porcine corneae model previously developed for loading live corneae with Calcein-AM.[4] Preliminary comparisons of the rate of penetration of the two viability dyes indicated that CMFDA had a significantly slower rate of penetration than Calcein-AM when used at recommended concentrations. Conversely, ethidium homodimer-1 was found to rapidly penetrate the corneae, loading dead cell nuclei well in advance of the CMFDA loading front.

To assess the penetration properties of CMFDA, central trephines (6mm diameter) were removed from four porcine corneae as previously described.[4] Each trephine was cut into two hemispheres and placed, stratified epithelia side down, into separate wells of two 24-well multiplates containing either Cornea Transport Media,[4,5] or Dulbecco's Phosphate Buffered Saline (D-PBS), and allowed to equilibrate at room temperature for 15 minutes. The media was aspirated from each well, replaced with fresh media or D-PBS containing 25μM CMFDA, and the stromal keratocytes loaded in the dark for either 4 or 24 hours at either 4°C or 34°C. The hemispheres were then gently washed three times with media, fixed for 10 minutes in freshly prepared 2% paraformaldehyde, and washed thoroughly with media. Each sample was equilibrated in a separate Epindorf tube containing media plus 20% dimethyl sulphoxide (DMSO), and stored frozen at –20°C. For sectioning, each hemisphere was remounted in an antero-posterior orientation, frozen in OCT (Tissue Tek, IN, USA), and 30μm cryosections collected serially through the whole thickness of the hemisphere. Cryosections were dried onto numbered slides coated with poly-L-lysine, and representative sections mounted under coverslips in VectaShield (Vector Laboratories, CA, USA) for qualitative assessment of dye penetration.

Loading Protocol

Since optimal loading and contrast of viable keratocytes throughout the dense corneal stroma was achieved at 4°C over 24 hours, the following general protocol was initially applied to all connective tissues sampled, and then secondarily modified to optimise the loading protocol for each specific tissue. Tissue explants and surgical biopsies were initially collected into the appropriate media and held at 4°C for transport to the laboratory where they were immediately trimmed of excess material, and rinsed in fresh media at 4°C. If necessary, metabolic studies requiring short term (6–24 hours) explant culture at 37°C were initiated at this time. Irrespective of the pre-treatment received, explants were placed in fresh media supplemented with 25μM CMFDA and 25μM ethidium homodimer-1, and the tissue incubated at 4°C in the dark with or without gentle agitation for 4 to 24 hours. Each sample was thoroughly rinsed in the appropriate buffer and fixed according to the specific experimental requirements of each preparation. Departures from this general protocol are noted for specific tissues detailed below.

Human, Porcine and Bovine Corneae

Human corneae found unsuitable for surgical transplantation were obtained from the New Zealand National Eye Bank, Division of Ophthalmology, Department of Surgery, University of Auckland. Porcine and bovine corneae were obtained from complete globes enucleated from 2–3 month old pigs, and 6–8 month old steers, supplied by the Auckland City Abattoir 1–3 hours after slaughter. Corneae were excised from the globes, loaded with fluoroprobe as described above (and in 4), and briefly fixed in 2% paraformaldehyde for 10 minutes. Washed corneae were either stored frozen in 20% DMSO, or embedded in OCT and cryosections collected in the lamellar plane as described above. Sections were immediately mounted under coverslips using VectaShield, a semi-permanent mountant containing anti-fading reagents to preserve fluorescence during detailed examination of keratocyte morphology by light and confocal microscopy.

Human Coronary and Internal Thoracic Arteries

Short segments (2cm) of the left anterior descending coronary and internal thoracic arteries were collected at autopsy from 14 individuals (12 males, 2 females) ranging in age from 15 to 81 years, with post-mortem times ranging from 10 to 23 hours.[6] Vessel segments were immediately placed in media M199 on ice for transport to the laboratory where they were cleaned of adherent muscle and fat.[6]

Initially, vessel segments were cut into small lengths (2–3 mm) and loaded according to the standard protocol described above. However, to more accurately assess the metabolic activity, and hence viability of CMFDA loaded cells, selected vessel segments were radiolabelled for periods ranging from 6 to 24 hours at 37°C in 3 ml of media M199 containing 35μci [^{3}H] glucosamine as previously described.[7] As a rule, CMFDA and ethidium homodimer were added directly to the incubation media 4 to 6 hours prior to the termination of radiolabelling to avoid additional loading incubations and temperature fluctuations which could influence cellular metabolism. Excess dyes and glucosamine were removed by media washes, and the vessel segments fixed in 2% paraformaldehyde for 2 hours at 4°C. Vessels were finally dehydrated in ethanol, cleared in propylene oxide, infiltrated and embedded in Polarbed 812 (Biorad, UK), and polymerised for 3 days at 60°C.[6]

Semithick resin sections (1.0 μm) were cut on a Reichert OMU2 ultramicrotome and heat annealed to glass slides. Slides were dipped in autoradiographic emulsion (K2, Ilford), dried for 3 hours, and stored at 4°C in the dark for 14 days. Slides were developed, and if required, stained briefly with erichrome black-T to reduce elastin autofluorescence,[8] prior to mounting under VetaShield. Confocal microscopy was used to correlate the distribution of autoradiographic silver grains with cells loaded by CMFDA or ethidium homodimer.

Articular Cartilage and Isolated Chondrons

Tibial plateaux of adult mongrel dogs, euthanised under veterinary supervision, were removed with their underlying bone attached. Individual plateaux (medial and lateral) were sawn into rectangular blocks no bigger than 2×5 mm^2, and held in Hams F-12 media at 4°C to equilibrate for 30–120 minutes. Cartilage explants were initially loaded as described above, but two slight variations were subsequently introduced; ethidium homodimer was ommitted from preparations destined for immunohistochemistry since it fluoresces in the same spectrum as Texas Red; on two occasions, the concentration of CMFDA was increased to 50 μM. At the conclusion of loading, cartilage explants were washed 3×30 min in PBS, fixed for 2–4 hours in 4% paraformaldehyde in PBS, washed three times and stored at –20°C in PBS containing 20% DMSO. Following thawing and removal of the subchondral bone, intact cartilage samples were either sectioned wet on a TPI Series 1000 vibratome at 25–100 μm, or embedded in OCT and cryosectioned at 10–20μm.

Chondrons were extracted from mature canine tibial cartilage as previously described,[9–11] and embedded in agarose monolayers for subsequent examination.[12] To load viable and non-viable chondrocytes in chondron-agarose preparations, Hams F-12 media supplemented with CMFDA and ethidium homodimer, was added to the culture dish and incubated for 4 hours at either 4°C or 37°C with little difference in loading. Chondron-agarose cultures were fixed in 4% paraformaldehyde, cut into small plugs approximately 1cm in diameter and stored at 4°C in PBS supplemented with 0.5% BSA and 0.05% sodium azide as previously described.[12]

Immunohistochemical analysis of extracellular macromolecules frequently involves enzymatic digestion to expose masked epitopes, and in the case of chondron-agarose plugs and thick vibratome sections, overnight extractions are generally required.[12,13] To assess the ability of CMFDA to withstand the attenuated immunohistochemical staining regimes required for thick confocal preparations, we immunolabelled CMFDA loaded chondron-agarose plugs for type VI collagen as previously described.[13]

Human Skin

Full depth samples of normal human skin (4mm^2), collected during mammoplasty, were freed of adherent fat and labelled overnight with CMFDA and ethidium homodimer as described above. Following washing and fixation in 4% paraformaldehyde for 4 hours at 4°C, the samples were equilibrated in PBS containing 20% DMSO and stored frozen at –20°C. Individual samples were thawed, washed extensively, and mounted in OCT for cryosectioning. Transverse sections showing the full thickness of the skin were examined by light and confocal microscopy.

Light Microscopy

All cryosections and tissue mounts were examined on a Leitz Dialux 20 light microscope equiped with phase-fluorescent and differential interference contrast oil-immersion objectives. Epi-illumination with a 50W mercury vapour lamp using a conventional (488nm) fluorescein filter block simultaneously excites CMFDA and ethidium homodimer. Light micrographs were recorded on Fujichrome 400 colour or T Max 400 black and white 35 mm film, and transfered to a Photo-CD (Kodak, New Zealand). The final colour plate were assembled using Adobe PhotoShop (Adobe Systems Inc., USA), exported to Aldus Freehand (Altsys Corp., USA) for addition of figure details and calibration bars, and the final plate printed using a dye sublimation colour printer.

Confocal Microscopy

Confocal images were obtained using a Leica TCS 4d Confocal Laser Scanning Microscope (Leica, Heidelberg, Germany) equipped with a Leica DM RBE upright microscope fitted with a motorised stage, transmitted and epi-illuminated light sources, a Krypton-Argon laser source and recommended filter sets, a range of infinity adjusted optics for oil and water immersion, a transmitted light detector fitted with DIC optics, and dual channel photodetectors. The Confocal system was operated in fluorescence mode to image the fluoroprobes, and reflectence mode to image silver grains in autoradiographs or silver enhanced gold particles used for immunohistochemistry.[13]

The confocal microscope was connected via ethernet to a high performance Silicon Graphics IRIS Indigo R4000 workstation with XS24 graphics operating VoxelView (Vital Images CA, USA) for image analysis. Maximum and minimum pixel intensities were optimised for the fluorochrome and photomultiplier channel selected, and the digital data sets collected were stored on rewritable magneto-optical disks mounted in a Parity magneto-optical disk drive interfaced with the Silicon graphics workstation.

Image Processing

The laser of a confocal microscope scans the specimen in the horizontal *xy* plane, but only collects data which lies within the focal plane. By precisely stepping the microscope stage in the vertical *z* axis it is possible to collect a series of thin (approximately 0.5μm), perfectly focused optical sections at different depths through the specimen, irrespective of section thickness.

The optico-digital data sets produced were processed in a variety of ways. Routinely, the image processing features of the Leica ScanWare software, which operates the confocal microscope, were used to prepare single optical sections, *z* series projections, and red/green stereo images. Specifically, *z* series projections represent computer averaged assemblies of all optical sections in the data set, producing a single, in-focus image of the full depth of the specimen. Similarly, red/green stereo images represent computer generated projections of all images in the data set, but in this process two images are created, one red and one green, which are tilted plus or minus four degrees about the *x* axis and projected as a single red/green image. Using red/green stereo glasses, the viewer is then able to appreciate the three dimensional organisation of stained material throughout the specimen scanned.

Alternatively, specialised image processing was performed on the Silicon Graphics workstation using Voxel View to produce a surface rendered three dimensional representation of the subject without the need for specialised viewing apparatus.

RESULTS

Dye Penetration and Staining Protocol

The results of dye penetration studies on intact living corneae are shown in Figure 1. Keratocyte loading at 34°C invariably produced excessive background staining of the extracellular matrix (Fig. 1a), particularly after extended incubation times, and was subsequently omitted from the general protocol. Conversely, loading at 4°C produced minimal background staining (Fig. 1b), which enhanced the contrast of loaded keratocytes.

Penetration of the dense stromal matrix proved to be extremely slow, and was optimised by the use of organ cul-

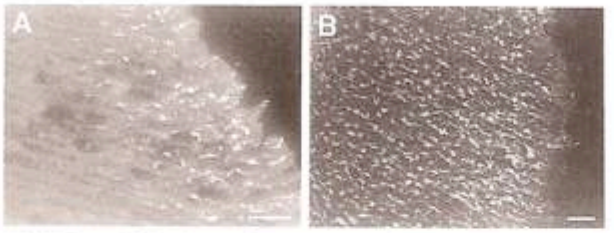

FIGURE 1 Light microscopic assessment of CMFDA penetration. (A) Transverse cryosection of porcine corneae loaded for 4 hours at 34°C. Note limited penetration and high degree of background fluorescence. Bar = 100 µm. (B) Transverse cryosection of corneae loaded for 24 hours at 4°C. Note the gradation in CMFDA loading and minimal background fluorescence. Bar = 50 µm.

ture media which minimised the tissue distortion observed when D-PBS was used. After 4 hours, regardless of the incubation temperature, the loading front had penetrated only a short distance (200–400 µm) into the cut margins of the cornea (Fig. 1a). After 24 hours incubation at 4°C, a gradient of dye intensity was still apparent (Fig. 1b), but keratocyte loading was sufficient to image the finest keratocyte cell processes throughout the entire corneal hemisphere.

The comparatively rapid penetration of ethidium homodimer-1 through the corneae was established in earlier studies employing Live/Dead Assay,[4] and the incubation time of 24 hours used in these studies was more than adequate to label dead cell nuclei throughout the corneal stroma. As a result of these trials, an optimised protocol employing cell or organ culture media as the dye vehicle, extended loading times (up to 24 hours), and incubation at low temperature (4°C), was initially adopted for general use on all the connective tissues detailed below. However we found that this basic protocol requires specific modifications to cater for the size and density of the individual tissue examined, and the requirements for downstream experimental analysis.

Human, Porcine and Bovine Corneæ

No apparent morphological differences were observed between living keratocytes loaded with Calcein-AM as previously described,[4] and keratocytes loaded with CMFDA as described above. Confocal examination of cryosections cut parallel to the epithelial surface of human, porcine and bovine corneae confirmed the presence of three morphologically distinct keratocyte sub-populations in the anterior, central, and posterior stroma.[4]

A low power *z* series projection of keratocytes in the anterior stroma is shown in Figure 2a. A dense network of flattened and irregular keratocytes which branch extensively before terminating on adjacent cell bodies and cell processes, is clearly evident. The ability of CMFDA to permeate the cytoplasm and define the finest details of cell process morphology is illustrated in Figure 2b. The high resolution serial optical sectioning capabilities of the confocal microscope, in combination with red/green stereo projection, reveals a degree of keratocyte inter-connectivity not previously available in thick tissue preparations.

Thoracic and Coronary Arteries

The standard protocol employing CMFDA and ethidium homodimer proved particularly suitable for studies on human internal thoracic and coronary arteries sampled at autopsy, and both fluoroprobes survived the dehydration and resin embedding procedures required for these studies. The resulting 1 µm thick resin sections provided high resolution light microscopic images (Fig. 2c), free of the optical interference and fluorescent flaring common in the thicker sections described elsewhere.

In all arteries examined, the distribution of live and dead cells was not uniform. In some regions of the vessel wall, all cells were viable, while in other regions of the same sample both live and dead cells were found together (Fig. 2c). Still other regions, including the cut margins, showed only dead cell nuclei stained with ethidium homodimer. The pale yellow autofluorescence of extracellular elastin was used as an anatomical reference point during routine examinations, and in general did not interfere with assessment of cell viability (Fig. 2c). However, masking of elastin autofluorescence with erichrome black-T did provide better contrast for loaded cells, and improved the resolution of smooth muscle cell processes.

Vessels labelled simultaneously with [^{3}H] glucosamine and fluoroprobes were used to validate the protocol, and to confirm that smooth muscle cells, endothelial cells and adventitial fibroblasts remained viable and metabolically active during the incubation period. Figure 2d shows a merged confocal reconstruction in which CMFDA was imaged using an FITC filter set, ethidium homodimer was imaged using a Texas Red filter set, and the silver grains in the autoradiographic emulsion imaged in reflectence mode. The high density of silver grains covering and surrounding viable cells confirmed the active synthesis of matrix proteoglycans, and was in sharp contrast to the very low levels of background glucosamine labelling observed amongst dead cell nuclei (Fig. 2d).

Articular Cartilage and Isolated Chondrons

Overnight loading of small cartilage samples at 4°C gave excellent staining of chondrocytes throughout the depth of

the matrix, with dead cells present almost exclusively at the cut margins of the explant. The viability of isolated chondrons was more easily evaluated since shorter incubation times were required for chondron cultures. Isolated chondrocytes cultured in agarose gel required a maximum of 1 hour at 37°C to become fully loaded with CMFDA or ethidium homodimer (Poole; personal observation).

A high resolution, single optical section through the centre of a living chondrocyte from the middle layer of adult articular cartilage loaded with CMFDA is shown in Figure 2e. In addition to clearly defining the shape and margins of the cell, variations in intracellular staining intensity correspond to key morphological features of mature chondrocytes. Hence regions of intense loading in the eccentrically placed nucleus were strongly contrasted against regions of weak loading which correspond to glycogen deposits and/or lipid droplets at the chondrocyte margin (Fig. 2e).

The response of CMFDA loaded chondrons to enzyme extraction and the attenuated labelling regimes required for type VI collagen immunohistochemistry are shown in Figure 2f. This dual channel, three dimensional reconstruction shows that CMFDA staining was remarkedly stable and durable once fixed, and provides an excellent contrasting fluorochrome for immunohistochemical studies demonstrating the relationship between viable cells and specific macromolecules in the pericellular microenvironment.

Human Skin Biopsies

Human skin samples were chosen to assess the potential clinical use of cell viability probes for immediate labelling of surgical biopsies. Using the standard protocol described above, we found that penetration into skin samples occured via the cut margins, with a gradual reduction in CMFDA loading intensity beneath the epidermis at the centre of the biopsy. In general, fibroblasts and smooth muscle cells within the papillary and reticular layers of the dermis stained intensely with CMFDA (Fig. 2g). In contrast, the epidermis was only loaded from the cut margins, with minimal dye penetration through the epidermal layers at the centre of the biopsy. Dead cell nuclei were predominantly localised at the cut margins of the biopsy, or associated with the cut ends of short segments of blood vessels and nerve fibres within the sample (Fig. 2g).

When imaged serially in fluorescence mode and viewed as a red/green stereo image, the precise microanatomy of

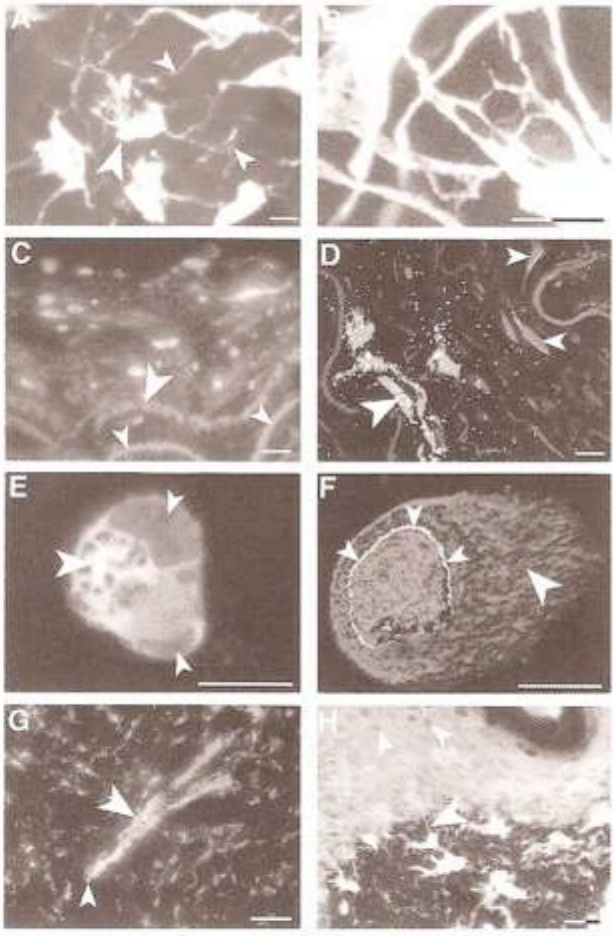

FIGURE 2 (Colour Plate I) (A–B). Intact porcine corneae. (A) A *z* series projection of 13 optical sections gathered by CLSM through 12.87 μm in the centre of the corneae to illustrate the morphology of the living keratocyte. Cell bodies stained intensely, but non-uniformly (large arrow), while the diffusion of CMFDA throughout the cytoplasm of extremely fine cell processes (small arrows) highlights the intricate morphology of the keratocyte network. Bar = 10 μm. (B) High-resolution red/green stereo image generated from 14 optical sections through 7.49 μm of the anterior stroma. Note the effectiveness of CMFDA in tracing the cytoplasmic components of cell processes which interconnect keratocytes within a three dimensional matrix of unstained lamallar collagen sheets. Bar = 10 μm. (C–D). Human internal thoracic artery. (C) Fluorescent light micrograph from a thin resin section of human artery loaded with CMFDA and ethidium homodimer. When viewed with an FITC filter set, the cytoplasm of viable smooth muscle cells fluoresces green while the nuclei of non-viable cells fluoresce red/orange. The internal (large arrow) and external (small arrows) elastic laminae show weak autofluorescence. Bar = 10 μm. (D) Triple channel confocal image from an artery labelled with [^{3}H] glucosamine, loaded with CMFDA and ethidium homodimer, and processed for autoradiography. Separate scans for viable cells (large arrow), non-viable cells (small arrows), and autoradiographic silver grains (white dots), were superimposed using Leica ScanWare to correlate viable, CMFDA loaded cells with the distribution of proteoglycans synthesized during active cellular metabolism. Bar = 10 μm. (E–F). Adult canine tibial cartilage. (E) Single optical section through a viable mid layer chondrocyte loaded with CMFDA and viewed unfixed by confocal microscopy. Glycogen storage granules (small arrows) show little intrinsic esterase activity, while substructures of the nucleus, possibly chromatin, show high levels of CMFDA reactivity (large arrow). Bar

CMFDA loaded cells in the dermis and epidermis was clearly evident (Fig. 2h). Fibroblasts in the reticular and papillary layers appeared to be connected by a syncytium of cell processes, while fibroblasts lying immediately beneath the epidermal basement membrane occasionally projected long cell processes which appeared to contact the basal cells of the stratum germinativum. The significance of this observation is not clear, but it serves to highlight the accurate portrayal of cell morphology which can be achieved with fresh surgical samples and fixable cell viability probes.

DISCUSSION

This study has shown that explants and biopsies of connective tissues can be loaded *ex vivo* with fluoroprobes which simultaneously reveal viable and non-viable cells within an unstained extracellular matrix. The protocol developed was optimised for loading viable keratocytes within the dense stromal matrix of intact corneae, and was subsequently adapted for use on a range of connective tissue explants destined for use in a wide variety of research applications. In general, cell or organ culture media provided the optimal vehicle for dye loading, with longer incubation times (up to 24 hours) and lower temperatures (4°C) providing the best compromise between loading intensity and background fluorescence produced by leakage of excess reaction product from overloaded cells.

The success of this protocol was largely attributable to the properties of the new generation fluoroprobes selected for this study. In general, most fluorescent probes used for cell viability assessment act as substrates for enzymes present in the cytoplasm of healthy cells where plasma membrane integrity and function remain normal. In contrast, fluoroprobes used to assess non-viable cells rely on poor penetrability through functional cell membranes, but a high affinity for nuclear DNA accessible through functionally compromised membranes. In the experiments described, the red fluorescent signal produced by ethidium homodimer clearly marked the nucleus, but not the cytoplasm, of non-viable cells, and was contrasted against the cytoplasmic distribution of the fluorescent green CMFDA in viable cells. It should be emphasised however, that excess unconjugated CMFDA reaction product can passively diffuse from the cells, particularly if they are loaded at higher temperatures, and care must be taken to ensure this excess is not secondarily complexed to thiol groups on extracellular proteins.

The dyes selected and the protocol devised therefore satisfy the four criteria considered essential for the development of a procedure which could allow differentiation between live and dead cells in fresh connective tissue explants used in a variety of experimental and clinical applications. Thus, while the size of the connective tissue explants obtained at biopsy, autopsy and from freshly euthanised animals did vary in overall dimensions, all were of a manageable size for collection, loading and experimentation. In general we found that explants with a maximum diffusion distance of 2mm to the centre of the sample loaded well with our protocol. However, penetration through connective tissue explants appeared to be dependent on tissue density and time of loading rather than incubation temperature, and as a general rule, the larger and denser the connective tissue explant, the longer the loading time required. Preliminary experiments employing higher concentrations of CMFDA also suggest improved loading characteristics for dense tissues such as cartilage (Poole and Gilbert; personal observation). It is therefore essential that the sample size and the penetration characteristics of individual connective tissues be assessed and optimised for each research or clinical application undertaken.

Optimal loading was achieved when viable cells were sufficiently stained so as to be able to resolve the fine cell processes characteristic of most connective tissue cells, and exemplified by the stromal keratocytes of the intact porcine corneae (Figs. 1, 2a, 2b). Ideally, CMFDA should fill the entire cytoplasm uniformly, but we have shown that a variety of intracellular organelles can display variable levels of glutathione S-transferase and/or esterase activity. For example, high levels of CMFDA labelling was associated with nuclear components in chondrocytes (Fig. 2e), and the mitochondria of keratocytes in the anterior stroma (Poole, Brookes; personal observation), while little intrinsic esterase activity was associated with storage areas such as the glycogen deposits and lipid droplets of chondrocytes.

= 10 µm. (F) Reconstruction of 150 dual channel confocal images through an isolated chondron loaded with CMFDA and immunolabelled for type VI collagen. The image was rendered and assigned natural colours using VoxelView, and demonstrates how contrasting fluorochromes can define chondrocyte volume (green), and the microanatomy of the chondron (red). Areas of overlapping label are shown in yellow (small arrows), and reflect the intimate relationship between the chondrocyte and its pericellular microenvironment. Bar = 10 µm. (G–H). Human skin biopsies. (G) Low powered light micrograph showing the distribution of viable (green) and non-viable (red) fibroblasts in the reticular layer of the dermis. A number of cells in a small blood vessel loaded intensely with CMFDA (large arrow), while dead cell nuclei predominate at the cut end of the vessel (small arrow). Bar = 100 µm. (H) High-resolution red/green stereo image generated from 16 optical sections through 8.8µm at the interface between the epidermis and the papillary layer of the dermis. Papillary fibroblasts loaded with CMFDA were interconnected by an extensive network of fine cell processes. Some processes project towards the epidermis (large arrow), and appear to make direct contact with basal epidermal cells in the stratum germinativum. The dark 'holes' in the epidermis (small arrows) represent unstained nuclei contrasted against the CMFDA loaded cytoplasm. Bar = 10 µm. (See Color Plate I at the back of this issue.)

The metabolic competence of CMFDA loaded cells was established using [^{3}H] glucosamine to monitor the synthesis and sequestration of matrix proteoglycans. Dense aggregations of autoradiographic silver grains were consistently associated with CMFDA loaded cells, while cells loaded with ethidium homodimer showed little or no evidence of [^{3}H] glucosamine incorporation above background levels.

One of the major advantages of the combination of CMFDA and ethidium homodimer over other fluoroprobes were their fixable properties, and subsequent stability during a variety of embedding, sectioning, and immunohistochemical processing techniques. Fixation was considered important for two reasons. First a number of biopsies were often collected and loaded at one time, but could not be cryosectioned immediately, introducing the possibility of degradative artifacts during storage in an unfixed condition. Second, fixation immobilises the dye-protein complexes formed, preventing potentially labile proteins from moving during sectioning and the prolonged tissue processing associated with resin embedding or immunohistochemistry. This protocol therefore offers a unique advantage in that living tissue explants can be loaded with contrasting dyes, and then stored indefinately for subsequent analysis and experimentation. Indeed, the morphological images produced in conjunction with confocal microscopy have already provided new insights into corneal keratocyte and dermal fibroblast morphology not previously available by traditional histochemical methods.

It is important however to emphasise the limitations of this protocol. First, the size and density of the sample is critical, and care must be taken to ensure that the smallest and most manageable sample size be determined for each tissue analysed. Second, CMFDA generally penetrates tissue blocks through the cut margins, and penetrability can be severely affected by the temperature and duration of loading, as well specialised tissues such as the epidermis where the high degree of exclusion coupling prevent adequate penetration. Third, ethidium homodimer emits in a similar spectrum to Rhodamine and Texas Red, and is best omitted from preparations optimised for immunohistochemistry. Finally, ethidium homodimer is thought to be cytotoxic and its use is restricted to research applications. Similarly, CMFDA is thought to be cytotoxic when excited with 488 nm blue light due to the formation of oxygen radicals. These features may therefore limit the use of these fluoroprobes for dynamic physiological studies, and in situations where stored human tissues are destined for transplant.

In summary, this study has shown that the fluoroprobes CMFDA and ethidium homodimer can be loaded into a wide range of living connective tissue explants to provide superior images of both viable and non-viable cells embedded in an extracellular matrix which remains unstained. These procedures have now been applied in a variety of experimental applications including quality assurance of human corneae stored prior to transplant, metabolic studies of autopsied human arteries, counterstaining of fresh surgical biopsies for histopathology, and as a contrasting fluorochrome for immunohistochemistry and confocal microscopy. Specific adaptations of the standard protocol established in this study could prove to be of value in a wide range of research and clinical applications which require differentiation between viable and non-viable cells in connective tissue and other organ explants.

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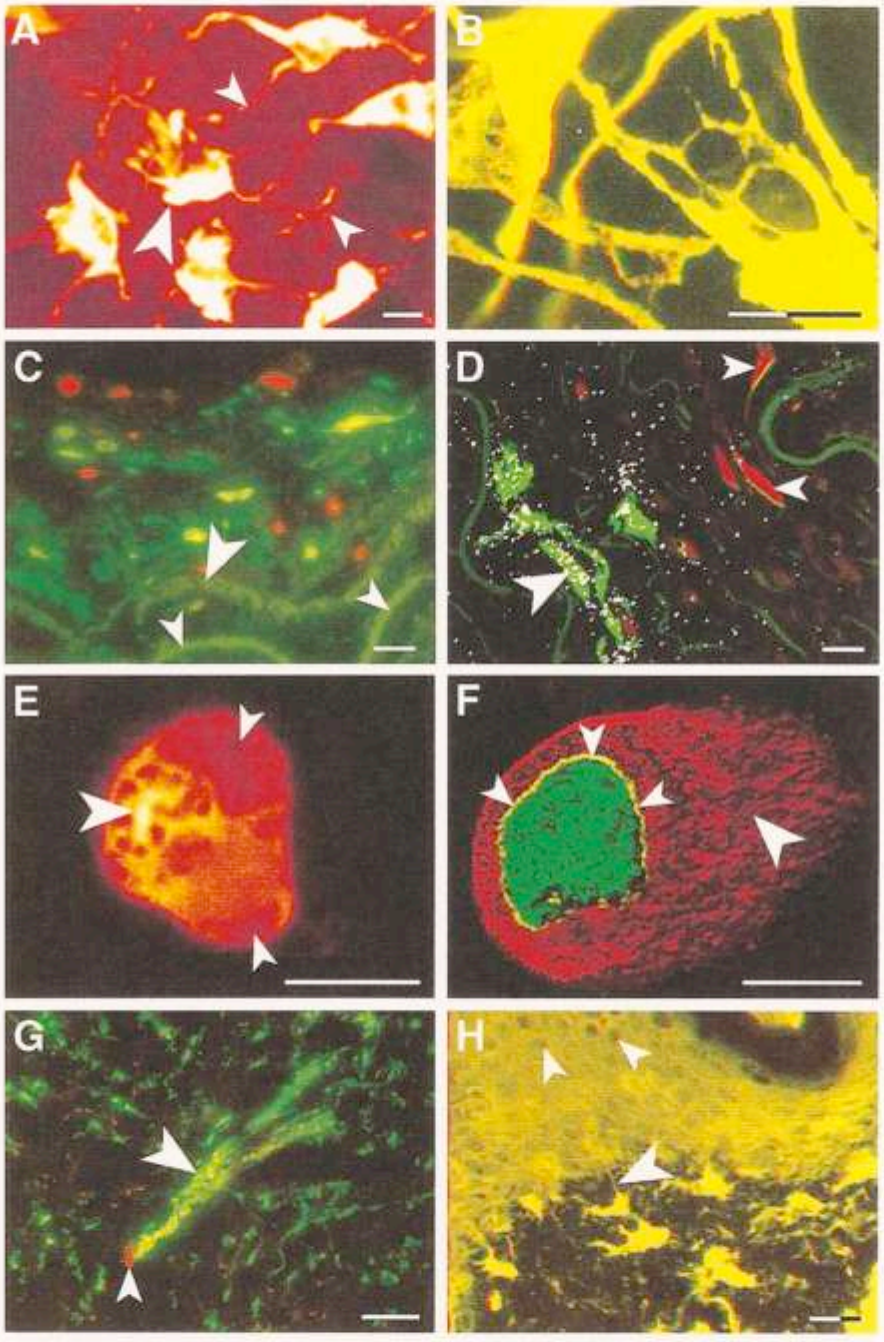

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COLOR PLATE I. See C.A. Poole *et al.*, Figure 2.