

# Cell Viability Mapping within Long-Term Heart Valve Organ Cultures

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**Background and aim of the study:** Organ cultures maintain cells within their native microstructural environment, and thus offer greater potential for studying tissue disease and remodeling than do monolayer cell cultures or pathological examinations of diseased tissue. To validate an in-vitro heart valve organ culture model, cell viability was examined within valve tissues over sustained culture periods.

**Methods:** Following culture of blocks of valve tissue for 1 to 49 days, cross-sections were cut with a vibratome, stained with a LIVE/DEAD kit, and imaged with confocal microscopy to quantify the number of live and dead cells present.

**Results:** In numerous organ cultures, valvular interstitial cells were found to be viable beyond 30 days.

Over the years, clinicians and researchers have used a myriad of approaches to study heart valve disease, which was reported to have affected over 100,000 people in the year 2001 (1). Although the most common methodologies have ranged from the pathological examination of surgically excised valves to the use of animal models, the use of interstitial and endothelial cells grown from heart valves provides a great opportunity for studies of valve disease mechanisms. Cells grown in monolayer cultures, however, are unlikely to behave in a similar fashion as when in their native three-dimensional matrix. Although some investigators have seeded valve cells into porous matrices to reconstruct the three-dimensional environment (2-4), this method may not reproduce the same in-vivo archi-

Live cells were abundant in the central region of the valve, but more sparse in the deepest central regions. Dead cells were found mainly on the surface of both fresh tissues and tissues after prolonged culture, with few dead cells occurring centrally.

**Conclusion:** This is the first reported mapping of cell viability within heart valve organ cultures, and results suggest that extended organ culture of valve leaflets is indeed possible. The derived viability staining methods have wide applicability for organ cultures of other tissues as well as tissue-engineered matrices.

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ture of normal or diseased tissues.

In order to overcome some of these limitations, the decision was made to study heart valve disease using an organ culture model that maintains the valvular cells in their normal microstructural environment. A similar organ culture approach has been used to determine the cell types involved in valvular wound repair (5-7). In an effort to validate this organ culture model for long-term use, attention was first focused on monitoring and mapping cell viability. Although the viability of valvular interstitial cells has been analyzed previously using flow cytometry (3,8,9), that technique cannot provide insight into the location of the viable cells within the tissue microstructure. Because valve leaflets are dense, heterogeneous, layered structures, the cells within each layer may have different viabilities. Particular concern was expressed with regard to the viability of cells located in the center of the cultured valve tissue, as these were furthest from the oxygen and nutrient supplies. Thus, the present study objectives were to develop and implement a protocol for mapping cell viability within organ cultures of valve tissues.

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## Materials and methods

### Organ culture preparation and maintenance

Heart valves were obtained from human heart valve surgeries, from explanted human transplant recipient hearts, and from laboratory animals (porcine, canine, bovine). The use of human tissues was approved by the Cleveland Clinic Institutional Review Board (#3278 and #4492), and the animal tissues were obtained from multiple non-valvular studies approved by the Cleveland Clinic Foundation Animal Review Committee. The valves were dissected from the hearts within 30 min of organ removal from the body, and the chordae trimmed from the mitral and tricuspid valves, and discarded. The leaflets then were thoroughly rinsed in an incubated buffered saline solution and sliced into  $1.5 \times 1.0$  cm culture blocks (5).

Each of these organ culture blocks was maintained in one well of a standard 12-well plate and fed with 4 ml Medium 199 with modified HEPES buffer (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B (Invitrogen, Carlsbad, CA, USA). Cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> environment, with the medium being changed at 48-h periods.

Following culture for 1 to 49 days, each organ culture block was sliced into three portions, each measuring approximately  $0.5 \times 1.0$  cm. One of these three sections was prepared for viability staining, a second was fixed in Histochoice (Amresco, Solon, OH, USA) for subsequent histology, and the third was reserved for a biochemical DNA assay.

### Viability analysis

The final overall approach for viability mapping involved first cutting thin cross-sections of the organ-cultured valve tissue. Next, the cross-sections were stained with viability dyes and immediately imaged with a confocal microscope. Finally, the images were analyzed to quantify the numbers of live and dead cells.

A vibratome (World Precision Instruments Inc., Sarasota, FL, USA) was used to cut the organ culture blocks into sufficiently thin cross-sections for confocal microscopy. The tissue was submerged in chilled Medium 199 with 15% FBS, positioned on edge and leaning against a 40% agarose gel (Invitrogen) fixed to the base of the vibratome organ bath (Fig. 1). The position of the tissue allowed the vibratome to cut an approximately 300  $\mu$ m-thick section which contained all three layers of the valve. Control sections were also cut at the same time. The negative control was unstained tissue, which was used to determine the tissue autofluorescence. The positive control for dead

cells was valve tissue that had been submerged in dimethylsulfoxide, water, or ethanol, and then exposed to ultra-violet light for 24 h. The positive controls for live cells were cultures of endothelial and valvular interstitial cells (obtained from other studies in the present authors' laboratory) grown in a 24-well plate.

The cut samples (both experimental and control tissues) and the control cell cultures were first rinsed in 37°C phosphate-buffered saline (PBS) for 20 min. The tissue sections or cell layers were then stained with 3  $\mu$ M calcein AM and 4  $\mu$ M ethidium homodimer-1 in PBS (LIVE/DEAD viability/cytotoxicity kit; Molecular Probes, Eugene, OR, USA). Ethidium homodimer-1 stained the nuclei of dead cells red, whereas calcein AM stained the membranes of live cells green. The sections were then incubated at 37°C for 30 min, with intermittent agitation in order to expose all tissue surfaces to the fluorescent dyes. The sections/monolayers were then rinsed in 37°C PBS for 20 min, and placed within Pap-pen wells (Newcomer Supply, Middleton, WI, USA) on glass slides and kept moistened with PBS.

Stained sections were immediately imaged using a Leica TCS-SP Laser Scanning Confocal Microscope (Mannheim, Germany GmbH). Ethidium homodimer was excited with an argon laser at 364 nm and emitted light from 400 to 480 nm was collected. Calcein AM was excited at 488 nm with an argon laser, and emitted light was collected from 500 to 550 nm. Images of the homodimer and calcein were collected at 364 and 488 nm sequentially. This prevented any overlap of the emission profiles from the two dyes that might have occurred if the images were collected simultaneously.

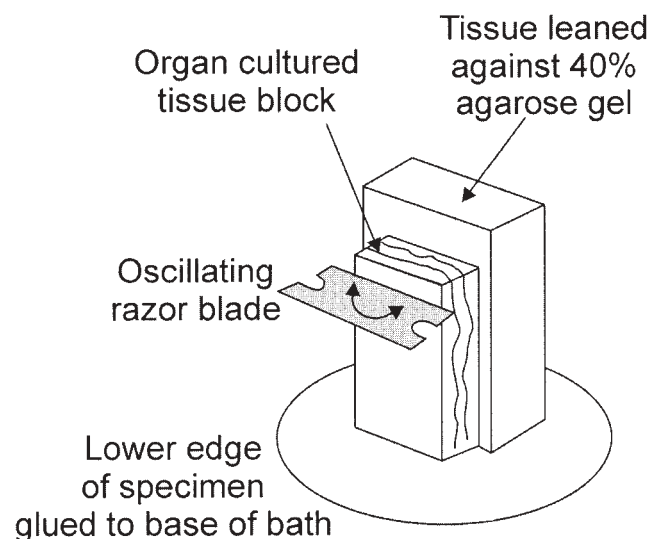


Figure 1: Diagrammatic representation of the vibratome sectioning of the organ culture. See text for details.

Serial optical sections were collected through various depths of the tissue and image stacks were then reconstructed. The reconstructed sections were up to 160  $\mu\text{m}$  thick, and contained both the central region of interest and all three layers of the valve. The positive and negative control tissues and cells were imaged according to the same protocol, although the cell monolayers were imaged with a Leica DMIRB inverted fluorescence microscope. All imaging parameters were kept constant for the duration of the study.

### Image processing

The stacked confocal images were analyzed using Scion Image (Scion Corp., Fredrick, MD, USA) to quantify the number of live and dead cells within the tissue. The images were first thresholded to a reference value chosen to maximize the visibility of the cells. Reference values of  $\pm 10$  pixel intensity units were also applied to determine the sensitivity of the thresholding. The 'analyze particles' function was then used to count the number of cells in each image. To account for edge effects, cells were counted with and without including the image edges. Lastly, a minimum and maximum particle size was set to minimize the effect of background noise. The average and standard deviation for the cell count were calculated from these various permutations, and analyzed to determine viability throughout the culture period. The images were further examined to determine if live or dead cells were located in specific patterns within the stained tissue.

The image data were also used to estimate the density of cells in the tissue blocks. The image area was multiplied by the height of the stacked images to obtain the analyzed tissue volume. A tissue density of 1.011 g/ml (previously measured in the authors' laboratory (10)) was used to estimate tissue mass and thereby calculate cell density in terms of cells per mg tissue wet weight.

### Histology

Specimens intended for histology were fixed in Histochoice for 24 h, embedded in paraffin wax, and cut into 5  $\mu\text{m}$ -thick sections. Sections were stained with hematoxylin and eosin and Movat's pentachrome for evaluation of tissue morphology and for qualitative comparison of cell density with the confocal microscopy images.

### Biochemical DNA measurement

Specimens intended for DNA measurements were weighed and dissolved in 100 mM ammonium acetate buffer (pH 7) containing proteinase-K (1 mg/ml; Invitrogen) at 60°C for 16 h. Duplicate aliquots of the dissolved samples were then subjected to a fluorescence Hoechst DNA assay (11) to calculate the mass of

DNA in the tissue block. Since each cell contains approximately 6 pg of DNA (11), these data were used to calculate the concentration of cells per tissue wet weight. These biochemically measured cell concentrations were compared with the cell concentrations estimated from the cell viability images.

## Results

Sixty-two valve segments were maintained in organ cultures for an average of  $18 \pm 13$  days (range: 0 to 49 days). These cultures included valves from 13 human hearts, three porcine hearts, one canine heart, and one bovine heart. In all of these cultures, the color of the culture medium changed from red to yellow-orange over the two-day feeding intervals, which provided preliminary evidence for the viability and metabolic activity of the cells. The majority of these organ cultures were used to derive the final viability mapping protocol, which was then successfully implemented for positive and negative controls, freshly harvested valves, and organ cultured valves (Fig. 2; Table I).

### Validation of viability stains

Valve sections that were cut with the vibratome but not stained with the viability dyes served as negative controls. When imaged with confocal microscopy, these sections demonstrated only tissue autofluorescence, with no fluorescent signal from either live or dead cells (data not shown). The positive control for the dead stain (dead valve leaflet tissue) demonstrated the red ethidium homodimer stain only (Fig. 2A), and the positive controls for the live stain (monolayer cell cultures) showed that both valvular interstitial cells and endothelial cells could be stained with the green calcein AM (Fig. 2B).

### Localization of viable cells

Live cells were found in higher densities in the central region of the valve tissue, and lower amounts externally (Fig. 2C-F). Dead cells were found mainly externally, with few occurring centrally. Interestingly, there were even dead cells present on the surfaces of valve tissues that were stained immediately after harvest (Fig. 2C). It was also noted that the density of viable cells in the deep central region of the valve was lower than in the more superficial regions in the 49-day culture (Fig. 2F).

### Histology

Movat's pentachrome-stained tissue sections showed that the cultured valve tissues maintained their heterogeneous layered structure. The amount of cells in the sections stained with hematoxylin and eosin appeared to be consistent with the images

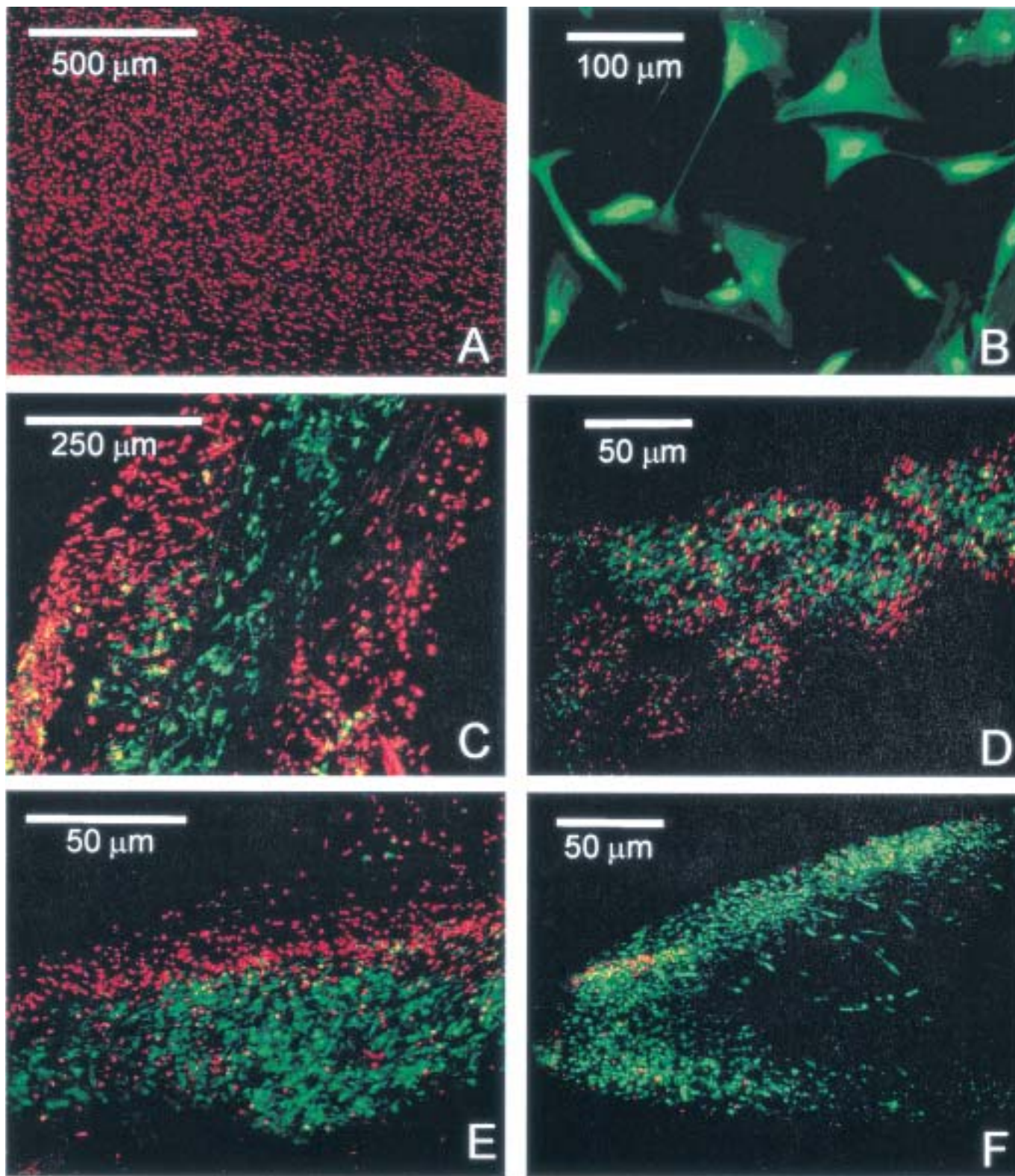


Figure 2: Viability-stained sections and positive controls. A) Positive control for dead cells (tissue soaked in dimethylsulfoxide); B) positive control for live cells (valvular interstitial cell monolayer); C) human pulmonary valve stained immediately after harvesting the valve from the transplant recipient heart; D) canine mitral valve stained after one day of organ culture; E) human aortic valve stained after 31 days of organ culture; F) bovine aortic valve stained after 49 days of organ culture.

obtained from the viability-stained sections.

### Cell concentrations

Live/dead cell counts were performed on the confocal images of seven valvular organ cultures (Table I). The 12 different image processing permutations yielded live and dead cell counts with an average coefficient of variation of 11%. In general, there were far more live than dead cells in the organ cultures (mean live:dead ratio = 1.64). There was no significant correlation between culture duration and ratio of live to dead cells ( $r^2 = 0.395$ ). When the total cell counts were normalized to the estimated mass of the confocal imaged tissue, the cell concentrations of the viability-stained valves fell into two categories - either overestimating or underestimating, by at least an order of magnitude - the cell concentrations calculated using the biochemical DNA assay (data not shown).

### Discussion

In the present study, methods were developed for mapping cell viability within organ cultures of valve tissues, and it was shown that cell viability could be maintained for up to seven weeks. Although longer culture times were not performed, extending culture duration past seven weeks appears promising. These data therefore support the use of organ culture as an alternative to cell monolayer cultures for the study of heart valve disease. Indeed, organ culture was previously used as a model for valvular wound repair by Lester et al. (5-7), although those studies were short-term in design (6 days) and did not examine cell viability. Extended duration organ cultures of valvular tissues, which have not been widely investigated in the past, would be particularly advantageous for studies of long-term valvular remodeling, chronic valvular diseases, and tissue engineering approaches. Furthermore, in-vitro organ cultures are simpler and less expensive experimental alternatives to animal

models, yet provide anatomic detail that is impossible to duplicate in monolayer cell cultures.

A surprising finding of the present study was cellular death at the edges of the organ culture, while the interstitial cells inside the tissues remained viable. This result was contrary to an original expectation that interior cells would be the least likely to survive, because these cells are located the furthest distance away from the oxygen- and nutrient-rich medium. Even in the 49-day culture, in which the deep central region contained fewer cells than did the more superficial central region, the deep cells that remained were alive. The differences in viable cell densities between the superficial and deep regions at 49 days, however, raises the possibility that some cells may have migrated from the deep center in order to be closer to the oxygen and nutrient supplies. This finding would be consistent with a previous study of oxygen consumption in aortic valves from young pigs by Weind et al. (12, 13). These authors proposed that valve leaflets thicker than 0.4-0.5 mm would become hypoxic, thus justifying the need for the capillary network that was found in these specimens (12,13). Their conclusion, however, was in contrast to that of Duran and Gunning (14), who suggested that because adult human heart valves (unlike young animal valves) are avascular, the cells inside must rely on diffusion for nutrient delivery. The influence of subject age and species, leaflet thickness, and oxygen/nutrient diffusion on cell viability, and possibly on cell migration, remains unclear.

Another interesting finding in the present study was that the biochemical measurements of cell number were poor matches for the cells counted from the viability-stained confocal image volumes, even though both of these methods are commonly used (15,16) and widely accepted. These differences can likely be attributed to the tissue heterogeneity - the DNA assay provides a measure of the average cell density in the entire tissue, but may not be representative of individual segments of the tissue. In contrast, the confocal images

Table I: Species, type of valve, duration of culture, and live/dead measurements for organ cultured specimens.\*

Species	Valve	Source	Days in culture	Live cells	Dead cells	Live/Dead ratio
Human	Mitral	Myxomatous repair	1	554 ± 378	455 ± 130	1.22
Canine	Mitral	Animal autopsy	1	856 ± 143	797 ± 54	1.07
Human	Tricuspid	Transplant	19	215 ± 9	179 ± 3	1.20
Human	Aortic	Transplant	19	1819 ± 235	894 ± 72	2.03
Human	Aortic	Transplant	31	1288 ± 322	1317 ± 45	0.98
Human	Pulmonary	Transplant	35	1083 ± 195	922 ± 77	1.17
Bovine	Aortic	Animal autopsy	49	3282 ± 212	681 ± 45	4.82

\*The seven specimens were imaged using confocal microscopy and analyzed for live versus dead cell counts.

demonstrate the cells in one small region, which may not be consistent throughout the tissue. Thus, the cell densities obtained from microscopic sections may be subject to sampling errors, and should be interpreted with caution.

The present finding of regional cell viability was specifically dependent upon the methodology used, whereby thinly cut sections of organ cultured tissues were stained *in situ*. Curtil et al. applied a similar *in situ* approach to examine their cell-seeding efficiency and cell viability within tissue-engineered valve matrices (4). Regional information such as this cannot be obtained by flow cytometry, which is more widely used to assess cell viability (3,8,9). Moreover, deriving the appropriate method for staining for the viability of cells located within the dense valvular matrix proved quite challenging. First, the fluorescent viability probes for live cells (such as calcein AM, fluorescein diacetate, or acridine orange) need to be applied to cells or tissues that have not been fixed - that is, to cells that are still alive. Furthermore, because these stains lack primary amines and thus cannot be anchored, stained cells need to be imaged immediately, and common histological methods such as fixing and clearing may diminish the fluorescence results. Although some residual fluorescence may remain after fixation and clearing - as shown by Poole et al. (17) and Curtil et al. (4) - it may not be representative of the original viability stain.

Another technical consideration was in determining how to cut sections so that the tissue cross-section could be imaged. Attempts to use a microtome, cryostat or scalpel blade were either unsuccessful, resulted in ragged tissue surfaces, or were incompatible with the requirement that cells be alive when they are stained and imaged. A vibratome was found to cut the tissues with the least amount of tissue damage, did not require prior fixation or embedding of the tissues, and resulted in tissue cross-sections with a smooth surface for confocal microscopy. Finally, the use of a confocal microscope with gain and offset controls permitted the substantial reduction of tissue autofluorescence, which can cause difficulties when imaging tissues rich in extracellular matrix (such as heart valve leaflets and collagenous tissue-engineered constructs).

### Study limitations

An initial limitation to this study was that only the viability of the cells was examined rather than their phenotype or metabolic state. Although the majority of cells remained alive, it remains to be shown whether the live cells maintained their normal cell phenotype, proliferative characteristics, and metabolism of oxygen and nutrients; these important issues are currently under investigation. The influence of tissue origin

(donor species and age) should also be examined in greater detail. In addition, the observation that cells at the periphery of the organ culture did not survive the extended culture period indicated that the culture medium may require further optimization. Another possible limitation is that amphotericin B may be toxic to endothelial cells at concentrations of 10-50 µg/ml (18-21), though levels of 1-5 µg/ml have been shown to be non-toxic (18,21). The amphotericin B level used in the present study was very low (0.25 µg/ml), but its inclusion was considered necessary to prevent contamination of the primary organ cultures. Finally, it is acknowledged that the finding of dead cells on the surfaces of valve sections stained immediately after harvesting might be due to adverse handling of the tissue samples.

*In conclusion*, a novel methodology has been developed to demonstrate the location of live and dead cells within valvular organ cultures, and viable organ cultures of heart valve tissues could be maintained *in vitro* for almost seven weeks. These methods may be applied to analyze *in situ* cell viability in a wide variety of dense collagenous tissues, organ cultures, or tissue-engineered materials.

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