

Explanation of Cell Counting Analysis files

(1) Morphometrical analysis

To evaluate the cellularity of the native ovine pulmonary roots (non-implanted), the implanted cryopreserved ovine pulmonary roots, and recellularisation of the explanted acellular porcine pulmonary roots, one section at each of the three levels from each explanted and non-implanted sample was stained with DAPI, or each of the antibodies for major cellular markers.

For sections stained with DAPI and antibodies to MAC, CD163 and CD80 sections from each level (Levels 1, 2 and 3) were subjected to cell counting as shown in Figure (1). Fields of view (FoV) were selected using a pre-determined template from the three different levels of each pulmonary root assigned for analysis. Cell counting was performed in nine areas of the pulmonary wall (three areas of each of the adventitial, medial and intimal regions, each at three levels) and two areas of the leaflet at 100 x magnification using "Image J" software. The total number of cells in the DAPI stained sections were counted and all the cells expressing a given marker stained with antibodies were counted within each FoV. The area of a field of view was 0.576 mm². The mean number of cells per FoV stained with DAPI (total cells) and each relevant antibody in the adventitia, media, intimal regions of the pulmonary artery wall and leaflets were calculated as indicated below. The mean number of cells per FoV were then multiplied by 1.47 to give the mean number of cells per mm².

- Adventitia mean of cell count for FoV's A level 1, 2 and 3; D level 1, 2 and 3 and G level 1,2 and 3
- Media mean of cell count for FoV's B level 1, 2 and 3; E level 1, 2 and 3, H level 1, 2 and 3
- Intima mean of cell count for FoV's C level 1, 2 and 3; F level 1, 2 and 3 I level 1, 2 and 3
- Leaflet mean of cell count for FoV's K level 1, 2 and 3 and J level 1, 2 and 3.

For sections stained with antibodies to CD3, CD34, CD19, CD271, CTGF, Ki67 and CD209, sections from Level 2 were subjected to cell counting as shown in Figure (13). Fields of view (FoV) were selected using a pre-determined template from Level 2. Cell counting was performed in nine areas of the pulmonary wall (three areas of each of the adventitial, medial and intimal regions) and two areas of the leaflet at 100 x magnification using "Image J" software. The total number of cells in the DAPI stained sections were counted and all the cells expressing a given marker stained with antibodies were counted within each FoV. The area of a field of view was 0.576 mm². The mean number of cells per FoV stained with DAPI (total cells) and each relevant antibody in the adventitia, media, intimal regions of the pulmonary artery wall and leaflets were calculated as indicated below. The mean number of cells per FoV were then multiplied by 1.47 to give the mean number of cells per mm²

- Adventitia mean of cell count for FoV's A level 2; D level 2 and G level 2.
- Media mean of cell count for FoV's B level 2; E level 2, H level 2.
- Intima mean of cell count for FoV's C level 2; F level 2; I level 2.
- Leaflet mean of cell count for FoV's K level 2 and J level 2.

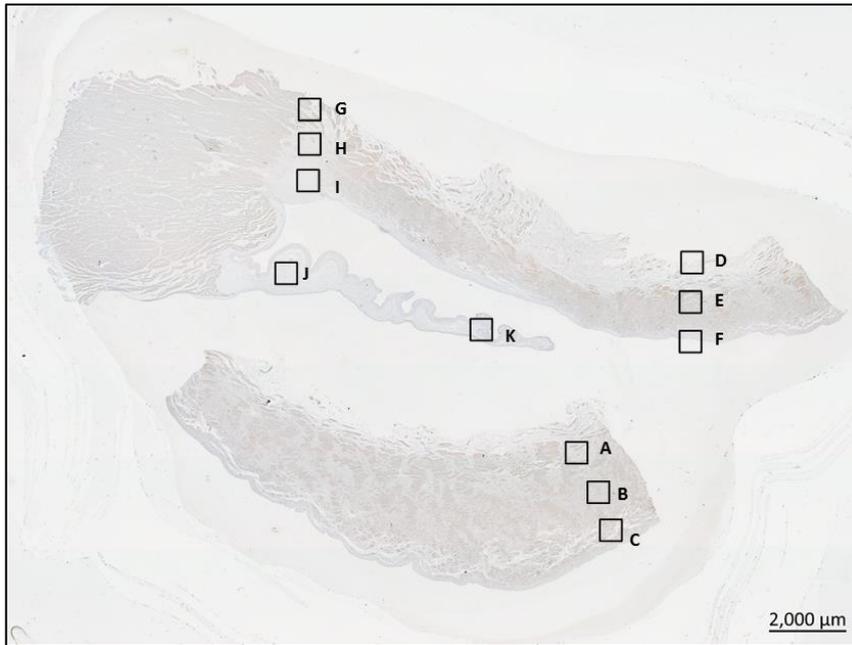


Figure (1) Scanned image of a section of native ovine pulmonary root showing the fields of view (100 x magnification) used for counting cell nuclei (DAPI) and positively labelled cells following immunohistochemical staining for cell markers. (A) Distal wall adventitia, (B) Distal wall media, (C) Distal wall intimal region, (D) Mid-wall adventitia (E) Mid-wall media, (F) Mid-wall intimal region (G) Junction adventitia, (H) Junction media, (I) Junction intimal region, (J) proximal leaflet, (K) Distal leaflet.

(2) EXCEL Files

Each file (one for each antibody) contains sheets:

- (1) NI ovine control (non-implanted ovine heart valves)
- (2) 1 month acellular (acellular porcine valves explanted from sheep at 1 month)
- (3) 3 month acellular (acellular porcine valves explanted from sheep at 3 months)
- (4) 12 month acellular (acellular porcine valves explanted from sheep at 12 months)
- (5) 12 month homografts (ovine homografts explanted from sheep at 12 month)

Which give:

The raw cell counts in each of the 9 areas shown in Figure (1) and counts adjusted to counts per mm²
 The mean of the counts per area of tissue (adventitia, media, intimal region and leaflet) and 95% CL

- (6) Data analysis

Welch`s ANOVA test applied to data (equal variances not assumed) followed by Games-Howell pairwise comparisons to determine individual difference between group means (significance level $\alpha = 0.05$) Where $n=4$ values in a group were all 0, it was necessary to assign one value as 0.1 to enable computation

- (7) Graphed data