Immunohistochemistry and double labelling immunofluorescence used standard staining protocols.

Briefly, the slides were dewaxed in xylene, and rehydrated in descending alcohol series. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide. A heat-induced epitope retrieval method (12 minutes at 800 watts microwave) in high pH9 Tris-based buffer (Connexin 43), or low pH6 Citrate buffer (GFAP, vimentin, adenosine A1 receptor) was used to unmask the antigens. The sections were blocked in 10% goat serum in PBS/T or ready-to-use 2.5% horse serum (Vector Laboratories) for 20 minutes at RT. This was followed by overnight incubation at 4°C with primary antibodies at a determined optimal concentration (see table) in diluent buffer (Dako, UK). The next day, the sections were incubated for 30 minutes at room temperature with universal anti mouse/rabbit HRP (DAKO Envision, UK) or ImmPRESS HRP secondary antibody (Vector Laboratories). Immunocomplexes were visualized with Dako DAB+ Chromogen (Dako, Glostrup, Denmark), following which the sections were counterstained with haematoxylin, and cover-slipped. The slides were washed with phosphate-buffered saline (Fisher Scientific, Ltd., UK) between all steps.

Double labelled Immunofluorescence

Detailed for GFAP/vimentin but similar method for GFAP/Cx43, Vimentin/Cx43, and Vimentin/A1 receptor

Slides were placed in xylene for ten minutes to dewax followed by rehydration in 100% Industrial methylated spirits (IMS) for 2x3 minutes and a final 3 minutes in 70% IMS solution. Following rehydration sections were placed in running water for five minutes then placed in 0.9% H_2O_2 for 15 minutes to block the endogenous peroxidase activity. After washing the slides in running water for a further 5 minutes, they were placed in a citrate-based unmasking buffer (pH 6.0) and microwaved for 12 minutes on high power for pre-treatment. The slides were then cooled, for 20 minutes on the counter-top. After cooling, slides were incubated with ready to use normal horse serum (2.5%) for 20 minutes to block any unspecific binding of the antibody. Next all the slides, except the double negative sections, were incubated with anti-GFAP, diluted at 1:1500, at 4°C overnight.

The next day the anti-GFAP antibody was washed off using 3x5 minute washes of PBS. Sections were then incubated with ImmPRESS HRP conjugated horse anti-rabbit antibody (Vector labs, Peterborough, UK) for 30 minutes. Again the antibody was washed off with 3x5 minute washes PBS. Next the slides were incubated with Tyramide signal amplification (TSA)-Fluorescein solutions for eight minutes. Tyramide signal amplification is required to amplify the signal from the primary antibody. The solution was made up of two thirds PBS, one third buffer and a dilution of 1:800. PBS washes were performed following the incubation. The slides were then incubated with anti-vimentin dilution 1:2000, overnight at 4°C. On the final day the vimentin antibody is removed by washing 3x5 minutes in PBS, and the HRP conjugated horse anti mouse antibody (Vector labs, Peterborough, UK) is added for 30 minutes. After PBS washes, TSA-rhodamine solution, made up the same as the fluorescein except with Cyanine 3, was added to the slides. They were incubated for 8 minutes at room temperature. The slides were then placed in 3 final PBS washes for 5 minutes each. Finally they were cover-slipped using Vectashield + 4',6-diamidino-2-phenylindole (DAPI) for counterstaining.

Cases included from the MRC sudden death brain bank : SD061/13, SD022/13, SD017/13, SD022/12

SD004/12, SD023/11, SD040/10