Assessing fibrinogen extravastion into Alzheimer's disease brain using high-content screening of brain tissue microarrays

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Abbreviations: tissue microarray, TMA; Phosphate buffered solution, PBS;

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Abstract

Background: Tissue microarrays are commonly used to evaluate disease pathology however ways to automate and quantify pathological changes from image analysis obtained from immunolabelled tissue microarray slides are limited.

Methods: This article demonstrates the utility of the VSlide scanner (MetaSystems) for automated image acquisition from immunolabelled tissue microarray slides, and subsequent automated image analysis with MetaXpress (Molecular devices) software to obtain objective, efficient and reproducible data from immunolabelled tissue microarray sections. Results obtained from a tissue microarray immunolabelled for fibrinogen is presented as an example. The observed changes were validated with Western blot analysis and gold standard blinded manual H-scoring.

Results: Significant increases in fibrinogen immunolabelling was observed in 29 Alzheimer's disease cases compared to 28 control cases analysed from a single tissue microarray slide. Western blot analysis also demonstrated significant increases in fibrinogen immunolabelling in 6 Alzheimer's cases compared to 6 control cases.

Comparison with Existing Method: VSlide Metafer software offers a 'tissue microarray acquisition' plugin for easy mapping of tissue cores with their original position on the tissue microarray map, replacing previous methods which depended on complex de-arraying algorithms. High resolution VSlide images are compatible with MetaXpress image analysis software. This article details the first time these two technologies have been coupled together to accurately and reproducibly analyse immunolabelled tissue microarrays within minutes, compared to the gold standard method of manual cell counting using H-Scores which is significantly more time consuming and prone to inter-observer variation.

Conclusion: Here, we couple brain tissue microarray technology with high-content screening and automated image analysis as a powerful way to address bottle necks in data generation and improve sensitivity to subtle but significant biological/pathological changes in brain disease.

1. Introduction

The use of multiple-tissue blocks in histology was first described over two decades ago (Battifora, 1986), and has since evolved into what is known today as a tissue microarray (TMA) (Kononen et al., 1998). A TMA consists of an array of cylindrical tissue cores extracted from different paraffin-embedded tissue 'donor' blocks, which are subsequently punched into a 'recipient' paraffin block. The utility of this technology far surpasses that of previous histological methods, particularly as it

enables tens, if not hundreds, of anatomical regions or cases to be arranged as small adjacent cores for assessment on a single glass slide (Decaestecker et al., 2009). This technology improves the processing time, reduces the amount of tissue resources and consumables required, and also minimises technical error (Kumar et al., 2004). Additionally, the sources of variation arising from immunolabelling protocols are consistent across all tissue cores on any given TMA section, allowing for standardisation of any technical error across all cases and/or anatomical regions (Kumar et al., 2004).

In diagnostic applications, there has been concern as to whether 0.6 mm or 1 mm cores are accurately representative of a tissue cross-section containing heterogeneous tumour cell populations. These concerns have been abated by studies showing a strong correlation between the results obtained from immunolabelled TMA sections and traditional larger tissue sections (Gillett et al., 2000; Kyndi et al., 2008; Sauter and Mirlacher, 2002). As a result, TMA technology has been particularly useful in cancer diagnostics (Hoos and Cordon-Cardo, 2001; Horvath and Henshall, 2001; Kallioniemi et al., 2001; Moch et al., 1999; Mohamed et al., 2007) and, in more recent years, it has been applied to the study of non-neoplastic intracranial disorders (Goldstine et al., 2002; Kauppinen et al., 2006; Martikainen et al., 2006; Qu et al., 2009; Sjobeck et al., 2003; Wang et al., 2002).

In addition, commercially available systems such as the VSlide scanner (MetaSystems) coupled with MetaMorph software (Molecular Devices) can

streamline image acquisition and analysis of cores. This coupling of scanner and software enables high-throughput processing of immunolabelled TMA sections, and thus avoids the effects of inter- and intra-observer subjectivity that is intrinsic to manual microscopical analysis (Cregger et al., 2006). This coupling also improves the objectivity and accuracy of the data obtained and accelerates data processing capability. Image analysis-based segmentation of staining/labelling and the biological parameters chosen for extraction of quantitative features are pivotal in this process and also for ensuring the reproducibility of the data generated (Decaestecker et al., 2009).

This article demonstrates the utility and importance of high-content screening of immunolabelled, paraffin-embedded TMA sections to quantify fibrinogen immunolabelling in middle temporal gyrus from post-mortem control and Alzheimer's disease cases. This specific example aids in the assessment of fibrinogen extravasation across a potentially leaky blood brain barrier in Alzheimer's disease cases.

2. Methods

2.1. TMA design, production and case information

There are several important factors to consider in the design and production of TMAs. An excellent resource which provides important guidelines and considerations for the use of TMAs is provided in this review (Ilyas et al., 2013). The typical

workflow used in our lab to prepare a TMA comprises of case selection, TMA template preparation, careful transfer of donor cores in to a recipient block and cutting sections using a sliding microtome (see Supplementary Fig. 1 for further details on the workflow involved).

Case selection was a critical step in TMA preparation. Matching control and disease group cases for post-mortem delay (PMD) and age at death was important in order to minimise variation caused by these parameters. Supplementary table 1 shows an example of a TMA, TMA#V, prepared using control and Alzheimer's disease cases that were matched for age at death (control, mean 75 years; AD mean 76 years), PMD (control, mean 14 hours; AD, mean 14 hours), days in storage (control, mean 2735 days; AD, mean 2284 days) and where possible, sex (control 28% female; AD 55% female). The table also provides details of the date of perfusion, corresponding to the storage time of cases in the Brain Bank.

All brain tissue used was obtained from the Neurological Foundation of New Zealand Human Brain Bank and in compliance with the University of Auckland Human Participants Ethics Committee. Brain tissue blocks used for the preparation of donor blocks were fixed, dehydrated and processed through standard histological protocols for wax embedding, as described previously (Waldvogel et al., 2008). Before preparation of the TMA, 7 μm sections were taken from each of the donor blocks and labelled using standard paraffin immunolabelling protocols for microtubule-associated protein 2 (MAP2). This ensured that all cases selected for the final TMA

were exempt of fixation irregularities and labelled accurately for an antibody against a ubiquitous protein for this anatomical region. Once cases were confirmed, an additional 7 μm section was taken from each case and labelled with Nissl. Nissl staining was particularly important for identifying specific cell layers of interest (for example, layer I-V of the cortical ribbon), to assist with choosing specific regions to core from the donor block. A TMA template was also prepared to assign the location of each case and of the blank reference points that served as orientation markers for mounting onto glass slides. An example of the template used to prepare TMA#V is provided in Supplementary Table 2.

The Advanced Tissue Arrayer, model VTA-100 (Veridiam) was used for the extraction of 2 mm tissue cores from different donor blocks and their insertion into the blank recipient paraffin block (see Fig. 1). Core cylinder lengths varied from 4-6 mm depending on the size of the donor tissue block. Careful, slow and consistent coring into donor blocks was imperative to avoid causing any cracks or breakages in the cores taken. Once all donor cores had been transferred, the recipient block was incubated overnight at 37°C, followed by 1 h at 60°C, in order to optimise the amalgamation of cores within the recipient block.

Once cooled, the recipient block was cut into 7 μm thick sections using a sliding microtome. The block was initially cut until all cases were represented as a single section. Approximately 200 sections were obtained from any given TMA. It was important to take care when mounting serial sections onto corresponding numbered

charged glass slides. Blanks were used to ensure consistency in the orientation of the section when mounted onto charged slides in water bath. Once mounted and dried overnight, the TMA sections were baked onto the slides by placing the mounted slides on a heat block (60°C) for at least 1 h. The slides were then stored at room temperature until further use.

2.2. Immunohistochemistry of paraffin-embedded sections

Standard immunohistochemistry was applied, as described previously (Waldvogel et al., 2008). Briefly, slides were dewaxed (1 h at 60°C), rehydrated (tissue slides submerged in xylene, then sequential decreasing concentrations of ethanol). Heat-induced antigen retrieval was achieved by heating slides, immersed in 10 mM citrate buffer, pH 6 for approximately 10 min at 121°C in an antigen retriever (model 2100-retriever, Pick Cell Laboratories). After cooling for 2 h, the slides were rinsed twice for 5 min in dH_2O. Endogenous peroxidases were quenched with a 20 min incubation in 50% methanol, 1% hydrogen peroxide (Calbiochem), followed by two 5 min rinses in dH_2O. Slides were then incubated in blocking buffer (PBS containing 0.02% Triton-X100 and 10% normal goat serum, Vector S-1000, Invitro Technologies) for 1 h at room temperature. This was followed by an overnight incubation in primary antibody at 4°C (Fibrinogen, raised in rabbit, DAKO, 1:100). The next day, after three 5 min washes in PBS, TMA slides were incubated in secondary antibody (anti-rabbit IgG, Sigma, 1:1000) for 3 h at room temperature, washed again and incubated in ExtrAvidin peroxidase (Sigma, 1:1000) for 1 h at room temperature. All blocking,

antibody and ExtrAvidin incubations were performed in a humidified chamber to prevent the sections from drying out. After three 5 min washes in PBS, slides were incubated in the peroxidase substrate, 3, 3-diaminobenzidine (final concentration 1x) diluted in 0.1% hydrogen peroxide and 0.4 M phosphate buffer for 20 min or until sufficient colour reaction could be visualised. Slides were washed three times for 5 min dH_2O and dehydrated (tissue slides were submerged in sequential increasing concentrations of ethanol and then xylene) and coverslipped.**Nissl staining of paraffin embedded sections**

Cresyl violet stains the Nissl substance of the endoplasmic reticulum of viable cells and was used to identify different cell layers of the cortex (Gittins and Harrison, 2004). Immediately before dehydration, slides were immersed in cresyl violet solution (0.025%; Janssen Chemica, in acetic acid) for 30 min or until sufficient staining intensity was reached and then briefly washed in water before the completion of the dehydration steps.

3. Western blot analysis

Materials and methods used for preparation of protein lysates and running/transfer of proteins utilised previously described Western blot techniques (Jansson et al., 2014; Narayan and Dragunow, 2010). Details of the cases used for Western blots are provided in Supplementary table 3. Where possible cases used were matched for age

at death (control, mean 60 years; AD, mean 74 years), PMD (control, mean 14 hours; AD, mean 10 hours), and sex (control, 33% female; AD, 50% female).

Membranes were blocked in TBS-T with 5% skim milk for one hour at room temperature. Then probed with fibrinogen antibody (1:250, DAKO, A0080, raised in rabbit) and for GAPDH (1:2,000, Abcam, ab9484, raised in mouse) diluted in TBS-T containing 5% BSA overnight at 4°C. Secondary antibody against the corresponding host species, goat anti-mouse Alexa 594 (1:2,000, Molecular Probes) and goat anti-rabbit Alexa 488 (1:2,000, Molecular Probes) were diluted TBS-T containing 5% skim milk for two hours at room temperature. Images were captured using the Li-COR imaging system or ChemiDoc MP imaging system (BioRad).

Fibrinogen protein bands were analysed using the region statistics drop-in in MetaXpress (Molecular Devices) software, where given the number of bands for fibrinogen labelling the integrated intensity for each lane was quantified and normalised to corresponding protein loading control band, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

4. Image acquisition and analysis using high-content screening technology

4.1. VSlide scanner (MetaSystems)

We demonstrate for the first time, the use of VSlide scanner (MetaSystems) coupled with MetaXpress (Molecular Devices) image analysis software to analyse tissue

microarrays in a cost effective and user friendly manner. Previous methods which have utilised other commercially available imaging platforms and analysis packages (Bolton et al., 2010; Braun et al., 2013; Camp et al., 2002; Dolled-Filhart et al., 2010; Ryan et al., 2011; Turashvili et al., 2009; Turbin et al., 2008) are listed in Table 1. It is important to note that these previous combinations require more complex software training and for users to have significant programming knowledge for basic analysis. In contrast, VSlide offers a customisable and user friendly tissue microarrayer tool enabling easy TMA acquisition by non-experts.

Immunolabelled cores were imaged using the VSlide scanner (MetaSystems) running Metafer4 (version 3.9.2) software. As shown in Fig. 2, an initial automated, low-power prescan of the TMA slide was obtained using the x2.5 objective. This prescan image was opened within the 'Microarray Tool' with Metafer 4 software, and threshold based segmentation was performed to identify the specific location of all labelled cores. User-specified dimensions (for example 6 x 10 cores) enabled a grid of interconnected dots to be placed on the thresholded x2.5 prescan thumbnail. Each dot could be selected upon a right mouse click and dragged to the desired location, which was placed in the centre of each corresponding core of interest. The location of each dot then guided the subsequent automated rescan at a higher magnification (x10, x20, x40 or x63 objectives could be used) of multiple sites (user specified) per core across the entire TMA slide in a high-throughput, standardised fashion. The platform employed the use of a motorised x, y and z stage, and could correct for

shading and background with automated background correction and auto-exposure options. Images acquired were automatically saved with individual filenames that corresponded to the core location (rows from left to right follow an A-F format and columns from the frosted edge of the slide to the end of the slide follow a 1-10 format, i.e the bottom left or left-most core closest to the frosted edge of a slide is always A1). Image acquisition took less than 15 min from start (low power prescan) to finish (grid assisted high resolution image resampling).

4.2. Segmentation obtained from MetaMorph image analysis (Molecular Devices)

The segmentation-based image analysis used to obtain semi-quantitative data from VSlide images of immunolabelled tissue microarray cores are shown in Fig. 3. This figure demonstrates the use of the MetaXpress analysis module 'Neurite Outgrowth' in conjunction with the MetaXpress dropin 'Integrated mophometry analysis' (IMA) to analyse fibrinogen immunolabelling using biologically relevant parameters such as area of staining as a percentage of total area of the image and integrated optical density corresponding to staining intensity of the antibody label. The 24 bit Tiff images from VSlide are first converted to 16 bit Tiff images, and based on user defined cell parameters such as size and staining intensity above back ground, the Neurite Outgrowth module generates accurate segmentation masks. The resulting masks are thresholded and used by IMA to measure and log the parameters of

interest from the original image directly to excel spreadsheets. Image analysis for 240 images per TMA slide took less than 20 minutes to complete.

4.3. Blinded H-Scores

A PDF document was created containing a representative image of each fibrinogen immunolabelled core from TMA#V. Images were randomised and presented to 2 independent volunteers who were blind to the case numbers corresponding to each image. The H-Score (Godbole et al., 2007) was used to obtain manual scores for staining across all the images. The manual scoring method involved all objects of interest in each image being counted for negative (0), weak (1), moderate (2) or high (3) immuno-staining levels, the totals for each intensity level were recorded and used to calculate the final H-Score for each image (Godbole et al., 2007). H-Score = ((0+1) x % of 'negative' cells) + ((1+1) x % of 'weak' cells) + ((2+1) x % of 'moderate' cells) + ((3+1) x % of 'high' cells). The blinded manual counting took the volunteers approximately 15 hours to complete for 60 representative images sampled from the original 240 images acquired with the VSlide, using the manual H-Scoring method.

4.4. Statistical analysis

Data obtained from segmentation based image analysis underwent statistical testing using GraphPad Prism version 5.03 (SmartDrawNet, 2009). Data sets were first tested for equal variances, if not significant, were tested for differences in the means

between disease and control groups using t-tests between groups to find where the significance originated. If not normally distributed then data sets were analysed with Kruskal Wallis tests with post-hoc Dunn's tests. Correlations between fibrinogen immunolabelling results and other variables (post-mortem delay or age at death) were tested using Pearson's correlation or an unpaired t-test for gender. Differences between groups were considered significant if the p-value obtained was less than 0.05, i.e. the observation was less than 5% likely to be due to chance alone.

5. Results

5.1. Significant increase in Fibrinogen load in AD brain compared to control

A low power scan (x2.5) of TMAV is shown (Fig. 4A) and representative images (x10) for control (H190) and AD (AZ65) cases are shown (Fig. 4B and 4C respectively). Strong fibrinogen immunolabelling is evident in AD brain and appears to surround microvessels, capillaries, cell bodies and processes dispersed throughout the cortical layers of the middle temporal gyrus, compared to very low levels in control brain. Automated image analysis TMAV demonstrated a significant ($P < 0.0001$) increase in total area of fibrinogen staining (Fig. 4D) and in the integrated optical density of fibrinogen immunolabelling (Fig. 4E) in AD (n=29) compared to control (n=28) middle temporal gyrus. Significant variation was also evident in AD cases compared to controls as shown by the larger error bars in the AD group.

Changes in fibrinogen immunostaining were found to be independent of differences in clinicopathological variables as there were no significant correlations found between fibrinogen immunostaining and age at death (Control, n = 28, r = -0.003, P = 0.99; AD, n = 29, r = -0.13, P = 0.50) or PMD (Control, n = 28, r = -0.05, P = 0.80; AD, n = 29, r = 0.32, P = 0.09) or length of storage in brain bank (Control, n = 28, r = -0.21, P = 0.27; AD, n = 29, r = -0.17, P = 0.38).

5.2. Correlations between automated analysis and blinded H-Scores

Significant correlations were found between the manual H-Scores obtained from the 2 volunteers (SL and CL) across control and AD cases (Fig. 5A; Control, n = 29, r = 0.52, P < 0.01; AD, n = 29, r = 0.54, P < 0.01), however the inter-observer variation between the H-Scores obtained from the respective volunteers was also significant, therefore Spearman's correlations were used. When H-Scores were compared to the measures obtained from automated analysis of fibrinogen immunolabelling, significant correlations were also obtained between H-Scores and integrated optical density (Fig. 5B; H-Score [SL], n = 60, r = 0.50, P < 0.0001; H-Score [CL], n = 60, r = 0.78, P < 0.0001) and between H-Scores and % of total area of fibrinogen staining (Fig. 5C; H-Score [SL], n = 60, r = 0.50, P < 0.0001; H-Score [CL], n = 60, r = 0.76, P < 0.0001).

5.3. Validation of immunolabelling results with Western blot analysis

Fibrinogen is a 340 kDa glycoprotein secreted from hepatocytes into the plasma. It is composed of 2 copies of 3 different polypeptide chains; Aα, Bβ and γ, interconnected by a complex web of disulphide bonds. After denaturation and separation on Western blots, polyclonal antibodies against fibrinogen detect 3 major bands (Fig 6; 67 kDa, 56 kDa and 47 kDa), corresponding to the 3 polypeptide chains: Aα, Bβ and γ, respectively (Okumura et al., 2002). Semi-quantitative analysis of these bands (normalised to GAPDH) demonstrated a statistically significant ($P < 0.05$) increase in fibrinogen load in AD cases compared to control cases also, corroborating with and in validation of immunolabelling results.

Discussion

This article demonstrates the utility and advantages of VSlide assisted TMA acquisition and MetaXpress™ image based analysis of TMAs as a powerful tool in evaluating pathological changes in post-mortem brain tissue for neurodegenerative disorders such as Alzheimer's disease. MetaXpress™ analysis enabled fast and accurate analysis of biologically relevant parameters, while also providing practical solutions for image and data integration, storage and retrieval. This method was used to investigate the level of fibrinogen extravasation across a potentially leaky blood brain barrier in Alzheimer's disease. The findings correlated with H-Scores obtained from volunteers who performed manual counting and demonstrated significant increases in fibrinogen deposition in Alzheimer's brain compared to controls.

Tissue microarrays have been used for over decade to provide a basis for adequate mapping and qualitative analysis of histopathological severity and type of lesions for a number of neoplastic and non-neoplastic disorders. In the context of neurodegenerative disorders such as AD, TMA technology has been used to characterise pathology in white matter (Sjobeck et al., 2003) and patterns of protein expression (e.g. Tau, Aβ, α-synuclein) or extent of gliosis in the brain with HIV induced encephalitis (Goldstine et al., 2002). We demonstrate a novel coupling of commercially available imaging and analysis platforms to obtain semi-quantitative data from TMAs.

It is important to note that TMA technology did not rule out all the problems associated with immunohistochemical quantification. For example, fixation conditions could differ between each original donor block. In addition, some cores were partially or completely lost during TMA preparation/processing. This is likely to have been the consequence of the varying core cylinder lengths between cases due to the variable thickness of original donor tissue blocks. As a result, upon sectioning the recipient TMA block, the shorter cylinders finished earlier than the longer cylinders. In addition, sampling bias of examining small core regions taken from large tissue blocks with the major assumption that the core will be entirely representative of the tissue block and/or the assumption that disease associated alterations are uniformly distributed with the anatomical regions/blocks of interest. Previous studies

have also shown that tissue found near the edge of a slide is susceptible to edge effects (Ciznadija et al., 2011).

Sjobeck and colleagues (2003) found that 2mm punched cores caused donor blocks to fissure several times, while 0.6mm core cylinders were found to be too fragile. They found 1mm punched cores produced the technically best sampling results (Sjobeck et al., 2003). This may be an important consideration for further TMA studies, with the added benefit that thinner cores enable a greater number of total cores to be included in the final TMA block.

If validated well, the advantages of automated image analysis coupled with tissue microarray technology significantly outweigh the limitations of this technology. Uniformity and consistency of staining conditions over the multiple brain regions and case numbers that can be processed on a single TMA slide (Wang et al., 2002) provide obvious benefits. TMAs reduce slide-to-slide variability that is inherent in most immunolabelling techniques, whether they are stained manually or with automated staining equipment. Digitisation of immunolabelled tissue samples also provides a permanent record of pathological data that can be easily shared by clinicians, pathologists and researchers. Digital pathology is becoming increasingly popular and necessary particularly for studies spread across multiple centres and countries.

Several studies have demonstrated that digital analysis correlates significantly with manual scoring methods, with the added advantage of removing human error/bias. It was necessary to validate the results obtained from automated analysis. This was done using Western blots and manual blinded H-Scores. Western blot analysis provided corroborating increases in fibrinogen protein bands (correlating to correct molecular weights of major chains of fibrinogen). Significant inter-observer differences in the H-Scores obtained from manual counts (statistical analysis required spearman correlations) were observed. This variation and subjectivity between counts obtained from two trained volunteers illustrates the risk of potential variation in qualitative histological assessment between different pathologists. In contrast, automated image analysis based methods provide objective, consistent and reproducible pathological assessment. Also, the time taken for automated analysis of a large number of samples is also a significant factor unparalleled by any form of manual counting and staining evaluation.

In summary, TMA analysis techniques are fast, use less consumables and tissue resources and immunolabelling and processing methods are standardised across all cores on a TMA section. In addition as a standalone, independent experiment, the data obtained are robust and representative of changes seen in a much larger sample size and sampling area.

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Table legends

Table 1. Previous combinations of imaging modalities and software packages used for analysis of tissue microarrays.

Figure legends

Figure 1. The 'Advanced Tissue Arrayer (Veridiam) was used to prepare TMA block. This figure illustrates how a core is taken from a paraffin embedded donor block and the core is transferred into the recipient block. An example of a final TMA block prepared using 2mm and 1mm cores is also shown.

Figure 2. VSlide imaging of TMA's is a three step process. Step 1, involves are low power scan of the entire TMA section (2.5x magnification). Step 2 utilises the 'Microarray Tool' available in Metafer software to apply a gray-value threshold that delineates all cores of interest in the TMA and enables the user to specify the accurate location of all cores using a grid, of interconnected dots. Each dot on the grid (green) can be moved to the centre of each corresponding core. The positions are automatically saved. Step 3, uses the grid positions from step 2 to acquire single or multiple sites per core across the entire TMA at higher resolution (a x10 image is shown as an example of one of 4 sites taken from a 2mm core).

Figure 3. A custom journal was written using MetaXpress (version 5.3.0.4) to measure area and optical density/integrated intensity changes from VSlide images of fibrinogen immunolabelling. The canned module: 'Neurite Outgrowth' was used to generate segmentation masks of specific fibrinogen immunolabelling. The segmentation mask was then thresholded and used by the MetaXpress dropin: Integrated morphometry analysis to measure attributes of the original image using the corresponding mask.

Figure 4. Fibrinogen immunolabelling is elevated in AD compared to control middle temporal gyrus. A low power scan (x2.5) of TMAV suggests gross differences between control and AD cases (A), these changes are clearly evident at higher resolution (x10), representative cases for control (H190) and AD (AZ65) are shown. Statistically significant differences ($P < 0.0001$) were found between groups (control, *n=28*; AD, *n=29*) for area of fibrinogen immunolabelling and immunolabelling integrated optical density.

Figure 5. Manual H-Score correlations to assess inter-observer variability and correlations with data obtained from automated analysis. The correlation between the two manual H-Scores was weaker than the correlation each respective manual score had with the automated analysis, as indicated by statistical significance ($P < 0.01$ compared to $P < 0.0001$ respectively) and average correlation coefficients (0.53 versus 0.64 or 0.63 respectively). Images of 3 blank cores were included in the blinded manual counts, raising the total sample size to n = 60.

Figure 6. Significant increase in fibrinogen immunolabelling was shown by Western blot analysis. Protein lysates obtained from inferior temporal gyrus demonstrated a statistically significant ($P < 0.05$) increase in fibrinogen load in AD cases (*n=6*) compared to controls (*n=6*), independent of changes in protein loading (semi-quantitative analysis was normalised to GAPDH loading control). Three major bands for fibrinogen were observed, corresponding to Aα, Bβ and γ chains.