Dual *in situ* hybridization and immunohistochemistry for quantitative analysis of house-keeping gene changes in normal and Alzheimer's disease brain tissue

Highet, B.A[1,2], Anekal, P.V.[3], Murray, H.C.[1,2], Coppieters, N.[1,2], Dieriks, B.V.[1,2], Faull, R.L.M.[1,2], Dragunow, M.[1,4], Curtis, M.A.[1,2]

[1] Centre for Brain Research, The University of Auckland
[2] Department of Anatomy & Medical imaging, The University of Auckland
[3] Biomedical Imaging Research Unit, The University of Auckland
[4] Department of Pharmacology, The University of Auckland

Background

In situ hybridisation (ISH) techniques are powerful tools for localizing gene expression in tissue samples. Combining ISH with immunohistochemistry (IHC) gives the cellular context of mRNA expression and spatial variation in expression patterns, which cannot be achieved with microarray or polymerase chain reaction.

We investigated the use of RNAscope® ISH (ACD Bio) in combination with fluorescent IHC on parraffin embedded human brain tissue from neurologically normal and Alzheimer's disease (AD) cases. We utilized tissue microarray (TMA) sections of middle temporal gyrus(MTG) to study the expression of three commonly used housekeeping genes offered by ACD Bio: ubiquitin C (UBC),peptidyl-prolyl cis-trans isomerase B (PPIB) and DNA-directed RNA polymerase II subunit RPB1 (POLR2A) to determine their suitability in the study of AD tissue. Furthermore, we set out to develop an automated analysis method for quantifying RNA ISH puncta across the total cell population and within neurons identified by NeuN$^+$ immunoreactivity. Overall, this project will provide a suitable platform to study changes in gene expression in diseased brain tissue with both cellular and anatomical context.

Methods

TMA sections comprising 2mm cores of middle temporal gyrus containing all cortical layers from approximately 20 normal and AD cases. Sections were processed for the RNAscope 2.5 HD Assay - RED before being subjected to fluorescent IHC for NeuN and counterstaining with Hoechst. Images were then acquired on a MetaSystems V-Slide slide scanner at 10x magnification, with puncta being imaged in both TxRed and under brightfield illumination, and DAPI and NeuN in their respective fluorescent channels. A custom macro was then developed which threshold masks cell and neuronal nuclei. The output is total puncta/total cell number as well as NeuN$^+$ puncta/NeuN$^+$ cell number.

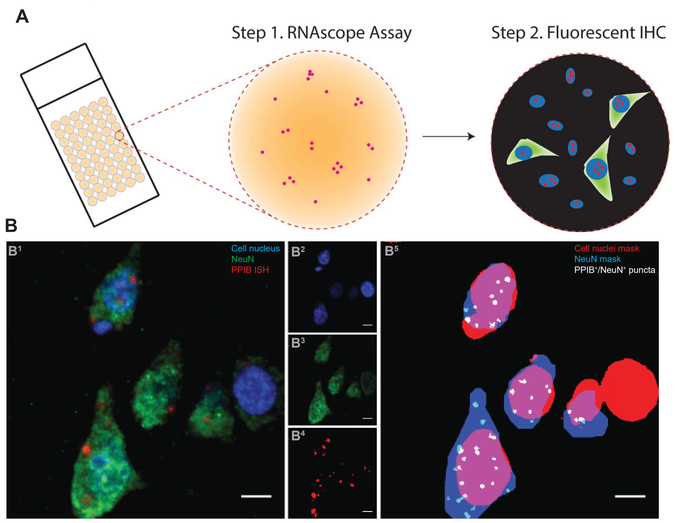

Figure 1: (A) Workflow for dual ISH + IHC protocol involves processing sections for RNAscope before conducting fluorescent IHC for the detection of NeuN$^+$ cells. (B$^{1\text{-}4}$) PPIB in situ signal obtained using the RNAscope 2.0 HD Detection Kit (Red) within NeuN-positive cells (green) in the middle temporal gyrus. (B^5) Threshold masking of the RNA puncta (white) and NeuN immunoreactivity (blue) as well as a dilated mask of the cell nuclei (red). These masks allow for the quantification of RNA puncta within all cells (defined by nuclei mask) and all neurons (defined by NeuN mask). Scale bar = 10 µm.

Results

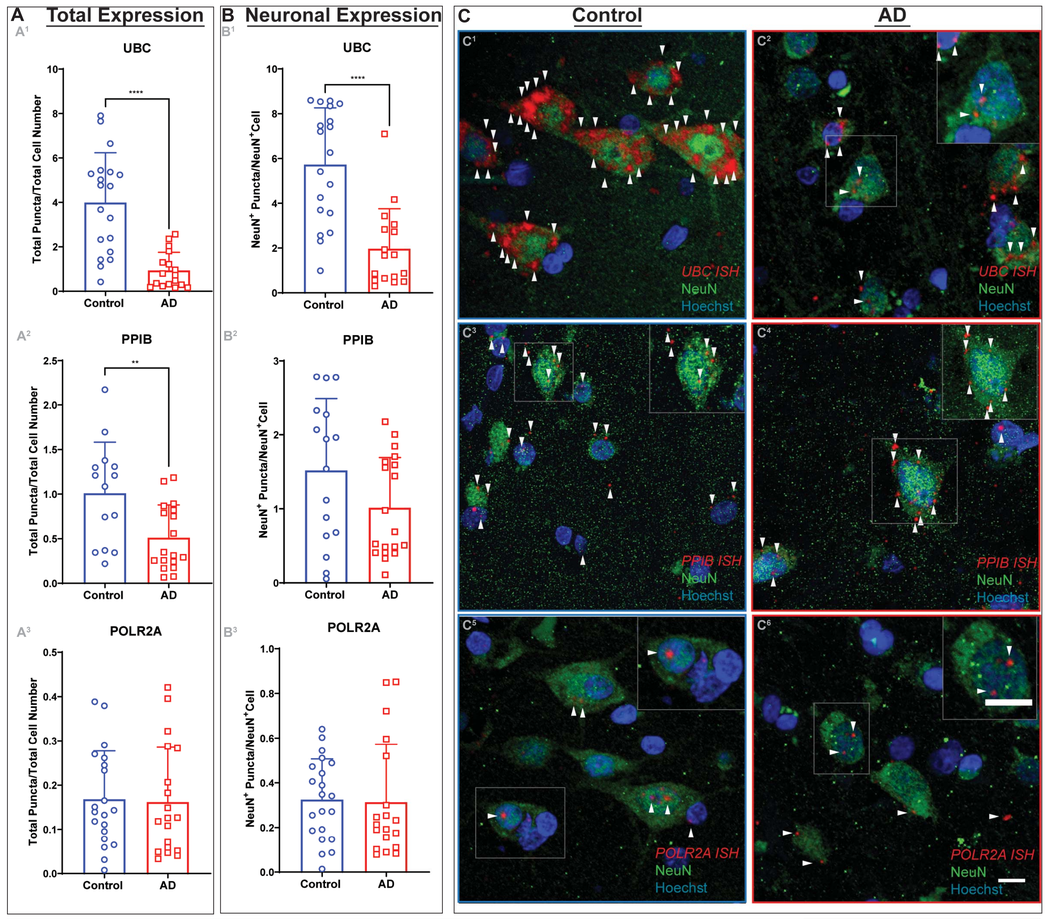

Figure 2. Total and neuronal specific expression of housekeeping genes wihin normal and AD MTG cores. (A) Total expression of housekeeping genes (Total Puncta/Total Cell Number). A significant decrease in both total UBC (A^1) and PPIB (A^2) expression was seen in AD (mean control UBC: 3.996, mean AD UBC: 0.9396, ****p < 0.0001. Mean control PPIB: 1.010, mean AD PPIB: 0.5111, **p = 0.0055), whilst no difference in POLR2A (A'") expression was seen (mean control POLR2A: 0.1683, mean AD PPIB:0.1621, p = 0.8679). (B) Furthermore, UBC (B^1) neuronal expression was also significantly decreased in AD(mean control UBC: 5.272, mean AD UBC: 1.973, ****p < 0.0001), whilst no significant difference in PPIB (B^2) or POLR2A (B^3) neuronal expression was seen (mean control PPIB: 1.518, mean AD PPIB: 1.015, p = 0.0811. Mean control POLR2A: 0.3256, mean AD POLR2A:0.3139,p = 0.8679). (C) Representative images of housekeeping gene expression within NeuN$^+$ cells (C^1: Control UBC, C^2: AD UBC, C^3: Control PPIB, C^4: AD PPIB, C^5: Control POLR2A, C^6: AD POLR2A). Scale bar = 10 µm. (D) Total and neuronal cell densities between control and AD cases:No significant changes in total cell density (D^1) or neuronal density (D^2) are seen between AD and control, however the proportion of cells that are NeuN$^+$ is significantly decreased in AD TMA cores (mean control total cell density: 1332 cells/mm^2, mean AD total cell density: 1466 cells/mm^2, p = 0.0941. Mean control neuronal density: 348.3 cells/mm^2, mean AD neuronal density: 283.1 cells/mm^2, p = 0.1304. Mean control neuronal proportion: 26.75%, mean AD neuronal proportion: 19.94%, **p = 0.0026).

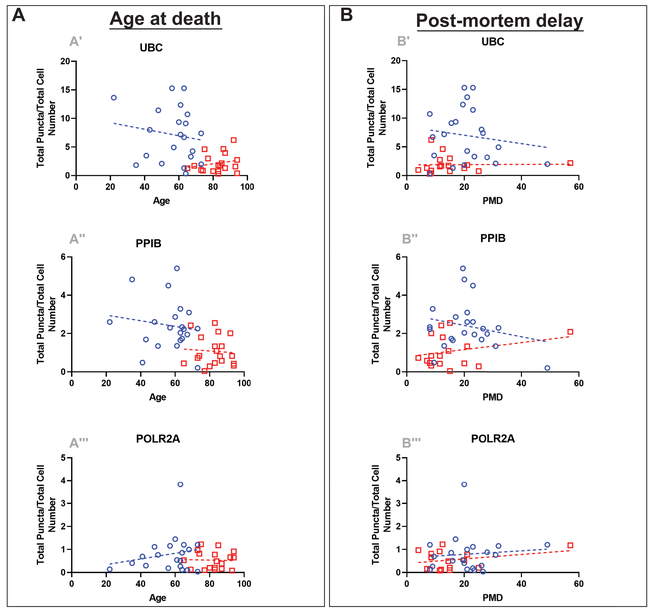

Figure 3: *In-situ* hybridization of housekeeping genes and correlations with age at death (A) and post-mortem delay (B). No significant correlations were seen between housekeeping gene signal and age at time of death (Control UBC: p = 0.4984,AD UBC: p = 0.3736. Control PPIB, p = 0.5246, AD PPIB: p = 0.772. Control POLR2A: p = 0.4140, AD POLR2A: p = 0.8990). Moreover, no significant correlations were seen between housekeeping gene signal and post-mortem delay (PMD) (Control UBC: p = 0.5059, AD UBC: p = 0.9700. Control PPIB: p = 0.3286, AD PPIB:p = 0.2837. Control POLR2A: p = 0.6986, AD POLR2A: p = 0.2435).

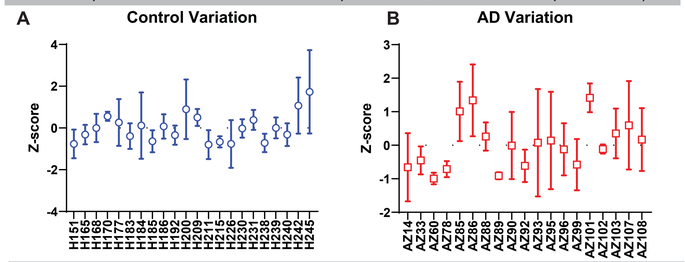

Figure 4: Normalised variation in the three housekeeping genes for each individual case comprising the TMA cores for both control (A) and AD (B). The majority of cases show a large amount of variation in expression for the 3 different housekeeping genes, suggesting that the *in situ* signal is dependent on differential expression of probes rather than overall RNA viability.

Discussion

- **Significant decreases in total cell expression of UBC and PPIB are observed in AD MTG cores.**
- **A decrease in neuronal-specific UBC expression is also observed**
- **No significant correlations between RNA ISH signal and age of death or post-mortem delay**
- **Therefore, UBC and PPIB are not suitable housekeeping genes for study of the AD MTG. Care should be taken when choosing genes for normalisation in other techniques such as PCR.**