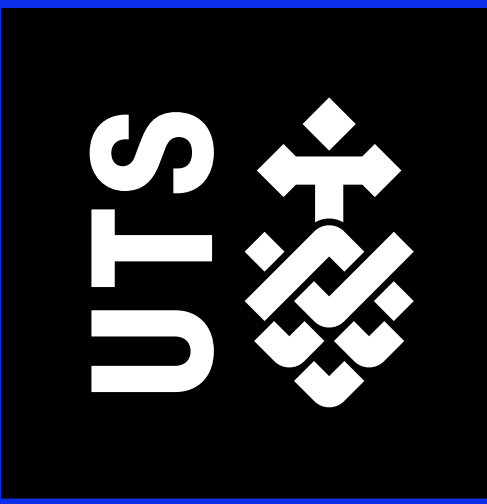




TyrRu: A Tyramide-Amplified Multimodal Ruthenium Tag for Elemental Mass Spectrometry and Immunofluorescence Imaging

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INTRODUCTION

- Visualization and quantification of proteins in tissue by immunohistochemical (IHC) techniques are difficult for low abundance proteins, not producing enough signal for accurate detection.
- Signal amplification strategies like tyramide signal amplification (TSA), uses the horseradish peroxidase(HRP) enzyme to convert labelled tyramide compounds into reactive intermediates capable of binding covalently with tyrosine residues present in tissue.¹
- Elemental mass spectrometry imaging (MSI) is used to image biomolecules in situ via metal-conjugated probes. Elemental MSI offers greater sensitivity, quantitative results, and higher degrees of multiplexing compared to immunofluorescence (IF), while being free of issues such as spectral overlap and autofluorescence.²
- TyrRu is a novel tyramide-functionalized ruthenium bipyridine complex developed for use as a multimodal TSA tag for IF and elemental MSI, increasing the detection sensitivity at higher image resolutions for protein targets in-situ.

AIMS

- To develop a multimodal TSA antibody staining protocol –
- Allow for biomolecule detection using LA-ICP-MS
 - Confirmatory detection by IF
- To apply and optimize TyrRu application and concentration to label Glial fibrillary acidic protein (GFAP) in mouse brain

METHODS

Staining protocol:

The staining procedure briefly involved fixing the tissue using 4% PFA, blocking steps for endogenous avidin, biotin, peroxidase and other native proteins, incubation of the primary antibody (GFAP), biotinylated secondary antibody, ABC-HRP complex and TyrRu to allow for the in-situ labelling of GFAP in murine tissue. Each step was followed by a 3 x 5 min wash with TBS + 0.01% Triton-X as the wash solution until the application of the ABC complex. Following this, a 0.1 M borate buffer with 0.003 % H₂O₂ was used as the wash solution and to dilute **TyrRu** for application.

Imaging techniques:

- LA-ICP-MS imaging was performed on an Elemental Scientific Lasers ImageBIO 266 nm (Kennelec; Mitcham, Australia) equipped with a TwoVol3 ablation chamber and coupled to an Agilent 7900 series ICP-MS (Mulgrave, Australia).
- High-resolution imaging was obtained using a 1 µm spot size and 0.9 µm spot overlap at a scan speed of 20 µm s⁻¹, with a 200 Hz firing rate and a fluence of 2 J/cm² (**Figure 1a**).
- Fluorescent images were taken using Nikon TiE2 inverted fluorescence deconvolution microscope using a Plan Apo λ 20x Ph2 DM (0.75 NA). An excitation wavelength of 488nm (6%, 150ms) was used, and the amount of emitted light detected was maximized by employing a pentaband filter.

RESULTS

The optimized conditions were applied to the analysis of GFAP in murine brain. A high degree of conformity between IF and iMSI was observed (Figure 1a and 1b), facilitating co-confirmatory screening of the protein target.

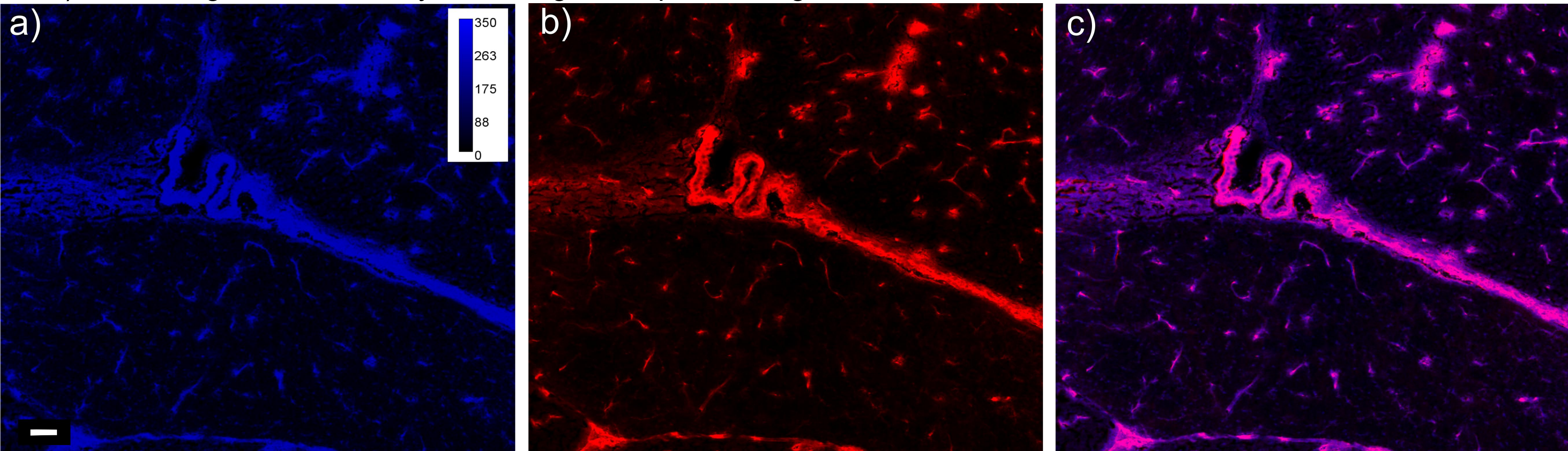


Figure 1: The optimized TyrRu protocol was applied to detect GFAP via (a) iMSI with the calibration bar units in in mg kg⁻¹, and (b) shows the IF image obtained on the same section prior to decoverslipping and iMSI. (c) The iMSI and IF images were superimposed to show the correlation between the complementary techniques, the scale bar depicted in a) represents 100 µm and is applicable to all images.

RESULTS

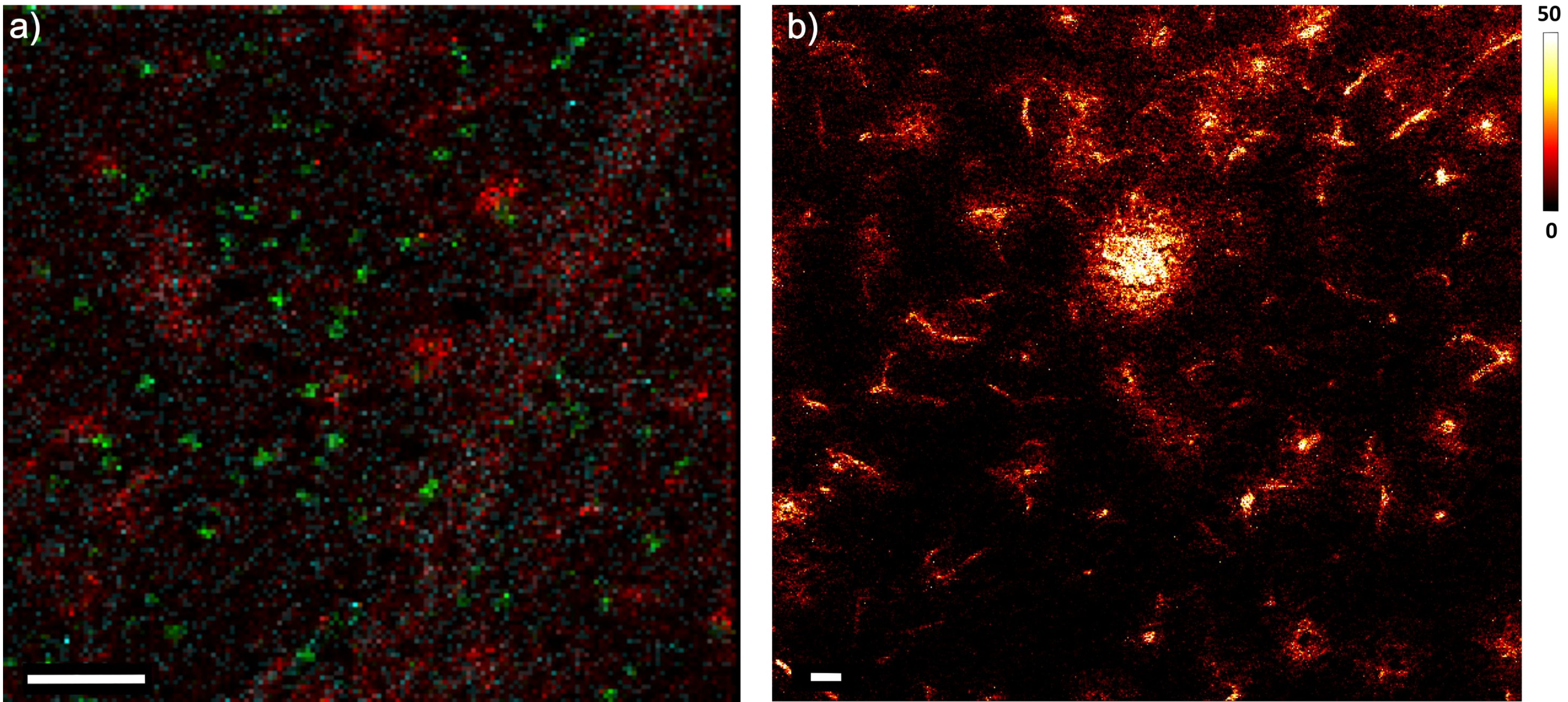


Figure 2: a) Multiplexed LA-ICP-MS image of GFAP (red) labeled with TyrRu, 170Er-NeuN (green), and Ir-DNA intercalator (cyan) in murine brain. b) Super-resolution reconstruction (SRR) image of GFAP labeled with TyrRu using the optimal conditions to obtain an image resolution of 250 nm. The scale bars represent 50 µm, and the calibration bar units are in mg kg⁻¹.

- We examined TSA amplification via TyrRu in a multiplexed application. GFAP in murine brain was again the target for TyrRu amplification, with the addition of an Er-conjugated anti-NueN antibody and an Ir-DNA intercalator (see Figure 2a).
- Application of TyrRu did not impact the quantification of the primary-labeled biomolecule. No significant differences in either variance or mean was shown when quantified in a multiplexed or individual application.
- Super-resolution reconstruction (SRR) involves the reconstruction of a higher-resolution image by combining multiple images ablated using consecutive offset orthogonal rasters that are acquired at subpixel distance offsets.
- Due to the increased detection sensitivity of TyrRu, we applied SRR using a 1 µm spot size for ablation. SRR allowed us to image at a resolution approximating that of a 40x IF, or 250 nm (Figure 2b).

DISCUSSION

- Indirect, amplified immunolabeling techniques had not been explored for elemental MSI applications.² TyrRu allowed for increased biomolecule labeling, facilitating both higher-resolution imaging and the investigation of low-abundance proteins.
- Its multimodality allows for initial screening and co-confirmatory imaging through its fluorescent properties, while also enabling relative quantification across samples.² It could be applied in a multiplexed analysis without impacting quantitative results.
- The amplification provided by TyrRu facilitated the use of SRR to obtain the highest resolution image of biomolecules by LA-ICP-MS. Imaging at subcellular resolution will allow unprecedented access to information on changes induced by disease and greatly reduce the complexity of such analyses.

CONCLUSIONS

- TyrRu was used as as a multimodal TSA analyte for the imaging and quantification of GFAP in mouse brain.
- TyrRu could be applied alongside primary-conjugated antibodies in a quantitative multiplexed analysis.
- The enhanced detection sensitivity facilitated the generation of an image with a resolution of 250 nm after super-resolution reconstruction, which is lower than that typically obtained by 40x IF microscopy.

References

1. Faget, L.; Hnasko, T. S. Tyramide Signal Amplification for Immunofluorescent Enhancement. In ELISA, Hnasko, R.; Springer: New York, NY, 2015; pp. 161– 172. DOI: DOI: 10.1007/978-1-4939-2742-5_16
2. Thai, A.; Lockwood, T. E.; Kohilas, I.; Bergin, R. J.; McDonagh, A. M.; Bishop, D. P. Elemental Mass Spectrometry Imaging of Biomolecules Using Metal-Conjugated Probes. Nat. Rev. Chem. 2025, 9, 672, DOI: 10.1038/s41570-025-00749-9