MULTIPLEX IMMUNOHISTOCHEMISTRY TO INVESTIGATE THE TUMOUR MICROENVIRONMENT

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Background

Multiplex immunohistochemistry (mIHC) allows researchers to phenotypically identify multiple cellular subsets within a single tissue section. At the Peter MacCallum Centre for Advanced Histology and Microscopy (CAHM), we are currently optimizing antibody panels for use in mIHC on both human and murine tissue sections. Multiplex IHC is accomplished through sequential rounds of antibody labelling followed by Tyramide Signal Amplification (TSA). The primary and secondary HRP (Horseradish Peroxidase) conjugated antibodies are removed after each antibody/TSA round by boiling the section, leaving the fluorescently conjugated tyramide densely populating the area immediately adjacent to the antigen (**Fig 1**).

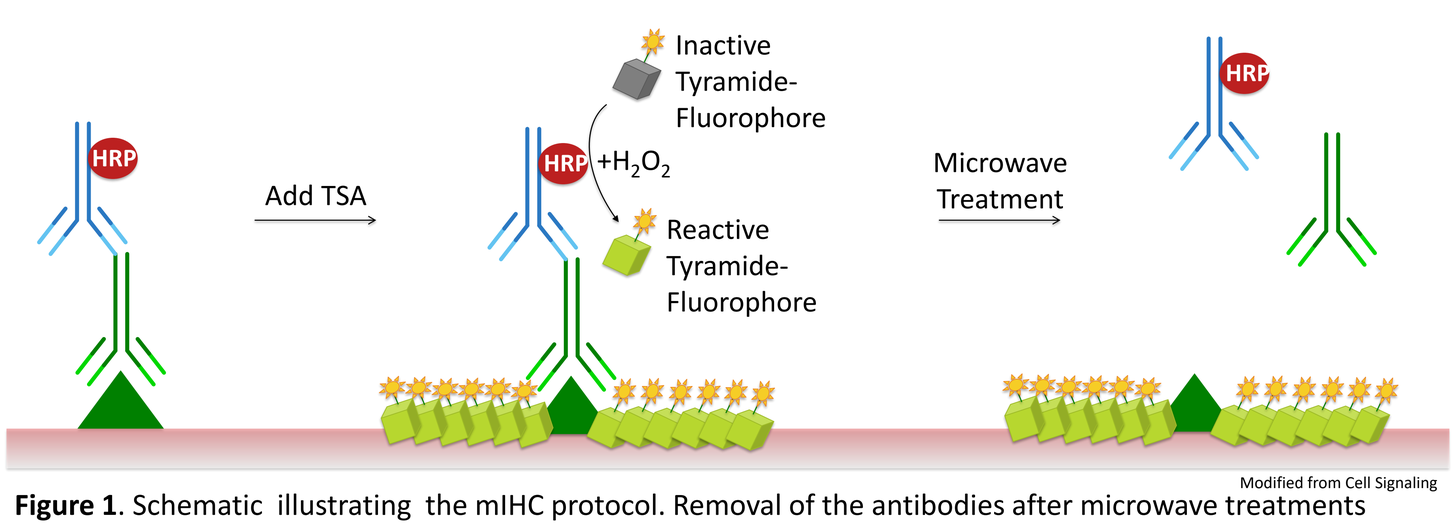


Figure 1. Schematic illustrating the mIHC protocol. Removal of the antibodies after microwave treatments facilitates being able to use antibodies from the same species.

Aims

1. Optimise the standard TSA mIHC protocol.
2. Optimise antibodies for use in mIHC for mouse and human tissues.
3. Compare spectral unmixing capabilities of the Perkin Elmer Vectra with confocal.
4. Develop new analysis protocols
5. Automate the staining process

Results and Discussion

1. The standard mIHC protocol is depicted in **Fig 2**. We compared Microwave treatment versus the use of the Pressure Cooker for removing the antibodies after labelling and found little difference between the two methods.

2. Each antibody was optimised (antigen retrieval method and dilution) and visualised using ImmPress signal amplification and the chromogen, 3,3' diaminobenzidine. These sections were useful controls for multiplexing. As a rule, for mIHC the less abundant antigens were labelled first followed by more abundant antigens. We utilise two panels for mIHC on mouse tissues and human tissues: an immune panel and a pan-immune panel (**Fig 3**).

3. The Perkin Elmer Vectra microscope is valuable for fast, high throughput imaging whereas spectral confocal microscopes offer high-resolution montages, and the flexibility to use >7 fluorophores and the newer infra-red fluorophores. The powerful Perkin Elmer 'Inform' software facilitates unmixing and segmentation of cells, tissue and stroma (**Fig. 4**). Using the image analysis software, Metamorph, we developed a macro to measure the distance of cells from the tumour margin (**Fig. 5**).

5. The Leica BOND Rx is currently being evaluated in our laboratory.

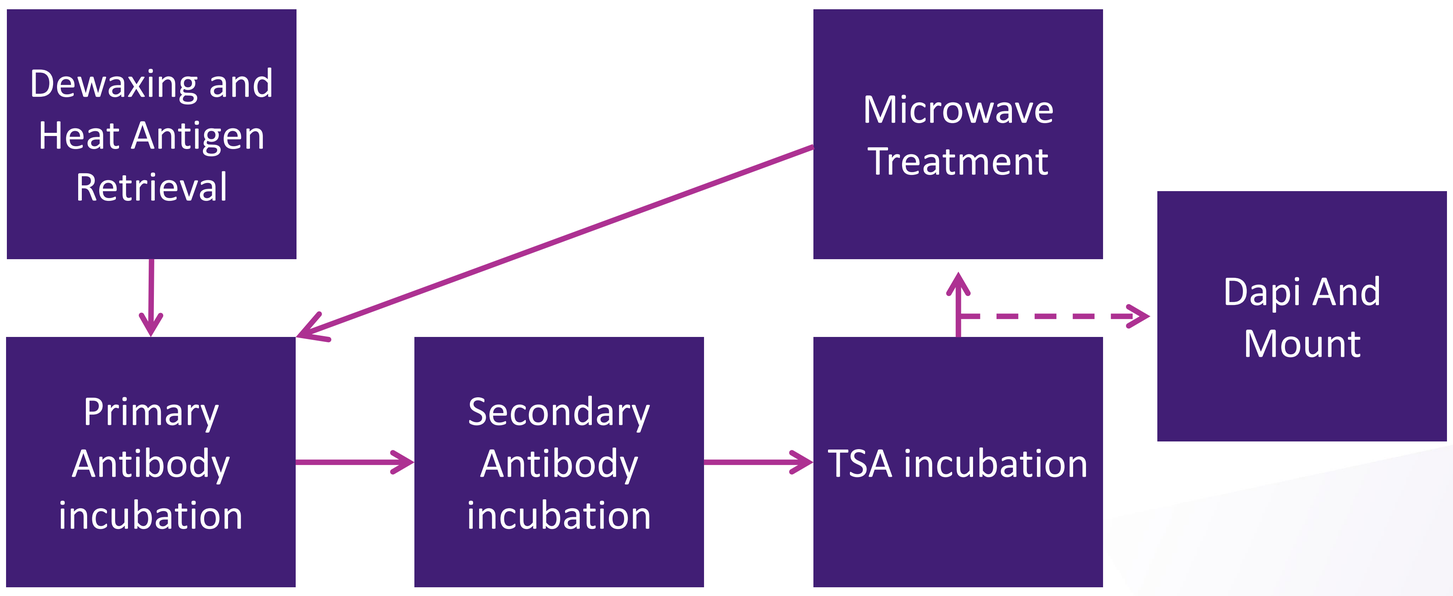
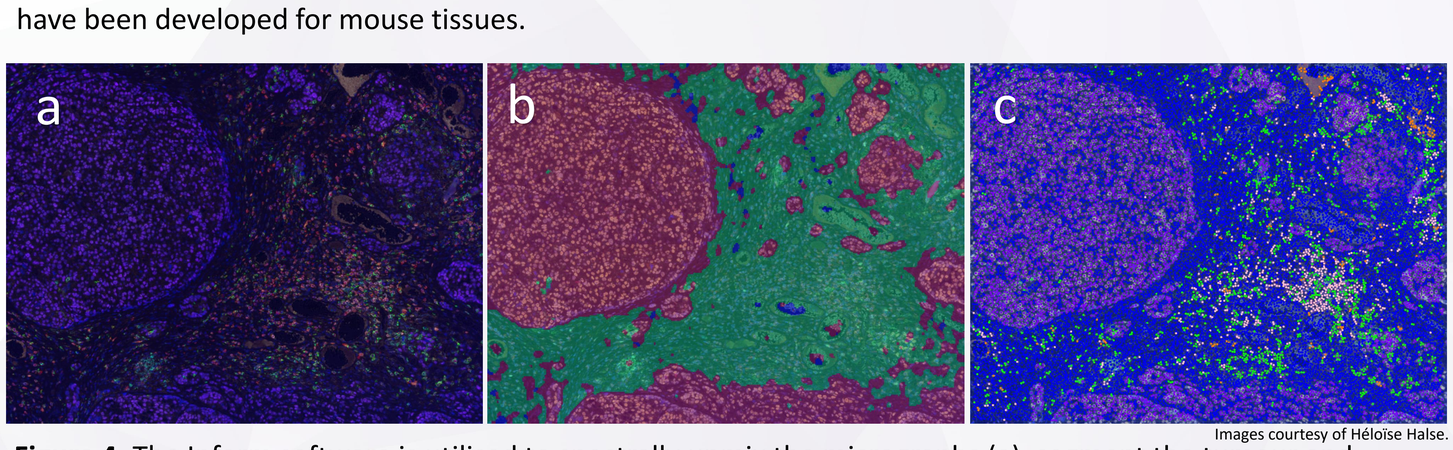


Figure 2. Simplified Opal Protocol.

T cell panel	Pan Immune panel
CD4 (T helper cells): pink	CD68 (Macrophages): white
CD8 (cytotoxic T cells): green	CD20 (B cells): orange
Foxp3 (Tregs): orange	CD11c (Dendritic cells): green
CD3 (T cells): red	CD3 (T cells): red
PDL1: yellow	PDL1: yellow
Tumour marker: purple	Tumour marker: purple
Dapi: blue	Dapi: blue

Figure 3. List of antibodies used in the human Immune and pan-Immune panels at Peter Mac. Similar panels have been developed for mouse tissues.



Images courtesy of Héloïse Halse.

Figure 4. The Inform software is utilised to spectrally unmix the micrographs (a), segment the tumour and stromal regions (b) and phenotypically segment the cells (c).

Summary

Multiplex IHC is a valuable tool for interrogating the tumour microenvironment. We are currently focussing on investigating the immune-infiltrates in various solid tumours and evaluating a system for the automation of Opal staining.

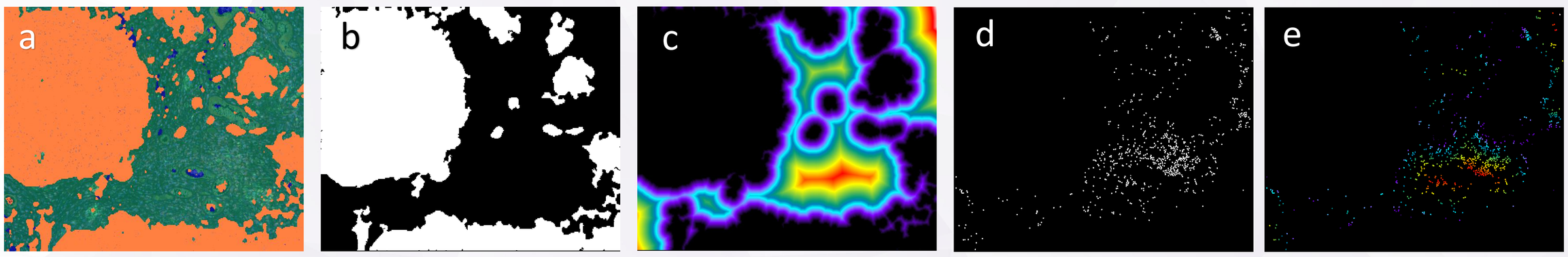


Figure 5. Using Metamorph, we can measure the distance of each cell from the tumour margin: (a) threshold the image then (b) binarise the result. (c) Use a Euclidean distance mask, which colours pixels depending on their distance from the tumour margin. Threshold the cells of interest and binarise (d). (e)Use image maths to overlay the result of (c) and (d). Output result to excel .