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RAPID COMMUNICATION

An automated staining protocol for seven-colour immunofluorescence of human tissue sections for diagnostic and prognostic use

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Summary

Multiplex immunofluorescence (mIF) allows simultaneous antibody-based detection and quantification of the expression of up to six markers, plus a nuclear counterstain, on a single tissue section. Recent studies have shown the potential for mIF to advance our understanding of complex disease processes, including cancer. It is important that the technique be standardised and validated to facilitate its transition into clinical use. Traditional approaches to mIF rely on manual processing of sections, which is time-consuming and a source of significant variation between samples/individuals. Here we determined if an automated diagnostic tissue stainer could be used for mIF incorporating tyramide signal amplification (TSA), and how the final image quality compared with sections stained semi-automatically or manually. Using tissue microarrays of fixed human breast tumour sections, we observed comparable antibody labelling between the diagnostic autostainer and manual technique. The diagnostic autostainer produced higher signal intensity with similar spectral unmixing efficiency. We also found that microwave treatment for antibody stripping during TSA labelling could be replaced by the heating option incorporated within the diagnostic-use autostainer. These data show that diagnostic autostainers used for traditional immunohistochemistry protocols can be readily adapted to achieve rapid preparation of high-quality sections using a TSA method for clinical mIF.

Key words: Automation; formalin-fixed paraffin-embedded tissues; multiplexing; multispectral; immunofluorescence; fluorescence-immunohistochemistry; imaging; immune cells.

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INTRODUCTION

Traditional immunohistochemistry (IHC) and immunofluorescence (IF) techniques allow visualisation of up to two and four markers on a tissue section, respectively, and require each of the primary antibodies used to be from different species. Not only is this largely impractical, these limitations also translate into missed opportunities to gain important prognostic and diagnostic information from patient samples. For example, in the case of cancer, we now know that the relative localisations, interactions and patterns of marker expression on cancer cells, immune cells, stromal cells and endothelial cells are key to the understanding of the disease.[1–3] Moreover, expression of certain molecules, such as programmed cell death receptor 1 (PD-1) specifically on tumour-infiltrating lymphocytes, is predictive of clinical outcome in several cancers,[4–7] and its ligand indicates likely responsiveness to treatment with the anti-programmed cell death ligand 1 (PD-L1) antibody (Atezolizumab/MPDL3280A).[8]

Improving clinical practice will require us to take advantage of such research findings, and the recent advent of multiplex immunofluorescence (mIF) could provide that possibility. This technique overcomes the limitations of traditional antibody-based approaches to immunofluorescence by allowing detection of up to six different markers, plus a nuclear counterstain, on a single tissue section.[9–12] To do so, mIF requires sequential rounds of antibody-labelling of individual epitopes, followed by horseradish peroxidase (HRP)-catalysed deposition of fluorophore-conjugated tyramide molecules around the epitope of interest. The deposited fluorophores become covalently bound, via the activated tyramide, to tyrosine residues on or immediately around the epitope, allowing both the primary and detection antibodies to be stripped from the section, eliminating the risk of antibody cross-reactivity when performing the next round of labelling.[13] Some studies have shown that it is possible to achieve reproducible mIF with a manual protocol utilising microwave exposure to strip antibodies between each round of marker labelling.[9–12] However, these protocols are labour-intensive and take several days to complete, potentially introducing the risk of multiple rounds of human error leading to unacceptable levels of staining variability in the final image.

For mIF to be widely adopted as a diagnostic and prognostic tool, staining and imaging protocols need to be standardised, automated and validated. Automated and semi-automated diagnostic stainers are relatively common equipment in clinical laboratories, but several questions exist around their

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potential application for mIF: is it possible to achieve high-quality and accurate staining without microwave exposure; how does the image quality compare to manual staining protocols; and can staining be completed in a timeframe that makes mIF practicable for clinical use?

Here we used tissue microarray sections prepared from human breast tumours to compare the image quality and processing time of mIF staining by hand, or by a semi- or fully-automated diagnostic tissue stainer, either with microwave or heat exposure. We present an optimised protocol for fully-automated seven-colour mIF that shortens the staining procedure from several days to one day; we also show how this procedure can be coupled with a multispectral imaging system for simultaneous detection of six tissue biomarkers plus a nuclear counterstain.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tissues from human breast cancer were obtained from the Department of Anatomical Pathology, Division of Pathology, Singapore General Hospital. Ethical approval was granted by the SingHealth Centralized Institutional Review Board (CIRB Ref. 2013/664/F and 2015/2199). The tissues had been previously fixed in Surgipath 10% neutral buffered formalin (Leica Biosystems Richmond, USA) for 6–48 h, in accordance with the American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guidelines.[14,15] Following fixation, tissues were processed using a Peloris Rapid Tissue Processor (Leica Biosystems Melbourne, Australia) prior to embedding in Surgipath Paraplast (Leica Biosystems Richmond). Four μm thick tissue sections were cut from tissue blocks onto glass slides (Gerhard Menzel, Germany) and heated at 80°C for 3 min, prior to staining with haematoxylin (Leica Biosystems Richmond) and eosin (Merck, Germany) (H&E) before mounting with DPX mountant (CellPath, Wales, UK).

Tissue microarray (TMA) construction

H&E slides of tumour samples were reviewed in order to select three representative areas of high cellularity from each tissue section. We used an MTA-1 Manual Tissue Arrayer (Beecher Instruments, USA) to construct tissue arrays by punching 1 mm diameter cores from the selected areas on the FFPE tissue block, and transferring the cores to recipient blocks. The TMA blocks were cut and stained with H&E as described above. Additional 4 μm TMA sections were cut onto Bond Plus slides (Leica Biosystems Richmond) and heated at 60°C for 20 min for mIF staining.

Antibodies and tyramide signal amplification (TSA)

Primary antibodies were initially optimised for IHC on a Leica Bond Max autostainer (Leica Biosystems Melbourne) via a polymeric/enzymatic HRP method. Briefly, antibody concentration was titrated on appropriate positive control tissues following antigen retrieval with either Bond Epitope Retrieval Solution 1 (pH 5.9–6.1) or 2 (pH 8.9–9.1) (Leica Biosystems Newcastle, UK). The optimal staining condition for each antibody was defined as the combination that exhibits the highest sensitivity and specificity on known positive and negative control samples, as judged by an experienced pathologist. Details of antibodies used are shown in Table 1.

Titration of Opal fluorophore-conjugated TSA buffer (PerkinElmer, USA) was performed using a modified IHC protocol which will be discussed in the following section. The TSA dilution factor that produced the highest specificity and signal intensity, and low background was used. The sequence of antibody labelling was decided based on the target antigen's stability to repeated heat-induced epitope retrieval (HIER). Each antibody was coupled with an appropriate fluorophore-conjugated TSA of specific spectrum based on the protein cellular localisation, to prevent potential signal crossover of co-localised targets.

Processing of TMA for mIF

TMA tissue sections were processed using a diagnostic-use Leica Bond Max Autostainer, a semi-automated Dako Autostainer Plus (Dako Colorado, USA) or by manual staining. The Dako Autostainer Plus automated only the slide staining steps. The HIER step between antibody stainings was carried out manually using a Milestone T/T Mega Histoprocessor (Milestone, Italy) laboratory microwave. Details of each staining method are shown in Table 2. In brief, deparaffinised 4 μm TMA sections were subjected to HIER, either using the domestic, Panasonic Inverter Straight Microwave Oven (Panasonic Appliances Microwave Oven, China) or laboratory microwave (for the semi-automated and manual protocols), or heating on the built-in slide heaters of the Leica Bond Max autostainer, before incubation with hydrogen peroxide to quench endogenous peroxidase activity that would otherwise interfere with the HRP-tyramide activation step. Antibody-based marker detection was then conducted sequentially according to the labelling order in Table 1, using an Opal Multiplex fluorescence IHC (fIHC) kit (PerkinElmer) as described.[9,16] Briefly, slides were incubated with a single primary antibody in antibody diluent containing background-reducing components (Dako North America, USA), followed by application of a polymeric HRP-conjugated secondary antibody. The appropriate Opal fluorophore-conjugated TSA was then added at 1:100 dilution. Slides were rinsed with washing buffer after each step. Following the heat-stable deposition of the TSA-conjugated fluorophore around the marker of interest, slides were again subjected to HIER to strip the primary and secondary antibodies bound to the tissue, ready for labelling of the next marker. These steps were repeated until all six markers were labelled; finally spectral DAPI (PerkinElmer) at 1:10 dilution was added as a nuclear counterstain. Slides were mounted in ProLong Diamond Anti-fade Mountant (Molecular Probes, Life Technologies, USA) and cured in the dark at room temperature for 24 h prior to storing at 4°C. Fluorescence images were acquired using a Vectra 3.0 pathology imaging system microscope (PerkinElmer), and analysed. Simulated IHC images were generated with inForm Cell Analysis software (PerkinElmer). The mIF workflow is illustrated in Fig. 1.

To adapt the Leica Bond Max for the seven-colour mIF, we had to work within its programming limitations. This autostainer utilises Bond Polymer Refine Detection and Bond Polymer Refine Red Detection kits (Leica Biosystems Newcastle) to perform double sequential IHC staining via HRP-DAB (3,3′-diaminobenzidine tetrahydrochloride) and alkaline phosphatase (AP)-Fast Red detection methods, respectively. In order to perform mIF with HRP polymers only, AP polymers were replaced with HRP polymers in a titration container and application of the DAB chromogen was omitted. Slides were also flushed with Fast Red chromogen for labelling of every second primary antibody to fulfil the machine requirement for usage of the Bond Polymer Refine Red Detection kit. As this machine offers full automation for staining of up to two antibodies, to achieve the six markers staining in the study, we had to manually restart the program after every two-antibody cycle, requiring a total of 3 cycles to complete the whole process. The application of the DAB chromogen was also skipped in the program of Dako Autostainer Plus for mIF

Table 1 Details of primary antibodies and Opal TSA used in mIF

No.	Marker	Clone	Cellular localisation	Antibody source	Dilution	Opal TSA
1	PD-L1	SP263	Membrane and/or cytoplasm	Ventana Medical Systems, USA	Ready-to-use	Opal 520
2	CD68	PG-M1	Cytoplasm	Dako North America, USA	1:100	Opal 650
3	CD163	10D6	Membrane	Leica Biosystems Newcastle, UK	1:200	Opal 570
4	FOXP3	236A/E7	Nucleus	Abcam, UK	1:200	Opal 540
5	CK	AE1/AE3	Cytoplasm	Dako North America, USA	1:200	Opal 690
6	CD8	1A5	Membrane	Leica Biosystems Newcastle, UK	1:30	Opal 620

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