Digital Pathology. Role of molecular diagnostics in cancers; multiple immunohistochemistry

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***Abstract*— Multiplexed platforms have become a standard feature of modern medicine in the field of histopathology in recent years. They have evolved into powerful technologies that enable image analysis of tumor tissues from formalin- fixed paraffin- embedded specimens, aiming for better assessment of morphology and distinctive alterations at the molecular level of the patient's sample, which is critical for the pathologist's diagnosis and classification, with significant implications for the following therapeutic options. And also, in order to gain a better understanding of the tumor microenvironment, which aids cancer prevention by simulating new therapy discoveries. And unlike traditional IHC, which can only identify one marker in a tissue sample, multiplex IHC may detect many markers in a single tissue sample while providing detailed information about the cell composition and spatial arrangement. Reviewing multiplexed technologies is to demonstrate their utility in the study of cancer tissue as well as their benefits for applications in cancer diagnosis, stratifying patients, and accuracy for treatment. Summary: Digital pathology plays a significant part in current clinical practice and becoming an increasingly critical technological necessity in the laboratory environment, Algorithms for image analysis and artificial intelligence have the potential to further increase the quality of diagnostics in pathology.**

Keywords*— *Digital pathology; Molecular diagnostics; IHC & MIHC Techniques; cancers.

Introduction

In histopathological practice, digitalization and cognitive data processing are becoming increasingly important. As it appears that simply identifying tumors based on shape and genetic profile is no longer sufficient, it is vital to extract as much information from tissue as possible and standardize the evaluation of various histopathological features [Huss R, Coupland S E]. And as a new era in our understanding of human cancer has begun with the realization that the immune system is capable of identifying and eliminating tumor cells, two examples of treatments that have demonstrated clinical effectiveness in treating malignancies that are resistant to traditional therapy; Cancer vaccinations and coinhibitory receptor inhibition [Lovitch SB, Rodig SJ]. Understanding the mainly unknown biology of carcinogenesis necessitates unravelling molecular mechanisms in premalignant lesions and identifying the drivers of the intralesional immune response during cancer formation [Mascaux C, Angelova M]. The immune microenvironment analysis in primary tumor tissue biopsy samples can be used to stratify patients based on clinical outcome, identify individuals who are likely to benefit from specific immunotherapies, and tailor combination immunotherapy to individual patients and tumour types. [Lovitch SB, Rodig SJ]. Early identification and therapy are crucial for improving cancer patient outcomes.

Molecular Diagnostics and Tumour Microenvironment Characterization

Understanding of tumour biology is fast transforming the pathology field, allowing for better prognostics and new therapeutics. This will most likely be motivated by a need for outcomes that cannot be reached through traditional approaches [Cregger M, Berger AJ]. However, techniques for studying the cellular and molecular components of the tumour microenvironment need to be improved [Blom S, Paavolainen L, et al.]. The majority of contemporary immuno-oncology research efforts are concerned with determining the involvement of distinct immune cell subsets in their natural location. The growing desire for more information on tumor microenvironment ''TME'' has resulted in the development of innovative methods such as multiplexed imaging, CyTOF mass cytometry, and single-cell RNA sequencing [Koh J, Kwak Y]. That the immune profiling is becoming a significant technique that identifies predictive indicators for immunotherapy response [Parra ER, Uraoka N].

Multiplex Immunohistochemistry: Principles and Techniques

And technical study indicated that multiplex markers may be utilized to assess the host immune state and type of treatment [Xie Youheng, et al]. Multiplex Immunohistochemistry and image analysis in phenotypic profiling of human cancer tissues is one of these molecular diagnostics, demonstrating the potential for molecular characterization of cancer biology and the discovery of prognostic biomarkers. The heterogeneity in individuals' clinical reactions, on the other hand, indicates the need for biomarkers to help in patient classification [Xie Youheng, et al]. In clinical practice,

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Immunohistochemistry (IHC) is frequently employed as a diagnostic method in the field of histopathology, but with a restriction, that only one marker can be labelled per tissue section. As a result, chances to gather crucial prognostic and diagnostic data from patient samples are lost. The simultaneous detection of several markers on a single tissue section is made possible by multiplex immunohistochemistry/immunofluorescence (MIHC/IF) technology [Tan WCC, Nerurkar SN, et al.]. As the demand for companion diagnostics with complex assessment requirements grows, we can expect to see an increase in the use of quantitative platforms, particularly those that can perform multiplexed analysis. That to better understand complicated pathological processes, the concept of molecular histopathology is switching from single-marker immunohistochemistry to multiplexed marker detection [Blom S, Paavolainen L, et al.

Multiplex immunohistochemistry (MIHC): Multiplexing are technologies used to detect the presence of many biological markers on a single tissue sample or a group of tissue samples [Parra ER, Francisco-Cruz A]. A modular technique can be used with any IHC-validated antibody combination, creating the framework for more map different investigation **(Figure 1).** It can aid in the preservation of microscopic tissue samples [Ozbek B, Ertunc O, Erickson A, et al.]. It is the process of performing numerous assays on formalin-fixed, paraffin-embedded (FFPE) materials, and Some assays are designed to discover diseases or improve pathological classification, while others are designed to guide therapy [Bolognesi M, Manzoni M and et al]. Multiplexed imaging techniques give unique biological information that cannot be obtained by conventional non-imaging approaches or single immunohistochemistry (IHC) procedures in many circumstances [Parra ER, Francisco-Cruz A]. MIHC also has a substantial benefit in assessing the host immune milieu, especially when minimal biopsy tissue material is available [Xie Youheng, et al.]. Multiplexed phenotyping assays are multiple assays, starts by applying the primary antibodies and ends by detecting the secondary antibodies with fluorescent labels, that multiple staining on the same section can be accomplished by using animal specific secondary antibodies connected with a reporter directed against antibodies generated in different animals (rabbit, goat/sheep, rat, and three to four mouse isotypes) [Bolognesi M, Manzoni M and et al].

Image Analysis and Interpretation

The image analysis protocol is described in depth by [Slik K, Blom S, et al (2018)]; Tissue microarrays (TMAs) were digitally evaluated for tumour budding, that TMA after being stained with multiplex IHC, which included EMT markers E-cadherin, ITGB4, ZO-1, and pan-cytokeratin. Blom et al. (2017) described how to perform multiplex IHC using fluorescently tagged secondary antibodies. The Metafer 5 scanning and imaging equipment (MetaSystems, Germany) was used to obtain five-channel fluorescent pictures with a X20 objective (NA 0.8). For image analysis, TIFF files were downscaled to 1:4 from the original resolution (a final resolution of 0.88 m/pixel), and cell image analysis software was employed (Cell Profiler version 2.2.0; Carpenter et al. 2006). Its primary phases are as follows: (1) spot perception; (2) epithelial cluster and bud perception; (3) channel intensity determination; and (4) data export**.** It has been also described by Lovitch et al. in 2016. That multiplexed immunofluorescence is a technique based on immunostaining FFPE samples with many antibodies and an equal number of fluorescently attached secondary antibodies, which are applied sequentially, followed by imaging the immune-stained sections with a microscope with particular spectrum filters or a multispectral imager. Simply then the visual processing and analysis are then utilized to create an image of the tissue in which the expression of each biomarker is identified and quantified separately [Blom, S.; Paavolainen, L].

Fluorescent reporters are increasingly being employed, not only with microscopes, but also with a range of other quantitative approaches (flow cytometry, image cytometry, confocal microscopy, etc.) that are better suited to the analysis of living or lightly fixed cells. [Bolognesi M, Manzoni M and et al]. MIHC has the benefit of being considerably easier to deploy than mass cytometry and single-cell transcriptome analysis since it does not require any extra instruments. Furthermore, bright-field imaging with MIHC allows for considerably higher resolution observation of the tumour and ‘’TME’’ in FFPE samples, which are the most often used specimens for cancer histopathology [Koh J, Kwak Y]. However, there are no systems allowing multiplexed IHC (MIHC) with high-resolution whole-slide tissue imaging and analysis, yet providing feasible throughput for routine use [Blom S, Paavolainen L, et al]. MIHC might play a more important role in both research and clinical pathology with further advancements in technologies for cell segmentation, IHC intensity estimation, and high-throughput picture processing [Koh J, Kwak Y, et al]. Multiplexed biomarker labelling in formalin-fixed paraffin-embedded tissue (epitopes, RNA, spectroscopically reactive chemicals) combined with imaging technologies and computer platforms presents the next-generation histopathology [Am J Transplant. 2020]. Multiplexed phenotyping assays of formalin-fixed paraffin-embedded (FFPE) tissues are "in the early stages of deployment and under highly active development," according to the researchers. [Montironi, R.; Cheng, L]. can be a helpful tool for tumour tissue immune-profiling, allowing many targets to be identified in the same tissue segment [Parra ER, Uraoka N]. This method provides for an objective assessment of '(a) biomarker expression in individual cells; (b) cell phenotype based on multiple biomarkers; (c) cell number based on phenotype; and (d) the geometric relationship between cells with same or distinct phenotypes. Lovitch SB, Rodig SJ]. The emergence of so-called "next generation IHC," a multiplex technology based

on isotope-tagged antibodies and in situ mass-spectrometry detection, may alter the landscape of multiplexing (reviewed in Rimm27), and with the Nano String technique, barcode tagged probes, such as antibodies, may allow for extremely wide multiplexing. However, neither system's ability to visualize single cells in a full slide image is known. [Bolognesi M, Manzoni M and et al].

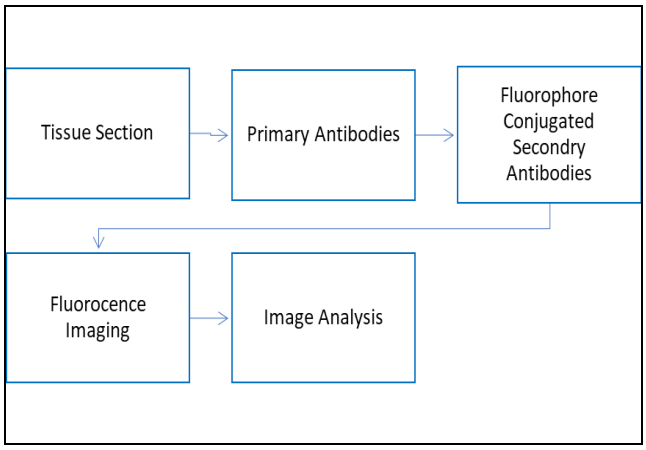

Figure 1. The Workflow of MIHC.

2)Traditional IHC vs MIHC (Table 1): The primary distinction between immunohistochemistry (IHC) and immunofluorescence ''IF'' is the use of light: absorption in IHC versus emission in IF. The diversification of fluorescent reporters is unnatural with ''IF'', as it is restricted by the source of light, the overlap of fluorescence spectra, the available fluorochromes, and, ultimately, the cost of the reagents [Bolognesi M, Manzoni M and et al]. The complexities of multiplexed immunostaining in comparison to ordinary immunohistochemistry is a disadvantage of the approach Lovitch SB, Rodig SJ. And unlike traditional IHC, which can only identify one marker in a tissue sample, multiplex IHC may detect many markers in a single tissue sample while providing detailed information about the cell composition and spatial arrangement. [Tan WCC, Nerurkar SN, et al.]. The most significant restriction of IHC is the inability to label more than one marker per tissue segment. And while IHC continues to be a highly practical and cost-effective diagnostic and prognostic approach, this single-marker method cannot tell the entire story of the complex immunological milieu. Another disadvantage of IHC-based biomarker evaluation is the substantial inter-observer variability. [Tan WCC, Nerurkar SN, et al.].

Table 1. Comparative Evaluation: MIHC vs Traditional IHC.

Feature	Traditional IHC	MIHC
Marker Detection	Single	Multiple (≥4–6)
Imaging	Bright-field	Fluorescence/multispectral
Tissue Use	High	Low (economical)
Analysis	Manual, subjective	Automated, quantitative
Clinical Utility	Diagnostic	Diagnostic + predictive

Applications in Cancer Diagnostics (Figure 2).

Multiplex immunohistochemistry and image analysis in phenotypic profiling of human cancer tissues is one of these molecular diagnostics, demonstrating the potential for molecular characterization of cancer biology and the discovery of prognostic biomarkers. In prostatic carcinomas Multiplex Immunohistochemistry applied to achieve an automatic classification of epithelial cells and glands (benign vs. neoplastic) with concurrent analysis of androgen receptor (AR) and alpha-methyl acyl-CoA (AMACR) expression at cell level resolution using multiplexed IHC with high-resolution whole-slide tissue imaging and analysis has to be done **[**Lovitch SB, Rodig SJ].

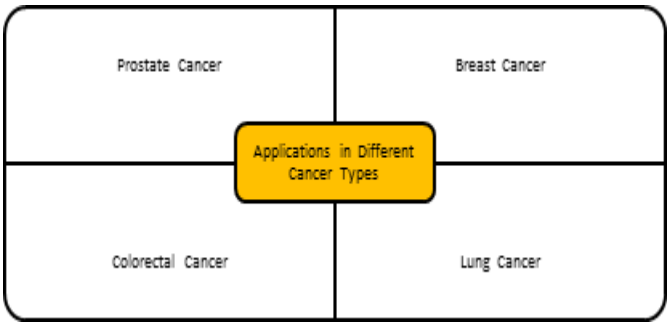

Figure 2. Applications of MIHC in different Cancer Types.

Advancements in Multiplex Immunohistochemistry (MIHC) for Cancer Diagnostics and Personalized Medicine

Multiplex immunohistochemistry (MIHC) has transformed cancer diagnostics and personalized medicine by enabling the simultaneous detection of multiple biomarkers within a single tissue section. When coupled with digital pathology and whole-slide imaging, MIHC ensures high-resolution tumor analysis, streamlining diagnostic workflows and reducing inter-laboratory variability. AI-driven image analysis enhances the accuracy of tumor classification, biomarker quantification, and prognostic predictions by automating complex data interpretation. MIHC facilitates precise biomarker quantification, improving tumor profiling and guiding clinical decision-making. Moreover, automation and digital platforms reduce costs, making advanced diagnostics more accessible, particularly in

underserved regions. The utilization of formalin-fixed, paraffin-embedded tissues, combined with enhanced antigen retrieval techniques, ensures high-quality tissue analysis while minimizing observer variability. MIHC's application in longitudinal studies supports the validation of prognostic markers and tracks tumor progression, offering insights into treatment efficacy and outcomes. Regulatory frameworks and validation studies further ensure the ethical application of MIHC and digital pathology, addressing concerns related to patient consent, data privacy, and the integration of AI in clinical practice.

Conclusion

In summary, the integration of digitalization and advanced data processing in histopathology is transforming the field. The shift to multiplex immunohistochemistry (MIHC) allows for the simultaneous detection of multiple biomarkers, enhancing our understanding of tumor biology and the immune response. This approach not only improves prognostic accuracy but also helps identify patients who may benefit from specific immunotherapies. The future of pathology is rooted in these advancements, promising significant improvements in diagnostics and therapeutic strategies in cancer care.

Limitations of Multiplex Immunohistochemistry

Despite its clinical promise, multiplex immunohistochemistry (MIHC) presents several limitations that hinder its widespread adoption. The technique involves complex protocols and relies on expensive reagents and imaging platforms, making it less accessible for routine use, particularly in low-resource settings. Fluorophore spectral overlap, tissue autofluorescence, and the limited number of validated antibody combinations restrict the extent of multiplexing. Additionally, variability in staining procedures, imaging parameters, and data interpretation methods across laboratories poses challenges for standardization and reproducibility. The analytical demands of MIHC also require advanced computational tools and technical expertise, which are not yet universally available in clinical pathology laboratories.

Future Perspectives in MIHC Implementation

Ongoing technological advancements are expected to address current limitations and facilitate the broader integration of MIHC into clinical practice. Innovations in fluorophore design and spectral unmixing techniques will enhance multiplexing efficiency and reduce background interference. Automation of staining and imaging workflows will improve throughput and reduce inter-operator variability. Artificial intelligence and machine learning are poised to streamline image analysis, support pattern recognition, and assist in the interpretation of complex biomarker data. Establishing standardized protocols, robust analytical pipelines, and accessible platforms will be essential to ensure consistency across institutions. As these developments progress, MIHC is set to become a foundational tool in precision oncology, offering more comprehensive tumour profiling and enabling personalized therapeutic strategies.

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