Journal of Posthumanism
2024
Volume: 4, No: 3, pp. 1187–1193
ISSN: 2634-3576 (Print) | ISSN 2634-3584 (Online)
posthumanism.co.uk

DOI: https://doi.org/10.63332/joph.v4i3.31911

An Overview of Histological Staining Techniques

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Abstract

Histological staining plays a central role in biomedical diagnostics and research by enhancing tissue contrast and enabling cellular and molecular visualization. Traditional staining methods such as Hematoxylin and Eosin (H&E) remain foundational for morphological assessment, offering clarity in distinguishing basic tissue architecture. However, the limitations of routine stains in detecting specific biomolecules have led to the development of specialized techniques, including Periodic Acid-Schiff (PAS), Trichrome, and silver-based stains, which improve visualization of carbohydrates, connective tissue, and microorganisms respectively. Immunohistochemistry (IHC) has further advanced the field by allowing antigen-specific detection using labeled antibodies, expanding diagnostic capabilities in oncology, infectious diseases, and biomarker profiling. Challenges in histological staining are multifaceted. Variability in reagent quality, fixation techniques, staining protocols, and human interpretation continues to impact diagnostic consistency. Artifacts introduced during tissue processing or sectioning can mimic pathology and compromise slide quality. Interobserver differences and subjective interpretations highlight the need for standardization and digital tools. Storage conditions and long-term stain stability also present issues, especially in retrospective analyses. Technological advancements are reshaping histological workflows. Automation in staining platforms ensures reproducibility and efficiency, while novel staining chemistries and microfluidic systems reduce processing time and reagent consumption. Digital pathology integrated with AI enables quantitative slide analysis and high-throughput biomarker detection. Multiplexing technologies now facilitate the simultaneous visualization of multiple markers within a single tissue section, revealing complex spatial relationships and cellular interactions. These innovations are driving a transformation in how tissue samples are evaluated, moving from descriptive morphology to data-rich molecular profiling.

Keywords: *Histological Staining, Immunohistochemistry, Special Stains, Digital Pathology, Tissue Diagnostics*.

Introduction

Histology, the study of tissues at the microscopic level, is foundational to understanding normal biology and pathological conditions. The visualization of tissue architecture relies heavily on staining techniques that enhance contrast and differentiate cellular and extracellular components. These staining methods are indispensable in diagnostic pathology, research, and medical education. Without them, the inherent transparency and uniformity of most biological tissues render detailed observation nearly impossible.

Staining in histology serves two fundamental purposes: to distinguish different tissue types and to identify specific cellular structures or molecules. Over time, numerous staining techniques have been developed, ranging from general stains that reveal broad tissue features to highly

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specific methods targeting particular proteins, lipids, or nucleic acids. The evolution of these techniques reflects advances in both chemical understanding and biomedical needs. Classical dyes such as hematoxylin and eosin (H&E) remain staples of routine histopathological practice due to their simplicity, efficiency, and diagnostic value. Hematoxylin stains cell nuclei a deep blue-purple, while eosin counterstains cytoplasmic components and extracellular matrix in shades of pink, offering excellent contrast and morphological detail (1).

Special stains have extended the capabilities of histology beyond the reach of routine methods. For instance, the Periodic Acid-Schiff (PAS) reaction is employed to visualize glycogen, mucopolysaccharides, and basement membranes, while the Masson's Trichrome stain helps differentiate muscle, collagen, and fibrin. These techniques are especially useful in pathology to highlight changes such as fibrosis, amyloid deposition, or fungal infections that are not easily seen with H&E alone (2). Silver stains, such as the Gomori methenamine silver (GMS), provide exceptional contrast for detecting microorganisms like fungi and certain bacteria within tissue sections (3).

Immunohistochemistry (IHC) represents a significant advancement in histological staining, combining immunological specificity with histological localization. IHC employs antibodies to detect antigens within tissue sections, and it has become critical in cancer diagnosis, infectious disease identification, and biomarker detection. The technique allows for the visualization of protein expression patterns with both chromogenic and fluorescent detection systems. Its power lies in specificity and flexibility, though it also introduces complexities related to antigen retrieval, antibody selection, and signal amplification (4). Despite their variety and application, staining techniques require careful standardization and quality control to ensure reproducibility and diagnostic accuracy. Technical variables such as fixation, section thickness, reagent quality, and staining protocols can significantly affect outcomes. Additionally, with the rise of digital pathology and automated image analysis, consistent and reliable staining becomes even more essential for quantitative evaluation.

Review

Histological staining techniques have continued to evolve in complexity and specificity, enabling more nuanced interpretations of tissue architecture and molecular composition. Traditional stains like H&E remain the gold standard due to their reliability and diagnostic clarity. However, their limitations in differentiating closely related tissue components or identifying specific biomolecules have led to the adoption of more specialized techniques. For example, immunohistochemistry offers the ability to target antigens with high specificity, allowing for precise localization of proteins within tissue samples. This technique has significantly enhanced the diagnostic capability in oncology and infectious disease pathology (5).

In parallel, advances in digital pathology have introduced automation and image analysis algorithms capable of quantifying staining intensity and distribution. This has improved reproducibility and minimized observer bias, especially in high-throughput settings. Fluorescent staining and multiplex immunostaining now permit simultaneous visualization of multiple targets, thus offering a more holistic understanding of tissue microenvironments. Such innovations not only improve diagnostic precision but also support biomarker discovery and translational research (6). Yet, these sophisticated methods demand rigorous standardization of protocols and high technical expertise to ensure accuracy and reproducibility across laboratories.

Comparative Analysis of Routine and Specialized Staining Methods

Histological analysis relies on an expanding toolkit of staining techniques, from simple dyes to complex, targeted systems. H&E staining, a foundational method in histology, provides clear contrast between nuclei and cytoplasm, making it ideal for general tissue architecture assessment. Its cost-effectiveness and ease of application have cemented its place in routine diagnostics. Still, H&E offers limited molecular insight. It cannot distinguish between tissue components with similar morphological features or detect proteins, carbohydrates, or lipids with specificity, making it insufficient in more nuanced diagnostic or investigative settings (7).

Specialized stains offer the precision needed for deeper tissue characterization. Techniques like PAS or Masson's Trichrome address specific biochemical targets. PAS reacts with polysaccharides and mucosubstances, illuminating structures like basement membranes or fungal cell walls. Masson's Trichrome separates muscle, collagen, and fibrin into three distinct colors, proving particularly useful in fibrosis evaluation. These methods, while more chemically complex than H&E, extend the resolution of histological investigations into the domain of biochemical composition and tissue organization. They have become essential in pathology labs that diagnose metabolic storage diseases, fungal infections, or fibrotic conditions (8).

Silver-based stains such as GMS or Warthin-Starry have demonstrated high sensitivity in microbial detection. In cases of fungal or spirochete infections, silver impregnation techniques outperform routine staining in visualizing organisms that otherwise remain indistinct. GMS, for example, is often used for Pneumocystis jirovecii pneumonia, where its high-contrast black-staining of organisms against a green background can reveal subtle colonization in alveolar exudates. These methods demand precise handling, with critical steps in fixation and silver solution preparation that, if mishandled, result in high background or poor specificity. Compared to H&E, the operational complexity increases, but so does diagnostic value, particularly in infectious disease pathology (9).

IHC, considered the benchmark of specialized staining, surpasses routine and classical special stains in its ability to localize specific antigens with high fidelity. Tumor typing, classification, and therapeutic marker evaluation often depend on antibody-based staining to detect nuclear, membranous, or cytoplasmic proteins. While a pathologist can determine cell morphology with H&E, IHC distinguishes between morphologically similar neoplasms using panels of markers such as cytokeratins, vimentin, or CD markers. The choice of primary antibody, antigen retrieval technique, and detection system all influence staining outcome, making IHC technically demanding. Yet, its adaptability has led to widespread adoption in cancer diagnostics, infectious disease identification, and prognostic stratification (10).

Recent shifts toward multiplexing and fluorescence-based detection systems have further distanced specialized staining from conventional dye techniques. Techniques such as multiplex immunofluorescence allow simultaneous visualization of several markers in a single tissue section. This has proven especially effective in tumor microenvironment profiling, enabling quantification of immune infiltrates and their spatial relationships with tumor cells. These methods are not without drawbacks: signal overlap, photobleaching, and complex image processing remain obstacles. Yet, the information yield significantly exceeds that of single-stain protocols. Advanced platforms integrating image analysis with staining protocols are now being used to automate feature detection and standardize quantification, a task nearly impossible with conventional stains (10, 11).

Cryosection staining introduces another layer of contrast in the comparative landscape. While H&E staining of formalin-fixed paraffin-embedded tissues is standard, frozen sections stained intraoperatively serve a different purpose. Here, speed is prioritized over morphology. Specialized rapid stains such as toluidine blue or modified H&E protocols are used to achieve interpretable results in minutes. The trade-off is lower resolution and occasional artifacts, but the utility in margin assessment or urgent pathological clarification is invaluable. Specialized rapid stains enhance the surgeon-pathologist interface, enabling immediate intraoperative decisions without compromising patient outcomes (12).

Challenges and Limitations in Histological Staining

Despite the utility of histological staining in diagnostics and research, its practice remains constrained by numerous technical, procedural, and interpretative limitations. These challenges can arise at multiple stages of tissue preparation, including fixation, embedding, sectioning, staining, and analysis. The fixation process, for example, must preserve both morphology and antigenicity. Formalin, the most widely used fixative, cross-links proteins effectively but may obscure antigenic sites and complicate subsequent IHC or molecular applications. Over fixation can lead to epitope masking, necessitating antigen retrieval protocols that may not restore target accessibility uniformly (13).

Reagent variability presents persistent concerns in staining reproducibility. Stains prepared in-house are vulnerable to fluctuations in concentration, pH, and degradation over time. Commercial staining kits offer greater consistency but come at a higher cost and may still suffer from lot-to-lot variability. The outcome of PAS or silver staining, for instance, can be markedly influenced by subtle shifts in reagent preparation or application timing. Batch effects across laboratories also complicate collaborative studies and large-scale research, where reproducibility across staining runs is essential for meaningful interpretation (14).

Interpretation introduces another layer of difficulty. Staining intensity is influenced by numerous factors beyond the presence of the target structure. Thickness of sections, duration of staining, counterstaining intensity, and dehydration protocols all contribute to the final appearance. Morphological detail may be obscured or exaggerated, especially in overstained or under-differentiated slides. Interobserver variability in reading H&E, PAS, or Trichrome slides is well-documented, especially in borderline or diagnostically ambiguous cases. Even with standardized protocols, subjective interpretation often plays a role, emphasizing the need for objective digital analysis tools that remain imperfect in handling complex staining patterns (15).

Storage conditions of stained slides affect long-term diagnostic reliability. Over time, chromogenic stains may fade, and coverslip adhesives can leach into tissue sections, distorting the image. Fluorescent stains, particularly in multiplex immunofluorescence, are vulnerable to photobleaching and require dark, cold storage environments. These limitations restrict the utility of archival specimens, especially when retrospective studies are planned or when litigation demands durable histological evidence. Protocols for slide preservation remain inconsistent across institutions, leading to variability in long-term stain retention (16).

Multiplex techniques bring technical complications beyond what routine histology faces. Staining multiple antigens simultaneously in a single section can result in cross-reactivity, signal overlaps, or differential antibody penetration. Fluorophores must be carefully selected to avoid spectral bleed-through, and sequential staining steps require precision in timing and washing to avoid background buildup. Signal amplification techniques such as tyramide-based methods

improve sensitivity but often increase non-specific staining. Fluorescence quenching and epitope loss over multiple rounds of staining can reduce the integrity of the data, especially in high-plex settings. These challenges are particularly pronounced in tumors with heterogeneous expression profiles, where low-abundance targets may be drowned out by dominant signals or masking from autofluorescence (17).

Artifacts also remain a common source of diagnostic confusion. Folds, tears, bubbles under coverslips, or incomplete section adherence introduce visual disturbances that may resemble pathological features. Tissue compression during microtomy can elongate nuclei or distort architecture. Contamination between specimens—particularly during automated staining or microtome can introduce foreign tissue fragments, leading to misinterpretation. Fungal elements from adjacent blocks or pigment deposition due to metal contamination during silver staining can produce false positives. These physical limitations persist even in digital workflows and must be identified by trained observers to avoid misclassification or diagnostic error (18).

Advancements in Staining Technologies and Automation

Recent developments in histological staining have redefined how tissues are labeled, processed, and interpreted, moving beyond manual application and interpretation into the realm of precision engineering, robotics, and digital analytics. Automated stainers now dominate diagnostic pathology labs, performing staining protocols with reproducible timing, reagent application, and washing steps. These systems minimize human error, improve batch-to-batch consistency, and reduce labor costs. Instruments such as the Ventana BenchMark and Leica BOND platforms allow full automation of H&E and IHC procedures, often integrating slide baking, deparaffinization, antigen retrieval, and staining into a seamless workflow (19).

Automation extends beyond basic mechanics to include smart software-driven optimization. Platforms integrated with machine learning algorithms can predict staining conditions based on tissue type or previous runs, suggesting protocol adjustments in real time. This adaptability enhances efficiency, particularly in high-throughput labs dealing with variable sample types. Advanced barcode systems ensure sample traceability and minimize risk of mislabeling during processing. These quality control layers, though initially costly, have proven essential for clinical accreditation and compliance with regulatory standards. They also support lean laboratory practices, where turnaround time and error rates directly affect patient care outcomes (20).

Non-traditional staining approaches have begun to emerge as viable alternatives to dye-based techniques. Label-free methods, such as stimulated Raman scattering microscopy and quantitative phase imaging, generate contrast based on intrinsic optical properties of tissues rather than chemical labeling. These techniques avoid artifacts introduced by fixation and staining, allowing analysis of live or freshly excised tissue in real time. They are particularly valuable in intraoperative settings where rapid decisions are required and where staining delays could compromise resection margins. While these approaches do not yet offer the molecular specificity of IHC, ongoing integration with computational pathology platforms continues to narrow this gap (21).

Digital pathology has facilitated the coupling of high-resolution slide scanning with algorithm-driven image analysis. Deep learning networks trained on thousands of stained slides can now detect patterns, quantify features, and classify histological subtypes with increasing accuracy. When paired with multiplex immunofluorescence, these tools enable spatial transcriptomics and protein co-localization analysis on a pixel level. Such integration offers a depth of information

unattainable by traditional light microscopy, especially in research fields involving tumor heterogeneity, immune cell profiling, or organoid modeling. Multi-omic histological maps are being developed to align morphological data with genomic and proteomic layers, bridging the divide between classic histology and systems biology (22).

Advances in reagent chemistry have also led to new classes of stains and detection systems. Nanoparticle-based chromogens provide greater signal amplification and longer stability than conventional dyes. Quantum dots, for instance, offer tunable fluorescence emission and resistance to photobleaching, allowing multi-target visualization over extended imaging sessions. Enzyme-labeled polymer detection systems have largely replaced traditional biotin-avidin complexes in IHC due to reduced background and improved sensitivity. Additionally, microfluidic staining devices are being explored as compact alternatives to full-sized autostainers. These chip-based platforms reduce reagent volume, accelerate diffusion, and enable localized staining of specific tissue zones without compromising integrity of adjacent areas (6).

Spatial biology platforms now use multiplex staining combined with advanced imaging and AI-based quantification to construct 3D tissue maps. Techniques such as CODEX and MIBI allow detection of over 40 markers within a single section, with spatial resolution sufficient to define microenvironments, cell neighborhoods, and interaction networks. These technologies surpass conventional staining not only in multiplexing capacity but also in their integration with computational infrastructure. Their implementation is growing in translational oncology, infectious disease research, and neuroscience, offering a granular view of cellular interactions previously hidden in bulk tissue sections (23).

Conclusion

Histological staining continues to evolve, bridging traditional techniques with emerging digital and molecular innovations. Automation and multiplex technologies are enhancing precision, scalability, and data integration. Despite persistent challenges in standardization and interpretation, advanced platforms are redefining tissue diagnostics. These developments are reshaping the future of histopathology across both clinical and research domains.

References

Bancroft JD. Bancroft's Theory and Practice of Histological Techniques: Expert Consult: Online and Print, 7e.

Kiernan J. Histological and histochemical methods: Scion publishing ltd; 2015.

Culling CFA. Handbook of histopathological and histochemical techniques: including museum techniques: Butterworth-Heinemann; 2013.

Ramos-Vara JA. Technical aspects of immunohistochemistry. Veterinary pathology. 2005;42(4):405-26.

Fisher C. Immunohistochemistry in diagnosis of soft tissue tumours. Histopathology. 2011;58(7):1001-12.

Stack EC, Wang C, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods. 2014;70(1):46-58.

Prophet E, Mills B, Arrington J, Sobin L. Laboratory methods in histotechnology Washington. DC: American Registry of Pathology. 1992.

Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. Cold spring harbor protocols. 2008;2008(5):pdb. prot4986.

Wang S, Lai J, Wu R, Zhang L, Huang M, Xiao Y, et al. Grocott methenamine silver staining is the optimal

approach to histological diagnosis of pulmonary cryptococcosis. Frontiers in Microbiology. 2022;13:885511.

Yaziji H, Barry T. Diagnostic Immunohistochemistry: what can go wrong? Advances in anatomic pathology. 2006;13(5):238-46.

Gerdes MJ, Sevinsky CJ, Sood A, Adak S, Bello MO, Bordwell A, et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. Proceedings of the National Academy of Sciences. 2013;110(29):11982-7.

Algadi HaH, Abou-Bakr AA-E, Jamali OM, Fathy LM. Toluidine blue versus frozen section for assessment of mucosal tumor margins in oral squamous cell carcinoma. BMC cancer. 2020;20(1):1147.

Shi S-R, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. Journal of Histochemistry & Cytochemistry. 1991;39(6):741-8.

Masood S, von Wasielewski R, Mengel M, Nolte M, Werner M. Influence of fixation, antibody clones, and signal amplification on steroid receptor analysis. The Breast Journal. 1998;4(1):33-40.

Shah KK, Lehman JS, Gibson LE, Lohse CM, Comfere NI, Wieland CN. Validation of diagnostic accuracy with whole-slide imaging compared with glass slide review in dermatopathology. Journal of the American Academy of Dermatology. 2016;75(6):1229-37.

Bolognesi MM, Manzoni M, Scalia CR, Zannella S, Bosisio FM, Faretta M, et al. Multiplex staining by sequential immunostaining and antibody removal on routine tissue sections. Journal of Histochemistry & Cytochemistry. 2017;65(8):431-44.

Tsujikawa T, Kumar S, Borkar RN, Azimi V, Thibault G, Chang YH, et al. Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis. Cell reports. 2017;19(1):203-17.

Ekundina V, Eze G. Common artifacts and remedies in histopathology (a review). African Journal of Cellular Pathology. 2015:1-7.

Taylor C, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. Histopathology. 2006;49(4):411-24.

De Matos LL, Trufelli DC, De Matos MGL, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomarker insights. 2010;5:BMI. S2185.

Orringer DA, Pandian B, Niknafs YS, Hollon TC, Boyle J, Lewis S, et al. Rapid intraoperative histology of unprocessed surgical specimens via fibre-laser-based stimulated Raman scattering microscopy. Nature biomedical engineering. 2017;1(2):0027.

Van der Laak J, Litjens G, Ciompi F. Deep learning in histopathology: the path to the clinic. Nature medicine. 2021;27(5):775-84.

Goltsev Y, Samusik N, Kennedy-Darling J, Bhate S, Hale M, Vazquez G, et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. Cell. 2018;174(4):968-81. e15