Beyond the H&E: Advanced Technologies for in situ Tissue Biomarker Imaging

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Abstract

For decades, histopathology with routine hematoxylin and eosin staining has been and remains the gold standard for reaching a morphologic diagnosis in tissue samples from humans and veterinary species. However, within the past decade, there has been exponential growth in advanced techniques for in situ tissue biomarker imaging that bridge the divide between anatomic and molecular pathology. It is now possible to simultaneously observe localization and expression magnitude of multiple protein, nucleic acid, and molecular targets in tissue sections and apply machine learning to synthesize vast, image-derived datasets. As these technologies become more sophisticated and widely available, a team-science approach involving subspecialists with medical, engineering, and physics backgrounds is critical to upholding quality and validity in studies generating these data. The purpose of this manuscript is to detail the scientific premise, tools and training, quality control, and data collection and analysis considerations needed for the most prominent advanced imaging technologies currently applied in tissue sections: immunofluorescence, in situ hybridization, laser capture

microdissection, matrix-assisted laser desorption ionization imaging mass spectrometry, and spectroscopic/optical methods. We conclude with a brief overview of future directions for ex vivo and in vivo imaging techniques.

Key words: biomarkers; fluorescence microscopy; immunohistochemistry; in situ hybridization; laser capture microdissection; MALDI; molecular pathology; quality control

Introduction

Light microscopy-based evaluation of tissues, typically by hematoxylin and eosin (H&E)-stained histology, remains the cornerstone of pathology data.^{1,2} The importance of comprehensively analyzing tissue composition and organization in determining regions of interest for further analysis has myriad downstream impacts. Routine histopathology is now just one component of a vastly more comprehensive tissue-based dataset attuned to biomarker visualization.³ To achieve multiparameter subcellular resolution, computer-assisted image analysis is needed.^{4,5} The simultaneous analysis of signal—indicated by chromogen, fluorophore, or unique spectral emission—and tissue structure is tremendously useful for biomarker discovery, validation, and assay development. Still, advanced imaging techniques are not performed in a vacuum, and imaging analysis data should serve to complement and corroborate findings across companion assays. Internal quality control and validation remain critical to generating meaningful data. Thus, the workflow for these techniques is iterative and involves investigators, pathologists, microscopists, and computer scientists.⁶ Herein, we discuss new developments and practical applications in five major areas of advanced tissue microscopy: immunofluorescence (IF), in situ hybridization (ISH), laser capture microdissection (LCM), matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS), and spectroscopic/optical techniques.

Immunofluorescence

The antigen specificity of antibodies has enabled detection of specific molecules in tissue sections for many decades. This technique has been used to gain an understanding of the molecular underpinnings that drive both physiological and disease processes by visualizing their presence and distribution cells and tissues. IF tissue staining has emerged as an alternative to chromogenic immunohistochemistry (IHC)⁷ due to a number of unique advantages, including multiplexing (acronym mIF or mxIF) of multiple antigens, quantitative detection, and therefore an improved multidimensional approach to analyzing the molecular composition of tissues.^{8–12} While IF is based on the same antibody-mediated detection of antigens as IHC, detecting antibody binding through fluorescence rather than chromogenic stains offers both quantitative and logistic advantages (Figure 1): (1) the spatial distributions of targeted molecules can be captured in a separate fluorescent channel and recorded separately from other visible components; (2) it delivers truly quantifiable information regarding content of molecules of interest (Figure 2); (3) it enables multiplexing through the detection of multiple, distinct fluorophores, and recent advances in cycling staining and/or multispectral imaging have removed previous limits on the dimensionality of antigen detection; (4) direct comparisons of two or more stains in the same physical location enables true co-localization of the antigens. The transition to digital imaging has increased the utility of IF exponentially as it enables sophisticated

quantitative analysis from whole tissue to single cells and quantitative correlations not possible through other means.

The principle of fluorescence was described in the 1850s and postulates that fluorophores excited at a defined wavelength emit light of a different wavelength in an amount that can be mathematically determined.^{13,14} Fluorescent microscopy was one of the first applications of fluorescence in the 1910s and allowed observatory works with autofluorescent objects. The purification and mass production of specific antibodies and their conjugation to fluorophores led to the IF methodology we know today. Visualization of IF starts with object (slide) illumination by an excitation source, which can be a broad wavelength (mercury arc, xenon arc, LED lamps) or discrete wavelength (lasers). To limit visualization to one fluorophore, filters are used to restrict the emission range from broad-spectrum sources or by selecting the appropriate laser wavelength. The target fluorophore absorbs the light and its energy level is raised to an excited state, decay from which yields photon emission. Emitted light is captured and filtered by efficient light-collecting optical elements such as lenses, mirrors, and filters. A detailed review is provided elsewhere.¹³

Despite many advantages offered by IF, the photosensitivity of fluorophores has deterred many that rely on stability and longevity of chromogenic staining to ensure robust and reliable analysis at the scope. Prolonged viewing of fluorescence, as is often desired during review of clinical sections or IHC scoring, can diminish and even ablate the fluorescence. While fluorescence (in the optical spectrum) can be viewed with our eyes and captured by camera, IF imaging was revolutionized when

Figure 1 Schematic representation of distinct approaches to immunofluorescent (IF) staining. In direct IF, binding of primary antibody to its antigen is visualized with a fluorophore directly conjugated to the primary antibody, while indirect IF visualizes antigen binding with a secondary antibody specific to the isotype of the primary antibody. The fluorescent signal from IF can be amplified by increasing the number of fluorescent labels by biotin-avidin complex binding to the antibody or by enzymatic amplification that enables deposition of fluorescent labels onto and adjacent to the antibody. Simultaneous multiplexing of IF is accomplished when multiple primary antibodies to distinct antigens are visualized with separate fluorophores. Serial multiplexing can be accomplished through sequential staining with distinct primary antibodies. To demonstrate the signal output, each technique is depicted by a schematic and a representation of the fluorescent output “image” is shown on the far right.

Figure 2 Quantitative imaging of immunostaining. A relative comparison of immunofluorescent (A) or chromogenic (B) staining is shown. A representation of the signal generated in each technique is illustrated graphically with optimal detection of three signals with increasing intensity. A hypothetical image at optimal exposure/development time is illustrated to show the detection of three objects of increasing signal intensity.

microscopes were equipped with digital imaging using charge-coupled devices (CCD) and photomultiplier tubes. Detection of photon energy by conversion into measurable electrical signal in a sensor not only enabled the recording of fluorescent stains but also unbiased quantitative analysis of this output. The direct correlation between the number of fluorophores attached to an antibody, the number of photons emitted from these fluorophores, and the amplitude of the electrical signal in the detector (i.e., CCD camera) enable accurate quantitative analysis of antibody binding (Figure 2). In today's research and clinical settings, robust, accurate, and fast imaging systems have made it possible to document IF stains easily and economically. The past decade has seen the rise of numerous commercial and open-source software systems that enable capture, storage, and analysis of digital images of IF. Significant advances in this space include the fluorescent whole slide scanners that quickly generate full-scale views of the fluorescent slides as tiled images that can be viewed either locally or online and analyzed with either commercial or open-source image analysis software (Table 1). In recent years, the digital pathology systems have evolved to leverage global, internet-mediated connectivity to enable large-scale, whole-slide viewing, manipulation, and analysis. This provides significant benefit, supporting globalization of information in science and medicine, enabling collaborative work on large data sets and complicated cases, allowing rapid, real-time access to digitally stored information, and facilitating data analysis and re-analysis.

The most common approach to fluorescent microscopy excitation is direct illumination of the entire imaging field, a process referred to as wide-field microscopy. While this maximizes both excitation and emission, fluorescent emissions in regions above and below the focal plane are also captured, leading to decreased

resolution and decreased signal-to-noise ratio. Alternative approaches to fluorescent microscopy, including confocal microscopy and two-photon microscopy, have been developed to overcome these challenges. While many advanced systems are available for detailed, high-resolution fluorescent imaging, wide-field fluorescent microscopy remains the preferred approach to imaging IF in tissue sections because it is a robust technique that is readily deployed in most histopathology environments.

While all immune-affinity-based techniques rely on antigen-antibody interaction, they vary greatly in the means by which antibody binding is detected. In IF, antibody-antigen binding is ultimately visualized through detection of a fluorophore. However, this fluorophore can be affiliated with the primary antibody through at least three mechanisms: (1) direct conjugation, in which the fluorophore is linked directly to the primary antibody; (2) indirect, in which the fluorophore is conjugated to a secondary antibody used to detect the primary antibody; or (3) amplified, in which the primary or secondary antibody is either conjugated to biotin and enables biotin:avidin complexes or is conjugated to horseradish peroxidase and enables tyramide amplification. Where possible, directly labeled primary antibodies are preferred to ensure specificity. Unavailability of conjugated primaries or loss of their activity often requires the use of a fluorescent secondary. Amplification of fluorescent signals is often appealing; however, these methods often lead to nonlinear increases in signal and require significant optimization for each tissue type and each antibody.

Since different fluorophores have a distinct spectrum of excitation/emission, they can be multiplexed, meaning that they are visualized in distinct channels and, if conjugated to distinct antibodies, enable simultaneous detection of multiple targets. This is not only a convenient time- and cost-saving strategy—it enables spatial correlation of unique antigens.

Table 1 Common software options for analyzing digital images of fluorescent stains

Software	Strength	Weakness
ImageJ/Fiji https://imagej.net/Fiji	Highly versatile, scriptable, customizable, with many predesigned functions, large and active online community	Steep learning curve, going beyond individual images requires scripting
CellProfiler http://cellprofiler.org	Highly versatile, scriptable, customizable, with many predesigned functions, large and active online community—with some specialized functions	More restricted workflow, little bioinformatics integration
Omero https://www.openmicroscopy.org/omero/	Scriptable, customizable, with some predesigned functions, large and active online community—with some specialized functions	Steep learning curve, difficult customization and deployment
Matlab https://www.mathworks.com	Highly versatile, scriptable, customizable, with many predesigned functions, large and active online community, commercial support, command-line available	Steep learning curve, custom language for scripting
KNIME https://www.knime.com	Highly versatile, scriptable, customizable, with many predesigned functions, large and active online community, commercial support, graphic workflow design, big-data integration	Integration of R, Python, MatLab, Java, etc., and therefore requires workflow optimization

Hitherto, multiplexing was most commonly used to determine colocalization of antigens, but more recently it has enabled improved analysis of spatial distribution of cell types (e.g., immune cells) and molecular activity (e.g., chromatin modification). Multiplexing can be achieved in a single staining cycle when each antibody is detected in a distinct fluorescent channel. In this setup, the scope of multiplexing is limited by the number of fluorophores that can be optically separated (generally 4, but up to 7). Recent advances in cyclic staining have overcome this restriction by eliminating the antibody and/or fluorophore after imaging and re-staining the tissue with new antibodies followed by subsequent imaging of the new stain. While technically advanced, the main challenge in cyclic staining is the optimization of serial detection because the already large range in fluorescent signal can be complicated by the increased handling and processing of the tissue. Moreover, nonspecific staining and artifacts will accrue during the processing and can compromise interpretation.

While antibody availability and specificity is the most immediate factor limiting immune detection of antigens, there are a number of common challenges when developing an immunofluorescent strategy. Initially, a high-affinity antibody with low cross-reactivity conjugated directly to a fluorophore reduces both time and cost of the assay while facilitating downstream analysis. Subsequent availability of the antigen for antibody binding can strongly influence the success of staining. Conventional antigen retrieval with heat, salts, and detergent generally opens the tissue sufficiently to enable binding.^{15,16} In some instances, post-translational modifications or crosslinking from the initial tissue fixation prevents antibody binding. While general access to the antigens can be achieved through many customizable methods, it is recommended to test additional antibodies rather than pursuing unconventional antigen retrieval techniques because they are likely to lead to poor reproducibility and inconsistent results. Thus, by selecting an antibody with high specificity and optimizing fluorophore conjugation, the chances of success in any immunostaining application will improve substantially.⁷ Antibodies for well-characterized proteins are readily available on market. However, IF applications for recently discovered proteins or protein modifications often require that investigators commit

to production of a new antibody. It is important to note that each antibody-antigen interaction is unique and that IF stains for the same protein with distinct antibodies can give very different results depending on the accessibility and affinity of an epitope.¹⁷ This has become evident for some proteins where the use of various epitopes for antibody generation has led to a large number of antibodies that vary greatly in performance and give rise to conflicting observations.

Possibly the greatest advantage of IF is the downstream data analysis that enables quantitative assessment of molecular features within tissues.¹⁸ Colorimetric IHC detection of antigen is very sensitive, reproducible, and robust in its visualization of an antigen, enabling target detection (present/absent) with ordinal quantitation of both intensity and cell number.^{7,19} This is contrasted with IF, wherein the nearly linear correlation between fluorescent emission and its concomitant antibody enables quantitation across a large and continuous dynamic range. The generation of pixel-based digital images from IF in conjunction with rapidly accelerating image analysis technologies has enabled detailed analysis of molecular features.^{20,21} These include traditional statistical measurements such as min, max, mean, median, and sum while extending to features that report on variations across pixel values within the image such as skewness and textures. Segmentation of the image to regions of interest, single cells, and even subcellular structures extends analysis to another quantitative dimension, as IF features among such image segments can provide unique insights into biology. In an era when complex relationships are thought to provide insight into the dynamics of disease progression, treatment responses, and evolution of resistance, computational processing of IF facilitates correction, normalization, and feature recognition.^{18,20,22} Such features can be used directly or form the basis for advanced algorithms and machine learning, which account for many more parameters than can be managed by a single person. While the emergence of so-called “artificial intelligence” in pathology is still controversial, there is no doubt that computer-assisted analysis (i.e., machine learning) is soon to become a powerful companion for the histopathologist that can transform both research and clinical analysis when applied appropriately (see Table 1 for common software options).^{23,24} Considering the quantitative properties of

IF (Figure 2), this strategy enables multi-dimensional analysis of complex molecular features and inter-relationships among features. This becomes particularly evident when IF is multiplexed and the measurement of individual antigens is converted from absolute measures to quantitative relationships between molecular features (i.e., relative abundance) within the tissue of interest. Such quantitative evaluations will not only enable unique analyses, they will also allow for the correction of intra-patient and inter-patient variability that has limited big data analysis in histopathology. Aeffner et al.⁴ discuss digital microscopy and image analysis in detail elsewhere in this journal issue. Altogether, IF offers a number of advances that not only enable us to address current histopathology challenges but also provide opportunities to develop solutions for the high-dimensional “big-data” centric nature of biomedical research (Table 2).

ISH

ISH is a powerful tool for detecting coding and noncoding RNA transcripts in cells from many preparations, including tissue sections, cell culture, viral particles, and stem cells. Multiple techniques have been developed, marked by steady improvements in sensitivity and specificity. The earliest methods relied on nucleotide radiolabeling.²⁵ Subsequent techniques employed riboprobe labeling and signal amplification by enzymes, digoxigenin, and biotinylation.^{26–29} A recently developed approach that has rapidly gained popularity is RNAscope (Advanced Cell Diagnostics, Hayward CA) (acdbio.com). This method permits detection of single mRNA transcripts of one to four unique target sequences with extremely high sensitivity and specificity.³⁰ Between one and four unique sequences can be detected by chromogenic or fluorescent tags, visible with standard brightfield, widefield, and confocal microscopy. A derivative technique by the same manufacturer, BaseScope, permits detection of shorter sequences down to ~50 base pairs (bp), sufficient to detect splice variants, mutations, circular RNA, and long noncoding RNA. RNAscope and BaseScope may be combined with IHC for co-localization of mRNA and protein.^{31,32}

The RNAscope approach is based on branched DNA ISH and signal amplification.^{33,34} High specificity is achieved by designing sequence-specific probes (Z-probes) that minimize nonspecific hybridization. Briefly, the lower region of each probe is a 25 bp oligonucleotide string that is complementary to the target sequence. The upper region is a 14 bp linker tail sequence, joined to the lower region by a 14 bp spacer sequence. These provide a 28 bp binding site for preamplifiers, which are built up during a multi-stage amplification cycle. In the final steps, chromogenic or fluorescent conjugates are bound to binding sites on each preamplifier. The net amplification greatly improves sensitivity compared to other methods.

In addition to its high sensitivity and specificity, an attractive feature of RNAscope is speed and ease of use. Single and multiplexed (2–4 probes) assays can be completed in 6 to 12 hours over 1 to 2 days with minimal equipment and preparation. Probes and reagents are provided in proprietary form (kits)

by the manufacturer along with detailed protocols for various applications. These are separately available for manual and automated assays.

The manual assay can be completed on a standard laboratory bench. Required equipment includes a hybridization oven capable of maintaining a constant temperature of 400°C, a slide rack, and staining dishes for fixative, buffer, and alcohol solutions. A carbuoy and assorted 1 L bottles are useful for storage of stock solutions. A typical setup can handle up to 20 slides at once. Assays have also been designed to work on two automated platforms: Leica and Ventana. Riboprobes are custom designed for each platform, along with specific reagents. These platforms are capable of high-throughput assays. Although sterile, RNase-free conditions are not necessary, we maintain those conditions at all stages, from sample acquisition through probe hybridization and amplification, to minimize mRNA degradation. Conservative technique can be especially important for detection of low-abundance transcripts and would be an important consideration in troubleshooting. In combining ISH with IHC, the ISH assay is typically performed first.^{31,32} Because pretreatment for ISH typically involves incubation in a protease solution, protocol optimization is necessary to maximize transcript detection and minimize loss of the target protein.

Probes are designed and synthesized by the manufacturer. The primer sequence is generally proprietary, although custom probes may be designed to target a specific sequence. It is recommended that probes be species specific, as sequence variations can be significant, leading to weak or nonspecific hybridization. A large catalogue of “stock” probes for numerous species has been developed. Probes not in the catalogue may be custom designed for a one-time extra charge, and these are eventually incorporated into the catalogue.

RNAscope and BaseScope can be performed on cells and fresh frozen, fixed, and formalin-fixed paraffin-embedded (FFPE) sections as well as wholemounts. Assays have also been developed for free-floating tissue sections.³¹ Each preparation requires a unique series of pretreatment steps prior to hybridization, but the subsequent amplification steps are fairly uniform. Recommended protocols for most preparations are provided by the manufacturer, but conditions must be optimized for specific applications.

Appropriate controls are essential for evaluation of probe specificity, troubleshooting, and quantification. The manufacturer recommends the concurrent use of positive and negative controls. Several positive controls, available from the manufacturer, are genes found in a majority of cell and tissue types across species. Since these controls are well described across tissues and conditions by the manufacturer, they are helpful in troubleshooting. We have also found it useful to include other highly characterized genes as positive and negative controls or cell markers in the tissue we mostly frequently assay (brain). These provide a check on tissue integrity and may also be used for normalization in quantification.³⁵

Typically, the RNAscope assay generates punctate labeling of “dots” approximately 1 micron in diameter. Each dot is reported

Table 2 Fluorescence versus chromogenic techniques for immunostaining

Feature	Sensitivity	Dynamic range	Quantitation	Multiplexing	Colocalization	Robustness	Accessibility	Optimization
Fluorescence	++	++++	Continuous	+++	+++	++	+	+++
Chromogenic	++++	++	Noncontinuous	+/-	–	++++	++++	++

to represent a single transcript.³⁰ The size is similar in both chromogenic and fluorescent assays. Depending on abundance and tissue type, as well as the application, visualization can be achieved at a wide range of magnifications. In Figure 3, a duplex chromogenic in situ hybridization assay (two probes: red and aqua) in mouse forebrain sections is illustrated. A low-magnification snapshot is shown in panel A. The high-abundance chromogenic signals are clearly visible even without magnification in this tissue. Higher magnifications (10x to 100x) are required to resolve or count individual “dots” (panels D–G). In Figure 4, images from a multiplexed fluorescent in situ hybridization (FISH) assay are illustrated. Four probes (*Adora1*, *VGAT*, *Vglut1*, *Vglut2*) plus the nuclear stain, DAPI, were imaged in different color channels. Panels B to F show each channel individually, merged in A. Panels G to L show transcripts in each channel at higher magnification. *Adora1* and *Vglut1* are expressed in most cells, whereas *VGAT* and *Vglut2* are expressed in a subset of cells.

Quantification of the signal may take many forms. Counting of transcripts (dots) is best achieved by imaging at 20x to 40x magnification, followed by automated signal capture using imaging software with appropriate capability, such as ImageJ, CellProfiler, or HALO. These provide a fairly direct estimate of transcript abundance for individual cells or a defined region of interest that can be quantified to detect regional differences or a change in expression.

RNAscope has been widely adopted by researchers and pathologists for evaluation of a wide range of tissue types, including cell cultures, tumors, and organ systems. Its popularity is evidenced by inclusion in over 1000 published articles at present. Specific applications include single-transcript detection and quantification, validation of transcriptomic data, gene editing, transgenic manipulations, quantification of mRNA abundance associated with development, aging, pathology and treatment, distribution of cells with positive or negative expression in a tissue sample, and cellular phenotyping by combining probes for a target sequence with cell type-specific markers (i.e., probes, antibodies, stain).

The most important advantage of the RNAscope approach is the high level of sensitivity and specificity that can be achieved with standard protocols. A second advantage is that the probes and reagents are provided by the manufacturer in ready-to-use kits. Probe design and manufacture are labor-intensive processes beyond the capabilities of many small laboratories; thus the commercial availability of probes removes a major barrier to the routine use of ISH. A third advantage is that, once optimized, the assays can be conducted in 1 to 2 days with minimal training or expertise. The main barrier for some labs might be cost. Catalogue probes are about \$500 and provided in quantities sufficient to conduct assays on 12 to 20 standard slides (depending on the total area to be reacted). Combined with the reagents and kits required, the total for a single-probe chromogenic assay on 12 to 20 slides would be about \$1500. Duplex chromogenic assays add approximately \$1000 to the assay cost, while multiplexed fluorescent assays (up to four probes) run up to \$3500, depending on the number of probes to be included. The recommended hybridization oven is about \$4000. Some of these costs are offset by the savings in labor and materials needed to synthesize and assess riboprobes in-house, as commercial availability is presently low.

An emerging technique that builds upon FISH is fluorescent in situ sequencing,³⁶ which allows for deeper, ultra-sensitive analysis, providing unbiased, quantitative, visual detection of RNA expression in FFPE cells and tissues, wholemounts, and organoids. It is the visuospatial counterpart to RNA-seq; architecture is preserved in tissue sections, or single-cell analysis can be performed. In fluorescent in situ sequencing, transcripts essential to a cell's function or type are enriched and used as internal controls rather than housekeeping genes. Without rRNA depletion, the lower limit of detection is approximately 200 to 400 mRNA molecules per cell; following rRNA depletion, this improves to about 10 to 20 mRNA molecules. In the protocol, in situ reverse transcription to cDNA is performed, followed by matrix generation, sequencing by synthesis or sequential ligation, imaging by epifluorescence or confocal microscopy, and analysis. Further details can be found in.³⁶

Figure 3 Duplex chromogenic in situ hybridization in mouse forebrain. Red and aqua “dots” indicate transcripts of two different genes. (A–C) Low magnification images show regional differences in expression. (D–G) High magnification images show transcript labeling in single cells. Black arrows, dual-labeled. White arrows, single-labeled. Scale bars: (A) 1 mm; (B–C) 250 μ m; (D–G) 20 μ m. Abbreviations: Aud, auditory; Ent, entorhinal; Hip, hippocampus; MG, medial geniculate; SC, superior colliculus; SN, substantia nigra.

Figure 4 Multiplexed FISH in mouse forebrain. Four probes (*Adora1*, *VGAT*, *Vglut1*, *Vglut2*) plus the nuclear stain DAPI were imaged in different color channels. Panels B to F show each channel at high magnification (merged in A). Scale bar: (A–F) 50 μ m.

LCM

LCM is a technique that utilizes an inverted light microscope equipped with a laser for precise collection of groups of cells or single cells in heterogeneous tissue. FFPE tissue, frozen tissue, cytology prepared samples, and cell cultures can be utilized as tissue sources for downstream analysis of DNA, RNA, or protein. This includes a wide array of molecular and proteomic assays including mass spectrometry, DNA genotyping and loss-of-heterozygosity analysis, RNA transcript profiling, cDNA library generation, proteomics discovery, and signal kinase pathway profiling.^{37–41} Prior to the introduction of LCM, researchers were limited to macrodissection of tissue from slides. Macrodissection is a crude method for tissue collection and often results in collection of adjacent unwanted tissue that can confound results. The innate heterogeneity of tissue samples and the inability to eliminate unwanted tissue is a major limitation of the macrodissection method. With LCM, the tissue collection is precise and limited to only the cells, organism, or material of interest.

In the mid-1990s, Dr. Emmert-Buck and colleagues at the National Institutes of Health developed the first LCM system.⁴² This instrument was commercialized by Arcturus and utilizes both ultraviolet (UV) cutting and an infrared (IR) laser capture system. In this system the tissue is captured on a membrane that is melted onto the tissue, which then lifts the tissue off the slide. Subsequently, the PALM Microbeam (Zeiss MicroImaging, Bernried, Germany) and the Leica LMD6000 (Leica Microsystems, Bannockburn, IL, USA) were introduced to the market. These instruments utilize only UV cutting systems that rely on gravity or catapulting the cells into a tube for collection and further analysis. The following discussion will focus on the Arcturus LCM system (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The Arcturus system utilizes a cap with a thermoplastic transfer film that directly overlies the tissue section. When the cells of interest are identified through the microscope, the IR laser is pulsed through the transfer membrane, which melts the membrane and bonds it to the target cells. Following the IR pulse, the transfer membrane returns to normal shape and lifts the adhered

cells off the slide. This process does not disrupt the biomolecular integrity of the captured cells. In this system, the UV cutting function can be used to ablate unwanted cells from the slide or utilized to dissect a large group of cells for collection. Following UV cutting around the perimeter of the cells of interest, several contact points are created with the IR laser, and groups of cells are lifted off the slide (Figure 5). IR capture and UV cutting can be performed on plain, uncharged glass slides. However, removal of large groups of cells is facilitated by utilizing PEN Membrane Glass Slides (Thermo Fisher Scientific, Kalamazoo, MI, USA).

LCM can be performed on FFPE, frozen sections, or cytology prepared. DNA recovery from FFPE tissue is easily accomplished, and as technology has advanced, capability of using FFPE in downstream RNA assays has expanded.⁴³ Frozen sections are still the optimal sources of material for RNA and proteomic work (Table 3). RNA degrades quickly when subjected to RNAases in the environment. Strict practices to minimize exposure to RNAases are employed during the microdissection process. The standard practice in our laboratory to maximize RNA yield is to complete microdissection of the frozen section within 90 minutes of cutting the slide. Tissue sections are typically cut at 2 to 15 microns, which maximizes the opportunity to collect full bodies. Thin sections may lead to increased number of sections needed for microdissection to achieve the desired sample size. Tissues that are too thick may not be completely collected from the slide, leaving key cellular components behind. The thickness can vary and is often dictated by the tissue type and how well the capture process is working with the particular tissue of interest. Tissues can be stained with a variety of stains including H&E, methylene blue, Wright-Giemsa, toluidine blue, cresyl violet, or immunohistochemistry (brightfield or fluorescence).^{39,40,44} Ready-made, nuclease-free staining kits that preserve RNA and DNA integrity are available from the vendor. The number of cells required for downstream assays can vary greatly depending on the assay itself or the quality of the tissue. The ranges are as follows: DNA 100 to 2000 cells, RNA 5000 to 10,000 cells, and

Figure 5 Laser capture microdissection, 200× magnification, Wright-Giemsa stain. (A) Liver, annotating UV cutting area around two vessels (red outlines at perimeter) and IR capture points (red dots along vessel walls). (B) Same liver section, post-UV cutting and IR capture. (C) Microdissected vessels extracted from tissue section. IR laser foci from A that fuse the tissue to the transfer film appear black.

Table 3 Sample types and preparations for laser capture microdissection specimens

Sample	DNA	RNA	Protein
FFPE	+	+	–
Frozen	+	+	+
Cytology	+	+/ ^a	– ^b

^aDepends on fixation; in air-dried, unfixed slides, RNA likely degraded.

^bRequires large numbers of cells, typically in the magnitude of 10⁴ or greater.

protein 4000 to 200,000 cells.³⁹ As new tissue types and assays are utilized, pilot runs and quality control analysis of specimens should be done to optimize the entire tissue preparation and collection processes.

The main limitation of LCM is the requirement that the user to be knowledgeable in microscopic anatomy and have the ability to identify and collect the correct cells of interest. This often requires collaboration with a pathologist or another professional trained in the microscopic anatomy of the tissue of interest. Additionally, processing, staining, and sectioning of tissue can be labor intensive and is another step that may require assistance from a professional histotechnologist. Also, although these instruments have been available for nearly two decades, access to the instrumentation may be an issue. Finally, the tissue preparation itself (i.e., over fixation, type of fixation, or poor tissue quality) can be a limiting factor to the type of downstream assays that are performed.⁴⁵

LCM is a robust technology that delivers precision tissue collection down to a single cell level in complex heterogeneous tissues that can be utilized in a broad spectrum of downstream molecular and “omic” assays. This powerful technology has opened doors to discovery across scientific disciplines and will continue to lead to advances in translational medicine.

IMS

IMS is an emerging analytical technique for the direct analysis of tissues. IMS combines the analytical power of mass spectrometry with the spatial power of histology to yield next-generation molecular histological data.⁴⁶ This approach is suitable for both fresh frozen and formalin fixed tissues. In these experiments, tissues are sectioned or directly mounted onto mass spectrometry-compatible surfaces. Some techniques require conductive surface, such as gold, stainless steel, or indium-tin oxides coatings. Others do not require conductivity, and analysis can be performed directly from microscope slides. Mass spectra are collected from defined x and y coordinates across the tissue section. This general process is depicted in Figure 6A. Peaks from the mass spectra can be false colored into heat maps, allowing for molecules to be spatially mapped

Figure 6 An overview of the imaging mass spectrometry workflow. (A) In step 1, biopsies are sectioned and placed on surfaces compatible with the ionization method, typically glass or stainless steel. In step 2, any sample preparation such as tissue washing or the application of MALDI matrix takes place. In step 3, the tissue surface is systematically interrogated using a mass spectrometer. Each pixel in the image represents a mass spectrum with defined x and y coordinates. An example MALDI image of a 1-week-old mouse pup (B). Each color in the image represents a unique small protein. Adapted with permission of John Wiley & Sons, Inc. from <http://onlinelibrary.wiley.com/doi/10.1002/rcm.8042/abstract>.

back to tissue surfaces. Figure 6B shows data from an IMS analysis of a whole body section of a mouse pup, where each color in the image represents a small protein. This approach is appealing for a number of reasons. First, it does not require specialized reagents, antibody labels, or any prior knowledge of the sample composition, making it ideal for discovery-based analyses. Second, many of the ionization techniques are non-destructive or minimally destructive to the tissue section. This allows for subsequent staining to co-localize histological features to IMS data sets.⁴⁷ Finally, it is able to analyze a wide range of analytes, including metals, metabolites, pharmaceuticals, proteins, and peptides, diversifying the chemical space for tissue analysis.^{48–52}

IMS can be coupled with a variety of ionization techniques, each requiring different analytical instrumentation. Secondary ion mass spectrometry offers the highest lateral resolution imaging capabilities, with sub-50 nm imaging reported.^{53,54} This technology uses a primary ion beam to strike the tissue surface, ejecting secondary ions that are detected with a mass spectrometer.⁵⁵ However, since this technology ionizes analytes using an ion beam, there can be significant fragmentation

of analytes in the process.⁵⁶ Matrix-assisted laser desorption/ionization (MALDI) IMS is a softer ionization technique, allowing for the ionization of small proteins and peptides using a pulsed laser beam.⁵⁷ MALDI IMS requires the sample be coated with a matrix to aid in the desorption and ionization of analytes, requiring additional sample preparation steps before analysis.⁵⁸ MALDI IMS is typically performed at a lateral resolution of 10 to 300 μm , though some papers have reported lateral resolutions of 1 to 3 μm .^{59–61} Some additional ionization techniques utilize solvents to extract analytes from the surface of tissues. One popular approach is desorption electrospray ionization, where a charged solvent stream is directed at a surface using a capillary.^{62,63} Analytes of interest are extracted from the surface and directed into the mass spectrometer. Desorption electrospray ionization IMS has been used to extract a large range of analytes but can have limited spatial resolution due to solvent spreading.⁶⁴

IMS does require specialized equipment to perform. A lab must have access to or purchase a mass spectrometer compatible with the desired ionization approach. In some cases, additional equipment is required to prepare the sample (e.g., matrix application devices for MALDI IMS).⁶⁵ Many mass spectrometers are available to perform such analyses and can vary widely in cost based on their performance. Factors to consider include resolving power, speed, and ability to perform fragmentation analysis (MS/MS).^{52,66–68}

Adequate controls are very important when obtaining IMS data. Since the strength of the technology lies in detecting molecular changes associated with diseases, healthy tissue is typically analyzed in parallel with disease. Data size can grow very large quickly because each pixel of the image contains both positional information as well as a mass spectrum. Specialized software is available to display the data as heat maps and is typically vendor specific. However, there are several free software packages available to process data including Cardinal and MSiReader.^{69–71} Vendor software is available that can handle multiple data formats from different manufacturers. There has been additional effort to standardize the format of IMS data for storage in repositories and to allow for data to be exchanged among researchers freely. This data format, called imzML, stores the mass spectra in a different file than the metadata, which are stored in XML files.⁷²

IMS has many advantages. One major advantage is the ability to directly analyze small molecules, lipids, and metabolites, a region of chemical space that is difficult to analyze with antibody or tagging-based approaches. Additionally, the chemical environment of the sample is unperturbed by *in vivo* radiolabels or fluorescent tags, allowing for endogenous measurements to be made.⁷³ This analytical approach does require specialized instrumentation, such as a mass spectrometer and qualified scientists to operate it, which presents a challenge for small laboratories. Additionally, as an emerging analytical technology, the field is still establishing the gold standards for data collection, reporting, and accessibility.⁷⁴ However, the ability of IMS to augment histological data with molecular information has made this tissue imaging approach appealing to many researchers.

Optical Imaging and Vibrational Spectroscopy

Advancements in nonlinear optical imaging and vibrational spectroscopy have begun simplifying, accelerating, and augmenting standard histopathological workflow and tissue analysis. Some of the techniques can be applied directly to unfixed

tissue specimens and computationally rendered into an H&E style image without significant preparation. In parallel, these modalities can offer additional tissue contrast and information to supplement structural details routinely observed with standard histology. Some of the prominent forerunners among these techniques include two-photon absorption fluorescence (TPAF), second harmonic generation (SHG), spontaneous Raman spectroscopy, coherent Raman scattering (CRS) microscopy, and Fourier transform infrared spectroscopy (FTIR).

TPAF was originally theorized by German-born physicist and Nobel laureate Maria Goepart-Meyer in 1930. TPAF is a commonly used biomedical research tool for deep-tissue fluorescence imaging of exogenous labels and endogenous compounds. TPAF involves simultaneous absorption of two lower energy photons to generate one higher energy photon, which is achieved using a high intensity of lower energy photons that have a higher tissue penetration depth. Thus TPAF imaging is highly advantageous in being able to image as deep as 800 μm in tissue with reduced photo-bleaching. TPAF images are typically reconstructed by having the laser raster-scan two-dimensionally through a sample. The generated fluorescence is directed onto a single-element photodetector through an array of focusing lenses, objectives, filters, and scanning mirrors—analogueous to laser scanning confocal fluorescence microscopy. The key requirement for this technique is essentially an ultrafast near-infrared laser (pico- to femtosecond pulse widths, MHz frequency range) to achieve TPAF. The usability of TPAF imaging systems is comparable to that of a laser scanning confocal fluorescence microscopy system once adequately trained. Understanding the technicalities of optics, microscopy, and image analysis are helpful but not necessary for end users. Depending on how the image acquisition software is configured, TPAF images are usually acquired as a TIFF file (a few hundred megabytes per image) and can be extended to multiple time series and color channels in a single image file. Analysis can be performed with any analysis software package, including open-source options such as ImageJ and CellProfiler, and are widely configurable based on users' needs.

TPAF images can be obtained from unfixed tissues and be rendered into H&E-like images using computational methods, yielding comparable results to standard histopathology.^{75,76} Beyond H&E, TPAF imaging can be applied for characterizing tumor margins in gross tumor biopsies^{76–78} while also providing insight regarding metabolism or three-dimensional structures in biological material. High costs of ultra-fast lasers required for TPAF imaging may prove deterring, as the expense can approach several hundreds of thousands of dollars. Recent advances in laser technologies with solid-state laser systems, fiber lasers, and supercontinuum sources are making TPAF imaging much more cost-effective. For further reading about TPAF, we direct the reader to detailed reviews and perspectives.^{79–81}

SHG occurs when a high-intensity laser is incident on a material that exhibits repetitive macromolecular structure that lack inversion symmetry. When these conditions are met, samples can generate a new wavelength of light at twice the frequency or one-half of the wavelength of the incident light. This distinct property is exhibited by certain structural proteins such as collagen, myosin, or actin that can be useful markers for tissue architecture or disease progression. Instrumentation for SHG imaging tends to be very similar compared to that utilized for TPAF imaging, and they are often performed in tandem. When using thin or clear samples, forward-detection through a high numeric aperture condenser may be necessary for ideal signal-to-noise. Once installed and optimized, image acquisition and analysis can be performed in a manner similar

to that with TPAF systems. As with TPAF, SHG too benefits from deep tissue imaging capabilities and does not exhibit photo-bleaching. Since SHG can only highlight structural constituents that lack inversion symmetry, it is often combined with TPAF to obtain complementary tissue information due to similarity in instrumentation between the two. SHG on its own or in combination with TPAF has thus been explored for studying structural changes during disease progression in tissue specimens^{80,82} or yielding H&E-like images from unfixed samples.^{75,76,83} A more in-depth discussion on the potential and applications of SHG can be obtained elsewhere.^{80,82,84,85} Spontaneous Raman spectroscopy, or simply Raman spectroscopy (RS), is a type of vibrational spectroscopy that probes the inelastic scattering of light. It was first demonstrated by Nobel laureate Dr. C.V. Raman in 1928. RS occurs when a photon collides and transfers energy to the chemical bonds in a sample, causing a small change in energy or wavelength of the incident light. Analyzing these changes in wavelength spectroscopically provides a “molecular fingerprint” of the vibrational bonds present in a sample (e.g., Amide I, Amide III, C = C resonance, P-O resonance). The ability to sense these changes in bond vibrations makes RS very sensitive to subtle biochemical changes, such as differences between normal and abnormal tissue types. RS is typically performed with a continuous-wave narrow linewidth diode laser incident on a sample. The scattered light from the sample is collected, optically filtered, and dispersed onto a camera with a diffraction grating. The camera then outputs intensities of light as a function of wavelength and one spatial dimension. These data are segmented and processed into a feature-rich spectrum in CSV format. Spectra are subsequently analyzed where each feature in the spectrum corresponds to the vibrational mode of a biochemical constituent in a sample.⁸⁶ Advanced analysis of Raman spectra can involve data reduction techniques, such as principal components analysis, coupled with multivariate-based or machine learning algorithms such as sparse multinomial logistic regression, support vector machines, or general linear models for normal and abnormal tissue classification. Alternatively, ratiometric spectral comparisons of peak intensities can offer insight to the biochemical dynamics across different samples. Further details on instrumentation, processing, applications, and previous work can be found in other reviews.^{87–89} Many tools for processing and analyzing spectral data have been automated through both custom and commercially available software packages, thereby requiring minimal end-user training. Raman microscopy or fiber-optic probe based systems are commercially available, or can be custom-built with research-grade components as per the specific research requirements. Due to its sensitivity for tissue biochemistry, RS has often been employed for in vitro or in vivo tissue characterization in the form of Raman microscopy or fiber-optic probe-based Raman spectroscopy, where Raman spectra can be obtained using point-based measurements within a few seconds. However, since Raman scattering is a weak optical phenomenon compared to fluorescence, RS imaging of an entire histological slide or tissue in vivo at cellular resolution would require lengthier acquisition times ranging from minutes to days. Spectral quality or number of spectra per sample can be traded off to speed up the process,⁹⁰ while others have employed ultra-fast lasers and sophisticated optics to obtain Raman-like information rapidly at subcellular resolutions for histological or in vivo applications. Among these techniques are Coherent Anti-Stokes Raman scattering (CARS) and Stimulated Raman Scattering (SRS), collectively referred to as CRS imaging.

The amount of tissue information derived from CRS is similar to RS and on par with the feature-richness of mass spectrometry. While both CARS and SRS provides image contrast stemming from Raman scattering that relies on the vibrational bonds present in the sample of interest, their mechanisms of action and detection differ. CARS signal relies on the interaction between a pump laser and a Stokes laser beam coincident on a sample. When the energy difference between the two laser beams matches a Raman vibrational resonance present in the sample, a new wavelength of light, or a CARS signal, is generated. CARS signal can be filtered and detected much like TPAF or SHG, and the laser differences can be tuned sequentially to acquire spectral information similar to RS. SRS also uses two lasers tuned to match Raman resonances and occurs alongside CARS signal generation. However, the detection method is a pump-probe based technique that looks for high-frequency changes in one incident laser line due to modulating the intensity of the other laser line using a lock-in amplifier. CARS has been utilized for in vivo histology on the skin surface through mosaic imaging, which constructs a region of interest, and facilitates differentiation between a nevus and a malignant neoplasm, for example, without biopsy.⁹¹

Some CRS permutations include single-band, narrowband, and broadband spectral imaging. Single-band CRS imaging typically selects a handful of notable peak resonances, indicative of protein, lipids, nucleic acids, or some combination of the three. Tuning the pump and Stokes beam differences to the distinct resonances of interest can allow for visualizing different Raman modes in a sample that can then be used to render H&E-like images. Instead of only single bands, ultrafast lasers can be used to probe short segments of the Raman spectrum to achieve narrowband CRS imaging, which yields hyperspectral images of samples over a few hundred wavenumbers of Raman spectrum. These images contain spatial and compositional information regarding the biochemical constituents in three dimensions that can be deconstructed and interpolated with advanced statistical methods.⁹² Broadband CRS is achieved by using high bandwidth or supercontinuum laser sources in conjunction with fast spectrometers to obtain access to the full bandwidth of Raman spectra, which can be used for extensive biochemical analysis of samples cellular level resolution, akin to Raman spectroscopic mapping but orders of magnitude faster.

While the flexibility and feature richness of CRS are potentially attractive, the laser costs are even greater than that for TPAF and SHG due to the need for multiple ultrafast laser lines and additional optics. There are a handful of commercial laser systems available for those interested in CARS and SRS, but many of these imaging systems are custom built by researchers with extensive training in optics and image processing. The exhaustive training required in CRS instrumentation often limits its accessibility to researchers. These techniques are relatively new, and work is underway to make CRS more accessible for user imaging systems. A more comprehensive outlook on the theory and applications of CRS can be found in other sources.^{87,93–95}

Similar to RS, FTIR is also a form of vibrational spectroscopy that relies on detecting changes in the vibrational frequencies in the sample upon light-sample interactions. However, while RS relies on inelastic scattering of the incident light, FTIR is based on the absorbance or transmittance of IR light in the sample. While yielding complementary information, RS signal depends on a change in polarizability of a molecule, whereas FTIR signal relies on the change in the net dipole moment of the molecule. An IR microscope can generate images that serve

as a suitable alternative to conventional histology. These images are essentially an IR map with an IR spectrum encoded in each pixel of the image, thus providing the biochemical composition of the specimen tested, as demonstrated for neurodegenerative diseases and cancer.⁹⁶ FTIR devices are less costly than RS setups and widely available with commercially available software that performs automated analysis of the generated data, making the technique user-friendly. FTIR is not affected by fluorescence from the sample that tends to interfere for RS. However, unlike RS that requires little to no sample preparation, specimens for FTIR testing require detailed sample preparation and can be heavily affected by sample uniformity, thickness, and water content.

Emerging Technologies

Multi-, super-, and hyperplex antibody-based labeling techniques are emerging for parallel biomarker analysis on a single slide. Broadly, approaches consist of fluorophore-, hapten-, or metal-coupled antibodies. Biotechnology enterprises offer customizable panels, such as Ultivue's InSituPlex (Ultivue, Inc., <https://www.ultivue.com/technology>), UltraPlex mxIF (Cell IDx, Inc., <https://cellidx.com/technology>), MultiOmyx hyperplexed IF (NeoGenomics Laboratories, Inc., <http://neogenomics.com/pharmaservices/lab-services/multiomyx/technology/hyperplexed-immunofluorescence-assay>), and CyTOF Imaging Mass Cytometry and Hyperion Imaging System (Fluidigm, <https://www.fluidigm.com/products/hyperion-imaging-system>). NanoString Technologies Inc. (www.nanostring.com), a forerunner in *in situ* hybridization hyperplexing, enables deep transcriptomic or proteomic analysis, with high spatial resolution and an impressive capacity for multiplexing up to 30 protein or RNA markers of human or mouse origin.⁹⁷ Specimens are run in one of three proprietary instruments (nCounter), which can function on as little as 25 ng RNA. Omic signatures are analyzed by companion analysis software (nSolver) that creates so-called molecular barcodes for tissue specimens. Appealingly, a single 5 μ m section of FFPE tissue is an adequate template for NanoString barcoding.⁹⁸ Body fluids, cell suspensions, and lysates are also acceptable. It is estimated that up to 800 targets will someday be assayable using their proprietary oligo-conjugated antibody cocktails. Currently, the company offers numerous themed (e.g., solid tumor, neuroinflammation), partially customizable gene and protein expression panels.

In vivo histology may be the holy grail of anatomic pathology, enabling unperturbed visual *in situ* subcellular structural analysis. Confocal laser endomicroscopy has been used in years for endoscopic or bronchoscopic diagnosis of alimentary and respiratory pathology, respectively. This approach allows for real-time

imaging to the micron level using a miniprobe and has been used to accurately diagnose inflammatory and neoplastic diseases, corroborated by histopathology, *in vivo*.^{99–101} Microscopy with ultraviolet surface excitation (MUSE) is another imaging technique that can generate histology-like images in select *in vivo* settings.

MUSE has proven valuable for nondestructive assessment of tissue surfaces. The potential of utilizing UV excitation for studying histology slides was discovered when tissue sections illuminated with UV wavelengths <300 nm provided microscopy images that had significantly higher clarity, sharpness, and contrast.^{102,103} The improved image quality is due to these UV wavelengths being absorbed within the few microns at the tissue surface and subsequently eliciting bright visible emission that generates images with better contrast and subcellular resolution. Instrumentation for MUSE consists of a couple of UV LED sources (wavelength <300 nm), a stage that allows UV transmission from the sources to the sample, conventional focusing optics, and an imaging sensor. As the light from the UV LEDs is focused onto the tissue surface, the resultant emission is collected by the focusing optics and directed onto either a gray-scale or color CCD camera. The acquired images are then saved in TIFF or JPEG formats and subsequently processed with open-source image processing-analysis softwares (ImageJ or GIMP).

Images obtained with MUSE can be made to resemble those from standard H&E slides (Figure 7) while also illuminating cytomorphological features not observable with conventional histology.^{104–106} The added value of MUSE is that it is a simple, nondestructive, slide-free technique that can provide high-resolution diagnostic histological images from fresh or frozen tissue sections within a few minutes compared to standard histology that may be delayed by days.¹⁰⁵ While commercialization of this technology is underway, the instrumentation involved for MUSE is relatively simple as described earlier, thereby requiring the end-user to have training similar to wide-field microscopy or LCSM to become proficient with MUSE. Due to limited tissue penetration depth of UV, MUSE can only assess specimen surfaces by a few microns and would thus require subsequent tissue sections to be made for imaging deeper layers.

Conclusion

Newly developed techniques for assaying biomarkers *in situ* are more sophisticated than ever, and most methodologies can be performed using readily available samples, such as FFPE. There are several emerging technologies not yet widely applied due to limited availability, cost, proprietary formulations, and unmet need for expertise. The relative novelty of some of these

Figure 7 (A) A MUSE image from the cut surface of a formalin-fixed section of porcine kidney stained with Rhodamine and Hoechst. (B) The same MUSE image has been converted to a virtual histology image. (C) Conventional H&E-stained slide from the same tissue specimen (not a serial section to the region visualized in A and B) after paraffin embedding and sectioning, demonstrating the similarity between virtual histology and conventional H&E staining. Figures courtesy of the Richard Levenson Lab, Department of Pathology and Laboratory Medicine, University of California, Davis.

advanced in situ microscopic techniques can pose a barrier to sufficient experimental validation and peer review. Eventually, utilization of such technologies in research applications may provide proof-of-principle for moving these modalities into the diagnostic mainstream of 21st-century personalized medicine.^{24,107–109} Nonetheless, in the discovery setting, techniques such as IF, ISH, LCM, MALDI IMS, and CRS serve to integrate anatomic and molecular pathology, wherein virtually any proteins, nucleic acids, or other endogenous or exogenous substances can be simultaneously identified and quantified in tissue sections.

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References

- Rosai J. Why microscopy will remain a cornerstone of surgical pathology. *Lab Invest*. 2007;87(5):403–408.
- Zarella MD, Breen DE, Plagov A, Garcia FU. An optimized color transformation for the analysis of digital images of hematoxylin & eosin stained slides. *J Pathol Inform*. 2015;6:33.
- Hamilton PW, Bankhead P, Wang Y, Hutchinson R, Kieran D, McArt DG, James J, and Salto-Tellez M. Digital pathology and image analysis in tissue biomarker research. *Methods*. 2014;70(1):59–73.
- Aeffner F, Wilson K, Martin NT, et al. The gold standard paradox in digital image analysis: Manual versus automated scoring as ground truth. *Arch Pathol Lab Med*. 2017;141(9):1267–1275.
- Di Cataldo S, Ficarra E. Mining textural knowledge in biological images: Applications, methods and trends. *Comput Struct Biotechnol J*. 2017;15:56–67.
- Aeffner F, Wilson K, Bolon B, et al. Commentary: Roles for pathologists in a high-throughput image analysis team. *Toxicol Pathol*. 2016;44(6):825–834.
- Ramos-Vara JA, Miller MA. When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry—the red, brown, and blue technique. *Vet Pathol*. 2013;51(1):42–87.
- Beutner EH. Immunofluorescent staining: The fluorescent antibody method. *Bacteriol Rev*. 1961;25(1):49–76.
- Hamashima Y, Harter JG, Coons AH. The localization of albumin and fibrinogen in human liver cells. *J Cell Biol*. 1964;20:271–279.
- Nakane PK. Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: A study on pituitary glands of the rat. *J Histochem Cytochem*. 1968;16(9):557–560.
- Nakane PK, Pierce GB Jr. Enzyme-labeled antibodies: Preparation and application for the localization of antigens. *J Histochem Cytochem*. 1966;14(12):929–931.
- Rimm DL. What brown cannot do for you. *Nat Biotechnol*. 2006;24(8):914–916.
- Lichtman JW, Conchello J-A. Fluorescence microscopy. *Nat Methods*. 2005;2(12):910–919.
- Matos LL Trufelli DC, de Matos MGL, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights*. 2010;5:9–20.
- Ramos-Vara JA, Beissenherz ME. Optimization of immunohistochemical methods using two different antigen retrieval methods on formalin-fixed paraffin-embedded tissues: experience with 63 markers. *J Vet Diagn Invest*. 2000;12(4):307–311.
- Zhang Y, Wang X-P, Perner S, Bankfalvi A, Schlücker S. Effect of antigen retrieval methods on nonspecific binding of antibody-metal nanoparticle conjugates on formalin-fixed paraffin-embedded tissue. *Anal Chem*. 2018;90(1):760–768.
- Rehman JA, Han G, Carvajal-Hausdorf DE, et al. Quantitative and pathologist-read comparison of the heterogeneity of programmed death-ligand 1 (PD-L1) expression in non-small cell lung cancer. *Mod Pathol*. 2017;30(3):340–349.
- Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med*. 2002;8(11):1323–1327.
- Hewitt SM, Robinowitz M, Bogen SA, et al. *Quality assurance for design control and implementation of immunohistochemistry assays: Approved guideline*. Wayne, PA: Clinical Lab Standards Institute; 2011.
- Gerner MY, Kastenmuller W, Ifrim I, Kabat J, Germain RN. Histo-cytometry: A method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity*. 2012;37(2):364–376.
- Schapiro D, Jackson HW, Raghuraman S, et al. histoCAT: Analysis of cell phenotypes and interactions in multiplex image cytometry data. *Nat Methods*. 2017;14(9):873–876.
- Bodenmiller B. Multiplexed epitope-based tissue imaging for discovery and healthcare applications. *Cell Syst*. 2016;2(4):225–238.
- Cabitz F, Rasoini R, Gensini GF. Unintended consequences of machine learning in medicine. *JAMA*. 2017;318(6):517–518.
- Ehteshami Bejnordi, B, Veta M, van Diest PJ, et al. Diagnostic assessment of deep learning algorithms for detection of lymph node metastases in women with breast cancer. *JAMA*. 2017;318(22):2199–2210.
- Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci USA*. 1969;63(2):378–383.
- Jin L, Lloyd RV. In situ hybridization: Methods and applications. *J Clin Lab Anal*. 1997;11(1):2–9.
- Kerstens HM, Poddighe PJ, Hanselaar AG. A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. *J Histochem Cytochem*. 1995;43(4):347–352.
- Kwon S. Single-molecule fluorescence in situ hybridization: Quantitative imaging of single RNA molecules. *BMB Rep*. 2013;46(2):65–72.
- Qian X, Lloyd RV. Recent developments in signal amplification methods for in situ hybridization. *Diagn Mol Pathol*. 2003;12(1):1–13.
- Wang F, Flanagan J, Su N, et al. RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*. 2012;14(1):22–29.
- Grabinski TM, Kneynsberg A, Manfredsson FP, Kanaan NM. A method for combining RNAscope in situ hybridization with immunohistochemistry in thick free-floating brain

- sections and primary neuronal cultures. *PLoS One*. 2015;10(3):e0120120.
32. Stempel AJ, Morgans CW, Stout JT, Appukuttan B. Simultaneous visualization and cell-specific confirmation of RNA and protein in the mouse retina. *Mol Vis*. 2014;20:1366–1373.
 33. Collins ML, Irvine B, Tyner D, et al. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic Acids Res*. 1997;25(15):2979–2984.
 34. Player AN, Shen LP, Kenny D, Antao VP, Kolberg JA. Single-copy gene detection using branched DNA (bDNA) in situ hybridization. *J Histochem Cytochem*. 2001;49(5):603–612.
 35. Hackett TA, Clause AR, Takahata T, Hackett NJ, Polley DB. Differential maturation of vesicular glutamate and GABA transporter expression in the mouse auditory forebrain during the first weeks of hearing. *Brain Struct Funct*. 2016;221(5):2619–2673.
 36. Lee JH, Daugharthy ER, Scheiman J, et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. *Nat Protoc*. 2015;10(3):442–458.
 37. Fend F, Emmert-Buck MR, Chuaqui R, et al. Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am J Pathol*. 1999;154(1):61–66.
 38. Golubeva Y, Salcedo R, Mueller C, Liotta LA, Espina V. Laser capture microdissection for protein and NanoString RNA analysis. *Methods Mol Biol*. 2013;931:213–257.
 39. Mahalingam M. Laser capture microdissection: Insights into methods and applications. In: Murray GI, ed. *Laser Capture Microdissection: Methods and Protocols*. New York, NY: Springer; 2018:1–17.
 40. Murakami H, Liotta L, Star RA. IF-LCM: Laser capture microdissection of immunofluorescently defined cells for mRNA analysis rapid communication. *Kidney Int*. 2000;58(3):1346–1353.
 41. Xu BJ, Caprioli RM, Sanders ME, Jensen RA. Direct analysis of laser capture microdissected cells by MALDI mass spectrometry. *J Am Soc Mass Spectrom*. 2002;13(11):1292–1297.
 42. Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. *Science*. 1996;274(5289):998–1001.
 43. Amini P, Ettlin J, Opitz L, Clementi E, Malbon A, Markkanen E. An optimised protocol for isolation of RNA from small sections of laser-capture microdissected FFPE tissue amenable for next-generation sequencing. *BMC Mol Biol*. 2017;18(1):22.
 44. Curran S, Murray GI. An introduction to laser-based tissue microdissection techniques. In: Murray GI, Curran S, eds. *Laser Capture Microdissection: Methods and Protocols*. Totowa, NJ: Humana Press; 2005:3–7.
 45. Espina V, Heiby M, Pierobon M, Liotta LA. Laser capture microdissection technology. *Expert Rev Mol Diagn*. 2007;7(5):647–657.
 46. Schwamborn K. The importance of histology and pathology in mass spectrometry imaging. *Adv Cancer Res*. 2017;134:1–26.
 47. Van de Plas R, Yang J, Spraggins J, Caprioli RM. Image fusion of mass spectrometry and microscopy: A multimodality paradigm for molecular tissue mapping. *Nat Methods*. 2015;12(4):366–372.
 48. Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: Localization of peptides and proteins using MALDI-TOF MS. *Anal Chem*. 1997;69(23):4751–4760.
 49. Corbin BD, Seeley EH, Raab A, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science*. 2008;319(5865):962–965.
 50. Cornett DS, Frappier SL, Caprioli RM. MALDI-FTICR imaging mass spectrometry of drugs and metabolites in tissue. *Anal Chem*. 2008;80(14):5648–5653.
 51. Prentice BM, Chumbley CW, Caprioli RM. Absolute quantification of rifampicin by MALDI imaging mass spectrometry using multiple TOF/TOF events in a single laser shot. *J Am Soc Mass Spectrom*. 2017;28(1):136–144.
 52. Spraggins JM, Rizzo DG, Moore JL, et al. MALDI FTICR IMS of intact proteins: Using mass accuracy to link protein images with proteomics data. *J Am Soc Mass Spectrom*. 2015;26(6):974–985.
 53. Chandra S. Quantitative imaging of chemical composition in single cells by secondary ion mass spectrometry: Cisplatin affects calcium stores in renal epithelial cells. *Methods Mol Biol*. 2010;656:113–130.
 54. Lechene C, Hillion F, McMahon G, et al. High-resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry. *J Biol*. 2006;5(6):20.
 55. Gamble LJ, Anderton CR. Secondary ion mass spectrometry imaging of tissues, cells, and microbial systems. *Micros Today*. 2016;24(2):24–31.
 56. Passarelli MK, Winograd N. Lipid imaging with time-of-flight secondary ion mass spectrometry (ToF-SIMS). *Biochim Biophys Acta*. 2011;1811(11):976–990.
 57. Cornett DS, Scholle MD. Advances in MALDI mass spectrometry within drug discovery. *SLAS Discov*. 2017;22(10):1179–1181.
 58. Norris JL, Caprioli RM. Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. *Chem Rev*. 2013;113(4):2309–2342.
 59. Kompauer M, Heiles S, Spengler B. Atmospheric pressure MALDI mass spectrometry imaging of tissues and cells at 1.4- μm lateral resolution. *Nat Methods*. 2017;14(1):90–96.
 60. Zavalin A, Todd EM, Rawhouser PD, Yang J, Norris JL, Caprioli RM. Direct imaging of single cells and tissue at sub-cellular spatial resolution using transmission geometry MALDI MS. *J Mass Spectrom*. 2012;47(11):i.
 61. Zavalin A, Yang J, Hayden K, Vestal M, Caprioli RM. Tissue protein imaging at 1 μm laser spot diameter for high spatial resolution and high imaging speed using transmission geometry MALDI TOF MS. *Anal Bioanal Chem*. 2015;407(8):2337–2342.
 62. Takáts Z, Wiseman JM, Gologan B, Cooks RG. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science*. 2004;306(5695):471–473.
 63. Takáts Z, Wiseman JM, Cooks RG. Ambient mass spectrometry using desorption electrospray ionization (DESI): Instrumentation, mechanisms and applications in forensics, chemistry, and biology. *J Mass Spectrom*. 2005;40(10):1261–1275.
 64. Nguyen SN, Sontag RL, Carson JP, Corley RA, Ansong C, Laskin J. Towards high-resolution tissue imaging using nanospray desorption electrospray ionization mass spectrometry coupled to shear force microscopy. *J Am Soc Mass Spectrom*. 2017;29(2):316–322. doi:10.1007/s13361-017-1750-8.
 65. Baker TC, Han J, Borchers CH. Recent advancements in matrix-assisted laser desorption/ionization mass spectrometry imaging. *Curr Opin Biotechnol*. 2017;43:62–69.
 66. Cumeras R, Figueras E, Davis CE, Baumbach JI, Gràcia I. Review on ion mobility spectrometry. Part 1: current instrumentation. *Analyst*. 2015;140(5):1376–1390.
 67. Prentice BM, Chumbley CW, Caprioli RM. High-speed MALDI MS/MS imaging mass spectrometry using continuous raster sampling. *J Mass Spectrom*. 2015;50(4):703–710.

68. Spraggins JM, Rizzo DG, Moore JL, Noto MJ, Skaar EP, Caprioli RM. Next-generation technologies for spatial proteomics: Integrating ultra-high speed MALDI-TOF and high mass resolution MALDI FTICR imaging mass spectrometry for protein analysis. *Proteomics*. 2016;16(11–12):1678–1689.
69. Bemis KD, Harry A, Eberlin LS, et al. Cardinal: An R package for statistical analysis of mass spectrometry-based imaging experiments. *Bioinformatics*. 2015;31(14):2418–2420.
70. Bokhart MT, Nazari M, Garrard KP, Muddiman DC. MSiReader v1.0: Evolving open-source mass spectrometry imaging software for targeted and untargeted analyses. *J Am Soc Mass Spectrom*. 2018;29(1):8–16.
71. Thiele H, Heldmann S, Trede D, et al. 2D and 3D MALDI-imaging: Conceptual strategies for visualization and data mining. *Biochim Biophys Acta*. 2014;1844(1 Pt A):117–137.
72. Römpf A, Schramm T, Hester A, et al. imzML: Imaging Mass Spectrometry Markup Language: A common data format for mass spectrometry imaging. In: *Data Mining in Proteomics. Methods in Molecular Biology*. New York, NY: Humana Press. 2011;696:205–224.
73. Palmer A, Trede D, Alexandrov T. Where imaging mass spectrometry stands: Here are the numbers. *Metabolomics*. 2016;12(6):107.
74. O'Rourke MB, Padula MP. A new standard of visual data representation for imaging mass spectrometry. *Proteomics Clin Appl*. 2017;11(3–4). doi:10.1002/prca.201600098.
75. Giacomelli MG, Husvogt L, Vardeh H, Faulkner-Jones BE, Hornegger J, Connolly JL, and Fujimoto JG. Virtual hematoxylin and eosin transillumination microscopy using epifluorescence imaging. *PLoS One*. 2016;11(8):e0159337.
76. Yoshitake T, Giacomelli MG, Cahill LC, et al. Direct comparison between confocal and multiphoton microscopy for rapid histopathological evaluation of unfixed human breast tissue. *J Biomed Opt*. 2016;21(12):126021.
77. Boppart SA, Brown JQ, Farah CS, Kho E, Marcu L, Saunders CM, and Sterenborg HJCM. Label-free optical imaging technologies for rapid translation and use during intraoperative surgical and tumor margin assessment. *J Biomed Opt*. 2017;23(2):1–10.
78. Cahill LC, Giacomelli MG, Yoshitake T, et al. Rapid virtual hematoxylin and eosin histology of breast tissue specimens using a compact fluorescence nonlinear microscope. *Lab Invest*. 2018;98(1):150–160.
79. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods*. 2005;2(12):932–940.
80. Thomas G, van Voskuilen J, Truong H, Gerritsen HC, Sterenborg HJCM. In vivo nonlinear optical imaging to monitor early microscopic changes in a murine cutaneous squamous cell carcinoma model. *J Biophotonics*. 2015;8(8):668–680.
81. Yue S, Slipchenko MN, Cheng J-X. Multimodal nonlinear optical microscopy. *Laser Photon Rev*. 2011;5(4). doi:10.1002/lpor.201000027.
82. Tilbury K, Hocker J, Wen BL, Sandbo N, Singh V, Campagnola PJ. Second harmonic generation microscopy analysis of extracellular matrix changes in human idiopathic pulmonary fibrosis. *J Biomed Opt*. 2014;19(8):086014.
83. Tao YK, Shen D, Sheikine Y, et al. Assessment of breast pathologies using nonlinear microscopy. *Proc Natl Acad Sci USA*. 2014;111(43):15304–15309.
84. Campagnola P. Second harmonic generation imaging microscopy: Applications to diseases diagnostics. *Anal Chem*. 2011;83(9):3224–3231.
85. Lombardo M, Merino D, Loza-Alvarez P, Lombardo G. Translational label-free nonlinear imaging biomarkers to classify the human corneal microstructure. *Biomed Opt Express*. 2015;6(8):2803–2818.
86. Mahadevan-Jansen A, Richards-Kortum RR. Raman spectroscopy for the detection of cancers and precancers. *J Biomed Opt*. 1996;1(1):31–70.
87. Austin LA, Osseiran S, Evans CL. Raman technologies in cancer diagnostics. *Analyst*. 2016;141(2):476–503.
88. Cicerone MT, Camp CH. Histological coherent Raman imaging: A prognostic review. *Analyst*. 2017;143(1):33–59.
89. Pence I, Mahadevan-Jansen A. Clinical instrumentation and applications of Raman spectroscopy. *Chem Soc Rev*. 2016;45(7):1958–1979.
90. Bergholt MS, Serio A, McKenzie JS, et al. Correlated heterospectral lipidomics for biomolecular profiling of remyelination in multiple sclerosis. *ACS Cent Sci*. 2018;4(1):39–51.
91. Weinigel M, Breunig HG, Kellner-Höfer M, et al. In vivo histology: Optical biopsies with chemical contrast using clinical multiphoton/coherent anti-Stokes Raman scattering tomography. *Laser Phys Lett*. 2014;11(5):055601.
92. Ji M, Lewis S, Camelo-Piragua S, et al. Detection of human brain tumor infiltration with quantitative stimulated Raman scattering microscopy. *Sci Transl Med*. 2015;7(309):309ra163.
93. Cheng J-X, Xie XS. *Coherent Raman Scattering Microscopy*. Boca Raton, FL: CRC Press; 2016.
94. Schie IW, Krafft C, Popp J. Applications of coherent Raman scattering microscopies to clinical and biological studies. *Analyst*. 2015;140(12):3897–3909.
95. Zhang C, Zhang D, Cheng J-X. Coherent Raman scattering microscopy in biology and medicine. *Annu Rev Biomed Eng*. 2015;17:415–445.
96. Malek K, Wood BR, Bamberg KR. FTIR imaging of tissues: Techniques and methods of analysis. In: Baranska M, ed. *Optical Spectroscopy and Computational Methods in Biology and Medicine*. Dordrecht, Netherlands: Springer; 2014:419–473.
97. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Curr Protoc Mol Biol*. 2011;Chapter 25:Unit25B.10.
98. Veldman-Jones MH, Brant R, Rooney C, et al. Evaluating robustness and sensitivity of the nanostring technologies ncounter platform to enable multiplexed gene expression analysis of clinical samples. *Cancer Res*. 2015;75(13):2587–2593.
99. Fritscher-Ravens A, Schuppan D, Ellrichmann M, et al. Confocal endomicroscopy shows food-associated changes in the intestinal mucosa of patients with irritable bowel syndrome. *Gastroenterology*. 2014;147(5):1012–1020.e4.
100. Fuchs FS, Zirlik S, Hildner K, Schubert J, Vieth M, Neurath MF. Confocal laser endomicroscopy for diagnosing lung cancer in vivo. *Eur Respir J*. 2013;41(6):1401–1408.
101. Kang D, Schlachter SC, Carruth RW, et al. Large-area spectrally encoded confocal endomicroscopy of the human esophagus in vivo. *Lasers Surg Med*. 2017;49(3):233–239.
102. Lin B, Urayama S, Saroufeem RMG, Matthews DL, Demos SG. Real-time microscopic imaging of esophageal epithelial disease with autofluorescence under ultraviolet excitation. *Opt Express*. 2009;17(15):12502–12509.
103. Lin B, Urayama S, Saroufeem RMG, Matthews DL, Demos SG. Characterizing the origin of autofluorescence in human esophageal epithelium under ultraviolet excitation. *Opt Express*. 2010;18(20):21074–21082.
104. Fereidouni F, Mitra AD, Demos S, Levenson R. Microscopy with UV Surface Excitation (MUSE) for slide-free histology

- and pathology imaging. In: *Optical Biopsy XIII: Toward Real-Time Spectroscopic Imaging and Diagnosis*, Vol. 9318. International Society for Optics and Photonics; 2015: 93180F. <https://doi.org/10.1117/12.2080408>, accessed on February 26, 2018.
105. Fereidouni F, Harmany ZT, Tian M, et al. Microscopy with ultraviolet surface excitation for rapid slide-free histology. *Nat Biomed Eng.* 2017;1(12):957–966.
 106. Ho D, Fereidouni F, Levenson RM, Jagdeo J. Real-time, high-resolution, in vivo characterization of superficial skin with microscopy using ultraviolet surface excitation (MUSE). *J Drugs Dermatol.* 2016;15(11):1344–1346.
 107. Bucur O. Emerging technologies in diagnostic pathology. Available online (<https://www.discoveriesjournals.org/discoveries/D.2015.02.PA-Dr%20Bucur.pdf>), 2015; accessed on January 10, 2018.
 108. Louis DN, Virgin HW IV, Asa SL. “Next-generation” pathology and laboratory medicine. *Arch Pathol Lab Med.* 2011;135(12):1531–1532.
 109. 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.