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Recent developments in multiplexing techniques for immunohistochemistry

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Abstract

Methods to detect immuno-labelled molecules at increasingly higher resolution, even when present at low levels, are revolutionizing immunohistochemistry (IHC). These technologies can be valuable for management and examination of rare patient tissue specimens, and for improved accuracy of early disease detection. The purpose of this mini-review is to highlight recent multiplexing methods that are candidates for more prevalent use in clinical research and potential translation to the clinic. Multiplex IHC methods, which permit identification of at least 3 and up to 30 discrete antigens, have been divided into whole section staining and spatially-patterned staining categories. Associated signal enhancement technologies that can enhance performance and throughput of multiplex IHC assays are also discussed. Each multiplex IHC technique, detailed herein, is associated with several advantages as well as tradeoffs that must be taken into consideration for proper evaluation and use of the methods.

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Keywords

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Introduction

The development of methods for cell and tissue identification has had a long and colorful history. Virchow's early microscopic studies of specimens, poorly prepared by today's standards, focused the attention of "naturalists" and physicians on the cell as the basis of tissue structure in living organisms and the source of diseases such as cancer. Coon's discovery of the immunohistochemistry (IHC) technique permitted the identification, by fluorescent labeling of one or two antigens in a tissue [1–3]. A significant increase in the number of color labels simultaneously used to identify tissue antigens can be achieved with modern processes, such as "multi-epitope ligand cartography" that allows the colorful visualization of up to 18 labels of antigens on tissue[4]. Attempts are presently being made to develop even more comprehensive IHC-based technologies that allow simultaneous visualization of an even larger number of antigens from a single tissue section, as well as to streamline, automate, and reduce the time expended on tissue staining and processing. Multiplexing methods help achieve these technological goals to ultimately enhance disease diagnosis and better inform timely patient care.

Multiplexed IHC can be broadly defined as technologies used to identify the presence of multiple biological markers on a single tissue sample or an ensemble of different tissue samples. Histological examination of many clinically relevant tissues types, such as sections of tumors, often require identification of complex expression patterns of multiple biomarkers. Low-level multiplexing IHC methods, including one that uses up to 4 labels with 2 colors on a single tissue type [5], have been developed from relatively standard IHC techniques. Over time, several technological advances have led to high-level multiplexing IHC techniques that permit simultaneous visualization of a larger number of markers.

In this review, we will briefly provide an overview of standard IHC methods (detailed reviews have been published by Ramos-Vara [1] and Taylor et al. [6]), including low level multiplexing methods, such as 2-color, 4-label multiplexing, that have been executed with relatively conventional reagent-based techniques. Then, we will detail methods to achieve high-level multiplexing (three or more color labels) by either whole section staining or spatially-patterned treatment of tissue sections. These technologies make use of a host of IHC tools, such as antibody-antigen dissociation reagents, spectroscopy, immunoblotting, microfluidics, micropatterning, and fluorescence photobleaching. Finally, we will touch upon signal enhancement techniques and automated systems that are needed to bolster the image clarity and data acquisition of highly multiplexed samples. An overview of these high-level multiplexing methods and a list of challenges they overcome is shown in Figure 1.

Standard immunohistochemistry

In the early 1940s, Alex Coon discovered that fluorescent labels could be conjugated to antibodies that were specific to particular antigens on tissues, a method referred to as immunohistochemistry [1], [2], [3]. Soon after, antibodies were conjugated to chromogen-reactive enzymes and metals. There are advantages and disadvantages associated with each type of color-based probe, and the method by which the antibodies are introduced to tissue samples.

Staining methods are categorized as being either “simultaneous” or “sequential,” with respect to the method of antibody application or readout of labeled antigens. In simultaneous IHC, multiple antibodies are used to label multiple antigens in one staining process. By contrast, sequential IHC involves several iterations of labeling single antigens, each with a different label or secondary antibody type (i.e. IgM vs IgG) until all desired antigens are visualized. The antigen labeling process can either be a one-step process or a two-step process. In the one-step process, the direct method, a reporter-coupled antibody is used to bind the antigen of interest specifically. In the two-step process, the indirect method, an antigen-specific primary antibody is sandwiched between a reporter-coupled secondary antibody and the antigen [1]. When more than one fluorescent label is used, spectral cross-talk among multiple labels, cross-reactivity between antibodies, photo-bleaching and fading of the fluorescent dye, and inherent autofluorescence of the tissue are major technical issues. Fluorescent IHC is typically restricted to three labels [7], and commonly used to visualize two antigens and the cell nucleus.

The emergence of antibodies tagged with chromogen-reactive enzymes enabled the visualization of antibodies with the use of bright field optical microscopes, increasing the use of IHC methods for labs and hospital facilities not equipped with fluorescence microscopes. Enzyme tags can circumvent some of the spectral issues encountered with the use of fluorescence-based IHC. The process involves tissue fixation, antigen retrieval, blocking of non-specific antigen species, application of a primary antibody, followed by washes, application of a secondary antibody conjugated with an enzyme, additional washes, then incubation with substrate for deposition of colored precipitate [1]. Enzyme or heat-based antigen retrieval is used to reverse alteration of antigen structure due to a fixation step; the antigen retrieval methods vary, based on the type of antibody used[1]. Chromogen deposits can remain intact following some antibody elution processes, and thereby yield a permanent stain without the presence of antibodies. Multiple cycles of the staining procedure, using different antibody-antigen complexes and contrasting substrate colors, permit sequential labeling of multiple antigens. Following each staining cycle, the stained tissue is imaged, and all subsequent imaging steps require microscopic realignment of the same field of view on the tissue. Issues arise from cross-reactivity due to co-application of two antibodies, cross-reactions between incompletely eluted antibodies and newly applied antibodies used in separate staining cycles, as well as harsh chemical treatments that diminish structural integrity of antigens and the tissue itself. Also, overshadowing of light shades of chromogen substrates by darker shades or heavier deposits hinders the visualization of colocalized or cellular compartmentalized antigens [8].

In some cases, it may be desirable to label tissue specimens that can be visualized with higher resolution imaging systems such as an electron microscope. Metals and electron dense chromogens, such as diaminobenzidine (DAB), have a two-fold application for optical microscopy and electron microscopy [2]. Immunoelectron microscopy uses nanometer-sized colloidal gold labels to map and quantify subcellular localization and distribution of proteins at high resolution and precision [9],[10]. The top and bottom faces of a tissue slice can be stained with separate primary antibodies, when using a different sized gold particle for each face. This double-face staining method is advantageous for colocalization experiments when using primary antibodies that were raised in the same species[11].

2- 4 Plex staining for enhanced discrimination of diseased tissue

Even when using a single-color label for imaging, combining multiple types of antibodies together can enhance staining. For example, the simultaneous use of two antibodies that are specific for separate markers of leukemia, CD11b and CD11c, but conjugated with the same color fluorescent dye, more clearly demarcates leukemia-afflicted tissues. This double antibody staining for different cancer-specific antigens, combined with the use of time-domain imaging to minimize auto-fluorescence, significantly enhanced the signal-to-background ratio [12]. Because one cannot distinguish the relative positions or abundance of the antigen labeled by each antibody, this type of unicolor and multi-antibody method is not considered multiplexing for the purposes of this review.

In an example of two-color multiplexing, one color stain was used for methylation status visualization and another color for a cocktail of antibodies specific for a group of non-cancerous cell antigens. The methylation status was determined with antibodies specific for O6-Methylguanine-DNA methyltransferase (MGMT), whereas the non-cancerous tissue antibody cocktail contained CD34, CD45 and CD68, which are respective markers for endothelium, lymphocytes, and macrophages. Both non-tumorous and tumorous tissue can express MGMT, but the methylation state of cancer cells is what is important in cancer prognosis. With this double-staining method, cancer cells with MGMT could be clearly identified and distinguished from non-cancerous cells [13].

In an inverse two-color quadruplex staining procedure, two lung cancer cell types, adenocarcinoma and squamous cell carcinoma, were readily identified, with the use of just two different colors by incorporating an inverse color scheme for the cytoplasm and nucleus of each cancer cell type. The procedure used two mouse primary antibodies against Napsin A and p63 and two rabbit primary antibodies against thyroid transcription factor and CK14. With this scheme, the nuclei (thyroid transcription factor) and cytoplasm (Napsin A) of adenocarcinomas were respectively stained brown and blue. Conversely, squamous cell carcinomas were labeled with a blue nuclear stain for p63 and a brown cytoplasmic stain for CK14 [5]. By using brown for staining of the nucleus of the first type of cancer cell and the cytoplasm of the second type of tumor cells, and by using blue to stain for the cytoplasm of the first type of cancer cell and the nucleus of the second type of tumor cells, two colors could be used to effectively visualize four different histological features.

Whole section multiplex staining

In this section we will describe multiplexing systems in which a staining process is uniformly applied to a whole mount sample (Figure 2).

Sequential multiplex staining

Stain erasing—Based on early sequential staining techniques, researchers created several variations of “stain-erasing” methods to clear the tissue specimen of one label, treat the tissue with a new and different label, and repeat the process to identify multiple antigens. Multi-epitope ligand cartography (MELC) is one such technique, which uses photobleaching to erase cellular labels to ultimately allow the visualization of > 18 markers [4] (Figure 2A). A limitation of this procedure is that it can only be applied to one microscope field of view of the tissue, as opposed to an entire slide [8]. However, one could envision that high-throughput application of MELC could be achieved using computational software that allows tracked application of MELC over an entire tissue slice, until all labels in a designated tracking pattern are exposed. MELC may not be the technique of choice, however, for tissue samples that display a high level of autofluorescence [8].

Sequential immunoperoxidase labeling and erasing (SIMPLE) is another sequential label erasing technique. As implied by its name, the method involves the labeling of a sample with peroxidase-based indirect IHC and subsequent erasing of the alcohol soluble substrate, 3-amino-9-ethylcarbazole (AEC). Also, an acid-based antibody elution method is used following alcohol-mediated removal of the substrate. Following each labeling, a given precipitate is assigned a pseudocolor, and all colors are overlaid at the end of the process to visualize all antigens. The SIMPLE method offers the advantage of allowing visualization of protein co-localization. A maximum of five markers can be used, although increased multiplexing is believed to be precluded by diminished physical tissue integrity due to specimen handling and cycles of ethanol dehydration, rather than acid-based antibody elution [8].

Simultaneous multiplex staining

Layered expression scanning (LES)—Gannot and colleagues developed a versatile layered expression scanning (LES) system (Figure 2B), in which antigens from different formats of biological samples, such as frozen tissue, tissue microarrays, and southern blots, are transferred to several membrane layers stacked together, each of which are subsequently separated and used to measure a different unique analyte [14], [15]. In demonstration of technique, Chung et al. transferred antigens from one tissue microarray (TMA) to a membrane stack. Afterward, using standard singlex IHC on each membrane, individual membranes were screened for the presence of a collection of proteins, including keratin, PSA, p53 and p300 [16].

A variant of LES is the indirect layer peptide array (iLPA) method. In this method, the tissue sample is treated with multiple primary antibodies, each specific for a different antigen. These tissue-bound antibodies are then transferred to a stack of membrane layers, each impregnated with unique peptide antigens that the different primary antibodies bind to [17],

[18]. Primary antibodies are released from the tissue sample by using a heated sodium phosphate buffer solution that facilitates their upward transport, via capillary effect, through the membrane stack [18]. Each primary antibody binds to a different membrane layer containing its complementary target peptides, while maintaining its original x-y position in which it was bound to the tissue sample. The resulting peptide-antibody complexes in each membrane can be detected using standard secondary antibody-based methods [17], [18]. While requiring rather special membranes and multi-step procedures, the iLPA method has the advantage of allowing visualization of multiple different antigens using just one tissue section and using multiple antibodies from the same species as the primary antibodies [15].

Imaging Mass Spectrometry—Mass spectrometry-based tissue-imaging techniques can vaporize molecules from within specific regions of tissues into gas phase-ions, and then measure their mass. By iteratively scanning across the entire tissue section, an image of the molecules that initially resided in each region of a tissue, prior to vaporization, can be reconstructed.

Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy can identify the presence of multiple proteins, peptides and small molecules within biological tissues in an unbiased manner, meaning without having to pre-select antibodies or other detection-biasing reagents. MALDI uses an organic compound matrix that when combined with laser irradiation promotes efficient desorption and ionization of molecules from the surface [19]. A particularly useful application of this method is the MALDI imaging of small-molecule drugs co-registered with antibody-based staining of drug target proteins for the investigation of target-binding properties of administered drugs [20]. A combination of IHC and MALDI techniques was used to colocalize anticancer drugs, such as afatinib, erlotinib, sorafenib, within the blood vessel [21]. Limitations of the direct MALDI imaging of tissues are the relatively low sensitivity of the method and the inability to quantitatively compare signals from different antigen molecules to each other due to differences in ionization characteristics.

MALDI can be combined with antibody staining of tissues by the incorporation of mass tags onto the antibodies [22]. Mass tags are molecules that have the same chemical formula and structure but different masses due to the difference in incorporation of heavier or lighter isotopes of carbon, nitrogen, or other atoms. The mass tag molecules are designed to be highly detectable by mass spectrometry, significantly increasing sensitivity. Because the mass tags are chemically identical except for their mass, their MALDI ionization characteristics are all the same. Thus, differences in mass spectra peak sizes between mass tags are due only to differences in original abundance and not because of any differences in ionization properties [23]. This allows quantitative comparison of levels of different mass tag tagged antibodies by mass spectrometry. Presently, there are multiple types of mass tags allowing at least 10-plex tagging.

Another mass spectrometry-based IHC technique, originally developed to access the level of metal induced toxicity in the brain [24], is laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), which generates quantitative maps of a panel of metals and non-metal elements in tissue sections [25], [24], [26]. In LA-ICP-MS (Figure 2C), a laser

beam, generated in an argon enriched atmosphere, evaporates a cryo-cut sample at normal pressure to dislodge ions from the tissue that are subsequently measured by mass spectrometry.

LA-ICP-MS can also be used to image metals attached to antibodies. This type of LA-ICP-MS IHC was first applied with low-level multiplexing for the screening of two cancer biomarkers: human epidermal growth factor receptor 2 (HER2) and MUC-1 in tissue samples using gold- and silver-labeled antibodies [27]. However, the method needed improvements in terms of sensitivity, and speed of analysis for detection of the isotopes. Recent advancements in the field combine the time of flight ICP-MS instrument with IHC and immunocytochemistry (ICC), to detect rare earth metal isotopes tagged to antibodies in a specimen of interest, and this coupled system can image up to 32 proteins and their modifications with a cellular resolution of 1 μm [28]. Following data acquisition, the individual isotope imaging signals are stacked together to form a high-dimensional image. Single cell marker expression can be achieved with the use of a watershed algorithm for computational cell segmentation.

Drawbacks to using spectroscopy-based IHC are the relative instability of metal-labeled primary antibodies, which can cause alteration of the binding properties, difficulty of metal-probe conjugation to antibodies, an increase in analysis time and labor, high cost of the mass spectrometer, and the usual cross-reactivity and non-specific binding of antibodies and probes.

Spatially-patterned multiplexing for multiple tissue regions

In a different approach from whole section staining of one tissue sample with multiple differentially tagged antibodies, researchers have used engineering advances to localize different antibody reagent solutions to different regions of a single tissue sample or to array multiple different tissues onto a single slide. The spatially restricted exposure of a tissue sample to different reagents is motivated by both the limited availability of some types of tissue sections as well as the desire to use fewer expensive reagents. The use of TMA is motivated by the desire to test one staining condition on many different tissues simultaneously. Other techniques presented in this section (Fig 3) use microfluidic devices to localize reagent solution manipulation or generate a spatial array of tissue

Microarrays

TMAs are commonly used for high-throughput assays in histochemical studies that incorporate different tissues (Figure 3A). A TMA can include similar tissues from a large number of different patients or from different tissue regions. [29] TMAs are useful for rapidly validating potential candidate biomarkers across many tissue sections (for reviews see [30], [31], [32]). The origin of the TMA is credited to Batifora (1986), who first developed a multi-tumor (sausage) tissue block, in which a number of rectangular rods from different tissues were compiled into a non-uniform array and then embedded in wax. The resulting tissue block slices, with a mosaic-like arrangement of tissue, were used to assess the distribution of a specific protein/antigen. However, the technique lacked a viable data analysis protocol to link the studied tissue to the patient's identity [33]. Later mechanization

of tissue placement into well-defined arrays within wax blocks enhanced the high-throughput ability of the original method [29].

The modern TMA is comprised of an organized array of round tissue rods, or cores [34], where core sizes range from 0.6 to 2 mm in diameter [35]. Mechanized hollow core punches on a turret are used to create a bore in a “recipient” wax block, and then to transfer a bore from a “donor” tissue block to the recipient block; this process is repeated until a full array of tissue bores is generated [29], [36]. It is imperative to ascertain the ability to track and retain information about the locations of excised material, and therefore, tissue of known morphology can be positioned at the head of an array to serve as a location marker. The tissue block is sectioned into slices just as a regular paraffin block would be cut [37]. Even though the possibility of generating a TMA with up to 1000 tumor cores, each 0.6 mm in diameter, has been demonstrated [34], some researchers have found that TMAs containing an excess of 500 cores pose technical challenges associated with tracking and evaluation of each core sample [29]. During the TMA formation process, up to 15 % of tissue cores can be lost. Also, several factors contribute to the efficiency of TMA-based evaluations, including core size [38], inter-core spacing [34], and tissue type [39], [40].

Microfluidic methods

Microfluidic platforms have gained interest for their high-throughput tissue analysis applications as they minimize cross talk [41], [42], permit manipulation of small fluidic volumes, and reduce reagent costs and tissue sample used, which are all desirable capabilities in biological multiplexing [43], [44]. A majority of the microfluidic devices used for IHC studies consist of parallel arrays of open channels that are reversibly bound atop a tissue section of interest. Gating valves are used to inject marker-specific labeling reagents into marker-specific channels [45]. The versatility of such devices allows the simultaneous manipulation of staining reagents and microscopic visualization of stained regions (Figure 3B1).

Before explaining microfluidic multiplex IHC, we introduce a singleplex microfluidic IHC example to illustrate basic advantages of microfluidics in IHC. Ciftlik et al. designed a microfluidic system to deliver reagents uniformly over a tissue by using a series of bifurcating inlet and outlets channels to permit homogeneous flow throughout the microfluidic chamber [45]. In this IHC microfluidic device, a glass/silicon micro-machined structure is positioned and clamped atop a tissue sample. The device integrates check valves with a hardware molded poly(methyl methacrylate) (PMMA) structure that is mechanically clamped to a glass slide. This device allowed the tissue to be exposed to high hydrodynamic conditions (10 μ l/s). Fast fluidic exchange coupled with uniform reagent distribution allowed researchers to use ultra-short incubation periods (less than 2 min), focusing on the proportionality existing between fluorescence signal strength and HER2 biomarker concentration at the early antigen–antibody binding stage. The authors reported the device’s ability to screen early stages of the binding process was responsible for a significant reduction in ambiguous HER2 quantification cases (fewer than 4%) [45].

A representative of microfluidic multiplex IHC is a four-channel microarray device used to identify breast cancer in human breast tissue samples, with four biomarkers, estrogen

receptor (ER), progesterone receptor (PR), HER 2, and ki-67, with one channel designated for each biomarker [46]. In addition to the 10-fold decrease in assay time and reagent consumption, automated microfluidic-mediated labeling permits control over the reagent flow rate, which can be optimized to increase receptor-ligand binding. This microfluidic array design has been scaled up to permit analysis of 10 biomarkers (Figure 3B1) in a single experiment [47]. This group also compared an 8-array microfluidic staining method, to label four breast cancer markers with quantum dots, to conventional multiplexing methods that were either sequential, using secondary antibodies from the same species for each cycle, or simultaneous, using a cocktail of antibodies from dissimilar species in single cycle. By allowing the staining of eight tissue regions, the eight-array microfluidic method surpassed the performance of the sequential method, which suffered from cross-reactivity, and the simultaneous method, which was limited to the identification of only two-to-three biomarkers [48]. Despite the improved multiplexing capabilities of these arrays, the reduced size of the microfluidic channels as well as the heterogeneity of the tissues analyzed made the positioning of the reagent delivery channels over the tissue's region of interest difficult. To address this issue, the group pre-stained tissue slides with QD525 labeled antibodies specific for keratin in tumor regions. Afterwards the microfluidic array could be precisely positioned over tumor-enriched regions to allow screening for ER, PR, and HER 2 [49].

In a non-array microfluidic approach, Lovchik et al. developed a microfluidic probe (Figure 3C) allowing local delivery of nanoliters ($2\text{--}30\text{ nl s}^{-1}$) of primary antibodies to micro-sized regions (few mm^2) of tissue, significantly reducing cross reactivity between staining reagent pools [50]. In detail, the microfabricated microfluidic probe consists of microchannels etched in a diamond-shaped silicon wafer, which is subsequently and permanently bonded to a $500\text{-}\mu\text{m}$ thick glass wafer. The wafer is tapered to allow two microchannel ports for injection and suction of reagents. The two microchannel ports are connected to two syringe pumps for controlled application of reagents. A computer-controlled X-Y-Z stage allows positioning of the microfluidic probe over the tissue sample with a resolution of $0.1\text{ }\mu\text{m}$. The simultaneous coordinated pumping out and drawing up of fluid allows localized exposure of tissue sections to staining fluid without long-range diffusion of reagent. This microfluidic reagent delivery tool was integrated with a standard IHC protocol to screen for the presence of three targets (ER, PR and p53) in breast cancer tissue.

Another simple yet clever device, is the multiplex-immunostain chip (MI chip), pictured in Figure 3B2, in which a whole mount is clamped, tissue side down, on top of a circular 50-well array chip. Each well, 2 mm in diameter, holds separate antibodies, and inversion of the set-up delivers each antibody solution to a distinctly confined region of the tissue. This configuration reduces time, effort and the expense of IHC analysis through the use of a single slide and inexpensive materials [51].

Aqueous two-phase system

An aqueous two-phase system (ATPS) forms when two aqueous solutions, each enriched with different polymers, or each with either a polymer or a salt, are mixed at sufficiently high concentrations. The solubilized polymers and salts cause the solutions to be immiscible, much like oil and water. A binodal curve is used to delineate the concentrations

of each polymer and/or salt in solution that leads to the formation of a single or two-phase system.

Aqueous two-phase systems are frequently used in separation and purification techniques in industrial-scale biological applications [52], which are facilitated by the fact that an ATPS has low interfacial tension, thereby preserving the structure and function of biomolecules, and because biomolecules frequently preferentially partition to one phase or the other. A partition coefficient is characterized for biomolecules in a given system according to the following equation:

$$K = \frac{C_{top}}{C_{bottom}}$$

where K is the partition coefficient, C_{top} is the concentration in the top phase, and C_{bottom} is the concentration in the bottom phase. Molecules that strongly partition have $K \gg 1$ or $K \ll 1$, whereas molecules with $K=1$ are distributed homogeneously between the two systems.

Aside from traditional purification techniques, ATPS has also been used for a variety of biotechnological applications. For example, transfection reagents were selectively delivered to a monolayer of cells to create an array of cell islands expressing green fluorescent protein, whereas the surrounding cell monolayer remained un-transfected [53]. Cell patterning of individual or multiple cell types using PEG/DEX ATPSs has been accomplished in a variety of formats including pipetting [54], [55], [56], acoustics [57], and microfluidics [57]. It was also demonstrated that PEG/DEX ATPSs could be combined with picoliter dispensing systems to achieve single cell or subcellular delivery of proteases and other biomolecules [56].

More recently, ATPS has been used to generate multiplex antibody-based assays [58,59]. Importantly, many antibodies demonstrate strong partitioning to the dextran phase in an ATPS composed of polyethylene glycol and dextran ($K \ll 1$). Frampton et al. demonstrated ATPS-based multiplex staining of cell monolayers and tissues for ICC and IHC [60]. Using ATPS, three different primary antibodies were confined to their respective dextran droplets and delivered to monolayers of MCF7 and HeLa cells. It was shown that the cells could be labeled for cytokeratin 7, E-cadherin, and histone 2B in a concentration-dependent fashion and that labeling was localized only to the approximately 1 mm² areas in which the antibody-containing dextran droplets were localized. In a demonstration of the ATPS-based IHC technique's capabilities, dextran droplets enriched with antibodies for α -smooth muscle actin and rat IgG, were used to focally stain sections of deparaffinized sections of rat aorta. ATPS was used to label the tunica media with alpha smooth muscle actin-specific antibodies, while the tunica intima, tunica media, and tunica adventitia, as well as thromboses found in the lumen, were all labeled with rat IgG-specific antibodies. The stains were only visible in regions where dextran droplets, and therefore primary antibodies, were localized (Figure 3D).

Signal enhancement

Quantum dots

The use of quantum dots (Figure 4A) is an effective signal enhancement method, often applied to multiplexed IHC, that offers several advantages over traditional fluorescent proteins and organic dyes [61], [62]. These semiconductor nanocrystals are usually <10 nm in diameter and exhibit a narrow fluorescence emission spectrum [63], high photostability [64], and a symmetric emission spectrum [65]. One commonly reported disadvantage of quantum dots, however, is the limited number of nanocrystals that possess the proper chemistry to attach themselves to their targeted molecule. Ideal quantum dots, however, are conjugated to biomolecules through electrostatic binding or direct covalent crosslinking, with minimal need for prior chemical modifications [66], [64] or probe purification that can reduce conjugated antibody yield [64]. New conjugation schemes for attaching quantum dot nanocrystals to targeted antibodies continue to be developed [63]. Quantum dot-conjugated antibodies can be expensive and require specialized antibody buffers [66]. Quantum dots are ideal complements to microfluidic multiplex IHC, as demonstrated by use of quantum dots to enhance the microfluidic array-mediated IHC [67].

Silver enhancement

Due, in part, to their high electron density, gold colloidal probes are used extensively to visualize tissue with electron microscopy. Gold nanoparticles are able to penetrate deep into tissue to reach obstructed antigens, and cause less steric hindrance than larger particles, permitting labeling of several epitopes. However, gold particles, being difficult to visualize with light microscopy, can be increased in size through a silver enhancement process for enhanced visual clarity. Silver enhancement requires a gold particle (solid core or clusters of nanoparticles), silver ions, and a reducing agent. The surface of the gold is a nucleation site upon which reduced silver ions are deposited. Through successive deposition, the overall particle diameter increases and can be more easily visualized as a result of enhanced contrast (darker stain), than with the use of gold alone (Figure 4B) [68].

The silver-enhanced metals particles can be detected through several modes that include absorption of light, electron scattering, Raman spectroscopy, and fluorophore interaction. In particular, surface-enhanced Raman scattering (SERS) detection offers several advantages. Compared to traditional and quantum dot enhanced IHC, SERS detection holds a greater potential for multiplexing due to its minimal spectral overlap as a result of its extremely narrow spectral peaks (<2nm). SERS detection is also not susceptible to photobleaching. Furthermore, in some implementations, only target-bound SERS nanotags produce bright signals; unconjugated SERS nanotags do not bind to the biomarkers, and thus do not produce a signal [69]. Currently, multiplex detection of up to 10 commercial nanotags has been achieved in a mouse model [69]. SERS is highly specific [70], and careful selection of reducing agents can lead up to a 10^5 -times amplification of signal, increasing SERS potential in use of multiplexed IHC [71].

To maximize the functionality of enhancement labels, correlative microscopy can be achieved with the use of enzyme labels, fluorescent markers and metal probes so that tissue

specimen labels can be visualized with multiple types of microscopy, including fluorescence microscopy, light microscopy and electron microscopy. The deposition of metal from solution is typically catalyzed by redox enzymes such as horseradish peroxidase. In the instance of correlative fluorescent and electron microscopy, the two types of probes should be carefully selected to prevent quenching of a fluorescent signal by gold particles [68].

Tyramide signal amplification

Tyramide signal amplification is an enzyme catalyzed method of detection that increases labeling of antigens through the deposition of a high density of probe-conjugated tyramide molecules. Tyramides can be conjugated to biotin or fluorescent labels, and are substrates for horseradish peroxidase (Figure 4C). Horseradish peroxidase catalyzes the formation of tyramide into extremely reactive tyramide radicals that covalently bind to electron-rich tyrosine moieties on the tissue. As the half-life of the tyramide radicals is short, the binding of tyramide to tyrosine is restricted to tissue surfaces in close proximity. Tissue surfaces with anchored biotinylated tyramide, must be further treated with fluorescent or enzyme tagged proteins that have a high affinity for biotin (i.e. avidin, streptavidin) prior to microscopic visualization [1],[72], and detection sensitivity is increased by as much as 10-times compared to standard biotin-based staining methods.

Perkin Elmer developed Opal™, a sequential multiplexing technology that uses probe conjugated tyramides to identify several antigens on tissue. Following each round of antigen labeling, the primary and secondary antibody complex is eluted from the tissue. This “stripping” step prevents crosstalk between primary antibodies from the same species [73].

Rolling circle amplification

Rolling circle amplification (RCA, Figure 4D) is a powerful DNA replication based tool that offers high sensitivity and enables subcellular level analysis within tissue without increasing background noise [1,74]. The RCA method involves the conjugation of the 5' end of an oligonucleotide (OLN) primer to an antibody, and a circular single-strand DNA template is annealed to the OLN primer. In the presence of DNA polymerase and nucleotides, the primer extends at its 3' end to create multiple copies of the circular DNA [75,76]. The resulting amplicon is labeled through hybridization with fluorescently tagged OLN [74–76]. RCA differs from the polymerase chain reaction in that the DNA replication is carried out at a specific antigen site, and in isothermal conditions, linear or geometric kinetics can be used to amplify round OLN probes [74,75]. The exponential and linear mode RCA amplification is respectively able to generate a 109- and 105 fold signal amplification in a short period of time[74].

Polymer based amplification

Polymer based amplification systems make use of a polymer chain that is linked to antibodies and enzymes, substantially increasing the number of enzymes, and therefore chromogen product in the vicinity of specific antigen on tissue. While the polymer-based labeling is usually executed in a two-step indirect procedure (i.e. Envision++, Figure 4E), where the second step involves binding the enzyme-antibody polymer complex to the primary antibody, one-step and three-step formats are also available. The one-step

procedure, in which the polymer complex directly binds to the antigen, suffers from limited availability of antigen specific labels. The three-step procedure is used to increase detection sensitivity. Some users have experienced low detection sensitivity with polymer-based IHC, an occurrence attributed to the spatial hindrance from large molecular-weight-polymer molecules. To address this issue of spatial hindrance, compact polymer complexes have been developed to reduce spatial hindrance, allowing tissue to be more accessible for chromogen penetration [6]. As the polymer system omits the use of biotin, nonspecific background staining, resulting from endogenous biotin expression, is circumvented [1,6].

Data acquisition and image analysis

Regardless of the IHC multiplexing method applied, comprehensive evaluation of the associated tissue specimens necessitates not only clear demarcation of antigens of interest, which can be afforded by the use of signal enhancement technologies, but also expedited image acquisition, intricate image analysis, and sound interpretation of results. A major shortcoming of standard pathological assessments of tissues is inter- and intra-observer variation [77], [78]. Equally undesirable is the long duration of the assessment process, inability to distinguish colocalization of proteins [77], unreliable manual tracking and archiving of histological slides [78].

A large cohort of image analysis systems, which bridge slide scanning and image analysis functionalities, are increasingly being employed to tackle the aforementioned image analysis challenges associated with multiplexed IHC. Existing within that cohort are Caliper Vectra [77], Hamamatsu Nanozoomer [79], [78], [80]), Bacus TMAScore, Dako ACIS III, Genetix Ariol, Aperio Image Analysis, 3DHistech Mirax HistoQuant, Bioimagene Pathiam [81], all of which can scan slides affixed to whole tissue or TMA slices prior to image analysis. For these technologies, the slide scanning speed can be as quick as 20 slides/h [77]. Additionally, IHC imaging algorithms and applications associated with Dako ACIS III, Aperio Image Analysis, and Bioimagene Pathiam platforms, among others, have received US Food and Drug Administration (FDA) clearance as diagnostic tools. Breast tissue is screened, with such tools, for one or more of the receptors known to be elevated in breast cancer cells, HER2, ER and PR [81]. Also, increasing system versatility, images from either fluorescent or chromogen-labeled tissue specimens can be acquired with Genetix Ariol, Mirax HistoQuant [81], Caliper Vectra [77], and Hamamatsu Nanozoomer [79], [78], [80].

Stand-alone tissue analysis software used to evaluate virtual slides include Definiens TissueMap, HistoRx AQUA, SlidePath Tissue Image Analysis [81] and CRi Nuance [77], [82], to name a few. Several of these software packages, however, are also compatible and used in conjunction with the aforementioned histological technologies for a variety of geometric, morphological and proteometric attributes. Notably, these systems provide tumor recognition and spectral deconvolution for fluorophores and chromogens for expressed proteins [77], [78], [81]. These software features are enabled through screening based on parameters including but not limited to hue value/width, intensity threshold scoring, morphology/geometrical characterization, pixel-count thresholding, and color saturation.

An appreciable number of labels can be interpreted for highly multiplexed histological specimens. For example, Vectra was used to scan TMA slides containing breast epithelium stained with ER-fluorescein, PR-A594, Her2-Cy5, Ki67-Cy3, CK-Coumarin, and DAPI, and InForm software was used to assess images acquired from this 6-plex study [73]. Most integrated scanning and analysis instruments can evaluate between four and six labels, enabling higher-level multiplexing for multivariate biomarker analysis. However, image analysis of highly multiplexed histological assays that incorporate greater than six labels could require the assessment of specific biomarker sets in sequential units [78].

Nonetheless, these highly advanced systems greatly exceed the ability of human researchers to identify and then evaluate biomarkers, by reducing inter- and intra-observer variability error [77], and are therefore ideal for clinical use. Furthermore, some of these technologies integrate IHC analysis platforms, such as the Bacus TMA score, to autonomously perform immunohistochemical scoring. For a more comprehensive summary of benefits and specs for a majority of the aforementioned histological assessment instruments and software, please see the review by Rojo et al. [81].

Conclusion

Timely diagnosis of disease and accurate assessment of response to treatment based on IHC is hampered by many method-dependent factors, including lengthy and laborious staining procedures, limited patient tissue samples, inefficient tracking of patient data, unreliable and ambiguous staining results, and sluggish data acquisition and analysis systems. It is imperative to develop more highly reliable and efficient IHC systems to address this need. Highly multiplexed IHC methods could provide an efficient means to expedite disease diagnosis and research. Some of these IHC multiplexing methods, such as SIMPLE and MI chip, were spinoffs from earlier developed IHC methods, while others, like the newly emergent ATPS, are borrowed from other fields. All of the methods discussed here are included in Figures 5 & 6, and allow the reader to determine the benefits of each method and consider the suitability and usefulness of each method for a particular application.

Expert commentary

With only five FDA-approved IHC-based biomarkers for cancer, several major issues preclude the general acceptance of IHC-based results as true and reliable indicators of disease states [83]. One major issue is the lack of standardization among all IHC methods [2], [84], and the user-to-user error. Many research labs as well as hospital-based laboratories have developed unique protocols for tissue fixation, embedding, slicing and labeling, where fixation time, embedding techniques, tissue depth, and reagent concentration can all contribute to variability in analysis, especially without an appropriate control tissue specimen. Moreover, characterization of results requires assessment by highly trained expert pathologists, and variability even exists among their evaluations [2]. Whereas a majority of the advanced highly multiplexed IHC methods and technologies (particularly the commercially available instrumentation) do not address all of the aforementioned IHC issues, the use of these IHC-based methods does allow users at various training levels to

reduce reagent costs, shorten experimental time, and preserve limited patient samples, once standardization of the IHC methods for a given lab is in place.

Multi-color and multi-layer multiplex IHC methods can allow up to > 30 discrete labels to be visualized in less time than traditional IHC methods. An advantage of these methods is the ability to observe co-localization of different antigens across the entire tissue sample. A major challenge for use of these methods, particularly as the number of different antigens being analyzed simultaneously increases, is the problem of antibody cross-reactions and optical cross-talk. To overcome antibody cross-reactions, it is often necessary to perform an iterative procedure that involves single antibody application, signal generation, single antibody stripping, and sequence repetition, using a different antibody each cycle. As another means to confront the challenge of spectral cross-talk, users also rely upon expensive imaging hardware and software to respectively visualize and analyze multiple biomarkers.

Spatially-patterned multiplexing methods offer a facile means to survey a small sample area of tissue for a larger assortment of disease markers using just one readout color and just one layer of a tissue section. However, a downside to these techniques is susceptibility to errors caused by tissue heterogeneity. Because the stained region of interest for any particular antigen can be small, there exists a danger of missing rare events or inaccurately quantifying histological characteristics. Furthermore, spatially-patterned multiplexing does not allow for reliable identification of protein co-localization, as each unique marker is stained for in a different tissue region.

Researchers have developed some workarounds. For example, MI chip reduces errors from “intratumor” heterogeneity by delivering the same antibody to at least 2–3 randomly positioned 2-mm-diameter staining regions of an array [51]. Also, Gulmann et al. claim that the 0.6-mm-diameter TMA cores specimens show high correlation (90%) with whole mount tissue samples [29]. The use of microfluidics or ATPS micropatterning methods, although technologically exciting as new ways to perform IHC, are less common and require tools and reagents that are not as readily available as other methods. In sum, the user will need to carefully consider the suitability of each technique for the nature of the disease specimens to be investigated and the institution’s available resources, and also optimize the techniques to achieve the highest possible correlation with analysis of whole mount specimens.

Five-year view

More and more multiplex IHC tools and methods are expected to emerge from research labs towards the clinic. Increase in user-friendliness, robustness, and cost-efficiency of multiplex IHC, together with more clinical validation of specific multiplex IHC tests, are areas of challenges and opportunities. Some of the methods discussed separately provide attractive opportunities for integration in the near future. In this regard, composite approaches can combine multi-color labeling strategies with spatially-patterned IHC methods. Development of these new methods, and the ability to capitalize on them, will require multidisciplinary training of a diverse-skilled research team, with members arising from different fields. For clinical use, these technologically complex methods would not only have to be of high

quality, but also will require automation to provide important clinically actionable information in an efficient, simple and easily understandable format, for the clinical pathologist who may not be versed with the use of high-multiplexing methods.

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Key Issues

- No single biomarker or protein can specifically and sensitively reveal all relevant histological correlates to pathological states.
- Multiplex immunohistochemistry allows visualization of more than one antigen at a time, but can suffer from antibody cross-talk, signal overlap, and procedural complexities.
- Multiplex immunohistochemistry can reduce inter-experiment error compared to performing several singleplex assays.
- Use of multiplexing methods is critical when available tissue samples are minimal in number or in tissue area.
- Increasing levels of multiplexing demand higher skills and experience from users or development of new automated approaches.
- With colorimetric and fluorescent multiplexing techniques, methods to minimize autofluorescence and deconvolute spectral overlap become critical.
- With spatially-patterned multiplexing techniques, it is difficult to know whether different antigens are co-localized or not, and there is a larger chance that rare markers may be missed.
- User-friendly and cost-effective multiplexing systems that improve upon signal sensitivity and specificity while reducing batch-to-batch and user-to-user variability are needed.

Figure 1. High-level multiplexing IHC systems. Listed are whole-section and spatially-patterned multiplexing immunohistochemistry systems and the key procedural challenges they address. Whole-section and spatially-patterned images reproduced with permission [30], [8].

Figure 2.

Whole section multiplex staining systems for IHC. A) Multi-epitope ligand cartography (MELC) technology consists in automated cycles of fluorescent staining, imaging, and photobleaching. Each cycle can target a different protein to produce a set of thresholded images mapping the different proteins' distribution. The resulting image represents a combinatorial molecular phenotype for any desired protein. B) The indirect layer peptide array (iLPA) is one variant of the layer expression system that positions a stack of antigen-coated membranes atop the tissue sample to capture antibodies detached from the tissue, and each free antibody binds to a complementary antigen-specific membrane. The original x-y position of each antibody is maintained. C) Schematic of a protocol combining LA-ICP-MS with metal tagged antibody capture using CyTOF capabilities and IHC/ICC techniques for subcellular resolution screening of biomarkers in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissues. IHC: Immunohistochemistry; ICC: Immunocytochemistry; MELC: Multi-epitope ligand cartography; iLPA: Indirect layer peptide array; LA-ICP-MS: Laser ablation inductively coupled plasma mass spectrometry.

Figure 2A. Reproduced with permission from Nature Publishing Group [85].

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Figure 3.

Spatially-patterned multiplexing systems for IHC. A) Tissue microarray manufacturing and use. Tissue microarrays are constructed by transfer of tissue cores from a "donor" paraffin block (a) to a pre-punctured "recipient" paraffin block (b) using a tissue microarrayer. A microtome can be used to cut slices from tissue microarrays. An adhesive-tape transfer system can be applied to the tissue microarray to keep tissue intact during transfer to and while mounted on the slide (c & d). B) 1. Microfluidic platform for multiplex screening of 10 biomarkers using 10 independently run microfluidic channels to deliver reagent-loaded solutions on a cancer breast tissue sample. 2. Multiplex-immunostain chip (MI-chip) uses flipping function to deliver antibodies to discrete regions of tissue. C) Microfluidic microprobe is a vertical probe actuated across a breast cancer tissue to deliver reagents for multiplex screening of cancer biomarkers. D) Multiplex primary antibody patterning of a cell monolayer using ATPS of Dextran microdroplets submerged in a bulk solution of polyethylene glycol (PEG) (left). ATPS technique was applied to a dorsal root ganglion (DRG) explant of a chick embryo, where Dextran droplets containing tetramethylrhodamine (TRITC) were localized to DRG axons (top right) or fluorescein isothiocyanate (FITC)-wheat germ agglutinin delivered to non-labeled DRG axons (bottom right). Reproduced with permission [60] Copyright 2015.

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Figure 4.

Signal Enhancements for Immunohistochemistry (A) Quantum dots are conjugated to secondary antibodies to enhance fluorescent signal. (B) In silver enhancement, nucleation of silver (Ag) around a gold (Au) core enhances visibility of the gold particles. (C) In tyramide signal amplification, horseradish peroxidase catalyzes the deposition of biotinylated tyramine in proximity to the tagged antigen (D) ImmunoRCA, enables easy visualization of antigens via surface-anchored DNA replication. OLN = oligonucleotide. Ab = antibody. Detection with 1:25,600 antibody dilution (E) Polymer-Based signal amplification involves binding a polymer backbone that is conjugated with multiple antibodies and enzymes for higher contrast visualization. Permissions were obtained for microscopy images of stained cells and tissues (enclosed in shaded boxes) [86], [72], [87],[74], [88].

Microscopy images of stained cells and tissues (enclosed in shaded boxes) are reproduced with kind permission from Nature Publishing Group (A) [86], Springer Science and Business Media (B) [87], Methods, 18(4) Soontornniyomkij V, Tyramide signal

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	SIMULTANEOUS		SEQUENTIAL	
	Mass Spectroscopy	Layered Expression Scanning (LES)	Sequential IP Labeling and Erasing (SIMPLE)	Multi-Epitope-Ligand Cartography (MELC)

Max # Labels	32	20	5+	18+
Stain Area	Whole mount or TMA	Whole mount or TMA	Whole mount or TMA	Imaged Region
Benefits	<ul style="list-style-type: none">• Subcellular resolution	<ul style="list-style-type: none">• Simple single process generation of replicas	<ul style="list-style-type: none">• IP based, minimize autofluorescence	<ul style="list-style-type: none">• IF based, large number of labels
Drawbacks	<ul style="list-style-type: none">• Onerous equipment	<ul style="list-style-type: none">• Variability between layers	<ul style="list-style-type: none">• Diminished physical tissue integrity with successive cycles	<ul style="list-style-type: none">• Limited to imaging region of sample
References	[24], [28]	[17], [18]	[8]	[4]

Figure 5. Comparison of whole specimen multiplexing methods for immunohistochemistry. All figure permissions have been obtained as follows: *mass spectroscopy*, reproduced with permission from John Wiley and Sons [24], Clinica Chimica Acta, 376(1–2); *SIMPLE*, reprinted by Permission of SAGE Publications [8]; *MELC*, reproduced with permission from Nature Publishing Group [4].

	Tissue Microarray (TMA)	Microfluidic Channels	Multiplex-Immunostain chip (Mi chip)	Microfluidic Probe	Aqueous Two-Phase System (ATPS)
Max # Labels	15	10	50+	3	3+
Stain Area	~0.3 - 3 mm ² /spot (up to 1000 spots)	Channel w * l = [several 100 μm] * [several mm] (up to 10 channels)	~3 mm ² /well (up to 50 wells)	~0.01 - 1 mm ² /region (1 x 3 inch glass slide)	~1 mm ² /dextran drop (1 x 3 inch glass slide)
Benefits	<ul style="list-style-type: none"> Highly multiplexing with low cost and labor 	<ul style="list-style-type: none"> Reduced operating time and reagent volume 	<ul style="list-style-type: none"> Simple single staining process 	<ul style="list-style-type: none"> Non-contact method Adaptable to TMA setting 	<ul style="list-style-type: none"> Non-contact Simple Reduced reagent consumption
Drawbacks	<ul style="list-style-type: none"> Not suitable for clinical environment 	<ul style="list-style-type: none"> Limited resolution over a single tissue 	<ul style="list-style-type: none"> Special preparation required for small tissue specimens 	<ul style="list-style-type: none"> Tissue loss 	<ul style="list-style-type: none"> Loss in staining area
References	[29], [30]	[46], [47]	[51]	[50]	[60]

Figure 6. Comparison of spatially-patterned multiplexing methods for immunohistochemistry. dia = diameter; w = width; l = length

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