Chromogens in Multiple Immunohistochemical Staining Used for Visual Assessment and Spectral Imaging: The Colorful Future

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Abstract

For the chromogenic visualization of immunohistochemical enzymatic reaction products, only a limited series of different enzymatic activities and chromogens are available. Consequently, combinations of two chromogens for double immunohistochemical staining are even more limited for visual assessment. The recent introduction of spectral imaging allows unmixing of multiple immunohistochemically stained tissue samples for the exclusive observation of colocalization and quantitative measurement of individual staining components. Because unmixing is based on spectral characteristics, multiple immunohistochemistry is no longer dependent on chromogens showing a high visual contrast. Although this allows greater flexibility for combining chromogens in multiple staining applications, it also appears that some chromogens do not have excellent spectral and microscopic properties. The present work provides an overview of currently applied chromogens for single and multiple immunohistochemistry. (*The J Histotechnol* 33(1):31–40, 2010)

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Key words: chromogen, immunohistochemistry, multiple staining, spectral imaging

Introduction

During the last decades, the use of polyclonal and monoclonal antibodies in immunohistochemical (IHC) methods for *in situ* cell and tissue typing at the light microscopical level has been developed to be a powerful tool for both research and diagnostic purposes. The use of enzymatic chromogens for producing permanently stained specimens has been widespread among many biological sciences. In many cases, investigators need to demonstrate two or more antigens in one tissue specimen. The use of serial sections for this purpose is considered extremely laborious and ineffective for small cell types. Because the study of serial specimens often fails or has an inconclusive result, the desire for multiple antigen visualization in one tissue specimen is almost as old as IHC itself. In fact, when Nakane and Pierce (1) described their first immunoenzyme single-staining procedure, they mentioned the option of multiple staining. One year later, Nakane (2) did indeed publish a multiple-staining method, applying three indirect immunoperoxidase techniques sequentially by using three differently colored peroxidase reaction products.

The study of colocalization (the presence of two antigens in one cell) is one of the main reasons for the performance of double staining. When the two antigens are present in the same cellular compartment, colocalization is marked by a mixed color. When the two antigens are localized in different cellular compartments (nucleus–cytoplasm, nucleus–cell membrane, cytoplasm–cell membrane), even at the light microscopical level, colocalization is observed mostly as two separate colors. For proper observation of colocalization, reliable multiple staining techniques are required. For multiple staining, there are two major approaches: immunofluorescence or immunoenzyme staining. Although the accuracy of immunofluorescence is well established and beyond dispute, this work is focused on the use of immunoenzyme chromogenic staining techniques for multiple staining.

In general, any IHC double-staining technique is a combination of two individual antigen visualization methods. For a double IHC staining protocol, two main problems have to be overcome: (1) determining how to prevent cross-reaction(s) between both individual detection methods and (2) identifying the color combinations that provide the best contrast between both individual colors and a mixed color at sites of colocalization. Many investigators (3–5) have tried to find solutions to these problems, resulting in a variety of double-staining procedures, color combinations, and self-labeling of primary antibodies. This work focuses on the different chromogens applied for multiple IHC procedures, both for observation with the unaided eye as well as spectral imaging.

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Aims for Double Staining

Direct Overview of the Localization of Two Different Cell Types in the Tissue Specimen

Double staining with the use of primary antibodies known *a priori* to be present in two different cell types results in two different colors; colocalization is not present in this case. Double staining reveals a direct overview of antibody localization and distribution in the tissue, or spatial contacts between two cell types.

Double Staining for the Identification of Cells

For many antibodies, it is unclear what cell type is positively stained. For example, when observing a few positive nuclei with the proliferation marker Ki-67, it remains largely unknown what cell type is proliferating (6). In a double-staining experiment, the antibody of interest is combined with antibody-staining structural cell markers: epithelial cell types, lymphocyte subsets, plasma cells, granulocytes, eosinophils, macrophages, fibroblasts, (smooth) muscle cells, endothelial cells, nerve cells, etc. In the case of newly developed antibodies, investigators would like to investigate which cell type is the target of the new antibody and whether cell biological data fit with the suspected (sub)cellular localization. For example, using the double-staining technique, De Boer and coworkers (7) elucidated interleukin-17 + cell types in atherosclerosis and Yapici and coworkers (unpublished data) enumerated interleukin-17 + cell types in renal allograft acute rejection.

Search for Colocalization when Combining Cellular Markers with Activation Markers

For example, T-lymphocytes can be present as "resting" T-cells or in an immunologically activated state. Because most "activation markers" not only stain T-cells, but also many other cell types, double staining with the ability to show colocalization is an important tool (8,9).

Saving Tissue Sections or Cell Specimens

In diagnostic pathology, reference laboratories regularly receive a few blank tissue slides of difficult cases for additional study. Furthermore, the amount of cytological material is sometimes very limited and only sufficient for a few cytospin specimens. With a limited number of tissue specimens available, multiple staining allows the use of more markers per slide.

From Visual Assessment to Spectral Imaging

The aim of many double staining experiments is colocalization: visualization of two markers in one cell or tissue constituent. The overlapping staining patterns result in a mixed color when colocalization is found at the same subcellular level (membrane, cytoplasm, and nucleus). For this purpose, many investigators perform double immunoenzyme staining, with horseradish peroxidase (HRP) and alkaline phosphatase (AP) activities confirmed by red and blue reaction products (Table 1), respectively (10). Under ideal conditions, colocalization is clearly visualized by a purple mixed color that contrasts fairly well with both basic colors. However, this mixed color might be very subtle or even missed if one of the colors overwhelms the other. For example, when a T-cell marker is combined with an activation marker, the T-cell marker stains all cells nearly at the same staining intensity, whereas the staining intensity of the activation marker will be quite variable depending on the onset of activation. Furthermore, it is much easier to distinguish single- and double-stained cells when the cells are spatially separated, rather than in cell clusters.

Recently, spectral imaging has been introduced for microscopic analysis (11). This technique, which so far has been applied in space and military technology, has now fully entered the microscopy world. By the use of spectral imaging, two or more colored reaction products can be "unmixed" on the basis of the spectral characteristics of the individual reaction products and chromogens. This means that in cases in which a small amount of chromogen A is colocalizing with a large amount of chromogen B, chromogen A can still be observed (and quantified) after unmixing, whereas colocalization would have been missed when observed by the unaided eye. For spectral imaging, the Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation, Woburn, MA) was connected to the microscope with a standard 1× c-mount. Data sets acquired from 420–720 nm at 20-nm intervals contain 16 black-and-white images, meaning that a full spectrum of color is captured at each pixel of the image. Spectral

Table 1. Characteristics of IHC chromogen reaction products and histological counterstaining reagents for multiple staining procedures

Chromogen	*Enzyme*	*Vendor*	*Reaction product*	*Mounting type*[a]	*Spectral variation at different intensities*	*Heat-step resistance*
DAB	HRP		Brown	Org/aq	++	Yes
DAB+Ni/Co	HRP		Blue-black	Org/aq	++	No[b]
AEC	HRP		Brick-red	Aq	++	No
VIP	HRP	Vector	Purple	Org/aq	+	No
Nova Red[c]	HRP	Vector	Brick-red	Org	++	No
TMB	HRP		Blue-green	Org	+	No
Vector Blue[d]	AP	Vector	Blue	Org/aq	±	No
Vector Red[e]	AP	Vector	Red	Org/aq	±	Yes
NBT/BCIP	AP		Dark blue	Org/aq	++	No
X-gal	β-gal		Turquoise	Org/aq	±	Yes
Hematoxylin	–		Blue	Org/aq	-	No
Eosin	–		Pink	Org	-	No
Methyl green	–		Green	Org	-	No

[a] Org = organic using either xylene-containing media or xylene-free (VectaMount, Vector Laboratories, Burlingame, CA); aq = aqueous.
[b] After heat-step colored reaction product returns to brown.
[c] Identical characteristics with: LV Red (Thermo/Labvision, Fremont, CA), Romulin Red (BioCare, Concord, CA).
[d] Identical characteristics with: Ferangi Blue (BioCare), Permanent Blue (Diagnostic Biosystems, Pleasanton, CA), LV Blue (Thermo/Labvision).
[e] Identical characteristics with: Vulcan Red (BioCare), Permanent Red (Diagnostic Biosystems), Liquid Permanent Red (Dako, Carpinteria, CA).

libraries of all chromogens in Table 1 were created from single staining. Spectral data sets acquired from multiple staining specimens were unmixed by the Nuance 2.10 software, fitting the algorithms of the loaded spectral libraries of the involved chromogens with every pixel of the spectral data set. This spectral unmixing results in monochrome component images of all involved chromogens. These individual component images can be subjected to standard image analysis: total amount of signal, signal intensity, surface, etc. (11). The software also provides an unmixed composite image that is basically built up in layers from the composite images. Furthermore, when one uses the Nuance software, exclusive images of colocalization as well as fluorescent-like pseudo-colored images can be created (5,11). There is a rapidly growing list of applications that use spectral imaging for the analysis of multicolored bright field specimens (5,12–19).

Procedures

Relative Sensitivity of Chromogens

For single-staining purposes, a primary antibody must be well titrated for optimal performance. This is usually done by testing dilution series in combination with different tissue pretreatments, applying a fixed detection system plus a chromogen system (20). Consequently, when that particular primary antibody is used in multiple IHC staining, it needs to be stained, perhaps by the use of another enzyme and chromogen. However, like IHC detection systems, the labeling enzymes and chromogens have their own sensitivity/efficiency characteristics (4,21). On the basis of many comparison tests and experience, Table 2 shows the relative sensitivity/efficiency of the chromogens mentioned in Table 1. Validation of primary antibodies for multiple IHC procedures is highlighted in Table 3.

Table 2. Relative sensitivity/efficiency of chromogen visualization systems

			AP (Vector Blue)	**AP** (Vector Red)		
HRP (TMB?)	**HRP** (NovaRed)	**HRP** (DAB+)	**HRP** (DAB)		**AP** (Fast Blue BB)	**β-gal** (X-gal)
AP (NBT/BCIP)		**HRP** (VIP)	**HRP** (AEC)			
High →						**Low**
			Primary antibody dilution			
1:500	1:200	1:100		1:50	1:20	

Table 3. Validation of primary antibodies in IHC triple staining

Make the following selections:

1. Double staining method. This selection is fully based on characteristics of the primary antibody (4,5).
2. Appropriate detection systems for each primary. NB: check for potential cross-reactions between the applied detection systems!
3. Color combination, which includes appropriate marker enzymes and substrates/chromogens.

Then, perform the following tests:

1. Primary antibody 1: dilution series using detection system 1 and chromogen 1
   Primary antibody 2: dilution series using detection system 2 and chromogen 2
   Primary antibody 3: dilution series using detection system 3 and chromogen 3
2. Select optimal dilutions for the given chromogens. When working with spectral imaging, these slides may serve later for acquiring pure spectra for the spectral library.
3. Perform 'half triple' staining procedures:
   primary antibody 1: detection system 1 and chromogen 1
   blank: detection system 2 and chromogen 2
   blank: detection system 3 and chromogen 3

   blank: detection system 1 and chromogen 1
   primary antibody 2: detection system 2 and chromogen 2
   blank: detection system 3 and chromogen 3

   blank: detection system 1 and chromogen 1
   blank: detection system 2 and chromogen 2
   primary antibody 3: detection system 3 and chromogen 3

   NB: only pure chromogen should be visible in these "half triple" staining slides! If not, cross-reaction among the individual detection systems is probably occurring.
4. Perform full triple staining procedure:
   primary antibody 1: optimal dilution using detection system 1 and chromogen 1
   primary antibody 2: optimal dilution using detection system 2 and chromogen 2
   primary antibody 3: optimal dilution using detection system 3 and chromogen 3

Best Chromogen Combinations for Traditional Visual Assessment with the Unaided Eye

When microscopically observing double-staining results with the unaided eye or by traditional RGB imaging, the chromogen combination is critical because visual contrast is the key requirement. Especially when the observation of colocalization is the main target, there must be an optimal contrast between the two basic colors as well as the mixed component. In the history of IHC, many different chromogen combinations for double staining have been proposed (4). However, only a few have proven to be suitable for direct visual observation of both the individual chromogens and a mixed color at sites of colocalization.

Red–Blue (from HRP and AP)

Initially, the red–blue color combination is composed of AP activity in blue by the use of Fast Blue BB/Napthhol-AS-MX-phosphate and HRP activity in red with 3-amino-9-ethylcarbazole (AEC) (10). Both reaction products dissolve in organic mounting media and therefore need aqueous mounting. Alternatively, two commercially available chromogens from Vector Laboratories (Burlingame, CA) can be applied: Vector Blue and Vector NovaRed, respectively (22), which can be mounted organically (VectaMount). A red–blue chromogen combination is also applied in the Thermo/Labvision Multivision™ kit, containing a cocktail of polymers, anti-rabbit (HRP or AP conjugated) plus anti-mouse (HRP or AP conjugated), and all chromogen reagents.

A counterstain coupled with the red–blue color combination must be tested per antibody combination. The most obvious choice would be methyl green (0.1% in acetate buffer pH 5.5), which yields weakly green nuclei. However, from personal observation, it appears that methyl green binds to the red reaction product, shifting its color to brown. As such, this lowers the color contrast and hampers the observation of colocalization by mixed colors. In some instances, a weak eosin counterstain (dip in eosin solution for 30 s, rinse in running tap water, and check microscopically until an optimal result is obtained) or a weak Nuclear Red counterstain may suffice and give a faint image of unstained tissue elements. Even a weak hematoxylin counterstain still contrasts relatively well with the blue AP-reaction product (personal observation). Visualizing AP activity with the use of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) is not recommended. NBT/BCIP yields a dark blue-purple reaction product that hardly allows the observation of a mixed color (personal observation).

Turquoise–Red (from β-gal and AP)

The turquoise–red color combination is composed of beta-galactosidase (β-gal) activity in turquoise and AP activity in red (3). At present, the β-gal marker enzyme is only available as a streptavidin-conjugate (Roche, Mannheim, Germany). The β-gal activity is visualized by the use of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) with ferro-ferri iron cyanide salts (23). The turquoise reaction product is very stable and does not dissolve in alcohols or other organic liquids (3,24). AP activity can be visualized in red using Fast Red TR or Fast Red Violet LB/Naphthol-AS-MX-phosphate (4,10) or other commercially available red AP visualization methods like Vector Red (Vector Laboratories), Liquid Permanent Red (LPRed, Dako), or Permanent Red (Diagnostic Biosystems). The red AP reaction product also allows organic mounting after skipping the dehydration in alcohols (specimens can be dried on a hot plate and coverslipped organically). A weak blue hematoxylin counterstain contrasts fairly well with the basic colors but is nearly identical in color with the blue-purple intermediate color at sites of colocalization. The problem with the turquoise–red combination is β-gal activity being certainly far less sensitive/efficient and more diffusely localized compared with red HRP or AP reaction products (3,24). For this reason, β-gal activity can be used only for those primaries that show an intense cytoplasmic staining at relatively high antibody dilutions, such as smooth muscle α-actin (clone 1A4).

Alternatively, a red–green combination can be obtained from green HRP activity visualized with 3,3′,5,5′-tetramethylbenzidine (TMB)/dioctyl sodium sulphosuccinate (25) and red AP activity (26). The blue–green reaction product is accurate and very sensitive/efficient; however, it is not stable after aqueous mounting (27). Because of the extreme sensitivity/efficiency of these chromogens, careful titration of primary and secondary reagents is strongly recommended. Generally, TMB-based visualization of HRP activity, including the commercially available chromogens TrueBlue (Kirkegaard and Perry, Gaithersburg, MD) and Vector TMB, are considered "difficult" by many users and seem not to work well with every antibody in each situation. A scientific explanation for these inconsistent and variable results is lacking thus far.

Red–Blue (from Sequential Double AP)

Recently, a new sequential double AP method was introduced (16) that combines two AP chromogens: Vector Blue and Vector Red. This sequential double-staining method is no longer dependent on animal species or mouse antibody isotypes/subclasses, and therefore allows mouse-mouse and rabbit-rabbit combinations of antibodies. This double-staining method can also be performed on human and animal tissues, provided that two noncrossreacting AP detection methods are available for two antigens. The red and blue reaction products can be readily distinguished from each other and the purple intermediate mixed-color by the unaided eye (16). A faint nuclear counterstain with 0.1% methyl green is appropriate and does not bind to either of the AP chromogens (16), as noted previously for Vector NovaRed. Theoretically, this sequential double-staining procedure with the intervening heat step can be applied with any chromogen combination, given of course that the first chromogen survives the heat step (Table 1).

All color combinations discussed so far share the drawback of containing at least one diffusely localized chromogen: Fast Blue BB, Vector Blue in both red–blue combinations, and X-gal in the turquoise–red combination. This drawback may compromise a crisp observation, with antibodies revealing small cellular constituents like dendrites, membranes, fibrils, etc.

Red–Brown (from AP and HRP)

The red–brown color combination is a result of HRP activity, with brown 3,3′ diamino benzidine (DAB) and a red AP reaction product. This chromogen combination ensures two sensitive/efficient enzymatic visualization procedures, each with a crisp microscopical appearance. A blue nuclear counterstain with hematoxylin is optional. For observation

with the unaided eye, this color combination has been applied for decades by many investigators (2,28) and is still used in several commercially available double-staining kit systems today (BioCare, Dako). Visualization of colocalization is not possible with this color combination because the red–brown mixed color cannot be distinguished from single-red or single-brown (4,10). Therefore, by visual assessment, the red–brown color combination is only useful for demonstrating two different cell populations or constituents without any colocalization. As an example, for prostate carcinoma diagnosis, initially a triple-staining cocktail (BioCare), was proposed composed of high molecular weight keratins (CK5 + CK14), p63 and AMACR (p504s), also known as the PIN-4 cocktail. Because these markers stain different tissue and cell compartments, all three markers were visualized with DAB in brown. In fact, this full brown triple staining is still used for diagnostic purposes. Today, the PIN-4 cocktail is developed in red (P504S) and both other markers in brown. The staining results readily reveal, even with the unaided eye, the areas with tumor cells expressing P504S (Figure 1). Spectral imaging is not needed in this case.

Best Chromogen Combinations for Assessment with Spectral Imaging

Because a spectral imaging system has the ability to unmix chromogens that are visually nearly indistinguishable, the visual color of a reaction product is theoretically no longer important. As long as there are no chemical interactions between the subsequent chromogen steps, and individual spectra are available from single staining for building a spectral library, multiple staining specimens can be unmixed into individual component images. The power of spectral imaging is demonstrated by unmixing three red chromogens: LPRed (AP), AEC (HRP), and Nuclear Red counterstain (Figure 2).

As emphasized previously in this work, the visual contrast between DAB and Vector Red after double IHC staining is poor, and the observation of colocalization by a mixed color is nearly impossible with the unaided eye. However, these exquisitely crisp enzymatic reaction products serve as a basis for analysis of multiple staining by spectral imaging (9,16,22). An important issue with chromogens for spectral imaging is that most HRP reaction products have the tendency to produce different spectra at different staining intensities (Table 1). Consequently, the Beer-Lambert law indicating a linear relationship between the optical density and concentration is no longer valid. AP chromogens like Vector Blue and Vector Red (16) hardly share this problem, making these AP chromogens very suitable for quantitation (5). From personal observation it is recommended that, when working with specimens to be analyzed by spectral imaging, all chromogens involved need to be only moderately to weakly stained, rather than strongly stained, to appear dark and intense. It is observed that, at high staining intensities, HRP-chromogens like DAB, NovaRed, and AEC, yield very dark, almost black, reaction products that cannot be subjected to spectral imaging (5).

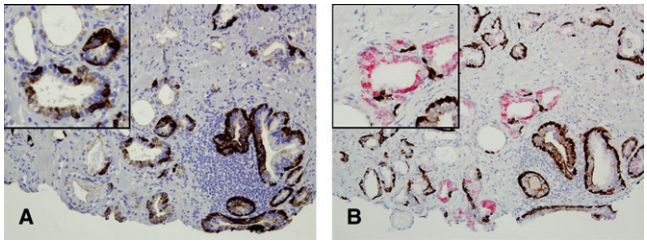

Figure 1. Semi serial formalin-fixed, paraffin-embedded (FFPE) tissue sections of a human prostate biopsy showing normal ducts and tumor after triple DAB PIN-4 cocktail IHC staining showing high-molecular-weight keratin (cytoplasmic, normal ducts), p63 (nuclear, normal ducts), and p504s (cytoplasmic, tumor) and a nuclear counterstain with hematoxylin (A). The same combination of antibodies, but staining p504s in red (AP, Vector Red), gives a much clearer overview of normal and tumor tissue elements (B).

Chromogen Combinations for Triple Staining and Beyond

In IHC literature, one can find only a few attempts to use triple IHC staining methods. Apart from the complexity of designing a staining method without cross-reactions among the individual detection steps, the visual observation can be difficult. Triple staining works best for the visualization of three different tissue elements without colocalization, for example, combining HRP-brown, AP-red, and β-gal-turquoise (4). This color combination has been used, for example, to demonstrate *Helicobacter pylori* in combination with two different mucins (29). By the use of a sequential approach with a second HIER step for removing antibodies from the first staining sequence, but leaving the colored reaction product undamaged (16), triple staining can be easily composed, for example: AP-blue–HIER step–AP-red, HRP-brown. As emphasized previously, color mixing of DAB-brown with either AP-red or AP-blue chromogens does not allow the observation of colocalization because of the lack of a distinct mixed-colored reaction product. Therefore, when it comes to the observation of mixed colors in multiple IHC-stained specimens, it is obvious that visual assessment is close to impossible. The application of spectral unmixing is an absolute necessity in this case.

Figure 3 shows IHC triple staining, combining three different macrophage markers tested on an advanced type of atherosclerotic plaque: CD68 (pan macrophage), CD14 (macrophages/monocytes), and CD163 (scavenger receptor on macrophage subset). When observing the RGB image of this triple staining (Figure 3A), it is clear from the different mixed-colors that different colocalizations and probably also triple-localizations are present. Quantitation of the different subsets and colocalizations with the unaided eye, however, is fully impossible. After spectral unmixing into the three different component images (Figure 3D–F) the measurement tool in the Nuance software allows one to draw an area of interest (Figure 3G) in which the average optical density can be calculated for each component image. This allows a comparison of staining intensity of these three different macrophage markers. Using the colocalization tool from the Nuance software, the percentage of different macrophages subsets, including colocalizations and triple-localizations, can be simply calculated.

When expanding from triple to quadruple IHC, a fourth chromogen needs to be included. Quadruple IHC is composed of two double staining procedures with a HIER step in between, for example: AP-blue–HRP-brown–HIER step–AP-red–HRP-purple (5) or AP-blue–HRP-brown–HIER step–AP-red–β-gal-turquoise. The quadruple IHC in Figure 4 follows this latter approach and demonstrates that quadruple IHC can be reliably performed, can be spectrally unmixed in

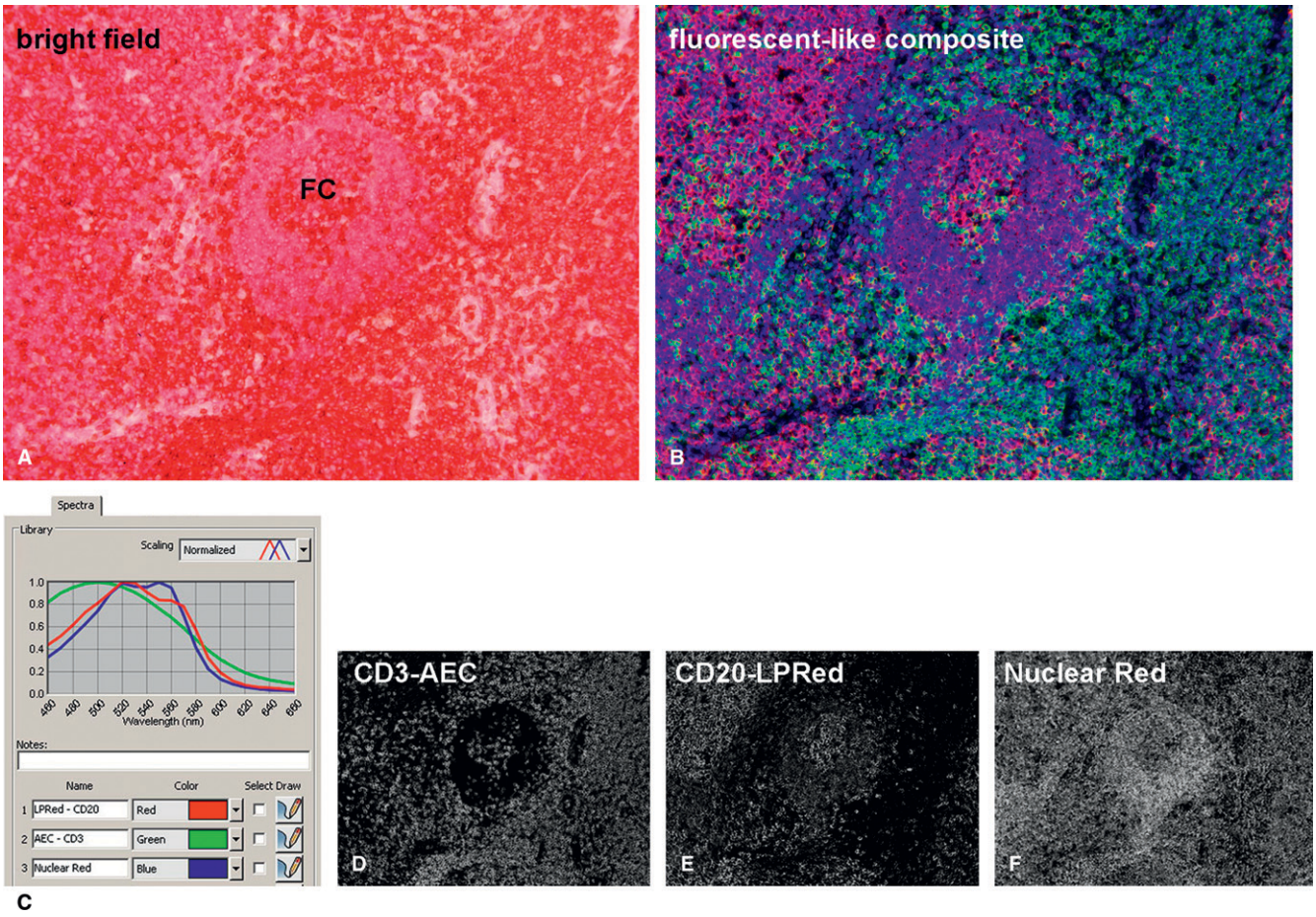

Figure 2. Detail of human tonsil FFPE tissue section showing a follicle center (FC) with surrounding T-cell areas stained with three red chromogens. T cells are marked with CD3 in red (HRP, AEC), B cells with CD20 in red (AP, Liquid Permanent Red), and a nuclear counterstain with Nuclear Red (A). After spectral unmixing using the spectral library (C), the unmixed composite (B) is a false-color, fluorescent-like image, showing high contrast among all components: CD3 in green, CD20 in red, and Nuclear Red in blue. This unmixed composite is built up (by the Nuance software) from the three unmixed composite images D, E, and F. The individual unmixed component black-and-white images (D–F) are TIF images that can be applied for all standard imaging purposes. Note the similar spectra of Liquid Permanent Red (red line), AEC (green line) and Nuclear Red (blue line) with maximum absorbance at 500–560 nm that still show good unmixing in panels D–F.

four component images, and that two different types of colocalization can be imaged and quantitated.

Figures 2–4 clearly illustrate that an objective analysis of IHC multiple staining is no longer a "magician's act." IHC multiple staining combined with spectral imaging forms a strong basis for multimarker tissue analysis. This capability approximates the results of fluoresence-activated cell sorting (FACS) analysis but with the obvious addition of tissue morphology.

Tips and Tricks for Adapting Single IHC Staining to Multiple IHC Staining

- The decision for multiple IHC staining usually comes after the examination of single IHC-stained slides. That means that the single-stained slides can be used later as references. Of course, the multiple-staining result per primary antibody should be highly comparable to the original single-stained slides.
- As emphasized previously, for multiple IHC staining, a moderate staining intensity is definitely preferred over intense staining. This can be accomplished by using one or two steps higher dilution—as compared with the original single staining—of the primary antibodies involved.
- Be aware that longer HIER may intensify the staining intensity of many primary antibodies. That means that primary antibodies involved in sequential multiple staining after the second HIER step might stain more strongly than usual.
- To validate primary antibodies for a multiple IHC procedure (Table 3), retitrate the primary antibodies involved with the detection systems applied in the multiple IHC staining protocol of choice, including the chromogens of choice. The dilution of the primary antibodies used in single IHC staining can be used as a starting point. Table 2 shows whether the primary antibody is expected to be either lower or higher dilution, depending on the sensitivity/efficiency of the chromogens used in multiple staining. Do not apply a hematoxylin counterstain. Check out the individual staining intensities and select the best dilutions for all primary antibodies. These single-stained slides can be used later for acquiring the individual spectra of the chromogens involved, thus building the spectral

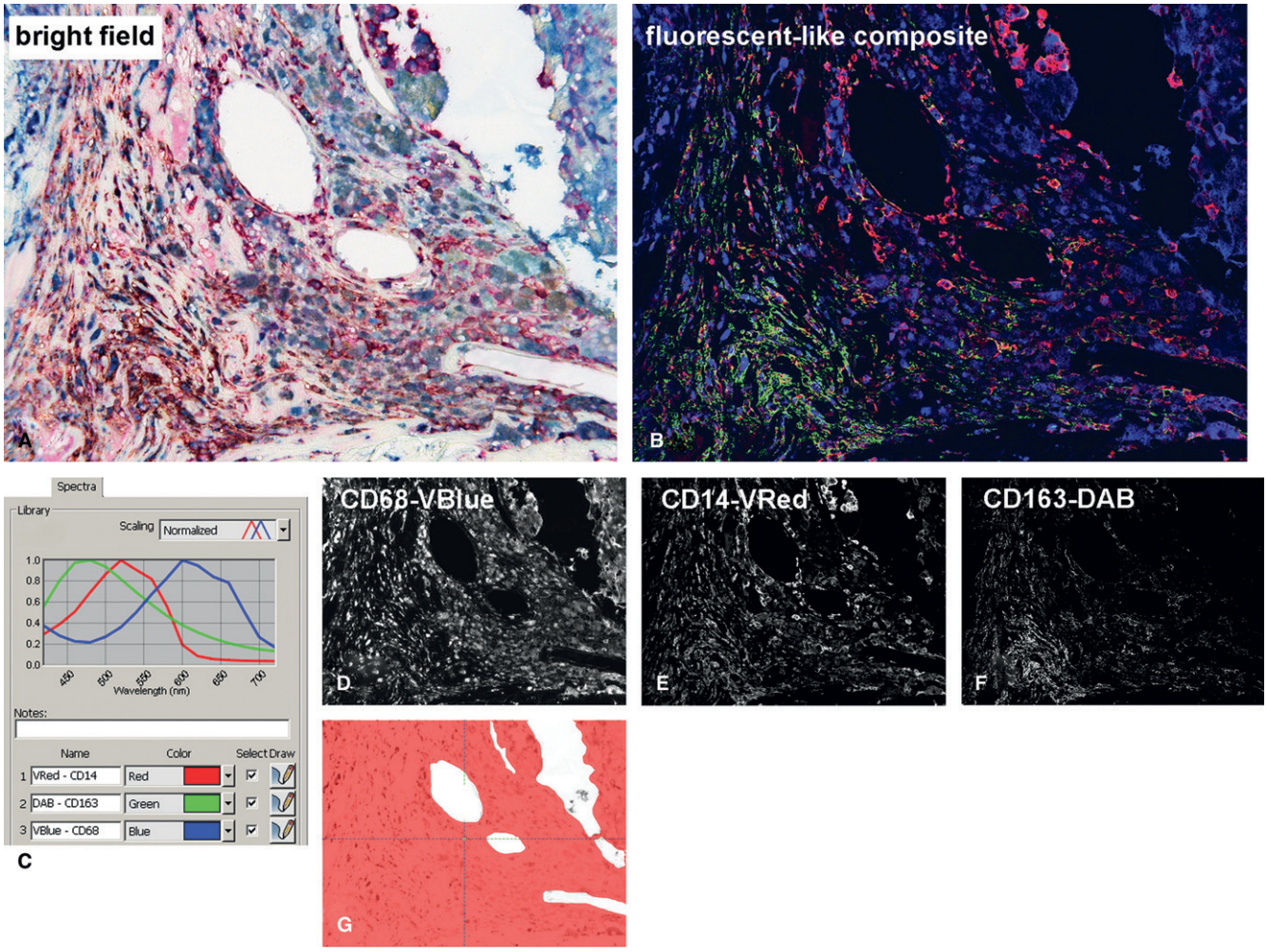

Figure 3. Detail of FFPE tissue section from an atherosclerotic plaque in human carotid artery showing IHC triple staining combining three macrophage markers: CD68 (pan macrophage) in blue (AP, Vector Blue), CD14 (macrophage/monocyte) in red (AP, Vector Red), and CD163 (scavenger receptor) in brown (HRP, DAB). With spectral unmixing using the spectral library (C) the unmixed black-and-white composite images (D–F) and unmixed composite (B) are created. The composite image (B) is fluorescent-like with false-colors for the three components: CD163 in green, CD14 in red and CD68 in blue. The red mask in G is a selection of tissue only (all vessel lumen are subtracted) that is applied to D–F for quantitation of all three components as well as enumeration of colocalizations and triple-localizations as shown in Table 4.

library. Avoid short visualization times (less than 5 min) by using higher dilutions of the primary antibody.

- Next, perform the full multiple staining protocol with only one primary involved and the others replaced by buffer. If this test yields acceptable results, perform the full multiple staining protocol.
- Using the optimal dilutions for the primary antibodies, perform two full multiple IHC experiments with all primaries involved, one with and one without hematoxylin counterstain. For a nuclear counterstain, use a 1:10–20 diluted hematoxylin solution (in tap water), thus ensuring weak to moderate staining intensity of the nuclei.
- When spectral imaging is involved, a good alternative for counterstaining is applying a 1:5 diluted (in tap water) eosin solution (1 min). The weak overall pink staining unmixes very well from all other enzymatic reaction products. It may serve to quantitate the actual tissue surface to avoid inclusion of empty areas, as in lung for example (18).
- For multiple IHC staining novices: do not handle too many slides or more than two antibody combinations in one experiment. Upon enzymatic activity visualization, one should be able to monitor the color development under the microscope at low magnification.
- Before the actual performance, draw a scheme of the planned multiple IHC staining and check carefully step-by-step if unwanted cross-reactions between the reagents may occur.
- Avoid the use of DAB as chromogen when brown tissue pigments are present, such as melanin, lipofuchsin, ceroid, and formalin pigment (occurring in bloody areas after using unbuffered formalin). The brown DAB reaction product and the brown tissue pigments are all scatterers of light, and they do not unmix well from DAB with spectral imaging.
- AP activity is strongly inhibited by phosphate ions from phosphate-buffered saline washing buffer. Use TBS throughout as a washing buffer, or if phosphate-buffered

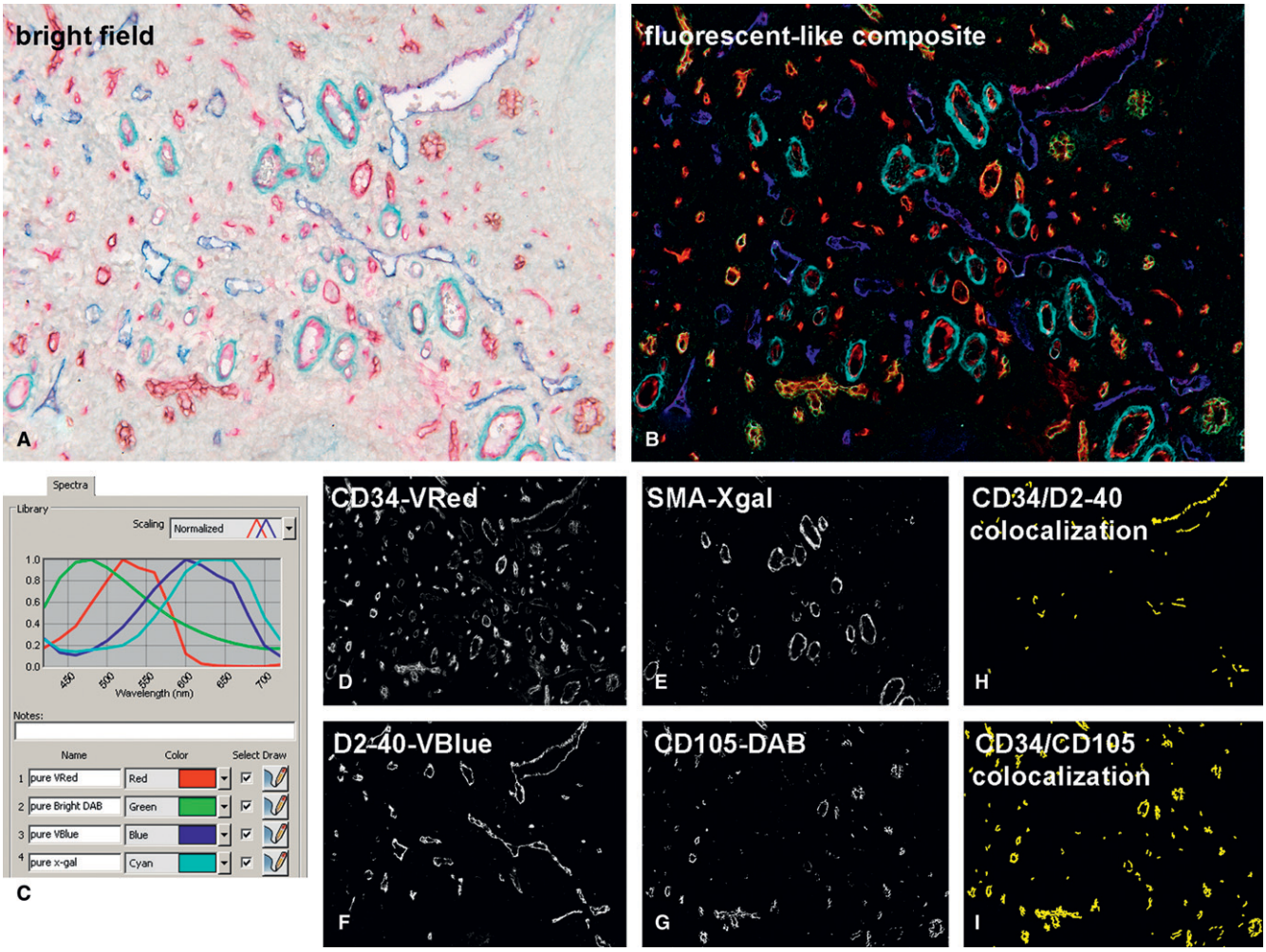

Figure 4. Detail of human tonsil FFPE tissue section showing the medulla after quadruple IHC (A) using smooth muscle α-actin in turquoise (β-gal, X-gal), CD34 (endothelial cells) in red (AP, Vector Red), D2–40 (lymph vessel endothelium) in blue (AP, Vector Blue), and CD105 (endoglin, recently formed endothelium) in brown (HRP, DAB). After spectral unmixing using the spectral library (C), the unmixed composite (B) is a false-color, fluorescent-like image showing high contrast among all components. D-G show unmixed composite images of the individual chromogens. H and I show exclusive images of colocalization; H: of all CD34, 5.6% is also D2–40 positive; I: of all CD34 21.2% is also CD105 positive.

saline is used, wash three times with a Tris-HCl buffer (100 mM, pH8.2) before AP visualization.

- HRP reaction product with Vector NovaRed must be mounted organically (dissolves quickly in aqueous mounting media). Dehydration of NovaRed reaction product in graded alcohols leads to a more brownish reaction product that does not contribute to the observation of colocalization by mixed colors (personal observation). Hence, rinse slide with distilled water and dry completely on a hotplate. Then use VectaMount (xylene-free, alcohol-free) for coverslipping.
- When combining β-gal (X-gal) activity with AP activity, be wary of the order of enzymatic development. Both red and blue AP reaction products (Vector Red, Vector Blue) bind to X-gal reaction product, likely due to a chemical interaction (personal observation). Therefore, AP activity should be visualized first, followed by X-gal.

Table 4. Component and colocalization analysis of triple IHC staining in Figure 3

Presence of markers	*%*[a]	*Staining intensity (average OD)*	*Surface (mm^2)*[b]
CD68	24.8	0.0655	0.31
CD14	16.7	0.0471	0.31
CD163	10.3	0.0208	0.31
CD68 + CD14	9.3		
CD68 + CD163	3.4		
CD14 + CD163	5.0		
CD68 + CD14 + CD163	2.6		

[a] Percentage of all pixels in selected red area of interest as drawn in 3G.
[b] Selected red area of interest as in 3G.

Future of Multiple IHC Staining

The present work showed that there is only a limited series of chromogens available for IHC staining in general (Table 1) and very limited possibilities to combine two chromogens for double staining. In fact, most chromogens in use for IHC staining are based on methods developed in the 1960s and 1970s for enzyme histochemistry. Innovation and original research in the development of new chromogens is

almost nonexistent. Because the application of some of the present chromogens is hampered by a rather diffusely localized reaction product (Table 1), further improvement of multiple IHC staining in combination with spectral imaging requires the development of a new generation of enzymatic chromogens with better microscopical properties: crisper, more transparent, and consistent in terms of exhibiting similar spectral characteristics at different staining intensities.

Undoubtedly, double IHC staining methods have played a significant role in many research projects. It was applied for qualitative confirmation of colocalization or for quantitating single- and double-stained cell populations. However, the introduction of spectral imaging certainly brings multiple IHC staining to the next level: multimarker tissue analysis. Spectral unmixing opens the possibility now to demonstrate colocalization as an exclusive image as well as further analyzing and quantitating the unmixed component images from triple or even quadruple IHC-stained tissue samples. Despite the significant investment required for hardware/software, spectral imaging will play an important role in the future of microscopical multicolor imaging, starting in the research world, and then gradually moving into the diagnostic arena.

At present, applications of multiple IHC staining in diagnostic pathology are still rare, and its applicability is under constant discussion by specialists in the field. However, the recent history of staining techniques leads one to believe that multiple IHC staining will play a significant role in the near future. It is hard to realize that less than 35 years ago many pathologists thought that IHC was unnecessary because standard histology staining and some classical enzyme histochemistry on cryostat tissue sections suited their needs completely! The FACS analysis technique initially began with single staining but has now progressed to procedures combining up to seven different cellular markers. These multiFACS staining methods are regularly used today, for example, in hematopathology diagnostics. It seems only a matter of time before the first parameters based on IHC multiple staining will be revealed. For example, many transcription, chemokine, cytokine, stem cell markers, etc. display broad IHC staining patterns, staining many different cell types, therefore making single IHC not always useful. When a particular combination of these markers demonstrates colocalization, which can be directly linked to diagnosis and prognosis or to selection of a successful therapeutic strategy, then multiple IHC staining also becomes an important tool for diagnostic purposes.

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