Conventional histological and cytological staining with simultaneous immunohistochemistry enabled by invisible chromogens

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Background information

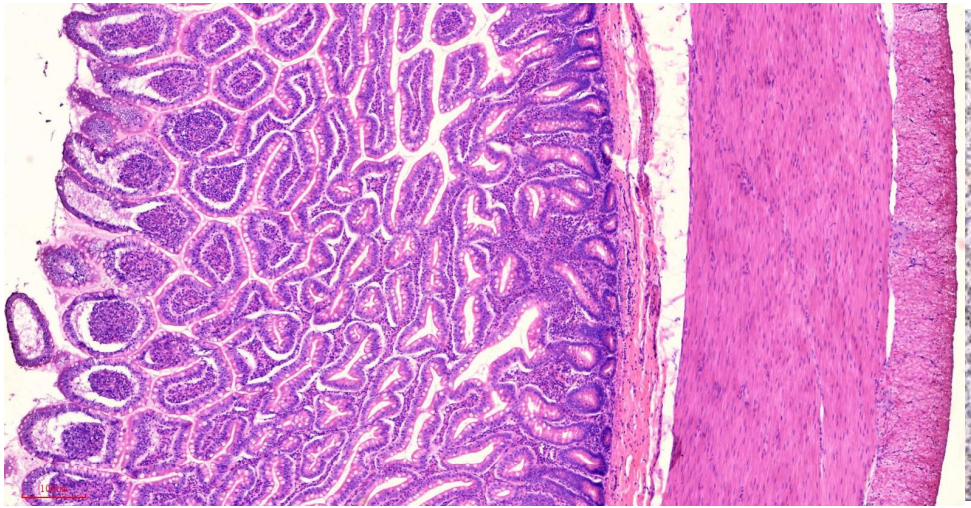
Chromogen

Chromogens for IHC staining are chemical compounds that produce a colored end-product that can be visualized under bright-field microscopy.

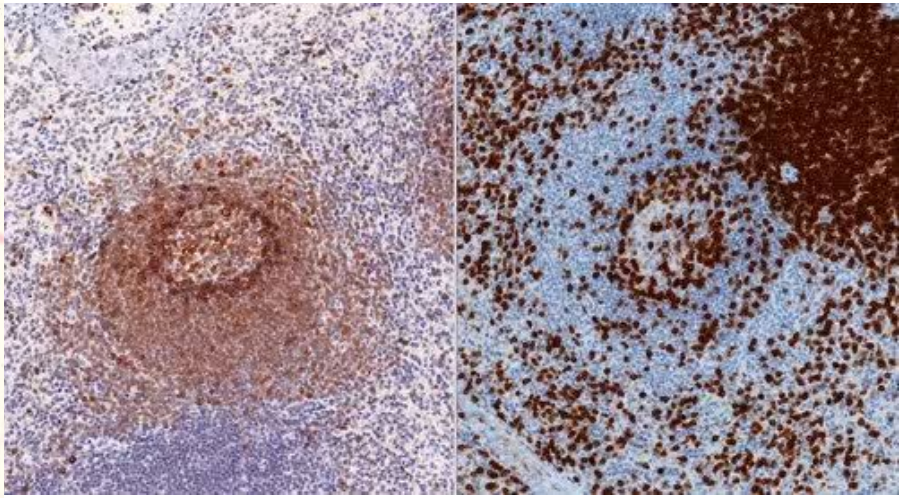
- a chromogen is a colorless chemical compound that is only converted to a colored compound through a chemical reaction

- In IHC this reaction occurs by the action of enzymes
  - horseradish peroxidase (HRP) - reacts with DAB
  - alkaline phosphatase (AP) - reacts with Fast Red

Common chromogens
3,3’-diaminobenzidine tetrahydrochloride (DAB)
Fast Red



H&E stained slide example
https://www.ihappysci.com/product/human-ileum-cross-section-histology-slides/



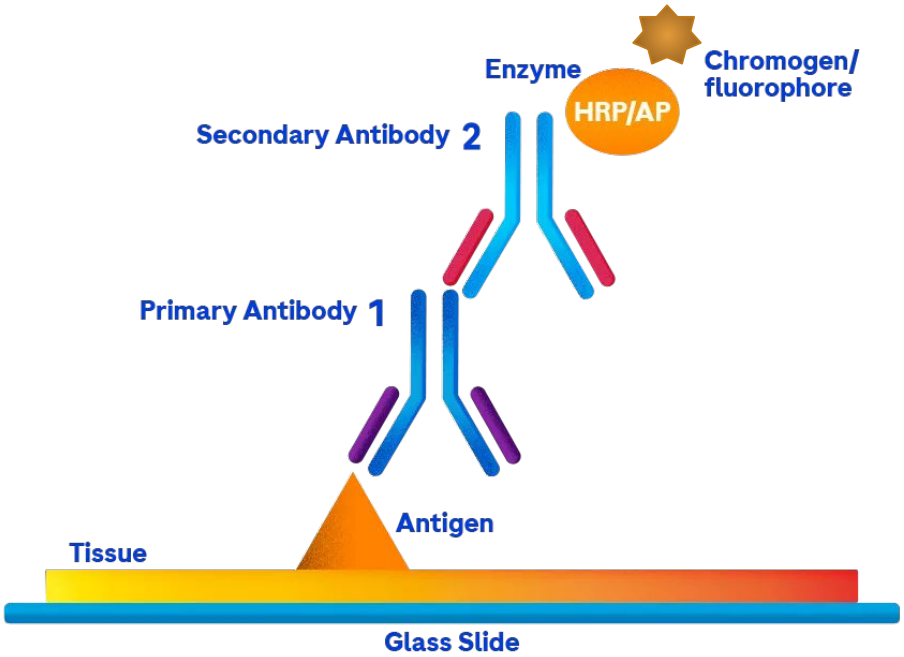
DAB IHC example
https://www.sigmaaldrich.com/US/en/technical-documents/protocol/protein-biology/immunohistochemistry/immunohistochemistry-protocol

The ultimate goal of IHC is to apply color to otherwise colorless cellular features. We also refer to this as staining. This IHC staining allows slides to be visualized under a microscope through the use of chromogens.

The stain produced by a dye may be visually similar to that of a chromogen, however their mechanism of action is quite different.

Background information

IHC protocol steps



https://diagnostics.roche.com/us/en/products/product-category/immunohistochemistry--ihc-.html

Analytical Steps

6. Staining is the analytical part of the IHC process. It encompasses antigen retrieval, application of the primary antibody and visualization system, ending with counterstaining:
   a. Antigen retrieval is performed to recover the antigens that may have been altered by fixation;
   b. Endogenous enzymes are blocked (this step can also be performed after primary antibody incubation);
   c. A primary antibody is applied that specifically binds to the antigen of interest;
   d. The secondary antibody carries the label (enzyme); upon application it binds to the primary antibody;
   e. Chromogen is applied to visualize the antibody/antigen complex;
   f. Counterstaining is performed to visualize nuclei and overall tissue architecture;
   g. Sections are dehydrated, mounted and coverslipped.

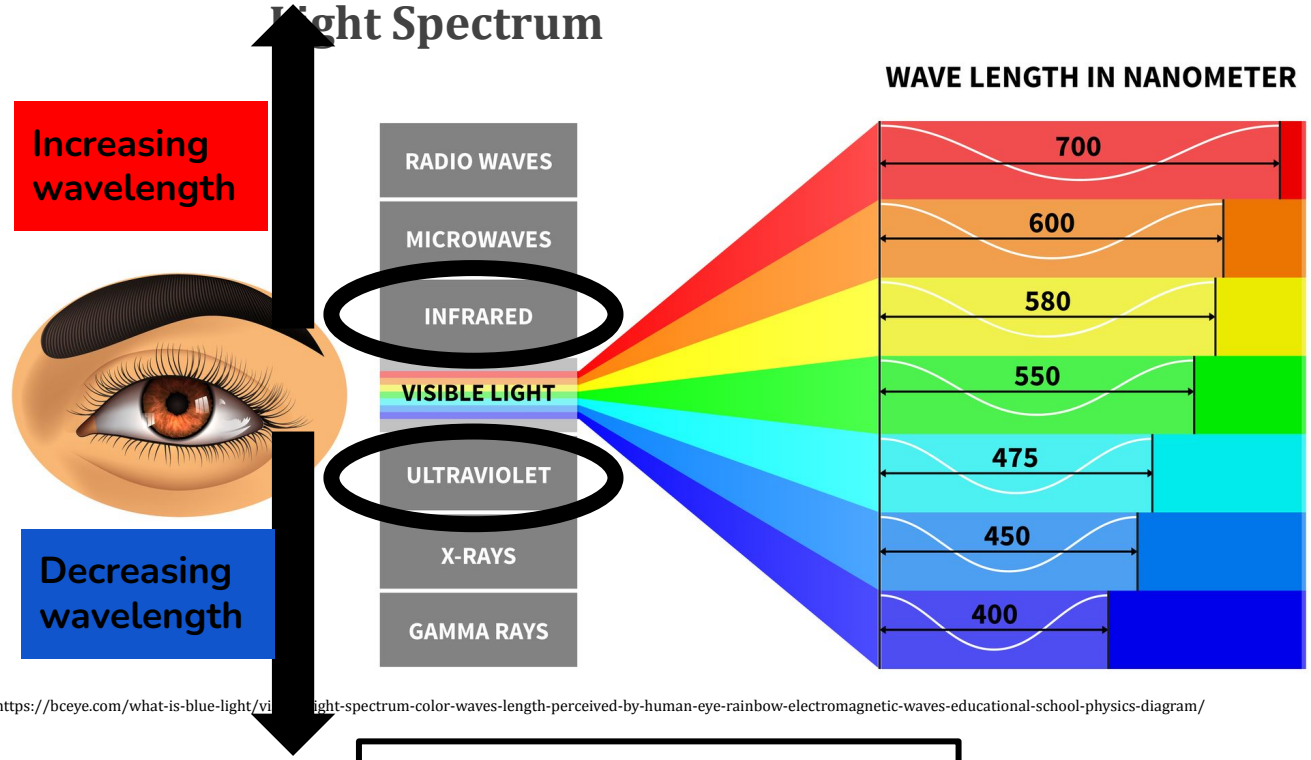
Page 12 *Immunohistochemical staining methods.* DAKO Corporation.

Background information

Invisible Chromogens

dyes deposited by enzymatic action, like conventional chromogens, but having absorbance in the ultraviolet (UV) or near infrared (NIR) predominantly outside the boundaries of human visual response.

Background information



https://bceye.com/what-is-blue-light/vi ight-spectrum-color-waves-length-perceived-by-human-eye-rainbow-electromagnetic-waves-educational-school-physics-diagram/

ROY G BIV

Visible Light Spectrum

- Wavelength range of
  400 nm (violet)
  to
  700 nm (red)

Ultraviolet

- Wavelength range of
  400nm
  to
  10 nm

Near Infrared

- Wavelength range of
  800nm
  to
  2,500 nm

A red shirt looks red because the dye molecules in the fabric have absorbed the wavelengths of light from the violet/blue end of the spectrum. Red light is the only light that is reflected from the shirt.

Background information

Chromogens for IHC staining are chemical compounds that produce a colored end-product that can be visualized under bright-field microscopy.

Invisible chromogens are defined as dyes deposited by enzymatic action, like conventional chromogens, but having absorbance in the ultraviolet (UV) or near infrared (NIR) predominantly outside the boundaries of human visual response.

H&E

~~Conventional histological and cytological~~ staining with simultaneous ~~immunohistochemistry enabled b~~y invisible chromogens

IHC using

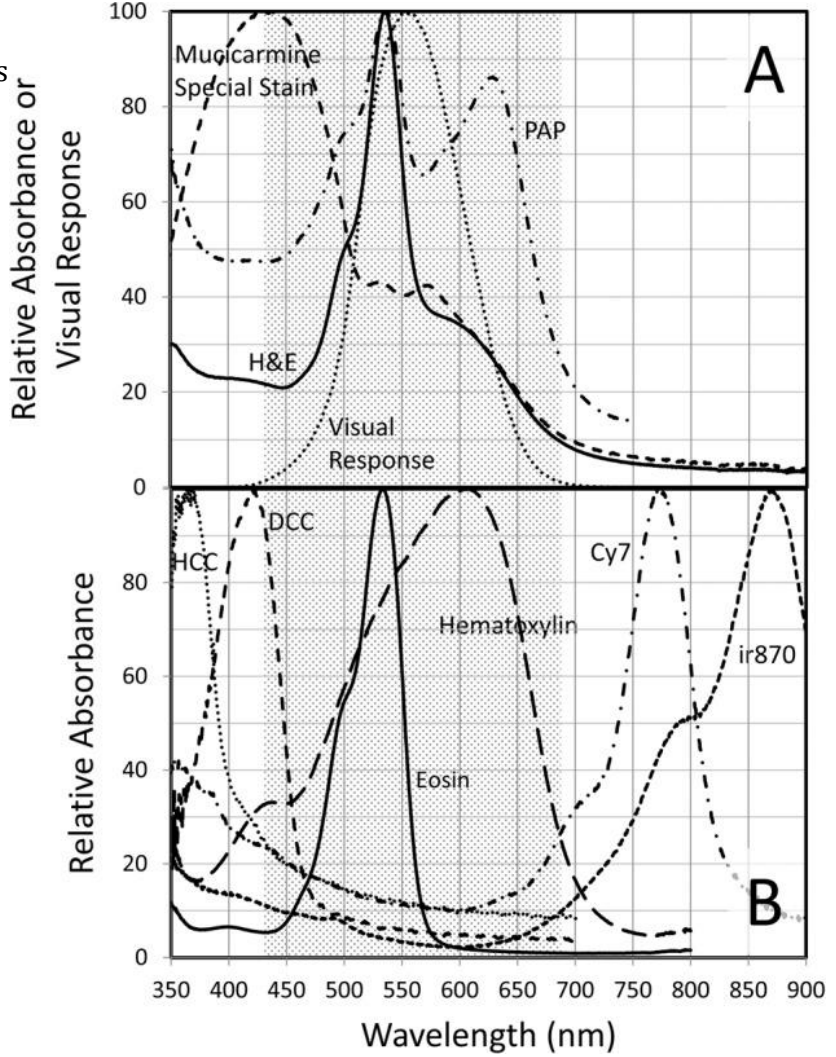
Simultaneous H&E and IHC staining using invisible chromogens

Authors: Larry E. Morrison, Mark R. Lefever, Heather N. Lewis, Monesh J. Kapadia, and Daniel R. Bauer from Ventana

Overview

- Pathologists gain an incredible amount of information from H&E stained tissues that supports diagnosis, prognosis, and prediction of therapeutic response.
- Currently, H&E and IHC require separate slides, because the stains would otherwise obscure one another.
- Performing H&E and IHC on different slides does not permit comparison of staining at the single cell level, since the same cells are not present on each slide, and alignment of tissue features can be problematic due to changes in tissue landscape with sectioning.
- The situation is worse for cytology specimens, for which alignment of information between two different specimen slides is not possible.
- Lighter application of the IHC stains would result in the H&E stain obscuring the IHC stains.
- Use of immunofluorescence in place of IHC is also problematic since eosin is strongly fluorescent and hematoxylin can quench fluorescence.
- The above problems can be alleviated by multiplexing 'invisible' IHC with H&E simultaneously on the same specimen slide.

- **Invisible chromogens** are defined for the present purpose as dyes deposited by enzymatic action, like conventional chromogens, but having absorbance in the ultraviolet (UV) or near infrared (NIR) predominantly outside the boundaries of human visual response.
- The visible range, approximated as 430 nm to 690 nm is shaded in the figure.
- Looking at the absorbance spectra plotted in Fig. **1A**, H&E absorbance declines outside the region of visual response, and is significantly reduced below 450 nm and especially above 700 nm.
- By using invisible chromogens, H&E and IHC stains are spectrally separate, so both types of stain can be present at the same time on the same specimen with minimal interference.
- The authors have taken advantage of a new class of chromogens, the covalently deposited chromogen (CDC), which simplifies chromogen development and permits dyes with essentially any desired spectral characteristics to be rapidly converted to chromogens suitable for IHC, including the invisible CDC.

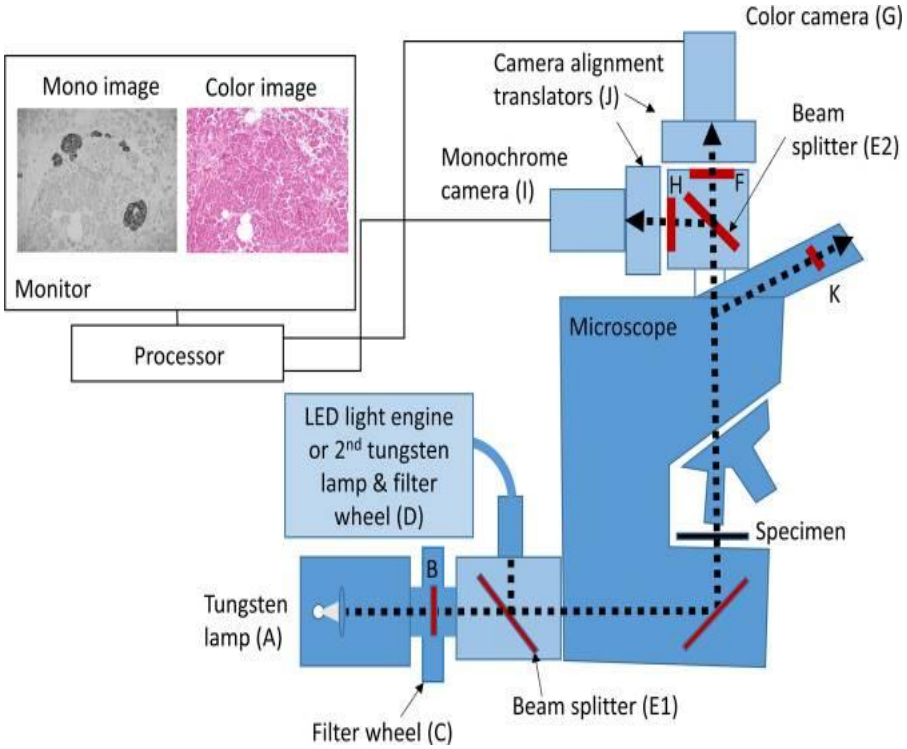


Covalently Deposited Chromogens (CDCs)

- Brightfield microscopy is the preferred method of pathologists for diagnosing solid tumors, utilizing common staining techniques such as hematoxylin and eosin staining and immunohistochemistry (IHC). (18)
- Historically, clinical evaluation of proteins and nucleic acids in tissue has relied upon *in situ* immunoenzymatic detection methods utilizing peroxidase and alkaline phosphatase to catalyze conversion of several well-known chromogenic stains, including 3,3′-diaminobenzidine (DAB), Fast Red, and Fast Blue, into insoluble products. (13)
- However, as our understanding of the complex tumor microenvironment grows, there is increasing demand for multiplexed biomarker detection. Currently, multiplexed IHC assays are almost exclusively based on immunofluorescence because brightfield techniques are limited by the broad spectral absorption of chromogens and a reliance on conventional 3-channel color cameras. (18)
- Common chromogens have absorbance peaks with full width at half maxima (FWHM) of 200 nm or more compared with fluorophores with FWHM typically between 30 and 60 nm in solution, with some broadening when deposited. (18)
- To address this problem, the authors employed covalently deposited chromogens (CDCs) that rely on enzymatic activation of dyes conjugated with tyramide and quinone methide precursors to produce stains covalently bound to cellular and tissue components surrounding the sites of targeted proteins. (18)
- CDCs have the significant advantage of rapid and facile development of new chromogens with desired spectral characteristics, thereby permitting development of chromogen stains with narrow and well-separated absorbance bands, similar to fluorophores. (18)

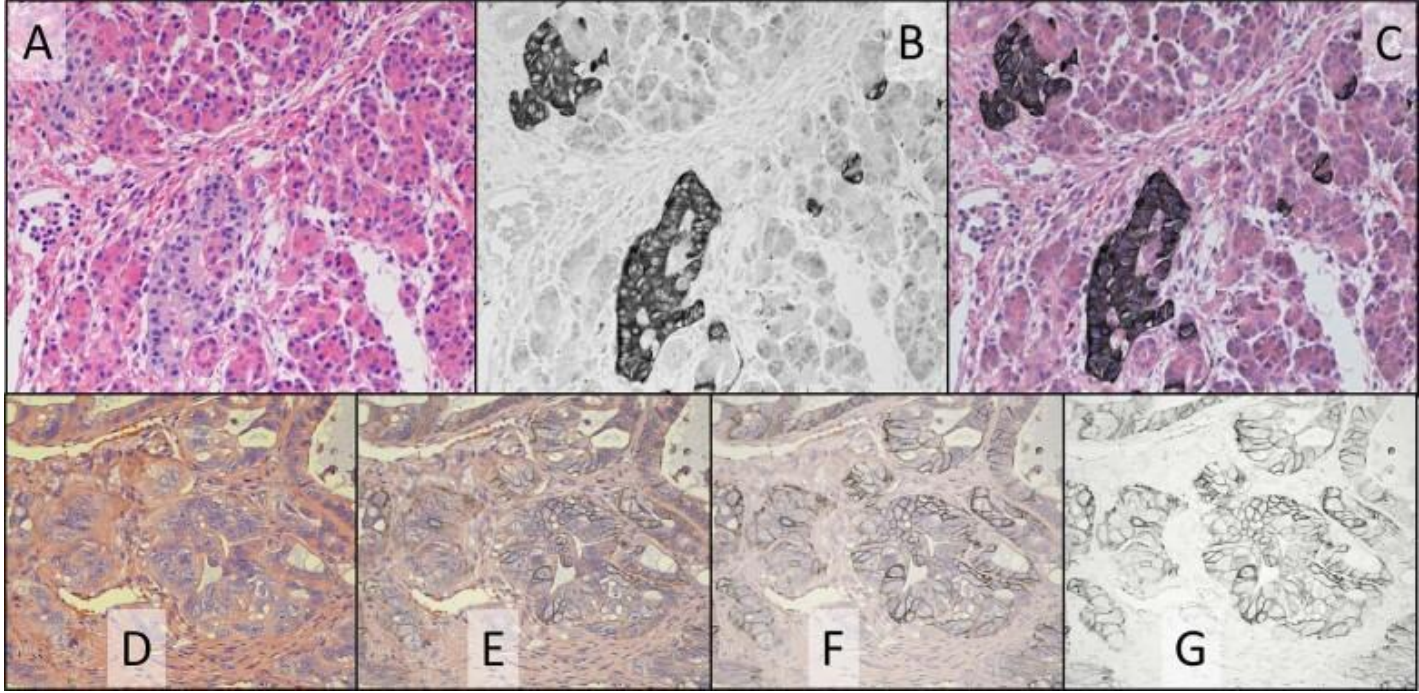
Imaging System

- Olympus BX-51 and BX-63 microscopes (Olympus, Waltham, MA) were adapted by replacing the conventional microscope lamp and camera.
- At the microscope lamp port, a dichroic beamsplitter (E1) combined visible illumination, from a tungsten halogen microscope lamp (A), with UV light and NIR light, from light emitting diodes (LEDs) or a second tungsten halogen lamp (D) with optical filtering to provide illumination channels at wavelengths near chromogen absorbance maxima.
- Light transmitted through the specimen was collected at the objective and split into the visible and invisible components with a second dichroic beamsplitter (E2) within a dual-camera mounting device (Thorlabs, Newton NJ USA) mounted at the camera port.
- In addition to the poor transmission of near UV light through the microscope optics, eye protection from invisible light was provided by custom barrier filters (K) inserted in the reticle space within the microscope eyepieces. (supplementary methods)
- Visible light was directed to a digital color camera (G) and the invisible light was directed to a monochrome camera (I).



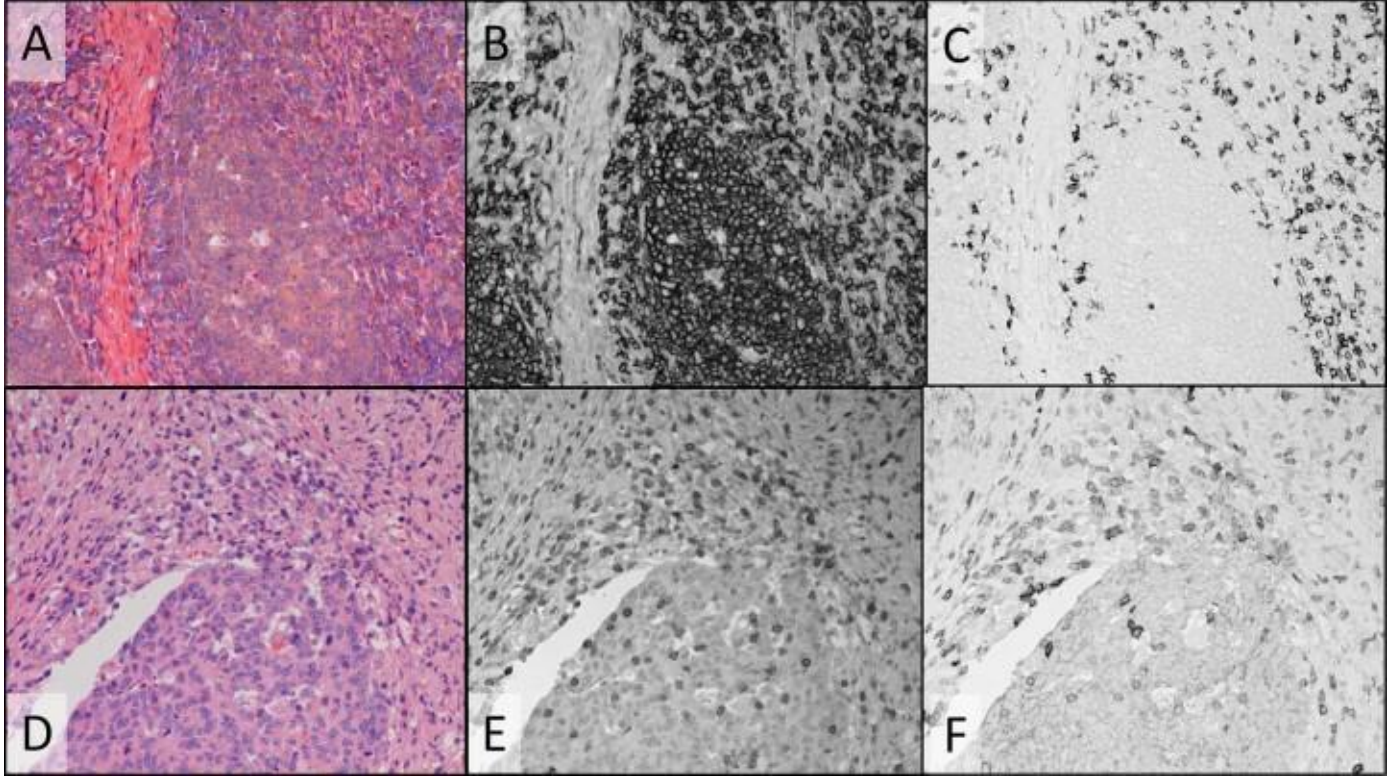
- The dual-camera imaging system is compatible with a pathologist's manual evaluation of specimens at the microscope since the H&E color camera and monochrome CDC biomarker videos can be viewed simultaneously on a computer monitor while scanning the specimen.
- Conventional visible stains and invisible IHC stains can be viewed and evaluated in real-time using a dual camera approach, or multispectral imaging and image processing can be utilized for spectral unmixing, quantitative analysis, and composite image formation.

Figure 3



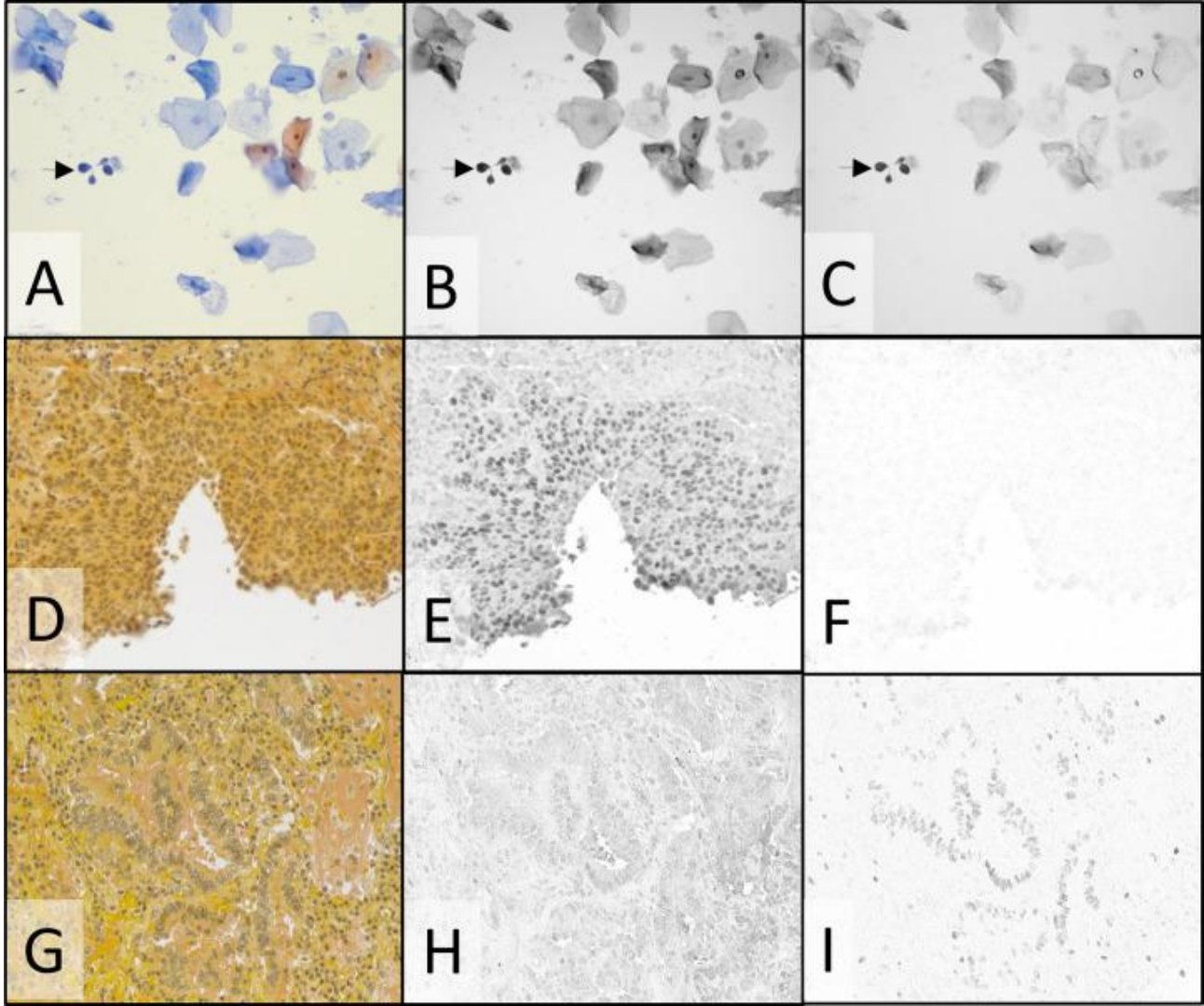
A. Color Camera sensing the visible light and reproducing the view through the oculars. H&E staining of a normal pancreas (450-700 nm).
B. Monochrome camera sensing the 769 nm light Cy7 CDC, showing the synaptophysin staining in the absence of H&E stain.
C. Multiplying the two images pixel-by-pixel provides a composite image showing both stains.
D-G. Several levels of weighting for FFPE tissue of a human breast tumor cell line (Calu-3) mouse xenograft stained simultaneously with H&E and HER2 IHC using Cy7 CDC. Overlay weighting is varied from 100% color image (Fig. 3D), to 100% monochrome image (Fig. 3G), and two intermediate weightings of the color and monochrome images (Figs. 3E and 3F).

Figure 4



A-C H&E and dual IHC staining of tonsil for CD20 & CD8.
D-F Colon tumor for CD3 (General T-cell marker) and CD8 (Activated T-cell marker).
B. Monochrome camera images of CD20 DCC CDC at 405 nm.
C. Monochrome camera images of CD8 Cy7 CDC at 769 nm.
E. Monochrome camera images of CD3 HCC CDC at 385 nm.
F. Monochrome camera images of CD8 Cy7 CDC at 770 nm.

Figure 5

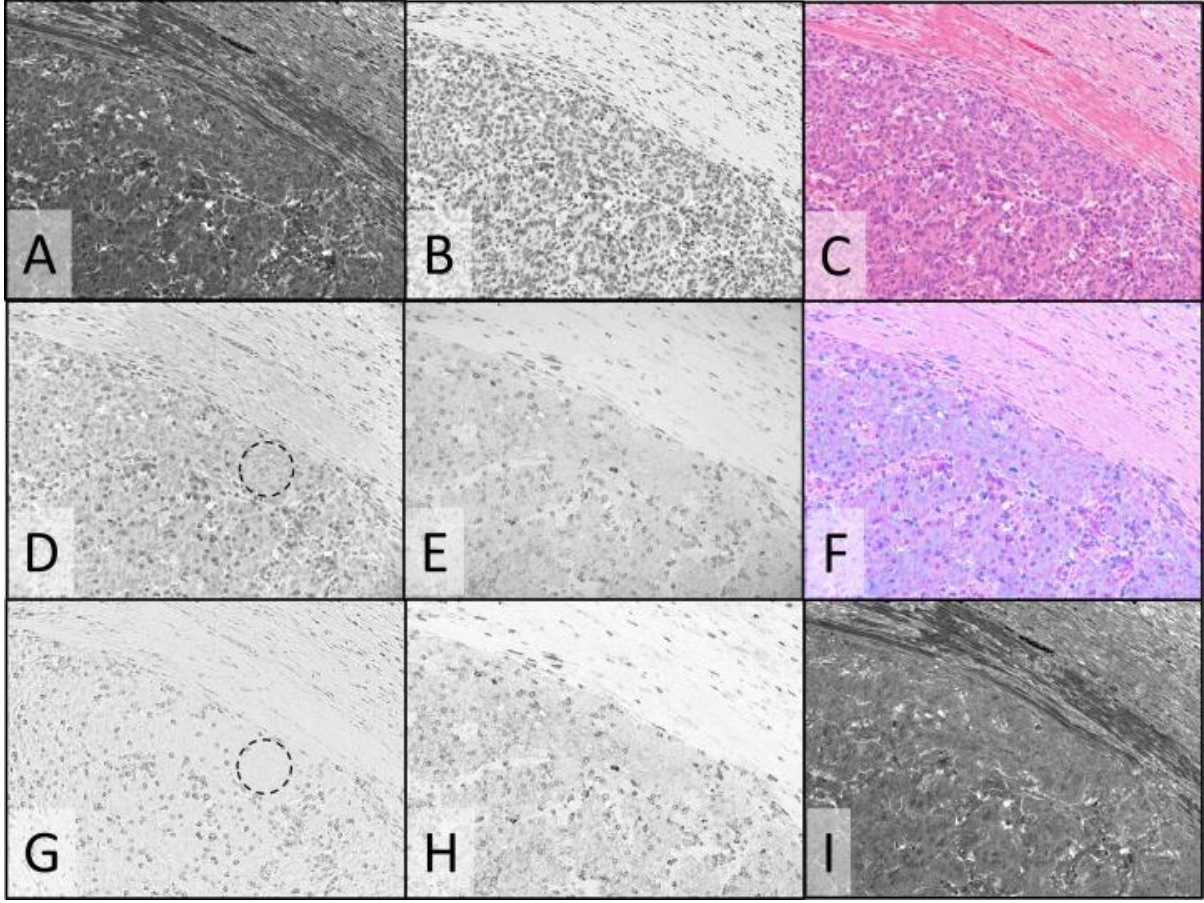


A. Color PAP image.
B. Monochrome Ki67 image 405 nm.
C. Monochrome p16 image 770 nm. (An arrow identifies a cluster of cells stained darkly with both IHC markers).
D. Color images of SCC tissue after application mucicarmine stain.
G. Color images of ADC tissue after application mucicarmine stain.
E&H. Monochrome of p40 Cy7 CDC 769 nm.
F&I. Monochrome of TTF-1 ir870 CDC 880 nm.

- Combining PAP and Ki-67/p16 duplex IHC, as shown in Fig. 5, panels A-C, enables the evaluation of PAP staining pattern, p16 expression, and Ki-67 expression in every cell, and may provide greater confidence in the final diagnosis.
- Mucicarmine special stain is often used to help identify ADC in NSCLC FFPE tissue by the pink coloration due to increased mucin production relative to SCC.
- TTF-1 and p40 IHC are also employed to distinguish ADC and SCC and duplex IHC for these two markers combined with mucicarmine special stain should re-enforce the carcinoma assignment on a single slide.
- Agreement between duplex IHC and mucicarmine special stain is evident in Fig. 5, panels D-F (SCC) and panels G-I (ADC) thereby providing greater confidence in their designations of SCC and ADC.
- Pink mucin staining is minimal in the SCC specimen (Fig. 5D) which also shows p40 positive cells (Fig. 5E), and mucin production is evident in the ADC specimen (Fig. 5G) in agreement with the presence of TTF-1 positive tumor cells (Fig. 5I).

Figure 6 - Multispectral imaging and image processing: composite color images and spectral unmixing in colon tumor.

A. The specimen was illuminated sequentially and imaged using four LEDs: **513 nm** - predominantly **eosin** absorbance.
B. 620 nm - primarily **hematoxylin** absorbance.
C. This color H&E image was created from the 513 nm and 620 nm images and faithfully reproduced the view through the oculars.
D. **CD3 390 nm** - primarily HCC CDC absorbance.
E. **CD8 770 nm** Cy7 CDC absorbance.
F. This color image was created by combining the CD3 image, pseudo-colored magenta, and the CD8 image, pseudo-colored cyan.
G&I. In particular, faint hematoxylin staining seen in the eosin image (Fig. 6A) and CD3 image (Fig. 6D) was eliminated in the unmixed eosin (Fig. 6I) and CD3 (Fig. 6G) images.
H. CD8 unmixed image was minimally altered by unmixing since there was minimal crosstalk.

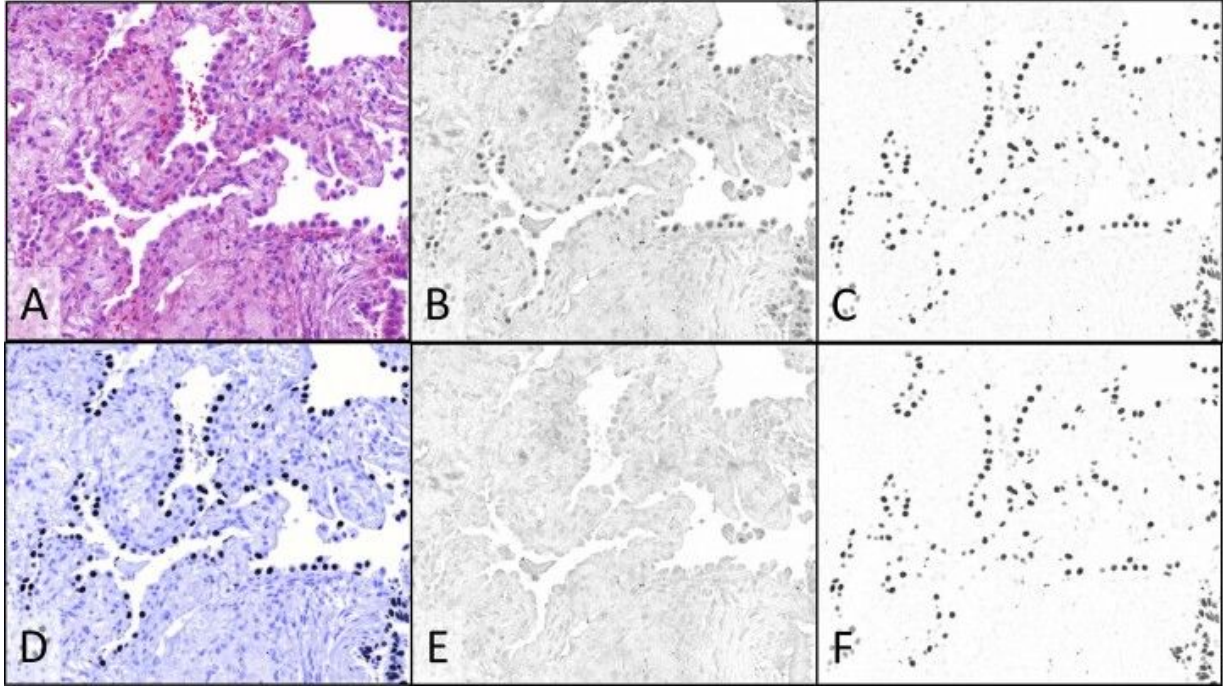


***Dashed circles in (D) and (G) emphasize regions with faint hematoxylin crosstalk that is removed after unmixing.**

- Quantification of CD3 staining can be improved by removing the faint nuclear stain, visible in Fig. 6D, to produce the unmixed image in Fig. 6G that is more effectively segmented for cell counting, measuring spatial relationships, and quantifying expression levels.
- Even the eosin image (Fig. 6A) is improved by removing faint hematoxylin crosstalk as seen in Fig. 6I.
- Fig. 6F in which CD3 and CD8 have been pseudo-colored magenta and cyan, respectively, producing blue in regions of CD3/CD8 co-expression. Note that cyan cells (CD8 only) are not apparent in the figure since any T-cells expressing CD8 should also co-express CD3, thereby producing the blue coloration.

Figure 7

A.The color H&E image was prepared from the unmixed 510 nm (predominantly eosin) and 599 nm (hematoxylin) images, reproducing the view through the oculars.
B. p40 images recorded using the 769 nm light channel - predominantly Cy7 absorbance.
C. TTF-1 images recorded using the 880 nm light channel - ir870 absorbance.
D. Two-color composite image formed from the spectrally unmixed hematoxylin (not shown) and TTF-1.
E. After unmixing - the particularly dark staining ir870 cells selected for this example have noticeable crosstalk into the 769 nm light channel (45% of 880 nm channel absorbance) which is eliminated after unmixing.
F. TTF-1 spectrally unmixed.



***Images of NSCLC ADC FFPE tissue stained with H&E, p40 IHC using the Cy7 CDC, and TTF-1 IHC using the ir870 CDC, illuminated with four filtered tungsten lamp channels: 510 nm (predominantly eosin), 599 nm (hematoxylin), 769 nm (predominantly Cy7), and 880 nm (ir870).**

- Image processing to unmix the TTF-1 and p40 multispectral images shows complete removal of the TTF-1 staining in the p40 image (Fig. 7, panel E vs panel B) to reveal the expected lack of p40 staining (Fig. 7E) in this ADC NSCLC specimen.
- When the eosin, hematoxylin, and chromogen images are unmixed, composite images can be prepared from any combination of these component images, such as in Fig. 7D in which only the hematoxylin and TTF-1 images are combined to provide a representation equivalent to a single TTF-1 IHC with hematoxylin counterstain.
- Image processing can be applied to multispectral images to remove spectral crosstalk and provide images of individual chromogens and stains with reduced or eliminated interference from other chromogens and stains, as shown in Figs. 6 and 7.

Discussion

- The invisible chromogens were developed using CDC technology that simplifies selection of chromogen spectral properties and allows IHC to be performed ahead of the conventional staining.
- CDCs are stable to subsequent conventional staining conditions due to the robust covalent attachment.
- To aid cell location, the H&E and IHC images can be combined into a single composite image, or a video composite image can be viewed while scanning the specimen using an overlay feature of the software.
- The colon tumor example demonstrates important advantages of performing H&E and IHC simultaneously on a single slide. It has been demonstrated in colon carcinoma that the distribution of CD3 and CD8 cells in the core tumor and invasive margin is a strong prognostic marker for disease-free and overall survival.

- Not all conventional stains are suitable for multiplexing with invisible IHC. These include black and some brown stains that absorb across the UV, visible, and NIR spectrum, such as iron-hematoxylin complex staining of elastin in Verhoeff’s Van Gieson stain.
- AI might be improved by enhancing certain cellular, stromal, and overall tissue features with invisible IHC, or identifying tumor-specific markers, providing additional information for diagnosis and correlation with outcomes.

H&E

~~Conventional histological and cytological~~ staining with simultaneous ~~immunohistochemistry enabled b~~y invisible chromogens

IHC using

Simultaneous H&E and IHC staining using invisible chromogens

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Questions