# Multispectral Imaging of Chromogenic Multiplex IHC Assays as an Alternative to Fluorescent Methods

Christopher A. Kerfoot, Sherif Girees, Lisa M. Dauffenbach
Mosaic Laboratories, Lake Forest, CA 92630, (949) 472-8000, www.mosaiclabs.com

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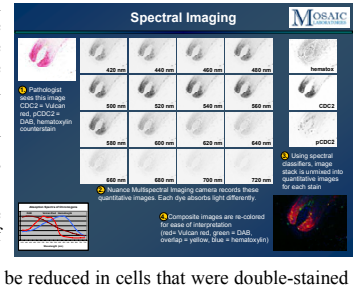
## Abstract

Detection and analysis of two or more co-localized antigens by light microscopy has been hindered by the difficulty in discerning and quantifying overlaying chromogens. Performing multi-analyte immunohistochemistry has many potential benefits and applications in the field of drug target evaluation and patient screening, including: 1) determination and quantification of immunoreactivity co-localization; 2) evaluation of spatial relationships between immunoreactive cell types; 3) signal transduction pathway activation studies; 4) reduction in tissue depletion; and 5) decreased reagent utilization. Multiplex immunofluorescence is not widely used as an analytical technique for clinical samples due to its lower sensitivity than chromogenic methods and concerns about autofluorescence in formalin-fixed tissue. Through the use of multispectral imaging, the absorption pattern of overlapping chromogens can be spectrally unmixed into quantitative individual dye components. This process works accurately when chromogens are developed to a lower intensity than typical for most standard pathology laboratories. The chromogens must be developed to a point where they do not scatter light or block subsequent chromogenic development. The current study was performed to define the accuracy of spectral unmixing of chromogens and evaluate whether interference existed between chromogens. Stained samples were imaged using multispectral imaging, and quantitative grayscale images for each analyte were created by spectral unmixing. The grayscale images were analyzed using image analysis software to determine the overall expression levels of each analyte. Development with Vulcan Red followed by DAB produced little interference, while high density development with DAB decreased the subsequent Vulcan Red staining intensity. Vulcan Red, DAB and hematoxylin unmixed properly and quantitatively. In summary, when multiplexed chromogenic IHC assays were developed using methods optimized for multispectral imaging, the technique demonstrated excellent performance characteristics without suffering from drawbacks commonly associated with fluorescence-based techniques.

## Introduction and Methods

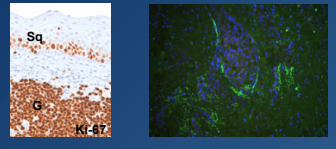
Multiplex immunohistochemistry (IHC) analysis was performed with Ki-67 and an antibody to Antigen #2 (target name withheld). Multiplex immunohistochemistry was performed using a cocktail of primary antibodies and polymer-based detection chemistry. Staining for Ki-67 was developed first with alkaline phosphatase and Vulcan Red chromogen. Staining for Antigen #2 was developed second using HRP and DAB. Samples were counter-stained with hematoxylin. If deposition of Vulcan Red masked the development of the second stain, there would be a reduction in the intensity of DAB where it co-localized with Vulcan Red, creating a "donut-like" appearance. If DAB caused inappropriate unmixing or masking of Vulcan Red, the Ki-67 staining intensity would be reduced in cells that were double-stained with DAB for Antigen #2 compared to cells that were only Ki-67 positive.




## Results


DAPI | Ki-67 = Alexa 488 | Color Merge

Ki-67 immunofluorescence using Alexa 488 labels basal layer of squamous epithelium (Sq) and germinal center B cells (G) in human tonsil. Note the autofluorescence (A) in formalin-fixed paraffin-embedded tissue.

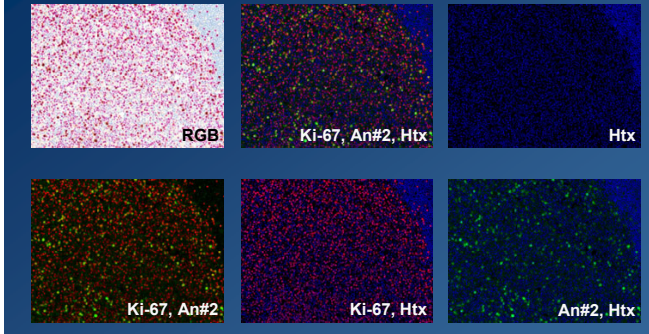



Strong green autofluorescence due to collagen and light green general autofluorescence in formalin-fixed paraffin-embedded tissue can interfere with analysis.

### Comparison of Immunofluorescence to Chromogenic Multiplex IHC

| | Fluorescent | Chromogenic |
|---|---|---|
| Stability | Dyes Photobleach and degrade over time<br>Quantum dots require "charging up" on fluorescent lightbox to reach "steady" intensity | Stable over time |
| Convenience | Requires imaging in the dark | Brightfield microscopy |
| Sensitivity | Limited or no signal amplification<br>Large quantum dots = steric hindrance (esp. nuclear stains) | Enzymatic detection provides signal amplification |
| Endogenous Stain/Fluorescence | Some autofluorescence can be subtracted out using Nuance, but it is not entirely spectrally uniform (e.g. collagen, elastin, lipofuscin). | Melanin in skin and other tissues can interfere with DAB-based detection |
| Number of Analytes | Theoretically, can perform 6-plex or greater | Have unmixed 4 chromogens successfully |
| Linearity | Linearity has been demonstrated | Linearity has been demonstrated |

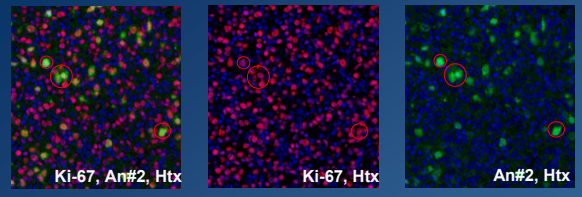
### Unmixed Multiplex Chromogenic Images




**Ki-67=Vulcan Red, Antigen#2 (An#2)=DAB, Hematoxylin (Htx) counterstain.**

**Multispectral cube taken and unmixed into components. Re-colored Ki-67=Red, An#2=Green, Htx=blue**

### Lack of Interference by First Round Stain

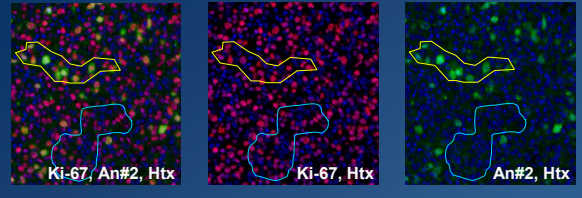



**Note the uniformity of staining for Antigen#2 (DAB), despite the underlying Ki-67 (Red) staining.**

**If the first round chromogenic deposition interfered with the development of the second round chromogen, the green would have lighter intensity in the center where it overlapped the red.**

**There is no evidence that the deposition of Vulcan Red reduces the staining for the second analyte.**

### Lack of Interference by Second Round Stain




**Note the uniformity of staining for Antigen#2 (DAB), despite the underlying Ki-67 (Red) staining.**

**The staining for Ki-67 (Red) was evaluated in regions containing a high fraction of DAB co-localization vs. regions with Ki-67 alone (demonstrated above).**

**Although variability exists between regions, there was no evidence that DAB masked the underlying red stain.**

| Region (ROI) | 0631-0728 | 0281-0938 | 0875-0479 | 0122-0417 |
|---|---|---|---|---|
| Desciption | NO DAB | NO DAB#2 | DAB | DAB#2 |
| Mean | 14.21 | 20.05 | 17.05 | 22.03 |
| StDev | 19.08 | 22.96 | 19.32 | 25.61 |
| Min | 0 | 0 | 0 | 0 |
| Max | 111 | 115 | 103 | 113 |
| Mode | 0 | 0 | 0 | 0 |
| Total | 31396 | 29483 | 40877 | 45098 |
| OD 20-39 | 13.55% | 17.66% | 18.60% | 15.11% |
| OD 40-59 | 7.33% | 9.21% | 9.82% | 10.48% |
| OD 60+ | 10.20% | 15.75% | 12.12% | 20.05% |

## Conclusions

- Development with Vulcan Red followed by DAB produced little interference.
- Vulcan Red, DAB and hematoxylin unmixed properly and quantitatively.
- Multiplexed chromogenic IHC assays were developed using methods optimized for multispectral imaging. The technique demonstrated excellent performance characteristics without suffering from drawbacks commonly associated with fluorescence-based techniques.