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# Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis

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ABSTRACT

Tissue sections offer the opportunity to understand a patient's condition, to make better prognostic evaluations and to select optimum treatments, as evidenced by the place pathology holds today in clinical practice. Yet, there is a wealth of information locked up in a tissue section that is only partially accessed, due mainly to the limitations of tools and methods. Often tissues are assessed primarily based on visual analysis of one or two proteins, or 2–3 DNA or RNA molecules. Even while analysis is still based on visual perception, image analysis is starting to address the variability of human perception. This is in contrast to measuring characteristics that are substantially out of reach of human perception, such as parameters revealed through co-expression, spatial relationships, heterogeneity, and low abundance molecules. What is not routinely accessed is the information revealed through simultaneous detection of multiple markers, the spatial relationships among cells and tissue in disease, and the heterogeneity now understood to be critical to developing effective therapeutic strategies. Our purpose here is to review and assess methods for multiplexed, quantitative, image analysis based approaches, using new multicolor immunohistochemistry methods, automated multispectral slide imaging, and advanced trainable pattern recognition software. A key aspect of our approach is presenting imagery in a workflow that engages the pathologist to utilize the strengths of human perception and judgment, while significantly expanding the range of metrics collectable from tissue sections and also provide a level of consistency and precision needed to support the complexities of personalized medicine.


## 1. Introduction

In the current push to drive a tailored approach to clinical care using clues hidden in tissue samples, there is significant effort underway to understand genomic alterations in order to match small molecule drugs to specific disease types. Yet genetics has not yielded the significant successes hoped for [1,2]. Perhaps the untapped contextual information, remaining in the tissue and captured through multiplexed labeling of proteins with subsequent image analysis, can help reveal additional needed information.

There have been various strategies employed in an attempt to characterize tissues from a histological perspective. Current pathology practice utilizes chromogenic immunohistochemistry (IHC) [3]. However, far more powerful multiplexed IHC (mIHC) approaches are now available, offering greater insights into disease heterogeneity and the characterization of systems biology mechanisms driving disease, as well as helping to conserve limited tissues. An added benefit of mIHC is improved accuracy through application of image analysis, with the use of landmark markers specifically to indicate tissue architecture. Landmark markers can also accelerate scan times. This workflow also can increase a pathologist's productivity by automatically measuring parameters hard to achieve reliably by eye, while needing the pathologist as an integral part of the workflow to review analysis results.

Though mIHC offers greater insight into molecular cascades and preserves tissue context, in current practice multiplexed stained samples can be difficult to interpret. Since mIHC often employs fluorescence, where multiple targets can blend together complicating resolution, this has the potential to muddle visual assessment. With formalin-fixed, paraffin-embedded (FFPE) tissues, there is also the potential for tissue autofluorescence, further complicating visual interpretation.

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Current imaging metrics can effectively address multiplexing complications, through multispectral unmixing strategies. Within oncology, this has broad potential for designing combinatorial therapeutic approaches by revealing co-expression, pathway configurations, and spatial relationships among cell types. With improved accuracy of data, automation, and faster scanning, multispectral mIHC has the capacity to induce a significant paradigm shift in tissue analysis. In this review, we will describe the varied methodologies that support multiplexing, with a particular focus on mIHC in the pathology workflow. In particular, we will assess a practical application of mIHC using Tyramide signal amplification (TSA) in conjunction with multispectral image analysis, which offers improved mIHC using similar species antibodies, while also providing quantitatively reproducible multiplexed data, batch to batch.

## 2. Review

### 2.1. Multiplexed staining methods

#### 2.1.1. Brightfield multiplexing

In order to perform mIHC on FFPE tissues in brightfield microscopy, chromogenic deposition of various chromogens/enzyme pairs is used. While this is useful when distinguishing different cell types, it is more challenging to assess when trying to co-localize targets within cells [4]. Some of the specific chromogens available for brightfield mIHC include: 3,3′-diaminiobenzidine (DAB) and nickel enhanced DAB (DAB-Ni), which produce an insoluble brown or black precipitate, respectively; 3-amino-9-ethylcarbazole (AEC), which produces a red precipitate that is susceptible to organic solvents; Vector VIP which produces an insoluble purple precipitate; and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP), which produces a deep blue precipitate (a more complete listing is available in Table 1). These chromogens can be visualized with either horseradish peroxidase (HRP; DAB, DAB + Ni, AEC and VIP) or alkaline phosphatase (AP; NBT/BCIP). In addition, several counterstaining dyes can enhance brightfield multiplexing, such as methyl green or hematoxylin, which stain nuclei green or blue, respectively.

While brightfield multiplexing is possible, there are several factors to consider. A primary concern remains the need to set up primary antibodies so that there is no cross reactivity, through separate conjugations with HRP or AP. But this limits multiplexing capacity, which can be partially overcome with sequential staining strategies [5–8]. This results in a labor intensive protocol where multiplexing is limited due to tissue degradation after successive serial IHC assays [9]. There is also the potential for chromogenic overlap, and the significant risk of obscuring one color by another. In particular, there is also overlap of chromogenic spectra, which limits the degree of chromogenic multiplexing in brightfield [4]. Hence while chromogenic IHC is a valuable tool and widely utilized in many pathology labs, the ability to practically multiplex beyond 3 targets is limited.

#### 2.1.2. Fluorescent multiplexing

Fluorescence mIHC takes advantage of light emission with different spectral peaks against a dark background. The basic principle behind fluorescent IHC relies on the ability of individual fluorophores to be excited by one wavelength and emit at a longer specific wavelength (a phenomena known as a Stokes shift). For IHC utilizing fluorophores as reporters, there are two basic ways this can be accomplished: via direct or indirect labeling. In direct IHC fluorescence, a fluorophore is directly conjugated to a primary antibody. In indirect IHC fluorescence, the fluorophore is conjugated to a secondary antibody, which is specific for the primary antibody. Indirect IHC fluorescence can also take advantage of amplification strategies, through either multiple secondary antibodies binding to a single primary, or via robust amplification approaches, such as the avidin–biotin complex (ABC).

There are multiple fluorophores available for IHC applications (such as Alexa or Cy dyes, see Table 2 for Cy dyes we have successfully multiplexed), and more recently, fluorescent quantum dot nanocrystals, which have narrower emission peaks when compared to standard fluorophores. In order to utilize fluorophores, there are certain microscopic requirements enabling proper visualization. These include a very bright light source, as well as paired excitation/emission filter sets specific to the fluorophores employed. For example, if using fluorescein, a filter set needs to provide an excitation wavelength ($\lambda$) of 494 nm, and an emission filter needs to pass an emission $\lambda$ of 517 nm. Similarly, a 525 nm quantum dot nanocrystal, where emission $\lambda$ is a function of nanocrystal size, requires an excitation filter that provides a $\lambda$ of $\sim$400 nm, and an emission filter for a $\lambda$ of 525 nm. Choice of filter sets is an important consideration, as it represents a physical limitation in multiplexing capacity. Often, the number of wavelength band passes that can be fit into the visible wavelength range will limit the number of fluorophores that can be utilized without crosstalk to 3 marker fluorophores, along with DAPI. Generally, number of filter cube sets each fluorescent microscope can accommodate is 4 or 5. Another consideration when planning any fluorescent mIHC assay is the potential for, and likelihood of, co-localization of different fluorophores. In the case where co-localization occurs, complications can occur in analysis if the co-localization causes colors to mix, as red and green might, to provide a degree of yellow. In this instance, the relative contribution of red and green is extremely difficult to determine using standard image analysis, and as such must be planned to achieve successful multiplexing.

Indeed, fluorescent mIHC has been successfully demonstrated in FFPE tissue in differing multiplex levels. Mason et al. [10] demonstrated 2-plex fluorescent IHC in FFPE tonsil interrogating CD79 and PCNA, while recently, Bogusz et al. [11] interrogated active BCR signaling in diffuse large B-cell lymphoma with various 2-plex combinations of CD20 coupled with either pLYN, pSYK, or pBTK. In contrast, 4-plex fluorescent IHC using quantum dots to interrogate the tumor microenvironment in gastric cancer has also been demonstrated [12]. In all instances, indirect labeling with primary-specific conjugated secondary antibodies was performed. This raises an important issue involving antibody species and the ability to

**Table 1**
Typical chromogens in multiplexed IHC assays.

| Chromogen | Catalytic agent | Deposition color |
|---|---|---|
| DAB | HRP | Brown |
| DAB + Ni | HRP | Black |
| AEC | HRP | Red |
| VIP | HRP | Purple |
| NBT/BCIP | AP | Deep blue |
| Vulcan Fast Red | AP | Red |
| Vector Black | AP | Black |
| Nova Red | HRP | Deep red |
| TMB | HRP | Blue |

**Table 2**
Sample fluorescent dyes for multiplexed IHC assays.

| Fluorescent dye | Excitation $\lambda$ (nm) | Emission $\lambda$ (nm) |
|---|---|---|
| Coumarin | 402 | 443 |
| Fluorescein | 494 | 517 |
| TMR | 550 | 570 |
| Cyanine 3 | 550 | 570 |
| Cyanine 3.5 | 581 | 596 |
| Cyanine 5 | 648 | 667 |
| Cyanine 680 | 669 | 688 |

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