Exp Oncol 2008
30, 4, 327–329

TECHNICAL ADVANCE

Experimental ONCOLOGY

IMMUNOFLUORESCENCE STAINING OF PARAFFIN SECTIONS: CREATING DAB STAINING LIKE VIRTUAL DIGITAL IMAGES USING CMYK COLOR CONVERSION

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Crystal violet treatment of formalin fixed paraffin embedded tissue slides greatly reduces the endogenous autofluorescence, and allows immunofluorescence (IF) staining with FITC or Alexa488 conjugated antibodies. Using cold CCD camera to capture the fluorescence images makes this staining method very sensitive. Here we show that combination of IF with the simultaneous recording of crystal violet induced red and Hoechst 33258 induced blue fluorescence permits the localization of the IF signal over a cytoplasmic: nuclear red:blue stain that visualizes the microscopic anatomy of the underlying tissue. To make the visual interpretation of the IF staining easier for microscopists, who are used to DAB staining over weak hematoxilin-eosin background, we created a simple color conversion procedure that turns the captured three-color fluorescence RGB (red, green, blue) images over a black background into four color CMYK (cyan, magenta, yellow, key color (black)) images.
Key Words: **immunofluorescence microscopy, paraffin sections, digital imaging.**

Immunostaining of formalin fixed and paraffin embedded sections is routinely carried out using enzyme conjugated secondary antibodies such as alkaline phosphatase or horse radish peroxidase (HPR). These enzymes produce insoluble chromogenic products from soluble colorless substrates that are deposited at the site of antigen:antibody complex. A frequently used substrate of HPR is diaminobenzidinetetrahydrochloride (DAB) that in combination with different metal ions such as silver or nickel produces dark brown or black deposits. The underlying tissue structures are regularly visualized by a weak hematoxylin or hematoxylin-eosin (HE) counterstaining. The resultant images can only be captured using color cameras. Due to the mixture of colors and the nonlinear nature of the dye deposition it is rather difficult to quantify the amount of antigen using this technique. Fluorescent dye conjugated secondary antibodies can overcome this problem by allowing monochromatic capturing of images over the dark background using highly sensitive cold CCD cameras. The signal amplification through double layer immunostaining with fluorochrome conjugated secondary antibodies and the image acquisition through black and white cameras allow a highly linear quantitation of the antigen. The greatest hinder for using immunofluorescence staining on formalin fixed paraffin embedded sections is the relatively high greenish autofluorescence that interferes with the most frequently used dyes such as Fluorescein isothiocyanate (FITC). The strength of autofluorescence among other factors is dependent on the tissue type, quality of fixative, time of fixation, thickness of the sections and the wavelength of excitation light [1]. The extent of interference between the specific immunostaining and background fluorescence can be controlled by several different means. One of the simplest methods is to use carefully selected high affinity antibodies in combination with good quality secondary antibodies to detect relatively abundant antigens and carry out the imaging in narrow focal planes using laser scanning confocal microscopy [2]. Alternative ways to decrease the signal to noise ration are: signal amplification by tyramid deposition [3, 4], using fluorescent dyes that has red emission [5], digital subtraction of separately measured background fluorescence [6] or use of spectral imaging in combination with linear unmixing. Alternatively the slides can be treated with different quenching agents, such as sodium borohydride followed glycine treatment [6], Sudan black ammonia [7] or Crystal violet [8–12]. This latter even allows quantitation of relatively weak signals such as proliferating cell nuclear antigen (PCNA) [13] or quantitative detection of apoptosis on highly autofluorescent liver sections [14].

In the present paper we tried to exploit the feature of the Crystal violet staining that creates a strong red fluorescence of all cellular structures when illuminated at 560 nm. Here we show that using this dye it is possible to visualize the underlying tissue architecture. When combined with DNA staining dyes such as Hoechst 33258 or DAPI this method can reveal very detailed cellular morphology. To demonstrate the feasibility oft the combination of immunofluorescence staining with Crystal violet: DAPI counterstaining we have carried out IF detection of p53 in the nuclei of neoplastic endometrium. 5 μm thick slides were deparaffinized in xylol and rehydrated in descending ethanol series. The epitopes were recovered by boiling

Received: November 10, 2008.
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Abbreviations used: CMYC – cyan, magenta, yellow, key color; DAB – diaminobenzidinetetrahydrochloride; FITC – fluorescein isothiocyanate; HE – hematoxylin-eosin; HPR – horse radish peroxidase; IF – immunofluorescence; RGB – red, green, blue.

the slides in 100 mM citrate buffer in microwave oven for 5 min. We used DO7 anti-p53 mouse monoclonal antibodies in combination with FITC conjugated rabbit anti-mouse secondary antibody (both from DAKO, Carlstrup, Denmark). The antibodies were diluted in the following blocking buffer: 0.2% Tween 20, 5% glycerol, and 3% BSA in PBS. Following the immunostaining the slides were treated with 0.5% Crystal violet in 0.9% NaCl (freshly diluted from 10% stock in methanol) for 10 min. After washing 3 times in 0.9% NaCl the slides were rinsed in PBS and mounted with 80% glycerol and 2.5% DABCO (pH 8.5). The images were visualized in a Leitz fluorescence microscope and were captured with a Hamamatsu C4880 CCD camera. We found that using RGB color-coding the green immunofluorescence signal can be conveniently overlayed on the red and blue cellular background. The drawback of this method is that the resultant images are on black background and do not resemble the color experience of conventional immunostaining. Here we show that a simple image conversion procedure can turn the original RGB image into a white background image that resembles a hematoxylin-eosin staining combined with black immunostaining deposits. The procedure that is summarized in Fig. 1 is the following:

1. An empty image with white background is created in CMYK mode in identical size to the RGB image.

2. The content of the red (R) channel is copied to the magenta (M) channel of the CMYK image.

3. The content of the green (G) channel is copied to the CMYK black (K) channel.

4. The content of the blue (B) channel is copied to the CMYK cyan (C) channel.

5. All channels of the CMYK image are selected and inverted.

6. The content of the yellow (Y) channel is deleted.

The advantage of the above described three color staining method is that fluorescent images that were captured by monohrome CCD cameras over a black background can be visualized in a way that is similar to the DAB enhanced immunostaining of routine immunohistology. The individual components (red cytoplasmic, blue nuclear and black immunostaining) of the resultant image are kept as separate information and can be freely toggled, both in the RGB, or in the CMYK mode, by switching on and off the corresponding channels as illustrated on Fig. 2. Importantly, the signal intensities of individual cells in the separate channels can be digitally quantitated with a 12 bit broad dynamic range. The described method also permits visualization of double immunostaining results obtained using e.g. FITC and Texas Red signals on sodium borohydride treated slides. In this case the crystal violet pattern in the red channel is replaced with the specific second (red) immunostaining. The resultant image shows the two specific antigens in black and red, respectively, over a blue nuclear background similarly to HRP and alkaline phosphatase double staining.

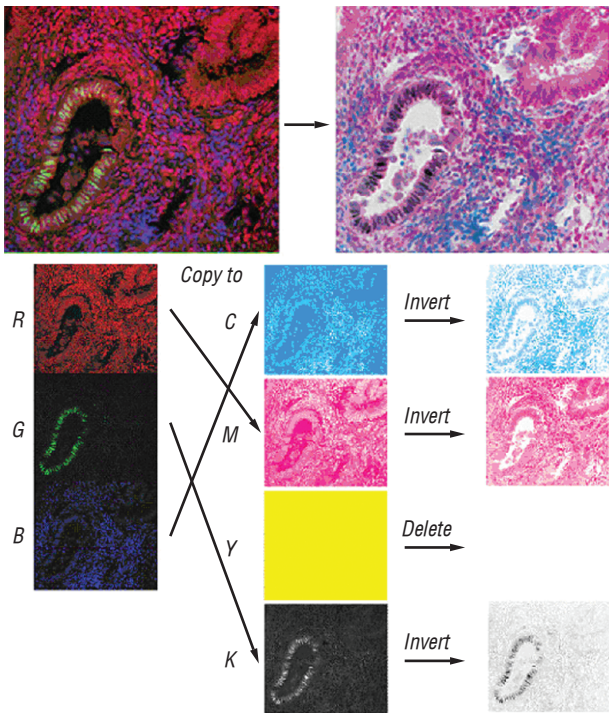

Fig. 1. Digital conversion of immunofluorescent RGB image into a "DAB combined with hematoxylin:eosin" like image. The example for RGB image is a section of a human displastic endometrium stained for p53 using FITC conjugated secondary antibody (green) with the fluorescence of the green autofluorescence quencher crystal violet (red) and nuclear staining with Hoechst 33258 (blue). The content of the red, green, and blue channels of the RGB image are copied into an identical size CMYK image, into the magenta, black and cyan channels respectively. The content of the CMYK channels are inverted and the yellow channel is deleted

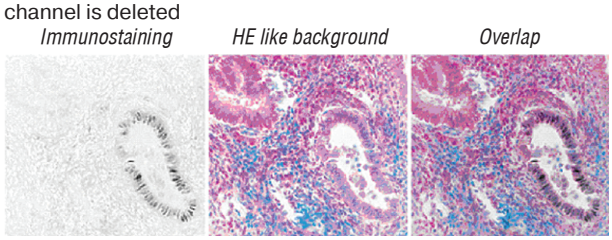

Fig. 2. The digitally created "DAB/HE" like image allows the separate presentation of the specific immunostaining (black) and the underlying histology (red/blue) as well as the combination of the two

Thus, we have developed an imaging method that allows vitalic in silico visualization of imunofluorescence signal on paraffin embedded slides in a manner similar to the conventional immunoperoxidase staining. The signal is quantifiable over a 4096 gray scale level. Moreover it can be freely toggled over an HE like nuclear:cytoplasmic background.

ACKNOWLEDGEMENTS

This study was supported by the Swedish Cancer Society (Cancerfonden), the Swedish Reseacrh Council and by Swedish Institute in the frame of Visby program.

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ИММУНОФЛУОРЕСЦЕНТНОЕ ОКРАШИВАНИЕ ПАРАФИНОВЫХ СРЕЗОВ: СОЗДАНИЕ ОКРАШИВАНИЯ DAB В ВИДЕ ВИРТУАЛЬНЫХ ЦИФРОВЫХ ИЗОБРАЖЕНИЙ С ИСПОЛЬЗОВАНИЕМ ЦВЕТНОГО CMYC-ПЕРЕХОДА

Окраска кристаллическим фиолетовым парафиновых срезов, полученных из ткани, фиксированной в формалине, значительно уменьшает явления аутофлуоресценции и обеспечивает иммунофлуоресцентную (ИФ) окраску антителами, конъюгированными с FITC или Alexa-488. Использование CCD-камер для регистрации флуоресцентных изображений делает этот метод очень чувствительным. Наша цель — разработать метод трансформации RGB (red, green, blue) — флуоресцентных изображений в CMYC (cyan, magenta, yellow, key color (black)) изображения. Показано, что комбинация ИФ с одновременной регистрацией красной и голубой флуоресценции, индуцированной соответственно кристаллическим фиолетовым и Hoechst 33258, позволяет определять ИФ-сигнал как цитоплазматично-ядерное красно-голубое окрашивание, которое визуализирует морфологические особенности прилегающей ткани. Для упрощения интерпретации ИФ-окраски патологами, привыкшими к окрашиванию ДАБ на фоне гематоксилин-эозина, нами была создана технология простого цветового перехода, который превращает зарегистрированные трехцветные RGB-флуоресцентные изображения на черном фоне в четырехцветные CMYC-изображения на белом фоне, используя программы работы с изображениями.

Ключевые слова: **иммунофлуоресцентная микроскопия, парафиновые срезы, цифровые изображения.**