WHOLE SLIDE, QUADRUPLE IMMUNOFLUORESCENCE LABELLING OF ROUTINELY PROCESSED PARAFFIN SECTIONS.

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ABSTRACT
Whole slide images (WSI) have acquired a stable place in diagnostic histopathology and immunohistochemistry. Immunofluorescence (IF) techniques hold a limited and selective role in diagnostics (e.g. renal and cutaneous pathology) and so far remain excluded from the digital pathology evolution, with notable exceptions, such as quantitative immunopathology. We explored the ability of a commercial fluorescent slide scanner to provide four-color immunofluorescent whole slide images from routinely processed tissues. With minor modifications and a careful match between filters and fluorochromes, we show that four-color IF WSI can be obtained from routine material with negligible autofluorescence, good sensitivity and diagnostic power.

KEYWORDS: Virtual slide; whole slide imaging; indirect immunofluorescence
INTRODUCTION

In the year 2000, David Mason published a brief report highlighting the advantages of immunofluorescent (IF) staining over double immunohistochemistry (IHC) (1): double IF is rapid, cheap, necessary if antigen co-localization is expected. This publication, which has been quoted 49 times, reflected a highly sophisticated, complex and advanced approach to immunolabeling for diagnostic and research use. However, single labeling in immunoperoxidase was so convenient, embedded in the routine use of light microscopy and immediately comparable to H&E in a matter of seconds, that the use of IF in routine histopathology has remained limited to renal and skin biopsies. The introduction of Quantum Dots, very bright, stable fluorescent particles with wide excitation spectra and narrow emission bands, were used only briefly in diagnostic pathology. The wide spectral range allows multiple co-labeling, which should be matched by spectral detectors and a software able to deconvolve the images (2). Six-color labeling of tonsil tissue with Quantum Dots was reported in 2006 (3), with little impact on the routine acquisition of this technology in the daily practice.

In summary, because of the use of pathologist unfriendly instruments (fluorescence microscopes) and awkward interactions with the media (visualization of one color at a time), indirect immunofluorescence has remained largely outside routine practice.

Elsewhere, multiple immunofluorescent labeling has evolved into complex imaging and analytical software tools (4), able to provide quantitative single-cell data extracted from tissue sections. These tools go with the term of “image cytometry”, in analogy to flow cytometry with which they share multiple points. The Yale group, headed by David L Rimm, has extensively used multiple IF labeling and image cytometry, in order to do quantitative assessment of relevant antigens in tissue sections, providing diagnostic and prognostic tools for the clinician and invaluable insight into the biology of common cancers (5, 6).

Whole Slide scanning and the production of virtual whole slides is revolutionizing the practice of pathology (7). Remote viewing and consulting, slide retrieval and archiving, integration with the patient’s records, teaching and imaging are all made possible by the introduction of slide scanners. Slides stained in single immunohistochemistry are imaged as well and there have been substantial effort to provide quantitative imaging tools to help pathologists to quantify and score stained slides. Scanner vendors have teamed up with image software companies to generate ad hoc software, which in some cases has gained FDA clearance for diagnostic use.

Double IHC labeling is used very sparingly in common practice, although it is gaining some foothold in specialized fields. Some of the imaging software allows separation of the images based on the color deconvolution of the immunohistochemical stains.

Taking from the 2000 David Mason’s paper, the availability of whole slide images on screen in immunofluorescence and in light microscopy, side by side, would allow the use of immunofluorescence in routine diagnostics, overcoming the shortcomings of instruments, software and media not familiar to the pathologist. We thus explored the use of multiple color indirect immunofluorescence on routinely processed sections and their evaluation on WSI.

MATERIALS AND METHODS

Tissues and cells
Leftover surgical pathology specimens were anonymized, fixed overnight at room temperature in 10% buffered formalin and embedded in paraffin. Serial sections were cut at 5 µm and collected on coated glass slides (Superfrost®, Thermo Scientific, Philadelphia, PA).

Filters
The Aperio ScanScope FL Slide scanner (Aperio, Vista, CA) is equipped with an Olympus 20X/0.75 PlanSApo objective, an XCite® exacte mercury lamp (Lumen Dynamics Group Inc., Ontario, Canada), a linear TDI sensor and a quad-band filter set in the Pinkel configuration (Semrock DA/FI/TR/Cy5-4X-B). The filter set consists of four excitation filters (FF01-387/11, FF01-485/20, FF01-560/25 and FF01-650/13) in a separate motorized filter wheel, and one filter cube in a motorized turret. The cube itself contains a quad-band emission filter (FF01-440/521/607/700) and a quad-band dichroic filter (FF01-410/504/582/669-Di01) (Semrock, Inc, Rochester, NY).

Two additional Semrock filters were separately purchased: an emission and dichroic orange filter (Cy3.5-A-Basic FF01-620/52-25 and FF585-Di01) and a broader excitation blue filter (FF01-377/50). All filters featured the zero-pixel-shift option. The spectral features of the excitation and emission filters are shown in Fig. 1A.

Five excitation and emission filter combinations were used thereafter: exc.377 – em.440 (blue); exc.387 – em. 440 (blue); exc.485 – em. 521 (green); exc.560 – em. 620 (orange); exc.650 – em.700 (red) (all nanometers). Blue, green and red emission spectra were collected with the quadband filter. The use of independent excitation filters with fixed multi-band dichroic and emission filters in a cube comprises the Pinkel Configuration (http://www.semrock.com/multiband-filter-set-terminology.aspx).

Fluorochromes and antibodies
Nuclear dyes and species and/or isotype-specific fluorochrome-conjugated secondary antibodies were purchased from Invitrogen/Life Technologies (Monza, Italy), Jackson Immunoresearch Europe (Suffolk, UK), and Biolegend (London, UK) (Table 1). Mounting media (Fluoromount G with and without DAPI) was purchased from Società Italiana Chimici, Roma, Italy. Primary antibodies were purchased from DAKO, Glostrup, DK (CD20, CD3, S-100, Ki-67) and from Santa Cruz Biotechnology, Heidelberg, DE (p16/CDKN2A). The excitation and emission spectra for the selected dyes and conjugates are shown in Fig. 1A.

Immunofluorescence staining
Five micron sections on slides were deparaffinized, rehydrated and antigen-retrieved as previously published (8). Subsequently, sections were blocked for 1 hr. with 5% defatted milk (Reire, Srl, Reggio Emilia, Italy) in Tris Buffered Saline containing 0.01% Tween-20 (TBS-T), then washed twice in TBS. Primary antibodies, optimally diluted in TBS containing 5% Bovine Serum Albumin (BSA, Sigma) were pooled (e.g. rabbit anti-S100 and mouse IgG1 anti Ki-67) and incubated overnight at RT. Pooled and diluted secondary antibodies (e.g. Donkey anti-rabbit Alexa 488 and Goat anti mouse IgG1 Alexa 680) were applied for 1 hr. After two washes in TBS-T, the primary pool / washes / secondary pool sequence was repeated once (9). After two final washes in TBS-T, the slides were counterstained with either Sytox Green (1 mM/ml in Fluoromount) or DAPI-containing mounting medium.

Visualization and image processing
Each whole slide image was acquired as a greyscale image and dowonloaded in a dedicated server. The images were cataloged in the Spectrum database software (Aperio), grouped by slide ID and automatically superimposed to create a composite pseudocolor image which was visualized with ImageScope (Aperio). ImageScope allows selective visualization, pseudocoloring, addition or removal of image layers as well as some contrast and brightness adjustments. Screenshots, single or
multifluorescent portions of the WSI were adjusted for contrast and downloaded with ImageScope. Composite images and labels for publication were edited with Adobe Illustrator and Adobe Photoshop CS3 (Adobe, San Jose, CA).

Quantitative detection on beads
Polystyrene beads coated with a defined amount of mouse Ig were purchased from DAKO (QIFIKIT® for Flow Cytometry, DAKO, Glostrup, DK). The mouse immunoglobulin coating the beads is a mouse CD5 IgG2a isotype antibody (CRIS-1); however, we discovered that the monoclonal bears IgG1 idiotypes as well, making the beads very versatile. 20 µl of QIFIKIT beads were stained for 30 min with saturating amounts of anti-mouse conjugated anti-IgG1 or anti-IgG2a antibodies in a test tube, washed twice in Tris Buffered Saline containing 0.01% Tween-20 (TBS-T), mixed with 1x10^6 methanol-fixed irrelevant cultured cell lines, 10% of egg albumin (Agricola Italiana Alimentare S.p.A., Verona, Italy) and cytopspun on glass slides (Shandon, UK). The slide was mounted with clear mounting medium and scanned on ScanScope. A small sample of the beads was stained and analyzed in flow cytometry as per instructions for sensitivity comparison.

RESULTS

Autofluorescence
Tissue autofluorescence, defined as a visible image due to endogenous fluorescent molecules, is due to flavins (10), hemoglobin and other tissue components with a molecular structure which emits in a broad range of wavelength when excited (11). Tissue autofluorescence in liver, kidney and lymphoid tissue was highest with the 482 nm excitation, followed by the 560 nm, 377 nm and 650 nm, as measured by the exposure time by which the image was acquired. While the instrument produced an image of the autofluorescent tissue in the green, orange and blue emission range, it could not produce an appreciable image in the red wavelength (650 nm), despite exposing the slide to the maximal time allowed (4000 ms (see Fig. 1B). This is consistent with the observed low autofluorescence in the red/far-red range (12). In order to avoid tissue autofluorescence, we exploited the differential between specific- and non-specific emission, by choosing specific nuclear DNA abundant staining vs non-specific background. In the UV channel, DAPI bound to DNA increases twentyfold its emission. The large amount of fluorescent DAPI molecules and the low background autofluorescence allows a good visualization of the nuclei. We then selected another DNA-binding molecule, Sytox Green, which is excited at 482 nm, emits at 504/523 nm and increases 1000-fold its fluorescence upon DNA binding. The differential visualization of the nuclei vs the background was even better, considering that the nuclear images were obtained in the most autofluorescent channel (see Fig. 1B).

LDS 751 is characterized by a very large Stokes shift (excited at 488/543 nm, emits at 712 nm) and enhances twentyfold its fluorescence upon DNA binding, therefore an interesting potential nuclear dye substitute, potentially adding a fifth channel to the existing four. However, despite the promising spectral characteristic, we found it unsuitable because on FFPE material, it binds to nuclear and cytoplasmic structures, stains fibrous tissue and bleeds into the green, orange and red channels, enough to raise the background signal and limiting the sensitivity of other specific fluorochromes. We found this dye a valuable cytoplasmic counterstain in frozen sections (not shown), where is known to label mitochondria (13).

Fluorochrome and filter optimization
In order to achieve maximal efficiency of the fluorescence elicited by a given filter combination, we used Searchlight (http://searchlight.semrock.com/), the online resource tool which estimate several parameters resulting from the combination of excitation and emission filters and fluorochromes. Assuming that the UV channel, no longer occupied by DAPI nuclear counterstaining, may be available for UV-emitting fluorochromes, we noticed that the default Aperio quad filter set was too narrow to optimally excite a number of fluorochromes in that range. We changed the excitation filter to a 377±50 nm filter, better suited for the biphasic absorption peaks of Alexa Fluor® 405 or BV421 (14) (see Fig. 1A). Despite the nominal gain in emission expected by broadening the excitation of these two dyes (Alexa Fluor® 405: from 1.1x1^{-7} mW with a 387/11 filter to 5.1x1^{-7} mW with a 377/50 filter), indirect immunofluorescence in the UV channel remained optimal only for very bright antigens. In addition, we could visualize upon long exposures (above 100 ms) a tail of the SYTOX Green fluorescence, this not expected from the published spectra (not shown).

Alexa Fluor® 488, FITC and Sytox Green were optimally excited with the available filter combinations. In the Orange channel, Rhodamine RedX had a considerable advantage, compared e.g. to Dylight 549 or Alexa Fluor® 568, when excited by the 560 nm filter (Dylight 549: 5.7x1^{-7} mW vs Rhodamine RedX: 1.2x1^{-6} mW).

Despite the fact that the light source excitation cuts off at 675 nm, Alexa Fluor® 647, Alexa Fluor® 660 and Alexa Fluor® 680 were all optimally excited by the 650 nm excitation filter. These three dyes were also excited, to a various extent, by the orange 560 nm filter, which made them bleed considerably in the orange detection filter combination, because of the quadband configuration. To avoid this, we added a Cy3.5-type, single band 620 nm emission filter, which allows the separate collection of orange (Rhodamine Red-X, Alexa Fluor® 568, DyLight 549 dyes) and red emission (Alexa Fluor® 647, 660, 680).

**Sensitivity**

To test the sensitivity of the configuration, comprising filters and fluorochromes, we selected the most favourable set, e.g. Alexa Fluor® 660 conjugates in the channel where autofluorescence was nil. We used a mixture of QIFIKIT stained beads and DAPI stained cell nuclei to focus and visualize both. The bead lot we used contained five 5 µm diameter bead population, expressing 2.500, 13.000, 63.000, 181.000 and 525.000 mouse IgG molecules per bead. When exposed to the maximal time allowed by the machine default, all the bead populations could be visualized, thus the sensitivity of detection of a simple indirectly stained antigen is about 2.500 antibody binding molecules per 5µm bead. (Fig 2) With similar preparations, background emerged in the orange and green channels, even on negatively stained beads, reducing the sensitivity of detection around 10.000 molecules/bead. The sensor is reported to have a linear response in that range (K. Gustashaw, personal communication), thus it may be used to directly quantify molecules in the tissue, provided that a discrete quantitative reference system is in place.

**Diagnostic applications**

To demonstrate the usefulness of multiple immunofluorescent staining of routine material, we selected unused blank sections from routine cases and stained them. One combination consisted of two mouse monoclonal antibodies (mouse IgG2a CD20/L26 and IgG1 Ki-67/MIB1) and a rabbit polyclonal antibody (CD3). In one single WSI, the pathologist could identify T and B cells and estimate the proliferative fraction of each (not shown). In a second multiplexed stain, a pigmented skin lesion was stained with a mouse IgG1 Ki-67/MIB1, a mouse IgG2a p16(CDKN2A)/JC8 and a rabbit anti S-100 (Fig. 3). Nevus cells stained positively for S-100 and p16, but none was proliferating, thus confirming a diagnosis of benign lesion. By toggling and combining the four color images in the image viewer...
application (ImageScope, Aperio), the pathologist can selectively visualize all pigmented cells (S-100+) and evaluate whether they express p16 and/or Ki-67, comparing them with internal controls. In both cases, the little tissue autofluorescence allowed the use of DAPI as nuclear counterstain.

DISCUSSION

We have replicated and confirmed all the points made by DY Mason and colleagues in year 2000 (1): double (and triple) labeling is feasible on routinely fixed sections, autofluorescence is not a major obstacle, the images obtained on fixed and embedded sections are often superior to the ones obtained on frozen sections, and the multiple IF technique is required to demonstrate antigens with overlapping subcellular distribution.

In addition, we propose a method to avoid tissue autofluorescence, if present, which avoids chemical treatment of the tissue (11), by shifting the nuclear counterstain to the most autofluorescent channel. A minor addition to David Mason’s contribution is the demonstration that the number of individual separate colors that can be used on routine material, without using spectral technology and Quantum Dots, may be four. The most interesting is the red fluorescence channel (emission >650 nm) because of its very low autofluorescence signal, thus a great sensitivity potential.

The ScanScope FL we have used contains a 6-position motorized excitation filter wheel and a 6-position motorized filter cube turret, allowing for greater flexibility in the choice of filters. This scanner acquires each fluorochrome channel sequentially, by employing the optimal excitation and emission filters. This is different from a flow cytometer, in which a beam of light simultaneously shines on all fluorochromes, and acquires all of the channels at once.

The combination of independent filter wheels and sequential acquisitions allows for even greater flexibility and the use of duochromes, such as Pe-Cy7 (15) and polymeric fluorochromes (14), and may increase the number of separate antigens detectable.

But the most intriguing results we have achieved, 13 years after that paper, is that a change in technology may allow a widespread use of what was proposed a while ago.

Virtual slides are viewed on the computer screen, differently from glass slides which are observed with a microscope. One of the consequences of the virtual slide technology is that the platform for observation in transmitted light and in immunofluorescence is the same, i.e. the pathologist’s computer screen. In addition, with the immunofluorescent slide being a whole slide, the pathologist can browse it in an identical fashion, comparing it to a routinely stained section. Being a WSI, the stains do not fade and the pathologist can select the area he wants to examine, without being limited to representative snapshots taken by somebody else in a dark room, unaware of what the H&E looks like. Furthermore, unlike double IHC, because of the total separation of colors, image analysis is much more straightforward and more complex analysis such as image cytometry can be applied. At least four scanner companies (Aperio, Leica, Hamamatsu and 3DHistech) plus other traditional microscope-based instruments now offer IF WSI capability.

Our future efforts will be twofold: aimed at standardizing antibody combinations and incubation conditions, so that the production of an optimally stained WSI is produced in a time frame competitive with the machine-stained routine IHC. Secondly, the suggestion that an IF WSI may produce quantitative measurement of antibody binding capacity in the tissue with little autofluorescence may open routine quantitation of relevant targets for therapy.
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FIGURE LEGENDS

Figure 1. **Optical filters and fluorochrome spectra.**
Filled peaks represents excitation (dashed) and emission (full line) spectra for Alexa Fluor® 405 or BV-421 (blue), Sytox Green (green), Rhodamine RedX (orange), Alexa Fluor® 660 (violet) and 680 (pink). The empty boxes, colored accordingly, represents excitation range for the respective filters in the nm range listed below. The black four-box set represents the emission spectra of the Quadband filter. The dark orange box located around 600-650 nm represents the emission range of the Cy3.5 filter. Dichroic mirror spectra are not represented for clarity. The image is obtained with the Searchlight webtool (Semrock).

B. **Tissue autofluorescence analysis.**
Four representative portions of WSI obtained from a fixed and embedded liver section, counterstained with Sytox Green and exposed for the time shown below each image. Each image is positioned below the spectral range at which was acquired (excitation at 387, 485, 560 and 650 nm; acquisition with a quadband 440/521/607/700 nm filter, respectively). A magnification of the nuclei is shown in the inset. Note the absence of autofluorescence in the green and red channel. Original magnification: 20x.

Figure 2. **Sensitivity measurement of the Aperio FL scanner.**
QIFIKIT beads stained with a Alexa Fluor® 660-conjugated antibody are shown (negative image). Five different intensity groups are shown, labeled from A to E. The same preparation was run on a FlowCytometer and the the histogram (shown in the inset) contains the same five populations, plotted on a log scale. For quantities on each population, see text. Cell nuclei in the slide, DAPI stained for focusing, are not shown. Original magnification 20x; each bead measures 5 µm in diameter.

Figure 3. **Four color immunopathology of a pigmented skin lesion.**
A whole slide, containing a pigmented lesion, is stained in quadruple immunofluorescence. Top left is a composite image of the four stains: p16 is pseudo-colored in white, S-100 in green, Ki-67 in red and the nuclei in blue. To better appreciate p16 and Ki-67 expression, only these two and DAPI are displayed in the top right image and in a magnified view (middle right). At the middle left is shown the entire WSI as it appears on the ImageScope screen; the area magnified in the composites above is represented by a white rectangle. The four single grayscale images are listed at the bottom. Note that a portion of the benign S-100+ nevus on the leftmost part do not stain for p16. No proliferating (Ki-67+) p16+ and/or S-100+ cells are seen. Original magnification 20x.
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