Eight markers, multiplex immunofluorescent staining with the NanoZoomer S60

(Multiple of 4 immunostains, DAPI and autofluorescence subtraction)

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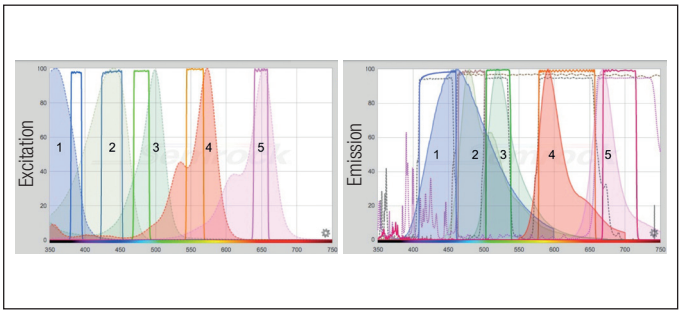
Today's pathology practice calls for an ever-shrinking size of tissue samples and an expanding need for immunostains. Fine needle biopsies are becoming the norm for diagnosis, staging and therapy. Cell block preparation is required to optimize the cytological diagnosis from fine needle aspirations. In solid tumors, such as lung cancer, there is a need to balance the necessity to save precious material for extractive molecular tests (EGFR) and the assessment of in-situ protein targets for advanced, personalized therapy aimed at activated oncogenes (ALK, BRAF, ROS), checkpoint inhibitors (PD-1, VISTA, PD-L1), etc. The cancer-driven impulse at immunomodulatory therapy has already impacted on the evaluation of biopsies obtained for autoimmune, inflammatory conditions such as systemic lupus erythematosus (SLE), chronic liver, intestinal and skin diseases, transplant rejection etc. These biopsies are traditionally minute, often unique tissue samples. Single cell analysis by cell suspension is not feasible and a relatively limited number of sections can be obtained in expert hands. Furthermore, formalin-fixed, paraffin-embedded (FFPE) material is almost invariably the only tissue available.

A solution to overcome these constraints is to do multiple stains on the very same section. Current protocols in IHC allow two-three stains on non-overlapping cellular/subcellular targets [1]. There are more choices by using immunofluorescence (IF) stains.

Most IF microscopes are equipped with three IF channels, conventionally named after the prototype fluorochrome DAPI, FITC, TRITC. A fourth channel may be added, Cy5. Traditional microscopes however do not record whole slide images (WSI), limiting the documentation to selected fields, after which the fluorescence on the section fades out and no additional fields can be examined. On the contrary, IF slide scanners record the WSI in multiple channels, allowing a dynamic, complete and retrospective evaluation of any tissue area of choice. In 2015, Hamamatsu Photonics introduced the NanoZoomer S60 brightfield and IF scanner, which can accommodate up to 60 slides. The S60 scanner has two six-filter wheels, one for excitation, the other for emission filters, a three-cube turret and is equipped with a Plan Apochromat Lambda 20x NA 0.75. Objective (Nikon), a Fluorescence Imaging Module L13820 equipped with a mercury lamp (Hamamatsu Photonics), and a ORCA-Flash 4.0 digital CMOS camera (Hamamatsu Photonics).



Choices for multiple IF stains are complicated by several limitations:

- Spectral unmixing of multiple excitation/emission wavelengths within the 350-750 nm span [2] requires ad hoc spectral IF microscopy apparatuses and software and can accomodate no more than 7 colors (including DAPI).
- DNA-barcode or directly fluorochrome conjugated antibody applications [3-5] require custom antibody conjugation, dedicated high NA, high sensitivity optics, multiple cycles of staining and quenching with only two fluorochromes at a time.
- Ion-tagged custom antibodies and in situ MALDI-type instruments can accomodate ~40 markers at 1µm/pixel resolution with a high cost of hardware investment [6].

Most of these techniques i) allow the staining of a single section per round, ii) do not support WSI, iii) reagents and instruments are so costly and time-consuming that staining of multiple single sections is discouraged, or one or all of these combined. Another property of FFPE material is tissue autofluorescence (AF), which has restrained the application of IF for diagnosis or research.

A method to sequentially stain and strip an FFPE routinely processed section has been published [7]. This method employs widely available primary unconjugated and secondary IF antibodies, double indirect IF staining and digital tissue AF subtraction [7]. By carefully selecting primary antibodies produced in various species and/or of different immunoglobulin isotypes (e.g. Rabbit Ig + Mouse IgG1 + IgG2a/b + IgG3 or Rabbit + Mouse + Rat + Goat Ig) the full extent of the IF span of the S60 can be exploited.

Four primary antibodies from one of the above mentioned selections can be visualized and acquired, in addition to the acquisition of the DAPI nuclear counterstain and the tissue AF for background subtraction. One single FFPE section is all that is required, different from a spectral unmixing acquisition, where for every desired wavelength a corresponding section is necessary for spectral identification and AF subtraction. To attain this goal, the standard S60 setting was modified to accomodate DAPI, BV480, FITC, TRITC, Cy5 and AF, as shown in Figure 1.

Figure 1: The composite panel represents the excitation filters and fluorochrome spectra (left) and the emission filters, dichroic mirrors and fluorochrome spectra (right). Excitation spectra are represented by a dashed profile, emission spectra by a solid profile. The filter profiles are represented by solid lines, the dichroic ones by a dashed line. 1: DAPI (359/461) [exc/em]; 2: BV480 (437/478); 3: Alexa 488 (499/519); 4: Rhodamine RedX (570/590); 5: Alexa 647 (652/668).

The filter combination depicted are DAPI: 387/11 – 435/40 [exc/em], BV480: 438/24 – 483/32; FITC: 480/17 – 520/28; TRITC: 556/20 – 617/73; Cy5: 650/13 – 694/44; AF: 438/24 – 617/73. The dichroic mirrors are: FF403/497/574-Di01 (triband), 458-Di02 and FF655-Di01.

Alexa® dyes are a Life Technologies patent. BV480 dye is a BD Biosciences patent. The spectra images are obtained with the Searchlight Semrock web application.

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To address the immune environment and the tissue architecture of SLE nephritis on FFPE renal biopsies, we set up an 8-marker panel in IF, inclusive of T cells (CD3), B cells (CD20), monocyte/macrophages (CD68, CD163), plasmacytoid dendritic cells (pDC) (IRF8), endothelium (CD34), interstitial or glomerular fibroblasts (CD248) and interferon response genes (MX1). The antibody clone, the Ig specific and the secondary IF reagent are shown in Table 1. This panel was divided into two four-marker rounds, with one stripping cycle in between. For stripping we used the beta Mercaptoethanol/SDS protocol [7]; however, a friendlier protocol has been published [7], based on non-toxic compounds. CD34 and CD248 stain non-overlapping stromal components in the interstitium and define the fine architecture of the glomerulus (Fig 2). In the presence of an autoimmune inflammation, MX1 is induced upon interferon signaling.

The immune infiltrate, both inside and outside the glomerulus is defined in its components (T, B, pDC, Mono/Mac) and the distribution relative to each other and the kidney structure (Fig 2). A complete 8-marker panel can be accomplished in three day staining and stripping cycles; up to 40 individual cases can be processed for this multiplex panel in a working week. The ability to perform 8 established stainings, on multiple individual single sections, with a turnaround time compatible with clinical needs, brings this technique close to a clinical application of the multiplex IF staining with the Nanozoomer S60. No other instrument/reagent combination available today can accomplish this task.

For research, sections stained with the 8-marker panel can be safely stored at -20 deg. C. in buffered glycerol [7] and sequentially stained and stripped in excess of 10 times (> 44 markers), bringing this technique up to high-plex IF staining ([7] and Bolognesi MM et al, in preparation).

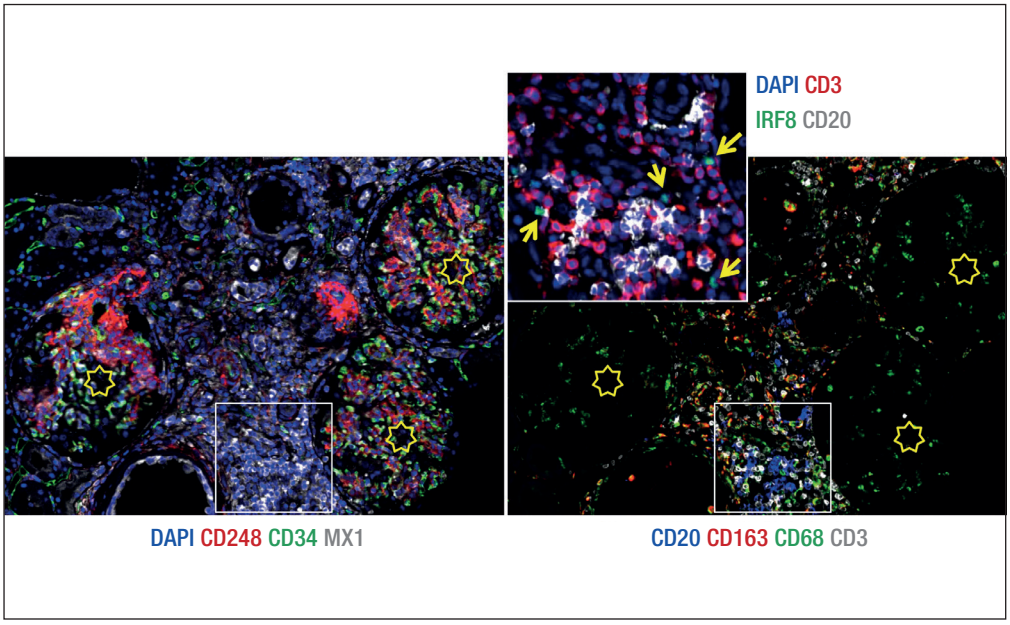

Figure 2: Eight markers (+DAPI) immunostaining of a renal biopsy with SLE. A selected field from a WSI is imaged as RGB+white composites.

Left: the image depicts three glomeruli (stars) containing CD248 mesangium (red) and CD34 endothelium (green). Sparse interstitial fibroblasts (red) and capillaries (green) are seen. MX1 (white) is induced in tubules, interstitial inflammatory cells and the endothelium of a vessel (lower center).

Right: the image shows macrophages (CD68, green) and histiocytes (CD163, red), admixed with CD20+ B cells (blue) and CD3+ T cells (white). CD68+ CD163- phagocytes predominate inside the glomeruli. The inflammation area in the white square, enlarged in the inset, shows IRF8+ plasmacytoid dendritic cells (green, arrowed) admixed with T cells (CD3, red) and B cells (CD20, white). DAPI (blue) counterstain. AF has been subtracted from individual grey level images before RGB merging.

Table 1

Primary and secondary antibodies used*

Antibody	Clone	Species	Isotype	Secondary Ab	Fluorochrome
CD3	-	Rabbit	Ig	Gt a Rb	Rhodamine RedX
CD20	L26	Mouse	IgG2a	Gta Mo IgG2a	BV480
CD163	10D6	Mouse	IgG1	Gt a Mo IgG1	Alexa 488
CD68	PGM1	Mouse	IgG3	Gt a Mo IgG3	Alexa 647
MX1	-	Rabbit	Ig	Gt a Rb	Alexa 488
CD248	B1/35	Mouse	IgG1	Gt a Mo IgG1	Alexa 647
IRF8	E9	Mouse	IgG2b	Gta Mo IgG2b	BV480
CD34	3A1	Mouse	IgG3	Gt a Mo IgG3	Rhodamine RedX

*Secondary antibodies are from Jackson Immunoresearch

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