Equivalence of Imaging Mass Cytometry and Immunofluorescence on FFPE Tissue Sections

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Introduction

Immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) sections plays a significant role in sample analysis in the areas of oncologic pathology, neuropathology, hematopathology and post mortem pathology. Recently, a handful of IHC-based diagnostic assays for biomarkers of cancers have been FDA-approved [9]. With the current developments of IHC tools and methods, it is reasonable to expect more use of this technique in clinical research and potential translation to the clinic. The availability of IHC methodologies capable of generating robust multiplexed, quantitative data would allow users to gain maximum information from tissue of limited availability such as patient biopsies.

Immunohistochemical staining can be a direct or an indirect process. The direct method is a rapid one-step staining method and involves a reporter-coupled antibody reacting directly with the antigen in the tissue sections. The direct conjugate procedure has the advantages of rapidity and ease of use. The two-step indirect method uses a labeled-conjugated secondary antibody directed against the unlabeled primary antibody. Although more time-consuming, indirect IHC provides more flexibility and signal amplification. It is the most common method in routine pathology practice.

Standard IHC labels include enzymatic (chromogenic immunohistochemistry) and fluorescent (immunofluorescence) moieties. Chromogenic detection is based on the activities of enzymes, most often horseradish peroxidase or alkaline phosphatase, which form colored, insoluble local precipitates upon the addition of substrate. The advantage of chromogenic IHC is the resulting permanent stain, compatible with histological dyes such as hematoxylin. The detection of multiple targets by chromogenic IHC is very challenging because of the limited choices of different enzymes and substrates. There is also the potential for chromogenic overlap and the significant risk of one color obscuring another. Variations of multiplexing staining methods, such as “stain-erasing” (involving successive staining and erasing cycles), can increase the number of targets that can be visualized simultaneously [8]. However, these methods are laborious and ineffective, so in practice, chromogenic multiplexing is limited to two targets. Beyond this relatively low multiplexing ceiling, chromogenic IHC is difficult to quantitate because of nonlinear optical effects and the enzymatic nature of the detection.

Immunofluorescence (IF) is based on fluorophores that are excited by one wavelength and emit at a longer wavelength (a phenomenon known as a Stokes shift). Fluorescent labels can provide a higher signal-to-noise ratio and a broader dynamic range than chromogenic labels and are more frequently used for simultaneous detection of multiple targets. A multitude of labels are available for IF applications, such as Alexa Fluor®, cyanine dyes and fluorescent quantum dot nanocrystals, which have narrower emission peaks when compared to standard fluorophores [7]. Multiplex studies are nevertheless challenged by the broad emission spectra of most organic fluorochromes, leading to overlap or crosstalk between channels. For appropriate results, the user must select combinations of dyes that have distinct, non-overlapping emission spectra, allowing visualization of four or five fluorophores simultaneously with standard equipment [8]. Post-processing of the signal to remove overlapping signal by spectral unmixing can expand the number of multiplexed fluorophores to eight [2]. This number can be further increased by using iterative staining and other variations of multiplex staining methods [5]. The linear nature of fluorescent signals is compatible with quantification, although autofluorescence of FFPE tissue sections and signal fading can affect the accuracy of the results [7].

A novel technique called imaging mass cytometry (IMC) uses metal-conjugated antibodies instead of fluorescent or enzymatic moieties [1, 3, 4]. The Fluidigm Hyperion™ Imaging System allows simultaneous detection of up to 50 parameters in a single tissue while eliminating the common challenges of conventional IF such as signal fading, spectral overlap and autofluorescence (see Table 1). The technique involves laser ablation followed by transfer of the ablated materials to the mass cytometer for time-of-flight detection of the metal ions. The technique allows us to gain spatial information, providing delineation of cell subpopulations and cell-to-cell interactions with further data interpretation and image analysis. The purpose of the experiments presented here is to compare IF and IMC techniques and to demonstrate their equivalency in human FFPE sections.

Materials and Methods

Tissue Section Preparation

FFPE tissue sections of human lymph node, breast and spleen were obtained from US Biomax (Cat. Nos. LYM181, MC245a) and human tonsil sections from Sigma-Aldrich (Cat. Nos. 104S, 168S, 275S). Slides were heated at 60 °C for two hours, then dewaxed in xylene and rehydrated in descending grades of ethanol. Antigen retrieval was performed at 95 °C for 10 minutes in basic Retrieval Solution (R&D Systems Cat. No. CTS013). Slides were left to cool at room temperature for 20 minutes, then successively washed in Maxpar® Water (Fluidigm Cat. No. 201069) and Maxpar® PBS (Fluidigm Cat. No. 201058) for 8 minutes each. Tissues were blocked in Maxpar PBS, 3% BSA in a humid chamber for 45 minutes, prior to IMC or IF staining.

IMC Staining

For imaging mass cytometry detection, slides were stained with assay-dependent concentrations of metal-tagged antibodies (Table 2–5) in 1X Maxpar PBS, 0.5% BSA at 4 °C overnight in a humid chamber. Slides were then rinsed twice in 1X Maxpar PBS, 0.2% Triton™ X-100 and twice in 1X Maxpar PBS for 8 minutes each with gentle agitation. Following a rinse in Maxpar Water during five minutes, tissues were stained with 0.3 µg/mL Cell-ID™ Intercalator-Ir (Fluidigm Cat.No. 201192A) in Maxpar PBS for 30 minutes at room temperature. The samples were then rinsed for 5 minutes in Maxpar Water and air-dried.

IF Staining

Fluorescent labeling of human FFPE sections followed the same staining protocol as described above, but instead of using metal-tagged antibodies, unlabeled antibodies or preconjugated fluorescent antibodies of the same clone were used following manufacturer-recommended concentrations (CD45 clone 2B11, eBioscience® Cat. No. 14-9457-82; CD20 clone H1, BD Biosciences Cat. No. 555677; CD68 clone KP1, BioLegend® Cat. No. 916104; CD3 polyclonal, Dako™ Cat. No. A0452; CD8a clone D8A8Y, CST Cat. No. 85336S; CD4 clone EPR6855, Abcam Cat. No. ab133616; Ki-67 Clone B56 Alexa Fluor 647, BD Biosciences Cat. No. 558615; pan-keratin clone C11 Alexa Fluor 488, Cell Signaling Technology™ (CST) Cat. No. 4523S; beta-catenin clone D13A1, CST Cat. No. 8814S; histone H3 clone D1H2, CST Cat. No. 4499S; vimentin clone RV202 Alexa Fluor 488, BD Biosciences Cat. No. 562338). Following overnight incubation at 4°C with the primary antibodies, the slides were washed twice in Maxpar PBS, 0.2% Triton™ X-100 and twice in Maxpar PBS for 8 minutes each. Detection of the unlabeled primary antibodies was performed using fluorescent-tagged secondary antibodies at 5 µg/mL (Donkey anti-Mouse DyLight 650, Thermo Fisher Scientific Cat. No. SA5-10169; Donkey anti-Rabbit DyLight 488, Thermo Fisher Scientific Cat. No. SA5-10038; Donkey anti-Rabbit DyLight 650 Thermo Fisher Scientific Cat. No. SA5-10041) incubated for 30

minutes at room temperature, protected from light. Slides were washed twice in Maxpar PBS, 0.2% Triton™ X-100 and twice in Maxpar PBS for 8 minutes each, then stained with 2 µg/mL DAPI for 30 seconds. After a final wash in Maxpar PBS, fluorescently labeled tissues were mounted with coverslips using VECTASHIELD® (Vector Laboratories Cat. No. H-1000) and sealed with clear nail polish.

Image Acquisition

Fluorescent images were acquired using a Leica® Dmi8 inverted microscope equipped with Leica FITC, Cy5® and DAPI filter sets. The images were captured with a Leica DFC365 FX camera using Leica Application Suite X.

For IMC image acquisition, immunostained and dried tissue slides were inserted into the ablation chamber of the Hyperion Imaging System, where a pulsed 200 Hz laser is focused over a user-defined region of tissue, ablating adjacent spots in 1 µm steps as the slide moves under the laser beam. The plumes of vaporized material are streamed by inert gas into the inductively coupled plasma of the Helios™ mass cytometer, where they are atomized, ionized and detected by a state-of-the-art time-of-flight mass spectrometer. Detected ions are then spatially assigned and pseudo-colored images are created that are identical in appearance to standard immunofluorescence images.

Results

The preparation and the staining of a section for an IMC experiment use the same protocols as for IF microscopy (Fig. 1). Once labeled, the tissue section is washed with high-purity water (Maxpar Water) and allowed to air-dry. The slide is then transferred to the laser ablation chamber of the Hyperion Imaging System for image acquisition.

To demonstrate the equivalency between IMC and IF, serial FFPE sections of various human tissues (normal or pathological) were directly stained with metal-conjugated primary antibodies for IMC. For IF, FFPE sections were either directly stained with fluorescent-labeled antibodies or stained stepwise with the purified primary antibody followed by fluorescent-labeled secondary antibody (see Materials and Methods

CD45, a general marker of immune cells, shows a widespread expression pattern in normal lymph node by immunofluorescence (Fig. 2, upper left). Imaging mass cytometry staining performed on the succesive section shows a comparable expansive tissue expression (Fig. 2, upper right). CD20, a common marker of the B cell lineage, has a dictinct distribution in the lymph node follicles where it is mostly concentrated in the germinal center. CD20 displays identical expression patterns in both IF and IMC samples, with

expression restricted to germinal centers and in a few cells scattered in the cortex of diffuse B-cell lymphoma tissue (Fig. 2, bottom).

Using normal tonsil tissue sections, we compared IF and IMC staining to identify different immune cell populations (Fig. 3).The tissue distribution of the various markers analyzed is equivalent in both IF and IMC experiments. CD68 is found in cytoplasmic granules of various cells of the macrophage lineage including monocytes, giant cells, and Kupffer cells. The IMC method clearly delineates the CD68-positive tingible body macrophages, responsible for removing debris of apoptotic cells in the germinal center. A corresponding IF experiment shows similar results. CD3 labels all T lymphocytes enriched in the diffuse lymphoid tissue of the tonsil. The cytoplasmic expression of CD3 in the expected areas of the tonsil is clearly shown by both IF and IMC. The expression of CD8a, marking cytotoxic T lymphocytes, shows the same discrete distribution between follicles on serial tonsil sections stained by IMC and IF. Similarly CD4, expressed by T regulatory and T helper cells, displays identical staining patterns by IMC and IF in tonsil.

IMC can also accurately identify tissue types and subcellular locations on FFPE sections. On tissue sections obtained from breast adenocarcinoma, cytokeratins and beta-catenin are respectively detected in the cytoplasm and membrane of breast epithelial cells, as confirmed by IF (Fig. 4). In the spleen, both IF and IMC experiments display the mesenchymal marker vimentin in the endothelial cells lining the sinuses (Fig. 4). Histone H3, as expected, is detected in all nuclei of the lymph node (Fig. 5, top). In the tonsil, Ki-67 is expressed in the nuclei of proliferating cells, mostly abundant in germinal centers, as shown by IMC and IF (Fig. 5, bottom).

Summary

The data demonstrate that imaging mass cytometry allows the detection of protein targets and provides general architectural details on FFPE sections of both normal and cancerous tissues. The resolution of the IMC permits the visualization of proteins in the membranous, cytoplasmic and nuclear cell compartments. The benefit over traditional IHC techniques is the ability to multiplex over 50 markers in a single experiment, enabling high-content analysis of human tissue with fewer tissue samples required. The IMC staining method follows a workflow similar to traditional IF staining and generates comparable results while eliminating issues such as autofluorescence and spectral overlap. In addition, IMC generates a linear signal over a much higher dynamic range than immunohistochemistry or immunofluorescence in a format that readily allows for quantification [4]. Comprehensive tissue cell segmentation can also be performed using Definiens® Developer XD platform for digital pathology [3, 4]. Users will thus be able to perform deep analysis of samples in limited supply (needle biopsies, small tumor tissue samples, mouse embryo development studies, etc).

References

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Table 1. Comparison of different immunostaining methods

	Chromogenic Immunohistochemistry	**Immunofluorescence**	**Imaging Mass Cytometry**
Number of markers identified per cell	2–3 identified simultaneously	3–6 makers identified simultaneously	Up to 40 co-localized markers simultaneously
Signal linearity	Chromogen absorbs light and scatters non-linearly.	Signal intensity is linear with fluorophore quantity.	Signal intensity is linearly proportional to number of metal-tagged antibodies.
Characteristics of absorption, emission spectra width	Chromogenic absorption spectra are wide.	Fluorophore emissions have spectral overlap	No overlap between mass channels
Signal imbalance /quantitation	Chromogen intensity relies on duration of enzymatic reaction, substrate concentration, and reaction conditions.	Difference in signal intensity between fluorophores can be more than 10-fold and impedes signal unmixing and quantitation.	Detected signal is generated from total amount of ablated tissue allowing absolute quantitation.
Importance of reliable cell segmentation and phenotyping for quantitation	Hematoxylin stain is often obscured by chromogenic stain, resulting in poor cell segmentation and unreliable quantitation.	Reliable nuclear stain with DAPI supports good image segmentation.	Multiple markers are used for nuclear and membrane images resulting in high quality cell segmentation and quantitation.
Logs dynamic range	1–2	2–3	3–4
Photobleaching	N/A	Highly problematic; avoided by TSA® Plus Opal system	None; long term stained sample archiving is possible
Autofluorescence	N/A	Significant	None
Sample type	5 µm FFPE; 10 µm cryosections	5 µm FFPE; 10 µm cryosections (for IF only, not TSA Plus Opal); cells immobilized on slides	5 µm FFPE; 10 µm cryosections; suspension cells immobilized on slides

Table 2. IMC reagents used to stain FFPE sections of lymph node

Marker	Clone	Catalog Number	Metal Tag	Working Concentration
CD45	2B11	3152016D	^{152}Sm	10 µg/mL
CD20	H1	3161029D	^{161}Dy	10 µg/mL
Nuclei	N/A	201192A	$^{191/193}$ Ir	0.3 µM

Table 3. IMC reagents used to stain FFPE sections of tonsil

Marker	Clone	Catalog Number	Metal Tag	Working Concentration
CD68	KP1	3159035D	^{159}Tb	10 µg/mL
CD3	Poly	3170019D	^{170}Er	10 µg/mL
CD8a	D8A8Y	3162035D	^{162}Dy	10 µg/mL
CD4	EPR6855	3156033D	^{156}Gd	2.5 µg/mL
Nuclei	N/A	201192A	$^{191/193}$Ir	0.3 µM

Table 4. IMC reagents used to stain FFPE sections of breast and spleen

Marker	Clone	Catalog Number	Metal Tag	Working Concentration
Pan-keratin	C11	3148020D	^{148}Nd	20 µg/mL
Beta-catenin	D13A1	3165032D	^{165}Ho	2.5 µg/mL
Vimentin	RV202	3143029D	^{143}Nd	10 µg/mL
Nuclei	N/A	201192A	$^{191/193}$Ir	0.3 µM

Table 5. IMC reagents used to stain lymph node and tonsil

Marker	Clone	Catalog Number	Metal Tag	Working Concentration
Histone H3	D1H2	3171022D	^{171}Yb	10 µg/mL
Ki-67	B56	3168022D	^{168}Er	10 µg/mL
Nuclei	N/A	201192A	$^{191/193}$Ir	0.3 µM

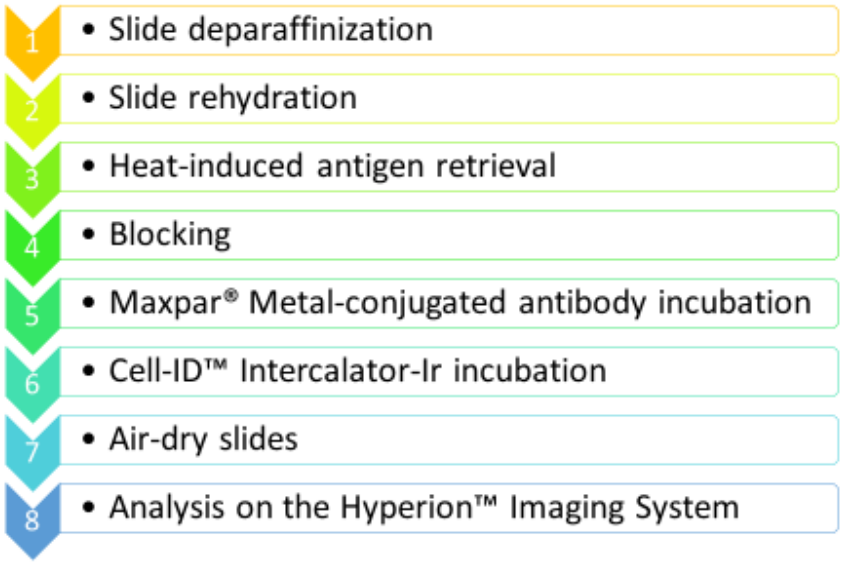


Figure 1. Summary of IMC staining protocol for FFPE tissue sections. An IMC staining experiment follows the same protocol as for IF.

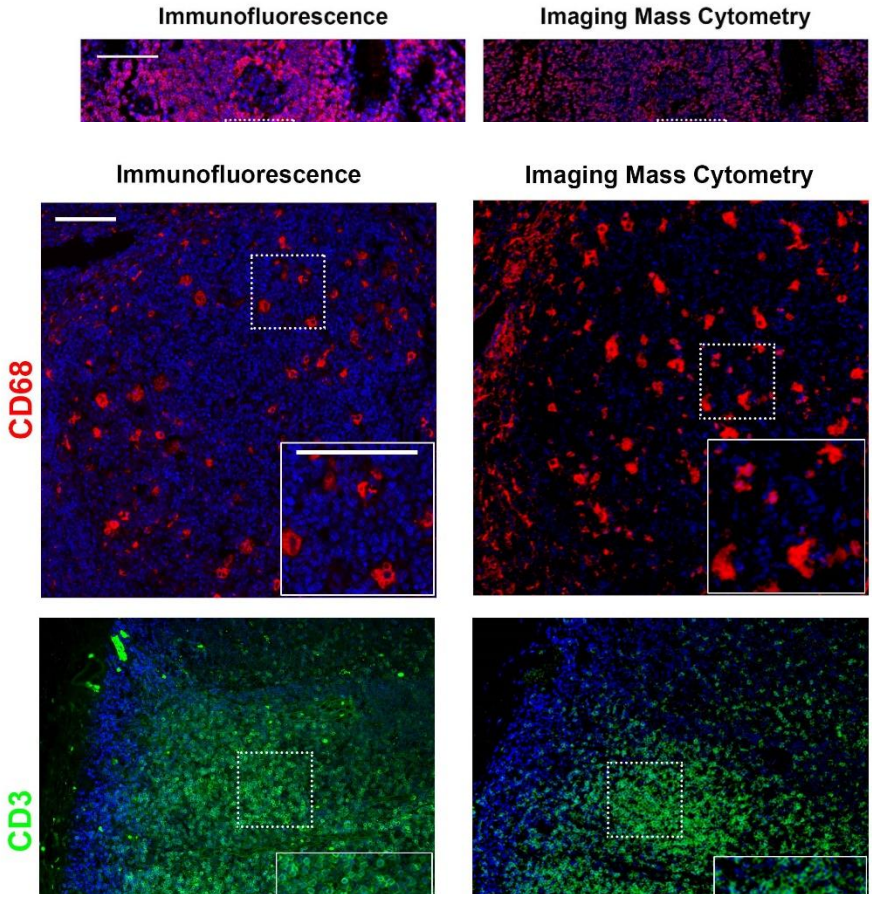


Figure 2. Comparison of IMC and IF on human lymph node. Protein targets were detected in serial sections of normal (upper row) and diffuse large B-cell lymphoma (lower row) lymph node using the same staining procedure and antibody clones (Table 2) for both IF (left) and IMC (right) images. All nuclei are displayed in blue. The dashed squares indicate the original position of the enlarged area displayed in the inset. Scale bars are equal to 100 µm and are shown only in the upper left image but are the same for all images.

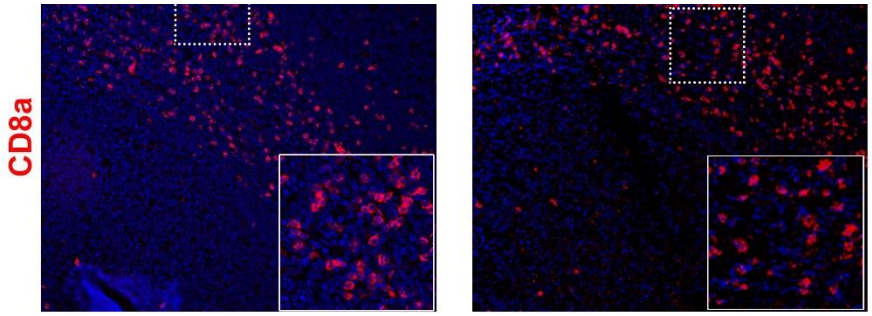
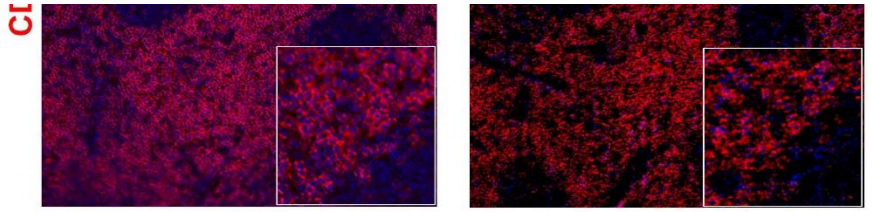


Figure 3 (next page). Comparison of IMC and IF on human tonsil. Protein targets were detected in serial sections of normal tonsil using the same staining procedure and antibody clones (Table 3), as shown in the IF (left) and IMC (right) images. All nuclei are displayed in blue. The dashed squares indicate the original position of the enlarged area displayed in the inset. Scale bars are equal to 100 µm and are shown only in the upper left image but are the same for all images.



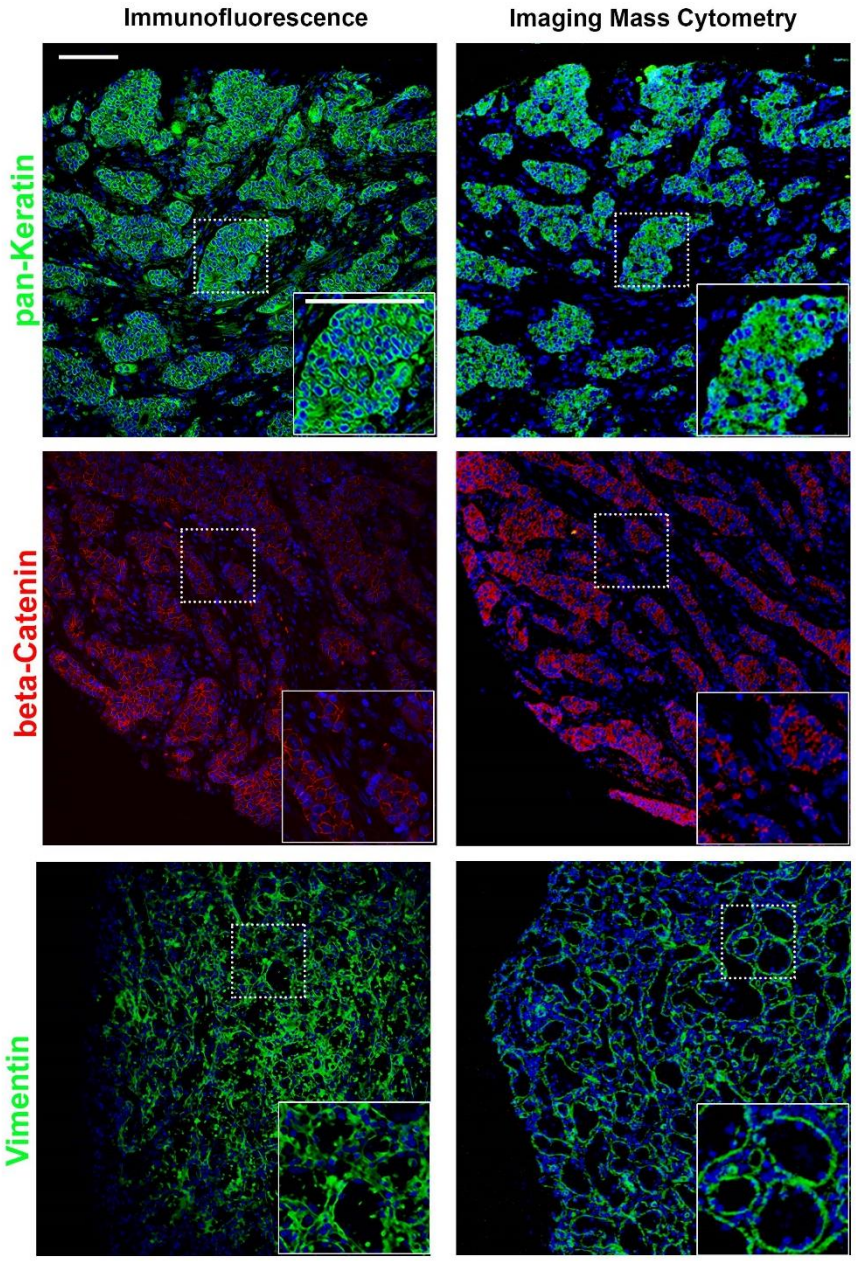


Figure 4. Detection of structural markers on FFPE tissue sections by IF and IMC. Protein targets were detected in serial sections of breast adenocarcinoma (first and second row) and spleen (third row) tissues using the same staining procedure and antibody clones (Table 4), as shown in the IF (left) and IMC (right) images. All nuclei are displayed in blue. The dashed squares indicate the original position of the enlarged area displayed in the inset. Scale bars are equal to 100 µm and are shown only in the upper left image but are the same for all images.

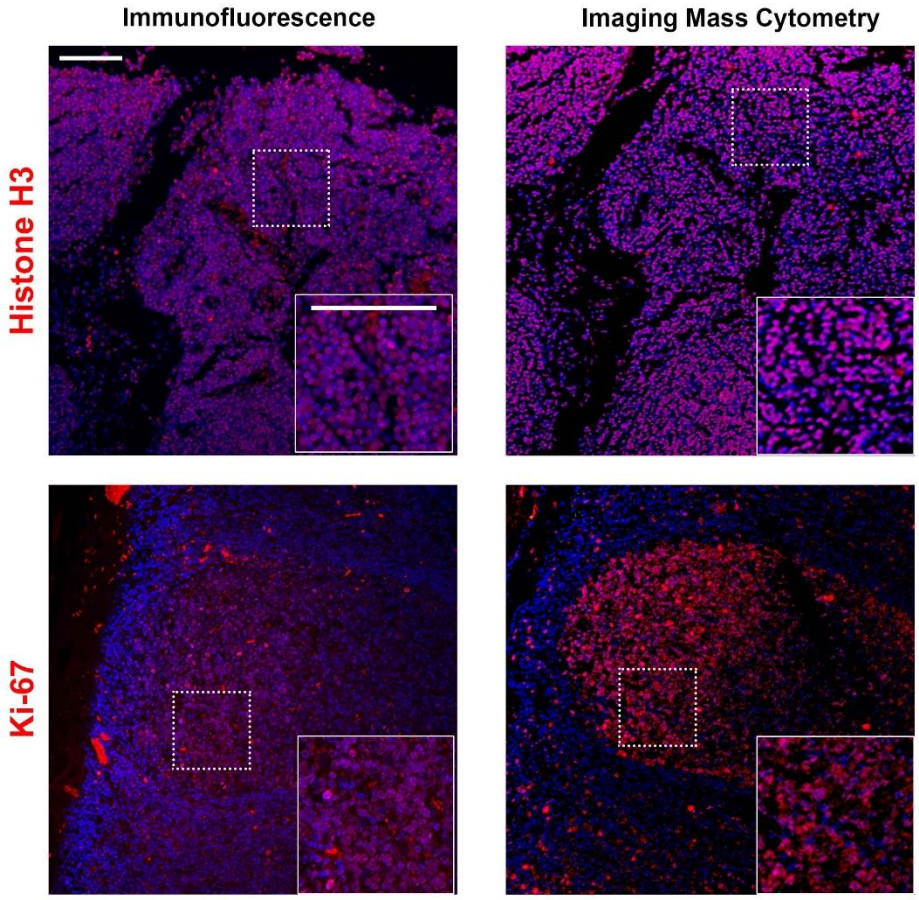


Figure 5. Detection of nuclear markers on FFPE sections by IF and IMC. Protein targets were detected in serial FFPE sections of normal lymph node (upper row) and tonsil (lower row) using the same staining procedure and antibody clones (Table 5), as shown in the IF (left) and IMC (right) images. All nuclei are displayed in blue. The dashed squares indicate the original position of the enlarged area displayed in the inset. Scale bars are equal to 100 µm and are shown only in the upper left image but are the same for all images.