White paper

U DISCOVERY 5-Plex IF procedure: A fully automated immunofluorescence multiplex solution

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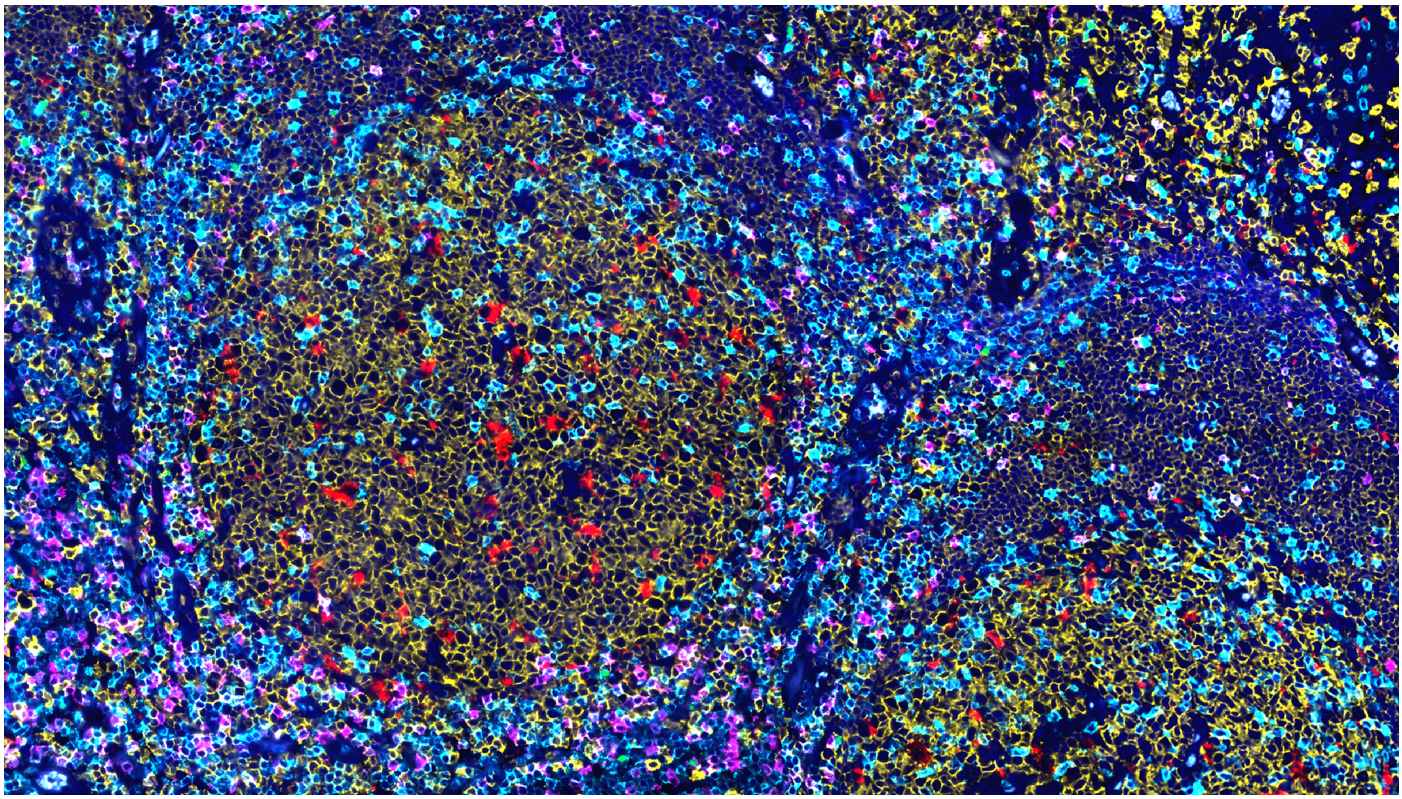

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Abstract

Recent advancement in the field of immunohistochemistry has led to the development of novel fluorescent multiplex technologies. Fluorescent multiplexed immunohistochemistry (IHC) assays inherently possess a higher order of complexities in terms of quality, reproducibility and reliability compared to single-plex IHC. To develop a robust staining platform, the key technical considerations are a) minimizing biomarker cross-reactivity, b) availability of stable detection chemistries, c) balancing the signal across a panel and d) applying standardized imaging parameters.

This paper discusses a fully automated Research Use Only (RUO) multiplexing technology developed for the VENTANA DISCOVERY ULTRA platform that provides flexible, easy-to-use software and stable reagents to enable complex assay development for the research community. The process involves 1-5 rounds of sequential application of unmodified primary antibodies with heat deactivation (HD) steps between each round. The fluorescent dyes in this multiplex procedure are carefully selected for spectral separation and provide optimal staining. The filters are designed to match the dye absorptions in order to minimize bleed-through of neighboring fluorophores. The order of fluorophore application is determined according to the stability of each tyramide-fluorophore to heat deactivation and allows for denaturation of the primary and secondary antibody conjugate, without affecting the detection adequacy. An approach to designing a 5-Plex assay is discussed.

Introduction

Growing awareness of the complexities of cancer and the emergence of personalized medicine necessitates the development of novel tools and technologies for cancer research. Powerful next-generation immunohistochemistry (IHC) platforms such as fluorescent multiplexing IHC technology are fast gaining ground and becoming an essential tool for life science research and the diagnostic market.[1,2,3,4] Unlike other competing technologies such as next-generation sequencing or flow cytometry, multiplex fluorescent IHC is capable of simultaneous detection of multiple biomarkers from a single tissue section without the loss of tissue context. Multiplex fluorescent IHC provides spatial information of complex protein and cellular interplay within the tissues, which is crucial for interrogating disease status and the tumor microenvironment.

Conventional chromogenic IHC, the most widely used methodology to evaluate *in situ* protein expression in tumor tissues, has the technical limitation of interrogating up to 1-3 biomarkers per section, thereby leading to repeated sampling of scarce tissues.[5] Growing demand to extract increasing biomarker information from small tissue samples is the key driver for the development of novel fluorescent multiplexing technologies. However, most of the current multiplexing staining methods available today are either manual or semi-automated. These long and complicated staining processes are usually labor-intensive, time-consuming and prone to human error.[6] The limited throughput hampers the generation of sufficient data from statistically significant sample sizes required for research or clinical trials.

For assay development, a stringent methodology is essential for a reproducible and reliable outcome. Many of the current manual techniques use non-standardized research reagents that affect robust assay development process. Without automation, implementation of an assay and comparison of multi-centric data severely hampers progress. Complexity and the sheer logistics of generating data using currently available technology are hindering the implementation of fluorescent multiplexing to the mainstream research market.

To meet the current challenges, a fully automated Immunofluorescence (IF) multiplexed assay, U DISCOVERY 5-Plex IF procedure, was developed to run on the DISCOVERY ULTRA platform (Roche Tissue Diagnostics). The technology enables simultaneous detection of 1-5 biomarkers on a single formalin-fixed paraffin-embedded (FFPE) tissue section. Stable fluorescent dyes are specifically developed for the assay. The automated staining procedure is paired with the stable fluorescent dyes in modular ready-to-use kits. The development of the assay procedure is described in details in the following pages.

Staining approach

The U DISCOVERY 5-Plex IF procedure is a fully automated multiplex technology developed to run on the DISCOVERY ULTRA platform to enable the detection of 1-5 biomarkers on a single slide. The process involves 1-5 sequential rounds of staining, each by a distinct biomarker. A heat deactivation (HD) step is inserted between each round, that removes the bound primary antibody and horseradish peroxidase (HRP) conjugated secondary antibody complex from the previous round and minimizes downstream cross-reactivity.

The staining procedure consists of 1) binding of an unmodified primary antibody to its target in the tissue section on a slide, 2) binding of the HRP conjugated anti-species antibody to the primary antibody, 3) HRP enzyme-mediated deposition of the tyramide-fluorophore that covalently binds to the tissue at the site of the reaction, 4) heat deactivation of the primary antibody and secondary antibody-HRP complexes, 5) repetition of steps 1-4 with the next biomarker in the queue of 1-5 biomarkers, 6) staining the tissue with DAPI counterstain, followed by 7) coverslipping with appropriate mounting medium. The stained slides are visualized using fluorescent microscopy.

The choice of reagents, sequence of detection kits and the primary antibody concentration play important roles in the optimization of staining quality. A schematic representation of the assay concept using the biomarkers in our model system (CD20, CD3, CD8, CD68 and FoxP3) is shown in Figure 1.

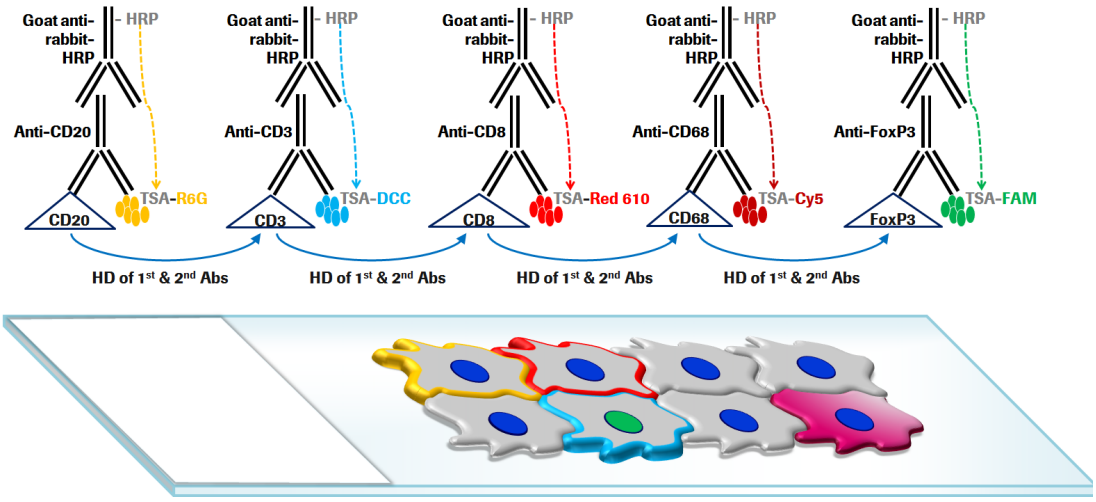


Figure 1: Staining approach for a 5-Plex assay on the DISCOVERY ULTRA platform depicting five rounds of sequential application of unmodified primary antibodies (CD20, CD3, CD8, CD68 and FoxP3), with HD steps between each round. A total of four HD rounds are performed. The detection sequence in the staining procedure is R6G, DCC, Red 610, Cy5 and FAM. Each antibody is paired with one fluorophore in the sequence. The 5-Plex stained slide represents membrane staining of CD20 (Rhodamine 6G: gold), CD3 (DCC: aqua) and CD8 (Red 610: red). The cytoplasmic stain represents CD68 (Cy5: far red) and FoxP3 (FAM: green) nuclear stain co-registers with blue counterstain DAPI.

Background

Development of the U DISCOVERY 5-Plex IF procedure for DISCOVERY ULTRA platform necessitated modifications to some of the basic on-instrument sub-processes to enable a robust automated assay. In a sequential staining procedure, deactivation of the primary antibody and secondary antibody-HRP bound to the first biomarker, prior to the application of subsequent biomarker(s) is critical to reducing cross-reactivity and facilitating downstream image analysis. The process must be effective at removing the bound primary and secondary antibody complexes from the prior round while preserving tissue integrity and the epitopes for the subsequent biomarkers. If primary antibodies from the same species are used, inadequate heat deactivation between cycles might lead to cross-reactivity. The composition of the reagent solutions used (including pH and surfactant), the temperature of heat deactivation process and incubation time can either enhance the staining or have severely deleterious effects on the staining and background.

To standardize this process, many reagent formulations, temperature ranges and durations of the heat deactivation steps were evaluated. It was determined that, for this fluorescent staining procedure, the BenchMark Cell Conditioning 2 (CC2) (Part number 950-123) was the most efficient reagent for deactivation of the bound primary antibody and secondary antibody-HRP while maintaining the integrity of tissue morphology and the subsequent epitopes (data not shown). The optimal temperature for effective heat deactivation was 100°C for a duration of 12 minutes. The heat deactivation process is followed by wash steps to remove the bound and unbound antibodies from the previous round.

Another important parameter for the success of a multiplex technology is the composition, stability and the method of deposition of the fluorophores. The selected fluorophores and the enzyme-mediated covalently-bound deposition method used in the U DISCOVERY 5-Plex IF procedure are designed to withstand components of the bulk reagents and multiple washes needed in the automated staining procedure. In addition, the fluorophores [Rhodamine 6G, DCC, Red 610, Cy5 and FAM] are subjected to multiple rounds of hydrogen peroxide and heat deactivation cycles that could potentially destroy their fluorescent properties. The stability of dyes was assessed by subjecting each fluorescent-stained slide through several rounds (from 0-4) of the hydrogen peroxide and heat deactivation cycles and quantification of staining intensity using internal software tool. The dyes were found to be stable in the order it is implemented in the final staining procedure.

To facilitate assay development and improve ease-of-use, the sequence of the fluorophores in the staining procedure is locked in the following order: 1) R6G, 2) DCC 3) Red 610, 4) Cy5 and 5) FAM. The order of the dyes has been selected to provide optimal stability while minimizing nonspecific staining and background. These dyes are available in ready-to-use pre-filled dispensers and currently have a real-time stability (RTS) of 120 days and ongoing when stored in 2-8°C refrigerator. Detailed information on the dye reagent part numbers is provided in Appendix 1.

The imaging was executed on the upright Zeiss AxioImager M2 and quantitative analysis was performed by an internal software package. A stable light source was implemented for the Zeiss AxioImager M2 system that was capable of standardized illumination at the sample plane with less than 1% variation. The microscopes and scanners were fitted with filter sets specific to the DISCOVERY fluorophores. The excitation/emission filters for imaging of the fluorophores are outlined in Appendix 2.

Staining procedure overview

The staining procedure interface on the DISCOVERY ULTRA platform is intuitive and flexible for customer ease-of-use. The tissue pretreatment options are selectable and include on-instrument slide baking, deparaffinization and cell conditioning process.

The staining procedure was optimized to use five fluorophores in ready-to-use detection chemistry kits (Appendix 1) and a counterstain. Each detection module (R6G, DCC, Red610, Cy5 and FAM; Appendix 3) includes (a) selectable/optional enzymatic digestion, (b) selectable primary incubation parameters (including time, temperature and manual application), (c) selectable anti-species HRP-conjugate (anti-rabbit, anti-mouse and anti-rat) and incubation time(s) and (d) selectable substrate incubation time(s).

The instrument can process up to thirty single-plex to 5-Plex slides in a single run. Depending on the primary incubation time, a 5-Plex assay has a turnaround time (TAT) of 10-12 hours. The "delay" option on the system and the walk-away automation makes it ideal for overnight runs, freeing up the system during the day for flexible workflow options. Appendix 3 provides details of the staining procedure software interface on the DISCOVERY ULTRA platform.

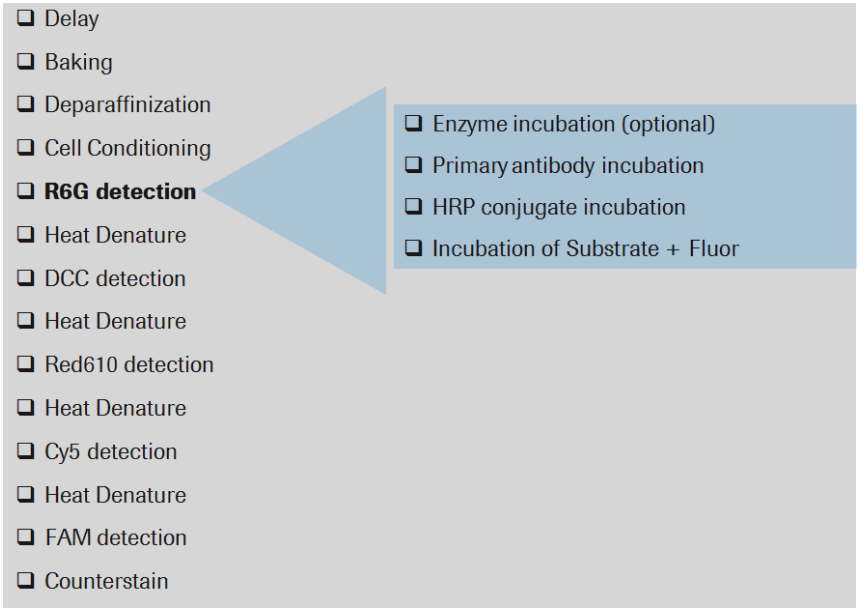
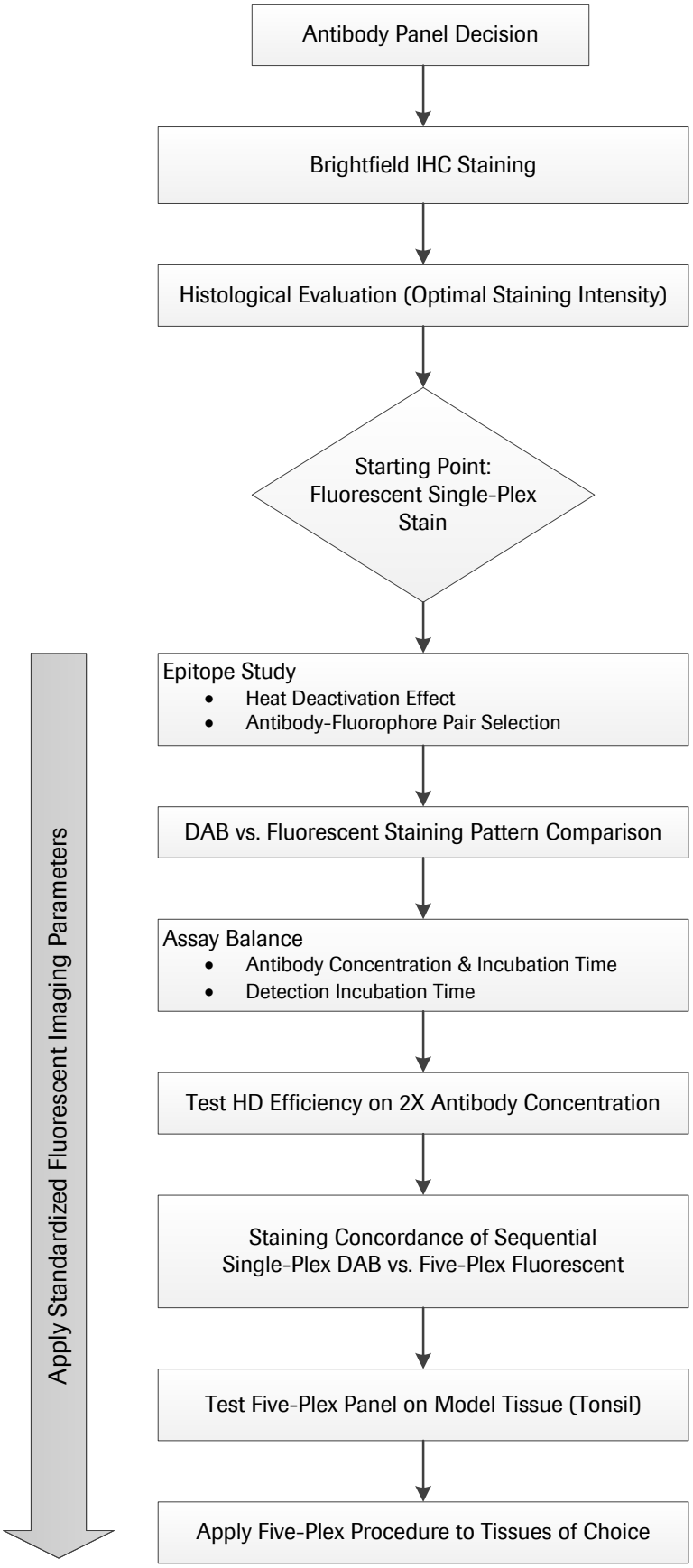


Figure 2. Staining procedure overview with five detection modules: R6G detection, DCC detection, Red 610 detection, Cy5 detection and FAM detection with total of four heat deactivation steps in between. Each detection module has options such as enzyme digestion and incubation choices for primary and secondary antibodies, and detection kits.

Optimizing the 5-Plex assay

The suggested steps toward developing a robust and reproducible multiplex IHC assay are: a) determination of each antibody titration for adequate staining and comparing to single DAB IHC b) evaluation of the effect of heat deactivation on each targeted epitope to decide on the optimal order of staining for each biomarker c) optimization and balancing the fluorophore signal in order to minimize interference of bleed-through and d) standardization of image analysis. Each of these steps was critical to obtaining reproducible and reliable data for research studies. The key steps required to develop a fluorescent multiplexed assay are discussed below, using the following panel of biomarkers as a model: CD20, CD3, CD8, CD68 and FoxP3. The reagents used for this optimization study were obtained from Roche Tissue Diagnostics or Spring Bioscience and a complete list is provided in Appendix 1. Both tonsil and tumor tissues were fixed in 10% Neutral Buffered Formalin and paraffin-embedded.

Workflow for creating a new 5-plex assay



Brightfield IHC staining

To characterize the expression of individual biomarkers, brightfield IHC staining was used as the gold standard. Each biomarker in the panel was first optimized on the DISCOVERY ULTRA platform using the DISCOVERY DAB detection chemistry (Anti-Rabbit HQ, Anti-HQ-HRP and ChromoMap DAB kit: Appendix 1). The epitope unmasking was standardized across all antibodies in the panel according to the package insert recommendations for tissue pretreatment. When developing the staining protocol, the most aggressive epitope retrieval conditions recommended for any of the antibodies in the panel was taken into account. Each antibody was titered to achieve optimal staining intensity (at least 3+), good signal-to-noise, and the best morphology. The DAB stained slides for each biomarker were assessed by a pathologist to evaluate appropriate staining pattern, staining intensity, cellular morphology and cell type. These antibody dilutions and protocol conditions were used as a starting point for the fluorescent staining optimization. The final dilution and DAB staining parameters are listed in Table 1 and Table 2.

Table 1: Protocol parameters for DAB staining

Biomarker	Cell conditioning (CC1) incubation Time (min)	Primary antibody incubation time (min)	Anti-Rabbit HQ incubation time (min)	Anti-HQ HRP-conjugate incubation time (min)	DAB + H_2O_2 incubation time (min)
CD20(SP32)	64	20	12	12	12
CD3(SP162)	64	20	12	12	12
CD8(SP239)	64	20	12	12	12
CD68(SP251)	64	20	12	12	12
FoxP3(SP97)	64	20	12	12	12

Table 2: Antibodies and dilutions

Antibody panel	Spring Bioscience catalog #	Package insert recommendation	Final dilution for DAB
CD20(SP32)	M3324	1:100	1:100
CD3(SP162)	M4624	1:150	1:150
CD8(SP239)	M5394	1:100	1:100
CD68(SP251)	M5514	1:100	1:100
FoxP3(SP97)	M3974	1:100	1:25

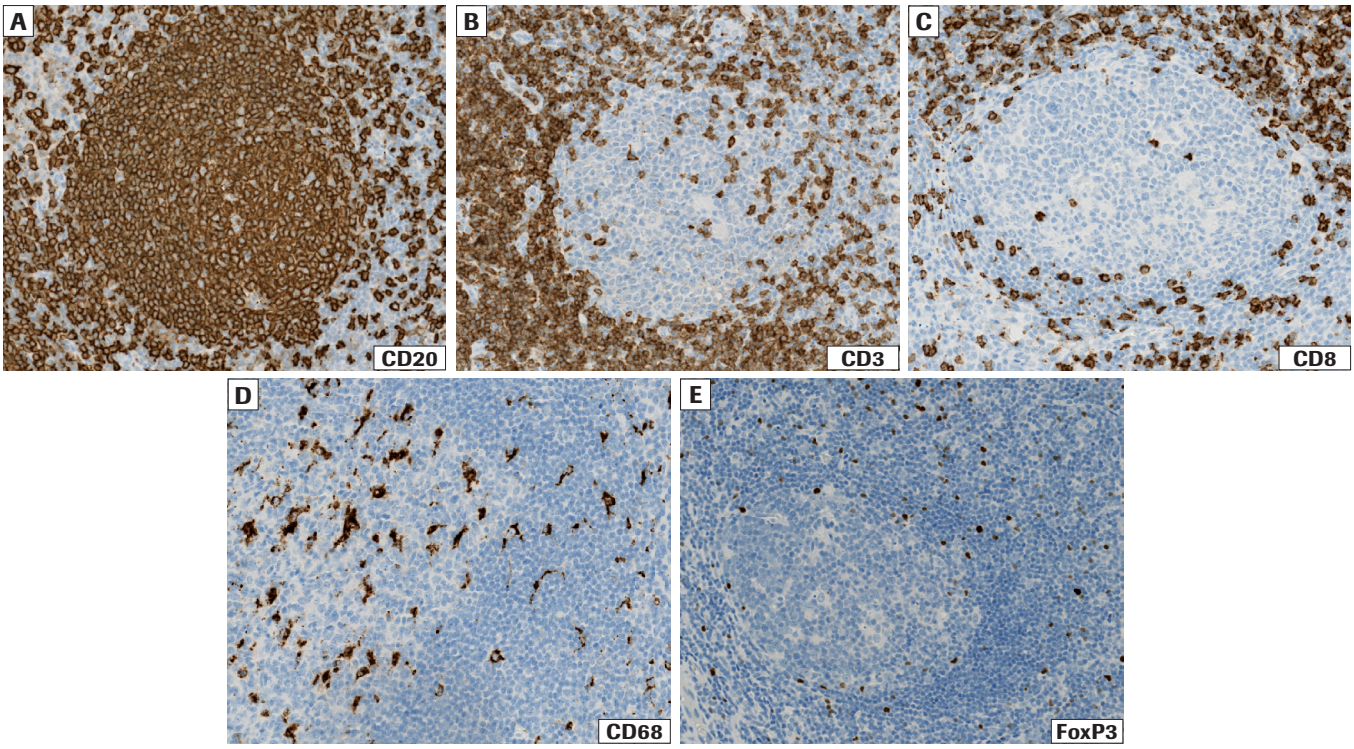


Figure 3. Optimized DAB staining on tonsil tissue to test the five biomarkers in the panel using the Anti-Rabbit HQ, Anti-HQ-HRP and ChromoMap DAB detection kits. Membrane staining patterns are observed in CD20, CD3 and CD8 positive cells. Cytoplasmic staining is seen in CD68 cells. FoxP3 shows discrete nuclei stain.

Standardization of imaging parameters for evaluation of fluorescence stain

Optimization of immunofluorescent staining for image analysis is dependent on both staining parameters and imaging parameters. Standardization of the imaging settings across the different biomarkers is essential to optimization in a multiplexed assay. It ensures that all biomarkers are adequately analyzed with minimal cross-reactivity between fluorescent dyes and facilitates downstream analysis. There are many approaches to balancing a multiplex assay and signal intensity across a panel of biomarkers such as balancing the staining, or alternatively, changing the imaging parameters. The approach taken here is a combination of both approaches.

To achieve balanced signal between biomarkers in a panel, the biomarker with the highest dynamic range from the DAB staining was selected as the starting point. Dynamic range refers to the difference between the brightest and the smallest signal that can be reliably measured. In the model panel used here, CD68 with DAB detection (Figure 3D) exhibits a wide range of signal intensity that correlates with its biological range of expression. The expression levels and staining intensities range from the very dark cytoplasmic stain at germinal centers to the fine filaments around the edges of lymphoid nodules of tonsil tissue.

In order to set the exposure time for the fluorophore channel with the highest dynamic range, the intensity capture range of the camera should be nearly filled. The exposure time should be adjusted such that the intensity values for the brightest pixels are within the range of approximately ⅔ to ¾ of the camera's maximum intensity value. For instance, a 12-bit camera will have a maximum intensity value of 4095 with the brightest pixel values around 3071 to be about ¾ of the available range (Figure 4). A 16-bit camera that typically has a larger dynamic range has a maximum intensity value of 65,535 with the brightest pixel value of around 49,151. The pixel values in the image can be visualized on a live histogram in image capture software that displays intensity values on the x-axis, and the number of pixels at a given value on the y-axis.

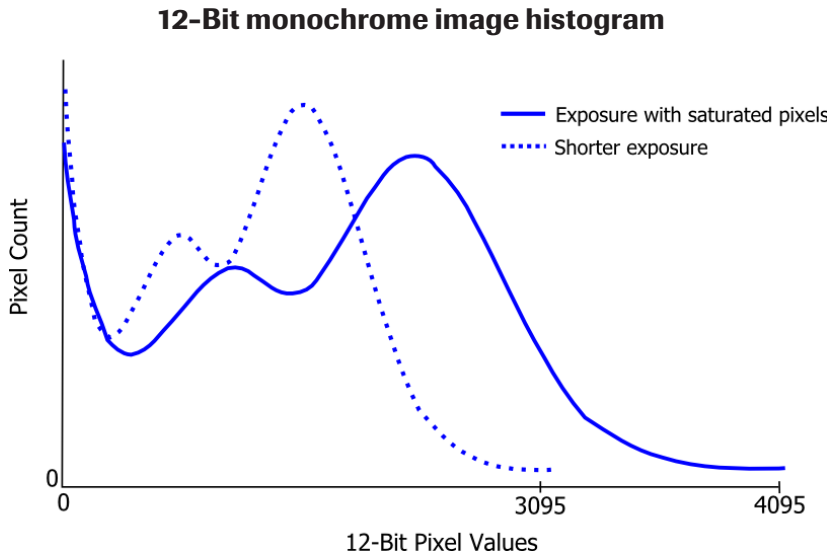


Figure 4. If a live-image histogram is available, the exposure time can be adjusted to ensure that the tail of the pixel distribution representing the brightest pixels ends at a value of approximately ¾ of the maximum intensity value of the camera (see graph above depicting a histogram with saturating pixels on a 12-bit camera [solid line], and the same histogram at a shorter exposure resulting in the brightest pixels at a value of 3095 [dashed line]).

Antibody titration for single-plex fluorescent stain of individual biomarkers

The optimal antibody dilution from the DAB staining for each biomarker was used as the starting point to transition from brightfield to single-plex fluorescence (Table 2). As discussed in the **Staining approach** section, the sequential staining in U DISCOVERY 5-Plex IF procedure (Appendix 3) involves a heat deactivation step between each round of visualization for a total of four heat deactivation steps. Since the heat deactivation may positively or negatively impact the epitope, determine appropriate placement in the sequence for each biomarker and pair it with the suitable fluorophore is critical. The sequence of the antibodies in the panel is based on the effect of the heat deactivation steps on the epitope.

Each biomarker is tested on the appropriate control tissue with each tyramide-fluorophores and its corresponding number of heat deactivation cycles. For example, to test an antibody with the DISCOVERY Rhodamine 6G detection kit, no heat deactivation step needs to be selected as it is the first module in the sequence. However, to test an antibody with the Cy5 kit, the three prior heat deactivation steps should be selected. In this model 5-Plex panel, no significant signal loss is observed from CD20, CD3, CD8 and CD68. However, FoxP3 shows improved signal intensity and distribution with four heat deactivation cycles and therefore is placed in the last round. A schematic representation of the process is shown below.

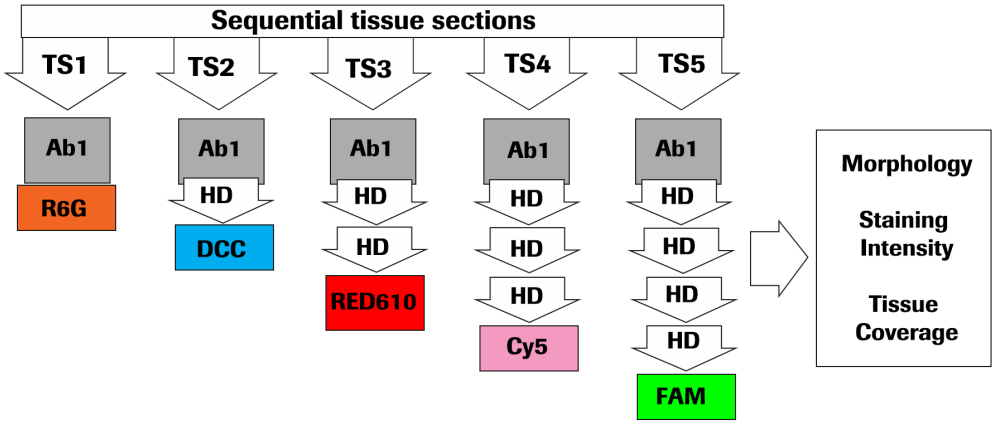


Figure 5. To evaluate the HD effect on epitope stability, sequential tissue sections were tested in the order shown here. Each antibody in the panel is stained separately with individual fluorophores that are locked in the procedure in the following order: R6G, DCC, Red 610, Cy5 and FAM. The number of HD prior to each detection module is selected to check for epitope stability in each cycle. The epitope is subjected no HD cycle when stained with Rhodamine 6G, one HD cycle with DCC, two HD cycle with Red 610, three HD cycles with Cy5 and four cycles with FAM. The results identify an antibody's position in the panel and pair it with the correct fluorophore for optimum staining.

Once the order of the staining was determined based on the epitope stability, further dilutions of each antibody were tested to fully optimize each single-plex. Table 3 provides the final dilution selected for individual antibodies and Table 4 provides the final sequence of antibody and fluorophore for the model panel.

Table 3: DAB and fluorescent antibody dilutions selected

Biomarker	DAB Ab final dilution	Fluorescent Ab final dilution
CD20(SP32)	1:100	1:400
CD3(SP162)	1:150	1:200
CD8(SP239)	1:100	1:300
CD68(SP251)	1:100	1:500
FoxP3(SP97)	1:25	1:25

Table 4: Paired biomarker and fluorophore sequence in 5-Plex		
Staining cycle	**Biomarker**	**Fluorophore**
1	CD20(SP32)	DISCOVERY Rhodamine 6G
2	CD3(SP162)	DISCOVERY DCC
3	CD8(SP239)	DISCOVERY Red610
4	CD68(SP251)	DISCOVERY Cy5
5	FoxP3(SP97)	DISCOVERY FAM

Optimizing signal balance across flourescence channels

For optimal separation of the signal in a multiplexed assay, it is important for the signal across all channel to be well balanced. To ensure that the signals for the individual biomarkers are consistent, when combined into a multiplex for imaging, minor adjustments in assay conditions can be made. Keeping the illumination level and exposure time of the microscope constant and following the above-discussed imaging strategy, slight modifications to the antibody dilution, incubation time and protocol parameters can generate a well-balanced signal with minimal cross-reactivity between the fluorophores. For example, to enhance complete membrane coverage with CD20 antibody at a dilution of 1:400, the primary antibody incubation time is increased from 16 to 28 minutes. In another study, additional FoxP3 stained nuclei were detected with a longer incubation time of 28 minutes. CD3 membrane staining at 1:200 dilution intensifies when incubation time for DCC detection is increased from 8 to 12 minutes.

The final assay parameters with fixed illumination and exposure time are recorded in Table 5.

Table 5 : Imaging parameters for balanced 5-Plex assay						
Biomarker titration	**Antibody titration**	**Primary antibody incubation (min)**	**Detection incubation (min)**	**Exposure: 100ms**	**Camera threshold**	**Bleed**
CD20(SP32)	1:400	28	8	Good morphology	> 2/3	Nominal
CD3(SP162)	1:200	28	12	Good morphology	> 2/3	Nominal
CD8(SP239)	1:300	28	8	Good morphology	> 2/3	Nominal
CD68(SP251)	1:400	28	8	Good morphology	> 2/3	Nominal
FoxP3(SP97)	1:25	28	8	Pick up more cells	> 2/3	Nil
Illumination level : constant						

Verification of heat deactivation efficiency

A key step in a sequential staining procedure is the effective heat deactivation between each round of biomarker staining to ensure adequate removal of the primary and secondary antibody complexes from the previous round. Incomplete heat deactivation (leaving residual antibody from the earlier rounds) will lead to cross-reactivity. A 2x concentration of the final working primary antibody dilution is tested to increase the safety margin of HD efficiency.

This process involves the application of a primary antibody of interest at 2x concentration followed by anti-species HRP-conjugate and enzyme-mediated deposition of the first fluorophore. Next, the tissue undergoes a heat deactivation step which should remove primary antibody and anti-species HRP-conjugate from the previous round of the staining. This is followed by the application of next primary antibody, the anti-species HRP-conjugate and enzyme-mediated deposition of the second fluorophore. Each antibody is tested at the chosen sequence of the panel as shown in a schematic (Figure 6). Ideally, the second primary antibody selected possesses a different expression pattern than the primary antibody used in the first round of staining. This serves as an internal control to rule out false negatives.

In the example depicted below, a nuclear staining antibody (FoxP3) is the second antibody of choice for testing HD efficiency of the membrane and cytoplasmic staining antibodies (CD3, CD8, CD20 and CD68). When HD of a 2x concentration of the primary antibody dilution is successful, only FoxP3 nuclear stain is observed on the tissue, verifying efficient heat deactivation (Figure 7).

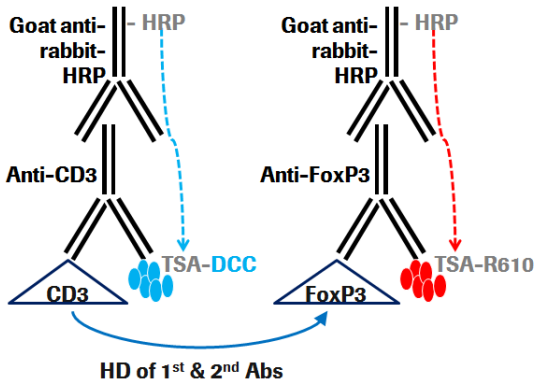


Figure 6. To increase the safety margin of HD efficiency, 2x antibodies bound to a tissue is checked for successful heat deactivation. A 2x concentration of CD3 primary antibody (rabbit monoclonal) is applied to tonsil tissue followed by anti-species HRP-conjugate and enzyme-mediated deposition of DCC. The tissue is then subjected to HD to remove the bound antibody complexes from the previous round, followed by incubation with a second primary antibody e.g., FoxP3 (rabbit monoclonal). Next, an Anti-Rabbit HRP secondary antibody is added, followed by enzymatic deposition of Red 610 fluorophore to detect optimum FoxP3 nuclear staining in the red channel.

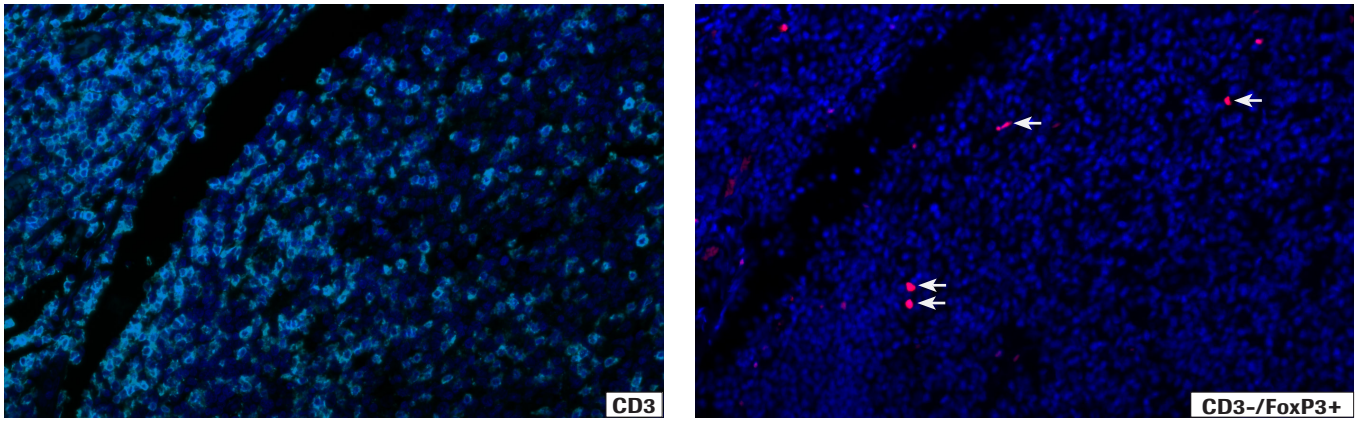


Figure 7. Heat deactivation of primary antibody on tonsil tissue. Control tonsil tissue slide stained with 2x concentrated CD3 antibody (rabbit monoclonal) shows bright membrane staining of CD3 positive cells (left). A sequential tissue section was incubated with the same CD3 antibody (2x concentration) followed by HD. The slide was next incubated with FoxP3 primary antibody and goat Anti-Rabbit HRP secondary antibody. The enzyme-mediated Red 610 detection step picked up FoxP3 nuclear stain (white arrows, right) but no CD3 membrane stain, indicating successful HD of the CD3 antibody from the first round.

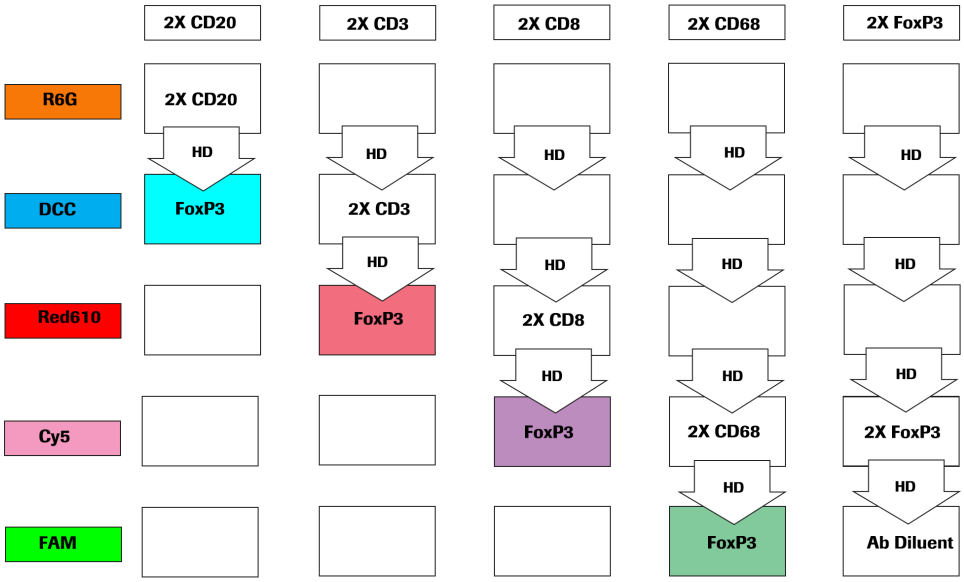


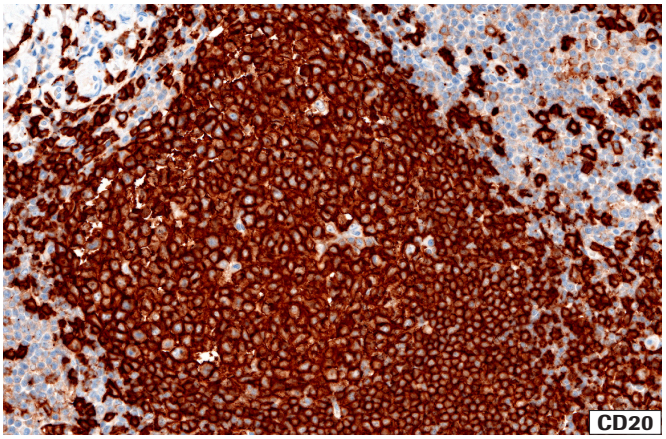
Figure 8. To test the successful removal of bound and unbound antibody from the previous round in a panel, this schematic plan is used. To maximize epitope retrieval, tissues are subjected to HD cycle prior to antibody incubation based on its position in the staining procedure. Primary antibodies (CD20, CD3, CD8, CD68 and FoxP3) are applied to the tissue at 2x concentrations followed by anti-species HRP-conjugate and enzyme-mediated deposition of fluorophores. Slides stained with CD20, CD3, CD8 and CD68 are subjected to HD, followed by incubation with FoxP3, goat Anti-Rabbit HRP secondary antibody and appropriate fluorophore predetermined in the staining procedure. Due to the absence of fluorophore after FAM in the sequence, antibody diluent is used in the FoxP3 slides.

Staining concordance between single-plex DAB and 5-Plex slide

The staining adequacy was evaluated for high-level staining concordance between a 5-Plex fluorescence IHC slide and the respective single biomarker chromogenic DAB IHC in sequential tonsil tissue sections. Cellular morphology, staining pattern and number of cells exhibiting positive staining in the same tissue areas are key criteria in evaluating staining concordance.
Six slides containing successive tonsil tissue slices were stained in the following order.

Slide1: CD20 [DAB]
Slide2: CD8 [DAB]
Slide3: 5-Plex [FL: CD20, CD3, CD8, CD68, FoxP3]
Slide4: CD68 [DAB]
Slide5: CD3 [DAB]
Slide6: FoxP3 [DAB]
Whole slide scans (WSI) of both single DAB stained slide and 5-Plex fluorescent slide were performed in Zeiss Axio Scan.Z1 and aligned in a split image viewer in Zeiss ZEN lite. The split image was zoomed in and out while moving the field of view to assess staining pattern and percentage of positively stained cells. Staining concordance was evaluated by a pathologist.

The membrane staining patterns and cellular coverages of CD20, CD8 and CD3 were comparable between single-plex DAB and 5-Plex fluorescent slides. CD68 staining of macrophages and dendritic cells in the 5-Plex slide and the corresponding DAB stained slide shows a similar dynamic range of staining pattern. The staining though visible in the image viewer is not well-represented during image capture. The FoxP3 nuclear stain is detected in similar areas of the tissue in both DAB and 5-Plex slides. The DAB-immunofluorescence staining concordance between CD20, CD3, CD8, CD68 and FoxP3 are shown in Figure 9.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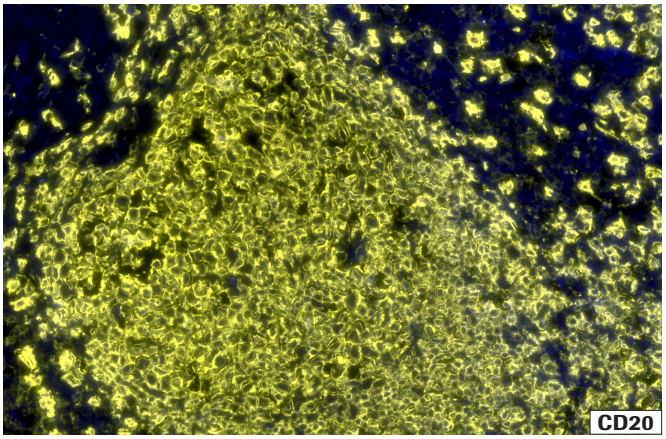
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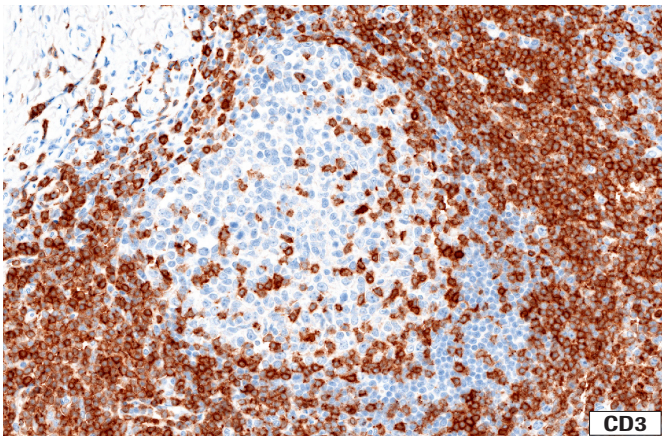
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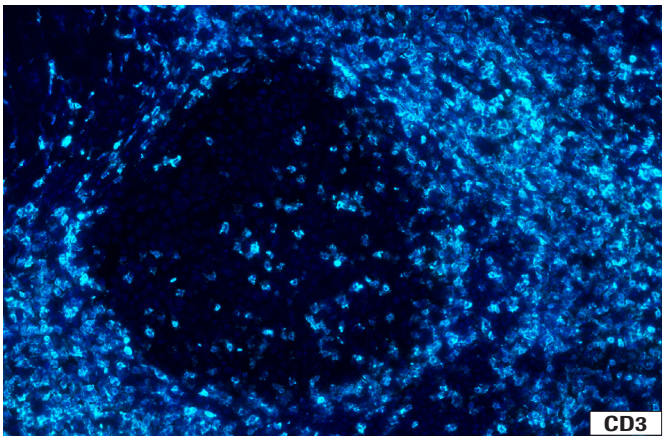
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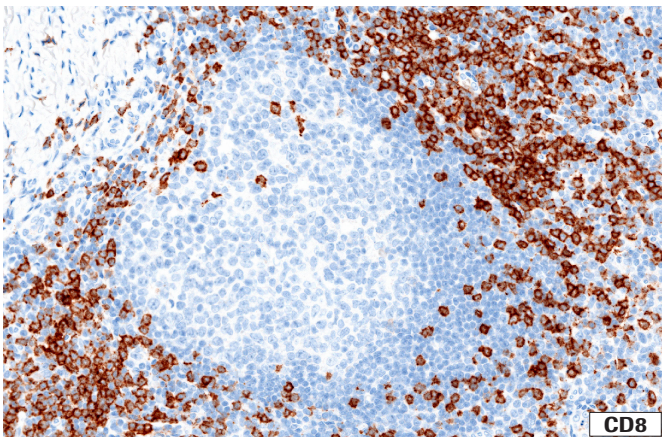
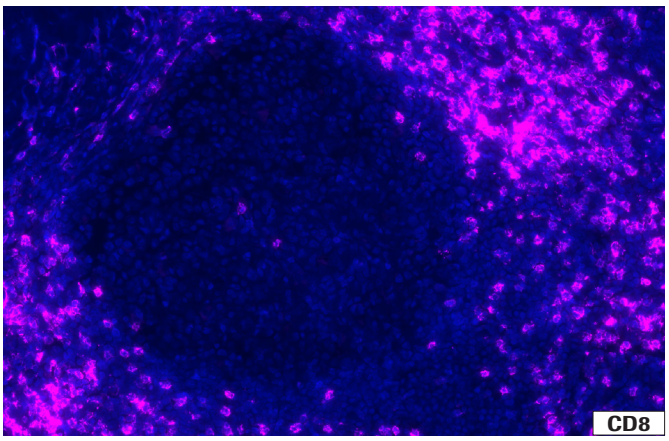
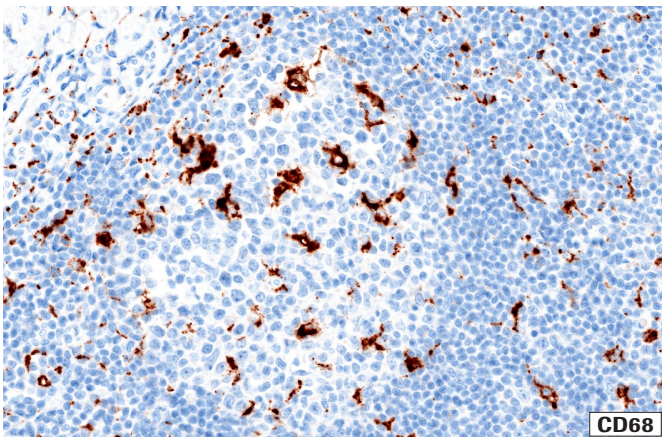
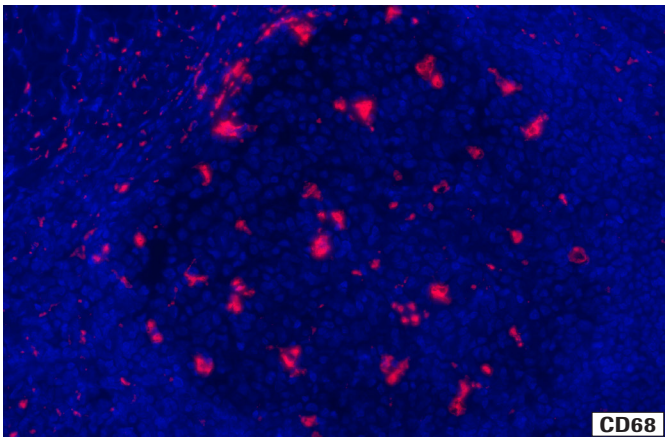

CD8


CD8


CD68


CD68

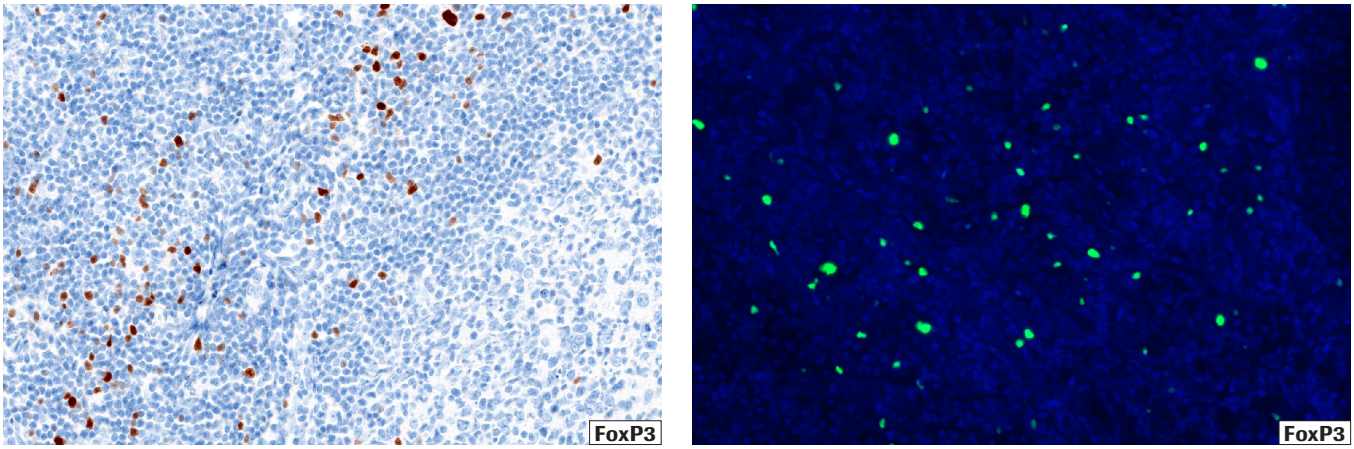


Figure 9. Staining concordance is evaluated between a 5-Plex fluorescent slide and a DAB IHC slide of individual biomarkers. Areas chosen from the same location of both DAB and fluorescent-stained slides were uploaded on a ZEN lite split viewer in the following order: CD20, CD3, CD8, CD68 and FoxP3. All five biomarkers from the 5-Plex slide show staining concordance with individual DAB IHC slides.

Test 5-Plex panel on tonsil tissue

After completion of individual biomarker optimization and their placement in the appropriate sequence in the staining procedure, they were combined into a 5-Plex assay and tested on tonsil tissue, as it is an ideal positive control for all the biomarkers used in this panel. The 5-Plex staining procedure, tested on tonsil tissue, evaluated signal intensity, morphology, cellular distribution and biomarker co-localization of CD20, CD3, CD8, CD68 and FoxP3 immune cells. The fluorescent image of the merged 5-Plex along with the images from each channel taken from a single tissue section is shown in Figure 10.

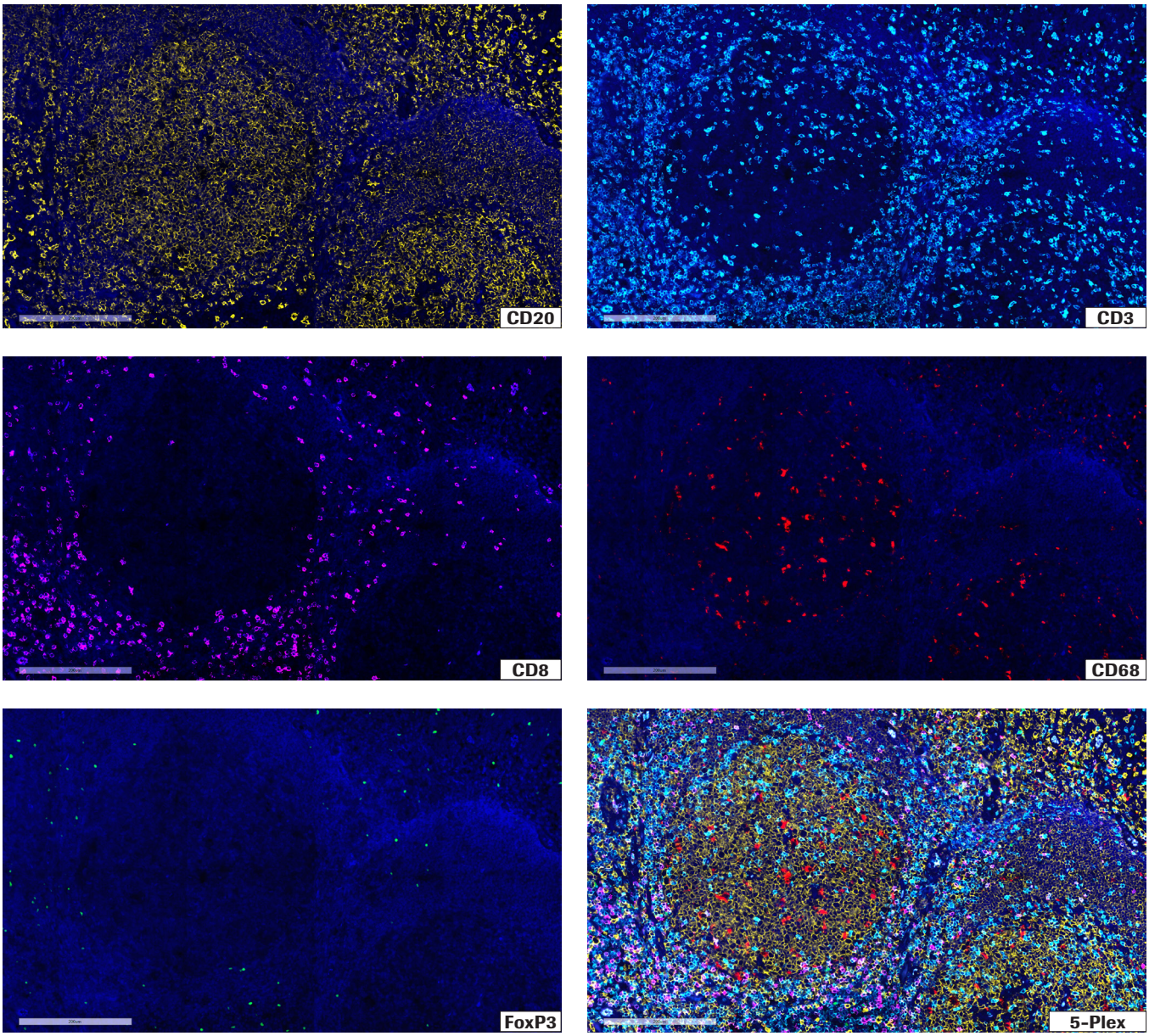
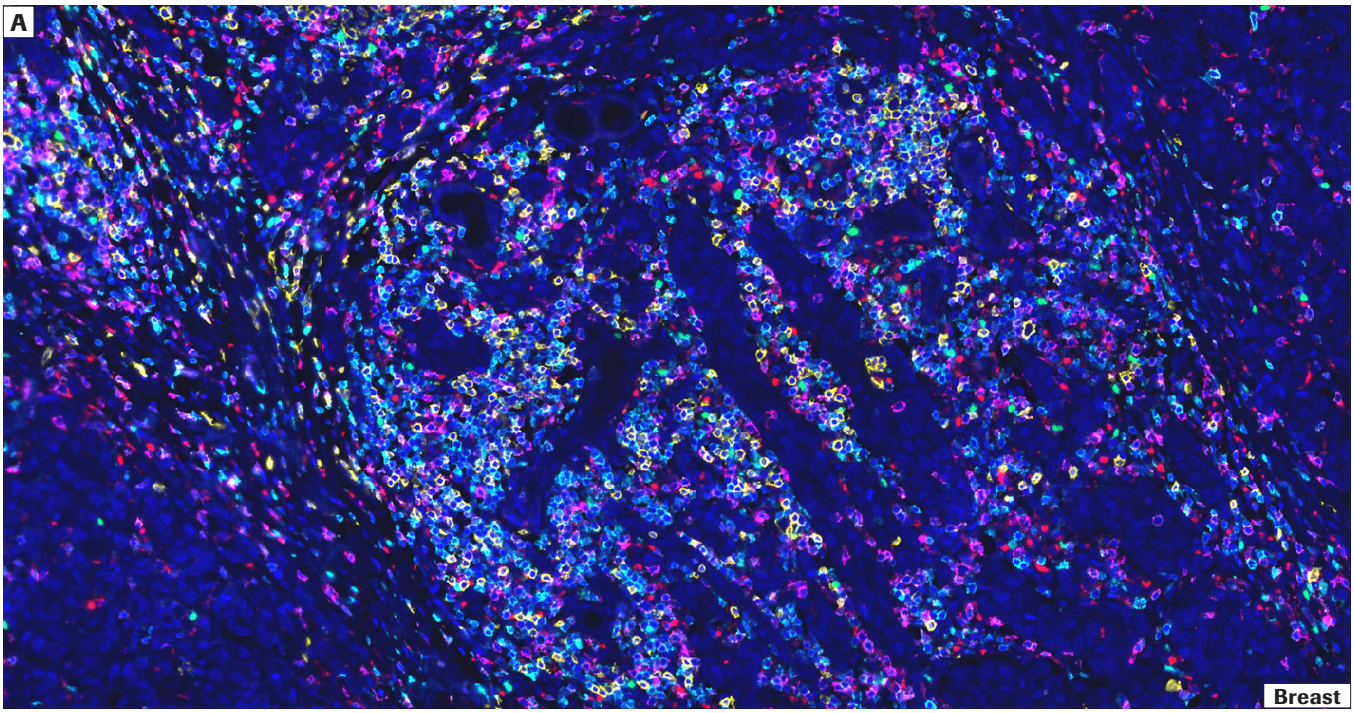
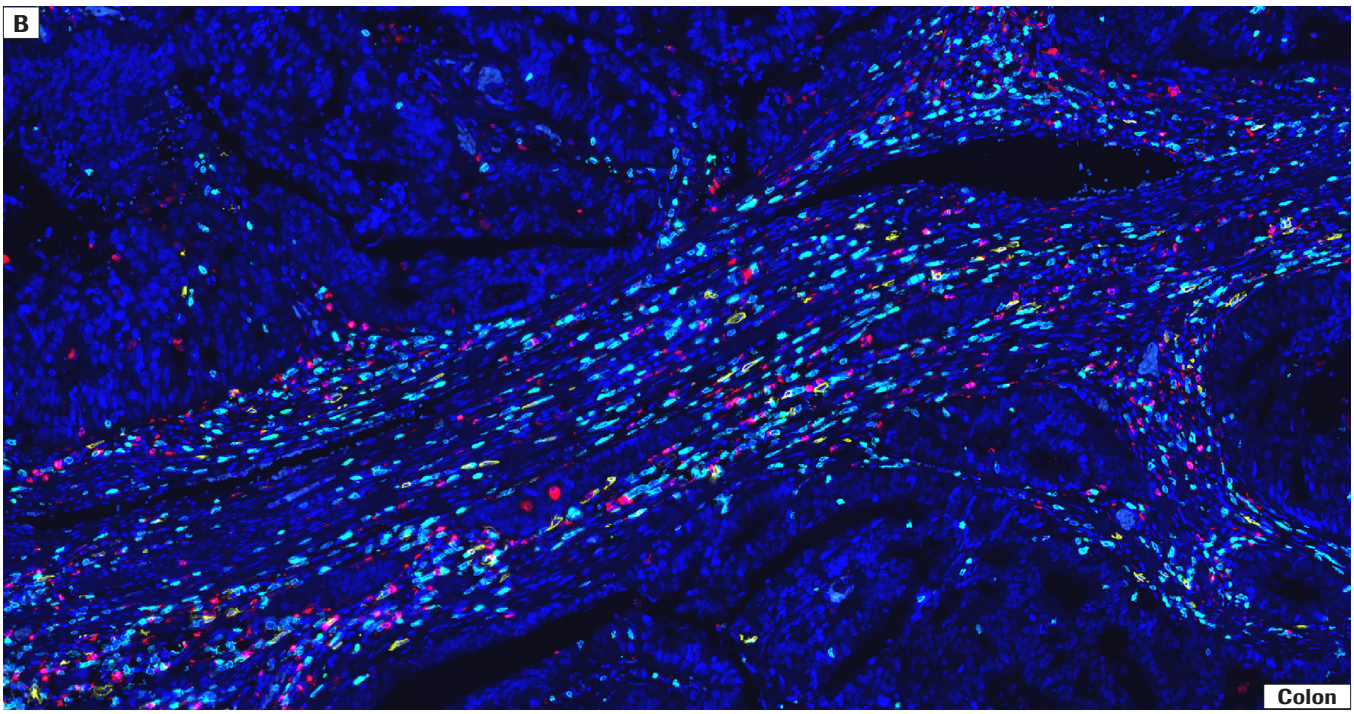


Figure 10: The staining procedure was tested on a model tissue (tonsil). Each biomarker stain is shown separately with DAPI counterstain. The biomarkers are represented with the following pseudocolors: CD20: gold, CD3: aqua, CD8: magenta, CD68: red and FoxP3: green. A merged 5-Plex image shows all five biomarker staining on the same tissue.

Application of the 5-Plex procedure to tissues of choice

To test the robustness of the staining protocol, random tumor tissues of breast, lung and colon carcinomas were stained with the 5-Plex staining procedure using the same protocol conditions utilized for tonsil tissue. Robust staining of all five biomarkers (CD20, CD3, CD8, CD68 and FoxP3) was observed in the tumor tissues tested. However, optimization is anticipated for different tissue types especially given that their pre-analytical conditions may be different. Figure 11 demonstrates the 5-Plex staining pattern of breast, lung and colon carcinomas.





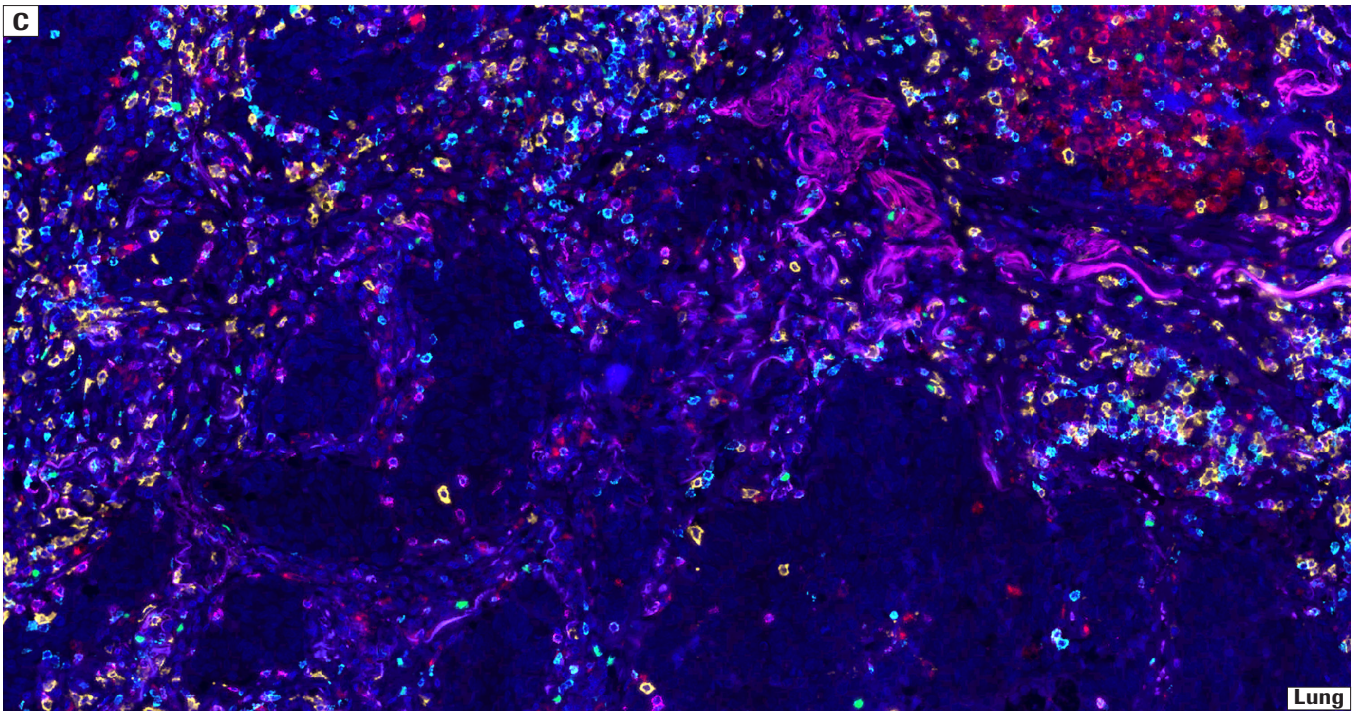


Figure 11 A, B, C: Representative fields from three tumors stained with 5-Plex panel. For each pseudocolored image CD20: gold, CD3: aqua, CD8: magenta, CD68: red, FoxP3: green, DAPI counterstain: blue. A: breast, infiltrating ductal carcinoma; B: colon, adenocarcinoma; C: lung, squamous cell carcinoma.

Conclusions

IHC is an essential tool routinely used in the diagnostic market and the research community. The increasing demands in the research market to interrogate multiple biomarkers from scarce tissues have led to the development of novel fluorescent multiplexing IHC technologies. In order to generate robust and reproducible multiplexed IHC assays, automated staining procedures are in great demand. The fully automated procedure developed on the DISCOVERY ULTRA platform facilitates high quality, reproducible 5-Plex immunofluorescent stain. To develop a robust procedure, antigen retrieval, temperature control, heat deactivation and incubation times were optimized. Careful consideration was given to bulk reagent choices, reagent compositions, and bulk capacities to facilitate a full run. The automated slide stainer can accommodate up to 30 one- to 5-Plex slides in a single run with a TAT of <12 hours.

The success of robust multiplexing staining is highly dependent on the availability of stable reagents. Deterioration of dyes can significantly hinder assay reproducibility and long range studies. The four new detection kits (Rhodamine 6G, DCC, Red 610 and FAM) were tested thoroughly to handle various components of the staining procedure. An ongoing RTS up to 120 days showed all TSA-fluorophores to be stable in the ready-to-use dispensers. The U DISCOVERY 5-Plex IF is an automated staining procedure on the DISCOVERY ULTRA platform demonstrated good reproducibility across the beta sites tested.

The ease-of–use and the flexibility of the staining procedure, in combination with ready-to-use reagent kits, can expand adoption of multiplex staining capability in small laboratories. The stability of the reagents in dispensers can standardize studies across sites. Improved throughput can generate sufficient data points for downstream image analysis and accelerate early research. Finally, the technology supports simplified design, optimization, and implementation of new multiplex assay panels with different biomarkers.

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APPENDIX 1: Reagents and part numbers		
Reagent Name	**Vendor**	**Part Number**
Primary Antibodies		
CD20 (SP32)	Spring Bioscience	M3324
CD3 (SP162)	Spring Bioscience	M3074
CD8 (SP239)	Spring Bioscience	M3164
CD68 (SP251)	Spring Bioscience	M5514
FOXP3 (SP97)	Spring Bioscience	M3974
Fluorescent Detection Reagents		
DISCOVERY OmniMap anti-Rb HRP (RUO)	Roche Tissue Diagnostics	760-4311 / 05269679001
DISCOVERY Rhodamine 6G detection kit	Roche Tissue Diagnostics	760-245 / 07988168001
DISCOVERY DCC detection kit (coumarin)	Roche Tissue Diagnostics	760-240 / 07988192001
DISCOVERY Red 610 detection kit	Roche Tissue Diagnostics	760-245 / 07988176001
DISCOVERY FAM detection kit	Roche Tissue Diagnostics	760-243 / 0798815001
Cy5 Kit, DISCOVERY detection kit	Roche Tissue Diagnostics	760-238 / 07551215001
DAB Detection Reagents		
Anti-Rabbit HQ	Roche Tissue Diagnostics	760-4815 / 07017812001
Anti-HQ HRP	Roche Tissue Diagnostics	760-4820 / 07017936001
ChromoMap DAB Kit	Roche Tissue Diagnostics	760-159 / 05266645001
Bulk Reagents		
Wash (RUO), DISCOVERY	Roche Tissue Diagnostics	950-510 / 07311079001
Liquid Coverslip (High Temperature)	Roche Tissue Diagnostics	650-010 / 05264839001
DISCOVERY CC1	Roche Tissue Diagnostics	950-500 / 06414575001
ULTRA Cell Conditioning (ULTRA CC2)	Roche Tissue Diagnostics	950-223 / 05424542001
Reaction Buffer (10x)	Roche Tissue Diagnostics	950-300 / 05353955001
Blockers		
DISCOVERY Goat Ig Block*	Roche Tissue Diagnostics	760-6008 / 07988214001
Inhibitor, DISCOVERY	Roche Tissue Diagnostics	760-4840/ 07017944001
Counterstain		
QD DAPI, DISCOVERY	Roche Tissue Diagnostics	760-4196 / 05268826001
Mounting Media		
Pro-Long Diamond Mounting medium	ThermoFisher	P36961
Other		
Antibody Diluent with Casein	Roche Tissue Diagnostics	760-219 / 06640002001
Tonsil control tissue, unstained	N/A	RT

APPENDIX 2: Wavelength interference filters used to image fluorescent multiplex stain					
Chroma Part #	**Filter Name**	**Fluorophore**	**Biomarker**	**Excitation**	**Emission**
49000	DAPI	DAPI	Chromatin	350 +/- 25	460 +/- 25
49302	Aqua	DCC	CD3	436 +/- 10	480 +/- 15
CUSTOM	Special Green	FAM	Fox P3	490 +/- 10	520 +/- 10
49304	Gold	Rhodamine 6G	CD20	546 +/-5	572 +/-10
49306	Orange	Red610	CD8	580 +/- 12	625 +/- 15
49009	Red	Cy5	CD68	640 +/- 15	690 +/- 25

APPENDIX 3: U DISCOVERY 5-Plex IF procedure

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