

Video Article

# Multiplexed Fluorescent Immunohistochemical Staining, Imaging, and Analysis in Histological Samples of Lymphoma

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URL: <https://www.jove.com/video/58711>

DOI: [doi:10.3791/58711](https://doi.org/10.3791/58711)

Keywords: Multiplexed immunofluorescence, multispectral imaging, quantitative analysis, biomarkers, lymphoma, digital pathology

Date Published: 12/19/2018

Citation: Hong, G., Fan, S., Phyu, T., Maheshwari, P., Hoppe, M.M., Phuong, H.M., de Mel, S., Poon, M., Ng, S.B., Jeyasekharan, A.D. Multiplexed Fluorescent Immunohistochemical Staining, Imaging, and Analysis in Histological Samples of Lymphoma. *J. Vis. Exp.* (), e58711, doi:10.3791/58711 (2018).

## Abstract

Immunohistochemical (IHC) methods for the *in-situ* analysis of protein expression by light microscopy are a powerful tool for both research and diagnostic purposes. However, the visualization and quantification of multiple antigens in a single tissue section using conventional chromogenic IHC is challenging. Multiplexed imaging is especially relevant in lymphoma research and diagnostics, where markers have to be interpreted in the context of a complex tumor microenvironment. Here we describe a protocol for multiplexed fluorescent IHC staining to enable the quantitative assessment of multiple targets in specific cell types of interest in lymphoma. The method covers aspects of antibody validation, antibody optimization, the multiplex optimization with markers of lymphoma subtypes, the staining of tissue microarray (TMA) slides, and the scanning of the slides, followed by data analysis, with specific reference to lymphoma. Using this method, scores for both the mean intensity of a marker of interest and the percentage positivity are generated to facilitate further quantitative analysis. Multiplexing minimizes sample utilization and provides spatial information for each marker of interest.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/58711/>

## Introduction

Lymphoid neoplasms are caused by the uncontrolled malignant proliferation of lymphocytes. These cells are vital components of the immune system and localize to the primary and secondary immune organs, such as the bone marrow, lymph nodes, spleen, and other mucosa-associated lymphoid system. Lymphoid neoplasms are a heterogeneous group of disorders who are classified based on a constellation of features, including morphology, immunophenotype, genetic features, and clinical presentation. While each parameter plays a part, lineage remains a defining feature and forms the basis for the WHO classification system which recognizes neoplasms derived from B cells, T cells, and natural killer (NK) cells<sup>1</sup>.

Key to the classification of lymphoma has been the characterization of the antibodies against leukocyte surface markers of the various subtypes of lymphocytes<sup>2</sup>. Immunohistochemistry (IHC) has been traditionally used for the analysis of such markers and is based on the principle of the specific antigen-antibody recognition to detect cell- and tissue-based molecules that can be visualized through the light microscope<sup>3</sup>. However, the identification of multiple targets on a single slide by conventional bright-field chromogenic multiplex IHC has limitations because it is often difficult to distinguish multiple color signals on a single tissue section reliably—especially for antigens with a very low expression<sup>4</sup>. Visual assessment and quantification of staining can also be subjective, causing variability in the analysis and data interpretation<sup>5</sup>.

Therefore, conventional IHC on formalin-fixed, paraffin-embedded (FFPE) samples is not feasible for the simultaneous detection of multiple targets in immunologically diverse diseases like lymphoma. Furthermore, distinguishing neoplastic lymphocytes from the surrounding immune cells is often imprecise. This hinders studies looking at the relevance of novel biomarkers in lymphoma. In this context, multiplex fluorescent IHC (mf-IHC) offers a promising alternative as it allows the quantitative assessment of antigen coexpression and a spatial relationship with higher precision while conserving limited samples<sup>6,7</sup>. When this imaging technology is partnered with the digital image analysis software, the data interpretation is made more efficient and facilitates the study of tumor and microenvironment heterogeneity<sup>8,9</sup>. In this protocol, a tyramide-based immunofluorescence (IF) multiplexing method is applied to amplify the signal and is compatible with any IHC-validated antibody from any host species, even those developed in the same species<sup>5,7,10</sup>. The tyramide-based protocol allows for the direct conjugation of the fluorophore to the

tissue of interest so that the primary and secondary antibody can be stripped after each step, allowing for the sequential application of multiple stains without antibody cross-reactivity.

A multiplexed strategy will be useful for predicting prognosis and treatment outcomes by identifying targets and their variant immunologic patterns in lymphomas. Multiplex fluorescent IHC has been applied in our lab for the study of a panel of T and B lymphocyte markers and T-follicular helper markers in angioimmunoblastic T-cell lymphoma (AITL), a subtype of a peripheral T-cell lymphoma characterized by aggressive clinical behavior and tumor heterogeneity<sup>11</sup>. The utility of this method is also illustrated in diffuse large B-cell lymphoma (DLBCL) where the increased signaling of a B-cell receptor with simultaneous C-MYC and BCL-2 expression suggests the potential therapeutic use of Bruton's tyrosine kinase inhibition<sup>12</sup>.

Here we describe the entire protocol from antibody validation to the selection of appropriate control tissues and multiplexing using lymphoma FFPE tissues, with an eventual analysis of stained slides using a scanning automated quantitative pathology imaging system.

## Protocol

All tissues used in this protocol were obtained under the Singapore NHG Domain Specific Review Board B study 2014/00693.

### 1. Selection and Validation of Antibodies

NOTE: Before proceeding with the establishment of any multiplexed panel, ensure that all antibodies stain robustly, identifying only the target antigen of interest. The aim is to select antibodies that specifically recognize the antigen of interest in tissue sections.

- For an antibody with a well-established research use, or routine clinical use in IHC, confirm conditions such as epitope retrieval and antibody dilution by performing conventional IHC<sup>5,13</sup> on positive and negative control tissue sections. For tissue sections of human origin, ensure that the appropriate ethics clearances are in place prior to initiating experiments.  
NOTE: Positive controls are tissues that are expected to express the antigen of interest, and negative controls are those that do not. Benign tonsil tissue is chosen as a good tissue control for lymphoma antigens because it contains a mixture of immune cells including B cells, T cells, and antigen-presenting cells, as well as stromal and epithelial cells. The latter serve as useful negative internal controls.
- For unknown targets or for commercial antibodies with insufficient published data, perform antibody validation by creating matched positive and negative control FFPE cell blocks<sup>14</sup>, using CRISPR knock-out or siRNA knock-down of the antigen of interest in an appropriate cell line through standard molecular biology techniques. Use these FFPE cell blocks in lieu of positive and negative control tissues for conventional IHC as per step 1.1.  
NOTE: HeLa or 293T cells are commonly used for antigens that are not cell-type specific, as lymphoma cell lines are difficult to transfect. For antigens that are lymphocyte specific, lymphoma cell lines can be used with viral transduction or electroporation as the mode of gene delivery (albeit with low efficacy).

### 2. Planning the Sequence of Antibodies and Fluorophores for the Multiplex Panel

- Plan the sequence of reagents for the final multiplex staining. For example, here the sequence for the multiplex protocol was initially planned as first, Bcl-6; second, Bcl-2; third, C-Myc; fourth, CD20; fifth, Ki67.  
NOTE: To decide on the sequence, antibodies with a weak affinity requiring higher concentrations should be stained first in the final multiplex staining. High-affinity antibodies that are likely to be resistant to multiple rounds of microwave stripping are applied last in the multiplex protocol to avoid nonspecific staining.
- Plan the fluorophore partner for each antibody in the panel. See **Table 1** and **Table of Materials** for the choices in this example.  
NOTE: To decide on the fluorophore partner for each antibody, choose spectrally distinct fluorophores for antibodies with similar patterns of localization within the cell and within tissue. This will minimize spectral overlap and difficulty with unmixing.

### 3. Monoplex Tyramide-based IF in a Simulated 5-plex Multiplex Panel

NOTE: In this example, the protocol for CD20 is discussed, which is planned as the fourth antibody in a multiplex sequence described above. The number of additional stripping steps will differ for the position of the antibody in the sequence.

- Cut 3  $\mu\text{m}$ -thin sections of positive and negative control tissue using a microtome and place the sections on poly-L-lysine-coated microscope glass slides.
- Dewax the sections using an appropriate clearing solution 3x for 4 min each. Rehydrate the slides in 100% alcohol, 90% alcohol, and 70% alcohol, 4 min each. Put the slides in distilled water for 2 - 3 min.
- Place the slides in a microwave-safe glass jar in a standard antigen retrieval buffer (commercially available) to immerse the slides. Perform heat-induced epitope retrieval (HIER) using a suitable microwave, in pH9 antigen retrieval solution at 98 °C for 25 min.  
NOTE: Any microwave with a pressure ranging from 800 - 1,100 Watts can be used, and HIER can be optimized accordingly. In this case, a multifunctional microwave tissue processor (open to air) was used for HIER and stripping. The initial settings for the retrieval of a particular epitope are based on the prior knowledge from conventional IHC.
- Perform additional rounds of microwave stripping (three in this case, for the fourth antibody in a panel), each round with 100% power to 98 °C and 20% power for 10 min to maintain at the same temperature. Cool down the slides in distilled water for at least 10 min.  
NOTE: Perform multiple rounds of microwave stripping during the monoplex IF step to expose the target antigen to the same number of heating steps as in the proposed multiplex. Since CD20 is planned as the fourth antibody, do microwave stripping 3x before and one time after doing the primary antibody incubation.

5. Check and replenish the buffer after every 5 min during additional microwave stripping. Importantly, do not let slides dry out during the multiple stripping procedures. When taking the slides out of the microwave, use forceps and heatproof gloves to place them into a separate jar of distilled water. Wait for the antigen retrieval solution to cool naturally.
6. Block the tissue peroxidase activity by using a commercial peroxidase block for 10 min (3% hydrogen peroxide can be used as an alternate reagent). Wash with Tris-buffered saline (TBS) and a nonionic detergent for 5 min.  
NOTE: TBS is composed of 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl. TBS to make TBS-D.
7. Block with antibody diluent or Bovine serum albumin for 15 min followed by incubation with the primary antibody of interest (e.g., CD20, 1:2,000 dilution in antibody diluent, see **Table of Materials**) at room temperature for 30 min. Wash 3x with TBS-D buffer for 5 min each time. There should be sufficient TBS-D buffer to immerse the slide completely in all washing steps.  
NOTE: Bovine serum albumin can be used as an alternate antibody diluent. The volume of antibody diluent depends on the size of the tissue, ranging from 50  $\mu$ L (for biopsy samples) to 400  $\mu$ L (for excision samples or tissue microarray samples).
8. Incubate with an appropriate horseradish peroxidase (HRP)-labelled secondary antibody, chosen on the basis of the species of origin of the primary antibody for 15 min at room temperature. Wash 3x with TBS-D buffer for 5 min each time.
9. Apply an appropriate tyramide-based fluorescent reagent (1:100 in amplification diluent) and incubate at room temperature for 5 min, to allow fluorophore conjugation to the tissue sample at the sites of primary antibody binding. Wash 3x with TBS-D buffer for 5 min each time.
10. Perform an additional microwave-based stripping to remove the primary and secondary antibody. Repeat as required based on the position of the antibody in the sequence.
11. Place the slide in distilled water to cool down. Incubate with DAPI (1:100 dilution in antibody diluent) for 10 min, followed by wash 3x with TBS-D buffer for 5 min each time. Dry the area on the slide without the tissue with wipes and mount the slide with appropriate mounting media.
12. Image the slide (see sections 6 and 7 of this protocol) and determine the appropriateness of the staining (as defined by clear discrimination of the positive and negative control tissue).  
NOTE: If the staining pattern is incorrect, redo the monoplex staining after adjusting one or more of the following parameters: the number of heat retrieval steps, the amount of heat retrieval, the incubation period/concentration of antibodies (primary and secondary), the position of antibody in the sequence, and the choice of fluorophore. The monoplex staining step typically requires multiple rounds of optimization to obtain a suitable set of parameters for appropriate staining. It is advisable to test a range of fluorophores with each primary antibody, as this will provide flexibility in deciding the final multiplex set.

#### 4. Repetition of the Monoplex for Each Antibody in the Multiplex Protocol

1. Repeat the monoplex staining for the other antibodies in a similar way by adjusting the number of microwave stripping steps, based on the position of the antibody in the sequence. For instance, when performing monoplex staining for the third antibody in a six-plex multiplex panel, perform two microwave stripping steps before and three microwave stripping steps after the primary antibody incubation.  
NOTE: It is important to appreciate that the microwave-based stripping of antibodies also exposes the sample to HIER. If a specific antibody requires a different epitope retrieval strategy, this needs to be taken into account when planning the multiplex sequence. In this example, Ki67 epitope retrieval requires pH 9, for 30 min. Since 25 min of HIER will be done prior to Ki67 staining in the sequential protocol, only an extra 5 min of HIER are needed.

#### 5. Multiplex Staining Protocol

NOTE: Proceed with the multiplex staining protocol only after all the components have been optimized using monoplex IF staining. Review the results of the monoplex staining and design a table showing the final layout of the order of multiplex and the choice of fluorophore for each antibody. The details of antibody concentration, the duration of staining, and the sequence and nature of heat retrieval for each antibody used here is provided in **Table 1**.

1. Cut 3  $\mu$ m-thin sections of positive and negative control tissue, as well as the target tissue of interest (here, FFPE samples of lymphoma), using a microtome, and place the sections on poly-L-lysine-coated microscope glass slides (see **Table of Materials**).  
NOTE: Inclusion of the positive and negative controls for each antibody in the panel is advisable, along with the actual samples for multiplex. This allows for a confirmation that the staining protocol was performed correctly.
2. Dewax, perform heat-induced epitope retrieval (HIER), and block tissue peroxidase activity as in the monoplex protocol steps 3.3 - 3.5.
3. Incubate with the first primary antibody of the optimized multiplex sequence, as previously determined using the monoplex IF step. In this example, BCL-6, at a 1:30 dilution in antibody diluent at room temperature for 60 min (see **Table of Materials**), was the first step of the multiplex protocol. Wash with TBS-D buffer for 5 min.
4. Incubate with an appropriate HRP-labelled secondary antibody based on the species of the primary antibody used in the prior step (see **Table of Materials**) at room temperature for 10 min. Wash with TBS-D buffer for 5 min.
5. Apply the optimized tyramide-based fluorescent reagent (Cy5 in this example, 1:100 in amplification diluent, see **Table of Materials**) with incubation at room temperature for 5 min. After the incubation, wash with TBS-D buffer for 5 min.  
NOTE: The choice of the tyramide-based fluorescent reagent is based on the optimized monoplex IF.
6. Check the efficiency of staining of the first antibody using an appropriate microscope (see **Table of Materials**).  
NOTE: Interim imaging checks can be done with ease if the sample is a TMA or single slide. If, however, the stain is being performed on multiple slides, this may be impractical, and this step may be omitted.
7. Perform microwave stripping for the second antibody in the protocol, using conditions optimized in the monoplex step. Then, proceed with the staining of the second primary antibody (here, BCL2) with the HRP-labelled secondary antibody and tyramide-based fluorescent reagent (here, 520, see **Table of Materials**). Check the efficiency of staining under a fluorescent microscope after the completion of the second round of staining.
8. Similarly, repeat the procedure using the third, fourth, and fifth antibodies in the sequence (here, c-Myc, CD20, and ki67, respectively, with fluorophores 570, 540, and 620, respectively). Perform imaging checks in between each step if feasible.
9. Add a nuclear counterstain (DAPI, 1:100 dilution in antibody diluent) for 10 min and, then, wash 2x in TBS-D for 5 minutes each.

10. Mount the slides using an appropriate mounting reagent.

## 6. Preparation of Spectral Library Slides

NOTE: Sections 6 - 8 of this protocol are unique to multiplexed experiments that are imaged using a spectral camera.

1. Create library slides (single-stain reference images) for each fluorophore, DAPI, and autofluorescence on the same control tissue, to be used for multispectral image analysis.
  1. Cut 2 x 3  $\mu\text{m}$ -thin sections of the tissue type of interest (here, a lymphoma sample or a tonsil as control) using a microtome and place the sections on poly-L-lysine-coated microscope glass slides.
  2. Process both slides using the monoplex protocol (with all stripping and washing steps), but without antibody or fluorophore addition.
  3. Stain one slide with DAPI as per step 5.9 and leave one slide unstained (for the generation of the tissue autofluorescence spectrum).  
NOTE: The optimized monoplex slides (with a single fluorescence dye, without DAPI) can be used as spectral library slides to generate spectra of each fluorescence dye.
2. Scan these set of slides using appropriate filters and upload them into the image analysis library.

## 7. Spectral Imaging

1. **Scanning monoplex slides**
  1. Choose the filters from available standard epi-fluorescence filters (DAPI, FITC, CY3, Texas Red, and CY5) appropriate for the employed fluorophore.  
NOTE: The recommended filter cubes for specific fluorophores used in this example are shown in **Table 2**<sup>15</sup>.
  2. Examine each marker in its corresponding fluorescence channel to identify a suitable exposure time to obtain a clean signal. Focus on the tissue component that is supposed to have the strongest signal for the marker.
  3. Determine a fixed exposure time for each analyte (antibody-fluorophore combination), to allow cross-sample comparison of pixel intensity (although some commercial platforms have normalization strategies to account for differences in exposure).
    1. To decide on a fixed exposure time for a given channel, use a live camera setting to adjust the exposure time until there are no overexposed areas in the live camera image, and repeat this for all channels.
  4. After scanning the monoplex slides, generate simulated brightfield images (if the function is available in the software used) to visually compare with the normal IHC pattern and to determine if the staining pattern is correct (**Figure 1** and **Figure 2**).
2. **Scanning multiplex samples (TMA slides/multiple samples)**
  1. Set up the optical parameters as described for scanning monoplex images (step 6.1). First, adjust the focus manually or automatically (follow the instructions of the specific image scanning machine). Secondly, adjust the exposure time for each fluorescent channel to ensure that all the cores on a TMA, or all areas on a slide, are appropriately exposed.  
NOTE: The area selected to adjust the exposure time is important. Users need to choose the strongest signal region for each filter (*i.e.*, the Ki67 signal is stronger in the tonsil germinal center region than in the nongerminal center region; hence, users should use the germinal center region set at an appropriate exposure time). Users can also adopt an oversaturation correction function of the imaging system if available.
  2. Scan TMA slides under a TMA scanning mode if available in the imaging system, with an appropriate autofocus algorithm.
  3. For whole-tissue section slides, get the slides reviewed by a qualified pathologist to select optimal images from the most representative tumor areas.  
NOTE: The protocol described here is based on a defined microscope/image analysis system (see **Table of Materials**). There are other machines and image analysis software that can scan and analyze multiplex IHC slides<sup>7</sup>.

## 8. Data Analysis

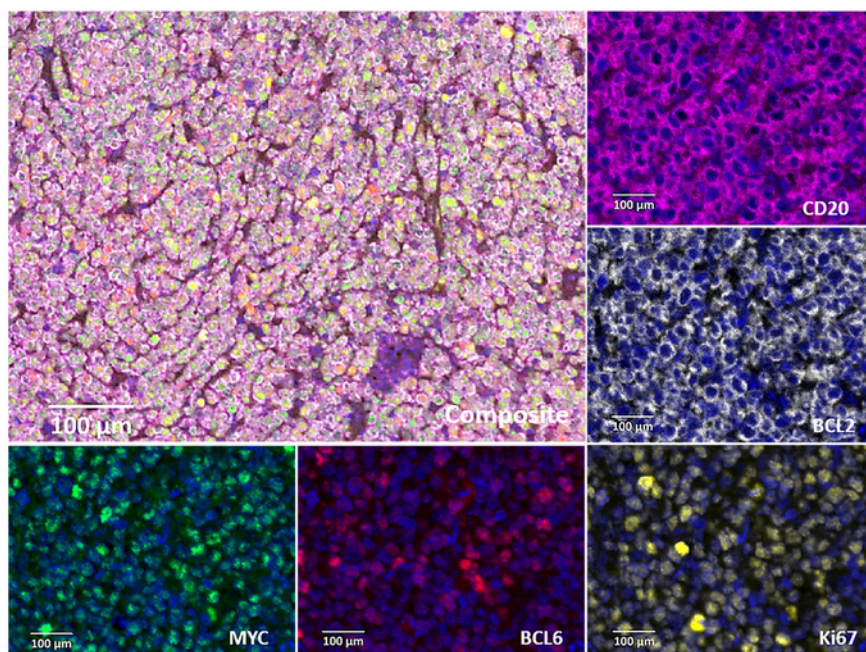
1. **Preanalysis assessment and planning**
  1. Use the reference slide for autofluorescence to subtract autofluorescence from the images scanned for analysis.
  2. Review the images before analysis to ensure that they are in-focus and without staining artifacts.
  3. Use a tumor marker (*e.g.*, CD20 in this example) to identify cells of interest and to proceed with cell segmentation, scoring, and batch analysis approaches. Select CD20-positive cells for the analysis.  
NOTE: For example, in the DLBCL samples analyzed here, the setting and parameters that are defined for cell segmentation are based on nuclear size and intensity but are specific to the image analysis software used (*e.g.*, a DAPI mean pixel intensity of at least 0.05; size in between 80 - 320 pixels, with a splitting sensitivity at 2 [this is a software-specific parameter which relies heavily on the morphology of the tumor: for small tumor cells, splitting of 0.7 - 1 is appropriate; for large tumor cells, splitting can be adjusted up to 4]).
  4. Request for qualified pathologist to review individual images and decide whether tissue segmentation is required to select tumor cell enriched regions and exclude regions with necrosis area/region or/and abundant reactive cells. If additional tissue segmentation is required, then select appropriate control regions (such as tumor cells/stroma/necrosis) and check that the image analysis software can correctly identify such regions
  5. Proceed with cell segmentation after tissue segmentation (if any), request a qualified pathologist to review the cell segmentation map to ensure the fidelity of the intended segmentation approach<sup>5</sup>.
  6. Determine the most biologically/clinically appropriate method of analysis for a given biomarker of interest (*e.g.*, percentage positivity, or mean intensity per cell).
  7. Select a cut-off value for each marker (for percentage positivity).

8. Determine the optical intensity positive cut-off value for each marker in conjunction with a pathologist. Generate histograms by analyzing the frequency distribution of the marker intensity/cell in appropriate statistics software (see **Table of Materials**).  
NOTE: An intensity value histogram can offer an overview of the distribution of the signal intensities.
  9. Determine an approximate cut-off from the histogram and verify this with a pathologist review, to correlate with manually determined cut-offs on selected images. In some situations, a uniform single cut-off will not be possible due to variability in staining, and a manual cut-off value for each sample will be required.  
NOTE: Section 8.1 should be done in image analysis software (see **Table of Materials**) unless otherwise specified.
2. **Marking positive and negative cells**
1. For each marker of interest, according to the cut-off number determined (through histograms or manually), use an "IF" or similar logic formula to mark positive and negative cells with marked value: number 1 for positive cells, number 0 for negative cells.
  2. For the positivity of all the markers, multiply the marked value of each marker (either 1 or 0) with the products in separate columns. If the product equals 1, it means the cell is positive for all markers. For the positivity and negativity of a specific marker, use an IF logic algorithm.  
NOTE: Section 8.2 needs to be done in statistics software (see **Table of Materials**).
3. **Generating percentage data and numeric data using a pivot table**
1. Generate percentage data for specific markers of interest, within defined cells (e.g., CD20-positive cells, or CD20-negative cells) (**Figure 6**).
  2. Insert a pivot table into the data sheet.
  3. To calculate the positivity percentage of a single marker, such as CD20, select the **SUM** function under the **Value** part of the pivot table to count the total number of CD20-positive cells using the sum of the CD20<sup>+</sup> cells divided by the total cell number. The result is the CD20<sup>+</sup> cell percentage within one core or one study number (depends on the selection of the pivot table row).
  4. Calculate the positivity percentage of multiple markers using the same method (**Figure 3**).
  5. Generate numeric data. Extract the median normalized count for each marker of each core or each study number by obtaining the mean intensity of each marker of interest in all the cells studied within a sample (**Figure 4**).  
NOTE: The median value cannot be derived in the pivot table directly. It can be derived using this formula: MEDIAN (IF (column of study number=specific study number, column of normalized value)).
4. **Plotting the data in a suitable graph**
1. Plot the results of the percentage positivity or median intensity per marker in a cell type of interest in an appropriate manner for further statistical testing and presentation.
  2. Create dot plots to provide a visualization of numbers and distribution.  
NOTE: Representing data with bar graphs does not convey information on distribution. Estimation plots are also recommended as a good method for data representation, with emphasis on the magnitude of difference between samples<sup>16</sup>.

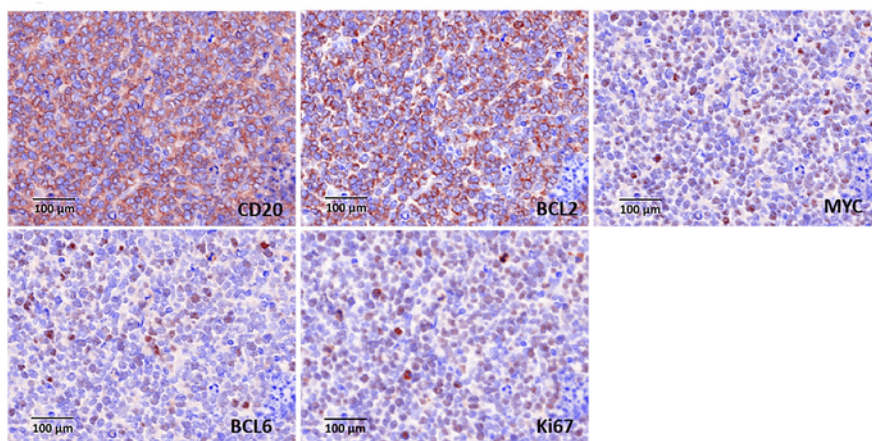
## Representative Results

mf-IHC images for a DLBCL sample with C-MYC and BCL2 gene rearrangement (double-hit lymphoma) are shown in **Figure 1**. **Figure 2** illustrates the simulated bright-field immunohistochemical images. **Figure 3** indicates the generation of percentage data. **Figure 4** displays the details of a median formula for the generation of numeric data. **Figure 5** shows the application of mf-IHC of a T-cell panel in angioimmunoblastic T-cell lymphomas. **Figure 6** shows the optimization image of the tonsil control sample and the data analysis for this sample.

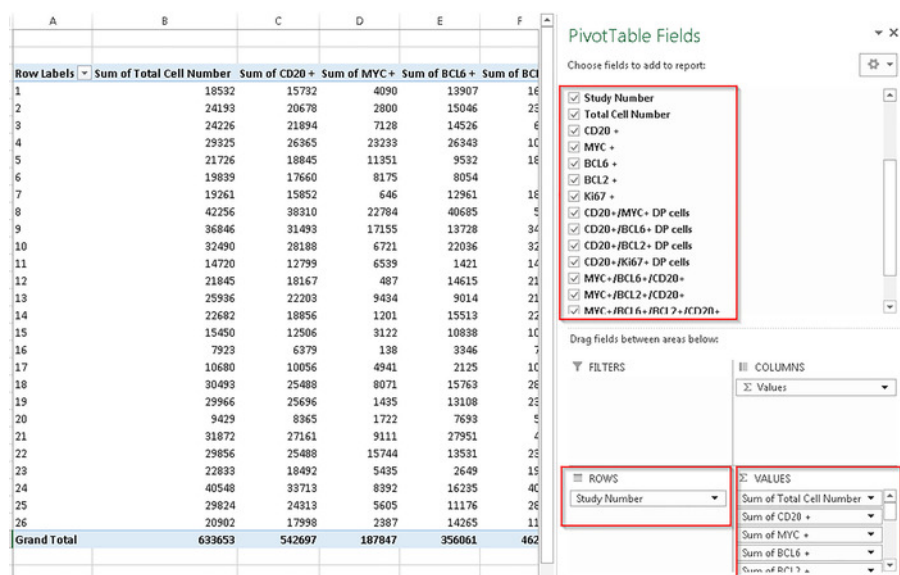




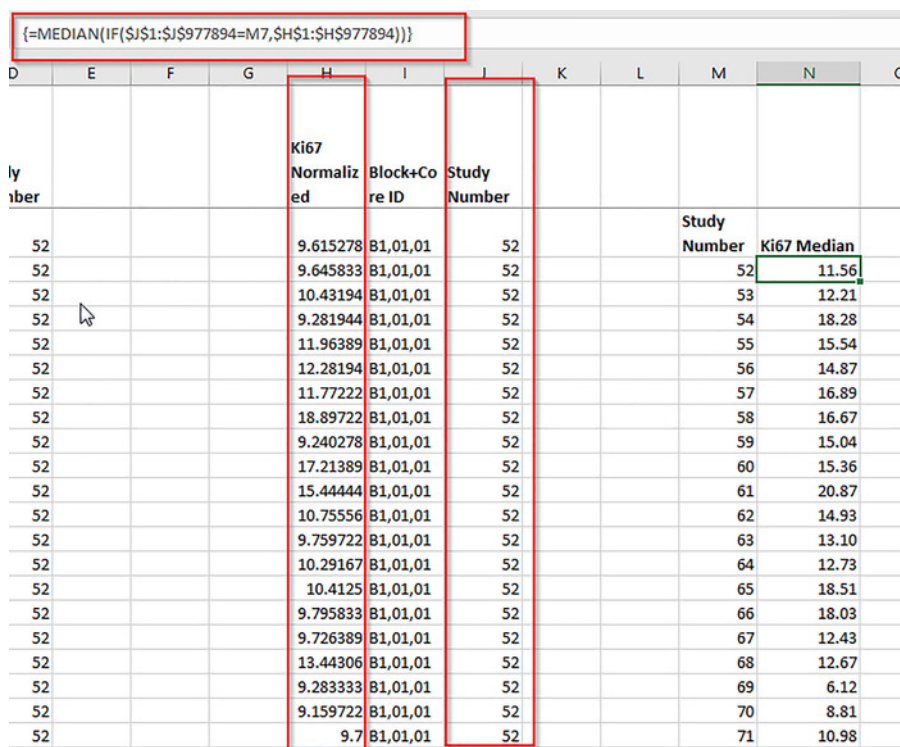
**Figure 1: B-cell multiplexed immunofluorescence panel images for a diffuse large B-cell lymphoma (DLBCL) sample with C-MYC and BCL2 gene rearrangement (double-hit lymphoma).** Magenta = CD20 (membrane); white = BCL2 (cytoplasm); yellow = Ki67 (nuclear); green = C-MYC (nuclear); red = BCL6 (nuclear), blue = DAPI (nuclear counterstain). The CD20-positive tumor cells show a high expression for C-MYC (80%) and BCL2 (>90%), and the Ki67 proliferation index is also high (90%). [Please click here to view a larger version of this figure.](#)



**Figure 2: Simulated bright-field immunohistochemical images (generated from Figure 1) of the same DLBCL sample with C-MYC and BCL2 gene rearrangement (double-hit lymphoma).** CD20 shows membrane staining in the tumor cells. BCL2 shows cytoplasm staining in >90% of the tumor cells. C-MYC positivity is about 80%. BCL6 shows nuclear staining in approximately 20% of the cells. Ki67 is positive in 90% of the cells. [Please click here to view a larger version of this figure.](#)

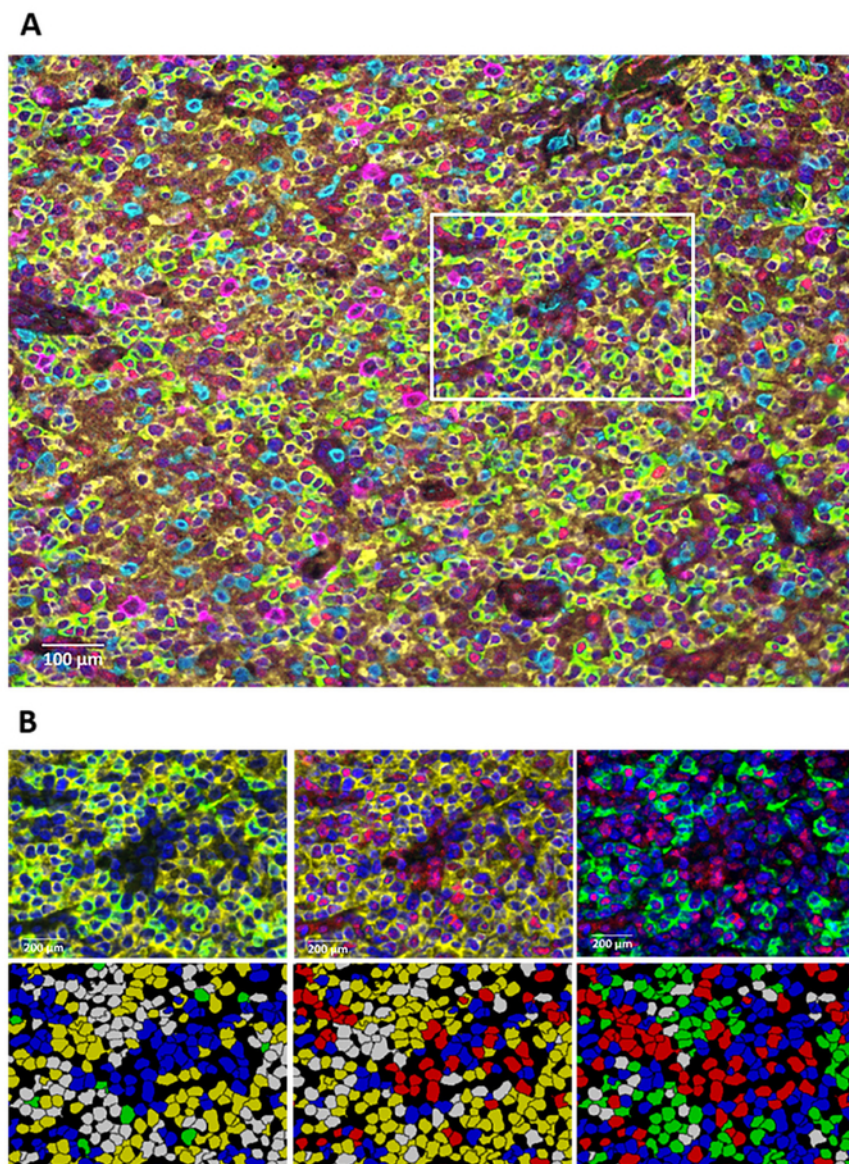


**Figure 3: Pivot table showing how to generate percentage data according to study number.** [Please click here to view a larger version of this figure.](#)



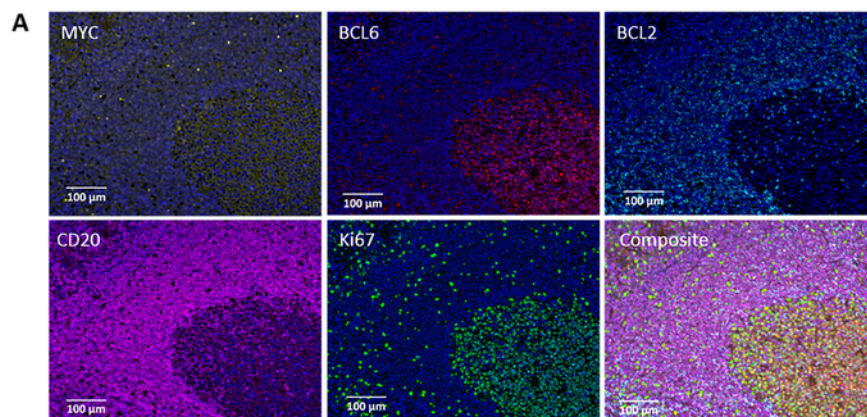
**Figure 4: Median formula for the normalized Ki67 OD value according to study number.** The IF statement finds all study numbers that are equal to a specific study number (which is 52 in this figure). Then, it returns the corresponding **Ki67 Normalized** value. **Ctrl + Shift + Enter** key combinations can be used to calculate the median (**Ki67 Median**) for these returned values, which is 11.56 for study number 52. [Please click here to view a larger version of this figure.](#)





**Figure 5: Multiplexed immunofluorescence panel images for an angioimmunoblastic T-cell lymphoma (AITL) sample. (A)** The composite image shows the cellular heterogeneity of an AITL sample. Magenta = CD20 (membrane); yellow = CD4 (membrane); green = PD1 (membrane); red = BCL6 (nuclear); cyan = CD8 (membrane); blue = DAPI (nuclear counterstain). **(B)** The upper row of images shows a magnified view of the region selected in the white box in panel A. The lower row of images shows the corresponding segmented cell masks: yellow/red/green showing single CD4/BCL6/PD1-positive cells, respectively. Blue represents negative cells, and white indicates double-positive cells. The images reveal that 50% of CD4<sup>+</sup> cells are PD1<sup>+</sup> (left, white), while 20% of CD4<sup>+</sup> cells are also positive for BCL6 (middle, white). The double positivity rate for PD1 and BCL6 is about 10% (right, white). [Please click here to view a larger version of this figure.](#)





**B**

A	B	C	D	E	F	G	H	I	J	K	L
Nucleus BCL6 (Cy5)	Nucleus MYC (Opal 570)	Nucleus Ki67 (Opal 620)	Membrane BCL2 (Opal 520)	Membrane CD20 (Opal 540)	Core ID	Total Cell Number	CD20+	MYC+	BCL6+	BCL2+	Ki67+
0.829	2.932	1.319	0.213	4.109	Tonsil control	1	1	0	1	0	1
0.977	4.685	2.28	0.326	5.178	Tonsil control	1	1	0	1	0	1
0.954	4.555	1.689	0.521	6.215	Tonsil control	1	1	0	1	0	1
1.033	3.596	2.073	0.196	4.286	Tonsil control	1	1	0	1	0	1
1.475	2.842	1.206	0.715	4.021	Tonsil control	1	1	0	1	0	1
1.382	3.708	1.312	0.349	4.849	Tonsil control	1	1	0	1	0	1
1.481	3.868	2.335	0.566	4.952	Tonsil control	1	1	0	1	0	1
1.573	3.825	2.671	0.189	3.902	Tonsil control	1	1	0	1	0	1
1.345	2.737	1.363	0.683	4.485	Tonsil control	1	1	0	1	0	1
0.756	5.889	2.668	0.448	4.959	Tonsil control	1	1	0	0	0	1
0.655	2.44	0.988	0.785	4.517	Tonsil control	1	1	0	0	0	0
1.767	2.594	5.509	0.192	11.644	Tonsil control	1	1	0	1	0	1
0.597	5.13	2.356	0.459	4.933	Tonsil control	1	1	0	0	0	1

**Figure 6: Multiplexed immunofluorescence optimization images for tonsil control tissue.** (A) The composite image shows the germinal center area of a tonsil control sample. Yellow = C-Myc (nuclear); red = BCL6 (nuclear); cyan = BCL2 (cytoplasm); magenta = CD20 (membrane); green = Ki67 (nuclear); blue = DAPI (nuclear counterstain). C-Myc is positive only in a few cells. BCL6 and Ki67 are positive mainly within the germinal center, while BCL2 is positive mainly outside the germinal center. CD20 is diffusely positive inside and outside the germinal center. (B) The table shows that germinal center CD20-positive cells are also positive for BCL6 and Ki67 but negative for BCL2. [Please click here to view a larger version of this figure.](#)

Sequence of antibody staining	Primary antibody(see table of materials)	Total HIER required	Actual pre stain HIER	Secondary antibody	Fluorophore	Post-TSA antibody stripping
1	Mouse anti- Bcl6 (1:30, 60min RT)	25minutes, Ph9	25minutes, Ph9	HRP-conjugated anti-mouse, 1:1000' for 10 '	OPAL Cy5 at 1:100	1 round 100%power 1 min, 20% power for 10min
2	Mouse anti-Bcl2(1:50,60 min RT)	25minutes, Ph9	None	HRP-conjugated anti-mouse, 1:1000' for 10 '	OPAL 520 at 1:100	1 round 100%power 1 min, 20% power for 10min
3	Rabbit anti-c-MYC(1:50,30 min RT)	25minutes, Ph9	None	HRP-conjugated anti-rabbit, 1:1000' for 10 '	OPAL 570 at 1:100	1 round 100%power 1 min, 20% power for 10min
4	Mouse anti-CD20(1:2000,30 min RT)	25minutes, Ph9	None	HRP-conjugated anti-mouse, 1:1000' for 10 '	OPAL 540 at 1:100	1 round 100%power 1 min, 20% power for 10min
5	Mouse anti-Ki-67(1:50,45 min RT)	30minutes, Ph9	5 minutes pH9	HRP-conjugated anti-mouse, 1:1000' for 10 '	OPAL 620 at 1:100	1 round 100%power 1 min, 20% power for 10min

**Table 1: Example of a finalized layout for multiplex IF staining.** This table provides an example of how to specify the amount of HIER and microwave-based stripping to be done at each step, once the monoplex stains have been optimized.

		Filter				
		DAPI	FITC	Cy3	Texas Red	Cy 5
Dye	DAPI					
	Opal 520					
	Opal 540					
	Opal 570					
	Opal 620					
	Opal 650					
	Opal 670					
	Opal 690					

**Table 2: Guide toward the filter selection for fluorophores.** This table provides a rough guide toward appropriate filters that can be used on specified equipment, to visualize fluorophores of interest. It is recommended to check the filter specifications of the microscope being used in relation to the emission/excitation profile of the fluorophores used.

## Discussion

mf-IHC has the potential to enable pathologists to refine diagnostic criteria in lymphoid pathology and to analyze the role of biomarkers in specific cell types toward a prediction of clinical outcome. As a new research method, mf-IHC is increasingly applied to the quantitative and spatial identification of multiple immune parameters of tumor cells<sup>17</sup>. The detection of mf-IHC for the co-expression of tumor biomarkers has been shown to be reproducible and reliable<sup>5</sup>. However, the technology remains nascent and subjected to variability arising from reagent- and/or tissue-related factors, such as those due to inconsistency in tissue fixation and processing.

Critical to the technique is the use of well-validated antibodies that are specific, sensitive, and give reproducible results. There are examples in the literature of antibodies originally described to be specific for their antigens and later demonstrated to recognize unrelated proteins through the use of knock-out models<sup>14</sup>. The knock-out/ knock-down validation method in which wild-type or cells with overexpressed antigens serve as positive controls while cells with the targets knocked out or knocked down by siRNA or CRISPR methods are used as negative controls.

Like the conventional IHC, mf-IHC has many critical variables that need to be optimized for every experiment, for optimal staining and results. These include the pH of the antigen retrieval solution, the antibody dilution, the assignment of a fluorophore to each marker, and the concentration of the fluorophore. The commonly used antigen retrieval solutions are of pH 6 and 9. It is worthwhile to test which pH gives the optimal staining pattern and intensity with less background.

There are no specific guidelines to decide on the sequence of antibody application in the multiplex experiment as it depends not just on the affinity of the antibody, but also on the strength of the opal fluorophores. In general, antibodies with a weak affinity often require higher concentrations based on the monoplex staining and are applied first in the multiplex sequence. Strong affinity antibodies that are likely to be resistant to stripping are applied last, to avoid nonspecific staining. With regard to the choice of a specific fluorophore, it is preferable to avoid using fluorophores with similar spectral wavelengths for antigens which colocalize in the same cellular compartments. Antigens with low expression levels are assigned with the brightest fluorophores and *vice versa*. If, in the study sample, the signal intensity is weak, adjusting the opal TSA dilution can be done to achieve the desired signal<sup>18</sup>. In some cases, trying different antigen retrieval methods or increasing the primary antibody concentration may work. The initial dilution of the primary antibody can be the same as that established in a conventional IHC experiment. However, if the IF signal is not clear, the testing of other antibody dilutions will be required, as the conditions optimized for IHC do not always translate to IF. Signal intensity is affected by the order or sequence of immunostaining. Some epitopes are overexposed after two or three rounds of MWT, while some may degrade due to excess MWT. Certain fluorophores can also be affected by MWT and need to be tested for attenuation.

This protocol focuses on the colocalization and measurement of the intensity of markers within subsets of cells in a malignant B-cell lymphoma. The key challenges in the development of the current protocol involve the generation of an optimized multiplex panel for antigens that colocalize in the same cellular compartments. There needs to be an accurate unmixing of fluorophores for precise quantification. Once the multiplex panel is in place, imaging of the stained slides is relatively straightforward. The next hurdle involves the analysis of the imaged data. Unlike in immune infiltration studies, where the quantitation of specific immune cell types within an epithelial tumor is straightforward, colocalization studies in lymphoma rely heavily on the definition of positivity by a trained pathologist for each marker of interest. Deriving an appropriate diagnostic cut-off which can be applied to routine clinical practice remains a work in progress. Further refinements in methods of data normalization and automated cut-off triangulation will be required before this technique can be integrated into routine diagnostics.

Despite current limitations, the potential applications of mf-IHC and the knowledge that may be gleaned from the study of tumor cells and their spatial relationship with the microenvironment make it an attractive tool, especially for challenging histological cancers such as lymphoid malignancy. Further work is needed to establish protocols in tissue fixation and tissue processing that are optimal for the proposed workflow, as well as an enhancement in staining techniques, to allow for the simultaneous analysis of an increased number of targets.

## Disclosures

A.D.J. has received funding from PerkinElmer toward travel to user-group meetings. The authors have no other conflict of interest to disclose.

## Acknowledgements

S.-B.N. and A.D.J. are supported by the Singapore Ministry of Health's National Medical Research Council Transition Awards (NMRC/TA/0020/2013 and NMRC/TA/0052/2016). The authors acknowledge a Yong Siew Yoon Research Grant to A.D.J. from the National University Cancer Institute of Singapore toward the purchase of a Vectra spectral imaging microscope. This study is approved by the Singapore NHG Domain Specific Review Board B (2014/00693).

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