Journal of Visualized Experiments

Multiplexed fluorescent immunohistochemical staining, imaging and analysis in histological samples of lymphoma

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58711R3
Full Title:	Multiplexed fluorescent immunohistochemical staining, imaging and analysis in histological samples of lymphoma
Keywords:	Multiplexed immunofluorescence; Multispectral Imaging; quantitative immunohistochemistry; digital pathology; Biomarkers; lymphoma
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Singapore, Singapore

To
Dr. Vineeta Bajaj and Jaydev Upponi
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Manuscript title: Multiplexed fluorescent Immunohistochemical staining, imaging and analysis in histological samples of lymphoma

Dear Vineeta and Jay,

Thank you for taking the time to read through the manuscript carefully. I have tried to address as many of your suggestions in this manuscript. Please review and accept the changes/ delete the comment boxes. I have not changed everything suggested, as some sections were requested by the reviewers, some sections are specific to a particular set of commercial projects (Opal-Vectra) and some sections are based on well-established methods (immunohistochemistry), for which we have provided references.

I would prefer not to make any further formatting amendments to this manuscript, unless there are valid scientific concerns about any section. I look forward to its acceptance and publication at the earliest.

With my best regards,

Dr. Anand D Jeyasekharan, *MBBS MRCP (UK) PhD*
Principal Investigator, Cancer Science Institute of Singapore,
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TITLE:

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

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KEYWORDS:

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

SUMMARY:

Here we describe a protocol for multiplex fluorescent immunohistochemical staining and imaging for the simultaneous localization of multiple cancer-associated antigens in lymphoma. This protocol can be extended to the colocalization analysis of biomarkers within all tissue sections.

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.

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[1].

Key to the classification of lymphoma has been the characterization of the antibodies against leukocyte surface markers of the various subtypes of lymphocytes[2]. 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[3]. 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[4]. Visual assessment and quantification of staining can also be subjective, causing variability in the analysis and data interpretation[5].

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[6,7.] 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[8,9]. 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[5,7,10]. 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[11]. 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[12].

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.

1.1. 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[5,13] 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.

1.2. For unknown targets or for commercial antibodies with insufficient published data, perform antibody validation by creating matched positive and negative control FFPE cell blocks[14], 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

2.1. 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.

2.2. 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.

3.1. Cut 3 µm-thin sections of positive and negative control tissue using a microtome and place the sections on poly-L-lysine-coated microscope glass slides.

3.2. 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.

3.3. 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 a domestic microwave was used with power ranging from 800 - 1,100 Watt for microwave stripping. The initial settings for the retrieval of a particular epitope are based on the prior knowledge from conventional IHC.

3.4. Perform additional rounds of microwave stripping (three in this case, for the fourth antibody in a panel), each round with 100% power for 1 min and 20% power for 10 min. 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.

3.5. 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.

3.6. 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. To prepare it, dissolve 6.05 g of Tris and 8.76 g of NaCl in 800 mL of H_2O. Adjust the pH to 7.5 with 1 M HCl and make a volume up to 1 L with H_2O. TBS is stable at 4 °C for 3 months. Add 1 mL of a nonionic detergent to 1 L of TBS to make TBS-D.

3.7. Incubate 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 µL (for biopsy samples) to 400 µL (for excision samples or tissue microarray samples).

3.8. Incubate with an appropriate horseradish peroxidase (HRP)-labelled secondary antibody, chosen on the basis of the species of origin of the primary antibody (1:1,000 dilution in antibody diluent) and DAPI (1:100) at room temperature for 10 min. Wash 3x with TBS-D buffer for 5 min each time.

3.9. 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.

3.10. Perform an additional microwave-based stripping to remove the primary and secondary antibody, for 1 min in antigen retrieval solution at 100% power and for 10 min at 20% power. Repeat as required based on the position of the antibody in the sequence.

3.11. Place the slide in distilled water to cool down. Dry the area on the slide without the tissue with wipes and mount the slide with appropriate mounting media.

3.12. 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

4.1. 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**.

5.1. Cut 3 µm-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.

5.2. Dewax, perform heat-induced epitope retrieval (HIER), and block tissue peroxidase activity as in the monoplex protocol steps 3.3 - 3.5.

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.

5.4. Incubate with an appropriate HRP-labelled secondary antibody based on the species of the primary antibody used in the prior step (typically, 1:1,000 dilution in antibody diluent, see **Table of Materials**) at room temperature for 10 min. Wash with TBS-D buffer for 5 min.

5.5. 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.

5.6. 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.

5.7. 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 previously optimized 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.

5.8. 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.

5.9. 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.

5.10. 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.

6.1. 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.

6.1.1. Cut 2 x 3 µm-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.

6.1.2. Process both slides using the monoplex protocol (with all stripping and washing steps), but without antibody or fluorophore addition.

6.1.3. 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.

6.2. Scan these set of slides using appropriate filters and upload them into the image analysis library.

7. Spectral Imaging

7.1. Scanning monoplex slides

7.1.1. 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**[15].

7.1.2. 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.

7.1.3. 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).

7.1.3.1. 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.

7.1.4. 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**).

7.2. Scanning multiplex samples (TMA slides/multiple samples)

7.2.1. 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.

7.2.2. Scan TMA slides under a TMA scanning mode if available in the imaging system, with an appropriate autofocus algorithm.

7.2.3. 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[7].

8. Data Analysis

8.1. Preanalysis assessment and planning

8.1.1. Use the reference slide for autofluorescence to subtract autofluorescence from the images scanned for analysis.

8.1.2. Review the images before analysis to ensure that they are in-focus and without staining artifacts.

8.1.3. 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]).

8.1.4. After segmentation, request a qualified pathologist to review the segmentation map to ensure the fidelity of the intended segmentation approach[5]. Review individual images, and decide if cell segmentation is adequate, or if additional tissue segmentation is required to select regions enriched for tumor cells/stroma/necrosis. If additional segmentation is required, then select appropriate control regions and check that the image analysis software can correctly identify such regions.

8.1.5. Determine the most biologically/clinically appropriate method of analysis for a given biomarker of interest (*e.g.*, percentage positivity, or mean intensity per cell).

8.1.6. Select a cut-off value for each marker (for percentage positivity).

8.1.7. 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.

8.1.8. 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.

8.2. Marking positive and negative cells

8.2.1. 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.

8.2.2. 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**).

8.3. Generating percentage data and numeric data using a pivot table

8.3.1. Generate percentage data for specific markers of interest, within defined cells (*e.g.*, CD20-positive cells, or CD20-negative cells) (**Figure 6**).

8.3.2. Insert a pivot table into the data sheet.

8.3.3. 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 $CD20^+$ cells divided by the total cell number. The result is the $CD20^+$ cell percentage within one core or one study number (depends on the selection of the pivot table row).

8.3.4. Calculate the positivity percentage of multiple markers using the same method (**Figure 3**).

8.3.5. 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)).

8.4. Plotting the data in a suitable graph

8.4.1. 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.

8.4.2. 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[16].

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 AND TABLE LEGENDS:

Figure 1: B-cell multiplexed immunofluorescence panel images for a diffuse large B-cell lymphoma (DLBCL) sample with C-MYC and CL2 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%).

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.

Figure 3: Pivot table showing how to generate percentage data according to study number.

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.

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 $CD4^+$ cells are $PD1^+$ (left, white), while 20% of $CD4^+$ cells are also positive for BCL6 (middle, white). The double positivity rate for PD1 and BCL6 is about 10% (right, white).

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.

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.

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[17]. The detection of mf-IHC for the co-expression of tumor biomarkers has been shown to be reproducible and reliable[5]. 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[14]. 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. Secondary antibodies can be optimized through the titration of concentration and the duration of incubation time. 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[18]. 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 at 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.

ACKNOWLEDGMENTS:

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).

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.

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Figure 1

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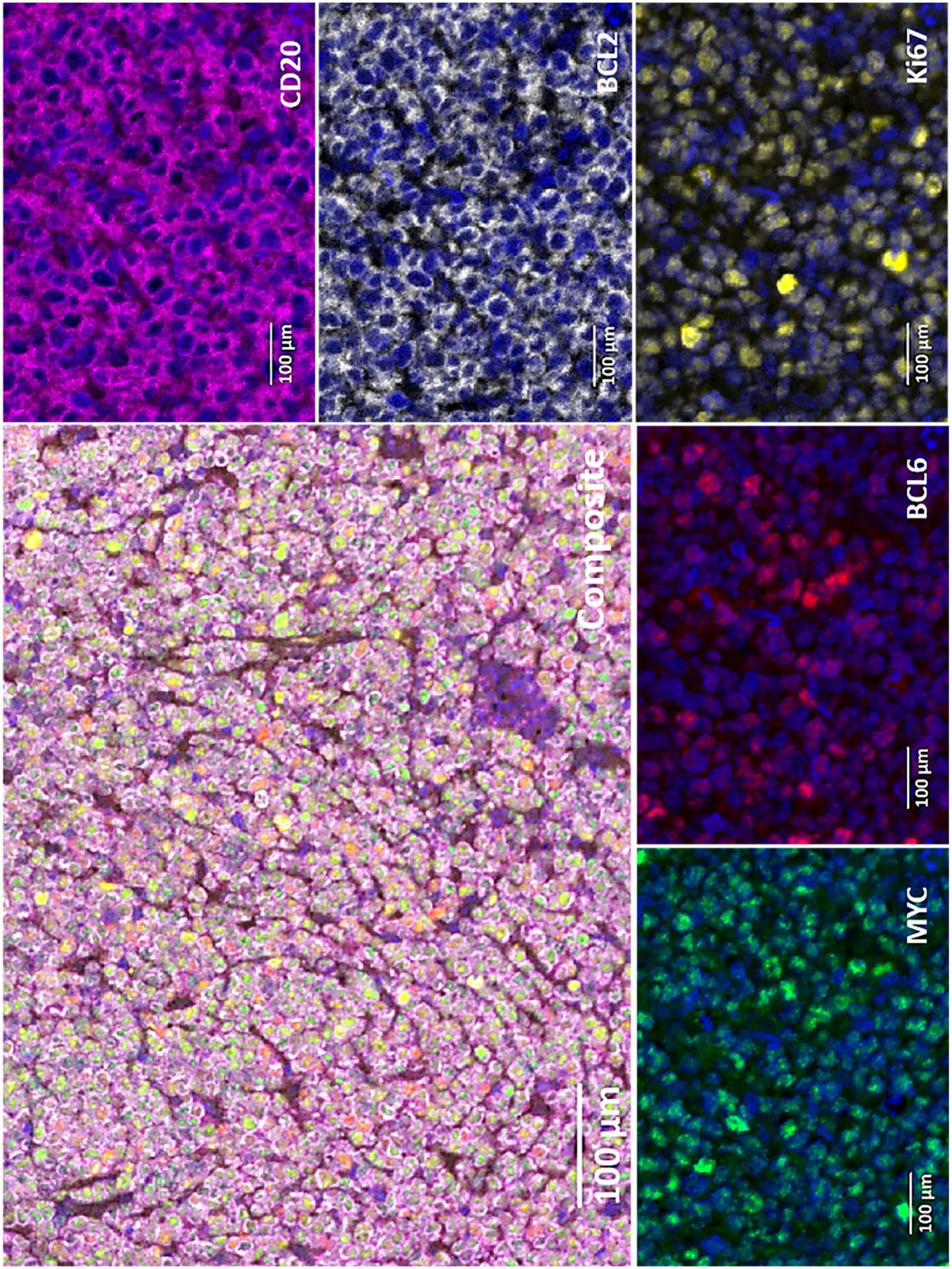

Fig 1.

Figure 2

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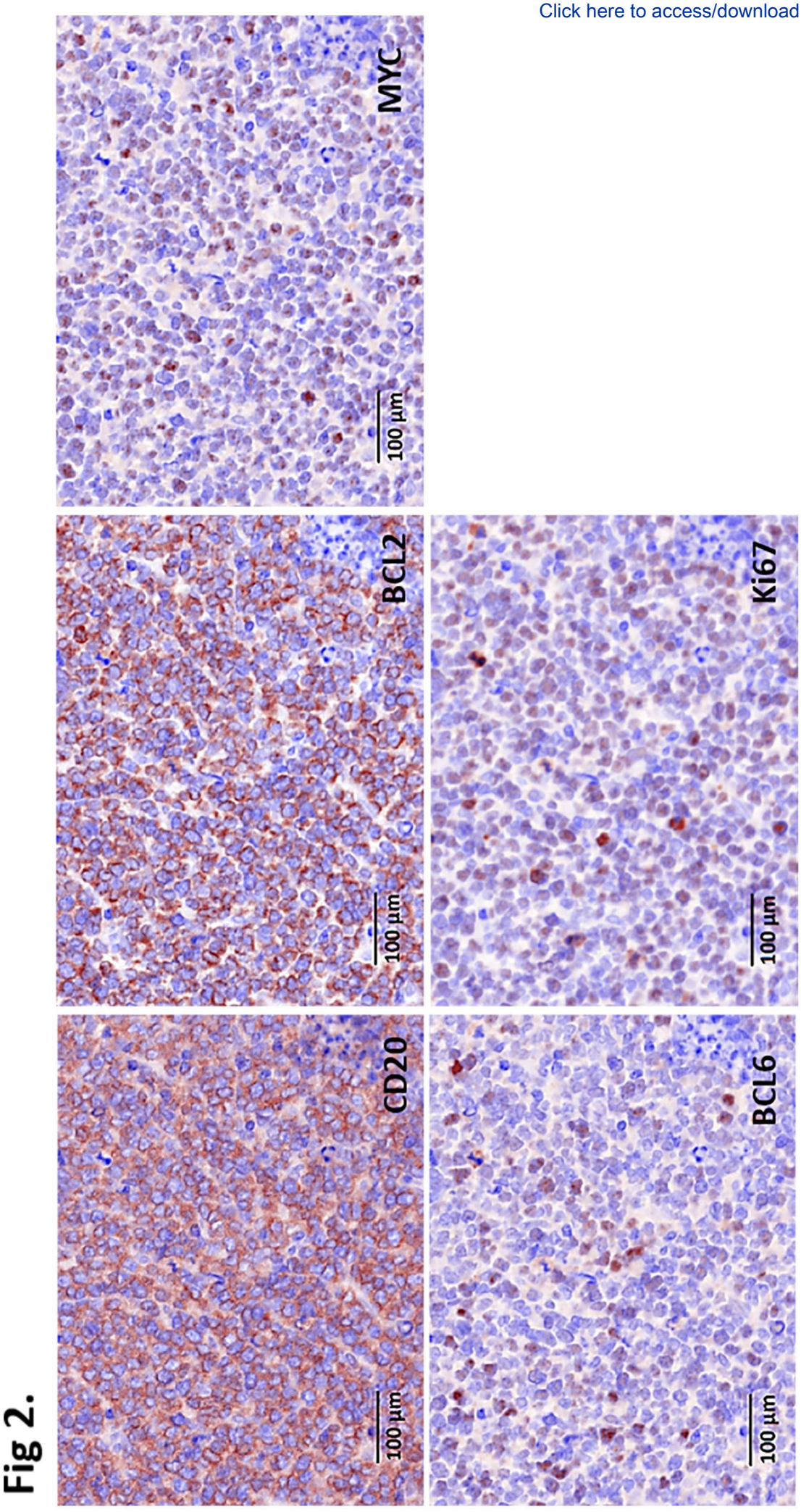
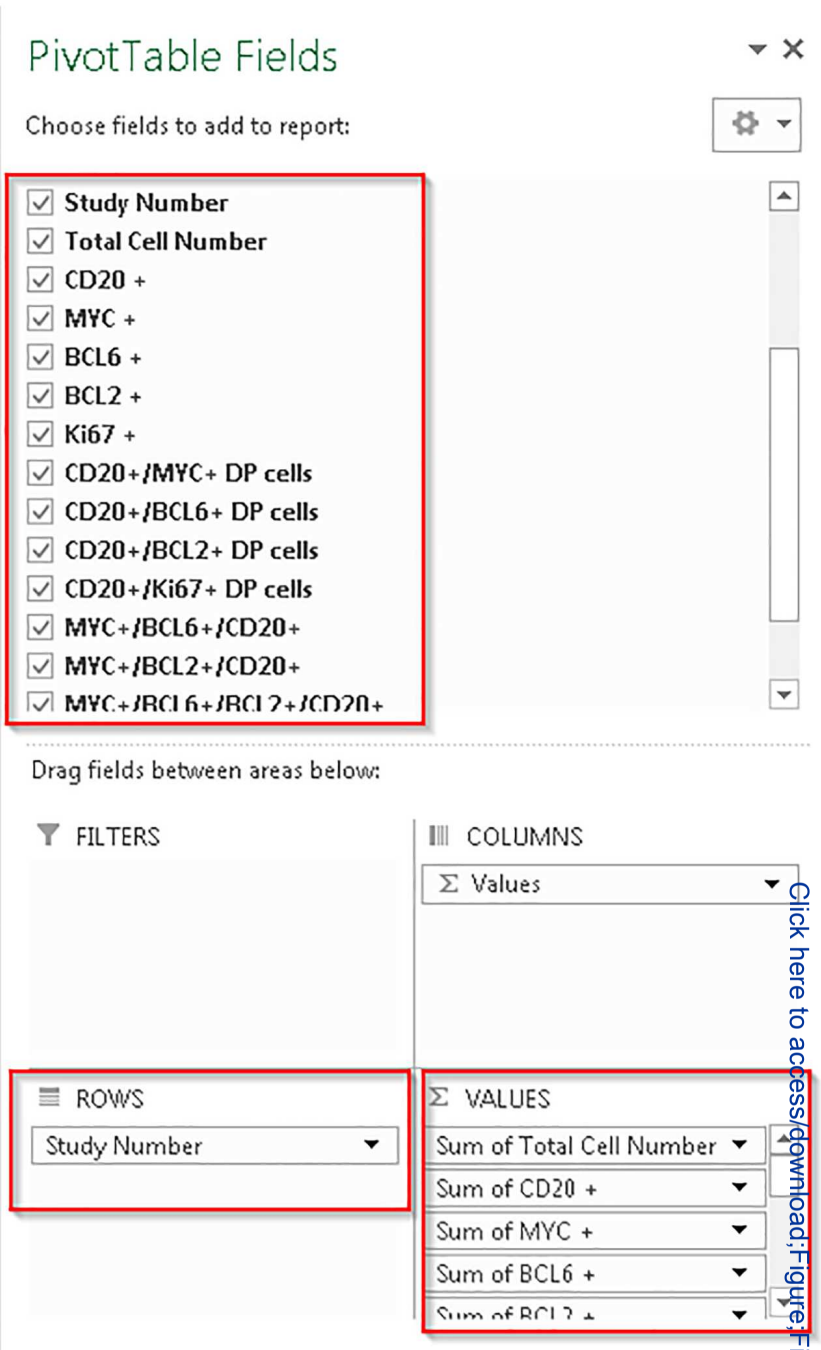


Fig 2.

Figure 3

Row Labels	Sum of Total Cell Number	Sum of CD20 +	Sum of MYC +	Sum of BCL6 +	Sum of BCI
1	18532	15732	4090	13907	16
2	24193	20678	2800	15046	23
3	24226	21894	7128	14526	6
4	29325	26365	23233	26343	10
5	21726	18845	11351	9532	18
6	19839	17660	8175	8054	
7	19261	15852	646	12961	18
8	42256	38310	22784	40685	5
9	36846	31493	17155	13728	34
10	32490	28188	6721	22036	32
11	14720	12799	6539	1421	14
12	21845	18167	487	14615	21
13	25936	22203	9434	9014	21
14	22682	18856	1201	15513	22
15	15450	12506	3122	10838	10
16	7923	6379	138	3346	7
17	10680	10056	4941	2125	10
18	30493	25488	8071	15763	28
19	29966	25696	1435	13108	23
20	9429	8365	1722	7693	5
21	31872	27161	9111	27951	4
22	29856	25488	15744	13531	23
23	22833	18492	5435	2649	19
24	40548	33713	8392	16235	40
25	29824	24313	5605	11176	28
26	20902	17998	2387	14265	11
Grand Total	**633653**	**542697**	**187847**	**356061**	**462**



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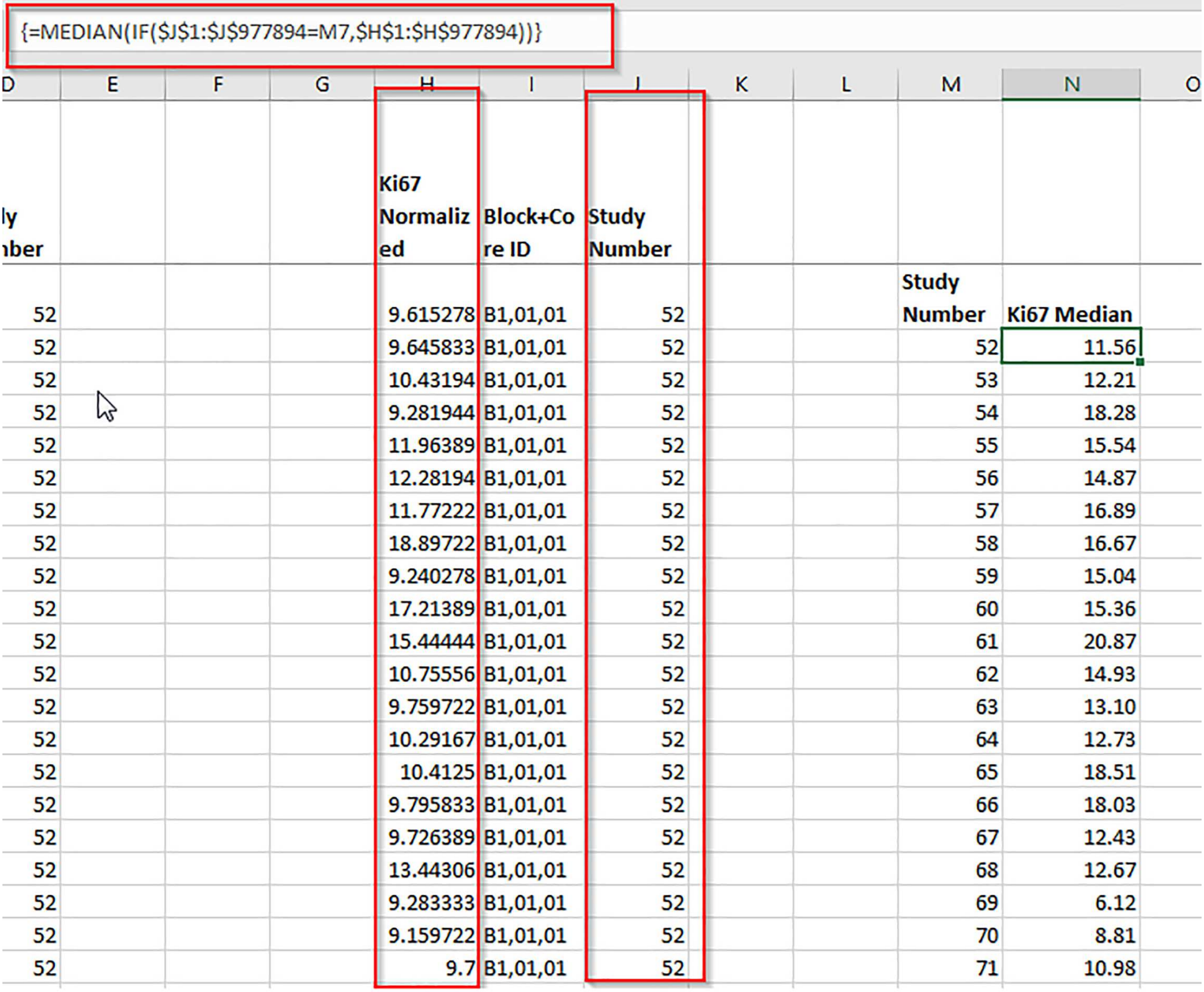

{=MEDIAN(IF($J$1:$J$977894=M7,$H$1:$H$977894))}

D	E	F	G	H	I	J	K	L	M	N	O
ly nber				Ki67 Normalized	Block+Core ID	Study Number					
52				9.615278	B1,01,01	52			Study Number	Ki67 Median	
52				9.645833	B1,01,01	52			52	11.56	
52				10.43194	B1,01,01	52			53	12.21	
52				9.281944	B1,01,01	52			54	18.28	
52				11.96389	B1,01,01	52			55	15.54	
52				12.28194	B1,01,01	52			56	14.87	
52				11.77222	B1,01,01	52			57	16.89	
52				18.89722	B1,01,01	52			58	16.67	
52				9.240278	B1,01,01	52			59	15.04	
52				17.21389	B1,01,01	52			60	15.36	
52				15.44444	B1,01,01	52			61	20.87	
52				10.75556	B1,01,01	52			62	14.93	
52				9.759722	B1,01,01	52			63	13.10	
52				10.29167	B1,01,01	52			64	12.73	
52				10.4125	B1,01,01	52			65	18.51	
52				9.795833	B1,01,01	52			66	18.03	
52				9.726389	B1,01,01	52			67	12.43	
52				13.44306	B1,01,01	52			68	12.67	
52				9.283333	B1,01,01	52			69	6.12	
52				9.159722	B1,01,01	52			70	8.81	
52				9.7	B1,01,01	52			71	10.98	

Figure 5

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Fig 5.

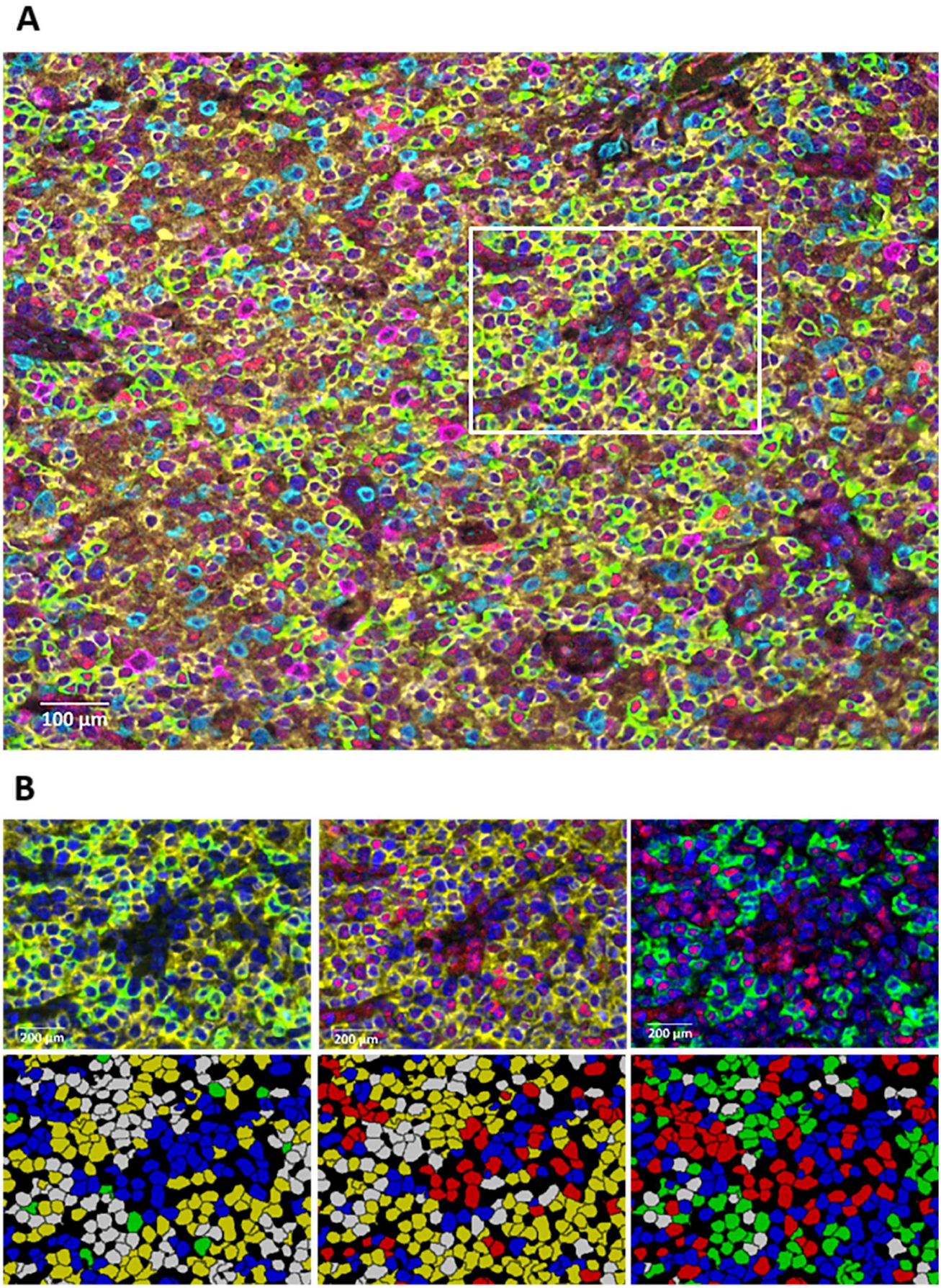
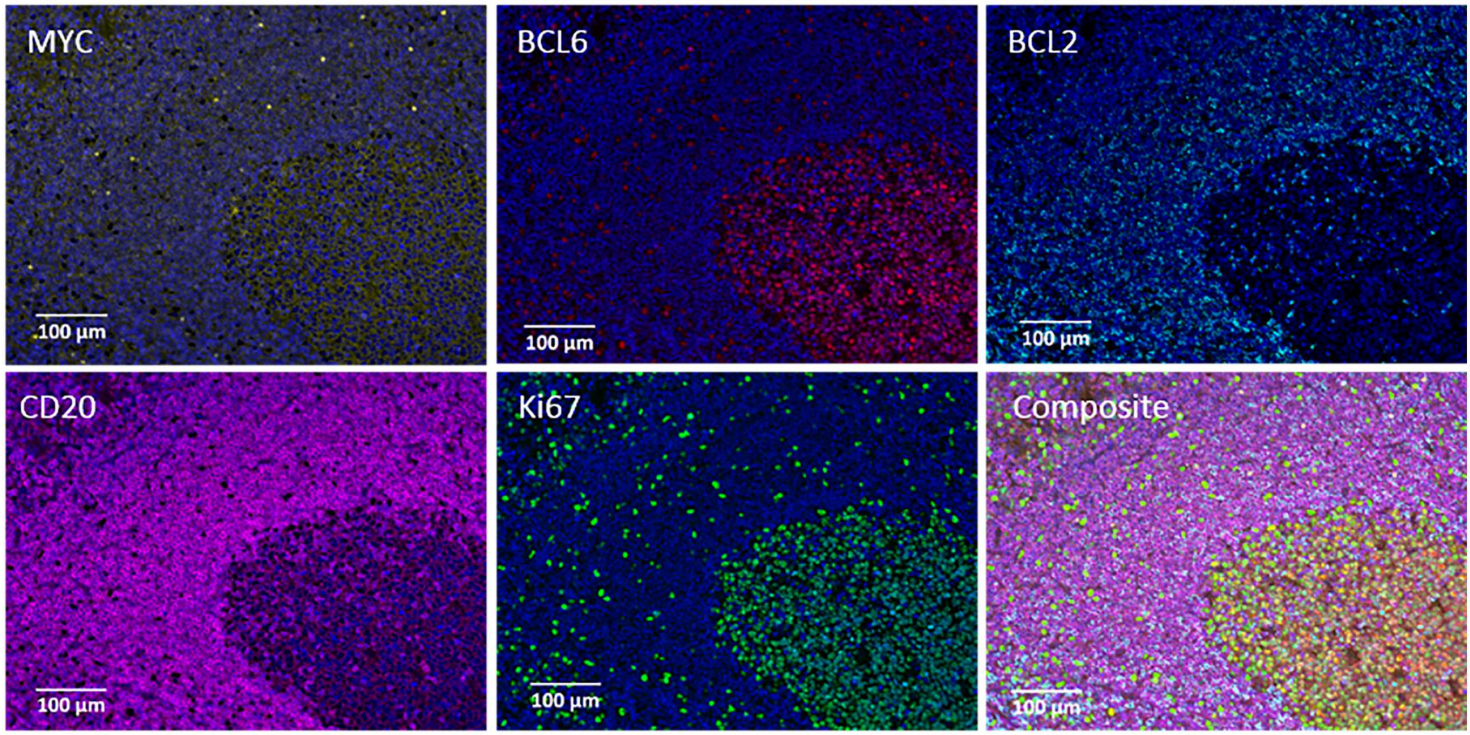


Fig 6.

A



B

A	B	C	D	E	F	G	H	I	J	K	L
Nucleus BCL6 (Cy5) Mean	Nucleus MYC (Opal 570) Mean	Nucleus Ki67 (Opal 620) Mean	Membrane BCL2 (Opal 520) Mean	Membrane CD20 (Opal 540) Mean	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

Table 1

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 2

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

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Antibody diluent	DAKO	REF S3022	Blocking
Peroxidase Blocking Solution	DAKO	S2023	For peroxide blocking
Vectra multispectral automated microscope	Perkin Elmer	Vectra2.0.8	Spectral imaging
absolute Ethanol	EMSURE	1.00983.2500	Ethyl alcohol for rehydration
Amplification Diluent	PERKIN ELMER	FP1135	Fluorophore diluent buffer
Anti-Mouse IgG [Goat] HRP-Labeled	PERKIN ELMER	NEF822001EA	Secondary antibody
Anti-Rabbit IgG [Goat] HRP-Labeled	PERKIN ELMER	NEF812001EA	Secondary antibody
BCL2	DAKO	clone 124 (Lot No. 20031561)(RRID-AB578693)	primary antibody
BCL6	LEICA	NCL-L-Bcl6-564(Lot No.6050438)(RRID-AB563429)	primary antibody
CD20	DAKO	Clone L26 (Lot No.20028627) (RRID-AB442055)	primary antibody
c-MYC	ABCAM	Y 69 clone ab32072 (Lot NO.GR29511133)(RRID-AB731658)	primary antibody
Cy 5	PERKIN ELMER	FP1171	Appropriate tyramide based fluorescent reagent
Graphpad Prism 7	Graph pad		Statisitcal software
HistoClear Clearing Agent	SIGMA	H2779-1L	Histoclear for dewaxing and clearing
inForm Advanced Image Analysis Software	Perkin Elmer	Inform Software 2.2.1	Data Analysis software

KI67	DAKO	Clone MIB-1 (Lot No.20040401) (RRID-AB2314699)	primary antibody
KOS MILESTONE multifunctional tissue processor	Milestone		Microwave for Heat induced epitope retrieval
Microwave	PANASONIC	NN-ST651M	Microwave stripping
Mowiol	SIGMA ALDRICH	81381 Aldrich Mowiol® 4-88	mounting media
Opal 570	PERKIN ELMER	FP1488	Appropriate tyramide based fluorescent reagent
Opal520	PERKIN ELMER	FP1487B21	Appropriate tyramide based fluorescent reagent
Opal540	PERKIN ELMER	FP1494	Appropriate tyramide based fluorescent reagent
Opal620	PERKIN ELMER	FP1495	Appropriate tyramide based fluorescent reagent
Poly-L-lysine coated slide	FISHER SCIENTIFIC	120-550-15	Slide for tissue section adhesion in routine histological use
Spectral DAPI	PERKIN ELMER	FP1490	nucleic acid stain
Target Retrieval Solution, pH9.0(10x)	DAKO	S2367	For HIER
Tris Buffer saline (TBS)	1st BASE	BUF3030 20X4L	for buffer wash
Tween 20	SIGMA ALDRICH	P1379-1L	Tween

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Article Title:	Multiplexed fluorescent Immunohistochemical staining, imaging and analysis in histological samples of lymphoma
Signature:	
Date:	28/6/18

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Rebuttal letter with point-by-point response to Reviewers/ Editorial team

Editorial comments:

Changes to be made by the Author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript and we are sure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

Please find the email address for each author as following:

1) Guo Hong: guohong_agzyy@outlook.com
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3. Keywords: Please provide at least 6 keywords or phrases.

The keywords for our manuscript are: Multiplexed immunofluorescence, Multispectral imaging, Quantitative analysis, Biomarkers**,** Lymphoma, Digital Pathology.

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We have deleted or modified all the commercial language.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Also see examples below:

1.1-1.3: Please describe how these steps are actually performed. They do not have enough detail to replicate as currently written.

We have modified these parts, please refer to 1.1-1.2 in the modified manuscript. The IHC experiments and cell block preparation mentioned here are performed with standard protocols, and referenced accordingly.

2.1: Please specify the source of tissue samples. What is used to cut into sections? Are the sections placed on slides?

This has been modified in the text. We have used tonsil and lymphoma samples from excision biopsies. Of them, tonsil is our control tissue for protocol optimization. We used a microtome to cut tissue into sections and placed the sections on adhesive microscope glass slides.

2.9(now 2.4): What volume of Dako antibody diluent is used?

This is specified in the text now. The volume of antibody diluent depends on the size of the tissue, ranging from 50 µl (for biopsy samples) to 400 µl (for excision samples or tissue microarray samples).

2.11, 2.13, 2.15, 3.9, etc.: What is the incubation temperature? Please specify throughout the protocol.

All the antibodies mentioned in this manuscript are incubated at room temperature. We have specified this condition throughout the protocol.

Step 4, 6.1.3: Please describe any specific actions being performed here so that we can adequately film this step.

We have expanded on these steps.

5.1.1: What are the criteria for choosing the different filters?

We have included the criteria for filter selection in 5.1.1, and Table 1 was added to illustrate this.

5.1.2: Please specify how to optimize the assay conditions.

We have described how these steps are performed in more detail.

6. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We agree with this comment. We have combined the shorter protocol steps.

7. Please include single-line spaces between all paragraphs, headings, steps, etc.

Single-line spaces between all paragraphs, headings, steps have been included.

8. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There are a 10 page limit for the Protocol, and a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have re-evaluated the length, the protocol is less than 10 pages and the highlighted parts are less than 2.75 pages.

9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have ensured that the highlighted part of the step includes at least one action.

10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have included all relevant details to perform the highlighted steps.

11. Figures 1, 2, 5: Please include a space between the numbers and their units of the scale bar (i.e., 100 µm).

We have modified all the scale bars in all the figures.

12. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

We have included the acknowledgements section.

13. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

We have included a disclosures section.

14. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

We have provided the required information in the Table of Equipment and Materials.

Reviewers' comments:

Reviewer #1:

I reviewed the manuscript, "Multiplexed fluorescent immunohistochemical staining, imaging and analysis in histological samples of lymphoma".

Below is the comments for each line indicated on JoVE58711R1.

We are grateful to the reviewer for their thorough review of our manuscript, and for all the suggestions for improvement.

Line 102: Transfection to these cells may be a bit tricky. However, using lentiviral particle to knockout or knockdown is also available. This kind of alternate procedure should also be described here.

We thank the reviewer for this comment. We have modified 1.1-1.2 sections.

Line122: The authors may refer Milestone's microwave. However, not all laboratory has same equipment. Therefore, the authors should describe at least the pressure (opened to air or the particular pressure).

We thank the reviewer for highlighting this point. We are using the microwave under normal pressure (opened to air).

Line 125: Again, this sounds like a necessity of buying a material from a particular company. Therefore, this should show H2O2 concentration as an alternate reagent

We agree with this comment. We have included additional note: 3% hydrogen peroxide can be used as an alternate reagent (please refer to 2.4).

Line126: TBS may be different in each lab, therefore the contents should be described.

We thank the reviewer for raising this point. We have provided more details in 2.4.

Line127: What can be an alternate for DAKO diluent? It should be described.
We appreciate the review's suggestion. Bovine serum albumin can be used as an alternate for DAKO antibody diluent. Please refer to 2.5.

Line128: Each microwave has different power setting. What power of microwave was used in this manuscript? 1100W? 1200W? Which reagent was used?

We thank the reviewer for raising this point. We have added the power of microwave as 800 -1100 Watt.

Lin2 127: and Line 128. If this order, blocking reagent could also be stripped by microwave. This is most probably because these lines are in wrong order.

We thank the reviewer for highlighting this part. The microwave stripping step was indeed in the wrong order, and has been revised.

Line135: This may be "concentrated" antibody from DAKO. It should be noted along with clone number.

We appreciate this comment. We have included the antibody clone number in the table of equipment and material.

Line 139, 141, and many others: washing step is only once? How much volume of TBS was used? No Tween added? (and no need for adding tween?) And Tween should not be added to stripping buffer (safety issue).

We thank the reviewer for highlighting this. We usually perform the washing steps with TBST for three times. Antigen Retrieval buffer is used for stripping. TBST is used as a wash buffer only, and this has been clarified in the manuscript.

Line143: If it does not damage the slide, it is fine. However, for the safety issue, it might not be safe to handle boiled solution. Something about safety should be noted here.

We thank the reviewer for this suggestion. We recommend users to use forceps and heatproof gloves to remove slides into a separate jar of distilled water. Please refer to 2.8.

Line162: antibody incubation time can be variable. It should be mentioned somewhere.

We appreciate the reviewer for raising this point. We have incorporated this into step 2.

Line167: Perkin Elmer's product is named as "Opal"+ number (Opal520 reagent). From this line, it is indicated as "Opal reagent"+ number. As far as this is the product from Perkin Elmer, the product name should be precise.

We agree with this comment. We have modified the reagent name in the table of equipment and material list.

Line233: What are the setting and parameters for this analysis? Especially, since checking cell segmentation does not need pathologist to check, what parameters were used in this study? (especially, typical intensity of DAPI, splitting sensitivity, minimum nuclear size are important.
And authors should describe if only Vectra can do this or there are some other alternative way to visualized these multiplex IHC samples (stained with Opal dyes). Furthermore, it is also important to describe if image taken by Vectra can be opened by software other than inForm.

We appreciate the reviewer's comments and have incorporated the changes into the text. We always have trained pathologists to help check cell segmentation. The pathologist needs to review individual images, and decide if cell segmentation is adequate, or if additional tissue segmentation is required to select regions enriched for tumour cells/ stroma/ necrosis. If additional segmentation is required, then select appropriate control regions and check that the image analysis software is able to correctly identify such regions. We have also included a line on other machines and image anlalysis software that can scan and analyze multiplex IHC slides, such as Aperio FL as an alternative for Vectra, HALO from Indica Labs for image analysis[1].

The appropriate changes have been made in the text

Figure1 is fine, but Figure 2 should show lymphoma and peripheral tissues.

We appreciate the reviewer for highlighting this. We stained the DLBCL cases using tissue microarray (TMA) sections. The TMA cores are all taken from tumour tissue with the highest tumour content and peripheral issues are not included in our TMA cores. Hence, we are not able to illustrate peripheral tissues for figure 2. However, we have added Figure 6 to demonstrate the staining in a normal tonsil control tissue sample.

Reviewer #2:

Manuscript Summary:
Hong et al, described the multiplex IF/IHC method that recent years quite widely use in the translational research and tried to address a technical difficulty by looking at lymphoma.
They already tried to provide their protocol on staining and analytic pipeline which is honest, open and generous.

We are grateful to the reviewer for their thorough review of our manuscript, and for all the suggestions for improvement.

Major Concerns:

In general, as a technical paper/protocol paper, i have several concerns about this paper:

1) the image provided not super high resolution, and in fact is quite blurry (maybe due to the copy that I am reviewing)

We apologize for the quality of the first submission. We have provided all the figures in eps format with higher resolution for the revised manuscript.

2) I would suggest the author to provide the same images with higher magnification too.

We appreciate the point being made. In this case, 20x images are routinely used for Vectra imaging, as the quantitative data is similar with 20x and 40x images, but the speed is much faster with 20x for large data sets. We have retained 20x images as they are a more accurate representation of the images that are typically used for quantitation.

3) the protocol is unclear about the difference between multiplex and single, is there any different condition of the same antibody that the authors adopted for single or multiplex? how was the optimization/validation done? on single, or on multiplex; on IHC or on IF? If there is optimization on IHC and IF done, please kindly provide the correspondent image too.

We thank the reviewer for highlighting this. Some of the images are at lower magnification to illustrate the spatial relationship of tumour cells with surrounding microenvironment (i.e. peripheral tissue as requested by reviewer 1). However, we have taken reviewer 2's suggestion and replaced some images with higher magnification, eg. Figure 5B. In general, the optimization is largely performed at the monoplex IF step, after which the conditions are replicated in the multiplex. Please refer to Figure 6 for multiplex IF optimization images.

4) can the authors show some internal control when they analyse the data? e.g. a cell that not suppose to be expressing one particular marker, will be showing 0 or 0% in their analysis.

We appreciate the reviewer for raising this point. For most lymphoma antigens, we use tonsil slides as our control tissue, where there is an admixture of cells, some of which serve as negative controls. We have added Figure 6 B to show germinal center CD20 positive cells are also positive for BCL6 and Ki67, while being negative for BCL2.

Minor Concerns:

1) I am not sure whether it is limited by the journal, the citation in this article is very limited. many places that needed citation are lacking or insufficient.

We thank the reviewer for this comment. We have added more citations within the manuscript.

2) the citation is obsolete. some of the reference about this technique is almost 10yrs ago which make this paper not so timely. I suggest the authors cite at least some papers on the field from 2017-2018.

We apologize for this oversight. We have added more recent citations in this regard.

Reference:

1 Parra, E. R. Novel Platforms of Multiplexed Immunofluorescence for Study of Paraffin Tumor Tissues. *Journal of Cancer Treatment and Diagnosis.* **2** (1), 43-53, doi:10.29245/2578-2967/2018/1.1122, (2018).