Flow cytometric sorting coupled with exon capture sequencing identifies somatic mutations in archival lymphoma tissues

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The enormous number of archived formalin-fixed paraffin-embedded (FFPE) tissues available are a valuable resource of material for research. However, the use of such tissues poses many challenges, among which is the difficulty of isolating different cell populations within the tissue. In this study, we used tissue from two types of non-Hodgkin lymphoma as a model to demonstrate a method we have established and optimized to separate FFPE samples into distinct tumor and nonmalignant populations. Using FFPE reactive tonsil sections, various approaches for antigen retrieval and labeling, and the effectiveness of flow cytometric sorting were tested. We found that, among the 11 cell surface or intracellular antigen markers investigated, CD3 ϵ , CD79A, LAT, PD-1, and PAX5 could be successfully labeled after antigen retrieval in Tris-EDTA buffer (pH 8.0) at 65 °C for 60 min, and 1.8–2.7 μ g DNA per million cells could be extracted after sorting with DNA quality similar to that of tissue without staining or sorting. To test whether we could perform next-generation sequencing using a custom capture platform on sorted cells, we used three lymphoma cases with FFPE tissues which had been stored for 1 to 4 years. We demonstrated that the DNA from sorted cells was adequate for exon capture sequencing. By comparing the sequencing results between neoplastic and normal populations, somatic mutations could be clearly identified in the tumor population with variant frequencies as low as 11.7%. The corresponding normal fraction clearly helps in the analysis of somatic mutations and the exclusion of artifacts. This study provides an approach using flow cytometric sorting to separate different cellular populations in paraffin-embedded tissues and to unambiguously distinguish somatic mutations from germline variants or artifacts. This approach is also useful in enriching the tumor component in samples with heterogeneous components and low tumor content.

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Formalin-fixed paraffin-embedded (FFPE) tissues are routinely utilized in lymphoma research,¹ as this preservation process is both ubiquitous and robust. However, the use of FFPE tissues for antigen-antibody labeling and next-generation sequencing (NGS) has consistently posed logistical challenges. First, the preservation process induces crosslinks that mask epitopes for antibodies. Heating FFPE samples to temperatures near 100 °C is often recommended for crosslink reversal and antigen retrieval. However, using such high temperatures for antigen retrieval may damage DNA and reduce its quality to the extent that it may no longer be suitable for downstream applications such as exon capture sequencing.

An additional difficulty of conducting sequencing analysis on lymphoma samples is the frequent absence of the corresponding normal samples and the heterogeneity of cells contained within the biopsied tissue.² Although many germline variants that match known variants present in the general population can be eliminated by comparison to databases such as the single nucleotide polymorphism database (dbSNP), rare germline variants may be missed in the absence of a corresponding normal sample. In order to have confidence of the somatic origin of the detected single nucleotide variants (SNVs) or other apparent mutations, NGS requires both the neoplastic cells and a corresponding sample of normal, non-neoplastic cells with which to compare.³

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A further problem is that the fraction of neoplastic cells is low in some non-Hodgkin lymphoma samples, resulting in a low variant allele frequency (VAF). In such cases the mutations may be entirely missed or present at such low frequencies as to be difficult to distinguish from background sequencing errors.⁴

Purification of tumor lymphocytes by flow sorting or magnetic separation can help to resolve these problems, but these procedures are usually done on freshly isolated cells.^{5–7} For FFPE tissue, laser microdissection can be utilized,^{8–12} but the technique is labor-intensive, and it is difficult to obtain a sufficient number of cells. Furthermore, prior labeling of the tissue sections may be necessary to facilitate the recognition of the desired cell populations to isolate, and routine antigen retrieval procedures may further degrade the DNA.

It is clearly of value to optimize a protocol for flow sorting and mutational analysis that separates samples into neoplastic and corresponding normal cells after antigen retrieval and immunostaining without compromising the integrity or DNA quality of the single cells. A few previous research reports have mentioned the value of isolating tumor cells for cancer studies on FFPE tissue through DNA ploidy and immunophenotyping analysis.^{13,14} However, these studies did not provide a robust protocol for ensuring effective antibody labeling and the isolation of DNA of sufficient quality for exon capture sequencing. In this study, we report a method for separating FFPE samples into distinct groups of neoplastic and non-neoplastic cells by gentle antigen retrieval, antigen labeling, and flow cytometric sorting. We confirmed the validity of our method by extracting DNA from the sorted FFPE cells and assessing it for quality. In addition, we performed exon capture sequencing on our enriched cell populations to determine whether our approach will help to identify tumor-associated mutations, especially those occurring in lymphomas with low tumor content.

MATERIALS AND METHODS

Materials

Four de-identified FFPE tissue blocks from the archives of the Department of Pathology at City of Hope Medical Center were utilized in this research, including one tonsil tissue stored for two years with reactive hyperplasia that was used for evaluating the conditions for cell isolation and antigen retrieval and labeling, one lymph node sample stored for one and a half years from a patient with follicular lymphoma (FL), and two lymph node samples from patients with angioimmunoblastic T-cell lymphoma (AITL; named as case 1 and case 2, stored for one year and four years, respectively). These lymphoid tissues were fixed for 4 h to overnight in 10% buffered formalin before further processing to prepare FFPE blocks. The diagnoses were confirmed by hematopathologists in the department. This study was approved by the Institutional Review Board of the City of Hope Medical Center. Thick sections of 50 micrometers were prepared.

Single Cell Isolation from FFPE Tissue Sections

For estimating cell recovery, FFPE tissue sections, after removing the surrounding paraffin, were weighed with GeneMate GM Research Balance (B-1850-60). Then the tissue sections were deparaffinized three times with xylene in a 1.5 ml Eppendorf tube, each with 10 min incubation and rotation at room temperature. This was followed by sequential 5 min rehydration in ethanol at decreasing concentrations, from 100% (twice) to 90, 70, 50, and 20%. Ethanol was washed out with water during the last step. Each time the tube was centrifuged at 10 000 g for 3 min and the supernatant was discarded carefully.

The tissue was added to 150 μ l PBS in a 1.5 ml Eppendorf tube and ground gently with a Fisherbrand™ Pellet Pestle (Fisher Scientific, catalog No. 12-141-364) into a crude cell suspension, which was then digested in 300 μ l PBS with a final concentration of 0.3% collagenase/dispase (Sigma-Aldrich, catalog No. 11097113001) at 37 °C for 10 min. Digestion was stopped immediately by adding EDTA to a final concentration of 5 mM, and the cell suspension was placed on ice. After that, the cell suspension was filtered using a 70 μ m nylon mesh (Fisher Scientific, catalog No. 22363548), followed by washing the filter with 10 ml cold PBA (1% BSA in PBS), and all filtrates were combined in a 50 ml centrifuge tube (BioPioneer, catalog No. CNT-50). Then the cell suspension was centrifuged at 500 g for 5 min, and the cell pellet was resuspended in 10 ml PBA and centrifuged again. After discarding the supernatant and resuspending the cell pellet in 1 ml PBA in an Eppendorf tube, cell concentration was estimated by Countess automated cell counter (Invitrogen). Single-cell recovery per milligram tissue was then calculated.

Antigen Retrieval

In order to ensure an adequate number of cells for sorting, we used 1×10^6 cells per tube. The cells were pelleted by centrifugation in an Eppendorf tube at 500 g for 5 min, and the supernatant was discarded. Tris-EDTA buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) was added, and the cells were treated with various incubation times and temperatures: 85 °C/25 min, 85 °C/15 min, 70 °C/80 min, 70 °C/60 min, 65 °C/60 min, 55 °C/60 min, 45 °C/60 min, and 37 °C/60 min. After centrifugation at 500 g for 5 min and removal of the supernatant, the cell pellet was resuspended in 400 μ l PBA and transferred to a 5 ml Falcon tube (Life Sciences, catalog No. 352054).

Immunostaining

Before incubation with antibodies, Triton X-100 was added to a final concentration of 0.1%. The cell suspension was mixed well and incubated at room temperature for 10 min. The cells were then added to 4 ml PBA and resuspended in 400 μ l PBA after centrifugation at 500 g for 5 min and discarding the supernatant. Fluorescent-labeled or unlabeled primary antibodies were added to the cell suspension and incubated for 2 to 4 h in a dark box at room temperature. Following

incubation, 4 ml PBA was added into the Falcon tube, and the supernatant was discarded carefully after centrifugation at 500 g for 5 min. The washing step was repeated twice in order to completely remove the unbound antibodies. Next, the cell pellet was resuspended in 400 μ l PBA, and fluorescent-labeled secondary antibody was added in some cases with incubation for 30 to 60 min, followed by washing once with 4 ml PBA and resuspension in 600 μ l PBA. Multiparameter flow cytometric analysis and cell sorting were performed using the BD Aria III cytometer with Diva software (BD Biosciences). The performance of the flow cytometer was monitored daily using CS&T research beads (BD Biosciences, catalog No. 650621), and the drop delay was set by Accudrop Beads (BD Biosciences, catalog No. 345249).

We tested antibodies to T- and B-cell surface or intracellular molecules, including CD3, LAT (linker for activation of T-cells), BCL11B, CD4, CD8, PD1 (programmed death-1), CD20, CD79A, PAX5 (paired box 5), and CXCR5 for cell labeling and flow cytometric sorting; for CD3 labeling, both a monoclonal antibody (UCH1) binding the ectodomain of CD3 ϵ and a polyclonal antibody binding its cytoplasmic domain were evaluated. Information regarding the antibody reagents used is listed in Table 1.

Stained cells were gated into distinct populations, and 50 000 to 200 000 cells were sorted. CD79A or PAX5 positive and CD3 or LAT positive populations were defined as B-cells and T-cells, respectively. To confirm that these sorted populations did, in fact, represent B- or T-cells, the sorted cells were stained with other T-cell and B-cell specific markers, and the sample was then immunophenotyped to assess for purity.

Genomic DNA Extraction and Sequencing

The sorted cells were incubated with 180 μ l tissue lysis buffer and 20 μ l proteinase K (QIAamp DNA FFPE Tissue Kit,

catalog No. 56404) at 65 °C for 16 h and then incubated for 4 h at 56 °C following the addition of another 20 μ l proteinase K. DNA was then extracted following the remaining wash and elution steps of the kit. DNA concentration was estimated by NanoDrop through measuring the absorbance at 260 nm.

The quality of the extracted DNA was assessed by 1% agarose gel electrophoresis and quantitative PCR (qPCR) assays with two primer sets, QD196 and QD295 (Table 2), that amplify products of 196 and 295 bp, respectively. Equal quantities of template for qPCR were added, and the relative quantities of amplified products were compared. As a control, DNA extracted from fresh cells was assayed simultaneously.

Target Sequencing and Sequence Alignment

NGS was performed on DNA determined to be of sufficient quality. The sequencing libraries were constructed according to the manufacturer's instructions. All coding exons of a panel of 334 genes (Supplementary Table 1) were captured using the Agilent SureSelect platform. Paired-end (2 $\times$ 100 bp) multiplex sequencing of the captured fragments was performed on the Illumina HiSeq2500 instrument.

Paired-end reads in FASTQ format were aligned to the human reference genome (build hg19) using the *Burrows-Wheeler Aligner* (BWA; v0.7.5a)¹⁵ with default parameters.

Table 2 Primers used in DNA quality assessment

Name	Sequence
QD196-forward	5'-CATGGCTCACTGGCTTACAA-3'
QD196-reverse	5'-TTGCCTCTTACAGAGGAGCAG-3'
QD295-forward	5'-GTCAGAAGACTGAAAACGAAGCC-3'
QD295-reverse	5'-GCTTGCCACACTCTTCTCAAGT-3'

Table 1 Lymphocyte related antibody reagents utilized in study

Antibody	Source	Catalog#	Clone	Format	Secondary antibody
CD3	Dako	IS503	Polyclonal	Purified	Alex Fluor(R)647 anti-rabbit IgG (Cell Signaling, 4414S)
CD4	Ventana	790-4423	SP35	Purified	Alex Fluor(R)647 anti-rabbit IgG (Cell Signaling, 4414S)
CD8	Leica	PA0183	4B11	Purified	FITC goat anti-mouse IgG (Santa Cruz, SC2010)
CD79A	Pharmingen	555934	HM47	Purified	FITC goat anti-mouse IgG (Santa Cruz, SC2010)
LAT ^a				Purified	FITC goat anti-mouse IgG (Santa Cruz, SC2010)
BCL11B	BioLegend	650601	25B6	Purified	FITC goat anti-rat Ig (Pharmingen, 554016)
PAX5	Pharmingen	562688	1H9	PE	
CXCR5	Pharmingen	562781	RF8B2	PerCP-Cy5.5	
CD20	BioLegend	302323	2H7	PerCP	
PD1	BioLegend	329903	EH12.2H7	FITC	
CD3(monoclonal)	STEMCELL	60011az	UCHT1	APC	

^aKind gift from Dr Pierre Brousset, Toulouse, France.

Genome Analysis Toolkit (v3.1)¹⁶ was used to re-align the reads around known indels, and *Picard* (v1.115, Broad Institute, Cambridge, MA, USA) was used to mark the PCR duplicates.

Variant Calling and Annotation

Genomic variants were called using VarScan (v2.3.6).¹⁷ The following filtering criteria were applied to remove artifacts: (1) Only reads with mapping quality assigned by *BWA* larger than 36 were used; (2) variant positions had to be covered by at least 10 reads, with the variant allele being supported by at least 4 reads; (3) the VAF had to be at least 5% in at least one sample; and (4) variants with more than 90% supporting reads from one strand were removed.

The variants were then annotated using ANNOVAR (version: 2015-12-14).¹⁸ Variants that were recorded in dbSNP database (build: 138) and had a non-reference population allele frequency larger than 1% were considered to be germline mutations and removed.

RESULTS

Single Cell Preparation

After disaggregating the FFPE tissues into single cell preparations, 1.5, 1.9, and 2.4 million cells were obtained per mg of FFPE lymphoid tissue with reactive hyperplasia in three experiments, and microscopic examination showed many single cells. The nuclei of all cells were stained by trypan blue due to disruption of the plasma membranes, but the cytoplasm of many cells was still visible (Figure 1).

Cell Labeling and Flow Cytometric Sorting

We assessed the labeling of different antigens for flow sorting after antigen retrieval with various temperature-time combinations. For identifying T-cells and B-cells, the antigens

targeted initially included two B-cell lineage-specific intracellular molecules (PAX5 and CD79A) and three T-cell lineage-specific intracellular molecules (CD3, BCL11B, and LAT). For CD3, two different antibodies were evaluated. Temperature-time combinations ranged from 85 °C/25 min to 37 °C/60 min.

Fluorescence was observed for four of the intracellular molecules of interest (CD3 (with polyclonal antibody), CD79A, LAT, and PAX5), showing that they were successfully labeled by their corresponding antibodies (Figure 2). However, the intensity of fluorescence varied depending on the antigen retrieval conditions used. A gradual reduction of fluorescence intensity was observed with decreasing time or temperature for antigen retrieval (Figure 2a–h).

Two of the antibodies to intracellular molecules specific to the T-cell lineage—antibody to BCL11B, a nuclear marker, and the monoclonal antibody (UCHT1) to CD3, an antigen expressed both on the plasma membrane and intracellularly—were not suitable for the separation of T-cells from B-cells, because BCL11B was found to be only partially positive in T-cells, while CD3 (UCHT1) was unable to label T-cells sufficiently from the FFPE tissues (Figure 3).

We also assessed the utility of membrane markers CD4, CD8, PD1, CXCR5, and CD20 (Figure 3). CD4 was found to be partially positive with low fluorescence, but there were not distinctly defined CD4-positive and -negative populations, limiting its utility in isolating T-cells. PD1 was found to be positive on a small population of cells in the case of reactive hyperplasia, but positive in a large subpopulation of cells in AITL cases (see below). The other antigens could not be labeled regardless of the conditions used, and these antigen labels were not pursued in subsequent experiments.

We compared the antigen retrieval results using Tris-EDTA and citrate buffer. There was no significant difference between the antigen retrieval effects of these two buffers using similar incubation conditions (Supplementary Figure 1). In addition, we found that using 0.3% collagenase/dispase digestion for 10 min when preparing single cell suspension did not result in a detectable decrease in the intensity of the cytoplasmic antigen markers we examined, but extending the incubation time to 30 min resulted in significantly lower CD79A staining (Supplementary Figure 2).

DNA Quality Assessment after Sorting

DNA was extracted from three types of cell samples: separated cells from FFPE reactive lymphoid tissue after antigen retrieval and sorting (based on our protocol described above), FFPE reactive lymphoid tissue without antigen retrieval, and cells from the Jurkat cell line (Clone E6-1, ATCC TIB-152) as control. The quality of extracted DNA was assessed and compared. Gel electrophoresis indicated that DNA derived from FFPE samples was generally of poor quality, with smears in the lanes, while high-molecular-weight DNA could be seen only on the lane with DNA from fresh cells (Figure 4).

Figure 1 Cellular morphology in suspension after cell isolation from FFPE tissue (×40, trypan blue stain, reactive hyperplasia tissue sample).

For qPCR detection, equal quantities (5 ng) of all DNA samples were used as templates. The quantities of amplified products from the FFPE samples were compared to that from

the fresh Jurkat cell line. We found that the amplified 196 bp products from FFPE tissue were around 12% of those from a fresh culture of the Jurkat cells (Figure 4), presumably due to

Figure 2 Identification of cell populations from lymphoid tissue with reactive hyperplasia by flow cytometric immunophenotyping using different antigen retrieval conditions. (a, c–g) PAX5-positive cells (B cells) and CD3-positive cells (T cells) were separated as shown on dot plots. Antigen retrieval time and temperature are indicated on the top right corner of each panel. (b, h) Antigens could not be labeled in conditions with short retrieval time or low temperature. For LAT and CD79A labeling, only results using the retrieval condition of 65 °C/60 min are shown (i, j).

Figure 3 Detection of lymphocyte-specific markers by flow cytometry after antigen retrieval at 65 °C/60 min in a case of reactive hyperplasia. BCL11B and CD4 were partially and dimly positive (a, b); CD3 (UCHT1), CD8, CD20 and CXCR5 could not be labeled (c–e). PD1 was positive on a small fraction of cells (e).

Figure 4 DNA electrophoresis (a) and percentage of amplification product compared to fresh cell line (in three experiments) (b). The qPCR products were obtained after TE buffer antigen retrieval, except the last one. The amplified products of both 196 and 295 bp from FFPE tissue after antigen retrieval under higher temperature or longer incubation, 85 °C/25 min or 70 °C/80 min, were obviously lower than those with other conditions. In addition, the amplified products of DNA after citrate buffer antigen retrieval were a little lower than after TE buffer.

DNA fragmentation and degradation. Quantities of amplified products following conditions of high temperature (85 °C/25 min) or long incubation time (70 °C/80 min) were obviously lower than with other antigen retrieval conditions. The same trend was observed for amplified products of 295 bp. Incubation at 55–70 °C for 60 min seemed to be the optimal temperature-time combination for antigen retrieval while also reducing genomic DNA damage compared with un-retrieved samples. Therefore, 65 °C/60 min was selected for further studies. In addition, DNA quality is better with pH 8.0 TE buffer antigen retrieval than with citrate buffer (Figure 4).

Similar observations were made on DNA quality assessed by Bioanalyzer 2100 (Agilent Technologies). The quality of DNA of samples obtained from FFPE tissues without antigen retrieval and with antigen retrieval at 65 °C/60 min was better than that obtained from FFPE tissue with antigen retrieval at 85 °C/25 min (Supplementary Figure 3). These results suggest that the conditions used in our protocol were optimal for antigen retrieval and flow sorting, without obvious additional negative effect on DNA quality.

DNA recovery with this optimized condition was estimated. In three experiments, the DNA yield per million cells after antibody labeling and flow analysis was 1.8, 2.3 and 2.7 µg. For further analysis, about 50 000 sorted cells were used and yielded at least 100 ng DNA, which is sufficient for NGS.

Sequencing Results

Lymphoma samples were treated with the optimized conditions. A single-cell suspension from the FL case was labeled with antibodies to CD79A and CD3 and sorted into B-cell and T-cell populations (59% and 40%, respectively). In immunohistochemical staining, it was estimated that there were ~70% B cells and 30% T cells. The purity of the two populations was then confirmed by immunophenotyping with PAX5 and LAT (Figure 5). DNA from these two cell populations was extracted and sequenced with a custom gene panel. After filtering variants, candidate mutations and VAFs were determined (Supplementary Table 2). True mutations were those found in much higher frequencies in tumor cells as compared to normal cells. Variants present in both of these cell populations at similar VAF (about 50%) could be excluded as somatic mutations and deemed germline variants.¹⁹ For the low frequency variants shared between the tumor and non-tumor fractions, we specifically checked the sequence data manually with Integrative Genomics Viewer (IGV) and found most of them to be alignment artifacts (Supplementary Figure 4). In this way, 28 true somatic mutations were identified (Figure 5d). Some of these mutated genes (*TNFRSF14*, *FMN2*, *ZNF608*, *KMT2D*, *CREBBP*, *BCL2*, *MEF2B*, *IRF8*, *ATM*, *B2M*) have been reported as recurrent mutations with frequencies of close to or >10%.^{20–24} Other mutations (*RFTN1*, *TNIK*, *TET2*, *NOTCH1*, *FAS*, *GNA13*, *DDX3X*, *TAF1*) have been reported

with relatively lower frequencies in FL and transformed FL.^{25–30} Three mutations (*MDN1*, *LRRN3*, *ZFP36L1*) have been reported only in DLBCL.^{31,32} Two mutations (*CUX1*, *HUWE1*) have not been reported in FL or DLBCL.

Cells from two AITL cases were separated into CD3+/PD1–, CD3+/PD1+, and CD3–/PD1– populations (Figure 6a). The CD3+/PD1+ population was considered to be tumor T cells because of the documented PD1 positivity in the lymphoma samples, as is typical for AITL.³³ In AITL case 1, CD3+/PD1– and CD3+/PD1+ populations were 47% and 16%, respectively (about 70% CD3+ cells by immunohistochemical staining). Extracted DNA was sequenced by NGS (Supplementary Tables 3 and 4). The tumor population of AITL case 1 was found to contain two *TET2* mutations, ie *TET2-E1320* and *TET2-R1516*, whereas the CD3+/PD1– and CD3–/PD1– populations showed only very low *TET2* VAF (Figure 6b). In AITL case 2, CD3+/PD1– and CD3+/PD1+ populations were 38% and 31%, respectively (about 80% CD3+ cells by immunohistochemical staining). *RHOA*, *TET2*, *KMT2C*, *DYNC1H1*, and *IDH2* mutations were confirmed to be associated with the CD3+/PD1+ population with much higher VAF than the CD3+/PD1– counterpart (Figure 6c). *RHOA*, *TET2* and *IDH2* have been reported as frequent mutations in AITL,^{34,35} while *KMT2C* mutation has been reported at low frequency.³⁴ *DYNC1H1* (with VAF of 11.7% in our study) has not previously been reported in AITL. We sequenced the unseparated tumor tissue with the original block of AITL case 2 and compared the mutations detected with the separated tumor cells. The same somatic mutations were detected with VAFs that were about half of those detected from CD3+/PD1+ cells (Figure 6c), indicating that the tumor cells were enriched by sorting. The frequencies of other SNVs, either germline or artifactual, were similar between CD3+/PD1+ and CD3+/PD1– cells. Furthermore, we did not detect any new somatic mutations, suggesting that the manipulations associated with the isolation procedure did not alter the mutation profile of the original tumor.

DISCUSSION

The goal of the present study is to isolate separate populations of neoplastic and normal cells from FFPE tissues so that comparative studies can be performed. There are several challenges that need to be overcome to obtain a robust approach to achieve this goal. Single-cell suspensions need to be prepared with sufficient preservation of structural integrity for subsequent manipulations. In our study, a collagenase and dispase mixture provided a sufficient yield of reasonably intact single cells, but it is important to verify the effect of enzyme digestion, as excessive digestion can lead to weaker staining of some cytoplasmic markers.

The single-cell suspension needs to be labeled for sorting to identify specific populations. Antibody labeling of FFPE tissue generally requires antigen retrieval, and routine antigen retrieval is a harsh process with marked degradation of cellular DNA. We assessed various antigen retrieval

Figure 5 Cell sorting and sequencing for FL case. Single cells from FFPE tissue were separated into B and T cell populations by CD79A and CD3 (a). The sorted cells were confirmed by LAT and PAX5 labeling (b, c). DNA extracted from sorted cells was sequenced, and variants were identified and compared between B-cell and T-cell fractions (d). φ True somatic mutation. * Variant recorded in dbSNP with population frequency lower than 1%. Some are proven pathogenic mutations in which case both are indicated against the gene. The Low VAF SNP present in all cellular fractions are probably artifacts containing that SNP.

conditions for sufficient staining and optimal preservation of DNA. It has been reported that 1 mM EDTA or Tris-HCl buffer (pH 8.0) was more effective than citrate buffer (pH 6.0) for antigen retrieval,³⁶ and by utilizing an alkaline solution for heat-induced retrieval, DNA without further degradation could be extracted.³⁷ Therefore, Tris-EDTA buffer with pH 8.0 was used for subsequent antigen retrieval experiments. We found that an antigen retrieval condition of 65 °C/60 min is suitable for many of the antigen markers we tested. We then compared the DNA quality of samples before and after this antigen retrieval condition and demonstrated that the DNA was of similar quality. Prior studies have indicated that the DNA quality of FFPE tissues is inherently much lower than that DNA extracted from fresh cells under identical conditions.³⁸ Our quality assessment using qPCR and the Bioanalyzer indicated that DNA is still of sufficient quality for downstream applications including NGS.

It is essential to determine which antigen markers were preserved for antibody recognition for flow cytometric analysis and sorting. Although the number of analyzed markers was small, we found that cytoplasmic or nuclear

antigens were more likely to be maintained and effectively labeled by their corresponding antibodies. However, cell surface markers showed variable and generally weaker results with regard to retrieval and labeling. Because most proteins on the cell surface are transmembrane molecules, the likelihood of the antigen to be maintained and labeled may be related to the epitope's location within the peptide chain. Antigenic determinants located on the ectodomain may be more readily lost in the single cell preparation step, whereas those located near the membrane or facing the cytoplasm may be more likely to be maintained. However, the suitability of an antigen/antibody pair for this purpose needs to be empirically determined, and it is important to indicate precisely which antibody clone (for monoclonal antibodies) is effective. In this study, we have identified a number of T- and B-cell markers whose antibodies worked in our protocol.

Exon capture sequencing using DNA isolated from FFPE samples is technically challenging due to the limited quantity of DNA extracted from these tissues. Sequence alterations and errors could be caused by formalin fixation and DNA crosslinking.^{39,40} False positivities due to sequencing artifact

Figure 6 Cell sorting and sequencing for two AITL cases. (a) Cells separated from AITL cases were sorted into CD3+/PD1+, CD3+/PD1-, and CD3-/PD1- populations. (b) DNA extracted from sorted cells was sequenced, and variants were identified. (c) DNA extracted from sorted cells and unsorted tissue of case 2 was sequenced, and variants were identified and compared. ϕ True somatic mutation. * Variant recorded in dbSNP with population frequency lower than 1%. Some are proven pathogenic mutations in which case both are indicated against the gene. The Low VAF SNP present in all cellular fractions are probably artifacts containing that SNP.

in DNA from FFPE tissue have been reported.^{41–43} Some studies^{38,44} have compared the results of paired fresh frozen and archival FFPE tissue. The results indicated that although high-confidence mutation calls in the FFPE samples are comparable to that in the frozen samples, caution should be exercised in terms of the artifact due to the background DNA damage of FFPE sample. We therefore tested three tumor samples using a custom capture platform that has worked well in our laboratory. The NGS results from the purified non-neoplastic population derived from the lymphoma sample served as the germline control which we could use to exclude

germline variants and sequencing artifacts. Thus, the true lymphoma somatic mutations can be confidently determined, even in samples without a corresponding normal control. It can be seen that even with a limited panel of lymphoma associated genes, there are germline SNVs that are not readily screened out without the help of the 'normal' population. Although most of the variants recorded in dbSNP are indeed germline variants, several are actually known pathogenic mutations. Conversely, some germline variants are not present in dbSNP, and are known as private SNPs. There were also some shared variants with low VAF in both tumor

and non-tumor fractions. Most of these variants represented artifacts resulted from false calls because some variant reads were actually from paralogous or repeat genomic regions that were mapped to the wrong genomic position. These artifacts might be erroneously considered true somatic mutations if only the unsorted tissue was sequenced.

AITL is notoriously difficult to study by NGS, as the tumor content is frequently low, and tumor cells may not be easy to separate from reactive T-cells in the background.⁴⁵ We have demonstrated that we can substantially enrich the tumor cell population, and the tumor-depleted population could be used effectively as the germline control. Almost all of the SNVs detected that showed a large difference in VAF between the tumor and non-tumor population are known mutations in AITL.^{34,35}

Our study demonstrated that it is possible to obtain single-cell suspensions from FFPE tissue and to obtain specific cellular populations through multi-parameter flow cytometric sorting. We have tested different antigen retrieval conditions and a number of antibodies to antigens in different cellular locations and have provided an optimized protocol for the procedure. We have also demonstrated the utility of this procedure in NGS for investigating gene mutations present in lymphoma samples containing heterogeneous components. Additional antibodies should be evaluated for labeling an extended range of antigen markers especially the labeling of membrane markers. This approach has the potential to unlock the huge archives of FFPE tissues for NGS investigations. This protocol can also be further modified for broader applications including single-cell assays and the objective measurement of non-malignant cells in the tumor micro-environment by flow cytometry.⁴⁶

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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