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Ocular Oncology

A Novel Manual "Centrifuged-Enhanced" Cytosmear Technique for Improving Hypocellular Cytology in the Diagnosis of Vitreoretinal Lymphoma

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Purpose: To develop a manual "centrifuged-enhanced cytosmear" technique for cytologic and immunohistochemical analysis of hypocellular vitreous biopsy specimens in vitreoretinal lymphoma (VRL).

Methods: Diffuse large B-cell lymphoma cells were diluted to simulate vitreous biopsy yields (1000–50,000 cells/100 µL) and fixed in PreservCyt. Samples were centrifuged in a standard laboratory centrifuge and deposited into 1000 cells/100 µL PAP pen–defined circles on charged slides. Smears were air-dried, methanol-fixed, and stained with toluidine blue and CD20 immunohistochemistry (IHC). Cell density per high-power field (40×) was quantified and compared with noncentrifuged smears. Reproducibility was assessed across replicates and between two operators. Genomic DNA concentration was measured.

Results: Centrifuged-enhanced cytosmears demonstrated significantly higher cellular densities than standard smears across all concentrations ($P < 0.05$), especially at 1000 to 2000 cells/100 µL, where standard smears failed to detect cells. CD20 IHC was successful without cell dropout. Genomic DNA yields ranged from 5.25 ng (1000 cells) to 143 ng (50,000 cells). Interuser variability was not significant. Comparison with cytospin clinical cases showed that centrifuged-enhanced cytosmears are of comparable cellularity to clinical cases.

Conclusions: This centrifuged-enhanced cytosmear technique reliably concentrates sparse cells for cytology and IHC. It is reproducible and cost-effective, requiring no specialized equipment. Genomic DNA yields at very low cell counts of 1000 cells/ 100 µL is potentially sufficient for adjuvant MYD88 mutation analysis and can be used to support cytology diagnosis.

Translational Relevance: The centrifuged-enhanced cytosmear offers an accessible alternative to cytospin preparations, enabling VRL diagnosis and IHC analysis in settings lacking cytology infrastructure or where cell blocks cannot be performed.

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Introduction

Vitreoretinal lymphoma diagnosis is made by vitreous sampling, either by a vitreous paracentesis or diagnostic vitrectomy.[1–3] As there are no standardized techniques for specimen handling, these samples can often become hypocellular due to the friable nature of the diffuse large B cells, making them insufficient for routine flow cytometry or cell block preparations, where the vitreous is centrifuged to form a cell pellet/block for routine histopathology diagnosis.[2,3] When a cell pellet is not visualized, cytocentrifuge techniques such as cytospins are often used to transfer cells onto slides for cytology and immunohistochemistry (IHC) characterization of tumor cells.[2,3] However, as ophthalmic pathology laboratories are typically small independent laboratories, such machines may not always be available, and samples may have to be transferred to larger cytology laboratories for processing. Such delays may reduce sample cellularity. Thus, we developed a novel manual cytosmear technique to "focus" cells from hypocellular samples, enabling rapid processing at smaller laboratories while still permitting standard automated formalin-fixed, paraffin-embedded (FFPE) IHC protocols to be performed. We used various dilutions of diffuse large B-cell lymphoma (DLBCL) cells to simulate hypocellular samples fixed with PreservCyt and to demonstrate the "centrifuged-enhanced" effect using standard smear techniques to increase cell density in the smears. We also aim to demonstrate the reproducibility of this technique between different users and evaluate IHC cellular dropout using a CD20 B-cell marker with harsher automated IHC protocols. This technique is low-cost, does not require significant technical expertise, and may be useful for laboratories without cytocentrifuge equipment or cytology expertise. Furthermore, this technique is not limited to vitreoretinal lymphoma (VRL) and can also be utilized for other hypocellular ocular cytologic diagnosis.

Materials and Methods

The DLBCL cell line OCI-Ly3 (ACC 761) was purchased from the Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in Iscove's modified Dulbecco's medium with GlutaMAX, supplemented with 20% heat-inactivated fetal bovine serum (all from Gibco, Waltham, MA, USA). Cells were incubated in a humidified incubator at 37°C with 5% CO_2, then harvested and divided into individual source samples containing 1000, 2000, 5000, 10,000, and 50,000 cells in 100 µL of culture media. They were then fixed with PreservCyt fixative (ThinPrep, PreservCyt; Hologic, Singapore, Singapore) using a 1:2 (sample volume: 2 PreservCyt volume) ratio.[4]

Equipment

- Benchtop Eppendorf Centrifuge 5425R (Eppendorf, Germany) (capable of $500\times g$)
- Vortex mixer
- Pipettes and sterile tips (2–1000 µL)
- Hemocytometer or automated cell counter
- Microwave (for agarose)
- Humidified black staining tray
- Slide warmer or 37°C oven
- PAP pen (Vector Laboratories, Newark, CA, USA)
- Timer
- 0.2-µm syringe filter

Reagents

Item	Catalog No	Supplier
PreservCyt, ThinPrep	70406-002	Hologic Sweden AB (Solna, Sweden)
Agarose (biotechnology grade)	BIO-1000-500G	1st Base Pte Ltd (Singapore, Singapore)
ImmEdge (PAP) pen	H-4000	Vector Laboratories (Newark, CA, USA)
Toluidine blue	198161	Sigma-Aldrich (St. Louis, MO, USA)
Sodium tetraborate	B9876	Sigma-Aldrich
Methanol	106018	Merck Millipore (Burlington, MA, USA)
Phosphate-buffered saline	BUF-2040-10 × 1L	1st Base Pte Ltd
Permount mounting media	SP15-500	Fisher Scientific (Waltham, MA, USA)

Consumables

Item	Catalog No	Supplier
Leica BOND Plus Slides slides	S21.2113.A	Leica Biosystems (Nussloch, Germany)
1.5-mL microcentrifuge tubes	JEB.CFT000015	Jet Biofil (Guangzhou, China)
28-mm diameter syringe filters, 0.2-µm Pore PES Membrane	C05/431229	Corning (Corning, NY, USA)
Coverslips #1 (24 × 50 mm)	11911998	Menzel Glaser, Braunschweig, Germany

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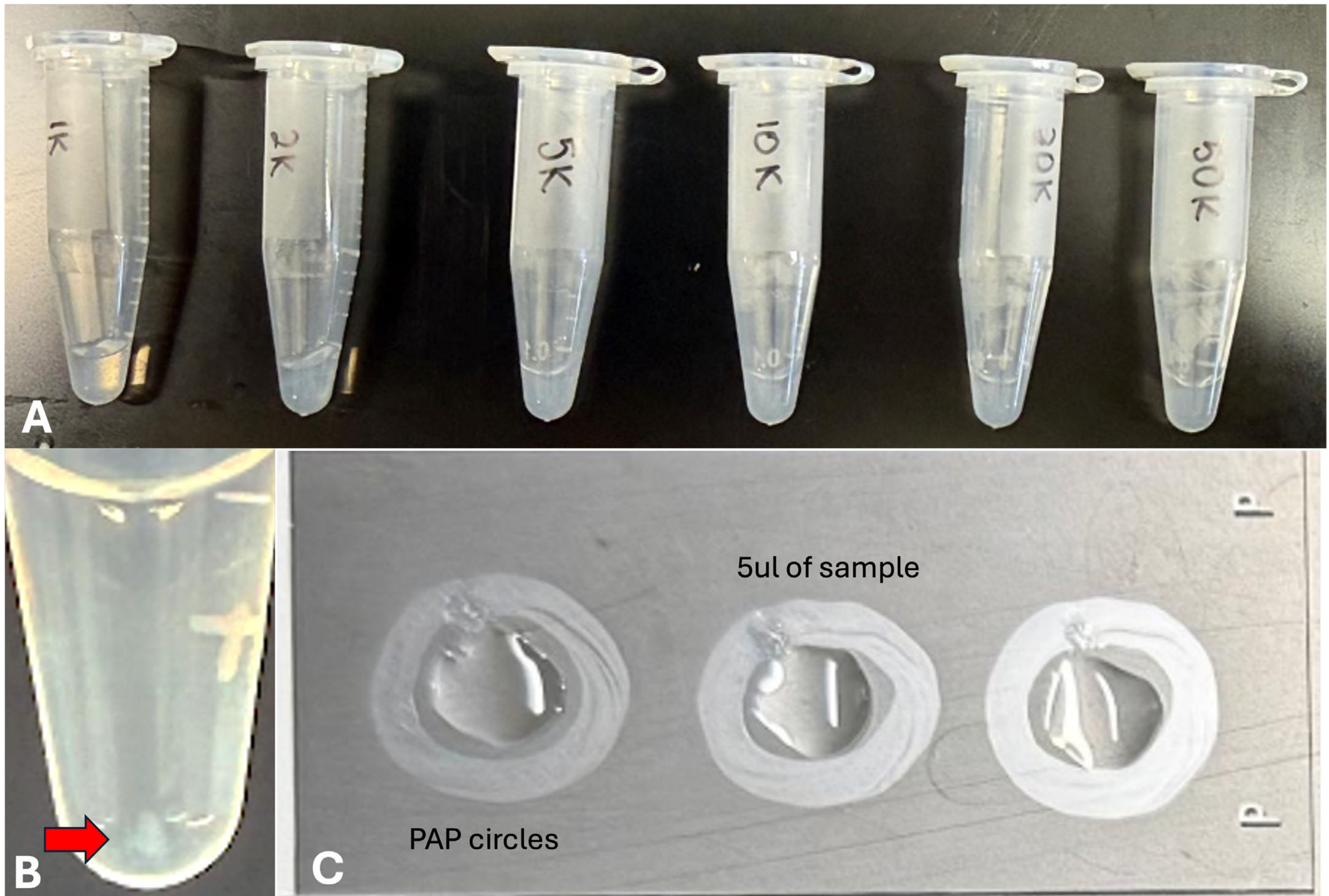

Figure 1. Summary of the centrifuged-enhanced cytosmear technique. Samples (**A**) were centrifuged at 500×*g* for 5 minutes. A small cell pellet was seen only in the 50,000 cell concentration (*red arrow*, **B**). PAP pens were used to manually draw circles 10 mm in diameter (**C**). After the excess supernatant was removed, leaving behind 15 µL of concentrated cell fluid, the sample was vortexed and 5 µL was pipetted out into each PAP circle and air-dried (**C**). Each set was repeated by two users (TLS, CEHH) to determine interperson variability and reproducibility.

Step-by-Step Protocol

1. Prepare and Fix Cells in PreservCyt (Fig. 1A)
   - Harvest and count cells.
   - Prepare suspensions of 1000, 2000, 5000, 10,000, 20,000, and 50,000 cells in 100 µL.
   - Add 200 µL PreservCyt to each tube (1:2 ratio → total 300 µL).
   - Vortex gently for 5 seconds.
2. Centrifuge Technique
   - Centrifuge remaining 200 µL at 500×g for 5 minutes at room temperature.
   - Carefully pipette supernatant without disturbing fluid, leaving behind 15 µL of solution; if a pellet is seen (Fig. 1B), remove extra supernatant, leaving behind 15 µL of solution without disturbing the pellet.
   - Vortex gently.
3. Smear (Fig. 1C)
   - Draw three circles on a polysine-coated slide using a PAP pen.
   - Add 5 µL of the sample into each circle.
   - Place slides in a 37°C oven for 12 to 30 minutes or until dry.
   - Stain for 30 seconds with 1% toluidine blue + 1% sodium tetraborate (filtered).
   - Apply one drop per circle in a humidified black staining tray.
   - Rinse gently with water and air dry.
   - Apply Permount mounting medium over the dried smear and carefully place a coverslip to seal.
4. Additional Step for IHC on Smear
   - After drying, add one drop of cold methanol to each smear.
   - Incubate for 10 minutes at room temperature.

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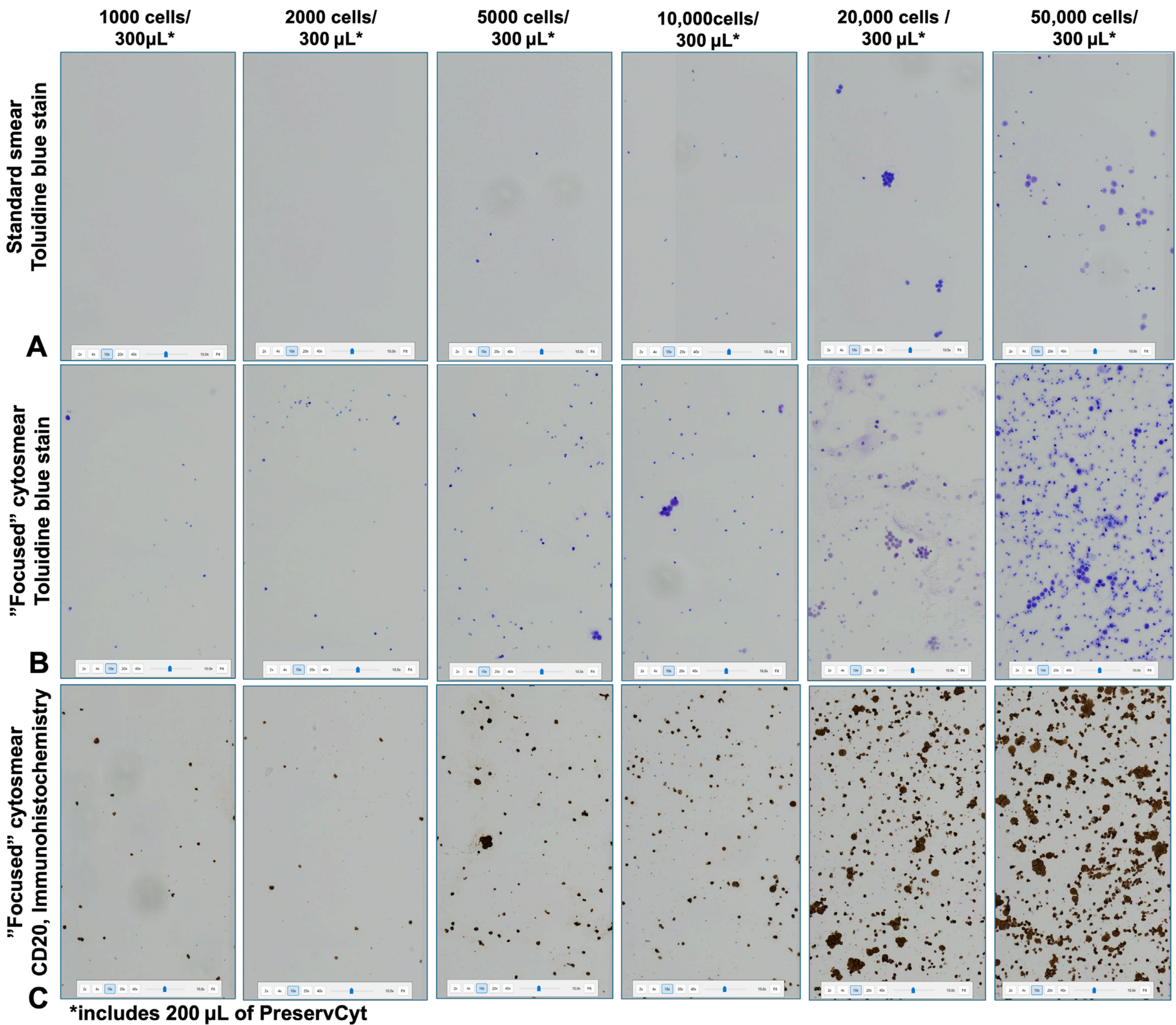

Figure 2. Cytology images (10× digital magnification, digital pathology; Motic Pathology Scanner, Hong Kong) with toluidine blue stain in standard smears (**A**) and centrifuged-enhanced cytosmears (**B**) and CD20 B-cell immunohistochemistry (**C**). This shows the cellular density seen in standard smears compared to the centrifuged-enhanced cytosmear. After methanol fixation on charged slides, the cells are retained and do not show fallout, even with automated IHC staining (**C**).

- Rinse gently in 1× phosphate-buffered saline before proceeding to IHC staining.

The above protocol was repeated in triplicate, measuring cell density within a 40× high-power field (field diameter 0.55 mm, BX43 microscope; Olympus, Singapore, Singapore). To test reproducibility, the protocol was performed by two different laboratory staff members, A (intern, TLS) and B (senior staff member, CEHH), separately using the same source samples. Non-centrifuged-enhanced, standard smears were also prepared using the above protocol, but with omission of the cell focusing step 2 (centrifugation) to compare the cellular quantity of a routine manual smear technique versus our cell "centrifuged-enhanced" smear technique (Fig. 2). Cytosmears' cellularity was compared to representative VRL and chronic inflammation clinical cytospin slides (Fig. 3).

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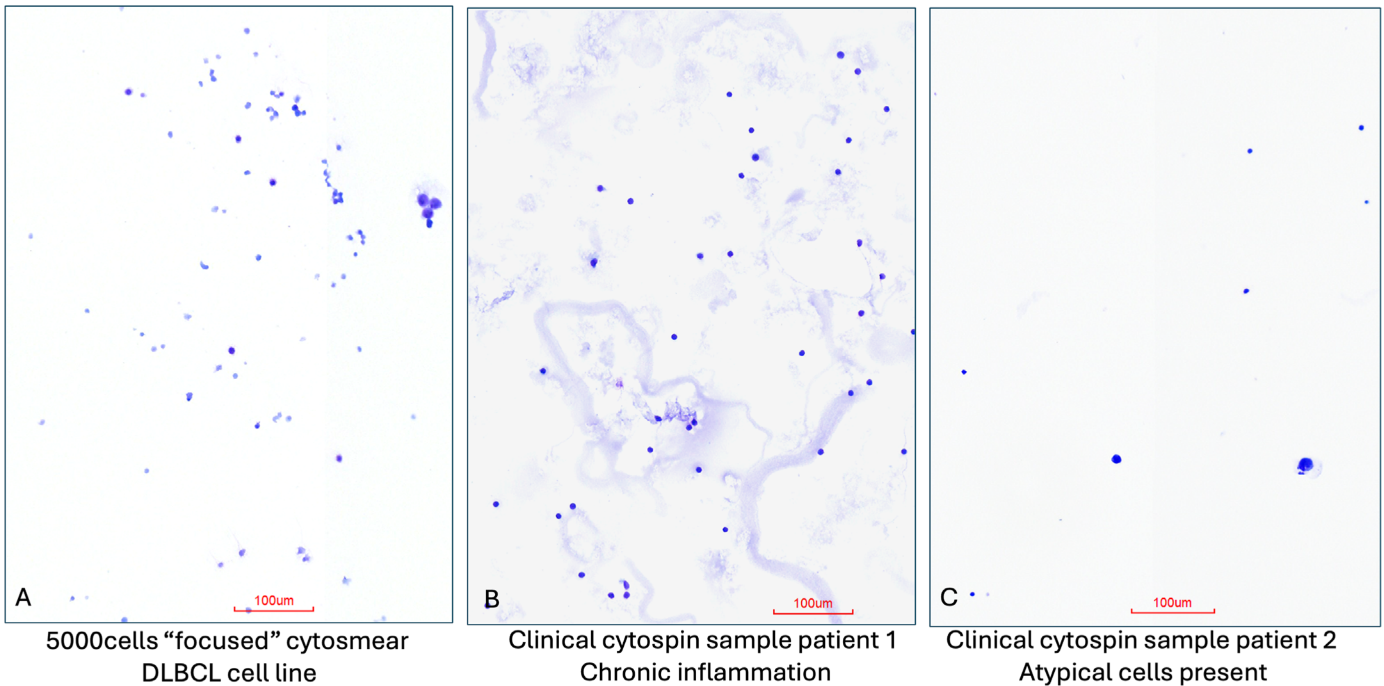

Figure 3. Centrifuged-enhanced cytosmears (5000 cells, **A**) in comparison to clinical samples (**B**, **C**). Comparison of our centrifuged-enhanced cytosmears (5000 cells/100 µL, A) with clinical samples shows that the cellularity is similar to a moderately cellular clinical vitrectomy sample diagnosed as chronic inflammation (**B**). In our cytosmear sample, large atypical B lymphocytes can be detected in a background of apoptotic cells, which also gives a clue to the diagnosis of VRL. In the cytospin clinical sample diagnosed with atypical B lymphocyte proliferation (**C**), the cellularity is very low. However, in a cytospin sample diagnosed with chronic inflammation, the cellular density is higher and similar to a centrifuged-enhanced cytosmear of 5000 cells/100 µL cellularity, Fig. 2B).

Results

Centrifuged-enhanced cell smears, concentrated by centrifugation, showed significantly higher cell densities than homogeneous cell smears without centrifugation (Table 1; Figs. 2A, 2B). At very low cell concentrations of 1000 and 2000 cells in fixative, centrifuged-enhanced cell smears revealed some cells that were not visible in homogeneous smears (Table 1; Figs. 2A, 2B). Tiny cell pellets were visualized only in samples with 50,000 cells (Fig. 1B).

Centrifuged-enhanced cell smears showed clear staining with FFPE IHC protocols without cell dropout (Fig. 2C). We compared reproducibility between two technicians and found no significant changes in the cell density between junior (intern) and senior laboratory officers using this centrifuged-

Table 1. Comparison of the Mean Cell Density Count (in 40× Field Diameter) and Genomic DNA Quantity in the Centrifuged-Enhanced Cytosmear Technique and Standard Smears

Mean Cell Density	1000 Cells/10 µL	2000 Cells/10 µL	5000 Cells/10 µL	10,000 Cells/10 µL	20,000 Cells/10 µL	50,000 Cells/10 µL
Standard smear, mean number of cells/40× hpf	0	0	1.7 ± 0.6	5.0 ± 1.0	9.7 ± 2.1	19.3 ± 4.2
Centrifuged-enhanced cytosmear, mean number of cells/40× hpf	9.3 ± 1.0	18.8 ± 2.8	24.3 ± 1.0	37.3 ± 1.9	46.0 ± 2.2	73.3 ± 3.9
P value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Total genomic DNA, ng	5.25	10.5	32	62	105	143

A significantly higher mean cell density was seen in the centrifuged-enhanced cytosmear samples. Of note, at low cell concentrations, standard smears could not demonstrate any cells. hpf, high-power field.

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Table 2. Interperson Variability in Cellular Density

	Interperson Variation					
Cellular Density Variability	1000 Cells/100 µL	2000 Cells/100 µL	5000 Cells/100 µL	10,000 Cells/100 µL	20,000 Cells/100 µL	50,000 Cells/100 µL
Technician A (lab intern)	9.0 ± 1.4	21.0 ± 1.4	24.0 ± 1.4	38.0 ± 2.8	47.0 ± 2.8	70.5 ± 3.5
Technician B (senior lab officer)	9.5 ± 0.7	16.5 ± 0.7	24.5 ± 0.7	36.5 ± 0.7	45.0 ± 1.4	76.0 ± 1.4
P value	0.70	0.06	0.70	0.54	0.47	0.18

To determine if the centrifuged-enhanced cytosmear could be performed by staff members of different training, we compared the cellularity density after the protocol was performed by two different staff members. This showed no significant difference in cellularity, suggesting that this can be readily reproduced.

enhanced technique (Table 2). The concentration of genomic DNA in each cell was also measured.

Conclusions

There is no standardized vitreous handling protocol for VRL diagnosis, and Figure 4 summarizes the current approaches used and highlights where our novel centrifuged-enhanced cytosmear technique may fit.[2,3,5] The gold standard for VRL diagnosis is still cytology and/or flow cytometry, although some ophthalmic laboratories can use elevated IL-10/IL-6 ratios to diagnose VRL.[6] However, with current guidelines that support systemic chemotherapy for VRL-only disease,[7] elevated IL-10/IL-6 ratios for VRL diagnosis only allow for local therapy as oncologists do not recognize VRL cytokine diagnosis for systemic treatment. While cytospins are currently the main alter-

Figure 4. Summary of current approaches and our novel centrifuged-enhanced cytosmear technique for VRL diagnosis. As the low cellular yield is the main challenge in VRL diagnosis, we highlight some of the pathways for low and hypocellular vitreous samples that may improve diagnostic yields.

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native for hypocellular vitreous cytology, our study shows that cytospins of moderately cellular samples are comparable to our centrifuged-enhanced cytosmears of 5000 to 10,000 cells/100 µL cellular concentrations (Fig. 3B) and, in hypocellular VRL yields (Fig. 3C), are comparable to our centrifuged-enhanced cytosmears of 1000 to 2000 cells/100 µL (Fig. 2B). This indicates that this technique is comparable to cytospins for detecting cells and is superior to standard smears (Fig. 2A).

MYD88^{L265P} mutations occur in up to 75% of patients with VRL.[8] In the recent World Health Organization Classification of Tumours Eye and Orbital Tumours,[9] VRLs have been reclassified as the MYD88/CD79B-mutated (MCD) class of DLBCL and, as such, share MYD88^{L265P} and CD79B mutations with immune-privileged site lymphomas such as central nervous system lymphomas. Thus, MYD88^{L265P} mutational analysis has been increasingly used as an adjunctive clinical test for VRL diagnosis,[10–13] especially when the cellular yield is too low for a confirmatory cytologic diagnosis. In this study, at very hypocellular concentrations of 1000 cells/100 µL, the genomic DNA concentration was 5.25 ng (Table 1), indicating that even when the cell yield is low, adjuvant MYD88^{L265P} polymerase chain reaction analysis can be used to support cytologic diagnosis and should be considered together with cytology.

For typical cell blocks to be performed, studies have suggested that a minimum of 1×10^6 cells is required for adequate diagnosis.[14] However, in this study, a very small pellet (Fig. 1B) was seen when the concentration of 50,000 cells/100 µL was spun down. Although some have reported that the cell block technique is efficacious for VRL diagnosis,[15] others reportedly prefer cytospins.[2] As our laboratory is familiar with both techniques, our experience is that cytospins are preferred when there is low yield or a very minute cell block, which may be at risk of being lost during processing. Due to the nature of FFPE preparation, shaving the FFPE for slides may risk losing cells. However, in very paucicellular yields or when a cytospin service is unavailable, it is still necessary to concentrate the cells before performing the cytospins. Our technique allows cells to be concentrated and replaces the cytospin step with a manual smear technique. This technique can further be modified by reducing the volume per PAP circle to give additional slides for IHC, or it can be used to concentrate cells before loading them to the cytospin machine, although the main role of this study was to develop a novel technique to allow rapid processing to reduce cell loss to improve VRL diagnosis. In this study, we demonstrated this novel technique's reproducibility between two users of different experience and showed that there was no statistical difference in the cellular density obtained (Table 2). Our novel centrifuged-enhanced cytosmear technique is a low-cost, easy-to-perform alternative to cytologic techniques for hypocellular yields as low as 1000 to 50,000 cells.

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Author Contributions: A.C.S.Y. and J.Y. conceived the project; C.E.H.H. and T.L.S. conducted the experiments and wrote the manuscript; N.Z.W. and F.W. designed the experiments and performed the IHC; W.M.H. assisted in cell cultures and edited the manuscript; W.M.M. supervised the project, performed the cell cultures and helped with data analysis and edited the initial draft. All authors reviewed the manuscript and helped with the final edits.

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