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Mechanical Microvibration Device Enhancing Immunohistochemistry Efficiency

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ABSTRACT

Immunohistochemistry (IHC) is a widely used technique in diagnostic pathology and biomedical research, but there is still a need to shorten the operation process and reduce the cost of antibodies. This study aims to assess a novel IHC technique that incorporates mechanical microvibration (MMV) to expedite the process, reduce antibody consumption, and enhance staining quality. MMV was generated using coin vibration motors attached to glass slides mounted with consecutive tissue sections. Multiple antibodies targeting various antigens were used to stain cancerous and normal tissues, with and without microvibration. Various parameters were tested, including incubation durations, temperatures, and antibody dilutions. The novel method showed the potential to achieve comparable or superior outcomes in significantly less time, utilizing over 10 times less antibody than controls. MMV improved specific staining quality, yielding stronger, and better-defined positive reactions. This was validated through a multicenter double-blind assessment and quantitative image analysis. The possible mechanisms were also investigated. MMV shortens immunohistochemical staining duration, reduces antibody usage, and enhances staining specificity, likely by accelerating antibody movement and diffusion. These improvements translate to time and cost savings, offering clinical and financial value for diagnostic pathology and biomedical research.

1 | Introduction

Immunohistochemistry (IHC) is a laboratory test that employs antibodies to detect specific antigens within tissue samples, revealing the distribution of proteins or peptides that are indicative of tissue morphology [1, 2]. It has been widely used in diagnosis, research, and forensic pathology [3–5]. Since its initial report [6], advancements have been made to optimize staining specificity, sensitivity, efficiency, and cost-effectiveness, such as the streptavidin–biotin complex technique and tyramine signal amplification technique [7, 8]. The invention of the antigen retrieval method further expanded the applicability of IHC to a wide range of formalin-fixed paraffin-embedded tissue specimens [9, 10]. Nevertheless, the substantial expense and lengthy

Abbreviations: AEC, 3-Amino-9-Ethylcarbazole; CK, cytokeratin; DAB, 3,3′-Diaminobenzidine; ER, estrogen receptor; ERBB2, Epidermal Growth Factor Receptor 2; hCG, human chorionic gonadotropin; HLA-G, Human Leukocyte Antigen G; IHC, immunohistochemistry; MMV, mechanical microvibration; P53, p53 tumor protein p53; PBS, phosphate–buffered saline; PD-L1, Programmed Cell Death-Ligand 1; PR, progesterone receptor; SDS, stain–decolorize–stain.

Summary

- The microvibration-assisted staining method demonstrated in this study is helpful to significantly save the amount of primary antibody used in the immunohistochemical staining process, improve the efficiency of antigen–antibody binding reaction, and shorten the staining turnover time.
- This technology has great application potential in both scientific research and clinical scenarios.
- Whether in manual staining operation or automatic staining platform, integrating this technology may save the cost of antibodies for basic research and clinical diagnosis, and reduce the economic burden of scientific research and the medical cost of patients.

incubation duration associated with antibodies pose a significant financial, temporal, and labor burden. It has been estimated that a typical hospital case requiring diagnostic IHC would involve the use of a panel of five antibodies, each with a cost of about $50 per antibody. The annual expenditure on IHC reached billions of dollars several decades ago in the USA alone [11], with antibody purchases comprising the largest proportion of these costs. The typical manual immunostaining process is intricate, involving multiple steps, and requires further improvement. A variety of physical engineering methods have been reported to reduce staining time, enhance antibody utilization, and refine staining optimization. These methods include, but are not limited to, microwave irradiation [12], microfluidic systems [13, 14], and alternating current electric fields [15, 16]. These methods play a role in accelerating the dispersion and movement of antibody molecules, as well as accelerating their specific binding to antigens. Mechanical microvibration (MMV) is also a potential method to play a similar role without requiring complex equipment systems and being environmentally robust, but it has not been systematically evaluated. Consequently, this study involved the development of a device capable of subjecting slides to MMV, which was subsequently evaluated across various tissue types using a diverse array of antibodies. The objective was to enhance the quality of staining, decrease incubation duration, minimize antibody consumption, and achieve comparable or superior staining outcomes compared to conventional manual protocol.

2 | Materials and Methods

2.1 | Specimens

Breast, rectal, ovarian cancer, and adjacent normal tissues, and human placenta tissue were obtained from the First Affiliated Hospital of Shantou University Medical College. The research protocol was approved by the Institutional Ethics Committee (Approval number: SUMC-2022-030-K) and samples were obtained with the consent of the patients. Comprehensive information regarding the samples can be found in supplement Table S1.

2.2 | MMV Device Fabrication

A prototype of the MMV device was assembled for experimental purposes. Specifically, a coin vibration motor (Need-For-Power Motor, NFP-FLAT-C1027) measuring 10 mm in diameter and 2.7 mm in thickness was attached to a sliding glass (CITOTEST, 80302-0001, 25 mm × 75 mm), serving as a vibration unit. Six of these vibration units were then mounted onto a bracket and formed a vibration module, as depicted in Figure 1. The microvibration demonstrates low amplitude, and reagent droplets (typically 50–100 μL) were able to remain stable on the slide of vibration units during the experimental process. An Arduino Uno Rev3 (Arduino, A000066) board was programmed by the Arduino Desktop IDE (accessed from https://www.arduino.cc/en/software) and controlled the intensity of each vibration unit. Based on the established performance characteristics of the vibration motor (Figure S1), the voltage utilized in all tests was set at 0.9 V. The device exhibited continuous and stable operation without any reduction in performance across a temperature range of 0°C–50°C. Prior to loading tissue slides onto the glass of the vibration unit, a drop of water was introduced to facilitate adherence between the upper glass of the tissue slides and the lower glass of the vibrating unit, owing to the siphon effect. Additionally, a manual fast rinsing apparatus was incorporated into the prototype. Following the completion of each antibody or reagent incubation, the sections were rinsed for a duration of 30 s before proceeding to the subsequent steps.

2.3 | IHC

For the preparation of paraffin-embedded sections, the tissues were initially fixed in 10% neutral-buffered formalin for a period of 24 h. Subsequently, the tissues were embedded in paraffin. Serial sections with a thickness of 4 μm were obtained using a Leica microtome and subjected to drying at a temperature of 65°C for 1 h. Prior to staining, the sections underwent deparaffinization through three consecutive 10-min incubations with xylene, followed by rehydration using a series of graded alcohol solutions. For antigen retreatment, immerse the tissues in a glass dish containing Tris-EDTA buffer (pH = 9.0), followed by heating the solution in a microwave oven and boiling it for 2 min. The temperature of the solution is then maintained at 95°C for 15 min through intermittent heating cycles. The temperature of the solution is monitored using a thermometer after each heating cycle. For frozen sections, fresh specimens were rapidly embedded using the Tissue-Teks OCT compound and submerged in liquid nitrogen for a duration of 20 s. The tissue was then sliced into thin sections measuring 6 μm and fixed with acetone that had been precooled to −20°C for a period of 2 min. For blocking steps, both types of sections were incubated in a 3% hydrogen peroxide solution and 5% bovine serum albumin blocking buffer for 10–20 min. Sections were then incubated with primary antibodies under varying conditions according to the testing design. Following the completion of the incubation period, the tissue sections were gently rinsed with phosphate–buffered saline (PBS) for 30 s using the fast-rising device. A secondary antibody was subsequently introduced to detect the primary antibody. Staining was visualized with AEC (3-Amino-9-Ethylcarbazole, ZSGB-BIO, China) or DAB kits (3,3′-Diaminobenzidine, Maixin Biotech, China).

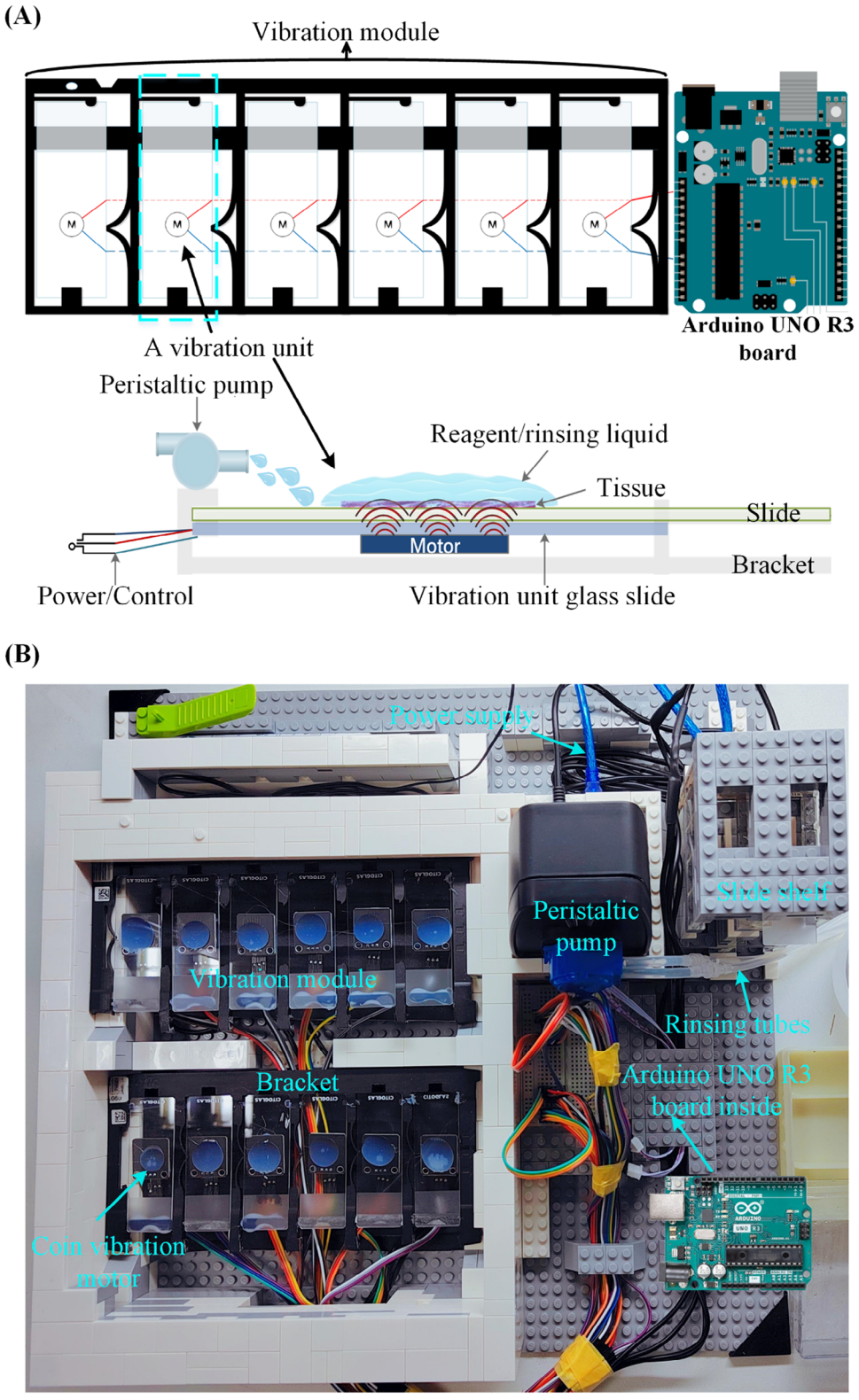

FIGURE 1 | Schematic of the MMV device and the prototype. (A) The schematic diagram illustrates the MMV device and a vibration unit with a fast rinsing tube. (B) The prototype MMV device with a manually controlled auxiliary fast rinsing apparatus. MMV, mechanical microvibration.

2.4 | Testing Design

The testing arrangement is illustrated in Figure 2. The experiments involved the establishment of three groups: the MMV group and matched control group, and the conventional group. A set of serial section slides (up to 13) were sequentially numbered and divided into groups. The MMV group included all the odd-numbered sections (up to 6), while the matched control group without vibration included all even-numbered sections (up to 6). Two tissue sections with adjacent numbers in the MMV or

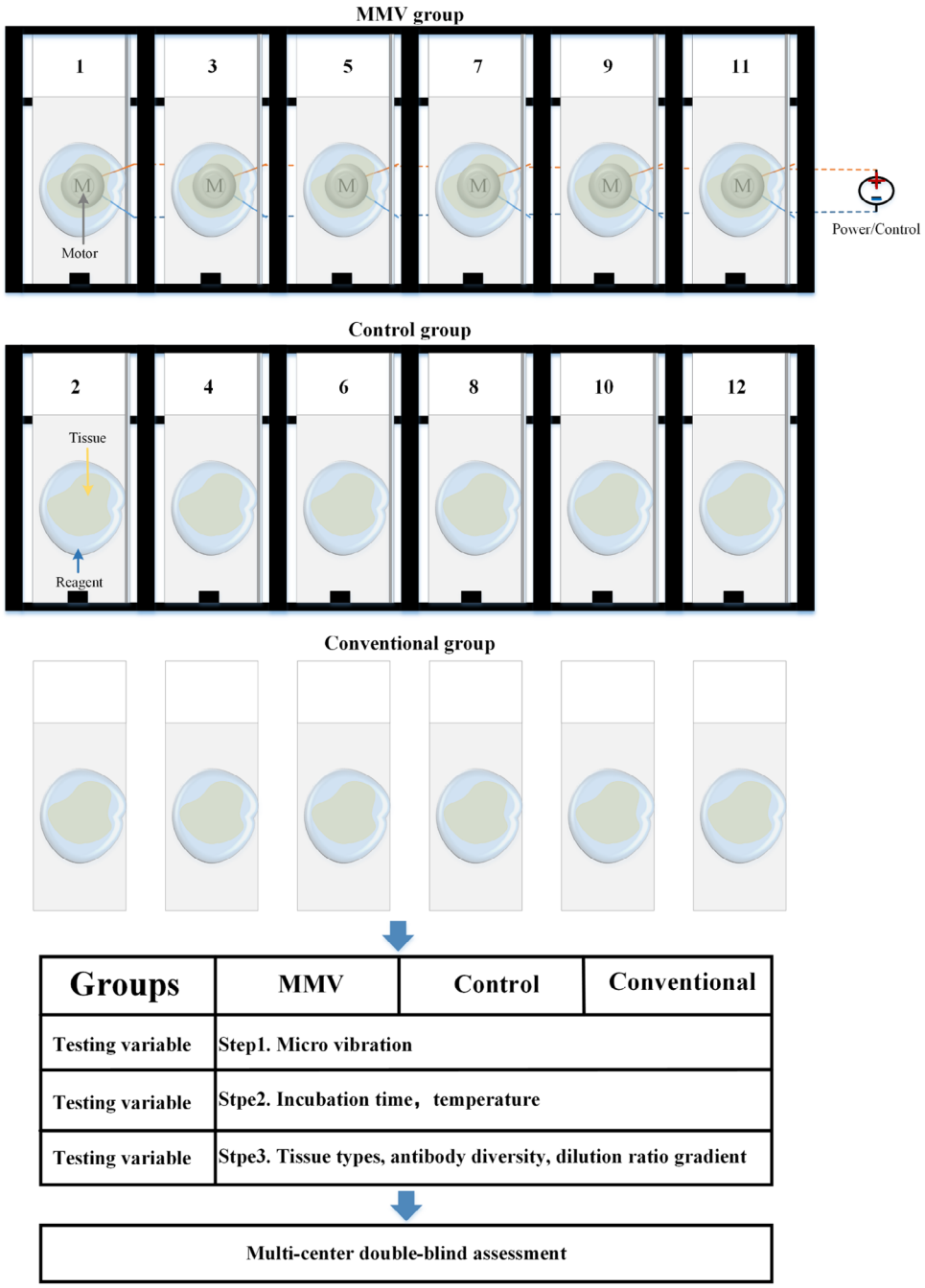

FIGURE 2 | Principle of testing.

control groups formed a pair. A separate section was assigned to the conventional group as the conventional manual staining control. Microvibration was exclusively applied to the MMV groups during the antibody incubation period, while the other groups did not receive such treatment. The primary antibody incubation time for the conventional group was overnight (16–18 h) at 4°C. The primary antibody volume, the secondary antibody incubation time, and color development time were consistent across all groups. Different factors were tested among the different section pairs, including primary antibody incubation time, incubation temperature, and antibody dilution ratio. The quality of paired section staining results of the three groups was assessed by multicenter double-blind evaluation. In the diagram, the yellow pattern represents the tissue, the blue pattern represents the reagent, and "M" stands for mechanical vibration motor.

The testing details comprised three main segments:

1. Effect of MMV on IHC staining: Frozen or paraffin-embedded breast tissue serial sections (3 cases for each type) were assigned to three groups. These sections were then stained with antipan cytokeratin (CK) (dilution ratio 1:100) or CK5/6 (dilution ratio 1:150). The primary antibody incubation

durations ranged from 10 to 30 min for both the MMV and matched control groups at 4°C.

2. Influential factors on MMV IHC method: Various tissues and antibodies were tested for staining effects. Breast, rectal, ovarian cancer or adjacent normal tissues, and placental decidua tissues were sectioned into 4–6μm-thick serial sections. Pairs of adjacent sections on separate slides were paired and allocated to MMV and control groups for comparison. Multiple sets of slides were prepared sequentially, forming a cohort of up to 12 slides, to examine factors such as incubation temperature, antibody/tissue diversity, and antibody dilution. Sections were incubated with primary antibodies at 4°C, room temperature (23°C), or 37°C for varying durations. For antibody diversity and dilution tests, a range of antibodies targeting cell membrane, cytoplasm, and nuclear antigens were employed. The predicted location of antigens was referred to the Human Protein Atlas Database [17]. These antibodies include anti-human chorionic gonadotropin (hCG), Ki-67, Epidermal Growth Factor Receptor 2 (ERBB2), estrogen receptor (ER), progesterone receptor (PR), CK5/6, tumor protein p53 (p53), and P120, were subjected to gradient dilution. The dilution of the antibodies starts from the recommended ratio, and every 10-fold increase was incremented as a gradient. Tissues from both the MMV and control groups were incubated with primary antibodies for 20 min at room temperature, either with or without microvibrating. A distinct section was allocated for the conventional group, serving as a control for standard staining.
3. Multicenter double-blind assessment: Stained sections from the three groups were scanned into whole slide images and evaluated in parallel by six senior pathologists from three pathological centers in a double-blind manner. Staining quality was graded on a scale ranging from 1 to 10, with higher scores indicating superior staining outcomes. Comprehensive information regarding reagents, antibody dilution gradient, and sample sizes is provided in Table S1.

2.5 | MMV-Assisted Stain–Decolorize–Stain (SDS) Technique

The MMV-assisted SDS technique was modified based on a previously reported protocol [18]. The SDS technique involves sequentially applying different antibodies on the same tissue section to achieve multiple immunostainings, serving as a model to test the staining quality and efficiency of the MMV technique. Placental decidua sections underwent routine pretreatment steps, including dewaxing, hydration, antigen retrieval, and blocking, as mentioned earlier (performed only during the initial round of staining). Sections were sequentially incubated with the primary and secondary antibodies at room temperature using the MMV device for 15 min. Conventional staining was used as a control. Positive staining was visualized with an AEC kit. Following hematoxylin counterstaining and mounting, the sections were scanned as digital slide images using a scanner (EasyScan, Motic, China). To achieve decolorization, the sections were immersed in 80% ethanol for 15 min, followed by transfer to an antigen retrieval buffer and subsequent microwave heating for 10 min, resulting in the restoration of the sections to an unstained state. The sections were then prepared for the subsequent round of staining, utilizing an antibody targeting a different antigen. This process of staining, scanning, decolorization, and restaining was repeated for a total of three cycles, enabling the identification of multiple antigens (Human Leukocyte Antigen G (HLA-G), CD3, and CD56) within the same section. Multiple round staining images on the same section were consolidated into a single image with each antigen-positive stain artificially assigned a distinct color.

2.6 | Measurement of MMV

The performance characteristics of the vibration unit were evaluated using a vibration meter (GM63A, Benetech, China), and the measurement results are presented in Figure S1. Additionally, the vibration waveform was recorded through high-speed microphotography. A micro scratch was designated on the glass surface of the vibration unit, and the movement trajectories of the scratch were captured using a digital microscope (Panthera, Motic, China) equipped with a high-speed camera (Moticam S20, Motic, China). The video (supplement video) was subsequently analyzed with MATLAB to determine the displacement of the marked dot along the *X*-axis (left and right movement) and *Y*-axis (forward and backward movement).

2.7 | Image Analysis and Statistical Treatment

The mean gray value of the staining signal was measured with the ImageJ program. Statistical analyses were performed using the Prism software (GraphPad Software, La Jolla, USA). A significance level of $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | MMV Shows Enhancement Effect on IHC Staining

An MMV prototype, including a fast-rising module, was assembled according to the specifications outlined in Figure 1. To validate the amplified impact of microvibration on IHC staining, frozen and paraffin-embedded breast tissue serial sections were subjected to staining with antipan CK and CK5/6 antibodies, both with and without the application of microvibration at a temperature of 4°C.

The findings in Figure 3 demonstrate that the slides subjected to microvibration exhibited more intense staining and improved clarity compared to the control group, regardless of the duration of incubation. As the duration of antibody incubation was prolonged overnight, the staining intensity in the conventional group became comparable to that of the MMV group. Nevertheless, the delineation of cytokeratin staining in the breast ductal and gland was notably enhanced in the microvibrated group, better demonstrating the tissue structure. The duration of MMV–IHC was significantly reduced compared to that of the

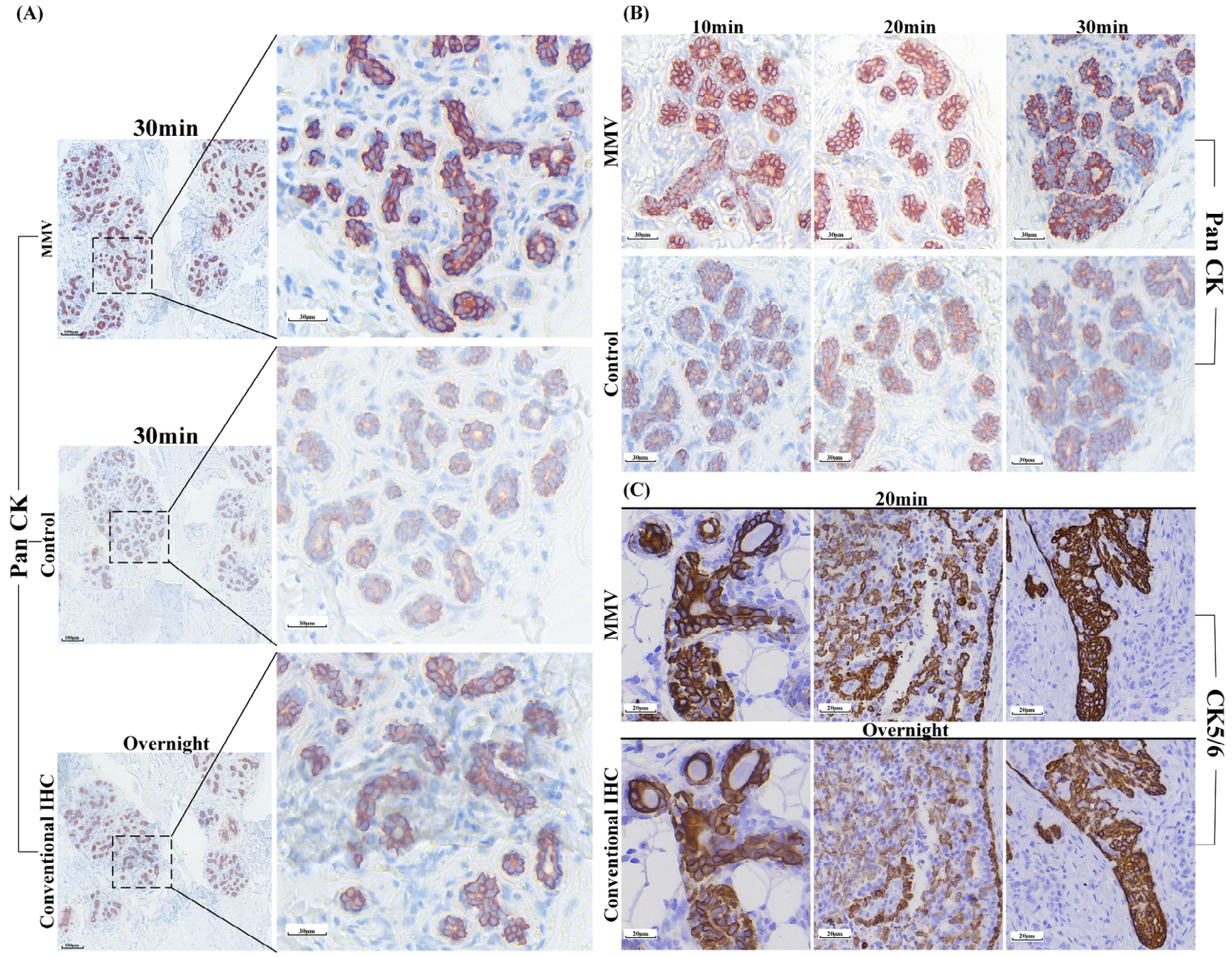

FIGURE 3 | Improvement of immunohistochemical staining with MMV. Frozen (A–B) and paraffin-embedded (C) breast tissue sections underwent staining with antipan CK or CK5/6 at 4°C, demonstrating that MMV led to enhanced IHC staining. Remarkably, the microvibration group displayed more pronounced staining and clearer antigen localization compared to the control and manual conventional groups. CK, cytokeratin; MMV, mechanical microvibration; IHC, immunohistochemistry.

manual conventional IHC method by 5–10 times while achieving the same or superior staining quality.

3.2 | MMV Confers Temperature Independence and Shortens the IHC Process

The MMV–IHC method underwent maximum performance tests and analyzed the effects of incubation temperature. Placental decidual and chorionic villus sections were divided into two groups and individually stained with Antiprogrammed Cell Death-Ligand 1 (PD-L1) and anti-hCG (Figure 4A) antibodies for analysis. The groups were subjected to incubation at temperatures of 4°C, room temperature (23°C), or 37°C. The results indicate that the staining efficacy of MMV–IHC surpassed that of the control group across all incubation temperatures. The increase in temperature further enhanced the staining; however, the impact was not significant when the antibody was subjected to a brief incubation period. Particularly noteworthy is the fact that the anti-hCG antibody dilution ratio (1:20,000) was increased to 200 times the recommended ratio, and the staining signal remained distinctly clear. These observations suggest that MMV exhibits promising prospects for enhancing antibody utilization.

In a separate examination of frozen breast cancer sections (Figure 4B), the effectiveness of MMV–IHC was evaluated using a 1:100 dilution of antipan CK antibody at a temperature of 37°C for 10 min. The staining signal was notably intensified under the synergistic influence of MMV and elevated temperature, indicating the prospective utility of this methodology for intraoperative frozen tissue section diagnosis.

A modified SDS staining technique, assisted by MMV, was employed to examine placental tissues, and detect the presence of CD3, CD56, and HLA-G sequentially on the same tissue sections (Figure 4C). The MMV–IHC staining process for each antibody incubation lasted only 15 min. The resulting images were merged and superimposed to visualize the distinct color-coded positive staining for each antigen. In contrast, the conventional SDS staining method requires more than 2 days, whereas the MMV-assisted process achieved comparable results in less than 10 h.

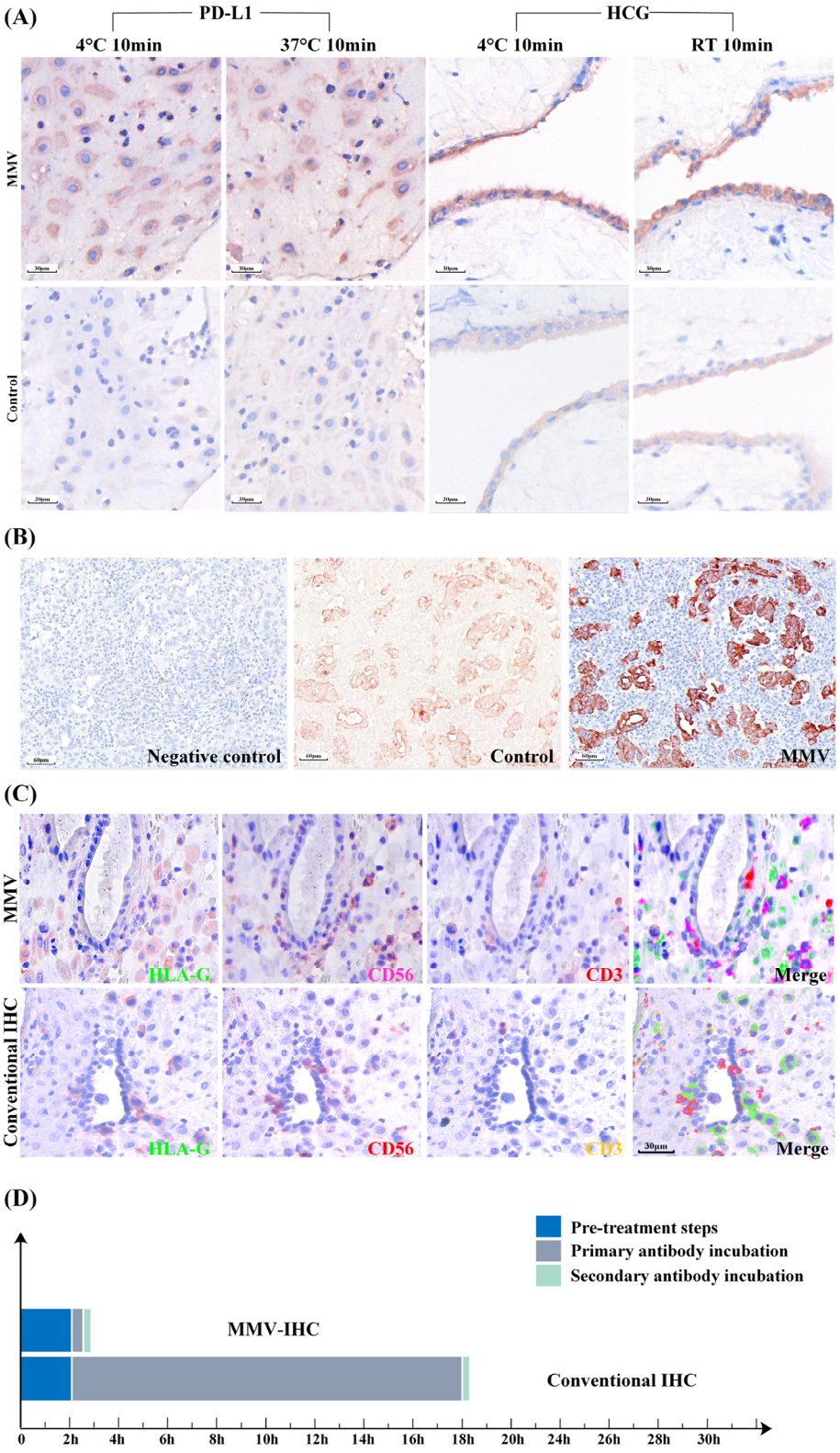

FIGURE 4 | MMV confers temperature independence and shortens the protocol for IHC Staining. (A) Placental decidua sections were stained with anti-PD-L1 at both 4°C and 37°C for 10 min. Chorionic villus sections were stained with anti-hCG at 4°C or at room temperature for 10 min. (B) Breast tissue frozen sections underwent MMV–IHC testing at a temperature of 37°C for 10 min. The microvibration negative control exhibited no unspecific staining, while MMV–IHC staining demonstrated superior results compared to the control without MMV. (C) CD3, CD56, and HLA-G were consecutively stained on a single decidua section using the MMV-assisted SDS technique, typically requiring 48–72 h but accomplished within 10 h. The obtained results were comparable to those obtained through the conventional protocol. Subsequently, the images were processed and merged to simulate multiple staining. (D) A comparison between the duration of MMV–IHC and conventional IHC. HLA-G, Human Leukocyte Antigen G; IHC, immunohistochemistry; MMV, mechanical microvibration; SDS, stain–decolorize–stain.

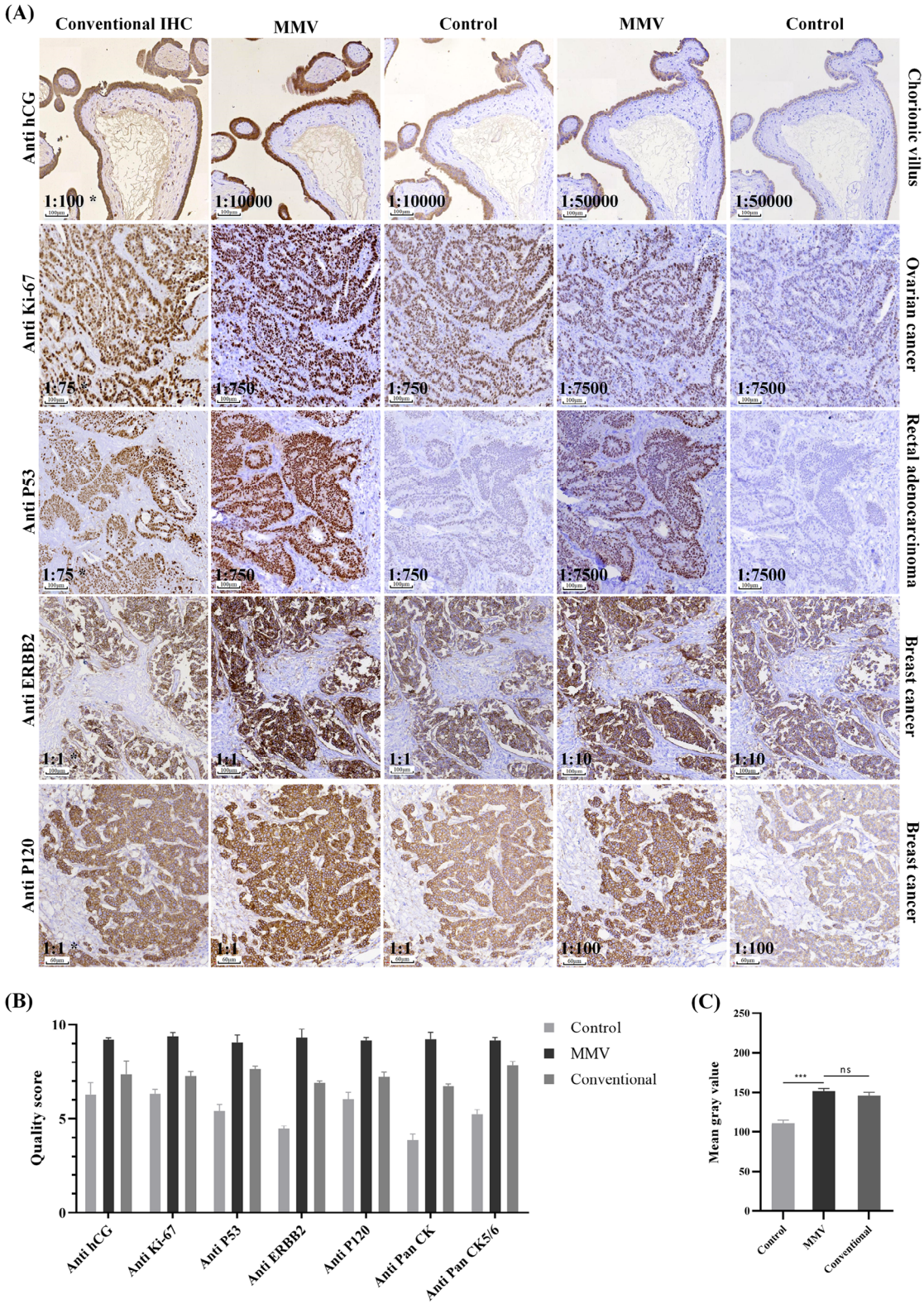

FIGURE 5 | Enhancement with MMV on IHC of different tissue types and antibody dilutions. (A) The recommended dilution ratios (with * indicated) for anti-hCG, Ki-67, p53, ERBB2, and P120 antibodies were employed to stain chorionic villus tissue, ovarian cancer tissue, rectal adenocarcinoma tissue, and breast tissue using conventional staining procedures. The dilutions of these antibodies were subsequently increased in 10-fold gradients, such as 1:1, 1:10, 1:100, and so forth, and separate MMV and control groups were established for each dilution ratio gradient, with a maximum of six gradients. The staining outcomes of representative gradient groups demonstrated that the staining efficacy of the MMV groups surpassed that of the control groups. In comparison to the conventional staining procedure, the staining effect of the antibody dilution ratio increased by more than 10 times in MMV groups and was still equivalent to or better than the conventional groups. All groups adhered to identical conditions for secondary antibody incubation, and the duration of color development was consistent. (B) A multicenter double-blind assessment was conducted to evaluate the staining outcomes of various antibodies in three distinct groups. The findings revealed that the staining quality observed in the MMV group was

Notably, tissues subjected to microvibration also exhibited an improved affinity for hematoxylin.

The duration comparison between MMV–IHC and manual conventional IHC is depicted in Figure 4D. The incubation periods for the primary antibody and secondary antibody in MMV–IHC staining were significantly shorter, taking only 15–30 min each, compared to conventional IHC (refer to Table S2). Additionally, the use of a fast-rising device further reduced the duration of PBS rinsing steps between antibody incubations to 30 s. Despite the shortened duration, the staining quality achieved with MMV–IHC was comparable or even superior to the conventional method.

3.3 | MMV–IHC Saves Reagents and Is Suitable for Multiple Tested Tissues and Antibodies

To extensively validate the enhanced effect of MMV on IHC, a comprehensive series of tests was conducted with breast, rectal, ovarian cancer tissues, and placental decidua tissues. Antibodies targeting various antigens were subjected to gradient dilution for testing purposes. The findings indicate that the enhancement by MMV was observed across all antibody and tissue types, as demonstrated in Figure 5. The staining achieved at a low dilution gradient in the MMV–IHC group was similar to that achieved at a high dilution gradient (e.g., anti-ERBB2 and anti-hCG) and superior to that of the control groups. The staining enhancement achieved by MMV was more pronounced at higher antibody dilution gradients. Even when using antibody dilutions that were 10 times greater than those used in the conventional method, comparable staining quality was achieved with the MMV–IHC method. These results indicate that MMV–IHC saves both costs and antibodies.

The multicenter double-blind evaluation confirmed that the MMV group exhibited superior staining results in terms of quality scores compared to the other groups (Figure 5B). The utilization of MMV demonstrated an enhanced staining effect on various tissues and antigens, which were distributed across distinct cellular positions. To assess staining intensity, three sets of slides were randomly selected (3 sections/group), each stained with the same primary antibody dilution. Grayscale values were measured in 10 high-power field views per slide, revealing that the MMV group exhibited stronger positive staining compared to the control groups and a similar background to that of the manual conventional groups (Figure 5C).

3.4 | MMV Waveform Analysis

The high-speed camera on a digital microscope was used to capture the trajectory of a micro scratch on the vibration unit slide, revealing a regular waveform on the horizontal plane (Video S1). Upon decomposing the waveform, it was observed that MMV exhibited periodic movements along both the *X*-axis and *Y*-axis. The amplitude of vibration on both axes was approximately 0.3–0.4 mm, and the vibration frequency was approximately 40 Hz. Figure 6 presents representative data illustrating these findings.

4 | Discussion

The demand for IHC has been experiencing significant growth, as exemplified by the annual examination of histopathological cases in the United Kingdom exceeding 13 million [19]. Implementing strategies to conserve antibody consumption and expedite the turnover process can effectively alleviate the economic burden associated with this technique and help meet the growing demand without compromising diagnostic quality.

Our findings indicate that the use of MMV can enhance the immunostaining reaction, decrease the duration of antibody incubation, and enable the utilization of a higher antibody dilution while maintaining comparable or superior staining quality. It is important to highlight that microvibration accelerated the antibody binding reaction and enhanced the efficiency of antibody utilization, without diminishing staining intensity or increasing background. Consequently, the employment of MMV would not compromise the interpretation of staining quality that is qualitatively evaluated and possesses significant implications for patient management or diagnosis (such as HER2, ER, or Ki-67 for breast cancer), as confirmed by assessments conducted by pathologists from multiple centers. On the other hand, enhanced visualization of tissue structure through MMV staining facilitates the assessment of tumor invasion extent and the identification of minute lesions, such as ascertaining the breach of the ductal basement membrane in breast cancer or the presence of micrometastases on frozen sections.

The SDS method, which is initially lengthy, is a good candidate to demonstrate the usefulness of MMV in expediting the IHC turnaround time. The modified MMV-assisted SDS allowed sequential immunostaining of several antigens on the same tissue section and shortened a 48–72-h traditional SDS procedure to less than a 1-day protocol with the same results. Moreover, the employment of MMV in the process of frozen section staining holds significant importance in clinical diagnosis, particularly due to the challenging nature and time constraints associated with frozen pathology diagnosis. Therefore, this MMV-assisted IHC technique would represent an improvement in the quality and efficiency of the extensively employed diagnostic and research technique.

Antibody costs remain the primary expense in both manual and automated IHC staining processes [11, 20]. If MMV could be integrated into automated staining systems, a 2-to-10-time

significantly superior to that observed in the control group, while maintaining consistent antibody dilution across all groups. (C) Ten high-power fields of view were randomly selected at the same position from adjacent consecutive slides within three groups. The mean gray level of the staining signal was measured with ImageJ. The results indicate that the staining intensity in the MMV group is significantly stronger than that of the control group. One-way ANOVA, ns indicates no statistical difference, *** indicates $p < 0.001$. ERBB2, Epidermal Growth Factor Receptor 2; MMV, mechanical microvibration; p53, tumor protein p53.

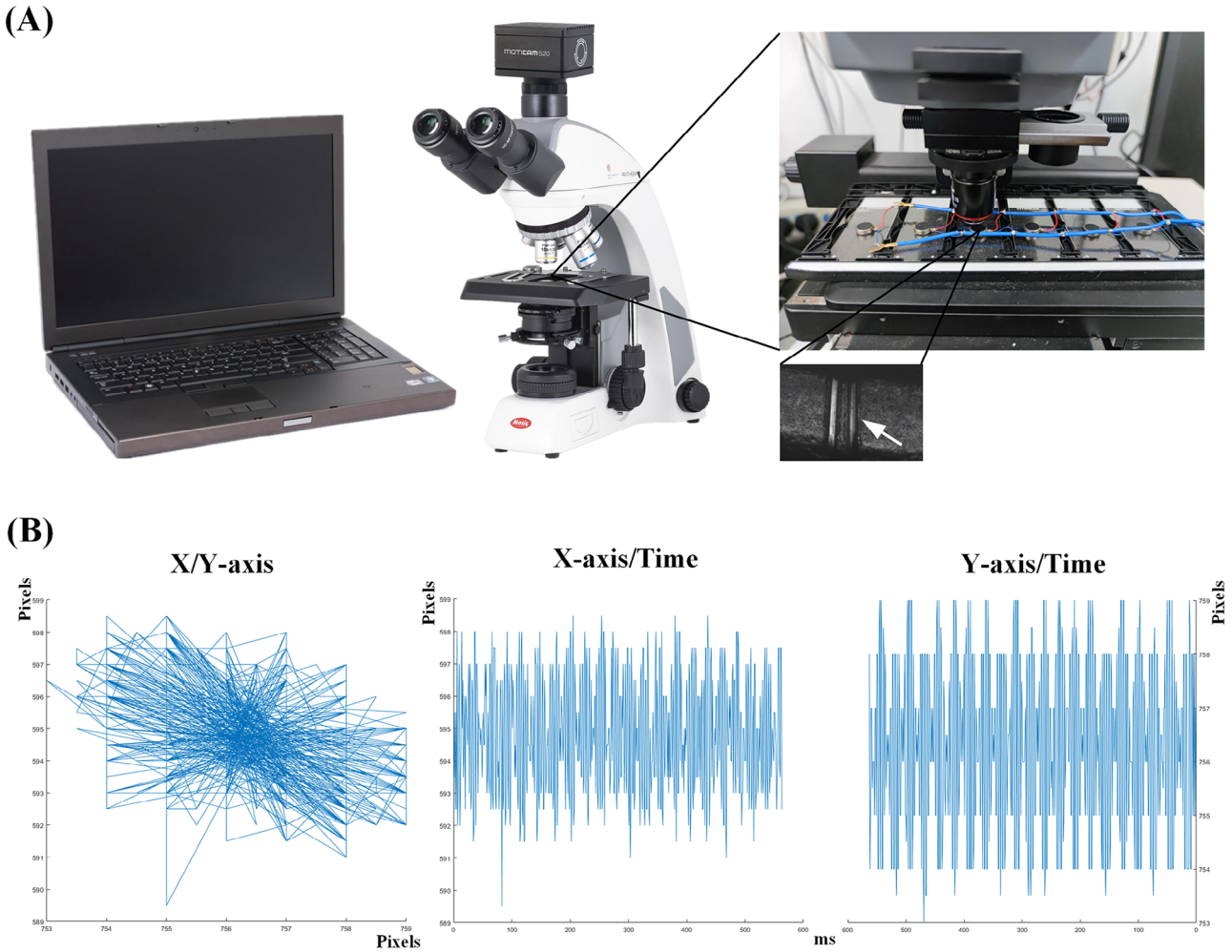

FIGURE 6 | Measurement of MMV waveform. (A) The measurement instruments used for capturing the waveform of MMV. A micro scratch on the vibration unit slide was tracked and recorded by a high-speed camera integrated with a digital microscope. Subsequently, the recorded video underwent waveform analysis with MATLAB. (B) The trajectory of the micro scratch was examined through analysis along the *X* and *Y* axes. MMV, mechanical microvibration.

higher dilution represents a considerable reduction in expense for each round of IHC, this saving would be significant for routine high-throughput IHC protocols performed in most pathological diagnostic labs. Automated IHC staining instruments have been widely adopted for their standardization and significant labor savings [21], offering excellent staining consistency across different systems [22]. However, they are criticized for their lack of flexibility and high reagent costs [23]. Most instrument manufacturers supply their own prediluted antibodies, with the labor savings offset by increased costs in these preformulated reagents. Many automated systems utilize an array or matrix configuration, with slides arranged in rows and columns within slots to form enclosed staining units, structurally analogous to the prototype developed in this study [24]. The distinction lies in the use of mechanical arms that traverse the array, distributing reagents to the slides. The integration of MMV into the staining units of automated platforms in the near future is feasible and could take various forms, such as incorporating vibration modules beneath or above each staining slot or employing a larger vibration unit to induce microvibrations across the entire array. However, the optimal integration method requires comparative validation. Existing platforms may necessitate the attachment of microvibration modules above the enclosed staining chambers, with customization adjustments required for different models.

Several ready-to-use antibodies (Table S1) tested in this study were designed to be compatible with automated staining platforms, and the staining results indicated a substantial redundancy in their volumes, consistent with previous reports [25]. Therefore, the integration of MMV in automated staining platforms could further mitigate the disadvantages of increased antibody costs (more than 7 EUR/per slide) and waste [20]. If validated, reagent manufacturers could further dilute existing ready-to-use antibodies to concentrations suitable for MMV-assisted automated staining platforms, significantly reducing the usage costs for end-users. Although not tested, techniques employing the same principle of antibody-antigen reactions should benefit from MMV. These would include immunofluorescence staining, ELISA, and Western blotting. Further tests are needed to evaluate the effects of MMV on these techniques.

The video captured by a high-speed camera revealed that the waves of MMV were periodic on the horizontal plane, and the peak height on the *Y*-axis is relatively higher than on the

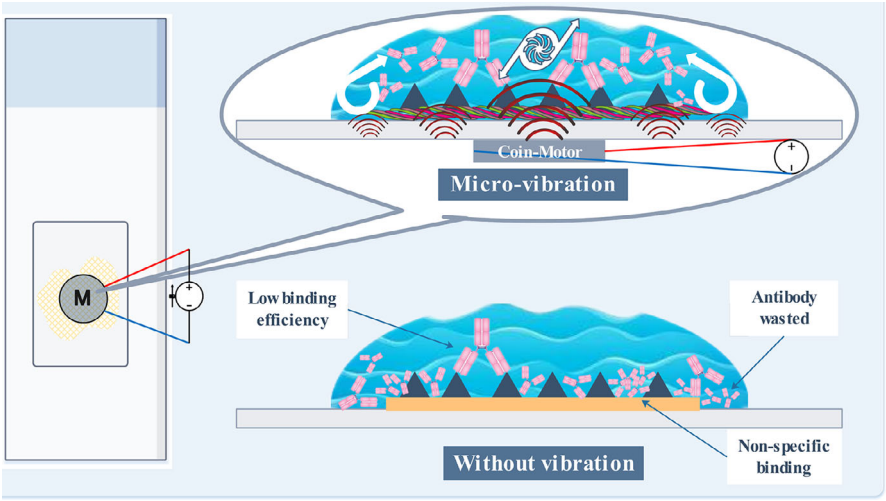

FIGURE 7 | Proposed mechanism of MMV enhancement effect on IHC. Excessive quantities of antibodies are often employed in IHC, resulting in suboptimal binding efficiency due to inadequate molecular mobility. Moreover, the surplus antibody may be deposited, leading to nonspecific binding and wastage. Conversely, MMV promotes antibody and tissue movements, enhancing the interaction of antibodies with target antigens, thereby augmenting binding efficiency and antibody utilization. Additionally, MMV may prevent nonspecific antibody binding, thereby reducing background and leading to superior staining quality. IHC, immunohistochemistry; MMV, mechanical microvibration.

X-axis. It appears that MMV increases the frequency of contact between antibodies and tissue antigens by promoting the flow of antibody fluid and synchronously vibrating tissues. It facilitates firm-specific binding between antibodies and antigens while inhibiting unstable nonspecific reactions, resulting in a better illustration of antigen localization and tissue structure.

Based on these observations, we propose a potential underlying mechanism elucidating the signal enhancement facilitated by MMV, as depicted in Figure 7. The MMV generated by the motors imparts heightened activity to the movement of antibodies within the confined liquid droplet on the tissue section. It promotes the interaction between antibodies and target antigens on the tissue sections, thereby accelerating the incubation process. Moreover, antibody molecules are activated, improving antibody utilization, and allowing higher dilution ratios.

Overall, this study conducted a thorough assessment of the impact of MMV on immunostaining enhancement, employing a wide range of controls. A mechanical vibration-assisted IHC protocol was developed and demonstrated its superiority in shortening incubation duration, increasing antibody dilution, and achieving superior staining quality. It is particularly suitable for long protocols, such as SDS and multiple staining techniques. The use of MMV can enhance the immunostaining reaction, and this effect is not affected by temperature. We proposed a possible mechanism for this improvement. This new protocol, employing a newly designed vibration incubation chamber, should find wide application in pathological diagnosis and research.

Author Contributions

Conceptualization, prototype device assembling, experiments designing, writing original draft, data analysis, and visualization: Weifeng Z. Tissues sample processing, IHC related experiments, and image data analysis: Jirui L, Fengshan X, and Liting Z. Multicenter double-blind evaluation and validation: Liangli H, Penghao L, Xiaomiao Y, Jingliang X, Meina D, and Jiongzhi H. Microvibration waveforms measurement and analysis: Dingrong Y and Jiahao X. Conceptualization, supervision, project administration, writing–review and editing, and funding acquisition: Liangli H and Jiang G. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The research protocols of this study were approved by the Shantou University Research Ethics Committee (Reference Number: SUMC-2022-030-K). The acquisition of samples has fully informed the patient.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. V. Schacht and J. S. Kern, "Basics of Immunohistochemistry," *Journal of Investigative Dermatology* 135 (2015): 1–4.

2. D. J. Dabbs, ed., *Diagnostic Immunohistochemistry* (Amsterdam, Netherlands: Elsevier Health Sciences, 2021).

3. A. Goldhirsch, W. C. Wood, A. S. Coates, et al., "Strategies for Subtypes–Dealing With the Diversity of Breast Cancer: Highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011," *Annals of Oncology* 22 (2011): 1736–1747.

4. K. Inamura, "Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification," *Frontiers in Oncology* 7 (2017): 193.

5. Y. Suehara, D. Alex, A. Bowman, et al., "Clinical Genomic Sequencing of Pediatric and Adult Osteosarcoma Reveals Distinct Molecular Subsets With Potentially Targetable Alterations," *Clinical Cancer Research* 25 (2019): 6346–6356.

6. A. H. Coons, H. J. Creech, and R. N. Jones, "Immunological Properties of an Antibody Containing a Fluorescent Group," *Proceedings of the Society for Experimental Biology and Medicine* 47 (1941): 200–202.

7. S. R. Shi, R. J. Cote, and C. R. Taylor, "Antigen Retrieval Techniques: Current Perspectives," *Journal of Histochemistry and Cytochemistry* 49 (2001): 931–937.

8. J. A. Ramos-Vara, "Principles and Methods of Immunohistochemistry," *Methods in Molecular Biology* 1641 (2017): 115–128.

9. S. R. Shi, Y. Shi, C. R. Taylor, and J. Gu, "New Dimensions of Antigen Retrieval Technique: 28 Years of Development, Practice, and Expansion," *Applied Immunohistochemistry & Molecular Morphology* 27 (2019): 715–721.

10. S. R. Shi, M. E. Key, and K. L. Kalra, "Antigen Retrieval in Formalin-fixed, Paraffin-embedded Tissues: An Enhancement Method for Immunohistochemical Staining Based on Microwave Oven Heating of Tissue Sections," *Journal of Histochemistry and Cytochemistry* 39 (1991): 741–748.

11. S. S. Raab, "The Cost-effectiveness of Immunohistochemistry," *Archives of Pathology & Laboratory Medicine* 124 (2000): 1185–1191.

12. T. Kumada, K. Tsuneyama, H. Hatta, S. Ishizawa, and Y. Takano, "Improved 1-h Rapid Immunostaining Method Using Intermittent Microwave Irradiation: Practicability Based on 5 Years Application in Toyama Medical and Pharmaceutical University Hospital," *Modern Pathology* 17 (2004): 1141–1149.

13. S. Brajkovic, D. G. Dupouy, L. de Leval, and M. A. Gijs, "Microfluidics for Rapid Cytokeratin Immunohistochemical Staining in Frozen Sections," *Laboratory Investigation* 97 (2017): 983–991.

14. R. D. Lovchik, D. Taylor, and G. Kaigala, "Rapid Micro-immunohistochemistry," *Microsystems & Nanoengineering* 6 (2020): 94.

15. H. Shirasawa, J. Kumagai, E. Sato, et al., "Novel Method for Immunofluorescence Staining of Mammalian Eggs Using Non-Contact Alternating-Current Electric-Field Mixing of Microdroplets," *Scientific Reports* 5 (2015): 15371.

16. H. Toda, Y. Minamiya, M. Kagaya, et al., "A Novel Immunohistochemical Staining Method Allows Ultrarapid Detection of Lymph Node Micrometastases While Conserving Antibody," *Acta Histochemica Et Cytochemica* 44 (2011): 133–139.

17. M. Uhlen, L. Fagerberg, B. M. Hallstrom, et al., "Proteomics. Tissue-Based Map of the Human Proteome," *Science* 347 (2015): 1260419.

18. J. Li, Y. Zhou, and J. Gu, "Stain-Decolorize-Stain (SDS): A New Technique for Multiple Staining," *Histochemistry and Cell Biology* 141 (2014): 251–262.

19. Report of the Review of NHS Pathology Services in England, accessed August 28, 2024, https://www.bipsolutions.com/docstore/pdf/14047.pdf.

20. M. Dordevic, M. Zivotic, S. Radojevic Skodric, et al., "Effects of Automation on Sustainability of Immunohistochemistry Laboratory," *Healthcare* 9 (2021): 866.

21. J. W. Prichard and A. K. Bitting, "Automated Immunohistochemistry Overview," in *Handbook of Practical Immunohistochemistry*, ed. F. Lin (Cham: Springer International Publishing, 2022), 41–46.

22. L. B. Guedes, C. L. Morais, H. Fedor, et al., "Effect of Preanalytic Variables on an Automated PTEN Immunohistochemistry Assay for Prostate Cancer," *Archives of Pathology & Laboratory Medicine* 143 (2019): 338–348.

23. J. W. Prichard, "Overview of Automated Immunohistochemistry," *Archives of Pathology & Laboratory Medicine* 138 (2014): 1578–1582.

24. E. Schenck and S. Renshaw, "Automated Immunochemistry" in *Immunohistochemistry and Immunocytochemistry*, ed. S. Renshaw (Hoboken, New Jersey, U.S.: John Wiley & Sons, Ltd, 2017): 157–168.

25. G. Wanqing, C. Yiming, T. Daoqiang, L. Qiang, and L. Zebing, "Study on the Cost Controlling of Automatic Instrument for Immunohistochemical Staining System," *Tumor* 42 (2022): 820–824.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.