

Intraoperative Label-Free Photoacoustic Histopathology of Clinical Specimens

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During cancer resection surgeries, intraoperative histopathologic examination of the surgical specimen is crucial for tumor margin identification. A conventional frozen-section analysis requires complex tissue processing, which prolongs surgery and potentially introduces interpretation errors. Here, as a novel approach to label-free intraoperative histopathology, a high-speed reflection-mode ultraviolet photoacoustic microscopy (UV-PAM) system employing a waterproof 1-axis microelectromechanical systems scanner is demonstrated. Label-free nuclear imaging is photoacoustically verified using tissue sections excised from mice and humans. Moreover, by imaging clinical specimens from cancer patients and numerically quantifying the histopathologic results, it is successfully demonstrated that the proposed UV-PAM system has great potential as an alternative intraoperative histopathology method with minimal tissue preparation processes.

1. Introduction

To definitively treat cancers, resection surgery is inevitable with intraoperative histopathology. During surgery, intraoperative histopathology enables differentiating cancerous tissues from non-cancerous ones, supporting proper surgical management in terms of diagnoses, prognoses, and further treatment processes. Conventional intraoperative histopathology is performed with frozen-section (FS) analysis, but this method presents several limitations in technical and clinical settings. FS analysis is complex: tissues are frozen, sectioned into thin slices (5–10 μm thick), mounted on plain glass slides, stained with

hematoxylin and eosin (H&E), and analyzed with a bright-field optical microscope. The procedures inevitably require several instruments, consumables (e.g., chemical reagents and glass slides), and skilled technicians to produce interpretable slides. In addition, the irreversible nature of the procedures involves tissue deformation, so samples cannot be preserved for standard postoperative histopathology such as paraffin-section (PS) analysis. The procedures are also destructive, and interpretation errors arise from tissue deformation, freezing artifacts, and staining variability.^[1]

In light of the limitations of FS analysis, various researches have been proposed to develop new examination methods, including reflectance confocal microscopy, third-harmonic generation microscopy, multi-photon microscopy, light-sheet microscopy (LSM), microscopy with UV surface excitation (MUSE), and stimulated Raman scattering (SRS) microscopy.^[2–7] However, reflectance confocal microscopy, third-harmonic generation microscopy, and multi-photon microscopy using intrinsic contrasts all have low image contrasts that bar them from visualizing cell nuclei specifically for histopathologic examination. For LSM and MUSE, an irreversible staining process is needed to visualize cell nuclei, which could make the tissue unusable for postoperative histopathology. More positively, label-free SRS microscopy has demonstrated accurate intraoperative tissue diagnosis of brain specimens on the basis of vibrational spectroscopy. Nevertheless, although label-free SRS microscopy has been validated by one-to-one image comparisons with FS analysis, its transmission-mode

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can be found under <https://doi.org/10.1002/lpor.202100124>

DOI: 10.1002/lpor.202100124

imaging configuration restricts its use to relatively thin specimens ($\approx 120\text{ }\mu\text{m}$).^[7,8]

Photoacoustic microscopy (PAM) is a promising imaging modality based on optical absorption and ultrasonic pulse generation by specific biomolecules.^[9–11] Because biomolecules emit ultrasound pulses in response to specific wavelengths of light, biological information about the molecules can be reconstructed without any contrast agent.^[12,13] In particular, cell nuclei can be observed with outstanding contrast because the DNA/RNA inside them have high optical absorption coefficients in the ultraviolet UV range.^[14] UV PAM has provided histopathology-like images of thin breast tissues by employing transmission-mode imaging in which the specimen was placed between the laser and the ultrasound transducer.^[15] An alternative UV-PAM system based on transmission-mode imaging with a micro-lens array has been proposed to implement high-speed imaging. However, the system architecture only demonstrated histopathology of optically thin specimens ($\approx 200\text{ }\mu\text{m}$ thick) impractical for clinical use.^[16] Moreover, this implementation required an expensive multi-channel data acquisition device. A recent non-contact approach, known as UV photoacoustic remote sensing (UV-PARS), was developed with a reflection-mode imaging configuration. The technique allowed for optical mapping without any water coupling medium, enabling histopathology of both animal and human tissues. UV-PARS systems demonstrated high resolution and signal-to-noise ratio (SNR) through in vitro experiments in the absence of light scattering.^[17–20] However, the optical heterogeneity in biological tissues inescapably scatters the light in both the excitation and probe beams of UV-PARS. It is thus difficult to achieve and maintain high acousto-optic efficiency in the turbid medium, leading to such unstable image quality. Although the high lateral resolution of the UV-PARS system ($1.2\text{ }\mu\text{m}$) was validated in water, only macroscopic architectural features with the size in hundreds of micron units, such as malignant nuclear groups, have been demonstrated with thinly sectioned tissues or paraffin-embedded tissues.^[21] Moreover, the UV-PARS systems require complex optical designs (multiple lasers, optically polarized components, etc.), making these instruments costly. The superior performances of the MEMS-UV-PAM were compared with the previously reported systems (Table S1, Supporting Information).

Herein, we introduce high-speed reflection-mode UV-PAM based on microelectromechanical systems (MEMS) scanning for label-free intraoperative histopathology. A fabricated 1-axis waterproofing MEMS scanner is embedded in a compact scanning module for high-speed examination. A 2-axis linear motorized stage is also employed for wide-field scanning, operating much like a commercial digital slide scanner. The developed MEMS-UV-PAM achieved a lateral resolution of $1.2\text{ }\mu\text{m}$, sufficient to clearly visualize individual cell nuclei. The variable imaging field-of-views (FOV) for wide ($10 \times 10\text{ mm}^2$) and magnified ($500 \times 500\text{ }\mu\text{m}^2$) regions had typical imaging times of 240 and 60 s, respectively. The imaging depth for clinical specimens was measured as $\approx 100\text{ }\mu\text{m}$. First, we verified label-free nuclear imaging capability with a frozen mouse brain tissue section. Then, we explored the system's clinical effectiveness by imaging formalin-fixed paraffin-embedded (FFPE) sections of human colon and liver tissues excised from cancer patients after deparaffinization. Moreover, we successfully demonstrated intraoperative

histopathology of the clinical colon and liver specimens from the cancer patients. Key histoarchitectural features of the tissues were highlighted, guiding pathologists' tumor margin assessment. We also provided histopathologic quantification of the liver tissues, illustrating nuclear differences between non-cancerous and cancerous tissues. Preserved by the non-destructive imaging characteristic of the MEMS-UV-PAM, the key histopathologic details were in high agreement with postoperative PS analysis. The MEMS-UV-PAM could potentially serve as the streamlined alternative to conventional intraoperative histopathology, eliminating tissue transfer from the operating room to a pathology laboratory and the need for complex procedures such as freezing, sectioning, staining, and mounting in FS analysis.

2. Results

2.1. Reflection-Mode UV-PAM Based on the 1-Axis Waterproofing MEMS Scanner

Our developed reflection-mode MEMS-UV-PAM incorporated the 1-axis waterproofing MEMS scanner and the 2-axis linear motorized stage (Figure 1a). Five major elements comprise the entirely self-contained system: 1) the UV pulse laser and related optical components, 2) the scanning module, 3) the 2-axis linear motorized stage, 4) a tissue holder, and 5) a computer for data acquisition, processing, and display. Compatible with both a pathology laboratory and an operating room, the fully integrated elements are transported on a movable clinical cart (Figure S1a, Supporting Information). The system operated synchronously in a predefined sequence (Figure S1b, Supporting Information). For a B-scan image, each pulse as triggers for the laser, linearized motor stage, and data acquisition of a PA signal were generated while the MEMS scanning was driven by with a sine wave. The integrated system features an inverted scheme of optical microscope, enabling users to locate tissues easily in a practical way. Moreover, the reflection-mode imaging configuration of the MEMS-UV-PAM can readily image large centimetre-sized clinical specimens for various histopathologic applications.

The MEMS-UV-PAM used a UV pulse laser at 266 nm, the primary absorption wavelength of DNA/RNA in cell nuclei, enabling label-free PA histopathology. The output beam from the laser was first split by the optical beam splitter, and the power fluctuation of each laser pulse was compensated for by continuously monitoring one split laser beam with the photodiode. The other beam was expanded by the beam expander, spatially filtered by the iris, and focused by the set of the lenses onto tissues, delivering an excitation pulse energy of $\approx 19\text{ nJ}$. To image tissue samples in the inverted scheme, the samples were first placed in the center of a custom-made tissue holder (Figure 1b). Then a small amount of deionized water was applied to the tissues to provide effective acoustic coupling between the tissues and a thin plastic membrane, which softly flattened the tissues. For wide-field scanning, this tissue holder was tightly fixed upside down to the 2-axis linear motorized stage. The optical and ultrasonic beam paths were coaxially and confocally aligned through the opto-ultrasound beam combiner (OUC), maximizing the image SNR (Figure 1c). To accurately position the tissue samples, we used coarse and fine knobs to move the tissue holder along the z-axis.

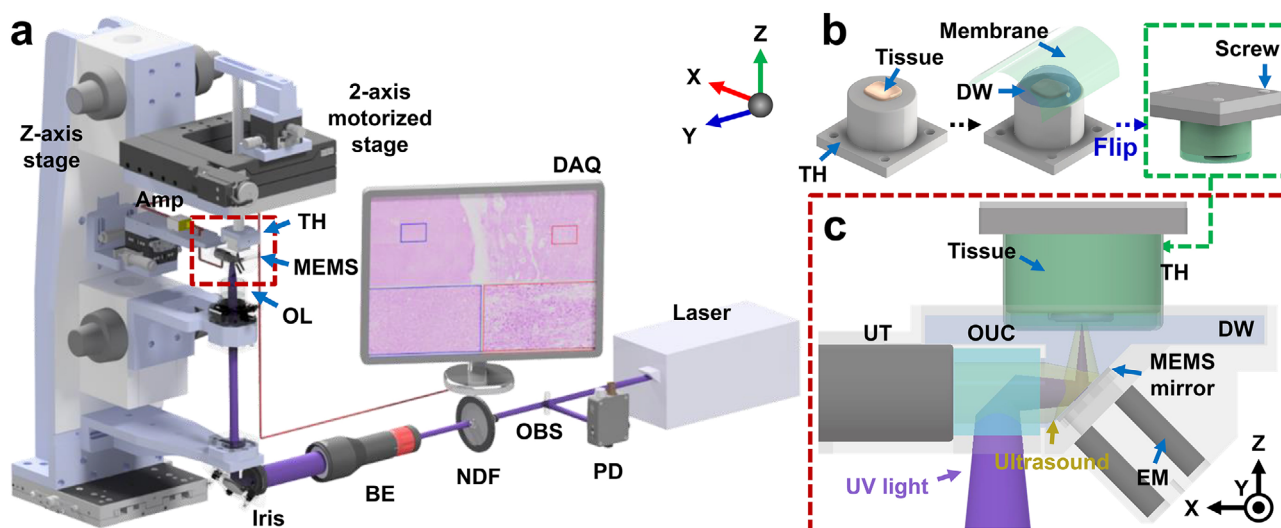


Figure 1. Reflection-mode MEMS-UV-PAM system for intraoperative PA histopathology. a) Configuration of the MEMS-UV-PAM system. b) Mounting a surgical specimen on a TH for PA histopathology. c) Magnified image of the scanning module with the TH shown in red dashed box in (a). PA, photoacoustic; MEMS, microelectromechanical systems; UV-PAM, UV photoacoustic microscopy; PD, photodiode; OBS, optical beam splitter; NDF, neutral density filter; BE, beam expander; OL, objective lens; TH, tissue holder; Amp, amplifier; DAQ, data acquisition board; UT, ultrasound transducer; OUC, opto-ultrasound beam combiner; EM, electromagnets; DW, deionized water.

To benchmark the performance of the MEMS-UV-PAM, we quantitatively estimated the lateral & axial resolutions, FOV, imaging speed, and SNR. Considering the lateral resolution of the system, we designed the optical components including the set of the lenses and the OUC and simulated the irradiance profile (Figure S2a, Supporting Information) with a ray tracing software (OpticStudio, Zemax, USA). Accordingly, we obtained a PA MAP image of a pattern edge on a quartz glass substrate patterned with chrome masking (Figure S2b, Supporting Information). Based on a Gaussian fitting of the line spread function from the cross-sectional profile L1 of the PA MAP image, the lateral resolution, defined by the full-width at half-maximum (FWHM), was $1.2\ \mu\text{m}$ (Figure S2d, Supporting Information). This value agreed well with the simulated value of $1.1\ \mu\text{m}$. To measure the axial resolution of the system, we performed PA imaging of a carbon fiber with a diameter of $6\ \mu\text{m}$. In a cross-sectional PA image of the carbon fiber (Figure S2c, Supporting Information), the PA amplitude profile across a line L2 was quantified with a Gaussian fitting, resulting in a FWHM of $65.1\ \mu\text{m}$ (Figure S2e, Supporting Information). The measured axial resolution was in close agreement with the theoretical value of $63.8\ \mu\text{m}$, determined by considering the effective acoustic bandwidth of the ultrasound transducer combined with the OUC.

The MEMS-UV-PAM was able to detect signals from a large area with a maximum FOV of $24 \times 24\ \text{mm}^2$ in the X-Y plane. To complement conventional intraoperative histopathology procedures, the scalable FOV of the MEMS-UV-PAM system was designed to enable users to scan an entire sample first and then to inspect a small FOV in detail. This process is analogous to the commonly used variable magnifications in conventional histopathological optical microscopy. Affected by the stiffness of the PDMS MEMS support structure, the scanning speed of the MEMS scanner could maximally reach $\approx 100\ \text{Hz}$. However, the

current laser pulse repetition frequency (PRF) of $10\ \text{kHz}$ limited the imaging speed. In the MEMS-UV-PAM, the imaging speed depended on two factors: the PRF of the laser and the number of pixels along a scanning range, which could be variable for multi-magnification viewing. For instance, the typically used a FOV of $10 \times 10\ \text{mm}^2$ (1000×1000 pixels) was obtained for wide PA histopathology within 240 s. For magnified views to visualize histopathologic details, a FOV of $500 \times 500\ \mu\text{m}^2$ (700×700 pixels) was acquired within 60 s. The SNR of the histopathologic images acquired by the MEMS-UV-PAM is addressed on each imaging results section below.

2.2. PA Imaging of a Mouse Tissue Section

To demonstrate that DNA/RNA can serve as a superior endogenous contrast for PA imaging of cell nuclei, a frozen mouse brain tissue section (Figure 2a) was photoacoustically imaged without additional staining procedures (Figure 2b). Then, the PA histopathology image was compared to a corresponding H&E stained microscopic image (Figure 2c). The brain slice sample was prepared as a FS as described in the Materials and Methods. The entire brain image was reconstructed through the multi-resolution spline algorithm. The PA image was pseudo-coloured by applying a customized colormap which resembled H&E staining. To visually illustrate structures with various nuclear distributions, we examined magnified views of representative architectures. The overall architectures of the brain section seen in the PA image were compared with those in the H&E image.^[22] As seen in Figure 2b1,c1, the cerebral cortex is identified on the outer part of the brain section, containing indistinct cell layers. The cornu ammonis and dentate gyrus are seen in the hippocampal region, where the nuclei are denser than other areas, with a characteristic horn-shaped distribution pattern

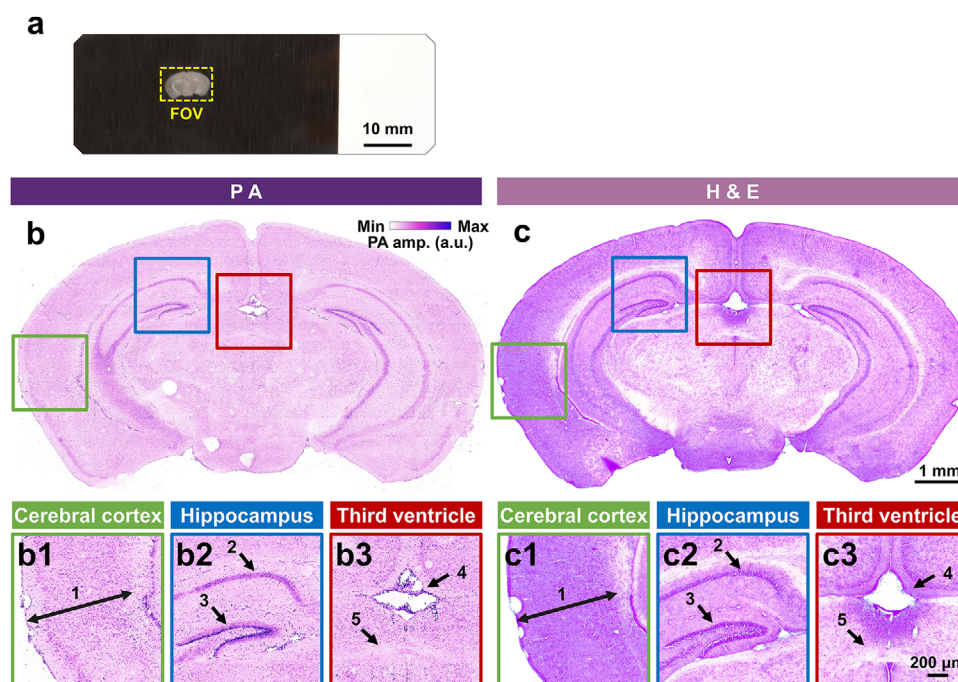


Figure 2. PA imaging of a frozen mouse brain tissue section. a) A frozen mouse brain tissue section on a slide glass. b) PA MAP image of the frozen mouse brain tissue section in (a), obtained by MEMS-UV-PAM without staining. c) Standard H&E stained image of the tissue section in (a), acquired by an optical pathology microscope. b1–b3) Magnified PA MAP images of the cerebral cortex, hippocampus, and third ventricle regions in (b), respectively. c1–c3) Magnified H&E stained optical images of the cerebral cortex, hippocampus, and third ventricle regions in (c), respectively. 1, cortex layers; 2, cornu ammonis 1 pyramidal layer of hippocampus; 3, dentate gyrus granule cell layer; 4, third ventricle; and 5, posterior commissure. MEMS, microelectromechanical system; UV, ultraviolet; PAM, photoacoustic microscopy.

(Figure 2b2,c2). The cell nuclei of the choroid plexus in the third ventricle, which creates and secretes cerebrospinal fluid, are well visualized in the PA image. A posterior commissure under the third ventricle is also shown, with a group of white fibres crossing the middle of the coronal section where no cell nuclei are observed (Figure 2b3). On the other hand, the choroid plexus cannot be seen due to the destructive nature of the FS analysis that might destroy the corresponding tissue region (Figure 2c3).

2.3. Label-Free PA Histopathology of Human Tissue Sections

To perform standard-of-care histopathology, colon and liver tissue specimens from cancer patients were processed as 10 μm thick deparaffinized FFPE sections, according to the earlier described clinical workflow (Figure S3, Supporting Information). Entire tissue sections (Figures S4a,b, Supporting Information) were photoacoustically imaged with a step size of 0.7 μm . The reconstructed PA image of a colon tissue section was pseudo-coloured (Figure 3a) to be familiar to pathologists, resembling a traditional H&E-stained image (Figure 3b). The PA image reveals the cellular and histoarchitectural features of the tissue section, permitting differentiation of non-lesional and lesional regions. To depict the features in detail, zoomed-in views of representative non-cancerous, boundary, and cancerous regions are illustrated (Figure 3a1–a3). The homogeneous structures of normal glands (or “crypts”) are circular or elliptical in an

organized pattern (Figure 3a1). Most of the epithelial cells in the glands are simple columnar epithelial cells. Both epithelial cell nuclei around lumens in the glands and stromal cell nuclei can be identified. Mucin vacuoles are also uniformly located within the gland, indicating that mucus secretion is not affected. As a connective tissue, lamina propria fills the space between the glands and contains various cells, such as lymphocytes and plasma cells. In the boundary region, there are fewer vacuoles in the glands nearer the cancerous region (Figure 3a2). Unlike the non-cancerous region, the cancerous region exhibits high variations in the morphology of glands. Neoplastic glands of adenocarcinoma are composed of numerous and highly packed nuclei exhibiting hyperchromatism and pleomorphism. In addition, the glands are filled with characteristic necrotic debris in several lumens (Figure 3a3). Moreover, the nuclear and histoarchitectural features have a pronounced concordance: the gland sizes, shapes, and number, as well as each nuclear aspect, are relatively identical between the PA (Figure 3a1–a3) and H&E images (Figure 3b1–b3).

A millimeter-scale area of a liver tissue section was also examined using the MEMS-UV-PAM (Figure 3c), and the observations were validated with a H&E stained image by an optical microscope (Figure 3d). In the high-magnification view, the non-cancerous region is primarily composed of hepatocytes, accounting for $\approx 80\%$ of the liver cells (Figure 3c1). The boundary of a hepatic lobule is also seen. In addition, fibrous demarcation between the non-cancerous and cancerous regions is discernible, with an irregular band of interweaving patterns (Figure 3c2).

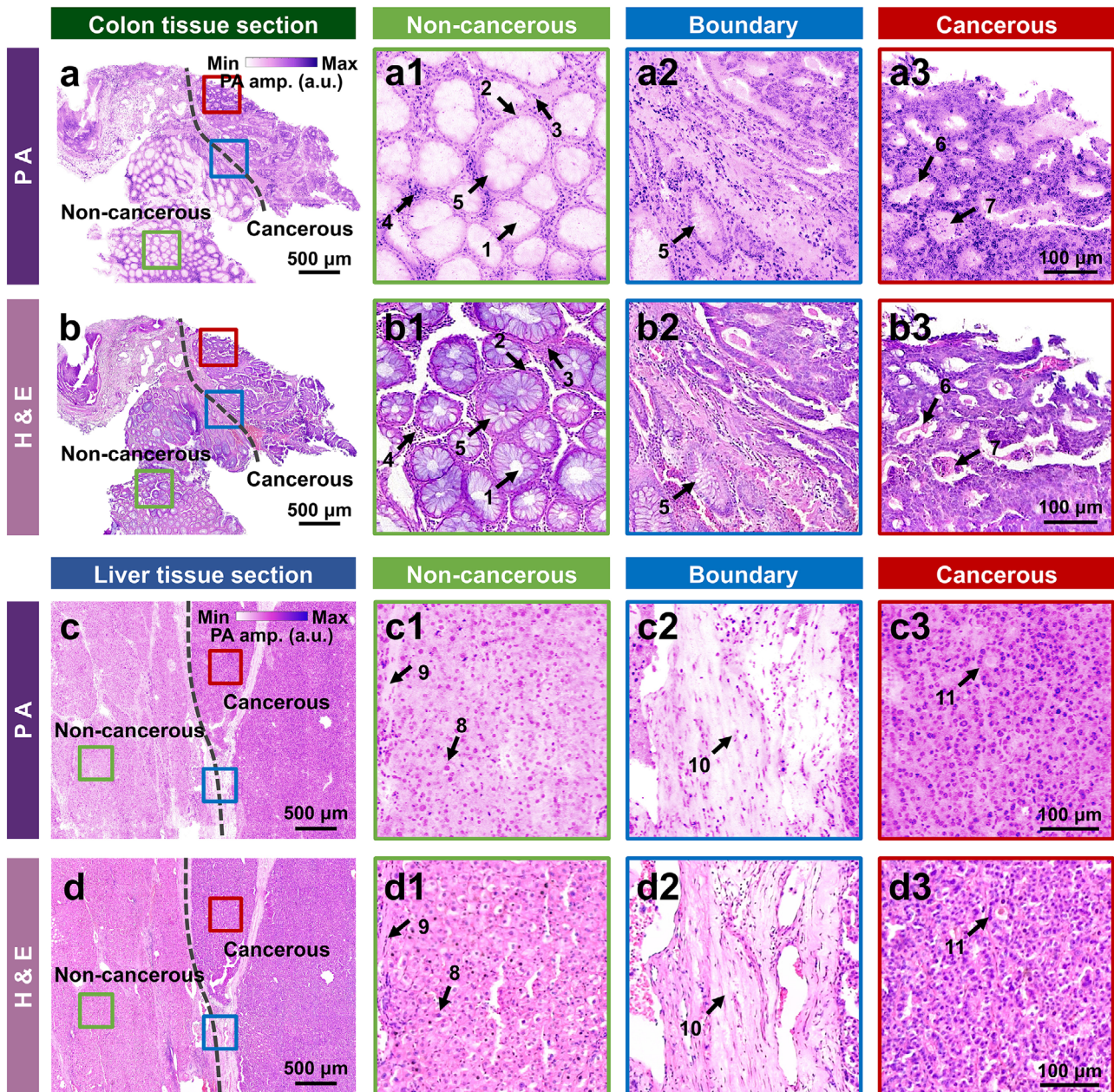


Figure 3. Label-free PA histopathology of deparaffinized FFPE human tissue sections. a,b) Label-free PA image of a) the deparaffinized FFPE colon tissue section and b) H&E stained optical pathology image acquired after the PA imaging experiment. a1–a3,b1–b3) Magnified histoarchitectural features of a1,b1) the non-cancerous, a2,b2) boundary, and a3,b3) cancerous regions shown in the green, blue, and red boxes in (a) and (b), respectively. c,d) Label-free PA image of c) the deparaffinized FFPE liver tissue section and d) optical pathology image acquired after the PA imaging experiment with H&E staining. c1–c3,d1–d3) Magnified histoarchitectural features of c1,d1) the non-cancerous, c2,d2) boundary, and c3,d3) cancerous regions shown in the green, blue, and red boxes in (c) and (d), respectively. The non-cancerous and cancerous regions are distinguished by black dashed lines in (a–d). 1, lumen; 2, epithelial cell nucleus; 3, stromal cell nucleus; 4, lamina propria; 5, mucin vacuole; 6, neoplastic gland; 7, necrotic debris; 8, hepatocyte; 9, boundary of hepatic lobule; 10, fiber accumulation; and 11, pseudo-glandular pattern.

In the non-cancerous region, MEMS-UV-PAM can visualize the morphological appearances of occasional pseudo-glandular pattern with elliptical shapes, which are a variant of bile ducts (Figure 3c3). The area and density of cell nuclei are also greater than in the non-cancerous region. The PA image features well match with those found in the subsequently performed H&E histology (Figure 3d1–d3).

2.4. Whole-Tissue PA Histopathology of Clinical Specimens

To demonstrate that MEMS-UV-PAM can support intraoperative histopathology of clinical specimens, we examined excised colon and liver specimens (3 to 7 mm in thickness) without any complex tissue preparation procedures (see Materials and Methods and Figure S5, Supporting Information). Initially, the ≈ 4 mm

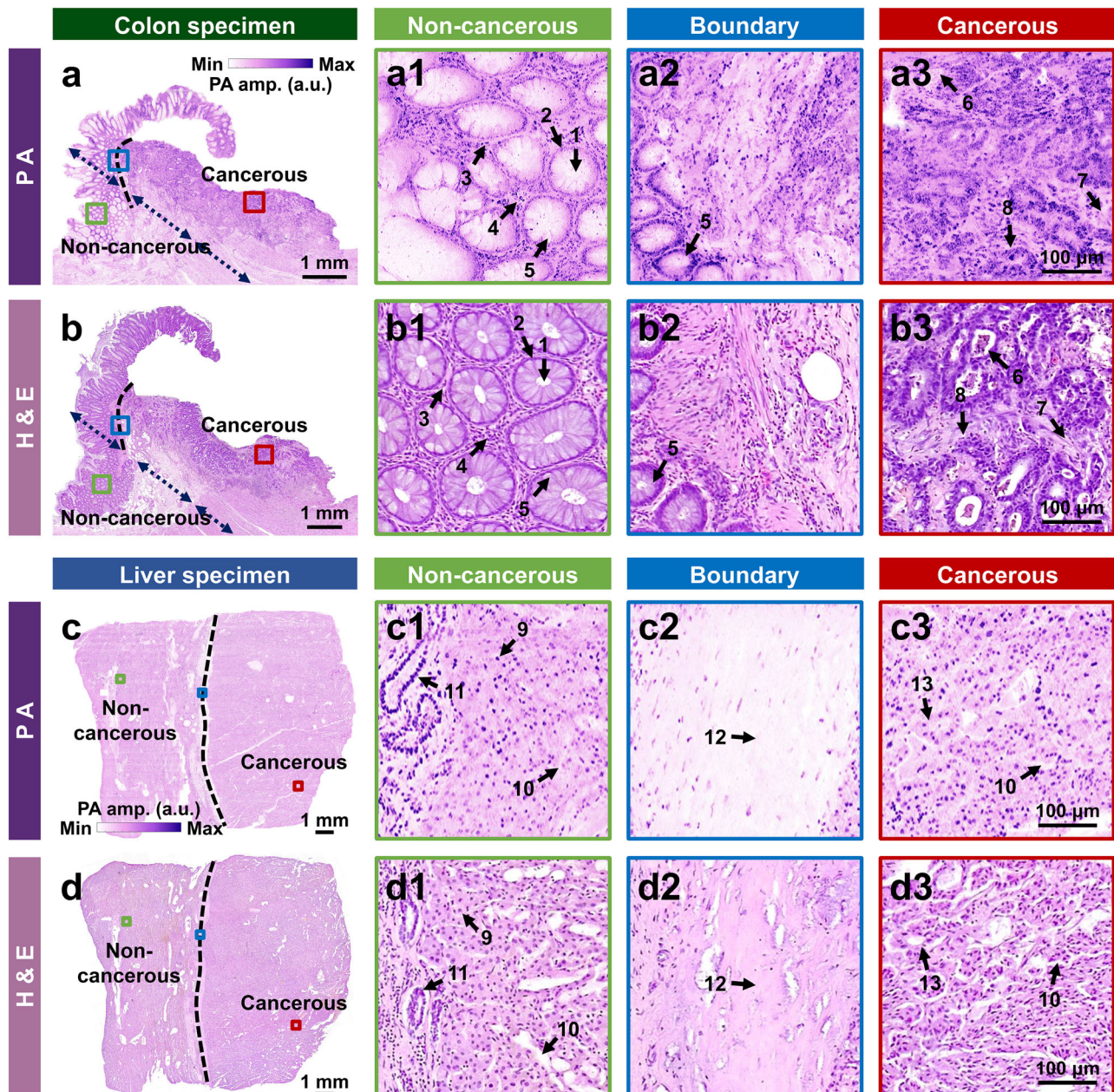


Figure 4. Whole tissue PA histopathology of clinical specimens from cancer patients. a,b) Label-free PA images of a) a colon specimen acquired from Patient 1 without complex procedures and b) an optical pathology image acquired after the PA imaging experiment with PS analysis and H&E staining. a1–a3,b1–b3) Magnified histoarchitectural features of a1,b1) the non-cancerous, a2,b2) boundary, and a3,b3) cancerous, regions shown in the green, blue, and red boxes in (a) and (b), respectively. c,d) Label-free PA image of c) a liver specimen obtained from Patient 4 without complex procedures and d) an optical pathology image acquired after the PA imaging experiment with PS analysis and H&E staining. c1–c3,d1–d3) Magnified histoarchitectural features of c1,d1) the non-cancerous, c2,d2) boundary, and c3,d3) cancerous regions, respectively shown in the green, blue, and red boxes in (c) and (d). The non-cancerous and cancerous regions are differentiated by black dashed lines in (a–d). 1, lumen; 2, epithelial cell nucleus; 3, stromal cell nucleus; 4, lamina propria; 5, mucin vacuole; 6, neoplastic gland; 7, desmoplastic reaction; 8, poorly differentiated cluster; 9, hepatocyte; 10, sinusoid; 11, bile duct; 12, fiber accumulation; 13, pseudo-gland.

thick colon tissue of Patient 1 was photoacoustically imaged (Figure 4a) and pseudo-coloured to be reminiscent of H&E staining. The SNR was calculated as 37 dB. Followed by PA imaging, the tissue was prepared for slide-based FFPE H&E histopathology (Figure 4b). Typical layers in the wall of the digestive tract are seen, such as the mucosa, submucosa, and muscularis externa

beneath the base of the glands. The non-cancerous tissue region (Figure 4a1) shows features of glands in regular patterns such as lumen, epithelial cell nucleus, stromal cell nucleus, lamina propria, and mucin vacuole. In the boundary tissue region, the gland pattern is irregular with the reduced number of the mucin vacuoles (Figure 4a2). Due to the invasive cancer, the structure

of the submucosa could not be seen in the cancerous region. As a region gets closer to cancer, it manifests an architectural complexity of the glands as well as cytologic atypia. Architecturally, characteristic gland formation could rarely be identified (<50% gland formation). Most shapes of the lumens are more angulated (Figure 4a3). The features attest to poorly differentiated adenocarcinoma (PDA). The PDA herein is prominent, with a crowded constellation of the irregular glandular structures, poorly differentiated clusters (PDC) composed of more than 5 cancer cells, and dirty necrosis in the glandular lumen. Muco-secretion loss is also indicated by the small numbers of vacuoles in the glands. Cytologically, nuclear atypia in the PDCs is severe, with highly packed cells that are more malignant-appearing than those in the non-cancerous tissue region. Desmoplastic reaction is shown by the proliferation of spindle cells intercalated within fibers near the glands. All the PA histoarchitectures of the clinical colon specimen are validated by those acquired in concomitant H&E-based histopathology (Figure 4b1–b3). Additionally, we performed PA histopathology of Patients 2 and 3 (Figure S6, Supporting Information). Compared to the PDA, the moderately differentiated adenocarcinoma shows 50–95% gland formation (Figure S6a,b, Supporting Information). The glands are inhomogeneous in size, indicating dysplasia. The other marked feature is nuclear pseudo-stratification, in which the cell nuclei appeared to be arranged in layers (Figure S6a3,b3, Supporting Information). The SNRs are calculated as 39 and 42 dB for Patients 2 and 3, respectively.

To further explore the potential of MEMS-UV-PAM, we conducted comprehensive label-free PA histopathology of large centimetre-sized liver specimens from three patients, seeking to differentiate HCC regions from non-cancerous regions (Figure 4c and Figure S7, Supporting Information). A PA image (Figure 4c) of a liver tissue specimen from Patient 4 was acquired and coloured to emulate H&E histopathology (Figure 4d). The image is also shown volumetrically in Figure S8, Supporting Information. The SNR is calculated as 42 dB. The non-cancerous region is composed of regular hepatocytes arranged in trabeculae (Figure 4c1). A portal tract, a standard histoarchitectural feature of the liver, is also observed in the non-cancerous region. The portal tract consists of three conspicuous tubular structures (portal vein, hepatic artery, and bile duct) surrounded by connective tissues, as detailed in the magnified images (Figure S9, Supporting Information). The portal vein, a primary source of hepatic blood supply, appears as a large area with a narrow wall. Compared to the portal vein, the hepatic artery is smaller in area, but has a well-developed entwined muscle wall. The boundary between the non-cancerous and cancerous regions is mainly filled with fibrous tissues (Figure 4c2). To the contrary, the cancerous region can be recognized as moderately differentiated HCC by architectural and cytologic atypia (Figure 4c3). The architectural pattern of a pseudo-gland, a deformed shape of bile ducts, is also observed. There are no portal tracts identified in the cancerous region, which is characteristic of HCC. Cytologic atypia manifest as an increased nuclear size (macrokaryosis) and nuclear irregularity, with various sizes and shapes of pleomorphism. The observed PA features agree well with the H&E-based histopathology (Figure 4d1–d3). In the moderately differentiated HCC of Patient 5, a trabecular growth pattern is also observed (Figure S7a3, Supporting Information). Broad trabecular patterns in sinusoid-like

blood spaces are present, with a thickness of 3 or more cells. Notable in the cancerous region are enlarged convoluted nuclei and invagination of cytoplasm, leading to nuclear pseudo-inclusion. In addition, the specimen of Patient 6 demonstrates the typical characteristics of well-differentiated HCC, such as prevalent fat vacuoles. The cancer cells are arranged in narrower trabeculae (≈ 2 cells wide) with minimal nuclear irregularity, almost like the nuclei in the non-cancerous region. However, the nuclei are enlarged, with prominent nucleoli (Figure S7b3, Supporting Information). The SNRs of the PA images from Patients 5 and 6 were measured as 43 and 37 dB.

2.5. Histopathologic Quantification by Cytologic Features in Clinical Liver Specimens

After segmenting cell nuclei from the clinical specimens of Patients 4–6, we estimated three cytologic features for histopathologic quantification: i) the nuclear area, ii) the nuclear density, and iii) the internuclear distance. These representative cytologic features were selected because they indicate obvious histopathologic changes that frequently occur in HCC and because MEMS-UV-PAM can fully reveal the features. The segmentation pipeline sequentially involved binarization, distance transformation, and application of a watershed algorithm (Figure S10, Supporting Information). The cytologic features on non-cancerous and cancerous regions were analyzed based on both the PA and H&E stained optical images. In the case of Patient 4, there is a significant difference ($p < 0.001$) in the nuclear area between the non-cancerous ($34.7 \pm 3.5 \mu\text{m}^2$) and cancerous regions ($41.6 \pm 3.4 \mu\text{m}^2$), which numerically verifies the macrokaryosis characteristic of HCC (Figure 5a). The cancerous region (643.6 ± 80.2 nuclei) also shows a significantly ($p < 0.001$) higher nuclear density than the non-cancerous region (499.7 ± 75.0 nuclei), indicating increased cellular proliferation (Figure 5b). The internuclear distance is weakly distinguished but statistically significant ($p < 0.001$) between the non-cancerous ($13.8 \pm 0.5 \mu\text{m}$) and cancerous regions ($12.6 \pm 0.4 \mu\text{m}$), which might be affected by nuclear crowding (Figure 5c). The quantitatively estimated features on the PA images show analogous trends to those of the H&E histopathology (Figure 5a–c). Furthermore, the cytologic features of the specimens from Patients 5 and 6 are substantially in agreement with those of Patient 4 (Figures S11 and S12, Supporting Information). For automated intraoperative classification of the clinical liver specimen, we implemented a support vector machine (SVM), a supervised learning model of a machine learning-based method, with the estimated diagnostic features as predictors. The PA histopathology clearly demonstrated the clinical potential to distinguish the cancerous region from non-cancerous region, with a decision hyperplane in the spatial distribution of the features. To compare the classification capability of the SVM models trained with the respective PA images (Figure 5d) and H&E images (Figure 5e), receiver operating characteristic (ROC) curve analyses of the SVM classifiers were evaluated for cross-validation (Figure 5f). The models with three clinical specimens achieved accuracies of $94.3 \pm 2.6\%$ for PA histopathology and $97.1 \pm 3.2\%$ for H&E histopathology. The analysis also shows sensitivities of $93.9 \pm 2.6\%$ for PA histopathology and $97.9 \pm 3.0\%$ for H&E histopathology. The

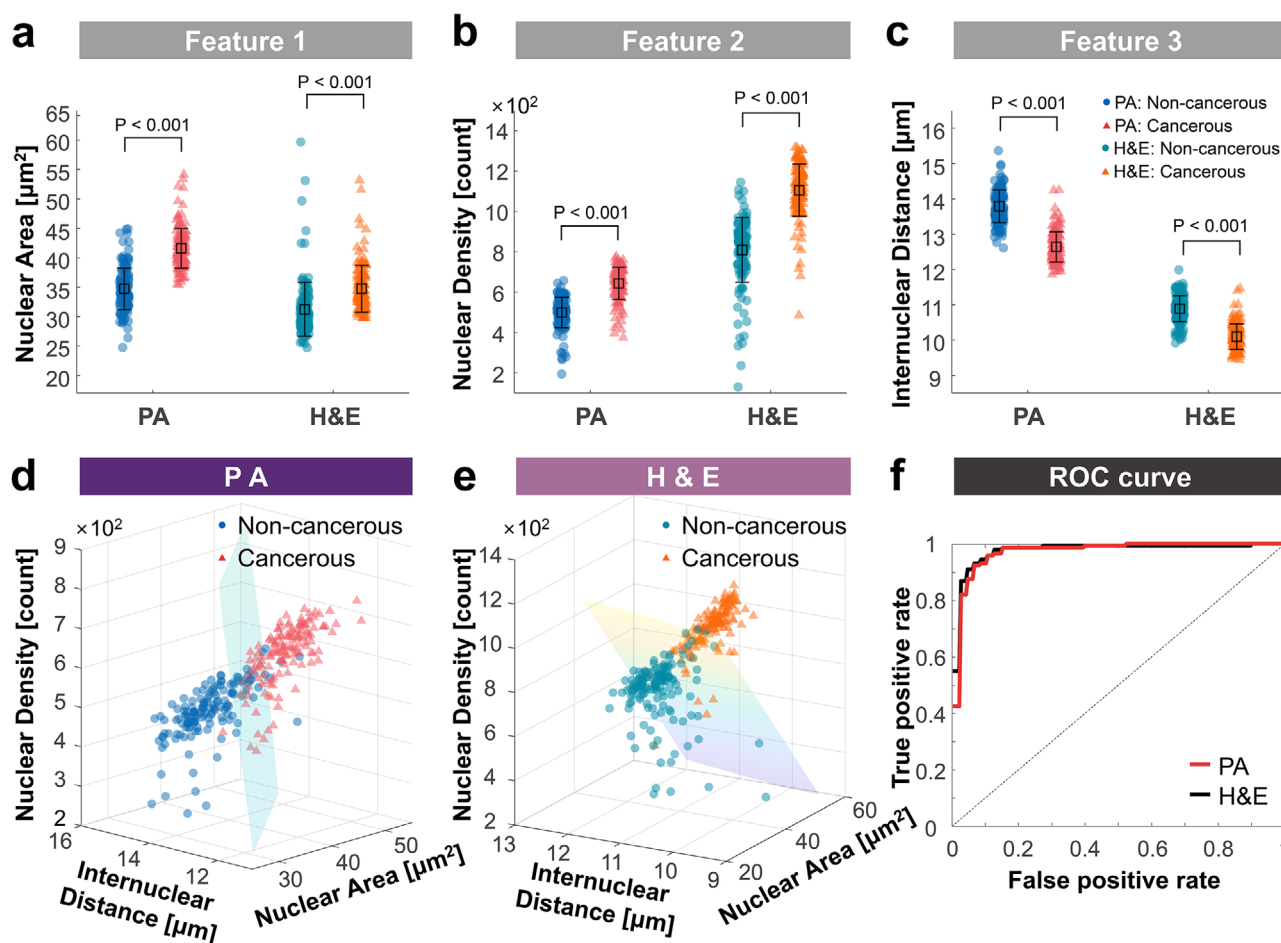


Figure 5. Histopathologic quantification by diagnostic feature extraction in a clinical liver specimen from Patient 4. a) Nuclear area, b) nuclear density, and c) internuclear distance calculated from the PA image (Figure 4c) and the H&E stained image (Figure 4d). Each feature was calculated within a reference area of $400 \times 400 \mu\text{m}^2$. d,e) 3D spatial distribution of each feature in the PA image and H&E image, respectively. For automated intraoperative classification between non-cancerous and cancerous regions with decision hyperplanes, a supervised machine-learning method of the SVM was trained with the diagnostic features estimated above. f) ROC curve analysis for liver cancer prediction in the PA image (red line) and H&E stained image (black line).

specificities are estimated as $94.2 \pm 2.7\%$ for PA histopathology and $97.9 \pm 3.0\%$ for H&E histopathology. The SVM classifier of H&E histopathology shows slightly higher performance than that of PA histopathology. While the nuclear area is a highly distinguishing feature between non-cancerous and cancerous regions in the H&E images, the feature in the PA images shows smaller difference between the two regions (Figures S11 and S12, Supporting Information). This difference might be a result of the limited lateral resolution and the shrinkage of cell nuclei during tissue processing for H&E histopathology, which requires several reagents (alcohol, xylene, paraffin-wax, etc.) leading to reduce cell volumes (Figure S13, Supporting Information).^[23]

3. Discussion

To further satisfy the clinical need for intraoperative histopathology, MEMS-UV-PAM can be improved in five aspects. First, in the current system, the scanning speed of the MEMS scanner is mainly determined by the PRF (10 kHz) of the UV laser because

intrinsic fluctuations in the laser pulse energy rapidly increase at a higher PRF. Considering that the MEMS scanner can operate at up to ≈ 100 Hz, it could be more fully utilized by generating 266 nm light using both a 532 nm pulsed fiber laser, with a PRF of up to 600 kHz, and a cesium lithium borate crystal²⁰, potentially providing high-speed clinical feedback to clinicians. Second, although it is sufficient for the diagnosis of intraoperative histopathology to visualize cell nuclei and structures of cytoplasm using one wavelength light, the system can be updated for multi-wavelength-based PA histopathology, which might provide additional guidance for the diagnosis.^[24,25] Especially, it is possible to point out cytoplasm more specifically by using light around the Soret peak (≈ 420 nm).^[26] Moreover, red blood cells, which have high absorption at 532 nm, are well-known indicators for cancer, such as peliosis in the liver. Third, the lateral resolution ($1.2 \mu\text{m}$) of MEMS-UV-PAM is currently restricted by the optical diffraction limit. Specifically, the coaxial alignment of the optical and acoustic beams via the OUC inevitably requires a working distance between the objective lens and each

component of the scanning module. An alternative PAM configuration for achieving coaxial alignment employs a ring transducer and a transparent ultrasound transducer (TUT).^[27] The ring transducer, however, has a limited central hole size that makes it difficult to increase the numerical aperture (NA). To overcome this bottleneck, a miniaturized TUT with high transparency in UV light can be fabricated to reduce the working distance and raise the NA. Fourth, histopathologic quantification of colon cancer is challenging due to the varying patterns of differentiation in abnormal glands, resulting in intra- and inter-observer variability. For colon cancer quantification, the glandular structure can be estimated via automatic segmentation, but this estimation is difficult with conventional computer vision techniques. A deep convolutional neural network architecture, such as U-net, can be applied to conduct pixel-based classification of each gland, producing a probability map of whether a specific pixel in the input image belongs to the gland or not.^[28] A best alignment metric can be used to measure the correlation of each segmented structure with the grade of the cancer. Such computer-aided histopathology might provide guidance in clinical environments where expert pathologists are vacant. In more fortunate settings, it could yield high consistency between pathologists in spite of the somewhat subjective nature of cancer grading and could reduce labor-intensive procedures as well.^[29] Finally, it is necessary to identify whether the fixation process affects the contrast of PA images for clinical scenarios in which specimens after excision surgery can be directly used for intraoperative histopathology.

4. Conclusion

Even though intraoperative FS analysis is standard and globally used, it inevitably creates artifacts such as uneven staining and tissue distortion (e.g., contraction, folding, and loss). These artifacts are generated repeatedly by the complex preparation procedures (freezing, sectioning, staining, and mounting), which might potentially obscure underlying cancer regions and negatively impact the histopathologic assessment. In contrast, by leveraging the superior optical contrast of cell nuclei, MEMS-UV-PAM can provide non-destructive and label-free histopathology of human colon and liver tissues with an image quality comparable to that of traditional slide-based H&E staining. It can intraoperatively highlight key histoarchitectural features of clinical specimens, minimizing interruptions to the surgical workflow. Moreover, by using digital image processing to identify cell nuclei in large liver tissue specimens, PA imaging also enables quantitative histopathology. Estimating the cytologic parameters of liver tissues, such as their nuclear density, area, and inter-nuclear distance, it can differentiate non-cancerous and cancerous regions for guidance in surgical margin assessment. The non-destructive and label-free advantage of MEMS-UV-PAM can be especially useful in a clinical situation where a minimally invasive needle biopsy is necessary which might damage the excised tissue after the conventional histopathology. Histopathology with MEMS-UV-PAM allows for subsequent postoperative examination of tissues, such as for immunohistochemistry or RNA sequencing. In addition, as a standalone microscopic imaging modality, it can contribute to various fields of biology, microanatomy, and chemistry.

5. Experimental Section

Preparation of Animal Tissue Samples: Male ICR mouse brain tissue was prepared through a FS protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Gachon University (approval no. LCDI-2018-0096). The mouse brain was first dissected out, frozen with aerosol sprays, and put into a cryostat containing a rotary microtome that sectioned it into 10 μm thick slices (bregma -2.46 to -2.8 mm), which were individually placed on coated slide glasses (cat# 5116-20F, Muto Pure Chemicals Co., Ltd, Japan). During sectioning, the temperature of the cryostat was equilibrated at -20 °C for ≈ 15 min to keep the tissue block from cracking. Each tissue section was air dried and adhered to a slide glass. PA experiments were performed in accordance with protocols approved by the IACUC (approval no. POSTECH-2016-0049-C1-R3) of Pohang University of Science and Technology (POSTECH).

Preparation of Human Tissue Samples: Two types of clinical human specimens, colon and liver tissues, were obtained from anonymous patients in the context of protocols approved by the Institutional Review Board (IRB) of Gachon Gil Hospital (approval no. GAIRB2018-430). Informed consent was obtained from all patients involved in the study. The cancerous specimens with a size of $\approx 15 \times 15 \times 5$ mm³ were excised along with a margin of non-cancerous tissue and fixed in 4% paraformaldehyde solution (cat # P1004, Biosesang, Republic of Korea) for four days at 4 °C. A subset of the excised specimens was prepared as deparaffinized FFPE tissue sections, using the following procedures. The specimens were washed with running water for 30 min, then dehydrated, cleared with 100% xylene, and embedded in paraffin wax (cat# 39 601 006, Leica, Germany) by using a tissue processor machine (Shandon Citadel, Thermo Scientific, USA) for 14 h. The paraffin tissue blocks were then sliced into 10 μm sections and put on coated slides (cat# 5116-20F, Muto Pure Chemicals Co., Ltd, Japan). The slides were incubated on a 37 °C heat plate overnight and then immersed in 100% xylene for 10 min to remove the paraffin around the tissues. The slides were then successively immersed in 100%, 95%, 90%, and 70% ethanol for 1 min at each concentration. After these steps, the slides were put in running water for 1 min to wash the ethanol from the slides and then preserved in water at room temperature. The remainder of the excised specimens did not undergo this procedure and were simply routed for PA imaging as thick clinical specimens. The deparaffinized FFPE tissue sections and clinical specimens were then transferred to POSTECH for PA imaging. No patient identifiers were provided to the researchers. For PA imaging, all procedures were conducted via IRB-approved regulations and guidelines of POSTECH (approval no. PIRB-2019-E013).

H&E Histopathology: After PA imaging of the frozen mouse tissue section and the deparaffinized FFPE human tissue sections, the slides were dipped in hematoxylin solution (cat# S330930-2, DAKO, USA) for 3 min and washed with running water for 1 min. The slides were then successively dipped in 0.8% ammonium solution (cat# A0627, Ducksan, Republic of Korea) for 30 s, in 95% ethanol for 30 s, and in eosin solution (cat# E4009, Sigma-Aldrich, USA) for 7 s. After H&E staining, the slides were washed with running water for 1 min and immersed successively in 70%, 90%, 95%, and 100% ethanol for 1 min at each concentration for dehydration. Next, the slides were immersed in 100% xylene for 1 min and mounted with a cover glass by using DPX solution (cat# 06 522, Sigma-Aldrich, USA). To validate the thick human clinical specimens, the tissues were processed after PA imaging by postoperative PS analysis. The clinical specimens were first paraffin-embedded, sectioned, and then stained following the above staining procedures.

Photoacoustic Scanning Module Based on the 1-Axis Waterproofing MEMS Scanner: A PA scanning module composed of an ultrasound transducer (V212-BC-RM, Olympus-NDT, USA; 20 MHz central frequency), a customized OUC, and a self-manufactured 1-axis waterproofing MEMS scanner were fabricated. The OUC was manufactured with two UV fused silica-based prisms sandwiching each hypotenuse face of the prism by optical contact bonding. Using vacuum electron beam evaporation, the hypotenuse of one prism was coated with a dielectric material, such as ZrO₂ and SiO₂, to increase its UV light reflectance. For focused acoustic detection, a concave surface was milled on the base side of the other prism and polished using a polishing machine to increase the transmission of UV

light. An effective ultrasonic bandwidth of 19.8 MHz was measured with the ultrasound transducer combined with the OUC. The MEMS scanner was fabricated by assembling an opto-ultrasound mirror structure and its driving electromagnets for the entire system. The entire fabrication procedure for the MEMS scanner was illustrated in previous articles.^[30,31] Additionally, to improve ultrasonic reflection, a silicon carbide (SiC) wafer coated with a 200 nm thick UV-enhanced aluminum layer was used, which had a higher acoustic impedance than the previously used silicon wafer. The SiC wafer was then diced into $6 \times 8.5 \text{ mm}^2$ pieces by femto-second laser micromachining for use in making the opto-ultrasound mirror. The support for the mirror was a soft polydimethylsiloxane (PDMS) structure, to which the mirror piece was attached with commercial silicone (KE-441K-T, Shin-Etsu silicone, Japan). The flexible PDMS structure was made by soft lithography in a mold shaped by a micro engraving machine (EGX-350, Roland, USA). The mold was filled by injection with a base material and a curing agent in a mass ratio of 10:1 (Sylgard 184 Silicone Elastomer Kit, Dow Corning, USA). After injection, the mixture was heat-cured in a vacuum oven at 70 °C for 4 h and stripped from the mold. Two neodymium magnets were stuck into holes with the silicone on either side of the PDMS structure. Lastly, two driving electromagnets were fabricated with soft cylindrical iron cores and air coils. To exert magnetic force on the neodymium magnets, a current to two driving electromagnets was applied via a high-current operational amplifier (TCA0372, ON Semiconductor, USA).

Reflection-Mode MEMS-UV-PAM System: The entire reflection-mode MEMS-UV-PAM system is shown in Figure 1a. The data acquisition program was developed in LabVIEW software (LabVIEW 2017, National Instruments, USA). The entire system was controlled using a multi-function data acquisition (DAQ) board (NI-PCIe 6321, National Instruments, USA) and based on user inputs. The DAQ generated external trigger signals concurrently with an 8-bit digital output to initiate four functions: irradiation from the UV pulsed laser (Wedge-HF-266, Bright Solutions, Italy), data acquisition of a digitizer (ATS9350, Alazartech, Canada), scanning by the 2-axis linear motorized stage (8MTF-75LS05, Standa, Lithuania), and steering the MEMS scanner. Each device was accurately operated in exact synchronization with the other devices. For UV light irradiation, 266 nm laser pulses with a width of 0.8 ns at a PRF of 10 kHz were generated. The collimated laser beam was first split by an optical beam splitter (BSW20R, Thorlabs, USA), then the reflected beam from the beam splitter was monitored by a photodiode (PDA25K-EC, Thorlabs, USA) to correct for the laser's pulse-to-pulse energy fluctuations. The transmitted beam was expanded by a beam expander (BE10-266, Thorlabs, USA) and spatially filtered by an iris. The filtered laser beam was focused onto the OUC by a meniscus lens (LE4197, Thorlabs, USA) and an aspheric lens (33 954, Edmund Optics, USA), reflected by the dielectric material-coated hypotenuse of the OUC and the MEMS scanner, and illuminated onto specimens with a focal spot diameter of $\approx 1.2 \mu\text{m}$. The focused laser beam was aligned with a generated ultrasound beam in a coaxial and confocal configuration by the OUC, providing high detection sensitivity. The scanning pattern of the MEMS scanner was controlled by the analog output signal of the DAQ, for example, a sine signal. The amplitude and phase of the signal were fine-tuned by considering the speed of the MEMS scanning. With sufficient electromagnetic force, the MEMS scanner could operate under water and steer both the laser beam and the ultrasound beam simultaneously, which enabled point-by-point imaging. Synchronized with the MEMS scanning along the x-axis, the 2-axis motorized stage was controlled to move the specimens along the y-axis, which acquired successive B-scan images for reconstructing a volumetric image. To extend the FOV, the motorized stage moved the specimens along the x-axis, and the MEMS scanning was repeated. For data acquisition, the generated ultrasound was focused by the acoustic lens on the OUC, transmitted through the OUC, converted into electrical signals by the ultrasound transducer, and then amplified by a low-noise pre-amplifier (ZKL-1R5+, Mini-Circuits, USA) with 40 dB gain. The high-speed digitizer card sampled the amplified electrical signal at 12-bit resolution, with a sampling rate of 250 MS s^{-1} .

Image Reconstruction and Histopathologic Quantification: Because the MEMS scanner was controlled by the operating sine signal, the B-scan images were linearly interpolated. The interpolated B-scan images were

sequentially converted from polar coordinates to Cartesian coordinates, based on the scanning geometry of the MEMS scanner. To create an entire seamless mosaic image, a multi-resolution spline algorithm was used to blend adjacent B-scan images.^[32] The resulting mosaic image was illustrated as a 2D maximum amplitude projection (MAP) image to generate a pseudo-coloured histopathology-like image.

To segment and detect cell nuclei in the 2D PA MAP image, it was first partitioned by binarization into a foreground and background. Before applying a segmentation algorithm, small components, such as noises in the binary image, were eliminated. The binary image was then transformed into a topographic map which represented location information by watershed segmentation based on distance transformation. For histopathologic quantification, diagnostic features, such as the nuclear area, nuclear count, and inter-nuclear distance, were extracted from both non-cancerous and cancerous tissues. The nuclear area was directly quantified in the segmented cell nuclei image. To measure the nuclear count and the inter-nuclear distance, centroids were detected by calculating the center of mass of the segmented binary image.^[33]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.W.B., H.K., and M.S. contributed equally to this work. This research was supported by Basic Science Research Programs (No. NRF-2019R1A2C2006269, 2020M3H2A1078045, 2020R1A6A1A03047902, 2020R1C1C1013549). It was also supported by the MSIP (Ministry of Science, ICT and Future Planning), Korea, under the "Artificial Intelligence Graduate School Program" (No.2019-0-01906) and BK21 FOUR project. It was also financially supported by the Smart Healthcare-based Thesis Research Grant Program of the Daewoong Foundation (DF-201901-0000001), Hyundai Motor Chung Mong-Koo Foundation. This paper was also supported by Korea Institute for Advancement of Technology (KIAT) grant funded by the Korea Government (MOTIE) (P0008763, The Competency Development Program for Industry Specialist).

Conflict of Interest

C.K. and J.Y.K. have financial interests in OPTICHO, which, however, did not support this work. All other authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

digital histopathology, intraoperative histopathology, label-free imaging, photoacoustic microscopy

Received: March 4, 2021

Revised: July 15, 2021

Published online: August 16, 2021

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