Research article

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A drawer-type abdominal window with an acrylic/resin coverslip enables long-term intravital fluorescence/photoacoustic imaging of the liver

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Abstract: The liver has a unique vascular structure and regional immunosuppressive characteristics closely linked to the occurrence and development of diseases. There are no long-term, large-field, and high-quality imaging methods to simultaneously obtain the structure of blood vessels and movement activities of immune cells in abdominal organs *in vivo*. This research developed a drawer-type abdominal window with an acrylic/resin coverslip named DAWarc and applied it to the intravital fluorescence/photoacoustic imaging of the liver for over 10 days. The liver lobe was inserted into the drawer holder of the DAWarc to physically fix the

liver, which decreased the imaging artifacts. The acrylic/resin material used as the coverslip has a high compatibility for fluorescence/photoacoustic imaging. Through intravital fluorescence/photoacoustic imaging, information on the structure of hepatic lobules, spatial distribution of nanopomgranate labeled Kupffer cells (KCs), the movement behavior of invariant natural killer T cells, and morphology of KCs were obtained. We also used a self-organizing map neural network to detect tumor metastases in the photoacoustic images automatically. Conclusively, the DAWarc model provided a powerful tool for intravital fluorescence/photoacoustic imaging of the liver; it helped us to better understand the structure of hepatic lobules and the distribution and function of immune cells during the occurrence and development of liver diseases.

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1 Introduction

As the body's largest solid organ, the liver is essential for performing metabolic and detoxification functions and is also considered a vital immune organ [1]. The liver is a high incidence organ for many diseases relative to its unique vascular structure and regional immunosuppressive characteristics [2–4]. Anatomically, the hepatic lobule is the basic structure of the liver, and the sinusoid is the capillary network in the hepatic lobule, which can reflect the typical microstructure of the liver. The occurrence and development of liver diseases, such as fatty liver, liver fibrosis, and metastatic tumor cell invasion, constantly destroy the hepatic sinusoid and cause a disorder in the structure of the hepatic lobule [5–7]. Detecting whether the hepatic lobule structure is regular or not could help evaluate the physiological and pathological status of liver diseases.

Liver disease processes usually involve many immune cell types, particularly liver-resident macrophages or invariant natural killer T (iNKT) cells. Kupffer cells (KCs), the largest proportion of resident macrophages located in the hepatic sinusoid, play a crucial role in immune surveillance [8–10]. Liver-specific iNKT cells can be involved in innate and adaptive immune reactions and play immune surveillance, regulatory, and killer roles in the occurrence and development of liver diseases [11, 12]. Typically, the function of immune cells in the liver is closely associated with their spatial locations and a given disease's progression [13–16]. Therefore, a long-term intravital imaging method for the liver to monitor microstructural changes of hepatic lobules and the spatiotemporal dynamic activities of immune cells in the lesion region (e.g., tumor microenvironment) need to be established.

Optical molecular imaging has unique advantages, such as multicolor, high spatiotemporal resolution, and 4D dynamic information, especially suitable for long-term dynamic monitoring of cellular and molecular events in tissue microenvironments *in vivo* [17]. However, intravital imaging of the abdomen with high spatiotemporal resolution often encounters a significant challenge: the irregular movement caused by respiratory fluctuation and heartbeat severely affects the stability of the focal plane of optical microscopy [18, 19], resulting in unclear images [20]. The early intravital imaging of the abdomen used instant adhesive agents [21] could solve this problem to a certain extent, but it usually requires complex post-image processing to minimize motion artifacts. In addition, instant adhesive agents induce severe inflammatory responses and a change in the physiological state of the imaged region. The imaging frequencies of these abdominal window models are limited (only 3–4 times) for abdominal imaging [22]. Consequently, these models cannot meet the demand for long-term dynamic monitoring of disease progression. In 2012, researchers developed an abdominal imaging window (AIW) model using “purse sutures” combined with titanium alloy rings suitable for long-term imaging of the liver [6]. However, it still did not solve the drifting of imaging's focal plane induced by respiratory fluctuation and heartbeat. An abdominal window model for long-term intravital imaging of liver suitable for high spatiotemporal resolution imaging and large-field imaging must now be developed [6, 23].

In vivo photoacoustic imaging has several advantages, including a superior cell resolution, deep imaging depth, large-field, and nonlabeling for blood vessel imaging [24–28]. We previously developed a self-assembled pomegranate structure-like nanoparticle named nanopomegranate to label KCs *in vivo* selectively. In that

research, the structure of hepatic sinusoids and criss-crossed arrayed hepatic lobules, along with the spatial location of nanopomegranate labeled KCs, were visualized simultaneously using dual-wavelength photoacoustic imaging with a high spatial resolution (1–2 μm) and a large field of view ($2 \times 2 \text{ mm}$) [29]. However, we had to pull the liver out of the abdominal and transient exposure for intravital optical-resolution photoacoustic imaging. This method is impossible to achieve long-term imaging for days.

Fluorescence imaging and photoacoustic imaging have good complementarity; the former monitors the dynamic activities of immune cells, and the latter is good at obtaining information on the structure and spatial distribution of blood vessels *in vivo* [8, 30, 31]. Combining these two imaging techniques provides a powerful tool for visualizing changes in the hepatic lobule microstructure and the dynamic activities of immune cells in lesion regions during disease progression [29]. Until now, the long-term intravital fluorescence/photoacoustic bimodal imaging with the high spatiotemporal resolution is not available, primarily because the cover glass on the abdominal window used for fluorescence imaging is not appropriate for photoacoustic imaging. This unsuitability is due to the strong attenuation caused by acoustic waves reflecting back and forth in the glass material. Finding a material that would effectively satisfy light and acoustic signal transmittance simultaneously remains challenging for long-term intravital fluorescence/photoacoustic imaging.

In this investigation, we designed a drawer-type abdominal window with an acrylic/resin coverslip named DAWarc for intravital imaging of the liver. DAWarc model allowed repeating fluorescence/photoacoustic imaging of the liver over a period of days to three weeks. To overcome the imaging artifacts caused by breathing and heartbeat jitters, the liver lobe was put into the drawer holder of DAWarc so that the imaging region of the liver was physically fixed. Through detection and screening of the optical properties of the transparent material, the acrylic/resin material used as the coverslip has a high compatibility for fluorescence/photoacoustic imaging. In a nutshell, the DAWarc model successfully achieved a long-term fluorescence/photoacoustic imaging of hepatic lobules and immune cells in the liver *in vivo*, which not only overcame the breathing and heartbeat jitters but also balanced the penetrability of light and acoustic signals. This model provides excellent value for investigating changes in microvascular structures and immunocyte activities in internal organs during disease progression and treatment.

2 Results and discussion

2.1 Designing of the DAW model for intravital imaging of the liver

To physically fix the liver lobe close to the coverslip and minimize tissue movement, we designed a DAW model made of a titanium ring, a thin semicircular fixation salver stuck at its bottom like a drawer for holding the liver lobe, and a coverslip glued at its top for optical imaging (Figure 1(A)). The bottom side of the titanium ring stuck a thin semicircular salver, forming a “drawer” to hold the

liver lobe. After incising the skin and muscle on the abdomen, the DAW was inserted into the gap between the skin and muscle layers, one of the liver lobes was placed carefully into the “drawer”, and then the DAW was secured with sutures and adhesives, hereto the abdominal cavity was resealed completely.

If this procedure is performed correctly, the window becomes an integral part of the abdomen, the abdomen is well sealed, and the liver tissue adheres closely to the window. One of the advantages of the DAW model is that it does not need to glue the liver tissue to the coverslip, minimizing any irritation and inflammation of the imaged region. A series pictures of the surgery process installing

Figure 1: DAWarc design, surgical procedure, and liver jitter amplitude detection.

(A) DAWarc window frame design. All dimensions are in millimeters. (B) Overview of the surgical procedure. (C) Photoacoustic imaging of mice with the DAWarc using an upright photoacoustic imaging system. (D) Fluorescence imaging of the same mice using an inverted confocal imaging system. (E) Schematic diagram of the detection of respiratory jitters in mice with the DAWarc or standard AIW using a laser displacement sensor, the laser wavelength was 655 nm. (F) Comparing amplitude curves of the respiratory jitters of mice with different windows: the black curve represents the respiratory jitters of mice using the standard AIW, and the red curve denotes the respiratory jitters of mice using the DAWarc. The quantitative unit of amplitude is millimeter (mm).

the DAW on the mouse were taken (Figure 1(B), 1–6). The mouse with the DAW for five days after surgery is shown in the 7th picture in Figure 1(B); the zoom in the picture displays a clear tissue, suggesting no change in the physiological state or inflammation of the imaged region (7th in Figure 1(B)).

To ascertain the DAW model's capability to overcome heartbeat and respiration jitter induced liver lobe movements, we evaluated the jitter disturbance from X-ray fluoroscopy imaging of the whole body of the mouse (Supplementary Figure 1, and Movie 1). To detect the effects of surgery installation and long-term carrying with the DAW on the mouse's physiological condition, we recorded the activity of the mouse with the DAW five days after surgery. The mice continued regular feeding, grooming, and nesting activities without abnormality in their locomotion (Supplementary Movie 2). As shown in Figure 1(C) and (D), we performed both upright and inverted microscopy imaging of the liver in the DAW model mouse using different imaging fixation boxes. We also used the laser displacement sensor to quantitatively compare mice's breathing and heartbeat jitters in the DAW model versus the standard AIW model [19]. The liver surface's amplitude in the mouse with the DAW was less than 0.5 mm, and that in the mouse with the standard AIW reached about 3.5 mm (Figure 1(F)).

These data indicate that the DAW model worked without adhesive agents and stably fixed liver tissues for imaging. This method successfully prevents the regular movements of the mouse liver in the z -axis while allowing natural heartbeat and regular respiration of the mouse without impeding its physiological state. The DAW model could also overcome the drifting of the focus plane during intravital imaging of abdominal organs and not rely on the subsequent image processing. Compared with abdominal imaging window fixed liver with glue, the DAW has several advantages: (1) reduce the unwanted inflammation and irritation of liver which caused by glue; (2) the surgery of DAW is easy to operate; (3) DAW allow the liver to be filled into the imaging window to obtain more imaging area; and (4) DAW is very suitable for long-time time-lapse imaging, stitching large-field images, and low temporal resolution imaging (such as photoacoustic imaging).

2.2 The DAW model efficiently eliminates the impact of respiration and heartbeat on the quality of imaging

To confirm the DAW model's ability to fix liver tissues, we prepared a liver metastasis model using tetrameric far-red

fluorescent protein-expressed B16 melanoma (tRFP-B16) cells and *Cxcr6*^{+/sfp} transgenic (CXCR6-GFP) mice. Time-lapse imaging was conducted using spinning-disk confocal microscopy with 1 frame per second. The data from time-lapse imaging showed that the stability of the imaging focus plane in the DAW, which does not need the complex processing of several frames of time-lapse videos to eliminate the slight motion artifacts, could obtain high-quality liver images (Figure 2(A), Supplementary Movie 3). Many CXCR6-GFP⁺ cells (in Green) accumulated in the tRFP-B16 tumor metastasis region (Blue); most of the CXCR6-GFP⁺ cells in the liver were iNKT cells [13, 32, 33]. Due to the liver's autofluorescence signal, the hepatic sinusoid (in light green) was also clearly displayed. The motion artifacts in the standard AIW model caused poor imaging quality; the morphologies of the hepatic sinusoid, iNKT cells, and tRFP-B16 tumor metastases were not distinctly observed (Supplementary Figure 2, and Movie 4).

Furthermore, we performed large-field fluorescence imaging of the region of interest of the liver using the DAW model. Large-field images of the livers of mice with or without tRFP-B16 tumor metastases were taken with the DAW (the imaging field range was $1327 \times 1327 \mu\text{m}$). As shown in Figure 2(B), we obtained distinct structures of the hepatic sinusoid and the distribution of iNKT cells in the normal liver (Figure 2(B), left panel) and the liver with metastases (Figure 2(B), right panel). In contrast, no clear image was captured for each liver frame examined with the standard window model, meaning we could not obtain high-quality and large-field images.

To quantitatively analyze the imaging stability and continuity of the DAW model, we measured relative displacement during time-lapse imaging, extrapolated from the image at the corresponding time point relative to the initial time point. The XY plane shift distance at each time point was calculated automatically by the FIJI's Registration plugin using the time-lapse imaging videos of the DAW model and standard AIW model (Supplementary Movies 3 and 4). The position was set to the (0, 0) point at the first time point, and each point in Figure 2(C) and (D) represents the image shift in the XY plane at different time points. The results indicate that the DAW significantly eliminated the XY plane shift during intravital liver imaging (Figure 2(C)) compared with the standard AIW (Figure 2(D)). Based on our findings, we concluded that the DAW model could overcome liver motion artifacts caused by the breathing and heartbeat jitters of living mice. This model could perform long-term intravital imaging and obtain a high spatiotemporal resolution and high-quality fluorescence images.

Figure 2: The DAW model enables confocal imaging to acquire high-quality, stable, and large-field images.

(A) The spinning-disk confocal microscopy produced 10-s split-frame images from time-lapse videos of liver metastases in CXCR6-GFP mice using the DAW 5 days after *tRFP-B16* cell injection. Blue: *tRFP-B16* tumor metastases; Green: GFP-labeled iNKT cells. Field of view: $331.8 \times 331.8 \mu\text{m}$; scale bar: $50 \mu\text{m}$. (B) The spinning-disk confocal microscopy acquired 4×4 large-field images ($1327.1 \times 1327.1 \mu\text{m}$); scale bar: $100 \mu\text{m}$. The left panel demonstrates the liver of normal mice with the DAW, and the right panel shows tumor liver metastases of mice with the DAW. (C–D) Displacement of confocal time-lapse imaging of the liver in mice with the DAW (C) and standard AIW (D). Scatter plots ($n = 3$ time-lapse videos) showing the shift in imaging at each time point in the liver area.

2.3 Long-term intravital fluorescence imaging of tRFP-B16 tumors, iNKT cells, and KCs in liver metastasis microenvironments

Long-term intravital fluorescence imaging is beneficial to understanding the movement activities and function of immune cells at the occurrence and development of liver disease. Here, we used the DAW to observe the long-term distribution and movement activities of iNKT cells and KCs in the tRFP-B16 liver metastasis model. We injected 4×10^5 tRFP-B16 cells into the hemispleen to generate a liver metastasis model in the CXCR6-GFP mice [34]. The DAW was installed on the mice three days after tumor cell injection, and long-term intravital fluorescence imaging was performed 3, 5, 7, and 9 days after tumor cell injection (Figure 3(A)). The growth process of liver metastasis was captured distinctly, as shown in Figure 3(B). On Day 3, only a few tRFP-B16 tumor cells colonized in the liver, along with each micro-metastasis lesion, contained several tumor cells (Figure 3(B), Blue). The size of the micro-metastasis was amplified on Day 5, continuously increased on Day 7, and the imaging field was extensively characterized by tumor metastases on Day 9. We found that the tumor cells colonized and orthotopically grew in the hepatic sinusoids on Day 3, disseminated spontaneously and destroyed the hepatic sinusoid on Day 5, and eventually caused a severe disorder in the structure of the hepatic lobule on Day 7 and 9. The distribution and movement activities of iNKT cells (CXCR6-GFP cells, Green) and the distribution and morphology of KCs (nanopomegranate labeled, Red) in the tRFP-B16 tumor metastases (Blue) were also observed (Supplementary Movie 5).

We quantitatively analyzed the distribution and movement behaviors of iNKT cells in the tumor microenvironment of liver metastases on Day 5. The microenvironment was partitioned into three regions: tumor parenchyma, tumor periphery, and out-tumor region. As Figure 3(C) shows, there was no significant difference in the density of iNKT cells in the tumor parenchyma and periphery (913.4 and 856.3 cells/mm²). The density of iNKT cells in the out-tumor area dropped to 82.8 cells/mm² but was not significantly different from the control group's (without tumor, 89.8 cells/mm²). The density of iNKT cells in tumor metastases was 11-fold and 10.1-fold higher than that in the out-tumor area and control group. These results suggest that the distribution of iNKT cells in the tRFP-B16 liver metastasis microenvironment has prominent spatial characteristics, and most of the iNKT cells accumulate in the parenchyma and periphery of tumor metastases.

Next, we quantitatively analyzed three parameters to describe iNKT cell motility properties on day 5: Mean speed, which represents the migratory speed; Confinement ratio, which indicates the ratio of the maximum displacement of each cell from its path length within a given time; Arrest coefficient, which denotes the percentage of the time each cell remained arrested [35, 36]. The data showed that iNKT cells accumulated at the periphery of the tumor with high motility (mean speed: 2.81 ± 1.92 μ m/min, confinement ratio: 0.24 ± 0.24 , and arrest coefficient: $45.2 \pm 30.8\%$; Figure 3(D)–(F), Day 5). When iNKT cells infiltrated the tumor parenchyma, their motility decreased significantly, with more confined trajectories and a significantly increased arrest coefficient (mean speed: 0.93 ± 0.81 μ m/min, confinement ratio: 0.19 ± 0.25 , and arrest coefficient: $70.5 \pm 29.8\%$; Figure 3(D)–(F), Day 5). Compared with the decreased movement activities of iNKT cells in the tumor parenchyma, iNKT cell mobility in both out-tumor and control mice was active (Out-tumor: mean speed: 3.83 ± 2.71 μ m/min, confinement ratio: 0.39 ± 0.32 , and arrest coefficient: $35.9 \pm 33.7\%$; Control: mean speed: 3.86 ± 2.20 μ m/min, confinement ratio: 0.40 ± 0.29 , and arrest coefficient: $31.9 \pm 28.0\%$). These data suggest that iNKT cell movement activities in a liver metastasis microenvironment also have spatial specificity. Compared with iNKT cells in the tumor periphery, out-tumor, and control group, the iNKT cells in the tumor parenchyma with the slowest mobility. The slowdown of iNKT cells is a hallmark of their activation, which suggests that the iNKT cells in the tumor parenchyma are activated and have long-term interactions with tumor cells.

We also analyzed the distribution and morphology of KCs in tRFP-B16 tumor metastases on Day 5. As shown in Figure 3(G), the density of KCs in the tumor parenchyma (224.9 cells/mm²) was much less than that in the tumor periphery (478.7 cells/mm²), out-tumor (267.4 cells/mm²), and control mice (296.9 cells/mm²), suggesting that the distribution of KCs in B16 liver metastasis microenvironments also has a spatial characteristic, and most KCs accumulate in the periphery of tumor metastases, rarely infiltrating the tumor parenchyma. We further quantitatively analyzed three parameters to describe the morphologic properties of KCs on Day 5: Area, which represents the areas of KCs; Aspect ratio, which describes the ratio of KC's major axis to the minor axis; Roundness, which indicates the shape of a KC close to a circle. The mean area of KCs in the tumor parenchyma (96.5 μ m²) was much smaller than that in the tumor periphery (144.1 μ m²), out-tumor (184.2 μ m²), and control group (187.8 μ m²). The aspect ratio of KCs in the tumor parenchyma (2.22) was similar to

Figure 3: Long-term intravital fluorescence confocal imaging of liver metastases.

(A) Schematics of the procedures and timeline of tRFP-B16 cell injection, DAW implantation surgery, and long-term fluorescence confocal imaging of liver tRFP-B16 metastases. (B) Intravital fluorescence confocal imaging of liver metastasis microenvironments at different times. Blue: tRFP-B16 tumor metastases; Green: GFP-labeled iNKT cells; Red: nanopomegranate labeled Kupffer cells (KCs). Top row: 2 × 2 large-field images; scale bar: 100 μm. Bottom row: images of the regions of interest from the top row; scale bar: 50 μm. (C) The density of iNKT cells in the tumor parenchyma, periphery, out-tumor regions, and control group on Day 5. The data are presented as the mean ± SEM ($n = 17$ –25 fields). (D–F) Scatter plots of the mean velocity (D), confinement ratio (E), and arrest coefficient (F) of iNKT cells in the tumor parenchyma, periphery, out-tumor regions, and control group. Each data point represents a single cell, and the red bars indicate mean values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant. (G) The density of Kupffer cells (KCs) in the tumor parenchyma, periphery, out-tumor regions, and control group. The data are presented as the mean ± SEM ($n = 17$ –25 fields). (H–J) Scatter plots of the cell area (H), aspect ratio (I), and roundness (J) of KCs in the tumor parenchyma, periphery, out-tumor regions, and control group. Each data point represents a single cell, and the red bars indicate mean values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant.

tumor periphery's (2.43) and smaller than out-tumor's (2.70) and control group's (2.83). Likewise, the roundness of KCs in the tumor parenchyma (0.53) was similar to the tumor periphery's (0.49) but was larger than out-tumor's (0.45) and control group's (0.43). These findings indicate that KCs in the tumor parenchyma have smaller cell areas and the same morphologic properties as KCs at the periphery, which tend to contract and are amoeboid. KCs in the out-tumor region have larger cell areas and similar morphologic properties as KCs in the control group, which tend to stretch and are spindle-shaped with ramifications.

2.4 The acrylic/resin material as a coverslip adapted to fluorescence and photoacoustic imaging

The liver has a rich capillary network, and its systemic structure is suitable for photoacoustic imaging. Compared with fluorescent confocal imaging of the liver (the depth is only 20 μm), the imaging depth of optically resolved photoacoustic imaging is much deeper, potentially up to 300 μm [29]. After fluorescence imaging of tumor metastases in the mouse liver using the DAW, we switched the imaging system to perform intravital dual-wavelength photoacoustic imaging of the same mouse. However, while using the glass as a coverslip is suitable for fluorescence imaging, it is inappropriate for photoacoustic imaging [37]. Therefore, we screened the acrylic/resin transparent material as a coverslip adapted to both fluorescence and photoacoustic imaging.

To detect the effectiveness of the acrylic/resin coverslip in photoacoustic imaging, we imaged a single hair covered by the acrylic/resin and glass coverslips using photoacoustic microscopy. Figure 4(A)–(C) presented the maximum amplitude projection of the hair under two materials and a blank group (without coverslip). The results showed that the image quality of the hair under the acrylic/resin coverslip was closer to that of the control group, which was more distinct than the glass coverslip, owing to the glass material has a stronger ability to attenuate the photoacoustic signal than the acrylic/resin. Then, we quantified the photoacoustic signals in the three groups, presented that acrylic/resin material was more suitable for photoacoustic imaging than glass. The average intensity of signal from acrylic/resin coverslip (Figure 4(A)), glass coverslip (Figure 4(B)) and blank group (Figure 4(C)) were 0.68, 0.28, and 0.96, respectively. Under the same laser intensity condition, the photoacoustic signal intensities across the acrylic/resin and glass materials were

attenuated for 30 and 70%, respectively, compared with the blank group. In order to show the penetration of photoacoustic signal in the acrylic/resin, glass, and blank group in the depth direction (Z-axis) intuitively, we extracted the cross-section images of the XZ plane. Figure 4(D)–(F) presented the cross-sectional images in the XZ plane through the yellow dashed lines in Figure 4(A)–(C). Compared with the cross-section image of acrylic/resin (Figure 4(D)), the cross-section image of glass (Figure 4(E)) presented greater signal attenuation and more artifacts (indicated by arrows) along the Z-axis. Thus, the penetration transmission ability of acrylic/resin for photoacoustic signals is significantly stronger than that of glass.

In order to accurately measure the value of acoustic signal attenuation caused by the reflection of acoustic waves back and forth in the acrylic/resin and glass coverslips, we imaged two coverslips with ultrasonic microscopy. The results showed that the acoustic signal transmission was stronger in the acrylic/resin coverslip than that in the glass coverslip. Ultrasonic waves passing through the coverslip were divided into two parts; the upper surface of the coverslip reflected one part, and the other part transmitted through the coverslip to form the ultrasonic echo of the lower surface received by the detector. In Figure 4(G), the ultrasonic waves reflected in the acrylic/resin coverslip were much lesser than those in the glass coverslip, thus, the detector could detect the ultrasonic echo on the lower surface. The unit pixel of the images is 3 μm and the echo interval between the upper and lower surfaces of the acrylic/resin coverslip is about 70 pixels. The thickness of the acrylic/resin coverslip was obtained (about 0.2 mm), which is consistent with the actual thickness of the acrylic/resin coverslip. In contrast, in Figure 4(H), due to the strong reflection of ultrasonic waves, only a few ultrasonic echoes transmitted the upper surface of the glass coverslip; it was difficult to detect ultrasonic echo on the lower surface of the glass coverslip.

In order to exclude the error of energy loss due to different attenuation when light is transmitted in the two materials, which results in different ultrasound signal intensities. We performed ultrasound microscopic imaging of acrylic/resin coverslips and glass coverslips under equivalent conditions. First, we quantified the ultrasound image intensities for both materials. The average intensity of ultrasound signals in the acrylic/resin coverslip (Figure 4(G)) and glass coverslip (Figure 4(H)) were 0.4 and 1.1, respectively, and the ultrasound signal attenuation in glass was 2.75 times higher than that in acrylic/resin. To further demonstrate the superiority of acrylic/

Figure 4: The photoacoustic imaging and ultrasound imaging of acrylic/resin and glass coverslips.

(A–C) Photoacoustic maximum amplitude projection (MAP) imaging of a hair covered by the acrylic/resin and glass coverslips. NPA represented normalized photoacoustic. Scale bar: 100 μm . (D–F) Cross-sectional images in the XZ plane corresponding to positions indicated by the yellow dashed lines in A–C. Scale bar: 200 μm . (G–H) Ultrasonic cross-sectional images of the acrylic/resin and glass coverslips in the XZ plane. (I) Ultrasonic signal profile through the yellow dashed lines in G–H. Scale bar: 200 μm .

resin coverslips, by extracting the ultrasonic intensity variation curves of both materials in the Z-axis, we can more visually demonstrate the penetration of the ultrasonic signal in the depth direction in acrylic/resin and glass. Figure 4(I) shows the ultrasonic intensity variation curves through the yellow dashed lines in Figure 4(G) and (H). The black and red curves represent the ultrasonic signals in the glass material and the acrylic/resin material, respectively. The ultrasonic echo inside the acrylic/resin

coverslip, indicated by the bright green double arrows, has smaller signal intensity than that inside the glass coverslip. These data presented that the penetration of the ultrasonic signal in the acrylic/resin was better than that in the glass. We also measured the transmission efficiency of light in the acrylic/resin and glass coverslips separately, and the results showed that the transmission of light in the acrylic/resin was about 94% of that in glass (Supplementary Figure 3).

2.5 Long-term intravital photoacoustic imaging of liver melanoma metastases and the SOM's automatic identification of metastases location

After tRFP-B16 tumor cell injection into the hemispleen to establish a liver metastasis model, we used dual-wavelength photoacoustic microscopy to monitor changes in blood vessel structures (by 523 nm pulsed laser) and in the distribution of KCs (by 744 nm pulsed laser) in the regions of liver metastases. The lateral and axial resolution of the photoacoustic imaging system are estimated to be 2.0 and 14.4 μm , and the imaging depth is estimated to be 1 mm,

and more detailed information of the photoacoustic imaging system is provided by Supplementary Materials. In Figure 5(A), large-field photoacoustic imaging ($2 \times 2 \text{ mm}$) shows the distinct structure of the hepatic sinusoid and lobule and growth of tRFP-B16 tumor metastases (shown as a dark region). The growing tumor metastasis first destroyed the morphology of the hepatic sinusoid on days 3 and 5 and then destroyed the structure of the hepatic lobule on days 7 and 9. The green cells in Figure 5(A) are nanopomegranate labeled KCs. The KCs were rarely observed in the tumor parenchyma and disseminated in the periphery and out-tumor regions, consistent with the quantitative analysis of the fluorescence imaging data in Figure 3(F).

Figure 5: Long-term intravital photoacoustic imaging of liver metastases and the SOM's automatic detection of metastases.

(A) Intravital photoacoustic imaging of tRFP-B16 liver metastases at different times. Red: hepatic sinusoid; Green: KCs; scale bar: 200 μm . (B) Schematic diagram of the experimental progress of the SOM's automatic detection of the regions of interest (ROI) of tumor metastases from photoacoustic images on Day 9. (C) The different shapes and sizes of four tumor metastases (labeled as 1, 2, 3, and 4) obtained after the SOM's automatic detection.

A series of image processing methods were applied to automatically detect tumor metastasis regions in the photoacoustic images. Due to the heterogeneity of the melanoma tumor metastases, some metastases expressed melanin and other metastases did not. As shown in Figure 5(B), the melanin expressing metastases have strong photoacoustic signals (No. 3 metastasis) and the nonmelanin expressing metastases have weak photoacoustic signals (No. 1, 2, and 4 metastases), which presented the hollow shapes in the photoacoustic images. For the acquirement of tumor metastases in photoacoustic images, it is necessary to denoise and enhance the contrast of photoacoustic images firstly. For the denoised and enhanced photoacoustic image obtained by the above preprocessing, it was divided into sub-blocks. In the research, the self-organizing map (SOM) neural network [38,39] was applied to acquire morphology information of tumor metastasis accurately and quickly, which is used unsupervised learning algorithm to classify the input vectors. The input vector of SOM is the corresponding feature value of each sub-block. Here, the image of uniformity and grayscale mean value were selected as the feature values of tumor extraction. Finally, SOM was used to classify each block to determine whether each block belongs to the tumor region or the background region, and then the sub-blocks belonging to the region of the tumor were combined by morphology to achieve the whole region of tumor extraction. The result of Figure 5(B) showed that the SOM could successfully identify different morphological tumor metastases with strong or weak photoacoustic signals. Figure 5(C) showed the final recognition result identifying the largest tumor (Figure 5(C), No. 1) with a length of 811 μm and a width of 575 μm and the smallest tumor (Figure 5(C), No. 4) with a length of 177 μm and a width of 172 μm . In Supplementary Figure 4, we also identified and analyzed tRFP-B16 liver metastases in large-field photoacoustic images on Days 5 and 7.

These results indicated that mice with the DAWarc can be evaluated with both long-term intravital fluorescence molecular imaging (Figures 2 and 3) and long-term intravital photoacoustic microscopic imaging (Figure 5). When performed the intravital imaging on a mouse with DAWarc, we need to use two different imaging systems: the spinning-disk confocal microscopy to do the fluorescence imaging and the photoacoustic microscopy to do the photoacoustic imaging. This dual imaging creates a shift in the imaging field of the liver, making it hard to match fluorescence imaging data with photoacoustic imaging data well. Some researchers have constructed multimodal photoacoustic and fluorescence imaging systems and applied them to biological multimodal imaging [40–42].

The acrylic/resin coverslip of DAWarc can be used in cranial windows, abdominal windows, and other imaging windows. Combining a new imaging system with the DAWarc model could achieve bimodal fluorescence/photoacoustic imaging of the same visual field of the liver in the same mouse; it will play an important role to investigate the dynamic changes in the vascular structures and immunocyte activities in internal organs during the disease progression and treatment process. We hope that integrated microscopy that combines both fluorescence and photoacoustic imaging could be built in the near future.

3 Conclusion

We created a drawer-type abdominal window with an acrylic/resin coverslip (arc) named DAWarc and applied it to intravital fluorescence/photoacoustic imaging of liver tumor metastases for over 10 days. The DAW model decreased breathing and heartbeat jitter-caused imaging artifacts. Using an acrylic/resin material as the coverslips efficiently decreased the amplitude of the ultrasonic echo in the glass material, making it suitable for intravital photoacoustic imaging. Long-term intravital fluorescence imaging of liver metastases in mice with the DAWarc provided information on the spatial location and movement activities of iNKT cells and localization and morphology of KCs in liver metastasis microenvironments. Long-term *in vivo* dual-wavelength photoacoustic imaging obtained information on the structure of hepatic lobules and spatial distribution of KCs (nanopomegranate labeled). Long-term intravital fluorescence/photoacoustic imaging data indicated that the structures of liver sinusoids and liver lobules were destroyed during the development of liver tumor metastasis, creating the spatial specificity of immune cell distribution, movement activities, and morphology. Additionally, the SOM neural network automatically detected tumor metastases in the photoacoustic images. In summary, we developed a DAWarc model suitable for long-term, large-field, and high-quality fluorescence/photoacoustic imaging of the liver *in vivo*. The DAWarc model is a powerful tool for understanding structural changes in hepatic lobules, as well as immune cell movement activities and function in liver diseases.

4 Supplementary Materials

The supplementary Figures, movies, and methods and materials will be available online on the journal's website.

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