Original Article

Combined indocyanine green and medical glue enables stable and precise position in animal studies: promising for fluorescence-guided pulmonary ground glass nodule resection

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Background: Accurate preoperative localization of pulmonary nodules is crucial for surgical treatment. The use of indocyanine green (ICG) for localization is prone to thoracic contamination and spread, resulting in the eventual failure of localization. By using medical glue combined with ICG, we can accurately and permanently locate various tissues in animal study, which can provide evidences for clinical translations.

Methods: A series of medical glue and ICG volume ratios of 2:3, 3:3, 4:3, 6:3, and 9:3 were mixed and injected immediately into subcutaneous tissues of BALB/c nude mice; either medical glue or ICG was injected singly in the control group. Fluorescence intensity over time and boundary sharpness were investigated to determine the optimal ratio. Then, fluorescence guided resection of tissue was performed *ex vivo* on the pig intestine utilizing optimal ratio. Further, localization agents with the optimal ratio were injected into the organs of living mice, and fluorescence imaging for accurate positioning was performed 24 hours later.

Results: The localization agents with a volume ratio of 4:3 showed the best boundary sharpness and the strongest photostability. With the guidance of fluorescence navigation, the marked tissues were accurately separated and removed from the surrounding tissue both on mice and on pig intestines. In the organs of living mice, the localization agents (ratio 4:3) realized accurate positioning of marked tissues. Additionally, the medical glue limited the diffusion of ICG, promising to enable more stable and precise positioning of the nodules during surgery.

Conclusions: The combination of ICG and medical glue presents a superior approach when compared to the individual use of either ICG or medical glue. This technique offers enhanced precision and durability and sealed the wound, thereby mitigating the risk of pneumothorax following puncture procedures. This innovative technique optimizes the properties of medical adhesive to augment tissue density while harnessing the real-time fluorescent endoscopic marking capabilities of ICG during surgical interventions. By employing this innovative technique, it holds significant promise for augmenting the accuracy of pulmonary nodule localization in thoracoscopic surgery within future clinical applications.

Keywords: Pulmonary nodule localization; indocyanine green (ICG); medical glue; mix and inject immediately; fluorescence-guided resection

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Introduction

With the increasing use of computed tomography (CT) imaging technology and the development of lung cancer screening, the detection rate of pulmonary ground glass nodules (GGNs) has been on the rise (1,2). Surgical treatment of pulmonary GGNs has become a crucial part of thoracic surgery (3). Among surgical procedures, video-assisted thoracoscopic surgery (VATS) is the primary approach for pulmonary nodule resection (4,5). However, the small size and lack of solid components in pulmonary GGNs makes them challenging to identify and position during thoracoscopic surgery (6,7).

To overcome the challenges associated with identifying and positioning pulmonary GGNs in thoracoscopic surgery, several auxiliary positioning methods have been developed. These include the widely used hook wire and microcoil, computer virtual navigation-guided, and printed navigational template-guided positioning methods (8-11). However, these methods have disadvantages such as pain and the risk of bleeding due to displacement of the hook wire and microcoil (12). Thus, the choice of the best localization method depends on the individual hospital and patient's condition, leading to a lack of uniform standards for the positioning of pulmonary GGNs.

Medical glue has been used preoperatively to increase the density of lung tissue containing GGNs and assist in nodule localization (13). However, CT-guided injection of medical glue has limitations such as the positioning glue deviating from the predetermined position or remaining unidentified during the operation (14). However, pure indocyanine green (ICG) positioning also has its shortcomings. Because it is in a liquid state, if the positioning is too shallow or the injection volume is too large, it is easy to cause chest contamination and diffusion, leading to positioning failure. A study about combining ICG with other positioning technologies, such as positioning hooks and other dyes (lipiodol, methylene blue), but still cannot completely overcome the shortcomings of ICG positioning (15).

To address these issues, we combined ICG and medical glue for positioning pulmonary GGNs. Medical glue was used to increase lung tissue density during preoperative navigation, whereas ICG was used to mark the target by fluorescence in real-time during surgery. Furthermore, medical glue can limit the diffusion of ICG, avoiding diffusive staining of normal tissue and enabling more stable and precise positioning of pulmonary GGNs during surgery. In this study, a mixture of medical glue and ICG was injected into the hind leg muscles of different animals (n=5), and either medical glue (n=1) or ICG (n=1) was injected singly into the control group (*Figure 1*). The results showed that this method prolonged the position time and reduced the diffusion of ICG in adjacent tissues than that achieved in the ICG group. The entrapment efficiency of medical glue-ICG was 81.65%±5.71% which much higher than the ICG group. This result also confirms that this method can achieve more precise positioning than medical glue. And we had confirmed in animal (n=1) that medical glue combined with ICG was very safe for living tissue localization. We injected a mixture of medical glue and ICG into various organs of mice 24 hours before surgery. We found that the mixture had a hemostatic effect, reduced local bleeding, and sealed the wound. At the same time, ICG and medical glue were mixed for one-time injection before surgery, which not only combined the advantages of both, but also reduced

Highlight box

Key findings

- Preoperative localization of lung nodule is a routine procedure for surgical guidance in ground glass nodule (GGN). Preoperative injection of indocyanine green (ICG) mixed with medical glue is promising to provide stable and precise intraoperative positioning of pulmonary nodule guided by fluorescence imaging.

What is known and what is new?

- Various techniques are employed to locate pulmonary nodules, yet challenges like pain, bleeding, marker displacement, and diffusion of localization agents persist.
- With the volume ratio 4:3, the mixture of ICG and medical glue showed optimal sharpness of fluorescence boundary and photostability, ensuring precise positioning.

What is the implication, and what should change now?

- This new technology allows physicians to perform quick and precise pulmonary nodule resection under the guidance of fluorescence. The mixture of ICG and medical glue can be used as an alternative to preoperative localization of pulmonary ground glass nodule.

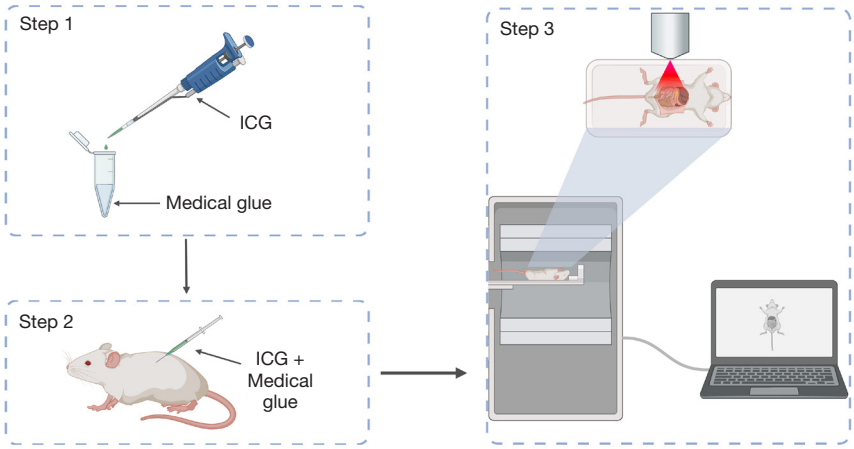

Figure 1 Flowchart of experimental procedures. Step 1, ICG and medical glue are premixed for injection. Step 2, the mixture of ICG and medical glue is injected into the subcutaneous area or organs of the mouse. Step 3, the injection sites are exposed to the excitation light and the fluorescent images are collected by a specific equipment. ICG, indocyanine green.

the number of punctures and sealed the wound. Overall, the combination of medical glue and ICG is very promising that it can be used clinically to locate pulmonary nodules before surgery in the future. We present this article in accordance with the ARRIVE reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-553/rc).

Methods

Reagents

The ICG used in this study was purchased from Dandong Medical Creation Pharmaceutical Co., Ltd. (Dandong, China), and each vial contained 25 mg of the compound. The medical glue used in this study was purchased from Jiangxi Bonerwell Biotechnology Co., Ltd. (China), and each vial contained 0.7 mL of the glue.

Imaging equipment

Fluorescence imaging was performed using the IVIS Spectrum Imaging System from PerkinElmer (Waltham, MA, USA) or Real-IGS FLI-10B from Nanjing Nuoyuan Medical Co., Ltd. (Nanjing, China).

Animals

A total of 24 (5–6-week-old) healthy BALB/c nude mice (half male/half female) were obtained from the Shanghai Jiesijie Experimental Animal Company [Animal License No. SCXK (HU) 2013-0006]. A protocol was prepared before the study without registration. Animal experiments were performed under a project license (No. IACUC-2105007) granted by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University, in compliance with institutional guidelines for the care and use of animals. Mice were kept in a specific-pathogen-free environment and given free access to sterile water and food.

In vivo fluorescence imaging using localization agents with different ratios

ICG was prepared in advance as aqueous solutions with concentrations of 2×10^{-3} mol/L. In the experimental groups, mixtures of ICG solution and medical glue were prepared with volume ratios of 2:3, 3:3, 4:3, 6:3, and 9:3, each with a total volume of 0.1 mL. The medical glue and ICG solution aforementioned were mix and inject immediately before injection. In the control groups, 0.1 mL of either ICG solution (2×10^{-3} mol/L) or medical glue alone was injected. In all groups, the injections were made in the lower right dorsal region of the mice (n=1 per group, total of 7 mice), and the fluorescence imaging was performed using the IVIS Spectrum Imaging System 1, 2, 4, 8, 12, and 24 hours after injection, and then with Real-IGS FLI-10B 27 hours after injection.

Fluorescence-guided resection of tissue containing localization agents

At 27 hours after injecting the localization agent with a volume ratio of 4:3 (ICG: medical glue), the mice were sacrificed via intravenous injection and the skin above the marked tissue was removed. The exposed labeled tissue was imaged using Real-IGS FLI-10B. Under the guidance of fluorescence imaging, the fluorescent tissue was excised entirely and imaged compared with the surgical field or the major organs using Real-IGS FLI-10B.

Verification of localization on porcine large intestines

Fresh porcine large intestines for *ex vivo* assays were purchased from the farmer's market. We injected an ICG aqueous solution and a localization agent with a volume ratio of 4:3 into fresh porcine colonic submucosa. Immediately after injection and 24 hours post-injection, we performed fluorescence imaging of the porcine colons using Real-IGS FLI-10B. After 24 hours, guided by fluorescence imaging, we removed the colonic tissue injected with the localization agent with a volume ratio of 4:3. We compared the resected fluorescent tissue with the remaining fluorescent tissue in the surgical field.

In vivo fluorescence imaging for identifying the positioning of the agents in the organs

The mice were anesthetized with 1.5% pentobarbital sodium. A mixture of ICG solution and medical glue was injected into the liver, lungs, and intestines of living BALB/c mice (n=3), with a total volume of 0.1 mL for each organ, using a volume ratio of 4:3. Then, 24 hours after agent injection, the mice were anesthetized and dissected to expose various organs. The marked positions in organs were observed, and the imaging was performed using the IVIS Spectrum Imaging System. After imaging, the organs in the positioning area were excised and compared to organs that were not marked. The mice were sacrificed by intravenous injection at the end of the experiment.

Statistical analysis

Statistical analysis was conducted by OriginPro 2016 (OriginLab Corp., Northampton, MA, USA, Version 9.3.266), ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA, Version 1.51j8, based on Java 1.8.0_112) and Living Image Software (IVIS Spectrum Imaging Systems, Living Imaging 4.5.5). Entrapment efficiency = $\text{Fluorescence}_{24h}/\text{Fluorescence}_{0h}$.

Results

In vivo fluorescence imaging using localization agents with different ratios

When combining medical glue and ICG for localization, it is necessary to mix the 2 components before use. However, ICG is a water-soluble anionic compound, whereas medical glue (α-cyanoacrylate butyl ester) is lipophilic, and the 2 are not compatible for direct injection. To address this issue, this study proposed to first prepare an aqueous solution of ICG at a concentration of 2×10^{-3} mol/L, and then to mix it with the medical glue before injection.

In vivo imaging was performed by injecting a mixture of ICG aqueous solution and medical glue at different ratios, and a control group was set up to inject either ICG aqueous solution or medical glue alone. As shown in *Figure 2*, 0.1 mL of the above samples were injected into the backs of the experimental mice and white light and fluorescence images were acquired after 24 hours of gelation of the medical glue.

In all experimental groups, the best imaging and localization effect was achieved at the volume ratio of 4:3 or 3:3, with almost no diffusion observed to the surrounding tissue. In the groups with the volume ratio of 6:3 and 9:3, the amount of ICG aqueous solution was significantly higher than that of medical glue, resulting in ineffective constraint of ICG by medical glue. As can be observed, in the ICG control group, ICG solution rapidly diffused and caused extensive boundary blurring. In the medical glue control group, using only medical glue increased the density of lung tissue near the nodule, which was beneficial for CT-assisted localization, but lacked the assistance of near-infrared fluorescence, resulting in difficulty in identifying the position of medical glue intraoperatively.

When magnified and translated into 3-dimensional (3D) mapping of fluorescence intensity 24 hours after, the marked tissue showed different sharpness of fluorescent boundary with different volume ratio of localization agents. The 3:3 and 4:3 group showed similar sharpness of fluorescent boundary and also best localization effect, as shown in *Figure 3*.

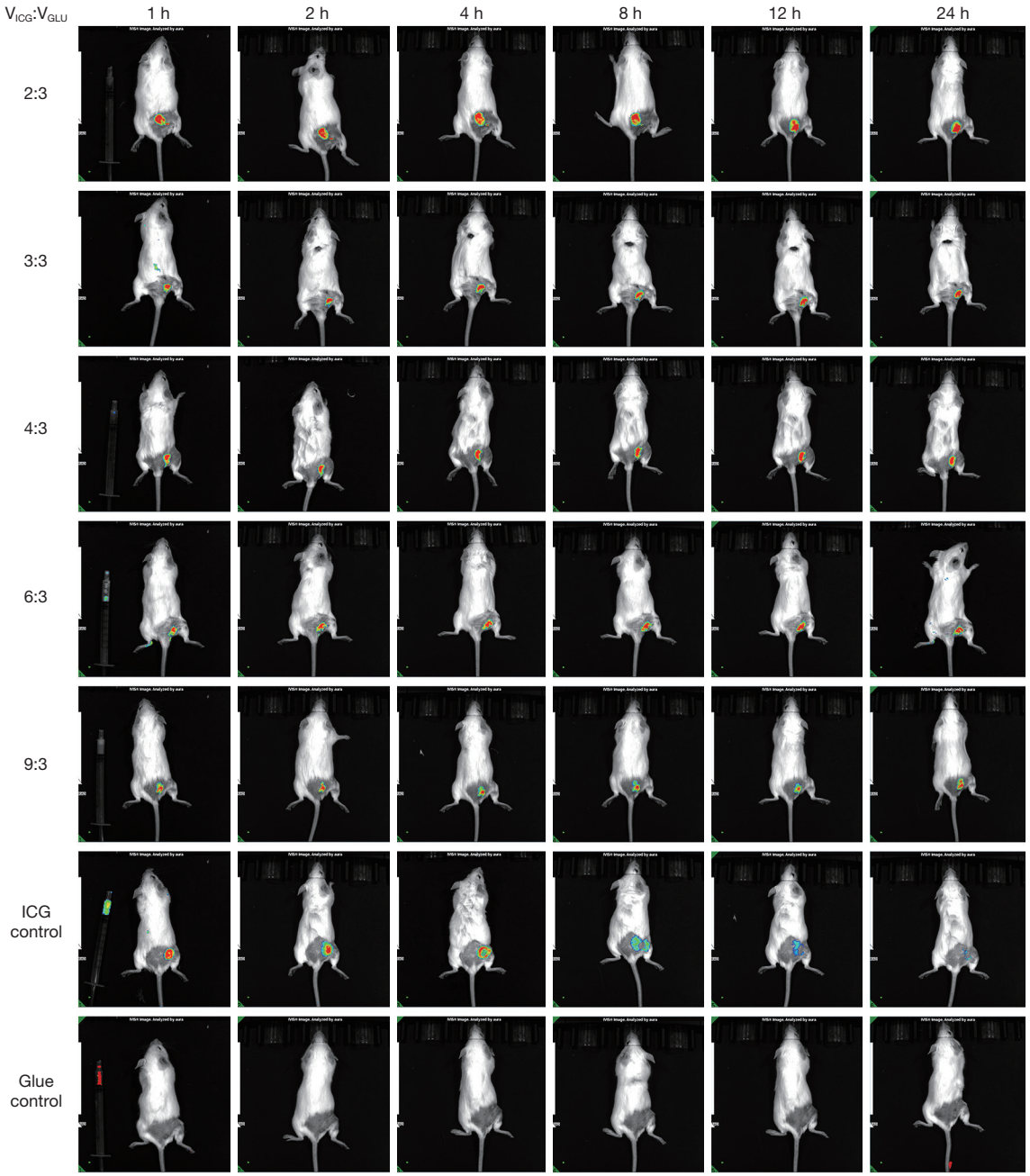

Figure 2 *In vivo* fluorescence imaging using localization agents with different ratios. The imaging results of the combined localization agents using ICG solution (2×10^{-3} mol/L) and GLU in different volume ratios were obtained. The different volume ratios of indocyanine green combining medical glue were better than that of the ICG group. In the ICG control group, it diffused too quickly, resulting in dispersed fluorescence and a rapid decrease in intensity. In the GLU control group, it was difficult to identify the location during surgery, and there was no fluorescent imaging. V, volume; ICG, indocyanine green; GLU, medical glue.

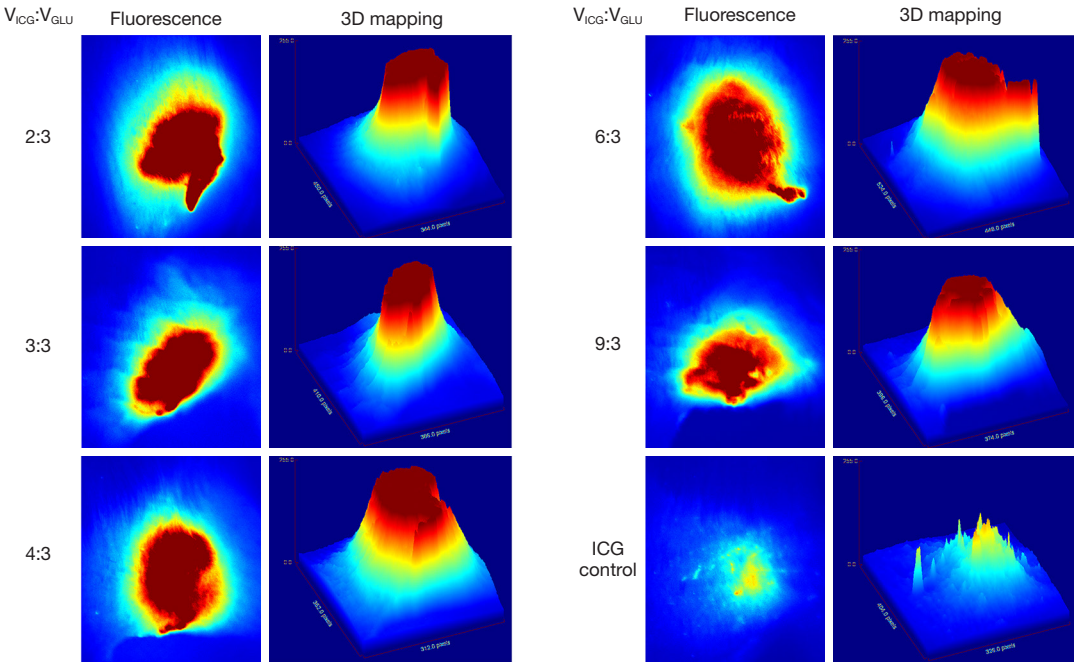

Figure 3 Local magnified color fluorescence and 3D display images of the localization agent-labeled positions. After 24 hours of injection of the localization agent with different volume ratios, the color fluorescence images of the labeled positions in the localized region were magnified and the region was displayed in 3D. The horizontal coordinates in 2 directions represent the position coordinates, and the vertical coordinate represents the fluorescence intensity. Overall, the fluorescence homogeneity and boundary sharpness of the 3:3 and 4:3 groups are similar. V, volume; ICG, indocyanine green; GLU, medical glue; 3D, 3-dimensional.

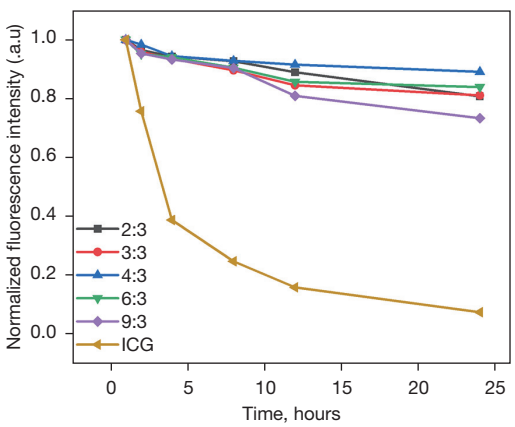

Figure 4 *In vivo* photostability of localization agents with different ratios. There was no significant difference in fluorescence decay over time between the 2:3, 3:3, and 6:3 groups. The 4:3 group had a slightly slower fluorescence decay rate, whereas the 9:3 group had a slightly faster fluorescence decay rate compared to the aforementioned groups. The ICG control group exhibited a significantly faster fluorescence decay. a.u., arbitrary unit; ICG, indocyanine green.

In vivo photostability of localization agents with different volume ratios

The fluorescence intensity of the marked tissue in *Figure 2* at different time points was further investigated, which could represent the *in vivo* photostability of localization agents. As can be seen in *Figure 4*, there were no significant differences in fluorescence decay over time among the 2:3, 3:3, and 6:3 groups. The 4:3 group exhibited a slightly slower fluorescence decay rate, whereas the 9:3 group showed a slightly faster fluorescence decay rate compared to the aforementioned groups. The ICG control group demonstrated a significantly faster fluorescence decay. The results indicated that the 4:3 group had the best photostability.

Fluorescence-guided resection of tissue marked with localization agents

At 27 hours after injecting the localization agent with a volume ratio of 4:3 (ICG: medical glue), the marked tissue

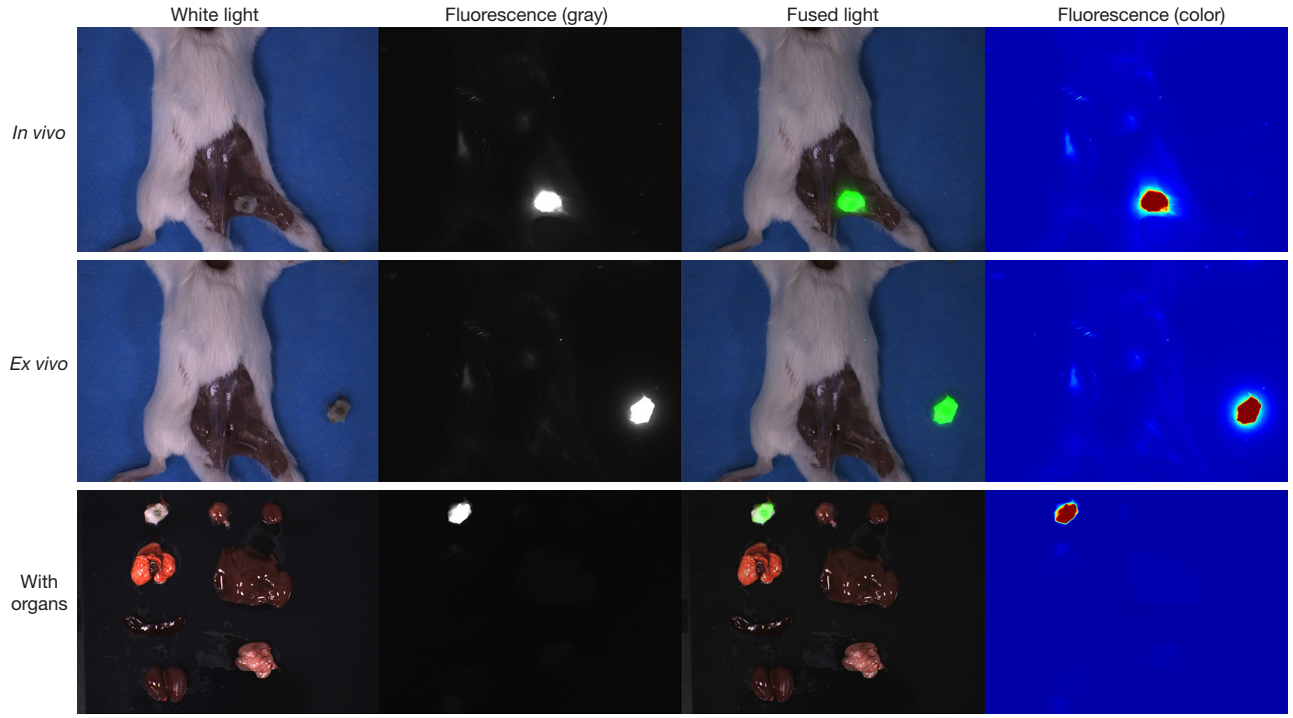

Figure 5 Fluorescence-guided resection of tissue marked with localization agents. The boundary of the marked tissue *in vivo* was clear, and the fluorescence intensity was uniform. With the guidance of fluorescence navigation, the marked tissue was completely removed, and there was no residual fluorescence after the removal. When the removed fluorescent tissue was imaged together with the major organs (heart, liver, spleen, lungs, kidney, and intestines), it was found that there was no obvious fluorescence in the major organs.

was imaged using *in vivo* fluorescence imaging after the skin was removed. As shown in *Figure 5*, the boundary of the marked tissue was clear, and the fluorescence intensity was uniform. With the guidance of fluorescence navigation, the marked tissue was completely removed, and there was no residual fluorescence after the removal. It is indicated that the contrast agent did not diffuse into the surrounding area. After excision, the fluorescent tissues exhibited a strong signal contrast compared to the major organs (heart, liver, spleen, lungs, kidney, and intestines), whereas the major organs showed minimal fluorescence.

Verification of the localization agent in the porcine colons

To compare the localization effect of ICG aqueous solution and the localization agent with volume ratio of 4:3, we injected them into fresh porcine colonic submucosa separately and investigated the fluorescent area after 24 hours. The fluorescent area of ICG group at 0 and 24 hours are shown in *Figure 6A*; apparently the ICG solution underwent extensive diffusion during the 24 hours, resulting in widespread fluorescence staining and imprecise localization within the surgical field. The fluorescent area of the localization agent group (volume ratio 4:3) at 0 and 24 hours are shown in *Figure 6B*, the fluorescent area at 24 hours appeared nearly identical to the initial injection site, indicating minimal diffusion. We further excised a partial sample of fluorescent tissue of the localization agent group; there was no fluorescence under the fluorescent tissue, which also indicated minimal diffusion in the vertical direction. Moreover, the boundary of excision was clear, indicating that even in cases where complete resection cannot be achieved in a single procedure, there will be no observed ICG leakage or contamination of the surgical field (*Figure 6C*).

Positioning of the agents in the organs with the aid of fluorescence imaging in vivo

To explore the position effect of the localization agents *in vivo*, a mixture of ICG solution and medical glue was injected into each organ with a total volume of 0.1 mL, using a volume ratio of 4:3. The mouse was anesthetized

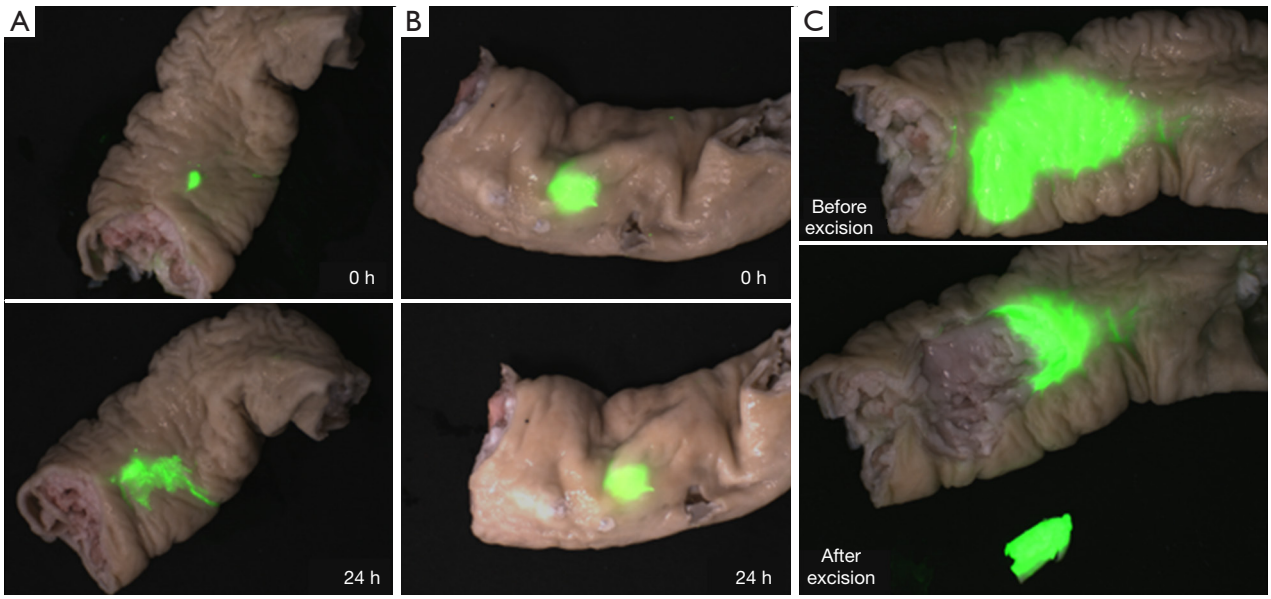

Figure 6 Verification of the localization agent on the porcine colons. (A) An ICG aqueous solution was injected into the submucosa of fresh porcine colon, and after 24 hours, extensive diffusion was observed. (B) In contrast, another fresh porcine colon was injected with a localization agent in a volume ratio of 4:3, and after 24 hours, no noticeable diffusion occurred. (C) Subsequently, a portion of the fluorescent tissue from the localization agent group was excised and compared with the surgical field, revealing no evidence of ICG leakage or vertical diffusion. ICG, indocyanine green.

and lied flat on the surgical cloth. A mixture of medical glue and ICG was injected into the lungs through between the ribs on the right side, into the liver under the xiphoid process, and into the intestines in the abdomen. Subsequently, 24 hours after injection, the marked organs (lungs, liver, and intestines) were exposed and the positioning points were clearly observed using the IVIS Spectrum Imaging System, whereas the unmarked organs showed no fluorescence (*Figure* 7). There were no exclusions in all experiments above.

Discussion

Accurate localization of nodules before thoracoscopic surgery is of great importance. The hook wire technique, which involves using a 20 G puncture needle with a barbed tip to pass through and anchor to a lesion, is now extensively utilized for localizing intrapulmonary nodules. This technique boasts a high success rate in accurately pinpointing the location of the nodules. However, it is worth noting that certain complications can arise during the localization process, including pneumothorax and dislocation and displacement of the hook wire (16). Methylene blue is a commonly used medical dye that can be injected into the lung parenchyma surrounding a lung nodule to stain the pleura. This staining process assists the operator in accurately locating the lesion during VATS. Methylene blue is an affordable option for this purpose; however, it has a rapid diffusion rate and its localization can be disrupted by pigmentation on the surface of the lung tissue. This can increase the challenge of achieving precise intraoperative localization using methylene blue (17). There are targeted fluorescent dyes for the visualization of GGN such as OTL38, which is not always accurate when discriminating tumors from benign lesions, especially between GGN and pneumonia (18). Spring coils are frequently employed for vascular embolization as well as for localizing pulmonary nodules. These coils consist of stainless steel wires that are positioned in the lung, forming a spiral circle that remains relatively stable and can be visualized using fluoroscopy (19). However, it is important to note that this technique necessitates a well-equipped operating room and can be costly. Additionally, there is a slight risk of a few spring coils detaching or falling off (20).

ICG does not interfere with hematoxylin-eosin (HE) staining and does not affect the pathologist's diagnosis (21). Currently, various localization agents containing ICG are utilized in the preoperative localization of pulmonary

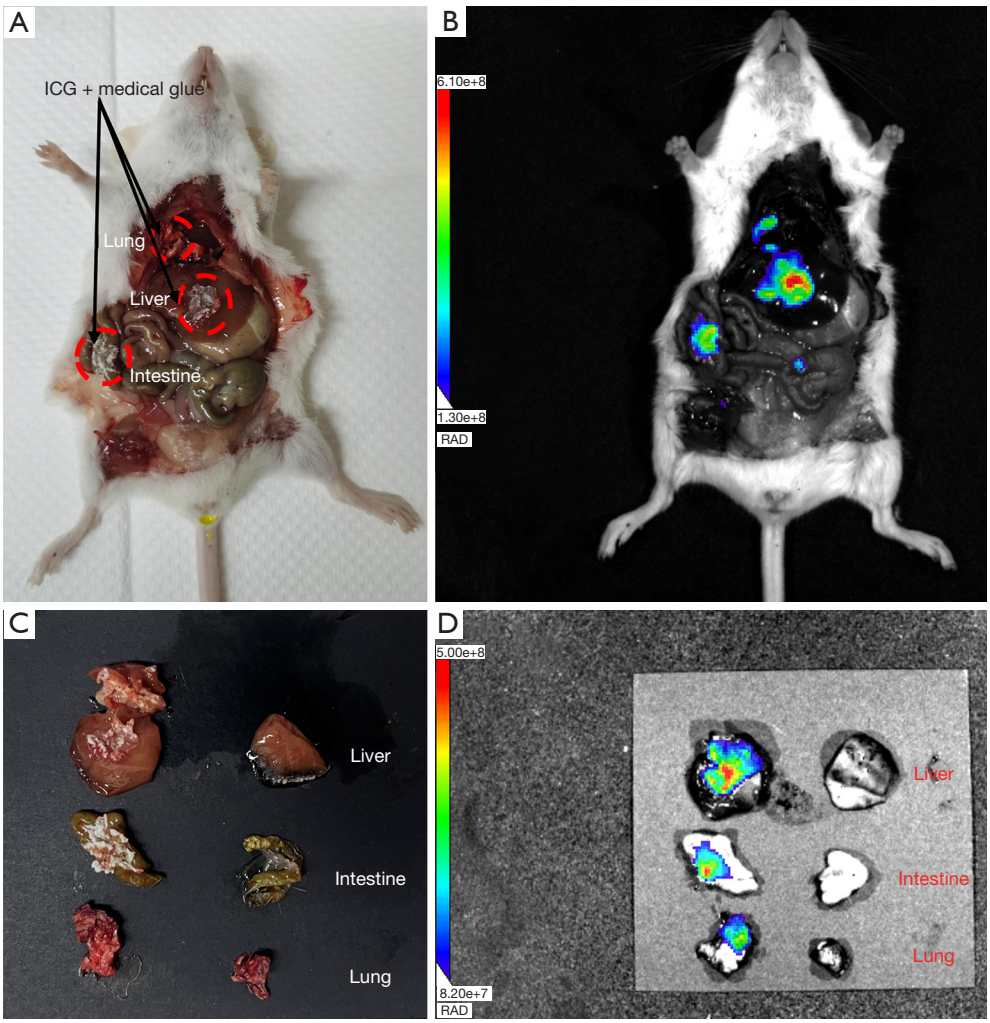

Figure 7 Positioning of the localization agents in the organs *in vivo*. (A) White light *in vivo* image. (B) Fluorescence *in vivo* image. A mixture of ICG solution and medical glue was injected into each organ with a total volume of 0.1 mL, using a volume ratio of 4:3. Twenty-four hours after that, the injection points in the liver [in the first line in (C) and (D)], intestines (in the second line) and lungs (in the third line) were fluorescent while other organs were not fluorescent. ICG, indocyanine green; RAD, radiation.

nodules. These agents include an emulsion composed of an ICG solution and lipiodol (ethiodized oil) (22), an ICG dye-soaked coil (23), or an ICG saline solution (15,24). Although these agents have facilitated precise localization and subsequent successful resection of pulmonary nodules, the unrestricted diffusion of ICG has often resulted in excessive dispersion and an expanded range of localization. This can potentially result in excessive resection of lung parenchyma. To address this limitation, efforts have been made to enhance the constraint of ICG during localization procedures. Several strategies have been explored, including the development of novel carrier materials and modification of the formulation of ICG-containing agents (25,26). These approaches aim to improve the retention and localization efficiency of ICG within the target area, thereby reducing diffusion-related issues. The optimization of ICG localization agents is crucial for achieving accurate and reliable preoperative localization of pulmonary nodules. By enhancing the constraint of ICG and minimizing its diffusion, the localization range can be effectively controlled, leading to improved precision during surgical interventions.

This study presents the application of a medical adhesive combined with ICG in the preparation of preoperative

localization materials for pulmonary nodules. To identify the optimal combination method, this study investigated the impact of different ratios of medical adhesive mixed with ICG injection on tissue localization. By using the medical adhesive and ICG in combination, the advantages of increasing lung tissue density and imaging enhancement are effectively combined, resulting in more accurate preoperative CT-guided localization of pulmonary nodules. In this particular application, the inclusion of medical adhesive serves 2 purposes. Firstly, it helps to limit the diffusion of ICG, ensuring a more concentrated and localized distribution of the dye. This controlled diffusion enhances the accuracy of detecting the adhesive localization nodules using fluorescence endoscopy. Secondly, the medical adhesive contributes to the improved photostability of ICG, preventing degradation or fading of the fluorescence signal. As a result, the combined effect of the medical adhesive and ICG enables more precise and reliable detection of the localized nodules during the fluorescence endoscopic procedure. The mixed medical adhesive and ICG are injected to locate the position of pulmonary nodules under preoperative CT guidance, eliminating the need for repeated percutaneous puncture injections.

During the combined administration of medical glue and ICG, we used only the sterilized water for injection to dilute the ICG to the optimal clinical concentration, maintaining its high stability and fluorescence quantum yield. No surfactants or organic solvents, such as methanol or ethanol, were used, and only clinically approved agents were prepared for immediate use.

Limitations

Further clinical verification needs to be carried out to examine the stability, safety, and positioning accuracy of the localization agent for pulmonary nodules. Future clinical research should also be controlled with traditional localization methods such as hook wire technique or methylene blue. Locating pulmonary nodules requires large animals or used clinically. We are also seeking clinical cooperation to do some research on pulmonary nodule localization, and also compared with other localization methods.

Conclusions

In summary, this positioning method does not involve unapproved drugs or medical devices, and thus should be safe, sterile, and easy to operate. The medical glue can enhance the photostability of ICG and reduce diffusion of ICG solution. In terms of clinical application, this method is promising to improve the accuracy of pulmonary nodule localization during surgery.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-553/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-553/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work

are appropriately investigated and resolved. Animal experiments were performed under a project license (No. IACUC-2105007) granted by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University, in compliance with institutional guidelines for the care and use of animals.

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