

Peer Review File

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Reviewer A

The authors present a foundational study on a new marking method using a material that combines indocyanine green (ICG) and medical glue. Traditional marking with indocyanine green has issues with diffusion and decomposition over time, but the authors demonstrated that mixing it with medical glue enhances its stability.

Comments:

1) The authors state that they achieved the best imaging and localization effect at a volume ratio of 4:3 or 3:3 as illustrated in Fig. 1. However, the images in Fig. 1 are too small, and while the change in fluorescence is noticeable in the ICG control, the differences in the experimental arms are hard to evaluate. Please replace with a clearer figure.

Reply 1: Thank you for the Reviewer's constructive and helpful view on the conciseness of the purpose of the research. We have carefully considered the above issue and briefly illuminated the aim of the study using the following revised paragraph highlighted in our re-submitted manuscript: Indocyanine green (ICG) is used for positioning. The fluorescence of indocyanine green has the advantage of not being affected by the color of the tissue itself. However, if the positioning needle is too shallow or the injection volume is too large, thoracic contamination and spread may easily occur. , the final positioning failed. We use medical glue as the drug-dissolving carrier of ICG, making it a non-flowing gel state, reducing diffusion within the tissue, prolonging the absorption time, making it easy to determine the positioning point, and improving the success rate of positioning. In Figure 1, it is impossible to determine which group has the best positioning effect just by looking at it, but it can be clearly determined that the positioning effect of indocyanine green combined with medical glue is better than that of the ICG group. Therefore, we need to correct our statement as follows: The different volume ratios of indocyanine green combining medical glue were better than that of the ICG group.

Changes in the text: Change "The 3:3 or 4:3 ratio group showed the best localization effect." to "The different volume ratios of indocyanine green combining medical glue were better than that of the ICG group." (Line 475-477)

2) In Fig.6, it's mentioned that marking was performed on the liver, lungs, and intestines. It's unclear how the authors identified and marked each organ in the live mice. Moreover, explanations about

which organ each part represents in Fig 6C and D are insufficient. Please provide a more detailed description.

Reply 2: Thank you for the Reviewer's valuable and kind tips here. We quite agreed with your suggestions and added the "The mouse were anesthetized and lied flat on the surgical cloth. A mixture of medical glue and indocyanine green 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." highlighted in our re-submitted manuscript. We also modified the picture to clearly label each organ. Change in the text: We added the content "The mouse were anesthetized and lied flat on the surgical cloth. A mixture of medical glue and indocyanine green 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." in our re-submitted manuscript. "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" (Line 512-524)

3) The authors suggest that this technique reduces the number of punctures and subsequently reduces the risk of pneumothorax. However, there's no clear evidence presented that supports this reduction in puncture numbers due to this method.

Reply 3: Currently, indocyanine green (ICG) was used for positioning. If the positioning needle was too shallow or the injection volume was too large, thoracic contamination and spread were likely to occur, and ultimately the positioning failed. Medical gel was liquid at room temperature and was fluid, making it easy to fuse with ICG. After being injected into the tissue, it changed to a non-flowing gel state, making it easy to confirm the positioning point and reducing chest cavity contamination caused by chest cavity overflow. 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, indocyanine green and medical glue were mixed for one-time injection before surgery, which not only combined the advantages of both, but also reduced the number of punctures and sealed the wound. Therefore, we believe that this technology can reduce the occurrence of pneumothorax and intrapulmonary hemorrhage after puncture in clinical practice in the future.

Changes in the text: We added the content "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. There are studies on 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." in our re-submitted manuscript. (Line 112-117) "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, indocyanine green and medical glue were mixed for one-time injection before surgery, which not only combined the advantages of both, but also reduced the number of punctures and sealed the wound." (Line 131-136)

4) In the abstract and the Highlight box, the authors conclude that the mixture of ICG and medical glue is effective for identifying lung ground glass nodules. However, this research is foundational, and I believe it's premature to draw such a conclusion. Additionally, in the conclusion of the main text, it's stated that the method is safe, sterile, and easy to handle. These are claims that are difficult to derive from this research. Please modify the wording accordingly.

Reply 4: Thank you so much for your helpful comments and kind reminder about the definition of the picture of our phylogenetic trees. We have carefully modified it and make it more readable.

Changes in the text: The combination of indocyanine green (ICG) and medical adhesive presents a superior approach when compared to the individual use of either ICG or medical adhesive. This combined approach 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 composite mixture as a localizing agent, it holds significant promise for augmenting the accuracy of pulmonary nodule localization in thoracoscopic surgery within future clinical applications. This development is pivotal in achieving precise localization of pulmonary nodules, which remains a critical aspect of thoracic surgery. (Line 62-72)

Preoperative injection of ICG mixed with medical glue is promising to provide stable and precise intraoperative positioning of pulmonary nodule guided by fluorescence imaging. (Line 80-82)

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. (Line 357-361)

Reviewer B

Innovative work with further possibilities. Experimental study with further clinical possibilities

Reviewer C

Lung tissue differs from other structures because it contains air and collapses during surgery. So, This new material should be tested in the lung tissue of large animals.

Reviewer D

First, the title needs to indicate the research design of this study, i.e., findings from an animal study. Otherwise, it is easy to mislead the readers.

Reply 5: Thank you for the Reviewer's valuable and kind tips here. We quite agreed with your suggestions and changed title to "Combined indocyanine green and medical glue enables stable and

precise position in animal studies: promising for fluorescence-guided pulmonary ground glass nodule resection”

Second, the abstract needs some revisions. The background did not explain why animal studies could answer this clinical question or just provide references. The methods need to describe the numbers of rates in the two groups and how the outcomes were measured, as well the statistical methods for comparisons. The results need to quantify the findings by reporting statistics and accurate P values. The conclusion needs comments for the generalizability of the current findings from animals to human patients.

Reply 6: Thank you so much for your helpful comments. We quite agreed with your suggestions and make some changes to the abstract.

Changes in the text: Background: Accurate preoperative localization of small pulmonary nodules is crucial for surgical treatment. The use of indocyanine green (ICG) for localization is prone to thoracic contamination and spread, resulting in eventual failure of localization. By using medical glue combined with indocyanine green, we can accurately and permanently locate various tissues in animal study, which can provide evidences for clinical translations. (Line 38-43)

Conclusions: The combination of indocyanine green (ICG) and medical adhesive presents a superior approach when compared to the individual use of either ICG or medical adhesive. This combined approach 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 composite mixture as a localizing agent, it holds significant promise for augmenting the accuracy of pulmonary nodule localization in thoracoscopic surgery within future clinical applications. This development is pivotal in achieving precise localization of pulmonary nodules, which remains a critical aspect of thoracic surgery. (Line 61-71)

Third, in the introduction, please analyze why animal studies could help answer the clinical question and also analyze what the potential limitations are.

Reply 7: Thank you so much for your helpful comments. There are currently clinical studies using indocyanine green and medical glue to locate pulmonary nodules. The materials we use are clinically approved. And we have confirmed in animals that medical glue combined with indocyanine green is very accurate, durable and safe for positioning tissues. Therefore, we feel that it is very promising to use this technology clinically in the future.

Changes in the text: Change “In this study, a mixture of medical glue and ICG was injected into lung nodules in xenograft tumor models, and either medical glue or ICG was injected singly into the control group. The results of our study showed that this method achieved more accurate positioning of pulmonary nodules during surgery than that achieved in the control groups. Overall, the combination of medical glue and ICG is a safer and more reliable method for positioning pulmonary GGNs. This method could reduce the risk of pneumothorax caused by repeated punctures and improve the accuracy of pulmonary nodule positioning during thoracoscopic surgery.”

to “In this study, a mixture of medical glue and ICG was injected into lung nodules in xenograft tumor models 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 of our study showed that this method prolonged the position time and reduced the diffusion of ICG in adjacent tissues achieved more accurate positioning of pulmonary nodules during surgery than that achieved in the control ICG groups. The entrapment efficiency of medical glue-ICG was (81.646±5.71)%. 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 indocyanine green was very safe for living tissue localization. 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.” (Line 123-138)

Fourth, in the methodology of the main text, please describe the sample size estimation procedures. I suggest the authors to use a flowchart to briefly describe the experimental procedures. The statistical analysis part is not informative. Please describe the outcome variables, test of their normality and how the comparisons were performed, as well as the P value for statistical significance.

Reply 8: We add a flowchart as Figure 1-revised to briefly describe the experimental procedures and renumber all the figures. Since local injection of fluorescent tracer hardly requires consideration of individual differences in animals, only one animal was included in each experimental group to minimize the amount of experimental animals and, consequently, alleviate some statistical considerations such as sample size estimation, test of variable normality and statistical comparisons.

Finally, some related papers need to be cited: 1. Zhang Y, Wu J, Zhang T, Zhang Q, Chen YC. Minute pulmonary meningotheial-like nodules: rare lesions appearing as diffuse ground-glass nodules with cyst-like morphology. *Quant Imaging Med Surg* 2021;11(7):3355-3359. doi: 10.21037/qims-20-676. 2. He J, Liang H, Wang W, Akopov A, Aiolfi A, Ang KL, Bertolaccini L, Cai K, Cao Q, Chen B, Chen C, Chen C, Chen D, Chen F, Chen J, Chen L, Chen M, Chen Y, Chen Z, Cheng C, Cui D, Cui F, Dai T, Dong Q, Ferrari PA, Flores RM, Fu J, Funaki S, Froudarakis ME, Gan X, Geng M, Guo J, Guo Q, Han Y, He J, He K, Hirai K, Hu J, Hu S, Huang J, Huang J, Jiang W, Kim KS, Kiss G, Kong F, Lan L, Leng X, Li B, Li G, Li H, Li H, Li H, Li J, Li X, Li S, Li Y, Li Z, Liang Y, Liang L, Liang W, Liao Y, Lin W, Lin X, Liu H, Liu H, Liu J, Liu J, Liu X, Liu Z, Lu X, Luo Q, Mao N, Pan Q, Pang D, Peng J, Peng J, Pompeo E, Qian R, Qiao K, Redwan B, Sang Z, Shao W, Shen J, Shen W, Sung SW, Tang W, Wang T, Wang G, Wang H, Wang H, Wang J, Wang W, Wang Y, Wang Z, Wei L, Wei W, Wu H, Wu J, Xia Z, Xu C, Xu E, Xu H, Xu N, Xu Q, Xu R, Xu S, Yang C, Yang H, Yang S, Yi J, Zhang G, Zhang H, Zhang J, Zhang M, Zhang X, Zhang Y, Zhang Z, Zhang Z, Zhao H, Zhao J, Zhao X, Zhou J, Zhou Y, Zhu C, Zhu S, Zhu X, Cui J, Yan Y, Chen KN. Tubeless video-assisted thoracic surgery for pulmonary ground-glass nodules: expert consensus and protocol (Guangzhou). *Transl Lung Cancer Res* 2021;10(8):3503-3519. doi: 10.21037/tlcr-21-663. 3. Peng JZ, Bie ZX, Li YM, Li B, Guo RQ, Wang CE, Xu S, Li XG. Diagnostic performance and safety of percutaneous fine-needle aspiration immediately before microwave ablation for pulmonary ground-glass nodules. *Quant Imaging Med Surg*

2023;13(6):3852-3861. doi: 10.21037/qims-22-1001. 4. Choi HS, Lee JH, Kim HK. Targeted intraoperative fluorescence imaging for the visualization of ground-glass nodules in the lung. *Transl Lung Cancer Res* 2022;11(11):2175-2177. doi: 10.21037/tlcr-22-751

Reply 9: Thank you so much for your helpful comments. We have added these related papers.

Changes in the text: Line 300-303 in the discussion, we add some discussion about targeted fluorescent dyes: “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)”.

Reviewer E

The paper titled “Combined indocyanine green and medical glue enables stable and precise positioning of target lesions for fluorescence-guided pulmonary ground glass nodule resection” is interesting. Combined use of ICG and medical glue is safer and more reliable than the use of either ICG or medical glue alone, and reduces the risk of pneumothorax that may be caused by repeated punctures. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) There have been many studies on ICG. What is the difference between this study and previous studies? What is the innovation? These need to be described in the introduction. It is recommended to add relevant content.

Reply 10: Thank you so much for your helpful comments. We have added relevant content in paper.

Changes in the text: Change “Conversely, puncture injection of chromogenic agents such as indocyanine green (ICG) is a simple method, but the easy diffusion of ICG results in high signal background overwhelming the target” to “However, pure 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. There are studies on 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.” (Line 113-119)

2) How effective is ICG in localization of pulmonary nodules? Suggest increasing the comparison of safety and effectiveness with other methods.

Reply 11: 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.

Changes in the text: Line 358-360 in the limitations, we add some content “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.” about future work.

3) Further prospective research with standardized protocols across multiple thoracic units is required in order to accurately validate these findings.

Reply 12: Our results showed that compared with the control ICG group, this method prolonged the localization time and reduced the diffusion of ICG in adjacent tissues, achieving more accurate localization of pulmonary nodules during surgery. The entrapment efficiency of medical glue-ICG was $(81.646 \pm 5.71)\%$, which was much higher than that of the ICG group. This result also confirms that this method can achieve more precise positioning than medical glue. We demonstrated in animals ($n=1$) that medical glue combined with indocyanine green is very safe for localization of living tissue. At the same time, the positioning edge can be accurately determined on pig intestine samples without spreading to the surroundings. Overall, the combination of medical glue and ICG is very promising and can be used for clinical preoperative localization of pulmonary nodules in the future.

Changes in the text: Change “In this study, a mixture of medical glue and ICG was injected into lung nodules in xenograft tumor models, and either medical glue or ICG was injected singly into the control group. The results of our study showed that this method achieved more accurate positioning of pulmonary nodules during surgery than that achieved in the control groups. Overall, the combination of medical glue and ICG is a safer and more reliable method for positioning pulmonary GGNs. This method could reduce the risk of pneumothorax caused by repeated punctures and improve the accuracy of pulmonary nodule positioning during thoroscopic surgery.” to “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 $(81.646 \pm 5.71)\%$ 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 indocyanine green 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, indocyanine green and medical glue were mixed for one-time injection before surgery, which not only combined the advantages of both, but also reduced 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.” (Line 123-138)

4) What is the preparation method of tumor imaging fluorescent probe based on ICG? How to perform function evaluation? It is recommended to add relevant content.

Reply 13: The medical glue and ICG solution were mixed for 5 seconds before injection. We compared the positioning effect by calculating the fluorescence intensity at the positioning position

at different time points, and then comparing the ICG entrapment efficiency after 24 hours. The entrapment efficiency of medical glue-ICG was $(81.646 \pm 5.71)\%$, which was much higher than that of the ICG group.

Changes in the text: We have added a formula to calculate coverage entrapment efficiency to perform function evaluation. (Line 208-209)

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Application of indocyanine green injection guided by electromagnetic navigation bronchoscopy in localization of pulmonary nodules, Transl Lung Cancer Res, PMID: 35070751”. It is recommended to quote the article.

Reply 14: Thank you so much for your helpful comments. We have added relevant content in paper.

6) Are there any other mixing methods besides the mixture method in this study? Suggest adding relevant discussions.

Reply 15: There are studies on mixing ICG with other positioning technologies, such as positioning hooks and other dyes (lipiodol, methylene blue), but they still cannot completely overcome the shortcomings of ICG positioning, according to reference 22 Rho J, Lee JW, Quan YH, et al. Fluorescent and Iodized Emulsion for Preoperative Localization of Pulmonary Nodules. Ann Surg 2021;273:989-96.