Response to Reviewer 2 Comments

1. Summary

Thank you very much for taking the time to review this manuscript. Please find the detailed responses below and the corresponding corrections in track-changes in the re-submitted files.

2. Point-by-point response to Comments and Suggestions for Authors

Comment 1: Dustin Greve et al. present their research entitled "Diagnostic benefit of molecular imaging in patients undergoing heart valve surgery for infective endocarditis." The authors conduct a retrospective study aiming to evaluate the potential of FISHseq as an additional molecular biological technique for diagnosing infective endocarditis, compared to conventional valve and blood cultures for pathogen detection. The results show that FISHseq demonstrates higher specificity and sensitivity for detecting certain pathogens. Below, I have some minor concerns regarding this manuscript.

Response 1: Thank you very much for your efforts in reviewing our manuscript and the positive feedback.

Comment 2: Figure 2: How does this black-and-white image indicate bacterial biofilm? It should be using the fluorescence-labeled specific FISH probe to detect the bacterial biofilm, right? I have a similar question regarding Figure 4.

Response 2: Thank you for this important question. In both Figure 2 and Figure 4, only DAPI stain (blue) but no FISH signal was detectable. We routinely visualize bacteria in a section of the sample simultaneously with the nucleic acid stain DAPI and with fluorescently-labeled specific FISH probes (each in a different fluorescence channel separated by narrow-band filter sets). DAPI stains all nucleic acids, so bacteria as well as host cell nuclei. Since we use a black-and-white camera at our fluorescence microscope for higher sensitivity, we take for each fluorescence channel a black-and-white image. The images are then overlayed or shown separately, shown either in black-and-white or, colored by the computer, in the respective color of the fluorescence dye.

If the bacteria have too few ribosomes to elicit a FISH signal with the FISH probes, they still show a DAPI signal. This happens in particular when the patients have been treated with antibiotics before, leading to inactive or dead bacteria. We can safely say that if we see a FISH signal, this in most cases corresponds to active bacteria with many ribosomes. If we only see the DAPI signal and no FISH signal, we cannot discriminate between inactive or dead bacteria. However, based on their morphology, density, quantity and formation in the host tissue, we can still conclude that bacteria are present, if they form a biofilm or not and interpret this as an old or treated endocarditis. The images of the DAPI signal are shown in black-and-white to have a better contrast for the readers' eyes as compared to a blue signal with dark background.

The image sections in Figure 2 and the figure legend have been adapted to make the constellation of findings easier to understand.

Comment 3: Table 2: Please check the sample size for the "Preoperative antibiotic treatment" group. Could you explain why the total number of patients in this category is less than 207?
Response 3: Infective endocarditis patients are often diagnosed in regional hospitals and then referred to our center. Unfortunately, due to incomplete documentation from referring hospitals, the exact duration of preoperative antibiotic treatment could not be traced in all cases. The analysis by duration of preoperative antibiotic treatment was therefore only carried out for n=201 cases. A footnote has been added to Table 2 to clarify this circumstance.

Comment 4: Figure 5: The pie chart indicates a sample size of n = 208. Please verify this.
Response 4: Thank you for your attention. We have reviewed the data for the pie chart and have noticed that we have made a mistake adding up the cases for 'valve culture negative and FISHseq positive'. The sum has been corrected from 84 to 83 and the total number of n=207 is now correct again.

Comment 5: The limitations section should include two additional points: 1) Surgery must be a prerequisite for the FISHseq assay. 2) FISHseq cannot provide information about antibiotic resistance.
Response 5: We agree that FISHseq is an assay for intraoperatively obtained valve material and therefore only available for IE cases requiring surgery. It is also true that FISHseq does not provide an antibiogram, which is why we see the procedure not as a competitor but as a complement to conventional microbiological culture. Both points have been added to the limitations section.

Comment 6: Please revise the format of the References section to comply with the citation requirements outlined in the "Instructions for Authors."
Response 6: Thank you for your attention, the citation style has been adapted according to the "Instructions for authors".