Response to Reviewers

Responses to Reviewer 1.

1. *Reviewer: P1 l88: give references for tetrazines labelled with 11C, 18F etc.*
   Response: The requested change has been made.

2. *Reviewer: Protocol part 1.2. Why so large amounts (high concentration) of ligands? These ligands are very potent and 100 times less would do it as well. This could save lengthy purification procedures. Comment about this in the discussion section.*
   Response: The high concentration of ligands was needed to maximize the radiochemical yield and reduce reaction times. In the literature, including Ref 21, 10^{-4} M of ligand is typically used for labeling this class of ligands. When the reaction was repeated with the tetrazine ligand at this concentration, radiochemical yields were low and degradation of the tetrazine was observed. As a result, higher concentrations of ligand were used, which significantly improved the radiochemical yield and reduced the synthesis time, and in turn minimized the degradation of the tetrazine. Notwithstanding the presence of additional ligand, the reported purification method is robust and produces the product in high effective specific activity. To ensure clarity for readers, the rationale behind the concentration used and the need for purification were added to the discussion.

3. *Reviewer: What is the chemical reason for steps 1.2.4 to 1.2.6? In step 1.2.3, the compound is formed and then purified from cold material in step 1.2.7. Comment about this.*
   Response*:* The purpose of these chemical reaction steps is to remove the t-butyl protecting groups on the imidazole portion of the ligands. Removing these groups results in carboxylic acid groups, which improves the solubility of the product and enhances the rate of clearance from non-target organs and tissues. This step is performed before the purification of the cold material to avoid the need for two separate purification steps which reduces the overall yield.

4. *Reviewer: Same question for the radiochemical yields after labelling the tetrazines. Why are the yields low? Given the ligand concentration in the labelling solution of about 0.3 mM, quantitative labelling is expected which is the strength of this approach. Side reactions? To underline the strength of this method, the labelling yield should be commented clearly in order to make the approach quantitative.*
   Response: Tetrazines are known to be sensitive to heating and the presence of highly basic groups. Consequently, we suspect that the modest yields are due to decomposition of the tetrazine. In addition, all yields reported are based on the amount of material obtained following HPLC purification where there is inevitably additional loss of material. The radiochemical yield based on HPLC analysis of the reaction mixture is much higher suggesting that the reactions reported are efficient. The yields reported however are isolated yields and are more representative of what one would expect to isolate with the methods reported.

Responses to Reviewer 2.

1. *Reviewer: Since the focus of the journal is on methods rather than on scientific data, it would be more appropriate to change the title by removing the word 'Evaluation' as this would imply some kind of experimental study.*
   Response: The focus of this manuscript is the methodology for both the preparation of the ^{99m}Tc-labeled ligands, as well as the steps taken to evaluate the tetrazines in vivo. We feel that the steps taken to evaluate these ligands are an important part of developing the technology. For this reason, we feel the title accurately represents what is being presented within the manuscript.

2. *Reviewer: For the same reason, it doesn't seem appropriate to include animal studies because these do not contribute to clarify the method. As explained in the Introduction, this paper is aimed at describing a pre-targeting method grounded in the bioothogonal Diels-Alder cycloaddition reaction between tetrazine and trans-cyclooctene (TCO) as a general tool for discovering new target-specific Tc-99m radiopharmaceuticals.*
   Response: We agree with the reviewer that the main focus of this manuscript is the synthesis and labeling of these ligands for a pre-targeting approach. However, we believe that demonstrating successful pre-targeting *in vivo* is a crucial step in validating the utility of any new tetrazine ligand*.*

3. *Reviewer: Thus, to limit its application to targeting bone metabolism falls short in showing its general applicability. In other words, it is supposed that the method must work and there's no need to demonstrate its effectiveness, but simply to precisely describe the full procedure. Actually, using this method to develop another example of bone targeting agent does not seem to stimulate any particular interest, as it would happen with other more attractive biomolecules such as antibodies. Moreover, the whole Tc-99m conjugate, assembled by carrying out the bioortogonal addition in solution before injection, is also expected to show some bone accumulation, thus making the pre-targeting approach unnecessary. To propose this chemistry as a method for developing an entire new class of target-specific Tc-99m radiopharmaceuticals, the whole procedure has to be very precisely outlined and this information should include the most optimal tetrazine derivatives as well as the most effective TCO derivatives, possibly being designed with the most favorable structural characteristics. Notably, this information on the composition of TCO-bisphosphonates was not reported in the paper. Unfortunately, the whole preparation looks very cumbersome, as it requires a remarkable number of steps comprising the use of non-aqueous solvents and evaporation, and a final purification by HPLC. It's hard to see how these complex manipulations could be translated in a clinical setting. The observed radiochemical yields ranged between 31−83%, thus suggesting a high variability of the coordination affinity of the various tetrazine ligands. Highly variable bone accumulation was also found for the different Tc-99m chelates, a fact that seems to indicate that the in vivo bioortogonal reaction does not always occur with the highest efficiency. All these limitations have not been properly addressed into the paper. In summary, to make the outlined procedure more solid and similar to a general-purpose method, some more optimization would be beneficial.*
   Response: The rationale behind using a bisphosphonate as the targeting ligand, as opposed to an antibody, is that it is a more convenient means of evaluating new tetrazines in vivo (lower cost, no need to use expensive tumor models, simpler experimental design, etc.). It was NOT to create a new radiopharmaceutical capable of targeting bone. Once pretargeting using a new labeled tetrazine and the TCO-bisphosphonate has been proven

effective, the new Tc-tetrazine complexes can be evaluated with other TCO-functionalized biomolecules (antibodies, peptides, etc). This approach can be used with any new labeled tetrazine (and to compare existing tetrazines head-to-head) and is therefore generally applicable; much more so than the TCO-derived antibodies used in the literature which are typically not widely available.

The biology clearly shows that each of the labeled tetrazine ligands can react with the TCO-bisphosphonate in vivo. The difference in accumulation of each Tc-ligand at the bone is due to the varying structures and polarities of each tetrazine, which is discussed within the manuscript. As shown between the five ligands, there is an increase in bone uptake from compounds 1-3, followed by a decrease in compounds 3-5 respectively. Therefore, significant effort around optimization of the compounds was conducted and discussed.

One of the key goals of the manuscript was to develop a robust method for producing tetrazines labeled with ^{99m}Tc, so that the utility of pretargeting with the most widely used isotope in diagnostic medicine could be evaluated preclinically. It is premature to develop a single step instant kit production method suitable for clinical use until a lead construct based on a TCO-derived biomolecule is identified. The method used here can be readily used in any radiochemistry laboratory opening the door to using pretargeting (or simply TCO-tetrazine chemistry) to create new technetium radiopharmaceuticals. As for the information on TCO-BP, the synthesis is presented in detail in reference 18. The details for all other procedures from labeling to in vivo testing are provided in the manuscript.

Responses to Reviewer 3.

1. *Reviewer: The appeal of this method is that the animal model is straightforward (normal mice pre-treated with TCO-BP, no need for xenografts) and yet challenging (need for the i.v. injected tetrazines to reach the sites of TCO accumulation, extravasate in high enough concentration, etc.), more than, for instance, the model of "blood pretargeting" used by Devaraj et al. in PNAS (2012). In such model, a side-by-side comparison provides useful information on tetrazine in vivo reactivity towards TCO, pharmacokinetics, and stability combined, in a short time, making a preliminary selection easy. However, a one-time point biodistribution is not enough to characterize the in vivo behavior of a new tetrazine and this preliminary evaluation must be followed by a more thorough in vivo characterization (blood kinetic, clearance from liver and kidney, excretion profile, etc.). For instance, in this study tetrazine 3 appears to be the most promising among the compounds tested. However, at 6 h post-injection compound 3 still has an elevated retention in gallbladder (ca. 90%ID/g) and kidney (> 10%ID/g), significantly higher than other tetrazines in the literature. Most likely such a probe would be not suited for pretargeted radioimmunotherapy, unless it clears effectively from non-target tissues at later time points, which needs to be assessed in a follow-up study. The authors somehow mention the screening nature of their approach in the introduction, but then this concept is lost in the discussion. In my opinion, the preliminary nature of this comparison should be stressed and, possibly, discussed further.*
   Response: The proof of concept nature of the results presented in the manuscript has been highlighted within the discussion section. The intention was to develop a method to create a new class of Tc(I)-tetrazine ligands and to show how a TCO-derived bisphosphonate could allow for simple, cost-effective and rapid screening of these new complexes in vivo. We feel it would be beyond the scope of a JOVE article and of lesser interest to readers to do an

extended time course study on each of the ligands. For clarity, the reasoning as to why the 6 h time point was chosen was added to the discussion section.

2. *Reviewer: One major concern is that in this study the authors show the biodistribution of 5 different tetrazines in mice pre-treated with TCO-BP and they conclude that the activity accumulation in joints proves tetrazine reaction with TCO in vivo. However, without negative controls one can only speculate on the (reasonably) TCO-specific tetrazine accumulation in joints. Especially in view of the scope of JoVE (the dissemination of scientific methods), the authors should present also tetrazine biodistribution in mice that were not pre-treated with TCO-BP or that were pre-treated only with bisphosphonates. Apparently biodistribution studies with tetrazines in the absence of TCO-BP were carried out by the authors but the data are not shown in this manuscript. In my opinion, this is key information to support any conclusion. In case the complete dataset is presented in the manuscript of Ref. 13 (submitted but not yet accepted), the authors should still give enough information here (or they should wait for acceptance of Ref. 13).*
   Response: A figure containing control biodistribution data of the labelled tetrazine compound alone (no TCO-BP administered to the animal) was added to the manuscript. The data showed no uptake in bone.

3. *Reviewer: The authors should report the specific activity of the 99mTc-tetrazines they injected in mice (or the moles they have injected). They report the amount of precursor used for radiolabeling (2 mg for all tetrazines, although their MWs are quite different) but then they do not disclose whether the excess (unlabeled) tetrazine was separated from the labeled ones during post-labeling HPLC purification. The administration of different amounts of tetrazines, if this is the case here, may be the reason for different uptake in joints, especially in the presence of a limited amount of TCO.*
   Response: The specific activity for the Tc-labeled Tz constructs has been added as requested. In addition, a comment regarding the ability of the reported purification method to remove unlabeled ligand was added to the manuscript.

4. *Reviewer: In the long abstract the author state that the 99mTc-tetrazines are stable in saline and PBS but then do not report stability studies. These experiments should be shown or a suitable reference should be given.*
   Response: A protocol for assessing stability, and a figure showing stability results has been added to the manuscript.

5. *Reviewer: In the introduction the authors should state the solvent that was used to determine the k2 value they report as K2 changes enormously in, for instance, methanol vs water.*
   Response: The solvent that was used has been added to manuscript.

6. *Reviewer: In the protocol section (1.2.8) after HPLC purification the authors evaporate the solvent with a rotavapor. To my knowledge the gold standard in radiotracer synthesis is solid phase extraction to eliminate all traces of MeCN and TFA before formulation. Is there a reason why this method was not applied to the tetrazines in this study?*
   Response: A V10 evaporator was used to evaporate HPLC solvent to save time during the labeling procedure rather than conducting solid phase extraction to remove MeCN and TFA. The latter can be done in the absence of a V10 evaporator but requires additional time.

7. *Reviewer: In the protocol section (2.2.9) the method that was used to calculate the %ID is not clear. To my knowledge, the gold standard is to pre/post-weigh syringes then weigh and count aliquots of the injected dose along with the tissue samples (method that does not require further decay correction when using modern gamma counters). Here I don't understand if all organs and tissues (including carcasses), and possibly the cage bedding, etc., were counted in order to estimate the injected dose. The authors should better explain what they did.*
   Response: The method the reviewer has suggested was similar to the one used to generate the data in the manuscript. Additional detail on the biodistribution protocols including the method by which %ID/g was calculated, and the use of standards for the gamma counter, have been added to the protocol section.

8. *Reviewer: In Fig 3 the authors don't show the tetrazine biodistribution in important non-target tissues (e.g. muscle and spleen). If available, these organs should be added to the figure for completeness of information.*
   Response: In a separate biodistribution study where a full organ list was used, there was no significant uptake in the muscle or spleen, along with other non-target organs. Therefore these organs were not taken during the reported biodistribution experiment. This explanation was added to the discussion section, as well as a figure of a healthy mouse biodistribution study to justify the decision. The key was to compare the extent of uptake in the shoulder and knee joints between the pre-targeted and control experiment, which is an indicator of whether or not the labeled tetrazine is able to combine with the TCO-bisphosphonate in vivo.

9. *Reviewer: Also in Fig. 3 the authors should replace the "*" used to indicate the bones with a different symbol as it is confusing (usually this symbol denotes statistical differences).*
   Response: The "*" symbol has been changed to the symbol "^" in the figure (now Fig. 4) and in the figure legend.

Response to Reviewer 4.

1. *Reviewer: This manuscript describes the methodology very well and is very helpful for the scientists who are using this method. No serious problem was found in this manuscript.*
   Response: We thank the reviewer for the positive comments.

2. *Reviewer: The authors indicated the place where the Figure 1 should be located, which is good. However, they didn't indicate the places for other Figures, which should be indicated.*
   Response: Approximate placing for figures 2-5 were added.

3. *Reviewer: Figure 2 and 3 are not seen, which looks like due to a technical problem of the on-line submission software.*
   Response: This issue seems to be specific to this reviewer as the other reviewers did not have this problem. All figure files that were part of the resubmission have been submitted as .tif files and their quality verified.