

Journal of Visualized Experiments

Harnessing the Bioorthogonal Inverse Electron Demand Diels-Alder Cycloaddition for Pretargeted PET Imaging --Manuscript Draft--

Manuscript Number:	JoVE52335R3
Full Title:	Harnessing the Bioorthogonal Inverse Electron Demand Diels-Alder Cycloaddition for Pretargeted PET Imaging
Article Type:	Methods Article - JoVE Produced Video
Keywords:	Positron Emission Tomography, Click Chemistry, Pretargeting, Tetrazine, Trans-cyclooctene, Inverse Electron Demand Diels-Alder Cycloaddition
Manuscript Classifications:	5.1.370.350: Diagnostic Imaging; 5.5.916.79: Click Chemistry; 8.1.181.529.776: Radiochemistry
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Abstract:	<p>Due to their exquisite affinity and specificity, antibodies have become extremely promising vectors for the delivery of radioisotopes to cancer cells for PET imaging. However, the necessity of labeling antibodies with radionuclides with long physical half-lives often results in high background radiation dose rates to non-target tissues. In order to circumvent this issue, we have employed a pretargeted PET imaging strategy based on the inverse electron demand Diels-Alder cycloaddition reaction. The methodology decouples the antibody from the radioactivity and thus exploits the positive characteristics of antibodies, while eschewing their pharmacokinetic drawbacks. The system is comprised of four steps: (1) the injection of a mAb-trans-cyclooctene (TCO) conjugate; (2) a localization time period during which the antibody accumulates in the tumor and clears from the blood; (3) the injection of the radiolabeled tetrazine; and (4) the in vivo click ligation of the components followed by the clearance of excess radioligand. In the example presented in the work at hand, a ⁶⁴Cu-NOTA-labeled tetrazine radioligand and a trans-cyclooctene-conjugated humanized antibody (huA33) were successfully used to delineate SW1222 colorectal cancer tumors with high tumor-to-background contrast. Further, the pretargeting methodology produces high quality images at only a fraction of the radiation dose to non-target tissue created by radioimmunoconjugates directly labeled with ⁶⁴Cu or ⁸⁹Zr. Ultimately, the modularity of this protocol is one of its greatest assets, as the trans-cyclooctene moiety can be appended to any non-internalizing antibody, and the tetrazine can be attached to a wide variety of radioisotopes.</p>
Author Comments:	
Additional Information:	
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TITLE:

Harnessing the Bioorthogonal Inverse Electron Demand Diels-Alder Cycloaddition for Pretargeted PET Imaging

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KEYWORDS:

Positron Emission Tomography, Click Chemistry, Pretargeting, Tetrazine, *Trans*-cyclooctene, Inverse Electron Demand Diels-Alder Cycloaddition

SHORT ABSTRACT:

The bioorthogonal inverse electron demand Diels-Alder cycloaddition has been harnessed to create an effective and modular pretargeted PET imaging strategy for cancer. In this protocol, the steps of this methodology are described in the context of a model system employing the colorectal cancer targeted antibody huA33 and a ⁶⁴Cu-labeled radioligand.

LONG ABSTRACT:

Due to their exquisite affinity and specificity, antibodies have become extremely promising vectors for the delivery of radioisotopes to cancer cells for PET imaging. However, the necessity of labeling antibodies with radionuclides with long physical half-lives often results in high background radiation dose rates to non-target tissues. In order to circumvent this issue, we have employed a pretargeted PET imaging strategy based on the inverse electron demand

Diels-Alder cycloaddition reaction. The methodology decouples the antibody from the radioactivity and thus exploits the positive characteristics of antibodies, while eschewing their pharmacokinetic drawbacks. The system is composed of four steps: (1) the injection of a mAb-*trans*-cyclooctene (TCO) conjugate; (2) a localization time period during which the antibody accumulates in the tumor and clears from the blood; (3) the injection of the radiolabeled tetrazine; and (4) the *in vivo* click ligation of the components followed by the clearance of excess radioligand. In the example presented in the work at hand, a ^{64}Cu -NOTA-labeled tetrazine radioligand and a *trans*-cyclooctene-conjugated humanized antibody (huA33) were successfully used to delineate SW1222 colorectal cancer tumors with high tumor-to-background contrast. Further, the pretargeting methodology produces high quality images at only a fraction of the radiation dose to non-target tissue created by radioimmunoconjugates directly labeled with ^{64}Cu or ^{89}Zr . Ultimately, the modularity of this protocol is one of its greatest assets, as the *trans*-cyclooctene moiety can be appended to any non-internalizing antibody, and the tetrazine can be attached to a wide variety of radioisotopes.

INTRODUCTION:

Over the last thirty years, positron emission tomography (PET) has become an indispensable clinical tool in the diagnosis and management of cancer. Antibodies have long been considered promising vectors for the delivery of positron-emitting radioisotopes to tumors due to their exquisite affinity and specificity for cancer biomarkers.^{1,2} However, the relatively slow *in vivo* pharmacokinetics of antibodies mandates the use of radioisotopes with multi-day physical half-lives. This combination can yield high radiation doses to the non-target organs of patients, an important complication that is of particular clinical significance since radioimmunoconjugates are injected intravenously, and therefore — unlike partial body CT scans — result in absorbed doses in every part of the body, irrespective of the interrogated tissues.

In order to bypass this issue, significant effort has been dedicated to the development of PET imaging strategies that decouple the radioisotope and the targeting moiety, thereby leveraging the advantageous properties of antibodies while simultaneously skirting their intrinsic pharmacokinetic limitations. These strategies — most often termed *pretargeting* or *multistep targeting* — typically employ four steps: (1) the administration of an antibody capable of binding both an antigen and a radioligand; (2) the accumulation of the antibody in the target tissue and its clearance from the blood; (3) the administration of a small molecule radioligand; and (4) the *in vivo* ligation of the radioligand to the antibody followed by the rapid clearance of excess radioligand.³⁻⁸ In some cases, an additional clearing agent is injected between steps 2 and 3 in order to accelerate the excretion of any antibody that has yet to bind the tumor and remains in the blood.⁵

Broadly speaking, two types of pretargeting strategies are most prevalent in the literature. While both have proven successful in preclinical models, they also possess key limitations that have impeded their clinical applicability. The first strategy relies on the high affinity between streptavidin-conjugated antibodies and biotin-modified radiolabels; however, the immunogenicity of the streptavidin-modified antibodies has proven to be a worrisome problem with regard to translation.^{5,6,9,10} The second strategy, in contrast, employs bispecific antibodies that have been genetically-engineered to bind both a cancer biomarker antigen *and* a small molecule radiolabeled hapten.^{3,11-14} While this latter route is certainly creative, its broad

applicability is limited by the complexity, expense, and lack of modularity of the system.

Recently, we developed and published a pretargeted PET imaging methodology based on the inverse electron demand Diels-Alder (IEDDA) cycloaddition reaction between *trans*-cyclooctene (TCO) and tetrazine (Tz; **Figure 1**).¹¹ While the reaction itself has been known for decades, IEDDA chemistry has experienced a renaissance in recent years as a click chemistry bioconjugation technique, as illustrated by the fascinating work of the groups of Ralph Weissleder, Joseph Fox, and Peter Conti among others.¹²⁻¹⁵ The IEDDA cycloaddition has been applied in a wide range of settings, including fluorescence imaging with peptides, antibodies, and nanoparticles as well as nuclear imaging with both radiohalogens and radiometals.¹⁶⁻²⁶ The ligation is high yielding, clean, rapid ($k_1 > 30,000 \text{ M}^{-1}\text{s}^{-1}$), selective, and — critically — bioorthogonal.²⁷ And while a number of types of click chemistry — including Cu-catalyzed azide-alkyne cycloadditions, strain-promoted azide-alkyne cycloadditions, and Staudinger ligations — are bioorthogonal as well, it is the unique combination of fast reaction kinetics and bioorthogonality that makes IEDDA chemistry so well suited to pretargeting applications in whole organisms.^{28,29} Along these lines, it is important to note that the recent report from our laboratories was not the first to apply IEDDA chemistry to pretargeting: the first report of pretargeted imaging with IEDDA arose from the work of Rossin, *et al.* and featured a SPECT methodology employing an ¹¹¹In-labeled tetrazine.³⁰

As we discussed above, the pretargeting methodology has four fairly simple steps (**Figure 2**). In the protocol at hand, a pretargeted strategy for the PET imaging of colorectal cancer that employs a ⁶⁴Cu-NOTA-labeled tetrazine radioligand and a TCO-modified conjugate of the huA33 antibody will be described. However, ultimately the modularity of this methodology is one of its greatest assets, as the *trans*-cyclooctene moiety can be appended to any non-internalizing antibody, and the tetrazine can be attached to a wide variety of radioactive reporters.

PROTOCOL:

ETHICS STATEMENT: All of the *in vivo* animal experiments described were performed according to an approved protocol and under the ethical guidelines of the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee (IACUC).

1. Synthesis of Tz-Bn-NOTA

1.1) In a small reaction vessel, dissolve 7 mg NH₂-Bn-NOTA (1.25×10^{-2} mmol) in 600 μL NaHCO₃ buffer (0.1 M, pH 8.1). Check the pH of the solution. If needed, adjust the pH of the solution to 8.1 using small aliquots of 0.1 M Na₂CO₃.

1.2) Add the NH₂-Bn-NOTA solution to 0.5 mg Tz-NHS (1.25×10^{-3} mmol) in a 1.7 mL microcentrifuge tube. **NOTE:** The Tz-NHS can either be weighed out dry or added from a stock solution of dry DMF or DMSO (< 50 μL).

1.3) Allow the resulting reaction solution to react for 30 min at room temperature with mild agitation.

1.4) After 30 minutes, purify the product using reversed-phase C₁₈ HPLC chromatography to remove unreacted NH₂-Bn-NOTA. The NH₂-Bn-NOTA can be monitored at a wavelength of 254 nm, while the Tz-NHS and Tz-Bn-NOTA are best monitored at a wavelength of 525 nm.

NOTE: Retention times are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 0:100 MeCN/H₂O (both with 0.1% TFA) to 100:0 MeCN/H₂O over 25 minutes and an analytical 4.6 x 250 mm C₁₈ column is used, the retention times of Tz-Bn-NOTA, Tz-NHS, and NH₂-Bn-NOTA will be around 15 min, 16.5 min, and 10 min, respectively. The product can be purified from the other reaction components in either a single run or multiple runs using a semi-preparative or preparative C₁₈ HPLC column. ¹H-NMR, analytical HPLC, and ESI-MS are all methods that can be used to verify the purity of the completed Tz-Bn-NOTA precursor.¹¹

1.5) Freeze the collected HPLC eluent using liquid nitrogen.

1.6) Wrap the frozen collection tube in opaque aluminum foil.

1.7) Place the frozen collection tube in a lyophilizing vessel overnight to remove the HPLC mobile phase.

1.8) Store the purified product (a bright pink solid) in the dark at -80 °C.

NOTE: This is an acceptable stopping point in the procedure. The completed Tz-Bn-NOTA precursor is stable for at least 1 year under these conditions.

2. Preparation of huA33-TCO immunoconjugate

2.1) In a 1.7 mL microcentrifuge tube, prepare a 1 mg/mL (2.7 mM) solution of TCO-NHS in dry DMF.

2.2) In a 1.7 mL microcentrifuge tube, prepare a 2 mg/mL (13.3 μM) solution of huA33 in 1 mL of phosphate buffered saline, pH 7.4 (0.01 M PO₄²⁻, 0.154 M NaCl).

2.3) Using small aliquots (< 5 μL) of 0.1 M Na₂CO₃, adjust the pH of the antibody solution to 8.8-9.0. Use either pH paper or a pH meter with a microelectrode to monitor the pH, and be careful not to allow the pH to rise above pH 9.0.

2.4) Once the antibody solution is at the correct pH, add a volume of the TCO-NHS solution corresponding to 8 molar equivalents of the activated ester. For example, add 7.9 μL of the 1 mg/mL TCO-NHS solution (1.07 x 10⁻⁷ moles TCO-NHS) to the 1 mL of 2 mg/mL huA33 antibody solution (1.33 x 10⁻⁸ moles huA33). Do not exceed 5% DMF by volume in the solution.

2.5) Gently mix the solution by inverting the microcentrifuge tube several times.

2.6) Wrap the microcentrifuge tube in opaque aluminum foil.

2.7) Allow the solution to incubate for 1 h at room temperature with mild agitation.

2.8) After 1 h at room temperature, purify the resulting immunoconjugate using a pre-packed disposable size exclusion desalting column. First, rinse the size exclusion column as described by the supplier to remove any preservatives present on the column during storage. Then, add the reaction mixture to the size exclusion column, rinse the column with 1.5 mL 0.9% sterile saline, and subsequently collect the product using 2 mL of 0.9% sterile saline as the eluent.

NOTE: This step will yield the completed huA33-TCO as a 2 mL solution.

2.9) Measure the concentration of the resultant huA33-TCO on a UV-Vis spectrophotometer.

2.10) If a higher concentration is desired, concentrate the huA33-TCO solution using a centrifugal filter unit with a 50,000 molecular weight cut-off.

NOTE: It is important to note that while huA33 and a variety of other well-known antibodies (*e.g.* bevacizumab, trastuzumab, cetuximab, and J591) are very tolerant of being concentrated, aggregation and precipitation can occur upon concentration in other cases. Researchers attempting this procedure with a new antibody should trust the literature or their own knowledge of the antibody in question with regard to whether or not to concentrate the antibody.

2.11) Store the completed huA33-TCO immunoconjugate at 4 °C in the dark.

NOTE: This is an acceptable stopping point in the procedure. The completed mAb-TCO conjugate should be stable for at least 3 months under these storage conditions.

3. ⁶⁴Cu Radiolabeling of Tz-Bn-NOTA

NOTE: This step of the protocol involves the handling and manipulation of radioactivity. Before performing these steps — or performing any other work with radioactivity — researchers should consult with their home institution's Radiation Safety Department. Take all possible steps to minimize exposure to ionizing radiation.

3.1) In a 1.7 mL microcentrifuge tube, prepare a 0.5 mg/mL (723 μM) solution of Tz-Bn-NOTA.

3.2) In a 1.7 mL microcentrifuge tube, add 10 μL of the Tz-Bn-NOTA solution (5 μg) to 400 μL of 0.2 M NH₄OAc pH 5.5 buffer.

3.3) In the interest of proper radiochemical note-keeping, measure and record the amount of radioactivity in the sample using a dose calibrator before and after the ensuing steps in the protocol below (3.4-3.8). This will help with the accurate determination of radiochemical yields.

3.4) Add 2000 μCi (74 MBq) of ^{64}Cu to the Tz-Bn-NOTA solution.

NOTE: Typically, $^{64}\text{Cu}[\text{CuCl}_2]$ is supplied in a small volume ($< 30 \mu\text{L}$) of 0.1 N HCl, and thus only small volumes ($< 10 \mu\text{L}$) of this stock solution are needed for the radiolabeling reaction. If larger volumes of the $^{64}\text{Cu}[\text{CuCl}_2]$ stock are needed, the radiolabeling reaction is tolerant of increasing the overall reaction volume. However, the pH of the radiolabeling reaction solution should be monitored carefully to ensure that it does not fall below pH 4.0.

3.5) Allow the solution to incubate for 10 min at room temperature with mild agitation.

3.6) After 10 minutes of incubation, purify the product using reversed-phase C_{18} HPLC chromatography. Retention times are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 5:95 MeCN/ H_2O (both with 0.1% TFA) to 95:5 MeCN/ H_2O over 15 minutes is used, the retention time of ^{64}Cu -Tz-Bn-NOTA should be around 9.8 min while free, uncomplexed ^{64}Cu will elute with the solvent front at around 2-4 min.

3.7) Using a rotary evaporator, remove the HPLC eluent.

3.8) Redissolve the ^{64}Cu -Tz-Bn-NOTA product in 0.9% sterile saline.

NOTE: Given the 12.7 h physical half-life of ^{64}Cu , this is **not** an acceptable stopping point in the procedure. Perform the synthesis of ^{64}Cu -Tz-Bn-NOTA immediately prior to the injection of the radioligand, and follow **Step 3.7** immediately by **Step 4.5**.

4. *In Vivo* Pretargeted PET Imaging

NOTE: As in *Protocol Section 3*, this step of the protocol involves the handling and manipulation of radioactivity. Before performing these steps researchers should consult with their home institution's Radiation Safety Department. Take all possible steps to minimize exposure to ionizing radiation.

4.1) In a female athymic nude mouse, subcutaneously implant 1×10^6 SW1222 colorectal cancer cells and allow these to grow into a 100-150 mm^3 xenograft (9-12 days after inoculation).¹¹

4.2) Dilute an aliquot of the huA33-TCO solution from *Protocol Section 2* to a concentration of 0.5 mg/mL in 0.9% sterile saline.

4.3) Inject 200 μL of the huA33-TCO solution (100 μg) into the tail vein of the xenograft-bearing mouse.

4.4) Allow 24 hours for the accumulation of the huA33-TCO in the tumor of the mouse.

4.5) Dilute the ^{64}Cu -Tz-Bn-NOTA radioligand to a concentration of 1.5 mCi/mL in 0.9% sterile saline.

4.6) Inject 200 μL of the ^{64}Cu -Tz-Bn-NOTA radioligand solution (300 μCi ; 11.1 MBq; 1.6 nmol of ^{64}Cu -Tz-Bn-NOTA, assuming a specific activity of 6.7 MBq/nmol) into the tail vein of the xenograft-bearing mice.

4.7) At the desired imaging time point (*e.g.* 2, 6, 12, or 24 hours post-injection), anesthetize the mouse with a 2% isoflurane:oxygen gas mixture.

4.8) Place the mouse on the bed of the small animal PET scanner. Maintain anesthesia during the scan using a 1% isoflurane:oxygen gas mixture. Prior to placing the animal on the scanner bed, verify anesthesia using the toe-pinch method and apply veterinary ointment to the eyes of the mouse to prevent drying during anesthesia.

4.9) Acquire the PET data for the mouse via a static scan with a minimum of 20 million coincident events using an energy window of 350-700 keV and a coincidence timing window of 6 nanoseconds. After completing the acquisition of the image, do not leave the mouse unattended and do not place it in a cage with other mice until it has regained consciousness.

REPRESENTATIVE RESULTS:

The initial three steps of the experiment — the synthesis of Tz-Bn-NOTA, the conjugation of TCO to huA33, and the radiolabeling of the Tz-Bn-NOTA construct (**Figures 3** and **4**) — are highly reliable. In the case of the procedure above, the Tz-Bn-NOTA construct was synthesized in high yield and purity. The huA33 antibody was modified with 4.2 ± 0.6 TCO/mAb, and Tz-Bn-NOTA was radiolabeled with ^{64}Cu to yield the purified radioligand in >99% radiochemical purity, >85% decay-corrected yield, and a specific activity of ~ 6.7 MBq/nmol (**Figure 5**). The reactivity of the huA33-TCO conjugate and the tetrazine radioligand can be tested using radioactive instant thin layer chromatography (iTLC). This is done by mixing the radiolabeled tetrazine (100 μCi ; 0.55 nmol, assuming a specific activity of 6.7 MBq/nmol) with a slight excess of huA33-TCO (50 μg ; 0.66 nmol) in phosphate-buffered saline (pH 7.4) at room temperature for 5 minutes. Then, approximately 1 μCi of the solution is spotted onto a reverse-phase C_{18} TLC plate and allowed to dry. The TLC is run in 9:1 MeCN:H₂O, and the plate analyzed using a radioactive TLC plate reader. If the click reaction works as planned, the ligated ^{64}Cu -NOTA-A33 should remain at the baseline; if, on the other hand, the reaction fails, free ^{64}Cu -Tz-Bn-NOTA will appear at or near the solvent front.

Moving on to the *in vivo* imaging experiments, in the protocol described above, athymic nude mice bearing A33 antigen-expressing, SW1222 colorectal cancer xenografts were employed. Both acute biodistribution ($n = 5$ per time point) and PET imaging ($n = 12$) experiments reveal that the pretargeting strategy is capable of delineating the colorectal tumor growth with excellent image contrast and high tumor-to-background activity ratios (**Figure 6**). Uptake of the ^{64}Cu -Tz-Bn-NOTA in the tumor is apparent at early time points: 3.5 ± 0.6 %ID/g

and 4.1 ± 0.6 %ID/g at 1 h and 4 h post injection, respectively. However, at these early points, it is easily obscured by the amount of radioactivity clearing through the intestinal tract of the mouse (11.9 ± 4.4 %ID/g and 8.8 ± 3.4 %ID/g in the feces at 1 h and 4 h p.i., respectively). Over the course of several hours, the excess radioligand clears through the feces (1.4 ± 0.5 %ID/g at 24 h p.i.), and the tumor becomes the most prominent feature in the image (4.0 ± 0.9 %ID/g at 24 h p.i.). At these later time points, the tumor is well delineated in the image, and the tumor-to-background activity ratios are quite high; for example, the strategy yields tumor:muscle ratios of 26.6 ± 6.6 at 12 h p.i. and 27.0 ± 7.4 at 24 h p.i. Not surprisingly, control experiments using only ^{64}Cu -Tz-Bn-NOTA, non-specific antibodies, or huA33 without conjugated TCO moieties all resulted in minimal uptake in the tumor.

As will be discussed further below, this pretargeting strategy — like all pretargeting strategies — has a number of variables that will require optimization when applied to new antibody/antigen systems. Two of the most important are the mass of mAb-TCO construct injected and the length of the interval between the injection of the mAb-TCO construct and the injection of the radioligand. If the amount of mAb-TCO conjugate is too high *or* the interval time between injections is too short, the amount of free mAb-TCO in the blood goes up and the likelihood of click reactions occurring in the blood rather than at the tumor increases. For example, in the ^{64}Cu /huA33 system discussed here, both the administration of 300 μg of huA33 (rather than 100 μg) or the use of a 12 h interval time (rather than 24 h) resulted in noticeable increases in the amount of radioactivity visible in the heart of the mouse (**Figure 7A** and **Figure 7B**, respectively). In both of these cases, the click reaction is still clearly occurring at the tumor, as illustrated by the amount of tumoral uptake at early time points; however, the formation of radiolabeled antibody in the blood is also apparent. While this is tempting to dismiss because the radiolabeled antibody formed in the blood will still eventually find its way to the tumor, this somewhat defeats the purpose of using a pretargeting methodology, as the radiolabeled antibody will circulate slowly before it reaches the tumor and thereby raise dose rates to non-target organs. Conversely, if too little antibody is used, the amount of uptake in the tumor will naturally suffer. Overly long interval times may also reduce levels of tumor uptake as a result of slow antibody internalization, transcyclooctene isomerization, or antibody/antigen shedding. The diagnosis of these problems is more challenging and cannot be accomplished simply through the examination of the PET data. Clearly, a delicate balance must be maintained. Therefore, it is recommended that any investigators attempting to apply this strategy to a new antibody/antigen system use large amounts of mAb-TCO construct (≥ 200 μg) and short interval times (≤ 24 h) as starting points and optimize from there.

Figure Legends:

Figure 1: The inverse electron-demand Diels-Alder [4 + 2] cycloaddition click ligation between tetrazine and transcyclooctene.

Figure 2: An illustration of the four steps of the pretargeting methodology. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013; 54:1389-

Figure 3: A scheme for the modification of huA33 with TCO-NHS.

Figure 4: A scheme for the synthesis and ^{64}Cu radiolabeling of Tz-Bn-NOTA. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013; 54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.

Figure 5: A radio-HPLC trace of purified ^{64}Cu -Tz-Bn-NOTA.

Figure 6: PET images of ^{64}Cu -Tz-Bn-NOTA/A33-TCO pretargeting strategy. Mice bearing subcutaneous SW1222 xenografts ($100\text{--}150\text{ mm}^3$) were administered huA33-TCO ($100\text{ }\mu\text{g}$) via tail vein injection. After 24 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA ($10.2\text{--}12.0\text{ MBq}$ [$275\text{--}325\text{ }\mu\text{Ci}$]) via tail vein injection and subsequently imaged 2, 6, 12, and 18 h after the administration of the radiopharmaceutical. Transverse (top) and coronal (bottom) planar images intersect the center of the tumors. High levels of uptake in the gut at early time points (*i.e.* 2 and 6 h) largely clear by 12 h, leaving the tumor (white arrow) clearly delineated from all non-target tissue by 12 and 18 h post-injection. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013; 54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.

Figure 7: PET images of suboptimal pretargeting experiments. (A) Mice bearing subcutaneous SW1222 xenografts ($100\text{--}150\text{ mm}^3$, arrow) were administered huA33-TCO ($100\text{ }\mu\text{g}$) via tail vein injection. After 12 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA ($10.2\text{--}12.0\text{ MBq}$ [$275\text{--}325\text{ }\mu\text{Ci}$]) tail vein injection. (B) Mice bearing subcutaneous SW1222 xenografts ($100\text{--}150\text{ mm}^3$, arrow) were administered A33-TCO ($300\text{ }\mu\text{g}$) via tail vein injection. After 24 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA ($10.2\text{--}12.0\text{ MBq}$ [$275\text{--}325\text{ mCi}$]) tail vein injection. In both cases, the mice were imaged 12 h after the injection of ^{64}Cu -Tz-Bn-NOTA. In both panels, transverse (top) and coronal (bottom) planar images intersect the center of the tumors. While the pretargeting strategy clearly delineates the tumor in both cases, the results in both of these images are sub-standard compared to those displayed in Figure 6. In both 7A and 7B, there is a significant amount of background activity uptake in the heart. Under the conditions of Figure 7A, this is most likely the result of the huA33-TCO construct not being given enough time to localize in the tumor. Under the conditions of Figure 7B, this is likely a consequence of injecting too much huA33-TCO and having excess immunoconjugate still circulating in the blood even 24 h after injection. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013; 54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.

DISCUSSION:

The principal advantage of this pretargeted PET imaging strategy is that it is capable of

delineating tumors with target-to-background image contrast at only a fraction of the background radiation dose produced by directly labeled antibodies. For example, in the colorectal cancer imaging system described here, data from acute biodistribution experiments were employed to perform dosimetry calculations for the ^{64}Cu -based pretargeting strategy along with directly-labeled ^{64}Cu -NOTA-huA33 and ^{89}Zr -DFO-huA33. These calculations clearly illustrate the dosimetric benefits of the pretargeting system, especially when compared to the more clinically relevant ^{89}Zr -labeled antibody. The effective dose of the pretargeting strategy is 0.0124 mSv/MBq, while that of ^{89}Zr -DFO-huA33 is over 30 times higher: 0.4162 mSv/MBq. The dosimetric benefit of pretargeting is less pronounced when comparing to the ^{64}Cu -labeled antibody (0.0359 mSv/MBq), though the advantageous effect still exists.

One of the most significant strengths of this IEDDA pretargeting methodology is its modularity: the *trans*-cyclooctene can be appended to any antibody that is not internalizing, and a wide variety of cargos can be attached to the tetrazine. Indeed, our principal motivation for writing this protocol is to enable other research groups to employ this method with different antibody/antigen/radioisotope systems. Along those lines, we believe it is critical to address a number of issues that researchers should consider when adapting this methodology for other systems.

First, the selection of the antibody is tremendously important. Simply put, the antibody must be non-internalizing or internalized at a very slow rate. While the ideal kinetic parameters have yet to be determined, the antibody — and the reactive *trans*-cyclooctene it carries — must remain on the outside of the cell, for the internalization and sequestration of the antibody prior to the injection of the radioligand would dramatically decrease the number of *in vivo* click reactions. In the system described here, huA33 antibody targets and binds to the A33 antigen, a transmembrane glycoprotein expressed in >95% of all colorectal cancers. Importantly, it has been shown that even after binding its target, the huA33 antibody/antigen complex remains on the surface of the cell for days.³¹⁻³³ While the necessity of a non-internalizing antibody is admittedly a limitation to the strategy, a wide variety of non-internalizing antibodies are known, perhaps most notably the TAG72-targeting CC49 antibody that Rossin, *et al.* have explored in their excellent pretargeting work.^{30,34,35}

Second, this pretargeting strategy — like any other — requires significant optimization. In addition to the identity of the antibody and the tetrazine radioligand, two critical variables must be considered: the amount of antibody injected and the interval time between the injections of the antibody and the radioligand. We have addressed both these variables in the *Representative Results* section above, but to reiterate briefly, if either too much antibody or too short an interval time is employed, significant amounts of mAb-TCO conjugate will remain in the blood at the time of the injection of the radioligand. This, in turn, will result in the *in vivo* click ligation occurring in the blood rather than at the tumor, forming circulating, radiolabeled antibody that will only slowly accumulate in the tumor over time. Conversely, if either too little antibody or too long an interval time is employed, the final amount of radioactivity in the tumor will be suboptimal. In our opinion, performing rigorous imaging — or, preferentially — acute biodistribution experiments with the directly labeled antibody itself prior to any pretargeting experiments is the most reliable way to learn about the amount of antibody needed and the ideal interval time after the initial injection of antibody construct. For different injected masses of radiolabeled mAb, these experiments will provide concrete data on both the clearance of the

radioimmunoconjugate from the blood and its accumulation in the tumor, allowing for the selection of the most promising conditions for the pretargeting experiments.

Finally, the pharmacokinetics of the tetrazine-based radioligand must be considered when choosing a suitable radioisotope. In the system described here, the radiolabeled Tz-Bn-NOTA moiety is excreted from the body via the gut with a biological half-life of approximately 3-4 hours, making ^{64}Cu the positron-emitting radioisotope with the most complementary physical half-life. Unfortunately, the biological half-life of the tetrazine moiety is too long for it to be compatible with the more rapidly decaying radiometal ^{68}Ga ($t_{1/2} = 68$ min). In this case, any radioactivity in the tumor would decay through several half-lives before the excess radioligand finished clearing from the body. As a result, images would have to be acquired at early time points, when tumor-to-background activity ratios remain low.³⁶ Ideally, future generations of tetrazine radioligands would be engineered — perhaps via PEGylation, glycation, or other means — to excrete from the body more quickly. This would allow for radiolabeling with more rapidly decaying radioisotopes such as ^{68}Ga and ^{18}F which would in turn further enhance the dosimetric benefits of the pretargeted imaging strategy. Ultimately, as researchers adapt this technology for use with other radioisotopes for imaging (e.g. ^{124}I , ^{111}In , ^{18}F , ^{89}Zr , ^{68}Ga , etc.) or therapy (e.g. ^{89}Y , ^{177}Lu , ^{225}Ac , ^{125}I etc.), new tetrazine-based ligands will need to be developed to incorporate different chelators or radiolabeling prosthetic groups. The thorough investigation of the pharmacokinetics of these novel constructs will be essential to ensuring advantageous matches between the clearance properties of the ligands and the physical half-life of the radionuclides.

In the end, we very much hope that other investigators see the promise of this pretargeting technology and employ it with new antibody/antigen systems. While the preceding paragraphs illustrate that this adaptation process may not always be simple, it is our belief that this methodology could have a significant impact on nuclear imaging, targeted radionuclide therapy, and beyond.

ACKNOWLEDGMENTS:

The authors thank Prof. Ralph Weissleder, Dr. Pat Zanzonico, and Dr. NagaVaraKishore Pillarsetty for helpful conversations and the NIH for funding (BMZ: 1K99CA178205-01A1)

DISCLOSURES:

The authors have nothing to disclose.

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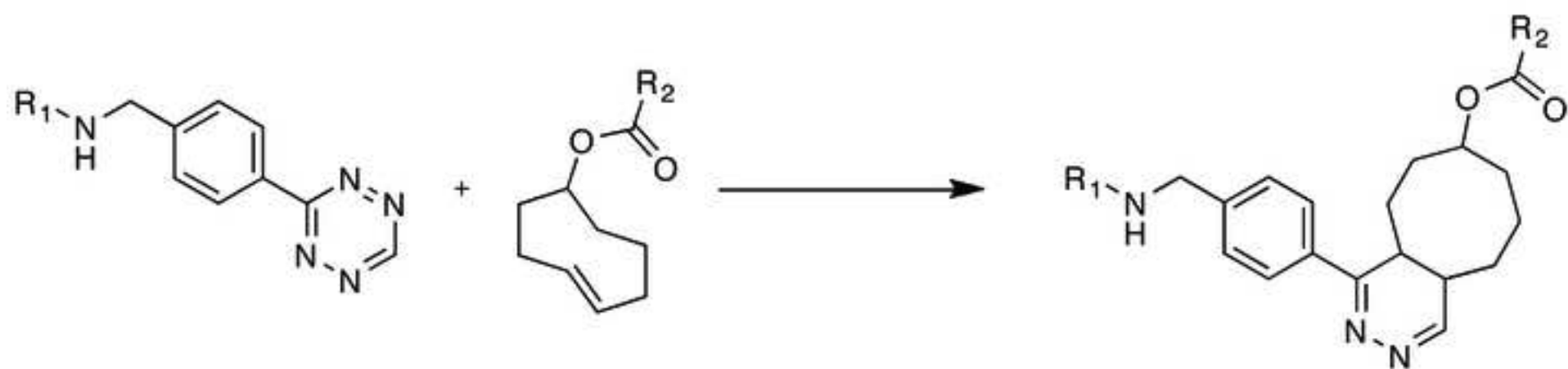


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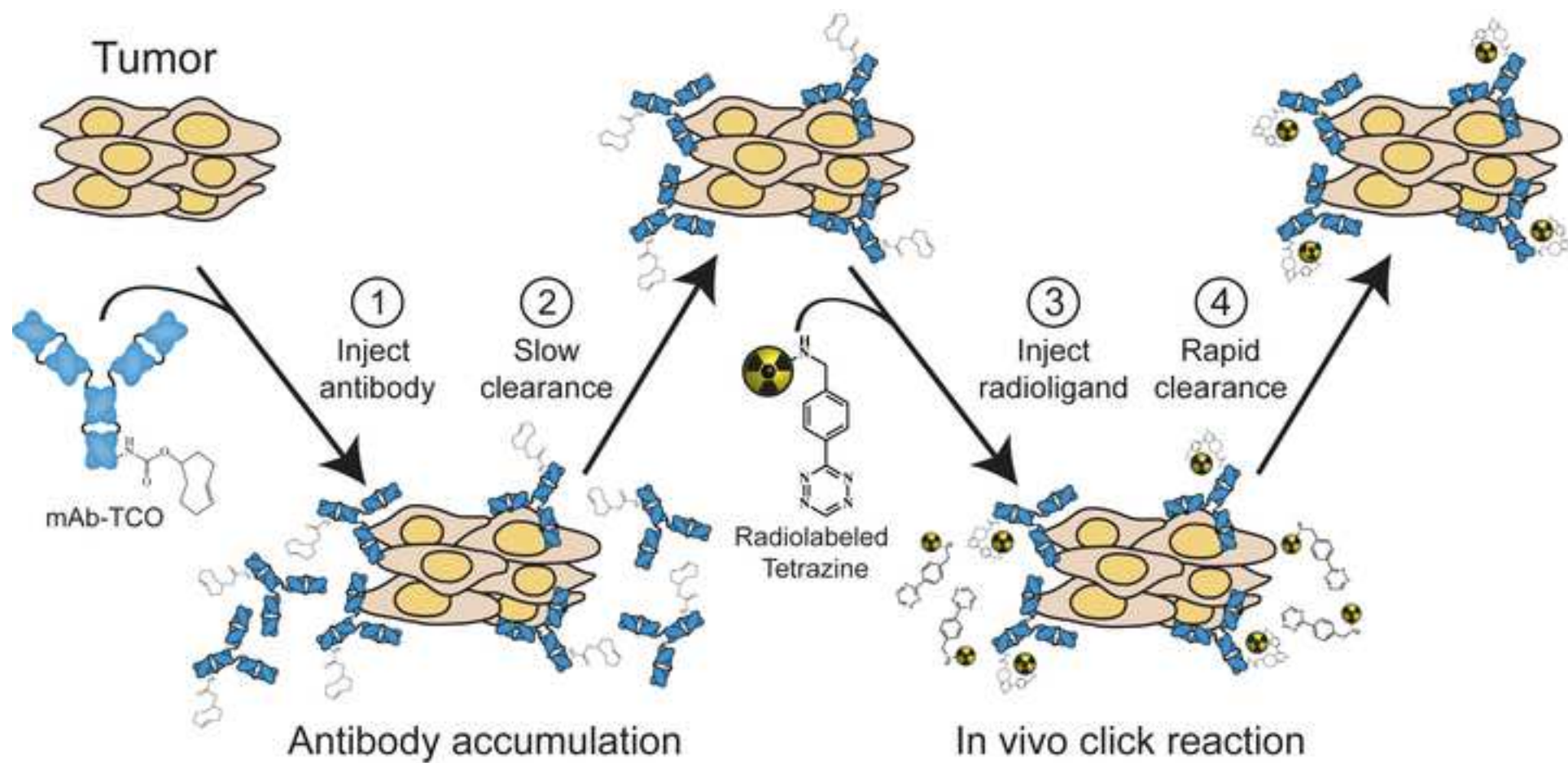


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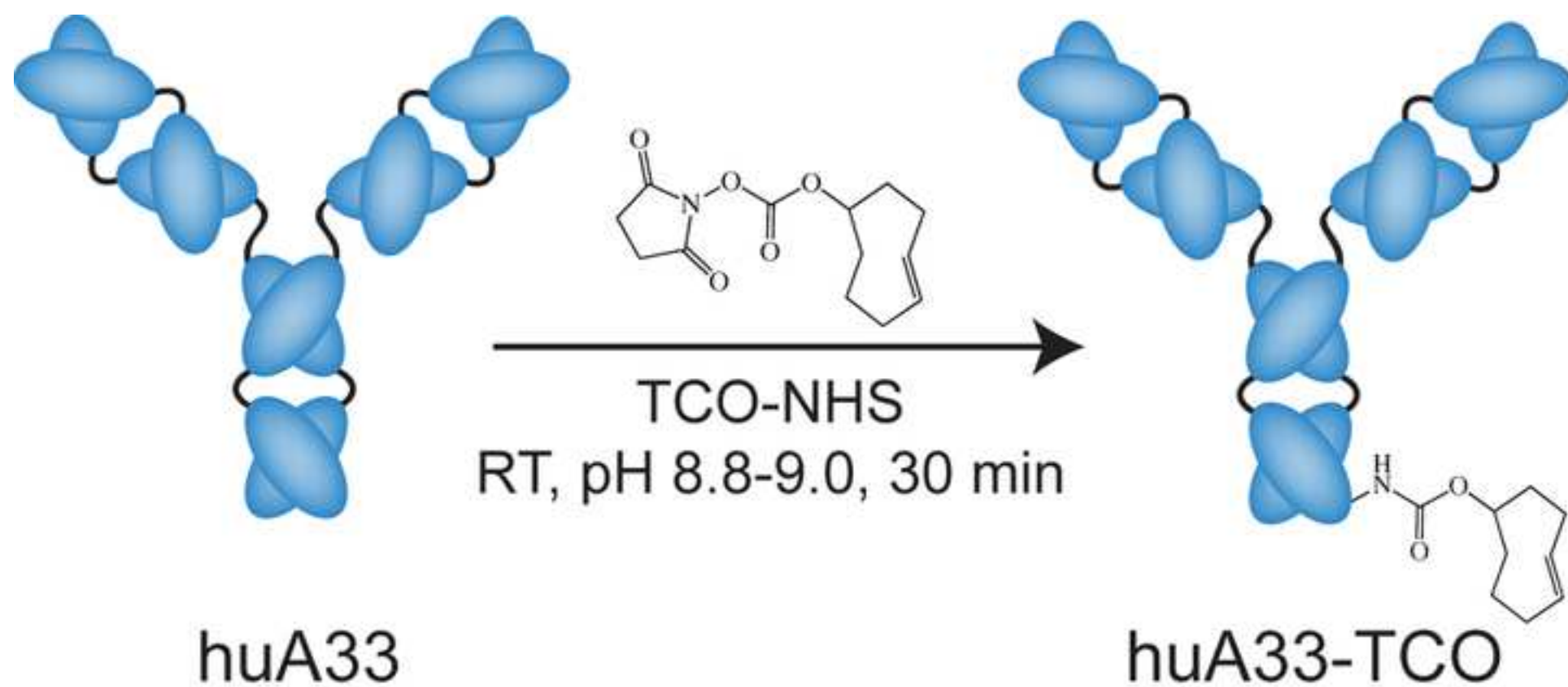


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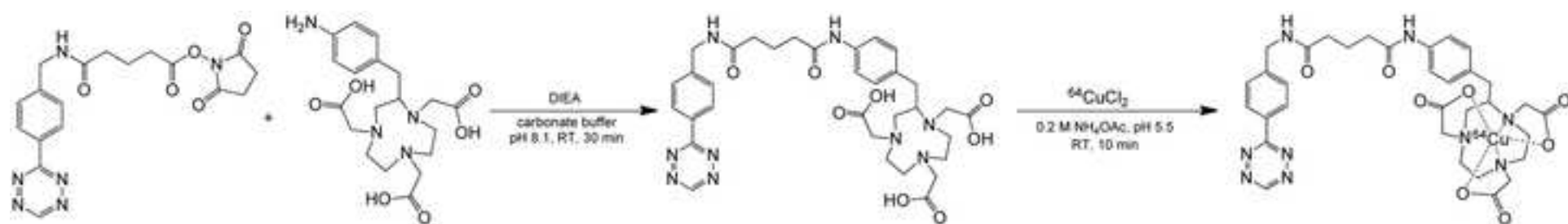


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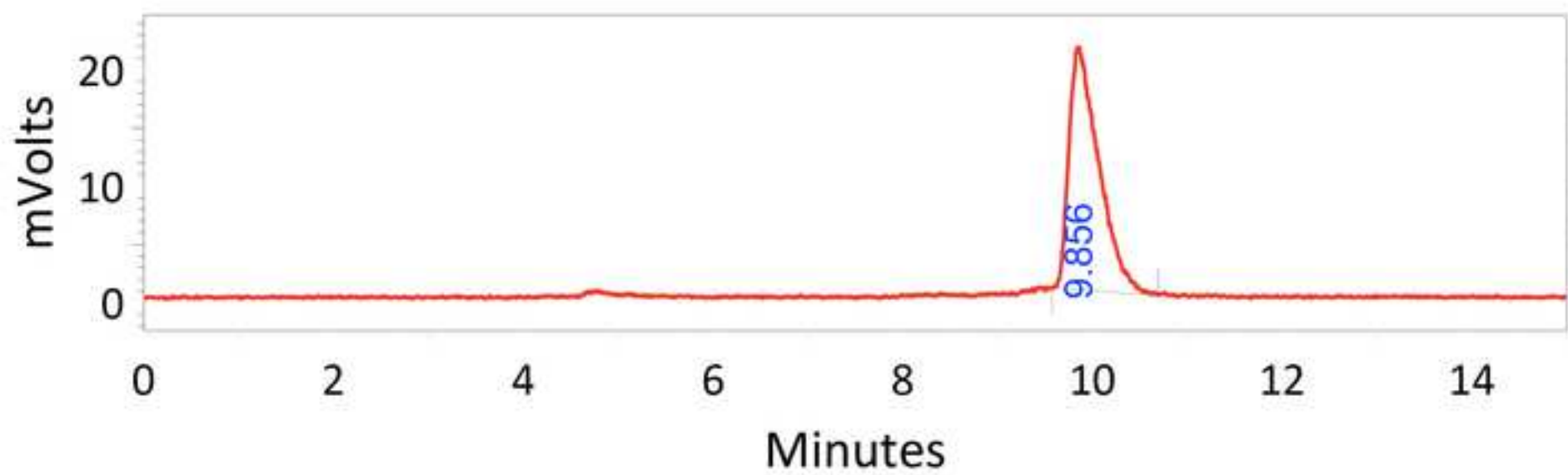


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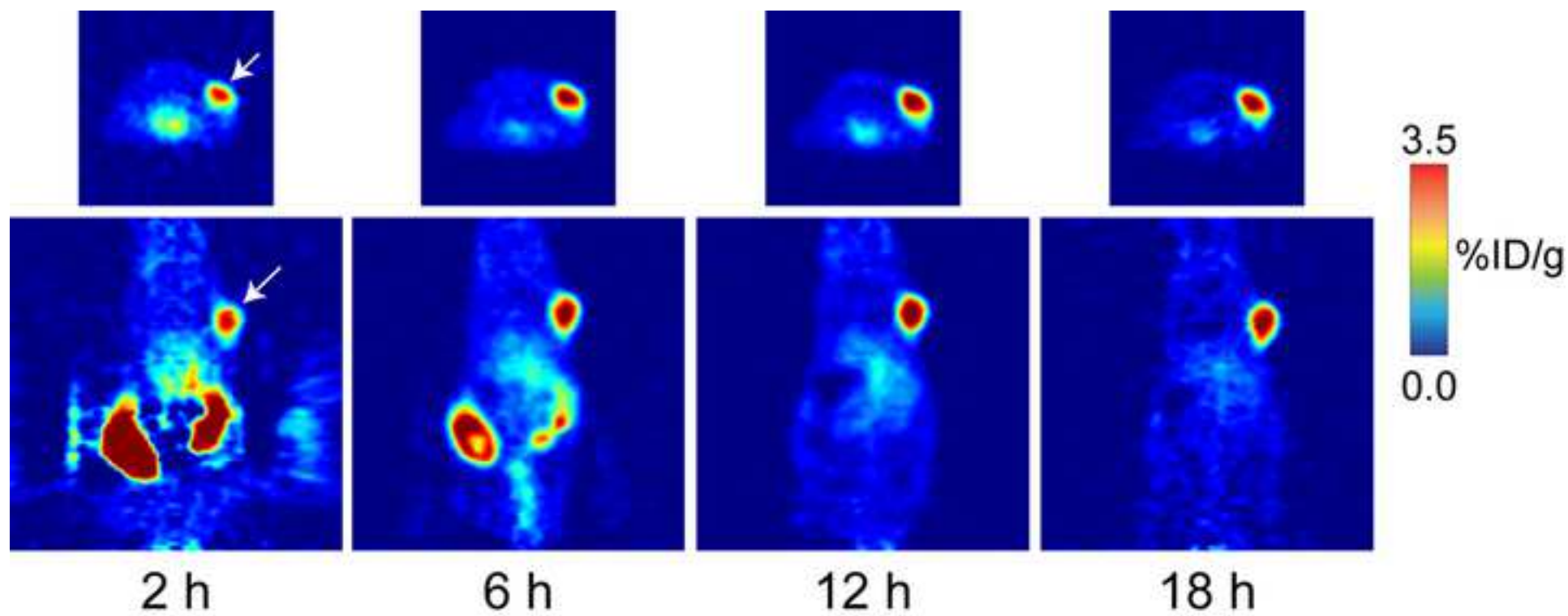
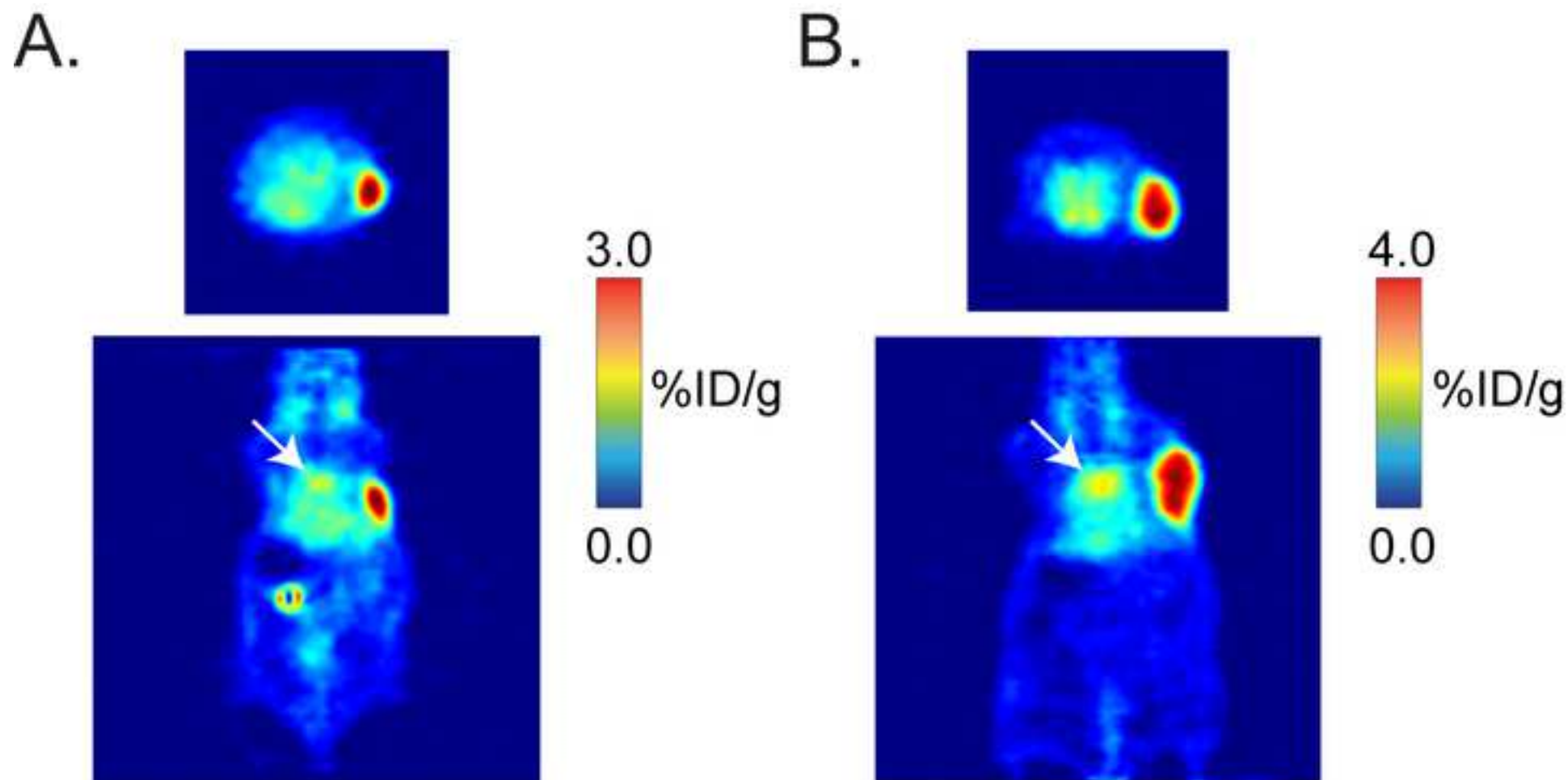


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August 8, 2014

Sephorah Zaman, PhD
Editor, *Journal of Visualized Experiments*

Dear Dr. Zaman,

Attached please find our revisions to the manuscript we have submitted entitled "*Harnessing the Bioorthogonal Inverse Electron Demand Diels-Alder Cycloaddition for Pretargeted PET Imaging*." Thank you very much for your consideration of our work and your careful review of the manuscript. We would also like to thank the three reviewers for their enthusiasm about the work and their very positive and thoughtful commentary. We have made a number of modifications based on the critiques offered by the work's reviewers. Below we have listed the reviewers' comments (in *blue italics*), our responses to said comments, and the changes we have made. In addition, we have listed the changes we have made to the manuscript requested by the editors.

Editorial Changes:

"All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the 'file inventory' to use for any subsequent changes."

Thank you! We have downloaded that version and are using it as the starting point for the revised manuscript.

"Prior to peer review, the length of the Short Abstract is exactly at our 50 word limit. If, in response to peer review comment, changes are made to the Short Abstract, please ensure that the final length does not exceed 50 words."

As none of the reviewers had any problems with the Short Abstract, we have not changed it. It remains exactly at the 50 word limit.

"Editor removed the highlighting from step 4.1, because as agreed, this step lacks sufficient detail for filming and will therefore, not be filmed. Highlighting was also removed from all steps describing anesthesia (step 4.7 and part of step 4.8)."

This is fine with us!

"In the Reference List, some journal names are abbreviated, while others are not. Please be consistent in either abbreviating or not abbreviating journal names."

We have changed the Reference List such that none of the journal names are abbreviated. We apologize for the error.

"Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version."

We have thoroughly proofread the revised manuscript.

"If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation].""

Figures 1, 3, and 5 are original. Figures 2, 4, 6, and 7 are modified from the *Journal of Nuclear Medicine* publication on which this article is based. The *Journal of Nuclear Medicine* allows authors to re-use their own figures in subsequent review publications. On the *JNM* website, one can read that "*JNM* authors retain the following nonexclusive copyrights, to be exercised only after the article has been published in final format in the print version of *JNM* ... (a) Reprint the article in print collections of the author's own writing ... (f) Reuse original figures and tables in future works created by the author." (<http://jnm.snmjournals.org/site/misc/permission.xhtml>)

In accordance with *JNM* recommendations, the figure captions have been altered to read "This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc."

Reviewer #1:

"Manuscript Summary: This manuscript provides a detailed method for using a previously developed "click" reaction for in vivo pretargeting. Overall the authors provide a clear description of the methodology used.."

We thank the reviewer for their careful reading of our manuscript.

"Step 1.8 mentions that this is a stopping point. How long is the compound stable in the given storage conditions (-80, dark)?"

This is a good point. Step 2.11 has been modified to include the line "The completed Tz-Bn-NOTA precursor is stable for at least 1 year under these conditions."

"Step 2.11 same question as above."

While we do not know *exactly* how long the mAb-TCO conjugate is stable in storage, we have stored it for multiple months at -80 in the dark. As a result, Step 1.8 has been modified to include the line "The completed mAb-TCO conjugate should be stable for at least 3 months under these conditions."

"Step 3.4 What is the composition of the ⁶⁴Cu solution (pH, volume) the researchers used? How tolerant is this reaction to increased volume?"

This is a great point. We have added the following to the discussion of Step 3.4: "Typically, [⁶⁴Cu]CuCl₂ is supplied in a small volume (<30 μL) of 0.1 N HCl, and thus only small volumes (< 10 μL) of this stock solution are needed for the radiolabeling reaction. If larger volumes of the [⁶⁴Cu]CuCl₂ stock are needed, the radiolabeling reaction is tolerant of increasing the overall reaction volume. However, the pH of the radiolabeling reaction solution should be monitored carefully to ensure that it does not fall below pH 4.0."

"Step 4.1 How many cells were implanted?"

We have altered Step 4.1 to read, "In a female athymic nude mouse, subcutaneously implant 1 x 10⁶ SW1222 colorectal cancer cells and allow these to grow into a 100-150 mm³ xenograft (9-12 days after inoculation)."

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“Typo: Line 83 “both are have” should be “both have””

This typo has been corrected. Thank you for noticing it!

Reviewer #2:

“*Manuscript Summary:* The manuscript is written clearly and the things you should take into account when you would like to apply the IEDDA to your own system are well explained. In the experimental procedure some details are missing, which I summarize hereafter. these should be addressed because otherwise researchers can not reproduce their procedure.

Thank you very much for your careful reading of our manuscript. We apologize for leaving the relevant details out of the protocol, and we have endeavored to add them where they are needed.

“Line 84: are should be removed”

This typo has been corrected.

“What is the molarity of the NaHCO₃ solution? Is a centrifuge tube necessary? Can this also be done in other types of tubes?”

We accidentally omitted the bicarbonate molarity, and we agree that the reaction can be done in other types of tubes. Step 1.1 has been altered to read “1.1) In a small reaction vessel, dissolve 7 mg NH₂-Bn-NOTA (1.25 x 10⁻² mmol) in 600 µL NaHCO₃ buffer (0.1 M, pH 8.1). Check the pH of the solution. If needed, adjust the pH of the solution to 8.5 using small aliquots of 0.1 M Na₂CO₃.”

“1.2 Is Tz-NHS a solid? Weighing 0.5 mg is pretty challenging”

We have never had trouble weighing out 0.5 mg of Tz-NHS on an analytical balance, but just in case using a stock solution is easier, we have amended Step 1.2 to read “1.2) Add the NH₂-Bn-NOTA solution to 0.5 mg Tz-NHS (1.25 x 10⁻³ mmol) in a 1.7 mL microcentrifuge tube. The Tz-NHS can either be weighed out dry or from a stock solution in dry DMF or DMSO (< 50 µL).”

“1.4 What is the retention time of unreacted NH₂-Bn-NOTA? what is the wavelength at which the compounds should be visible? What is the flow of the HPLC purification? Can the whole product be purified in one run? Or must this be done in multiple runs? Details are missing!”

“1.8 Are there any analytical measures that can be taken to be sure that the product is pure? NMR, HPLC, TLC, MS?”

While we agree that the details are missing, we believe that the HPLC purification of a compound is highly dependent on the equipment set up of the lab. We have given example retention times for the compounds, and have added the wavelength at which the compounds can be monitored, but the flow rate and whether the compound can be purified in a single run or multiple runs is dependent on the column that the end user possesses. Step 1.4 has been revised to read, “1.4) After 30 minutes, purify the product using reversed-phase C₁₈

HPLC chromatography to remove unreacted NH₂-Bn-NOTA. The NH₂-Bn-NOTA can be monitored at a wavelength of 254 nm, while the Tz-NHS and Tz-Bn-NOTA are best monitored at wavelength of 525 nm. The retention times and flow rates are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 5:95 MeCN/H₂O (both with 0.1% TFA) to 95:5 MeCN/H₂O over 30 minutes and an analytical 4.6 x 250 mm C₁₈ column is used, the retention times of Tz-Bn-NOTA, Tz-NHS, and NH₂-Bn-NOTA will be around 10.5 min, 9.5 min, and 6 min, respectively. The product can be purified from the other reaction components in either single run or multiple runs (depending on the loop) using a semi-preparative or preparative C₁₈ HPLC column. ¹H-NMR, analytical HPLC, and ESI-MS are all methods that can be used to verify the purity of the completed Tz-Bn-NOTA precursor.¹¹

“2.4 What is the max. volume of DMF that can be used?”

This is an excellent point. Step 2.4 has been amended to include “Do not exceed 5% DMF by volume in the solution.”

“2.8 Purification of the immunoconjugate is not completely described. Suggestion for addition: rinse the size exclusion column as described by the supplier to remove any preservative present on the column. Apply the reaction mixture to the size exclusion column, rinse with xx mL 0.9% NaCl and collect the product hereafter with xx mL 0.9% NaCl.”

We completely agree with this point by the reviewer. However, as we were not allowed to refer to any specific products (*i.e.* GE PD-10 size exclusion columns), we felt it was not appropriate to list the steps we'd take if we were using such products. However, now that the reviewer has pointed this out, Step 2.8 has been modified to include the following passage: “First, rinse the size exclusion column as described by the supplier to remove any preservatives present on the column during storage. Then, add the reaction mixture to the size exclusion column, rinse the column with 1.5 mL 0.9% NaCl, and subsequently collect the product using 2 mL of 0.9% sterile saline as the eluent. Note: This step will yield the completed huA33-TCO as a 2 mL solution. “

“2.8 How can other researchers check whether there is no unreacted TCO present in the immunoconjugate?”

While there is no reliable way to check if there is any unreacted TCO present in the immunoconjugate mixture, it is extraordinarily unlikely that the TCO-NHS moiety (MW = 267 Da) will elute from the size exclusion column along with the mAb-TCO construct (MW > 150,000 Da). Therefore, no modifications to the manuscript have been made in response to this query.

“2.10 Apparently huA33 can be concentrated. Maybe good to mention that it can be troublesome for other mAbs and aggregation can occur. Other researchers should be aware of this problem. Not every antibody can be concentrated endless.”

This is a good point. We have added the following passage to Step 2.10: “It is important to note that while huA33 and a variety of other well-known antibodies (*e.g.* bevacizumab, trastuzumab, cetuximab, and J591) are very tolerant of being concentrated, in other cases, aggregation and precipitation can occur upon concentration. Researchers attempting this procedure with a new antibody should trust the literature or their own knowledge of the antibody in question with regard to whether or not to concentrate the antibody.”

“3.4 ⁶⁴Cu in ?? Please add details about the acid in which it is supplied. What is the maximum volume of ⁶⁴Cu allowed? The pH is important for the radiolabeling, so there should be limitations on the volume and concentration of acid in which ⁶⁴Cu is supplied.”

This is a great point, and Reviewer #1 made a similar comment. We have added the following to the discussion of Step 3.4: “Typically, [⁶⁴Cu]CuCl₂ is supplied in a small volume (<30 μL) of 0.1 N HCl, and thus only small volumes (< 10 μL) of this stock solution are needed for the radiolabeling reaction. If larger volumes of the [⁶⁴Cu]CuCl₂ stock are needed, the radiolabeling reaction is tolerant of increasing the overall reaction volume.

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However, the pH of the radiolabeling reaction solution should be monitored carefully to ensure that it does not fall below pH 4.0.”

“Line 212: A h is missing after 12.7”

The “h” has been added in the manuscript.

“3.6 see comments to 1.4 regarding details that are missing.”

Again, we believe that the exact details regarding the retention times of the radiolabeled ^{64}Cu -Tz-Bn-NOTA will be highly dependent on the HPLC set-up of each laboratory. We have added additional detail to Step 1.4 in response to the reviewer’s concerns. To Step 3.6, we have added that “... free, uncomplexed ^{64}Cu will elute with the solvent front ($T_R \sim 2\text{-}4$ min)”. We do not believe that the inclusion of additional details here will be particularly helpful to researchers trying to repeat this work. Step 3.6 now reads, “3.6) After 10 minutes of incubation, purify the product using reversed-phase C_{18} HPLC chromatography. The retention times and flow rates are obviously highly dependent on the HPLC equipment setup of each laboratory (pumps, columns, tubing, *etc.*). However, to present an example, if a gradient of 5:95 MeCN/ H_2O (both with 0.1% TFA) to 95:5 MeCN/ H_2O over 15 minutes is used, the retention time of ^{64}Cu -Tz-Bn-NOTA should be around 9.7 min while free, uncomplexed ^{64}Cu will elute with the solvent front at around 2-4 min.”

“4.6 maybe good to add the amount of ^{64}Cu -Tz-Bn-NOTA in nmol.”

This is a great point. It is dependent on the specific activity of the ^{64}Cu -Tz-Bn-NOTA, but we have adjusted Step 4.6 to read “4.6) Inject 200 μL of the ^{64}Cu -Tz-Bn-NOTA radioligand solution (300 μCi ; 11.1 MBq; 1.6 nmol of ^{64}Cu -Tz-Bn-NOTA with specific activity of 6.7 MBq/nmol) into the tail vein of the xenograft-bearing mice.”

“Line 261: the IEDDA can be tested using equimolar amounts of mAb and TCO. Can is the ideal concentration for this? This information is missing.”

There is no “ideal” concentration for testing this reaction, as it works over a wide range of concentrations. However, we did omit example concentrations in this discussion. Therefore, we have added the following passage, “This is done by mixing the radiolabeled tetrazine (100 μCi ; 0.55 nmol, assuming a specific activity of 6.7 MBq/nmol) with a slight excess of huA33-TCO (50 μg ; 0.66 nmol).”

“line 273: how many mice are used?”

We have now included this information in the following sentence: “Both acute biodistribution ($n = 5$ per time point) and PET imaging ($n = 12$) experiments reveal that the pretargeting strategy is capable of delineating the colorectal tumor growth with excellent image contrast and high tumor-to-background activity ratios (**Figure 6**).”

“Additional Comments to Authors: It would be interesting to get some information on how to determine whether a mAb can be used for IEDDA. Clearly it should be non-internalizing. But how should this be determined and what is an acceptable internalization rate?”

This is an excellent point. Unfortunately, however, because the technology is so new, neither us nor anyone else knows what the exact parameters for the antibody should be. As the technology develops, we are confident that we will determine these parameters, but currently, they do not exist, and therefore we cannot discuss them in the manuscript. We have, however, modified a sentence to confer this, “While the ideal kinetic parameters have yet to be determined, the antibody — and the reactive *trans*-cyclooctene it carries — must remain on the outside of the cell, for the internalization and sequestration of the antibody prior to the injection of the radioligand would dramatically decrease the number of *in vivo* click reactions.”

Reviewer #3:

“Page 9, 388 ff Tz-Bn-NOTA is not a radioligand, it is a precursor, please reword. Explain what biological half-life 6-8 hrs) means in this context; is it blood clearance (sic)? If yes, please discuss why the clearance is so slow, protein binding? Also discuss how to improve this; PEG spacer? glycation? This tetrazine-based radioligand does not appear to be suitable for clinical translation. Is the slow clearance (via feces) also due to circulating TCO-Mab?? Would a clearing agent be necessary?”

These are excellent points. The “biological half-life” to which we refer is not blood clearance but rather clearance from the body as a whole. We agree that this tetrazine ligand is not ideally suited for clinical translation, and we are indeed working on developing novel tetrazines with faster clearance properties as the reviewer suggests. To answer the final question, the slow clearance (via the feces) is *independent* of the mAb-TCO conjugate and seems to simply be a property of the tetrazine radioligand. To address these concerns, this paragraph of the Discussion has been amended to read as follows:

“Finally, the pharmacokinetics of the tetrazine-based radioligand must be considered when choosing a suitable radioisotope. In the system described here, the radiolabeled Tz-Bn-NOTA moiety is excreted from the body via the gut with a biological half-life of approximately 3-4 hours, making ^{64}Cu the positron-emitting radioisotope with the most complementary physical half-life. Unfortunately, the biological half-life of the tetrazine moiety is too long for it to be compatible with either the more rapidly decaying radiometal ^{68}Ga ($t_{1/2} = 68$ min). In this case, any radioactivity in the tumor would decay through several half-lives before the excess radioligand finished clearing from the body. As a result, images would have to be acquired at early time points, when tumor-to-background activity ratios remain low.³⁶ Ideally, future generations of tetrazine radioligands would be engineered — perhaps via PEGylation, glycation, or other means — to excrete from the body more quickly. This would allow for radiolabeling with more rapidly decaying radioisotopes such as ^{68}Ga and ^{18}F which would in turn further enhance the dosimetric benefits of the pretargeted imaging strategy. Ultimately, as researchers adapt this technology for use with other radioisotopes for imaging (e.g. ^{124}I , ^{111}In , ^{18}F , ^{89}Zr , ^{68}Ga , etc.) or therapy (e.g. ^{89}Y , ^{177}Lu , ^{225}Ac , ^{125}I etc.), new tetrazine-based ligands will need to be developed to incorporate different chelators or radiolabeling prosthetic groups. The thorough investigation of the pharmacokinetics of these novel constructs will be essential to ensuring advantageous matches between the clearance properties of the ligands and the physical half-life of the radionuclides.”

“Typo: Line 83 “both are have” should be “both have”

This typo has been corrected. Thank you for noticing it!

“Page 7, line 300 why is too long an injection interval a problem, this antibody appears to stay on the cell surface for very long”

This is a great point. Overly long interval times are less of a problem than overly short ones, but they still may lead to decreased tumor uptake. To explain this further, we have added the following sentences to the work, “Conversely, if too little antibody is used, the amount of uptake in the tumor will naturally suffer. Similarly, overly long interval times may also reduce levels of tumor uptake as a result of slow antibody internalization, trans-cyclooctene isomerization, or antibody/antigen shedding.”

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“100 ug A33-TCO per mouse means about 400 mg per human?? Any concern of an immune reaction? TCO conjugated to a slow clearing protein?? 10 MBq per mouse, what activity to patients? 40 GBq??, please discuss.”

The reviewer makes an interesting point. However, it is important to note that this PET imaging system, like all radiopharmaceuticals, will not be scaled from mice to humans based simply on mass or surface area. Radiopharmaceuticals (e.g. ^{89}Zr -DFO-mAbs) are typically tested in animals using 5 – 10 MBq. As the reviewer points out, when this is scaled to humans based only on mass/surface area, it results in 20 - 40 GBq. As it turns out, the typical dose for a human in the clinic is 10 mCi (370 MBq). In the case of this pretargeting system, the most likely human doses are 10 mg of A33-TCO and 10 mCi (370 MBq) of ^{64}Cu -Tz-Bn-NOTA.

All that said, this protocol *does not* discuss any clinical matters and thus represents simply a guide to the preclinical approach. Therefore, we do not think discussing these translational matters is within the scope of the protocol, and we have not made any changes to the manuscript. If the editors believe it to be important to include this discussion

“The legends to Fig. 6, 7 need to be improved. What does the reader see?”

Excellent point. The legends have been improved. They now read:

“Figure 6: PET images of ^{64}Cu -Tz-Bn-NOTA/A33-TCO pretargeting strategy. Mice bearing subcutaneous SW1222 xenografts (100–150 mm³) were administered A33-TCO (100 µg) via tail vein injection. After 24 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA (10.2–12.0 MBq [275–325 µCi]) via tail vein injection and subsequently imaged 2, 6, 12, and 18 h after the administration of the radiopharmaceutical. Transverse (top) and coronal (bottom) planar images intersect the center of the tumors. High levels of uptake in the gut at early time points (*i.e.* 2 and 6 h) largely clear by 12 h, leaving the tumor (white arrow) clearly delineated from all non-target tissue by 12 and 18 h post-injection. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.”

“Figure 7: PET images of suboptimal pretargeting experiments. (A) Mice bearing subcutaneous SW1222 xenografts (100–150 mm³, arrow) were administered A33-TCO (100 µg) via tail vein injection. After 12 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA (10.2–12.0 MBq [275–325 µCi]) tail vein injection. (B) Mice bearing subcutaneous SW1222 xenografts (100–150 mm³, arrow) were administered A33-TCO (300 µg) via tail vein injection. After 24 h, the same mice were administered ^{64}Cu -Tz-Bn-NOTA (10.2–12.0 MBq [275–325 mCi]) tail vein injection. In both cases, the mice were imaged 12 h after the injection of ^{64}Cu -Tz-Bn-NOTA. In both panels, transverse (top) and coronal (bottom) planar images intersect the center of the tumors. While the pretargeting strategy clearly delineates the tumor in both cases, the results in both of these images are sub-standard compared to those displayed in Figure 6. In both cases, there is a significant amount of background activity uptake in the heart. Under the conditions of Figure 7A, this is most likely the result of the huA33-TCO construct not being given enough time to localize in the tumor. Under the conditions of Figure 7B, this is likely a consequence of injecting too much huA33-TCO and having excess immunoconjugate still circulating in the blood even 24 h after injection. This figure is based on research originally published in *JNM*. Zeglis, B. M. *et al.* A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *Journal of Nuclear Medicine* 2013;54:1389-1396. © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.”

“Legend to Fig.6, 326, it is 275-325 microCi not mCi, see also line 335. The imaging time point is missing in the legends.”

This typo has been corrected, and the imaging time points have been added to the legends. Thank you for noticing it!

“Page 6, line 259, please give specific activity as MBq/nmoles not per μ g. Without having the molecular weight it has little meaning.”

As we discussed in the response to Reviewer #2, the specific activity values have now been changed to MBq/nmol.

“Page 7, line 265, what is a radioactive plate scanner; please be more precise, Phosphor imager?”

We were somewhat limiting in defining the radioactive plate scanner because we were not allowed to mention name brands (*i.e. Bioscan*). To clarify things somewhat, we have amended the text to read “radioactive TLC plate reader”.

In addition to all of these changes, we have made a few other minor changes to correct typographic errors found during our subsequent proof-reading of the manuscript. These changes have been noted in the revised manuscript file using “Track Changes”.

Thank you again, and please let me know if you require any more information.

Respectfully,

A handwritten signature in black ink, appearing to read 'B Zeglis', is shown on a light-colored background.

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