DEPARTMENT OF ENERGY FINAL SCIENTIFIC/TECHNICAL REPORT

1. DOE AWARD NUMBER: DE-SC0001220

Name of Recipient: **Anna M. Wu, Ph.D.**

Project Title: **F-18 Labeled Diabody-Luciferase Fusion Proteins for Optical-ImmunoPET**

Principal Investigator: **Anna M. Wu, Ph.D.**

Co-Principal Investigators: **Clifton Kwang-Fu Shen, Ph.D., Arion Chatziioannou, Ph.D.**

Other Personnel: **Wei-Yu Lin, Ph.D., Kan Liu, Ph.D., Tove Olafsen, Ph.D., Zoo Hwang, and Scott Knowles**

Project Period: **09/15/2009 – 09/14/2012**

Date: **December 2012**

2. DISTRIBUTION NOTICE: Unlimited Announcement.

3. EXECUTIVE SUMMARY:

The goal of this project was to develop novel, dual modality imaging agents, based on an engineered antibody framework; such an approach provides a platform for rapidly producing imaging tracers specific for a variety of targets. The approach employes an antibody fragment called the diabody; it is the smallest, bivalent antibody-based fragment, consisting solely of two sets of the antibody binding regions. In this project, diabodies with a total of three different specificities (recognizing cancer targets HER2, CEA, and PSCA) were fused a luciferase to provide an optical signal. Variant luciferases and bioluminescent substrate were employed to provide color discrimination. Finally, novel and streamline technologies have been developed for radiolabeling the diabody-luciferase fusion proteins with F-18 for PET imaging. Innovations include microfluidic chips for radiolabeling, and novel chemistry for bioorthogonal, site-specific radiolabeling chemistry. In parallel, we have begun evaluating a prototype combined optical-PET instrument for simultaneous detection of F-18 radioactivity and bioluminescence.

a) This research provides a means for producing very versatile imaging agents for a variety of purposes. By using antibodies, we can develop agents that detect virtually any specific target. We are also tagging these antibodies with a radioactive as well as an optical label, so that either or both detection method can be used. When both are combined, we will be able to detect and quantitate two different targets simultaneously.

b) Each of the steps (constructing the diabody luciferase proteins, microfluidic labeling, bioorthogonal labeling, and simultaneous optical and radioactive detection) has been achieved separately. However, we faced challenges in purifying sufficient amounts of protein to conduct all the steps on the antibody-luciferase fusion protein. In particular, the luciferase activity seemed quite sensitive to acid and other conditions that were tried during purification.

c) This research will have broad applicability in a number of areas involving detection and imaging of trace substances. Availability of improved luciferases will enhance optical detection and can be applied in diverse research areas including plant and microbial biology as well as human biology. Rapid, inexpensive microfluidic chip-based labeling will be of general use in the radiotracer field. Development of these combined optical/nuclear imaging probes will have direct applications to observation and quantitation of biological targets. In particular, these multimodality probes will enhance nuclear medicine imaging, especially in PET, by enabling simultaneous detection of more than one molecular target.

4. GOALS/OBJECTIVES AND ACCOMPLISHMENTS:

1. **Produce Engineered Antibody-luciferase Fusion Proteins with Longer Wavelength Bioluminescence Emissions**. **Goal:** We will develop target-specific diabodies fused with RLuc2, GLuc or GLuc27/97 and they will be screened using several coelenterazine analogues (V or CLZ-V4) to obtain a larger bathochromic-shifted emission and enhance small animal optical imaging in the near-IR region. **Accomplishments:** We constructed a total of 28 different engineered antibody-luciferase fusion proteins. One set was based on an antibody recognizing HER2 and the other set, CEA. Five different luciferase fusion partners were used: Renilla luciferase 8 (RLuc8), red-shifted R-Luc8.6-535, Gaussia luciferase (GLuc), and two GLuc fragments (GLuc 27/97 and /GLuc 98/168. Bifunctional ELISA assays demonstrated retention of antigen binding as well as bioluminescence of these fusion proteins. Purification of sufficient amounts of protein for labeling posed challenges.

2. **Develop a Novel Method for ^{18}F-labeling of Biomolecules using Microfluidics. Goal**: We propose to apply microfluidic droplet generator/mixers and a ^{18}F-tag, N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB), for radiolabeling of diabody-luciferase fusion proteins to produce dual label PET probes. **Accomplishments:** Routine labeling of engineered antibody fragments using ([18F])-SFB on a microfluidic platform has been established.

3. **Develop Site-specific and Bioorthogonal Dual Labeling Methods. Goal**: We propose two novel radiolabeling methods: (1) using sortase mediated ligation (SML) for introduction of azido groups site-specifically into LPXTG-tagged diabody-luciferase fusion proteins. It can be later radiolabeled with a universal cyclooctyne ^{18}F-tag by a strain-promoted, copper-free click reaction; (2) using another bioorthogonal reactions, namely the inverse electron demand Diels-Alder (IED-DA) reaction, to conjugate ^{18}F-labeled tetrazine to a luciferase-fused diabodies which has been modified with the strained dienophile (norbornene or *trans*-cyclooctene) via SML. **Accomplishments:** Sortase-tagged diabody-luciferase fusion proteins were produced, although purification challenges limited the studies that could be done. Several amino ligands posessing tetrazine or azadibenzocyclooctyne moieties were synthesized. [^{18}F]-SFB was produced by one-pot synthesis and coupled to produce five different tags for bioorthogonal labeling. [^{18}F]TBFB proved most stable and reproducible. A Her3-specific minibody was conjugated to norbornene and the IDE-DA reaction used to produce [^{18}F]-minibody.

4. **Imaging Proof-of-Concept Biological Systems. Goal**: After evaluating target binding affinity and bioluminescence efficiency of our dual labeled diabody fusion proteins *in vitro*, we propose to image tumor xenografted mice using OPET. Such dual modality imaging can provide accurate spatial localization of tracers and allow better quantitative measurement. To use luciferases with different spectral properties also might enable imaging of more than one target simultaneously. **Accomplishments:** Phantom studies have been conducted using the Optical-PET scanner. A dual imaging study using a phantom containing ^{18}F and anti-HER2 scDb RLuc 8.6-535 crude supernatant verified the ability of the canner to obtain PET and tomographic optical imagins of the same sample, setting the stage for further work on dual modality OPET imaging.

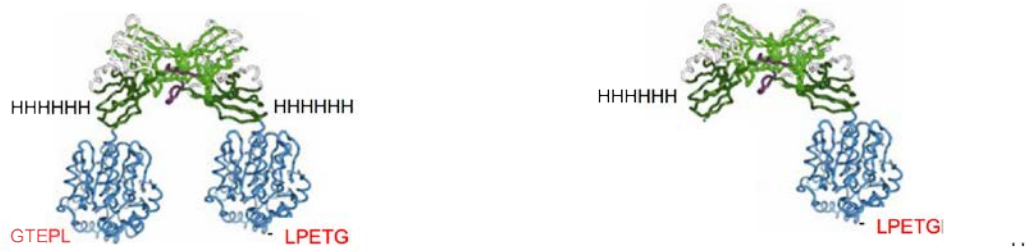
5. SUMMARY OF PROJECT ACTIVITIES FOR THE ENTIRE FUNDING PERIOD

Goal 1. Engineered antibody-luciferase fusion proteins.

Initial production of the diabody luciferase fusion proteins focused on production of anti-HER2 and anti-CEA diabodies (Db) and single-chain diabodies (scDb) fused with 5 different luciferases – Renilla Luciferase 8 (Rluc 8), red-shifted Renilla luciferase 8 (Rluc 8.6-535), Gaussia Luciferase (Gluc) and two Gaussia Luciferase fragments each previously shown to bioluminescence on their own (Gluc 27/97 and Gluc 98/168). Each of these constructs had a C-terminal His-tag for purification and an N-Terminal Sortase tag for site specific radiolabeling (see **Figure 1**)

These constructs were each cloned into a pEE12 mammalian expression vector and NS0 mouse myeloma cells were transfected by electroporation. Single cell clones were hand picked from a 96 well plate and were selected using Glutamine free media. Production of the diabody luciferase fusion proteins was detected using a luciferase assay and western blotting using anti-Rluc or anti-Gluc antibodies respectively (**Figure 2** A). A bifunctional ELISA, using immobilized antigen (CEA or HER2) followed by incubation with the Db-Luc fusion proteins and detection using a luciferase assay were performed and verified that the Db-luc fusion proteins both bound to their antigen targets and bioluminesce (**Figure 2** C).

A



Diabody-Luciferase scDiabody-Luciferase

B

Variant	Size (kDa)	Peak Emission with CLZ (nm)	Red-shifted Peak Emission with CLZ-V (nm)
Rluc 8	36	481	522
Rluc 8.6.535	36	535	570
Gluc27/97	7	470	
Gluc98/168	7	470	
GlucΔ17	17	470	

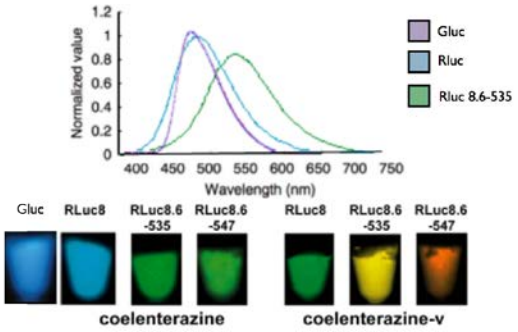


Figure 1. A. Diabody-luciferase fusion proteins consist of a non-covalently dimerized V_L-V_H-Linker-Luciferase (100-125kDa). Single-Chain-Diabody-Luciferases (61-90kDa) are made with two V_L-V_H segments connected by a long enough chain ($SGGGG_5$) to allow the scDb to fold over on itself to cross pair. B. Diabodies and ScDiabodies luciferase fusion proteins were made incorperating a variety of luciferases, allowing us to optimize protein size and luciferase brightness and color for in vivo targeting

After production and concentration of supernatants, we attempted to purify the diabody luciferase fusion proteins using their C-terminal His-tags using an AKTA Purifier. However, little or no binding to either Ni-NTA or Cobolt HisPur columns was detected indicating that the His-tag was likely buried inside the fusion protein making it unavailable for purification. Purification of the Db-Luc fusion proteins was then attempted using a Protein L column on the AKTA Purifier and eluting with a gradient of 0.1M Glycine buffer at pH 2.5. While purification of the protein was completed and verified by SDS-PAGE and Western blotting, we found that the Renilla Luciferase fusion proteins were sensitive to low pH and by the time the proteins disassociated from the protein L column the ability of the Renilla Luciferase proteins to bioluminescence had been lost. Despite a wide ranging search for other elution buffers (3.5M $MgCl_2$, 0.1M Citrate Buffer pH 2.5, Gentle Ag/Ab Elution Buffer, pH 6.6 [Peirce]) or other purification methods (Cation exchange, hydroxyapatite), we were never able to purify Renilla Luciferase fusion proteins that still had the ability to bioluminesce.

We then decided that our His-Tag was likely crucial to the purification of functional protein and decided that switching from a C-terminal His-tag to an N-terminal His-Tag would likely solve our purification problems. During the course of our production efforts we determined that the Gluc and Rluc8.6-535 single-chain diabody based constructs were the most promising as they provided brightest luciferase and the most red-shifted luciferase, respectively and hence had the greatest posibities for in vivo imaging where the number of red photons emitted from the tumor is the most important factor for detection. We also determined that the CEA Db and scDb based constructs did not express well. We therefore decided to focus on two new constructs consisting of anti-HER2 scDb-Rluc 8.6-535-Sortase-His (anti-HER2 scDb Rluc 8.6-535) and an anti-PSCA scDb Δ17Gluc M43LM110L-Sortase-His (anti-PSCA scDb Gluc) based construct based on an anti-PSCA diabody that we knew through previous experience produced much better than the anti-CEA diabody. The combination of anti-HER2 and anti-PSCA based constructs was also advantageous as it could take advantage of prostate cancer models that express both proteins that existed in our lab. The two point mutations in Gluc had recently been shown to prolong the flash kinetics of Gluc bioluminescence and would allow us a longer in vivo optical imaging window than with the wild-type protein.

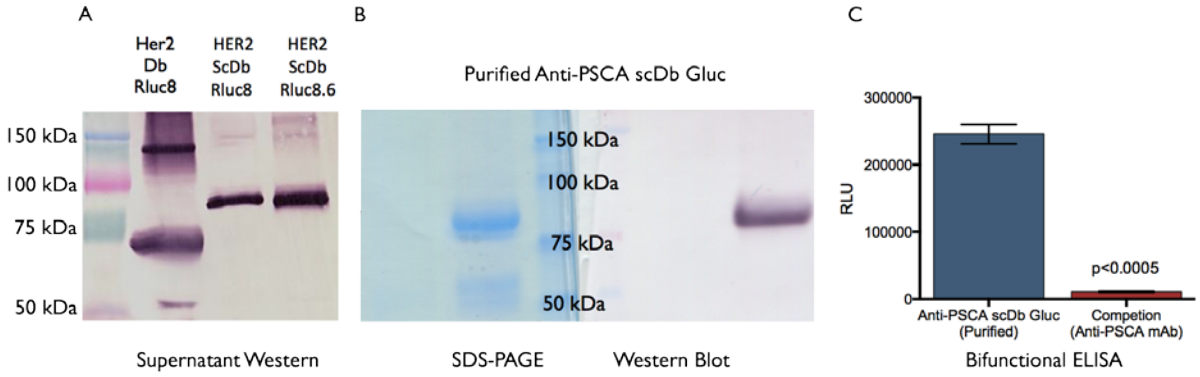


Figure 2. A. A variety of constructs were produced in the C-Terminal His-Tag format. Here a western blot using Anti-Renilla Luciferase detects the Her2 Db and Her2 scDb attached to both RLuc 8 and the red shift Rluc 8.6-535. These constructs could not be purified without losing luciferase activity. B. The reformatted Anti-PSCA scDb Gluc Protein could be purified on protein L C. The purified Anti-PSCA scDb Gluc protein retained its ability to both bind to PSCA and to bioluminesce as demonstrated here with an ELISA on immobilized PSCA with luciferase detection. Competition with an anti-PSCA antibody verifies the interaction is specific to PSCA.

Transfection and selection of the new anti-HER2 scDb Rluc 8.6-535 and anti-PSCA scDb Gluc was done via a novel pEE12-IRES-GFP expression vector that allowed for selection of stable producing clones via FACS cell sorting greatly reducing the amount of manual cell culture and selection required by lab personnel. The single-chain Diabody luciferase fusion proteins with an N-Terminal His-tag were found to bind to HisPur Ni-NTA columns and could be purified by this process. However, the purification process caused the constructs to dimerize and exist as dimer of scDbs. While the anti-PSCA scDb Gluc protein could be purified as a monomer on a protein L column and retained activity after elution (see **Figure 2** B and C), no strategy was found that could purify Anti-HER2 scDb RLuc8.6-535 monomer that retained the luciferase activity, and hence two-color in vivo work could not be pursued.

Goal 2. ^{18}F-labeling of biomolecules using microfluidics.

We have designed and fabricated new droplet generator/mixers for radiolabeling of diabody-luciferase fusion proteins using N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) to produce dual label PET probes. The microfluidic droplet generator was implemented in a two-layer polydimethylsiloxane (PDMS) chip and its core consists many feasible microchambers which allow adjustment of precise volume of each reagent (see **Figure 3**). These chambers are each filled with one reagent and then their contents are merged into a single end-to-end multi-component droplet with well-defined composition. Each droplet can be then assayed to obtain the desired properties of PET probes. In order to increase throughput and facilitate scale-up, a multi-channel version applying parallel operation was also developed (**Figure 3**). Furthermore, we also implemented a liquid sensor system allowing one to load [^{18}F]SFB and protein solutions with volumes as small as 1 µL, automatically. Thus, this microfluidics chip can take up the concentrated [^{18}F]SFB in PBS buffer from microwave radiochemical synthesizer and protein to be radiolabeled from a sample vial, very precisely. These chips can also be used for high-throughput screening of self-assembly conditions of superamolecular nanoparticles. Therefore, at this stage, we have finished the development of microfluidic platforms necessary for this project.

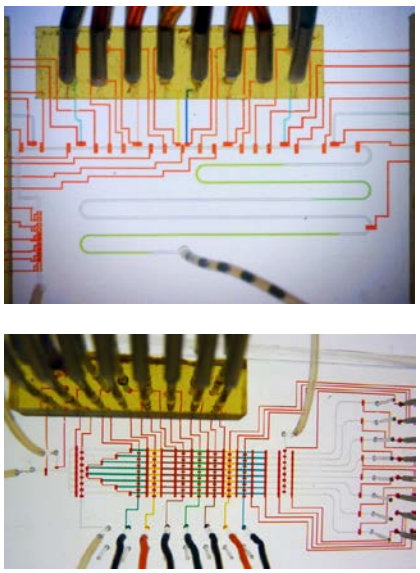
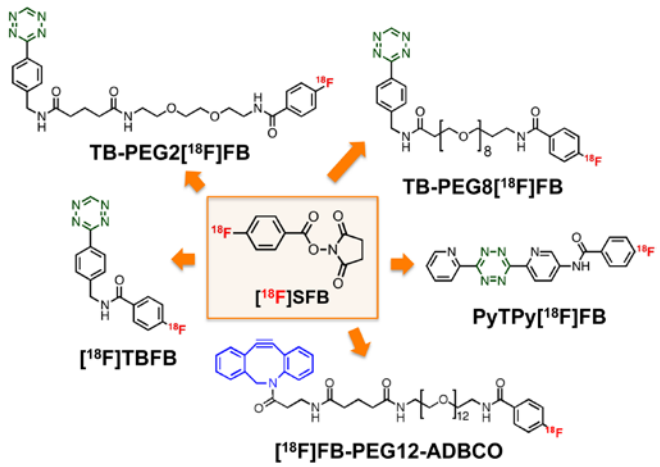


Figure 3. Microfluidic droplet generator/micromixer chips (top: single-channel; bottom: multi-channel) loaded with dyes to show control channels (red) and reagent loading channel (blue, yellow, green, brown, etc.).

Goal 3. Bioorthogonal radiolabeling labeling of biological molecules.

Efficient labeling of biological molecules with F-18 could result in an important class of PET probes. However, direct incorporation of F-18 into fragile biomolecules, such as proteins, peptides, or antibodies, is very changeling due to the lack of functional groups in the biomolecule required for a nucleophilic ^{18}F-fluorination and, more importantly, the harsh reaction conditions (high temperature, strongly basic) during the radiofluorinations could cause denaturation and decomposition of the sensitive biomolecule. Even with the application of ^{18}F-labeled prosthetic group, the radiolabeling yields are generally very low. Therefore, ^{18}F-labeled biological molecules are generally difficult to make, especially at high specific activities. Bioorthogonal reactions are emerging as a unique research tool for introducing various tags to target molecules in a complex biological environment. Due to their extremely high selectivity,



Scheme 1. Five different 18F-labeled tags derived from [^{18}F]SFB. Four tetrazine-based tags ([^{18}F]TBFB, TB-PEG2[^{18}F]FB, TB-PEG8[^{18}F]FB, PyTPy[^{18}F]FB) can react with norborene (Nb) or trans-cyclooctene (OCT) motif. Molecules with azide functional group can conjugate with the azadibenzocyclooctyne-based (ADBCO) tag ([^{18}F]FB-PEG12-ADBCO).

great functional group tolerance and usually very fast reaction kinetic in aqueous buffer solution, such reactions may become a powerful means for ^{18}F-labeling biomolecules *in vitro* or even *in vivo*. In the past two years, Dr. Clifton Shen (co-PI)'s lab has developed several ^{18}F-labeled tetrazine- and dibenzocyclooctyne-based tags (see **Scheme 1**) for testing the feasibility of efficient productions of ^{18}F-labeled biomolecules via the corresponding bioorthogonal ligations.

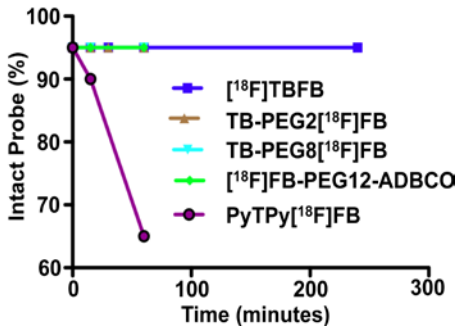
a. Properties of Novel ^{18}F-labeled Tags for Bioorthogonal Labeling of Biomolecules

Synthesis

We have designed and synthesized several amino ligands that possess either tetrazine or azadibenzocyclooctyne for the subsequent bioorthogonal ligation. They were radiolabeled by N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) to produce the corresponding [^{18}F]fluorobenzoyl tags (see **Scheme 1**). The coupling of those amino derivatives with [^{18}F]SFB**,** which was synthesized by a convenient one-pot microwave-assisted synthesis (developed by our group) was generally carried out in DMSO at 37°C for 30 min**.** After HPLC purification, [^{18}F]TBFB, TB-PEG2[^{18}F]FB, TB-PEG8[^{18}F]FB, and PyTPy[^{18}F]FB were obtained in a decay-corrected radiochemical yield (RCY) of 20-65% in < 90 min with radiochemical purity (RCP) >95 % and good chemical purity.

Probe stability

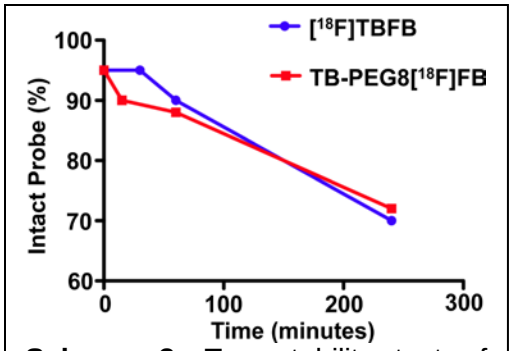
Due to the consideration of chemical instability of tetrazine derivatives in aqueous solution, we have tested our five new [^{18}F]FB-labeled tags in PBS buffer solution to access their decomposition rate. Besides PyTPy[^{18}F]FB, the rest of tetrazine-based were stable enough in aqueous media (<5% decomposition) after 4h in PBS at 37ºC (see **Scheme 2**). In addition, [^{18}F]TBFB and TB-PEG8[^{18}F]FB showed <20% decomposition after 2h and <30% after 4 h in cell culture media with 1% FBS (see **Scheme 3**). Those data indeed suggested their potential for conducting in situ radiolabeling of certain biomolecules with norborene (Nb), trans-cyclooctene (TCO), or azides handles, in buffer solution or cell culture media.



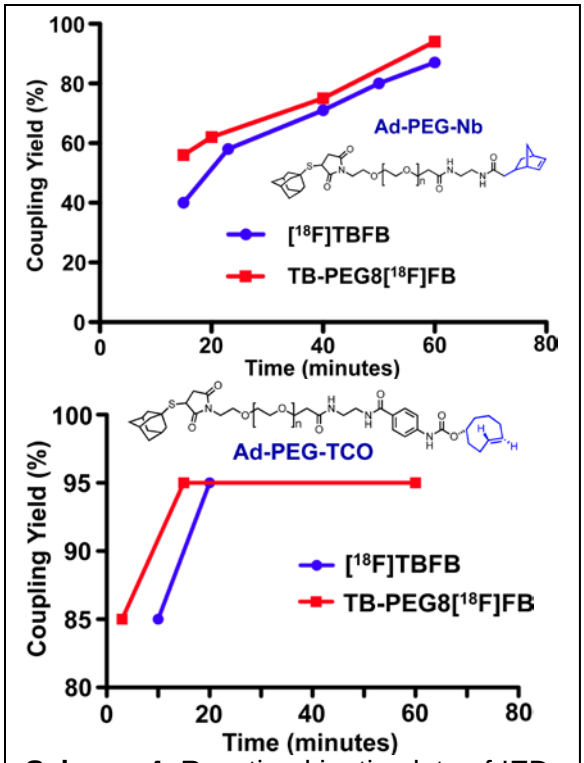
Scheme 2. Tag stability test in PBS buffer solution (pH 7.4) at 37ºC.

Reaction Kinetics

In order to measure the reaction kinetics of a series of [^{18}F]TBFB derivatives, two oligoPEGlyated polymers (Mw of PEG chain is approximately 5,000 Da) were made—one with norborene terminal (Ad-PEG-Nb) and one with trans-cyclooctene terminal (Ad-PEG-OCT), respectively. Radio-HPLC was used to determine the coupling yields of given reactions at the various time points. First, we tested the reaction speed of bioorthogonal ligations between [^{18}F]TBFB, TB-PEG8[^{18}F]FB and Ad-PEG-Nb (10 mg/mL) in PBS buffer. The data were shown in **Scheme 4**. TB-PEG8[^{18}F]FB proceeded the inverse electron demand Diels-Alder (IED-DA) reaction upon norbornyl group (Nb) slightly faster than [^{18}F]TBFB. The reason could be due to its better water solubility with the PEGlyated chain. It took about 18 minutes for TB-PEG8[^{18}F]FB to reach 50% coupling yield while [^{18}F]TBFB required 22 minutes. After 1 hour, the coupling yield of [^{18}F]TBFB and Ad-PEG-Nb was 80% and that of TB-PEG8[^{18}F]FB was 90%. To further accelerate the reaction, a more strained cyclic system, TCO, was introduced. Our results indicated that the IED-DA ligation was almost complete within 10 minutes in the case of TB-PEG8[^{18}F]FB. The time required for the [^{18}F]TBFB- Ad-PEG-TCO pair to finish coupling reaction was 20 minutes (see **Scheme 4**). The TCO-tetrazine system seems to be a better candidate for the next in vivo experiments.



Scheme 3. Tag stability test of [^{18}F]TBFB and TB-PEG8[^{18}F]FB in cell culture media with 5% FBS at 37ºC.

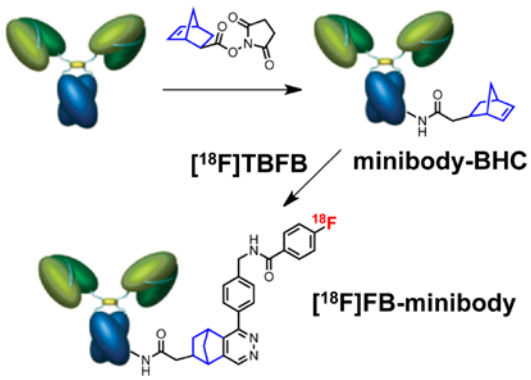


Scheme 4. Reaction kinetic plots of IED-DA ligation using Ad-PEG-Nb (top) and Ad-PEG-TCO (bottom) with two tetrazine-based tags, [^{18}F]TBFB and TB-PEG8[^{18}F]FB, respectively.

b. Bioorthogonal ^{18}F-labeling of biomolecules

The advent of chemical biology tools for imaging and tracking of biomolecules in their native environment is providing unique insights into cellular processes that are not achievable with traditional biochemical or molecular biology methods. Bioorthogonal labeling of biomolecules has proven particularly useful for the detection and study of glycans and lipids, based on a highly selective reaction between an abiotic functional handle and a designed chemical tag. With respect to the abiotic handle, there are many choices. For example, azide has been used extensively because of its straightforward chemical introduction, small size, and relative inertness. However, its reactivity and reaction rate, either via Staudinger ligation or strain-promoted cyclooctyne cycloaddition, are still not speedy enough for in vivo pre-targeting experiments. Often the reaction kinetics has required a high dose and large excess of secondary reagent to achieve detectable binding. Applications in molecular imaging, esp. PET, which require low doses and semi-equimolar conditions at low concentration have therefore remained unrealized. Recently, Robillard and Weissleder *et al* reported the fast reaction kinetics and selectivity in vitro & in vivo of the IED-DA reaction

between electron-deficient tetrazine and strained norborene & TCO derivatives suggest its potential for carrying out effective bioorthogonal reaction at low concentrations.



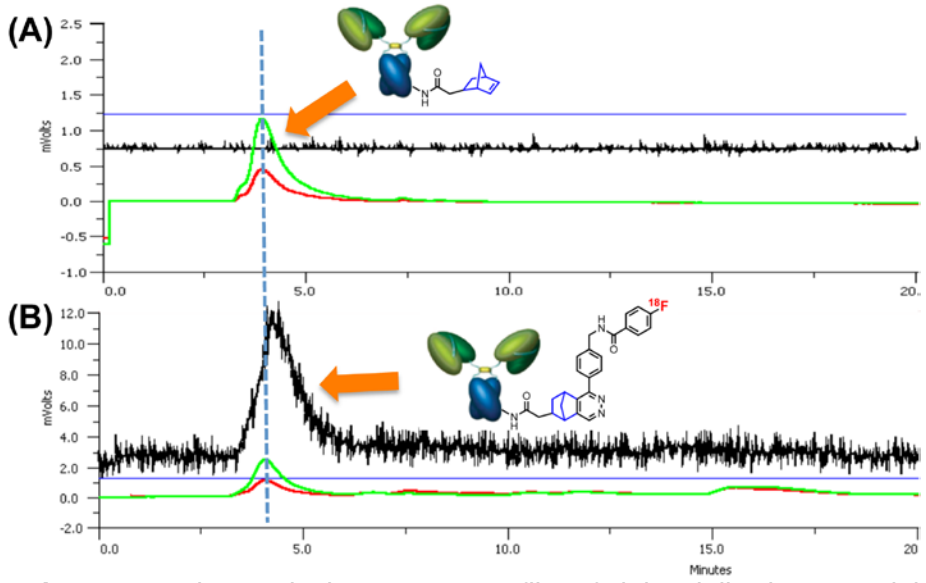
Scheme 5. Synthetic route to produce [^{18}F]FB-minibody via the bioorthogonal IED-DA reaction using minibody-BHC, which was synthesized from minibody and norbornyl NHS ester.

Installation of bioorthogonal handle on biomolecules

Although the reaction rate of tetrazine derivatives between TCO is faster than that of norbornyl ones, norbornyl NHS ester is commercially available and it will be easy for us to use it as a proof-of-concept system to test the feasibility of our bioorthogonal ^{18}F-labeling strategy. To start, bicyclo[2.2.1]hept-5-ene-2-carboxamide-labeled minibody (minibody-BHC) was prepared by direct conjugation of the activated ester of norbornene (norbornyl NHS ester) and anti-HER3 minibody (**Scheme 5**).

Bioorthogonal ^{18}F-labeling of anti-HER3 minibody

After purification using dialysis, minibody-BHC was radiolabeled via bioorthogonal IED-DA reaction using [^{18}F]TBFB. All the radiochemical processes were first carried out and developed in a manual setup then they will be further transferred into an automated microwave-assisted module. [^{18}F]TBFB was applied to finish the conjugation in two different solutions (PBS pH 7.4 or PBS with 1% FBS) to afford **[**^{18}F]FB-minibody (see **Scheme 5**). The progress of IDE-DA reaction was monitoring by i-TLC and size-exclusion radio-HPLC (see **Scheme 6**), which later provided feedback for optimizing coupling conditions. After 15 minutes, almost complete conversation of minibody-BHC into [^{18}F]FB-minibody was observed. The control experiments were also carried out to rule out the possibility of non-specific binding. With pristine minibody without BHC modification or PBS solution alone, the formation of [^{18}F]FB-minibody could not be found.



Scheme 6. Size-exclusion HPLC profile of (A) minibody-BHC; (B) [^{18}F]FB-minibody. (Black: radioactivity; green: 254nm, red: 280nm).

Summary

We have synthesized several novel ^{18}F-tags for bioorthogonal ligation and also successfully demonstrated to ^{18}F-label BHC-minibody and cRGDfk-BHC (data not shown) bioorthogonally using [^{18}F]TBFB via IED-DA reactions. The conversion was completed very rapidly and highly selective. Our preliminary results show great potential and high feasibility to utilize the [^{18}F]TBFB/BHC-modified biologics system for in vitro and in vivo experiments. More

detailed in vitro/in vivo studies using ^{18}F-labeled anti-HER3 minbody and cRGDfk are well underway. PEGylated [^{18}F]TBFBs with varied ethylene glycol linkers (TB-PEG2[^{18}F]FB & TB-PEG8[^{18}F]FB) are also being investigated in vivo using dynamic microPET in searching of suitable linker length that can fulfill pharmacokinteic requirements for in vivo pre-targeted immunoPET imaging.

Goal 4. Dual-modality optical and PET imaging using the prototype OPET scanner.

Initial work with the Optical PET Scanner and a phantom containing ^{18}F and anti-HER scDb RLuc 8.6-535 crude supernatant verified the ability of the scanner to obtain PET and tomographic optical images of the same sample (**Figure 4**), however in order to pursue the possibilities this technology opens further work is needed to obtain a red-shifted luciferase fusion protein variant that is stable enough to allow for robust purification.

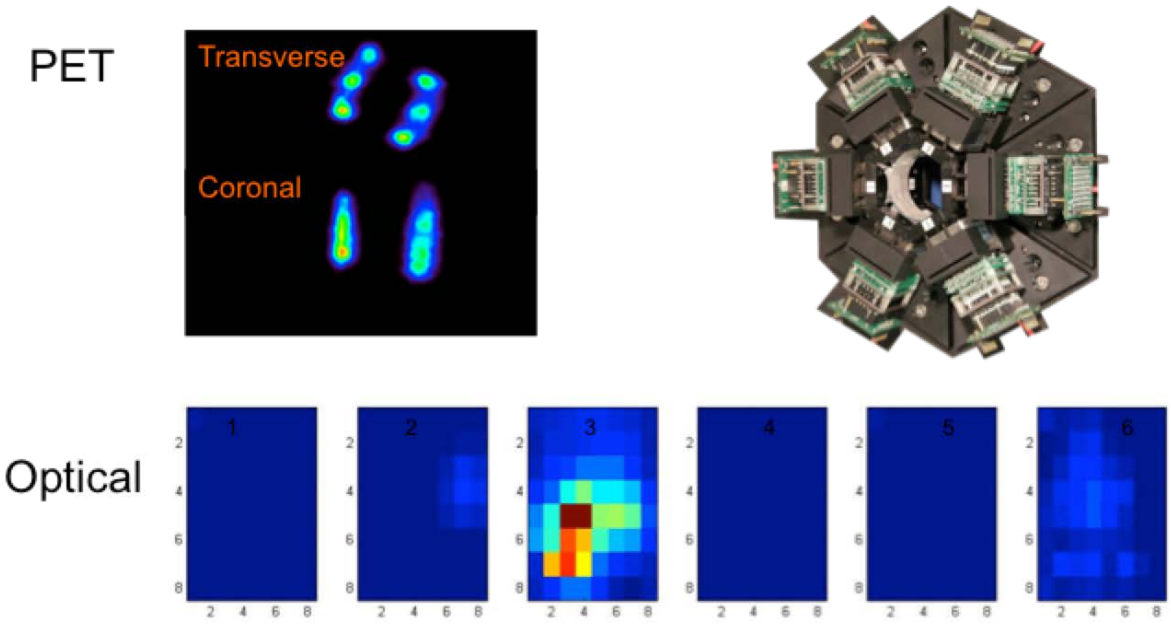


Figure 3. The OPET scanner can detect both PET and Optical data from a phantom containing ^{18}F-FDG and anti-HER scDb RLuc 8.6-535 with added coelenterazine.

6. PRODUCTS DEVELOPED/PUBLICATIONS:

1. Olma, S.; Liu, K.; Chen, Y.-C.; van Dam, R.; **Shen, C. K.-F.*** "Microfluidic Droplet Mixer for Fluorine-18 Labeling of Biomolecules" *J. Label. Compd. Radiopharm.* **2009**, *52(S1),* S10.
2. Olma, S.; Sirk, S. J.; Lepin, E.; Olafsen, T.; **Wu, A. M.**; van Dam, R.; **Shen, C. K.-F.*** "Convenient N-Succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) Synthesis and Labeling of Biomolecules" *J. Label. Compd. Radiopharm.* **2009**, *52(S1),* S166.
3. Olma, S.; Lambert, J.; Barnhardt, E.; Liu, K.; **Shen, C. K.-F.***; van Dam, R. "A Compact Microwave System for Rapid, Semi-automated Radiosyntheses" *J. Label. Compd. Radiopharm.* **2009**, *52(S1),* S509.
4. Lin, W.-Y.; Olma, S.; **Shen, C. K.-F.** "Rapid Radiolabeling of Biomolecules with Novel F-18 Tags" (UCLA Case No. 2009-339; Application # 61/116,902).
5. Wang, M.-W.; Lin, W.-Y.; Liu, K.; Masterman-Smith, M.; **Shen, C. K.-F.*** "Microfluidic for PET Imaging Probe Development" *Mol. Imaging* **2010**, *9(4)*, 175-191.

6. Liu, K., Chen, Y-C, Xia, C., Tseng, H.R., **Shen, C. K.-F.***, van Dam, R. M.* "Microfluidic device for reliable generation of liquid-in-air slugs and controllable generation of multi-component fluid segments separated by air", *Microfluid. Nanofluid.,* **2010***, 9(4-5)*, 933-943.
7. Liu, K.; Lepin, E. J.; Wang, M.-W.; Guo, F.; Lin, W.-Y.; Chen, Y.-C.; Olma, S.; Phelps, M. E.; Zhao, X.-Z.; Tseng, H.-R.; van Dam, R.M.*; **Wu, A. M.***; **Shen, C. K.-F.*** "Microfluidic-based ^{18}F-labeling of Biomolecules for ImmunoPET" *Mol. Imaging* **2010**, *10,* 168-176. DOI: 10.2310/7290.2010.00043.
8. Liu, K.*; Wang, H.*; Chen, K.-J.; Guo, F.; Lin, W.-Y.; Chen, Y.-C.; Phung, D.-L.; Tseng, H.-R.*; **Shen, C. K.-F.*** "A Digital Microfluidic Droplet Generator Produces Self-assembled Supramolecular Nanoparticles for Targeted Cell Imaging". *Nanotech.* **2010**, *21(44)*, 445603.
9. Wang, H.; Liu, K.; Chen, K.-J.; Lu, Y.; Wang, S.; Guo, F.; Lin, W.-Y.; Kamei, K.-I.; Chen, Y.-C.; Ohashi, M.; Wang, M.; Zhao, X.-Z.; **Shen, C. K.-F.***; Tseng, H.-R.* "A Rapid Pathway Toward a Superb Gene Delivery System: Programming Structural and Functional Diversity into a Supramolecular Nanoparticle Library". *ACS Nano*. **2010**, *4(10)*, 6235-6243.
10. Lin, W.-Y.; Liu, K.; Wang, M.; Girgis, M. D.; McCracken, M.; Tomlinson, J. S.; **Wu, A. M.**; **Shen, C. K.-F.*** "Bioorthogonal ^{18}F-labeling of Biomolecules via Inverse Electron Demand Diels-Alder Cycloaddition", World Molecular Imaging Congress (WMIC) 2010, 0863B.
11. Liu, K.; Lepin, E. J.; Wang, M.; Lin, W.-Y.; Tseng, H.-R.; **Wu, A. M.***; **Shen, C. K.-F.*** "Using Digital Microfluidics to Optimize ^{18}F-labeling Conditions of Diabodies", World Molecular Imaging Congress (WMIC) 2010, 0888B.
12. Olma, S.; **Shen, C. K.-F.** "Simplified One-pot Synthesis of [^{18}F]SFB for Radiolabeling" PCT Int. Appl. (2010) 10/26/1010/26/10.
13. Wang, S.; Liu, K.; Liu, J.; Yu, Z. T.-F.; Lee, E. Y.; Xu, X.; Reiss, J.; Chung, L. W. K.; Huang, J.; Rettig, M.; Pantuck, A.; Silegson, D.; Duraiswamy, K. N.; **Shen, C. K.-F.**; Tseng, H.-R. "Highly Efficient Capture of Circulating Tumor Cells Using Nanostructured Silicon Substrates with Integrated Chaotic Micromixers". *Angew. Chem. Int. Ed.* **2011**, in press.
14. Hou, S.; Phung, D. L.; Lin, W.-Y.; Wang, M.-W.; Liu, K.; Olma, S.; **Shen, C. K.-F.*** "Microwave-assisted one-pot synthesis of [^{18}F]-N-Succinimidyl-4-Fluorobenzoate ([^{18}F]SFB)". *J. Vis. Exp.* **2011** http://www.jove.com/details.stp?id=2755 doi: 10.3791/2755.
15. Liu, K.; Wang, M.; Lin, W.-Y.; Phung, D.-L.; Grigis, M.; **Wu, A. M.**; Tomlinson, J.*; **Shen, C. K.-F.*** "Molecular Imaging Probe Development using Microfluidics" *Curr. Org. Syn.,* **2011**, *8,* 473-487.
16. Knowles, S. and **Wu, A.M.*** "Advances in ImmunoPET: Applications in oncology" *J. Clin. Oncol*. **2012**, *30*, 3884-3892.