SUPPORTING MATERIAL

IN VIVO EXPERIMENTS

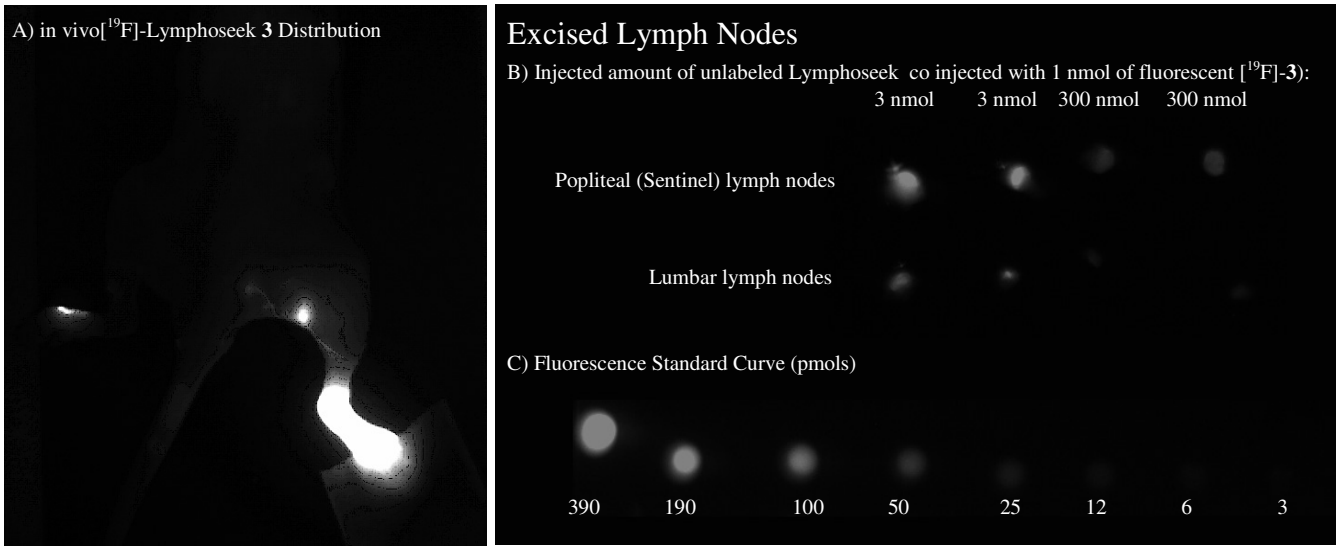
Multi Modality Imaging:

PET/CT image fusion:

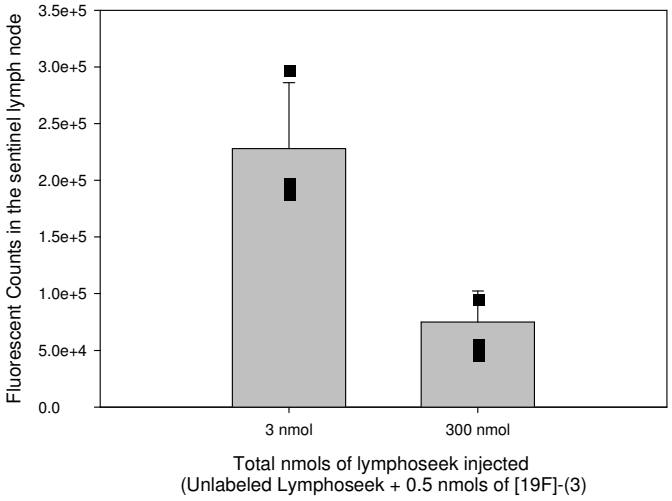
PET/CT image fusion was carried out with amide 0.9.1 open source software (http://amide.sourceforge.net). CT data in the DICOM (Digital Imaging and Communications in Medicine standard) format and PET data in .hdr (high dynamic image) format were imported into amide 0.9.1, as a new study. Inverted PET images were reflected around the x axis (right click study in study window →rotate (tab)→ change coordinate (j,y) from 1 to -1), and data was plot using a hot metal color table (right click study in study window →Colormap/Threshold (tab)→ Color Table → hot metal). Generally, PET thresholding between 1.0 and 0.0 percent was used (right click study in study window →Colormap/Threshold (tab)→ Set Max Threshold, Set Min Threshold). CT images were colormapped using a hot blue color table so that CT and PET data could be easily distinguished. Thresholding between 40 and 50 percent was sufficient to show soft tissue CT data.

To align PET and CT images, fiducidal marks were placed at appropriate locations (generally the bladder, hind right foot, right popliteal lymph node) and the alignment wizard (Tools → Alignment Wizard) was used to reconstruct an overlaid image. The overlaid image was cropped to a single mouse using Amide's cropping tool (Tools → Crop Active Data Set) and projections were generated (View → Volume Rendering). The rendered projections were exported as .jpeg files. All displayed PET images are PET/CT fused images. A sample .XIF file (PETCT.mpg) is included with this submission (Mouse 006, 1 nmol injection of **3**, note that amide 0.9.1 available at http://amide.sourceforge.net is required to open this file. This file possesses a .mpg extension and must be renamed with an .XIF extension to open.)

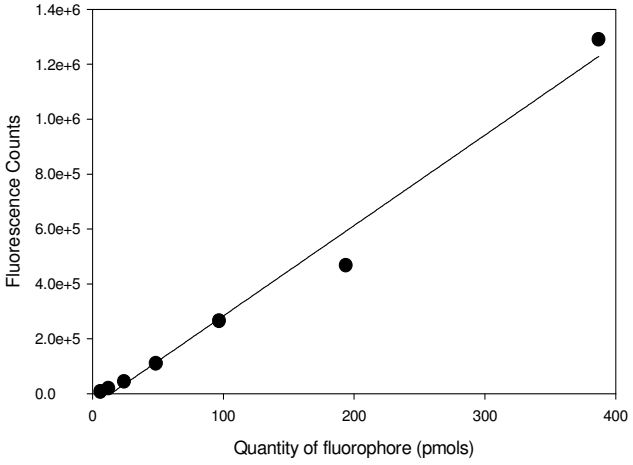
Proof of [^{19}F]-Lymphoseek-3's receptor specific binding activity



E) [^{19}F]- **3** accumulation in the **SLN** at 3 and 300 nmol



D) Standard Curve: Fluorescence Counts vs. Quantity



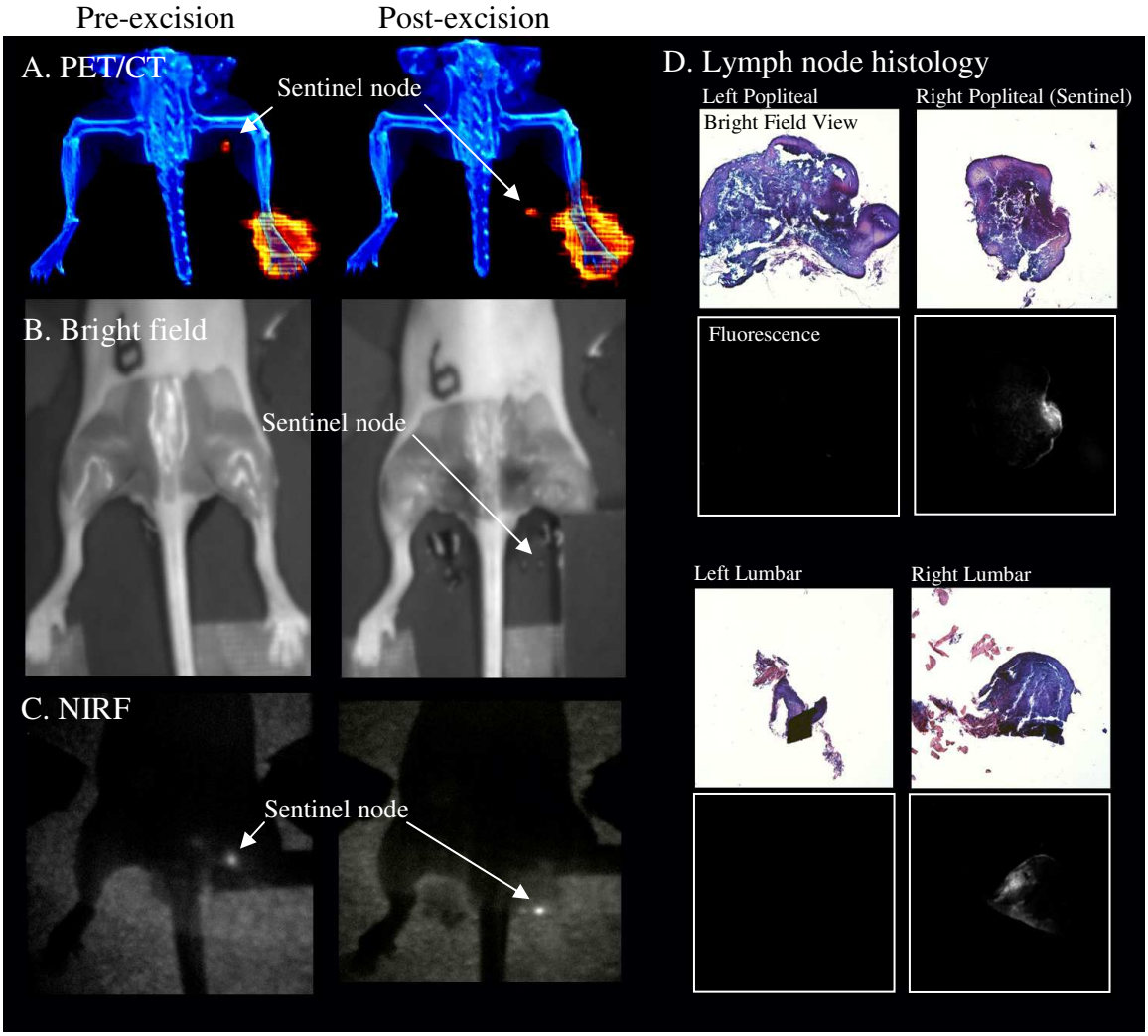
In vivo demonstration of [^{19}F]-Lymphoseek 3's receptor specific activity. Two solutions containing either 3 nmols (0.24 µL of a 12.5 nmol/µL solution) or 300 nmols (24 µL of a 12.5 nmol/µL solution) of unlabeled Lymphoseek were added to solutions containing 1 nmol (5 µL) of fluorescent [^{19}F]-Lymphoseek 3. These mixtures were diluted with water, injected into the rear footpad of mice at 30 µL volumes, and were allowed to distribute for 80 min. Three mice were injected at each dose. A) A 1 hour image skin-off in vivo image of [^{19}F]-Lymphoseek 3 prior to node extraction captured with a Maestro (CRi) 5 sec exposure. Note the clear visibility of the lymph tracks linking nodes to the foot's rear fat pad. B) Lymph nodes were extracted from mice and placed on a bed for analysis. The expected result, receptor specific accumulation, is observed. Greater accumulation of fluorescent [^{19}F]- Lymphoseek 3 is seen in the sentinel lymph nodes in 3 nmol injections than in 300 nmol injections due to increased competition with unlabeled Lymphoseek for sentinel lymph node mannose receptors. C) and D), Fluorescence [^{19}F]-3 standard curve generated with [^{19}F]-3 dissolved in 4 µL water droplets. Note that fluorescent photon quantization in tissue and standard curve conditions may differ due to the different media scattering properties, therefore fluorescence quantities in the standard curve are only relative representations of the fluorescence quantities within lymph nodes. The fluorescence response in the standard curve D) is linear therefore

fluorescence quenching at these quantities are ruled out. E) Bar graph of Lymphoseek distribution [^{19}F]-3 in the SENTINEL NODE at 3 and 300 nmol doses of Lymphoseek. A 3.5x contrast ratio is observed between the sentinel nodes in the different injection conditions (n= 3 mice per dose, P= 0.015 two tailed, two sample equal variance). Images B-C were generated with a 4 second exposure on the CRi Maestro imaging system.

[^{18}F]- 3 experimental data:

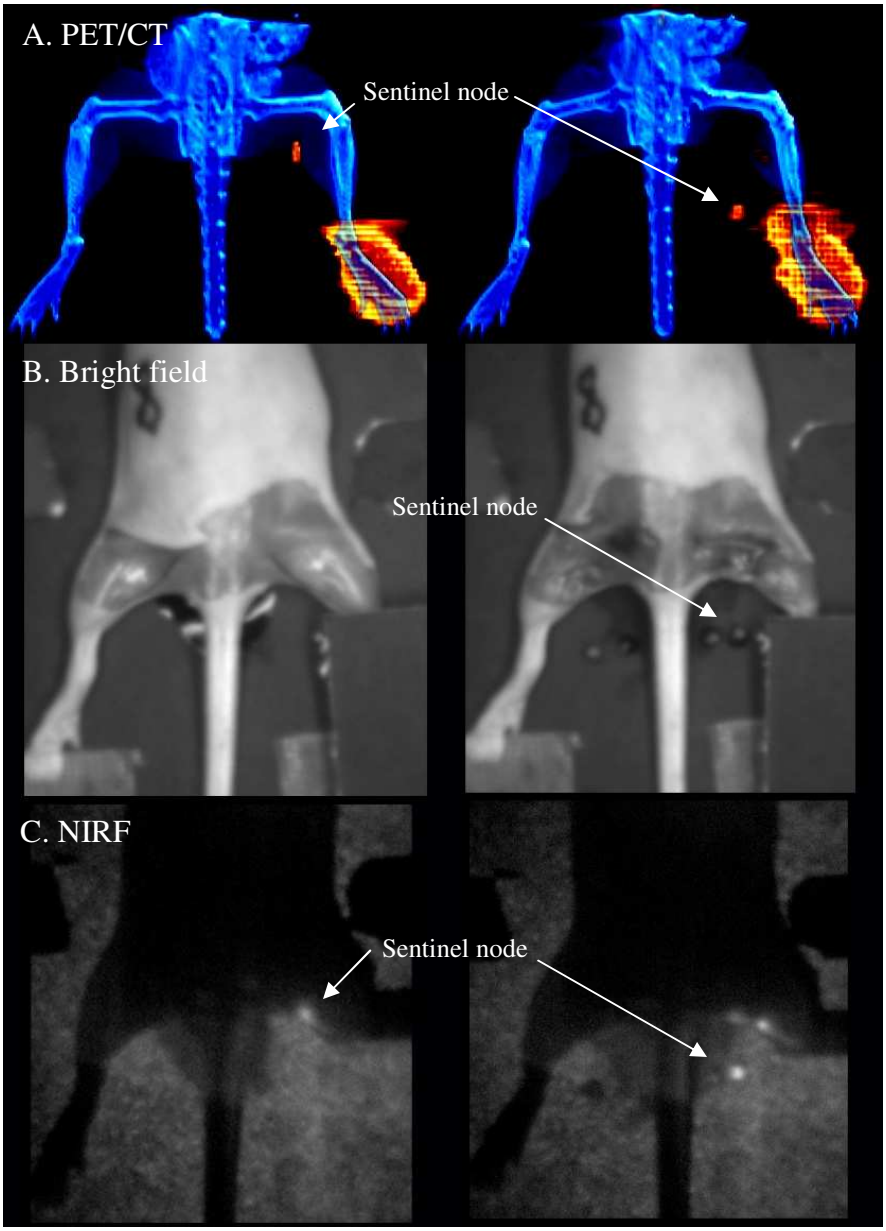
Low dose 1 nmol [^{18}F]-Lymphoseek **3** injections:

Mouse 1 of 3 low-dose 1 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 006)



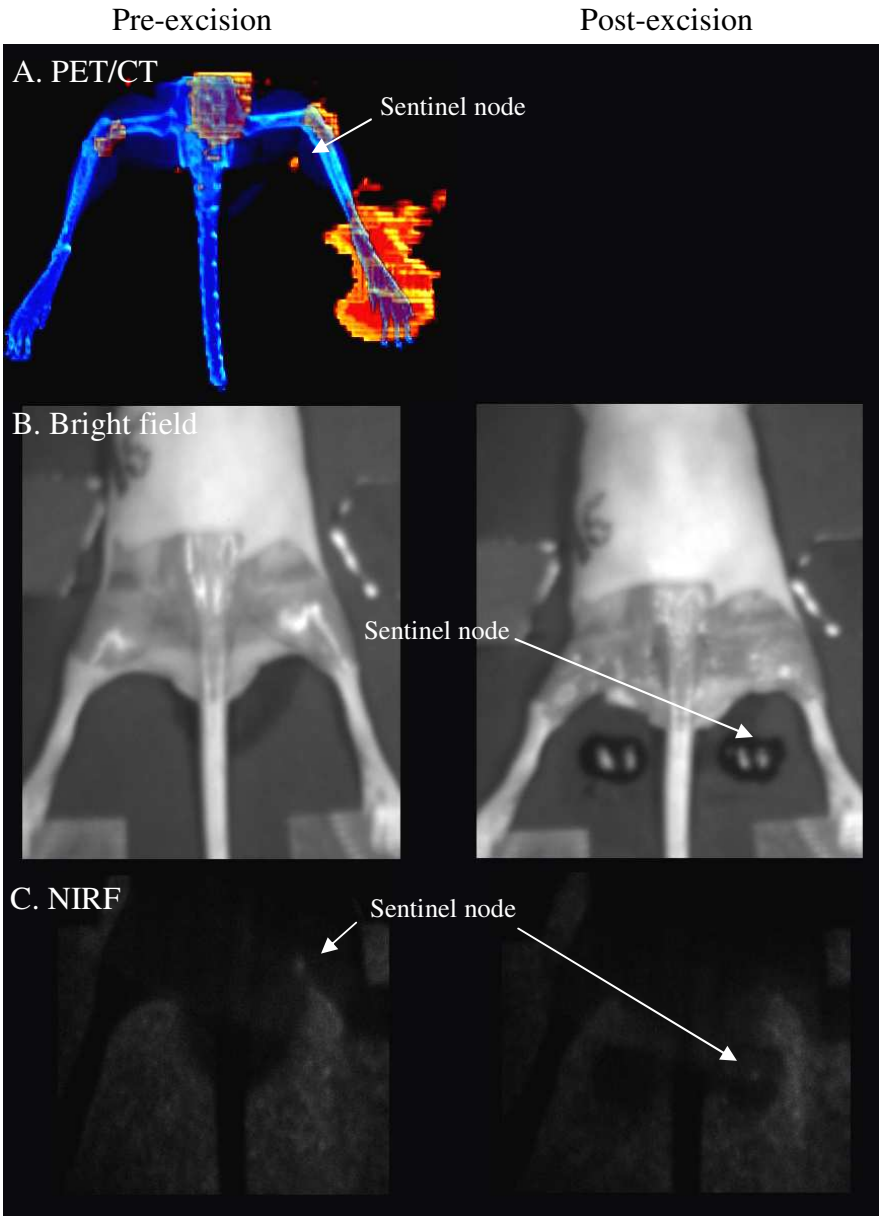
Mouse 006 was injected with a 10 µL, 1 nmol dose of [^{18}F]-labeled Lymphoseek **3** corresponding to a 48.1 µCi, 0.048 Ci/µmol ^{18}F injection at 10:19 AM, the **Time Of Injection (TOI).** The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 33.0 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. 20 min PET scan was carried out 2 hours and 57 min vs. TOI on 15.8 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 33 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow. *D.* Fluorescent and hematoxylin and eosin stained bright field verification of lymph node excision through histological cross sectioning of the nodes excised in B.

Mouse 2 of 3 low-dose 1 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 008)



Mouse 008 was injected with a 10 µL, 1 nmol dose of [^{18}F]-labeled Lymphoseek **3** corresponding to a 42.1 µCi, 0.042 Ci/µmol ^{18}F injection at 10:50 AM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 28.8 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. 20 min PET scan was carried out 3 hours and 7 min vs. TOI on 13.0 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 30 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

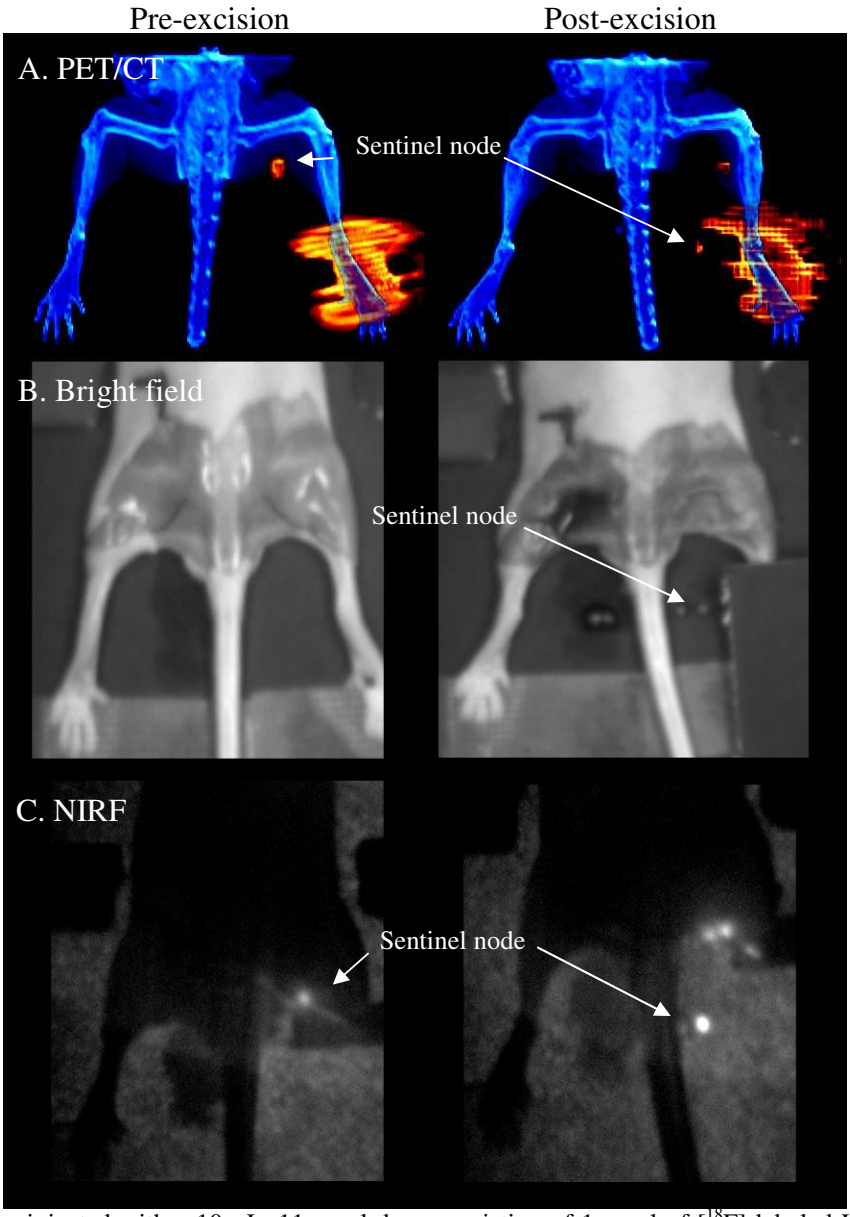
Mouse 3 of 3 low-dose 1 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 016)



Mouse 010 was injected with a 10 µL, 1 nmol dose of [^{18}F]-labeled Lymphoseek **3** corresponding to a 9.7 µCi, 0.010 Ci/µmol ^{18}F injection at 3:15 PM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 6.6 µCi of activity (decay corrected). The mouse did not possess sufficient activity for a post-excision PET scan at 3 hours and 59 min vs. TOI (2.2 µCi of activity remained, decay corrected) but sufficient activity remained in extracted nodes for scintigraphy in biodistribution studies. *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 24 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

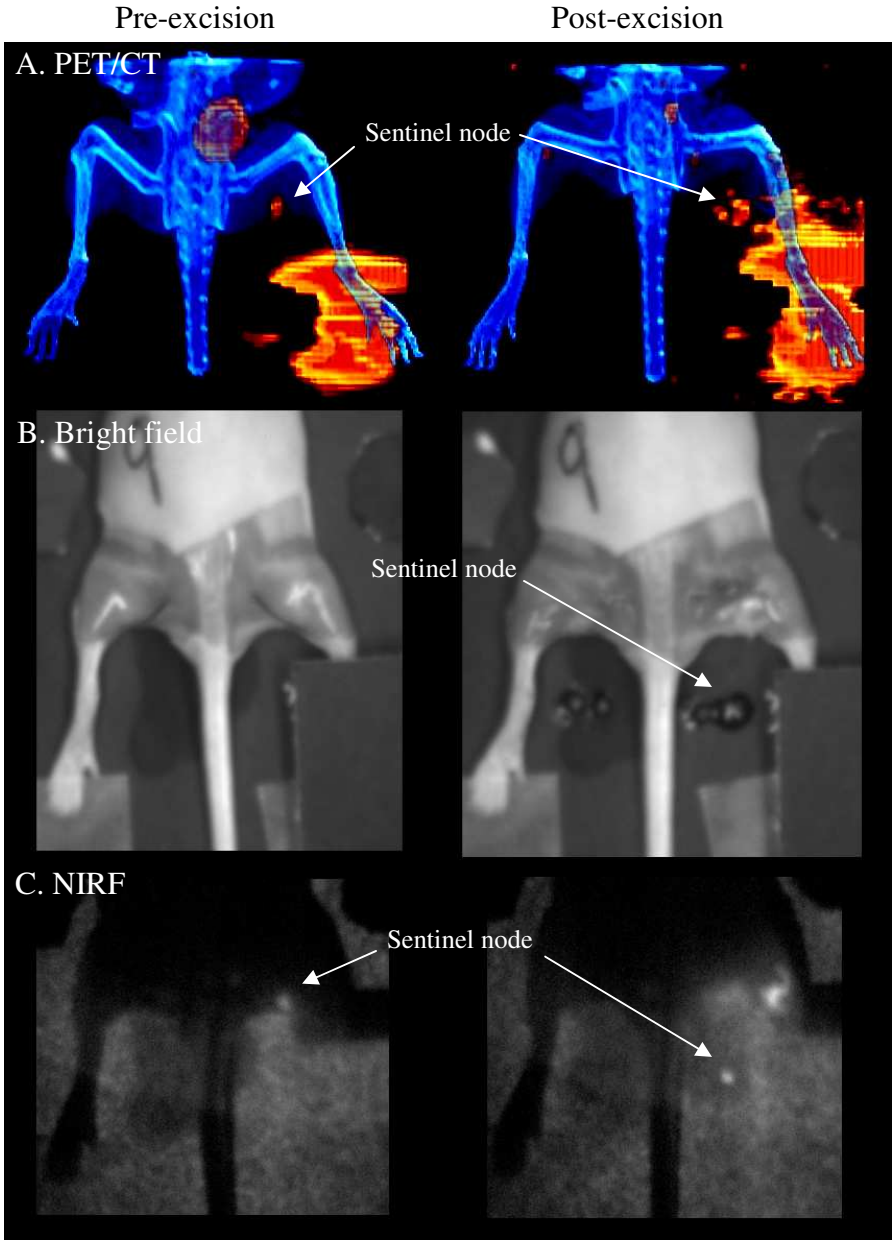
High dose 11 nmol [^{18}F]-Lymphoseek **3** injections:

Mouse 1 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 007)



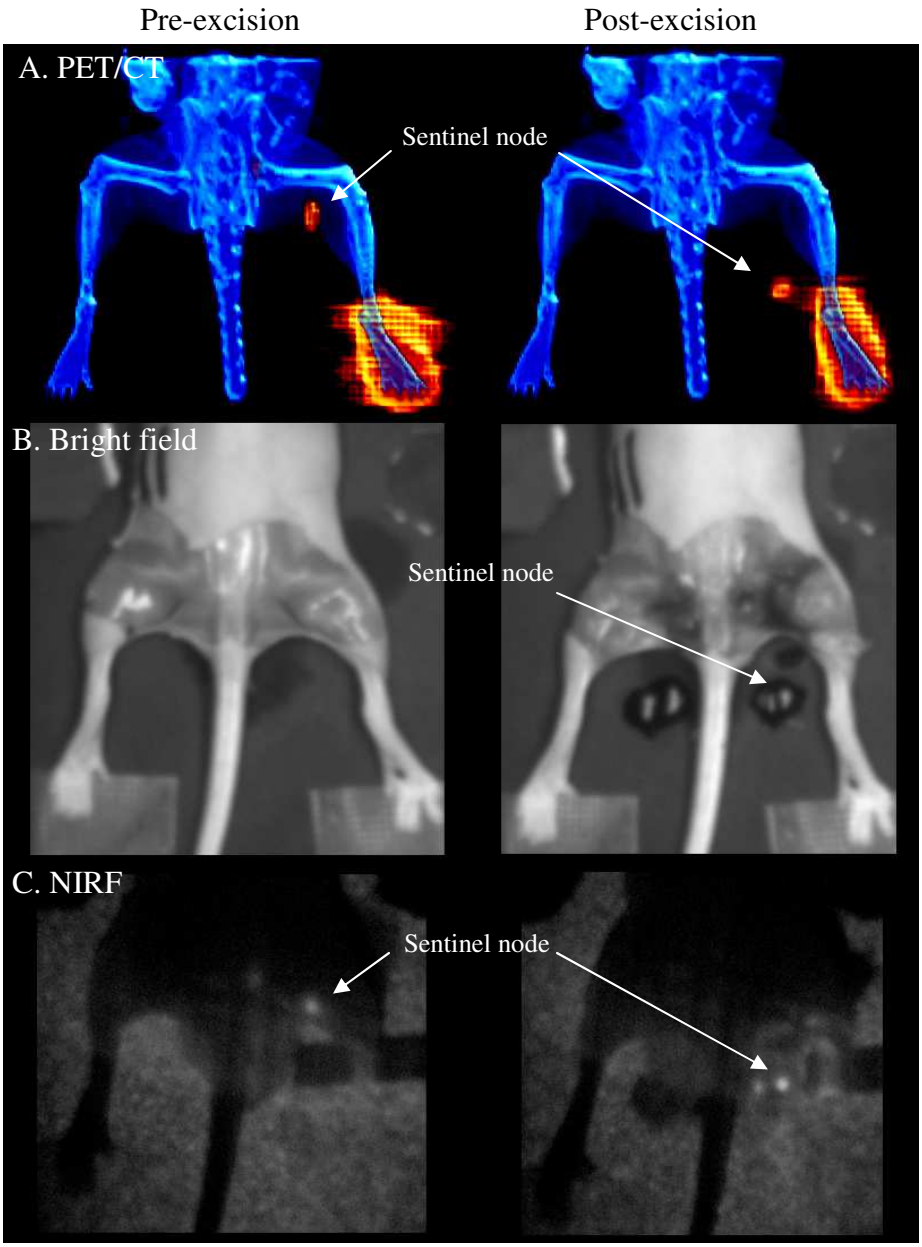
Mouse 007 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained a 40.7 µCi, 0.041 Ci/µmol of ^{18}F at 10:21 AM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 27.9 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. A 20 min PET scan was carried out 2 hours and 57 min vs. TOI on 13.3 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before (left) and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 33 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Mouse 2 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 009)



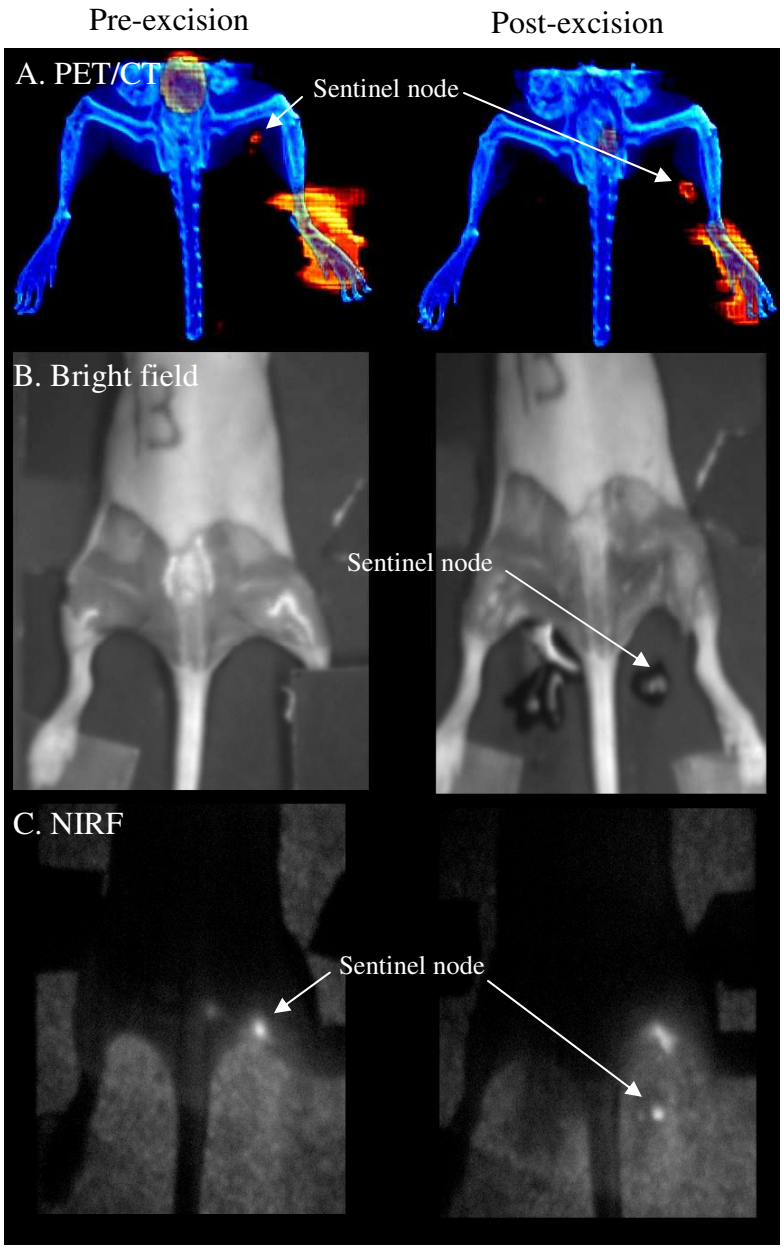
Mouse 009 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained a 31.3 µCi, 0.0031 Ci/µmol of ^{18}F at 10:51 AM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 21.4 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. A 20 min PET scan was carried out 3 hours and 7 min vs. TOI on 9.6 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 30 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Mouse 3 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 011)



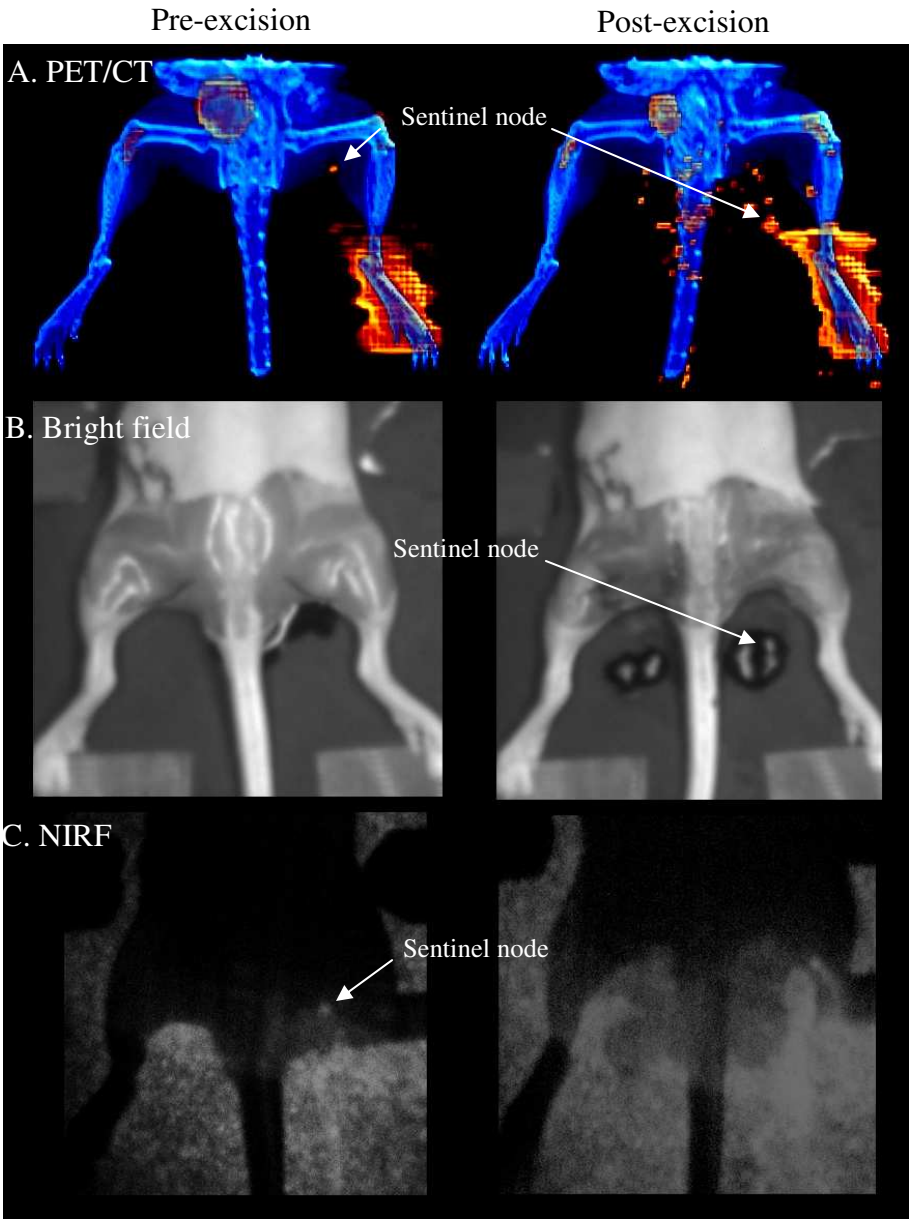
Mouse 011 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained a 23.2 µCi, 0.0023 Ci/µmol of ^{18}F at 11:19 AM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 15.9 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. 20 min PET scan was carried out 3 hours and 21 min vs. TOI on 6.5 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 27 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Mouse 4 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 013)



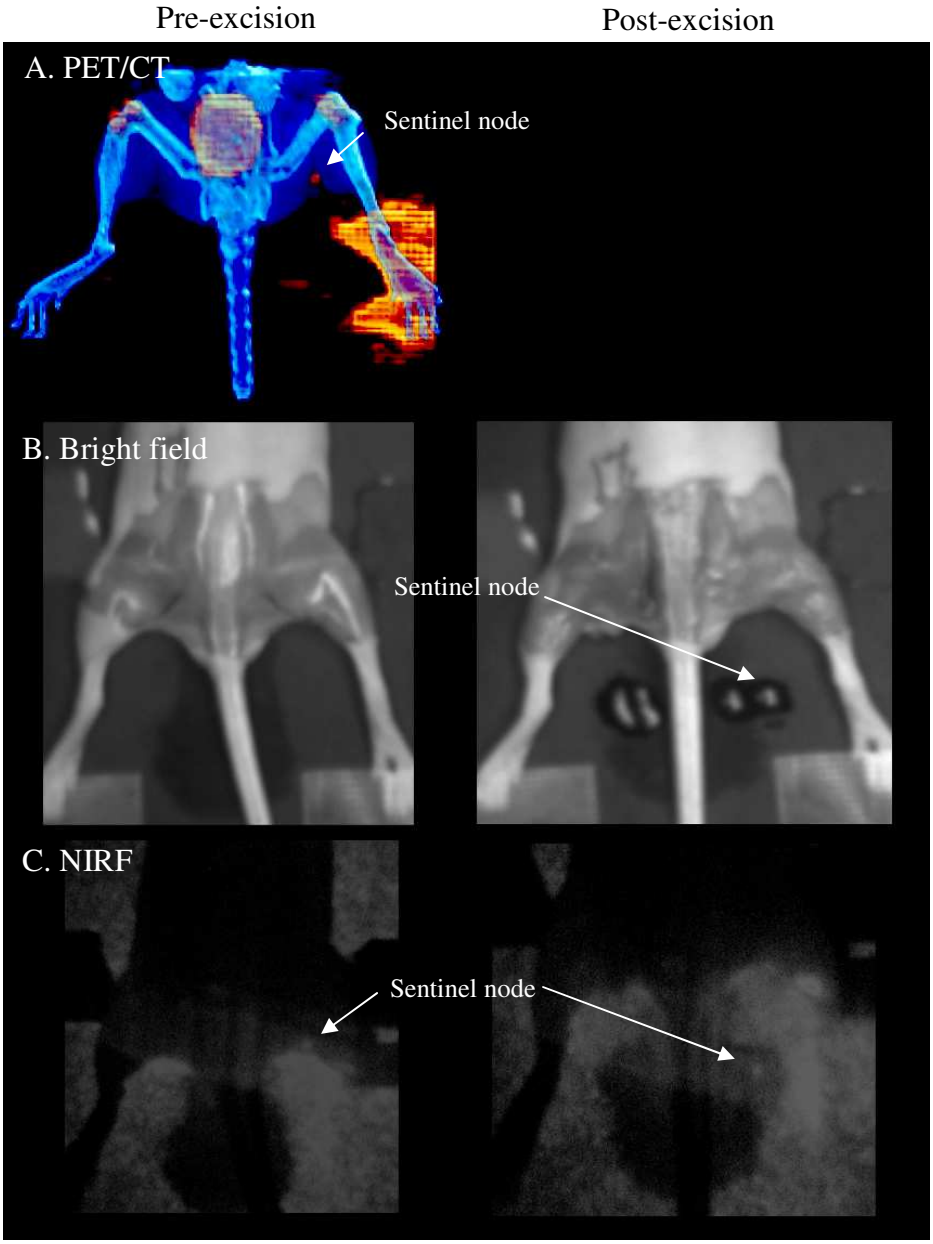
Mouse 013 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained a 14.6 µCi, 0.0015 Ci/µmol of ^{18}F at 2:11 PM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 9.9 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. 30 min PET scan was carried out 3 hours and 9 min vs. TOI on 4.4 µCi of activity (decay corrected). *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 24 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Mouse 5 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 015)



Mouse 015 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained a 11.4 µCi, 0.0011 Ci/µmol of ^{18}F at 2:45 PM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. A. top left, A 20 min pre-excision PET scan carried out 1 hour vs. TOI on 7.8 µCi of activity (decay corrected). *A. top right,* Post-excision PET scan of the mouse with resected nodes placed in the field of view. 30 min PET scan was carried out 3 hours and 19 min vs. TOI on 3.3 µCi of activity (decay corrected). The high background in this scan is due to contamination by urine and the low amount of ^{18}F activity that was used in the scan. *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 17 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Mouse 6 of 6 high-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek 3 (Mouse 017)



Mouse 017 was injected with a 10 µL, 11 nmol dose consisting of 1 nmol of [^{18}F]-labeled Lymphoseek **3** that had been diluted with 10 nmols of unconjugated, unlabeled Lymphoseek. This dose contained an 11.0 µCi, 0.0011 Ci/µmol of ^{18}F at 3:16 PM, the time of injection (TOI). The mouse was sacrificed by isofluorane overdose at 1 hour and 20 min vs. TOI. *A. top left,* 20 min pre-excision PET scan carried out 1 hour vs. TOI on 7.5 µCi of activity (decay corrected). The mouse did not possess sufficient activity for a post-excision PET scan at 3 hours and 59 min vs. TOI (2.4 µCi of activity remained, decay corrected) but sufficient activity remained in extracted nodes for scintigraphy in biodistribution studies. *B.* Bright field image of the mouse with skin removed before *(left)* and after node resection with all resected nodes (right and left lumbar and popliteal) placed in the field of view *(right). C.* NIRF images taken at 1 hour and 24 min vs. TOI on a custom full field IR camera before *(left)* and after *(right)* lymph node resection. When identifiable, the location of the sentinel lymph node is indicated with a white arrow.

Biodistribution:

Lymph node scintigraphy data:

Processed count data for each mouse is shown in the table below:

Mouse #	Organ	%ID	Net Wt. (g)	%ID/g	Average %ID	Average Mass (mg)	Average %ID/g
Low-dose 1 nmol injections of [^{18}F]-labeled Lymphoseek (3)							
6	Foot (Right)	92.42	0.1904	485.42	58 ± 11	179 ± 5	320 ± 60
8	Foot (Right)	85.96	0.1870	459.68			
10	Foot (Right)	16.70	0.1801	92.73			
12	Foot (Right)	45.90	0.1714	267.80			
14	Foot (Right)	52.53	0.1873	280.46			
16	Foot (Right)	54.21	0.1617	335.24			
6	Popliteal Lymph Node (Right)	0.37	0.0010	370.83	0.28 ± 0.09	1.9 ± 0.4	170 ± 50
8	Popliteal Lymph Node (Right)	0.38	0.0019	197.67			
10	Popliteal Lymph Node (Right)	0.11	0.0028	39.72			
12	Popliteal Lymph Node (Right)	0.64	0.0033	194.37			
14	Popliteal Lymph Node (Right)	0.14	0.0020	72.43			
16	Popliteal Lymph Node (Right)	0.07	0.0006	124.19			
6	Lumbar Lymph Node (Right)	0.07	0.0016	46.83	0.03 ± 0.01	1.4 ± 0.2	22 ± 6
8	Lumbar Lymph Node (Right)	0.04	0.0020	22.17			
10	Lumbar Lymph Node (Right)	0.02	0.0019	10.96			
12	Lumbar Lymph Node (Right)	0.01	0.0010	9.55			
14	Lumbar Lymph Node (Right)	0.02	0.0010	16.26			
16	Lumbar Lymph Node (Right)	0.03	0.0010	29.02			
6	Ratio Popliteal/ Lumbar (Right)	4.949	0.625	7.919	16 ± 10	1.5 ± 0.4	8.3 ± 2.6
8	Ratio Popliteal/ Lumbar (Right)	8.471	0.950	8.917			
10	Ratio Popliteal/ Lumbar (Right)	5.341	1.474	3.624			
12	Ratio Popliteal/ Lumbar (Right)	67.192	3.300	20.361			
14	Ratio Popliteal/ Lumbar (Right)	8.911	2.000	4.455			
16	Ratio Popliteal/ Lumbar (Right)	2.567	0.600	4.279			
High-dose 11 nmol injections of [^{18}F]-labeled Lymphoseek (3)							
7	Foot (Right)	82.92	0.1769	468.76	68 ± 6	177 ± 5	390 ± 40
9	Foot (Right)	84.37	0.1654	510.09			
11	Foot (Right)	77.37	0.1705	453.78			
13	Foot (Right)	49.59	0.1972	251.45			
15	Foot (Right)	53.59	0.1876	285.66			
17	Foot (Right)	58.74	0.1669	351.95			
7	Popliteal Lymph Node (Right)	0.90	0.0010	897.71	0.36 ± 0.14	1.5 ± 0.3	300 ± 140
9	Popliteal Lymph Node (Right)	0.35	0.0010	348.03			
11	Popliteal Lymph Node (Right)	0.58	0.0014	412.69			
13	Popliteal Lymph Node (Right)	0.28	0.0030	92.62			
15	Popliteal Lymph Node (Right)	0.03	0.0017	17.22			
17	Popliteal Lymph Node (Right)	0.04	0.0007	59.67			
7	Lumbar Lymph Node (Right)	0.07	0.0010	70.08	0.07 ± 0.02	0.95 ± .06	75 ± 30
9	Lumbar Lymph Node (Right)	0.05	0.0007	75.24			
11	Lumbar Lymph Node (Right)	0.18	0.0009	200.53			
13	Lumbar Lymph Node (Right)	0.07	0.0011	65.22			
15	Lumbar Lymph Node (Right)	0.02	0.0011	16.92			
17	Lumbar Lymph Node (Right)	0.02	0.0009	21.77			
7	Ratio Popliteal/ Lumbar (Right)	12.809	1.000	12.809	5.0 ± 1.7	1.5 ± 0.3	4.1 ± 1.8
9	Ratio Popliteal/ Lumbar (Right)	6.608	1.429	4.625			
11	Ratio Popliteal/ Lumbar (Right)	3.201	1.556	2.058			
13	Ratio Popliteal/ Lumbar (Right)	3.873	2.727	1.420			
15	Ratio Popliteal/ Lumbar (Right)	1.573	1.545	1.018			
17	Ratio Popliteal/ Lumbar (Right)	2.132	0.778	2.741			

Summary of Scintigraphy Data:

Tissue	1 nmol Lymphoseek injection			11 nmol Lymphoseek injection		
	% ID	Mass (mg)	% ID/g	% ID	Mass (mg)	% ID/g
Tissue Biodistribution						
Foot (Right)	58 ± 11	179 ± 5	**320 ± 60**	68 ± 6	177 ± 5	**390 ± 40**
Popliteal Lymph Node (Right)	0.28 ± 0.09	1.9 ± 0.4	**170 ± 50**	0.36 ± 0.14	1.5 ± 0.3	**300 ± 140**
Lumbar Lymph Node (Right)	0.03 ± 0.01	1.4 ± 0.2	**22 ± 6**	0.07 ± 0.02	0.95 ± .06	**75 ± 30**
Foot (Left)	0.26 ± 0.06	168 ± 3	**1.6 ± 0.4**	0.28 ± 0.06	158 ± 4	**1.7 ± 0.3**
Popliteal Lymph Node (Left)	0.004 ± 0.004	1.3 ± 0.2	**5 ± 3**	0.010 ± 0.005	1.5 ± 0.2	**8.8 ± 3.5**
Lumbar Lymph Node (Left)	0.010 ± 0.002	1.4 ± 0.2	**8 ± 2**	0.016 ± 0.006	0.87 ± 0.09	**23 ± 10**
Blood	0.15 ± 0.02	270 ± 30	**0.61 ± 0.08**	0.16 ± 0.02	250 ± 30	**0.7 ± 0.1**
Liver	1.6 ± 0.4	520 ± 60	**3.0 ± 0.6**	0.65 ± 0.07	360 ± 30	**1.8 ± 0.2**
Kidney	0.38 ± 0.09	360 ± 20	**1.1 ± 0.3**	0.7 ± 0.2	340 ± 10	**2.2 ± 0.6**
Spleen	0.14 ± 0.01	72 ± 5	**2.0 ± 0.2**	0.12 ± 0.01	69 ± 5	**1.7 ± 0.2**
Bone	0.11 ± 0.02	39 ± 4	**3.2 ± 0.8**	0.12 ± 0.02	39 ± 3	**3.4 ± 0.7**
Fold accumulation of ^{18}F-3 in the sentinel node over the distal node	16 ± 10	1.5 ± 0.4	**8.3 ± 2.6**	5.0 ± 1.7	1.5 ± 0.3	**4.1 ± 1.8**
% ^{18}F-3 Activity extracted in the sentinel node vs. all extracted lymph nodes	87 ± 7 (%)			74 ± 7 (%)		

Tissue biodistribution of [^{18}F]- Lymphoseek 3 in mice that were sacrificed 1 hour and 20 min following injection. Biodistribution data from 12 mice were used to compile this table. Data from 6 mice were obtained per injected Lymphoseek dose. "Fold accumulation" is defined as the ratio of activity in the sentinel node to the distal node in individual mice. The "% activity extracted" is defined as the difference in counts between the sentinel and distal lymph nodes minus the sum of counts in the sentinel and distal lymph nodes ((Count $s_{popliteal}$ – Counts $_{lumbar}$)/ (Counts $_{popliteal}$ + Counts $_{lumbar}$). Error = $\sigma/(n=6)^{1/2}$. The probability associated with a student's t-test (one-tailed distribution, two-sample unequal variance) = 0.022.

CHEMICAL SYNTHESIS:

General synthetic methods:

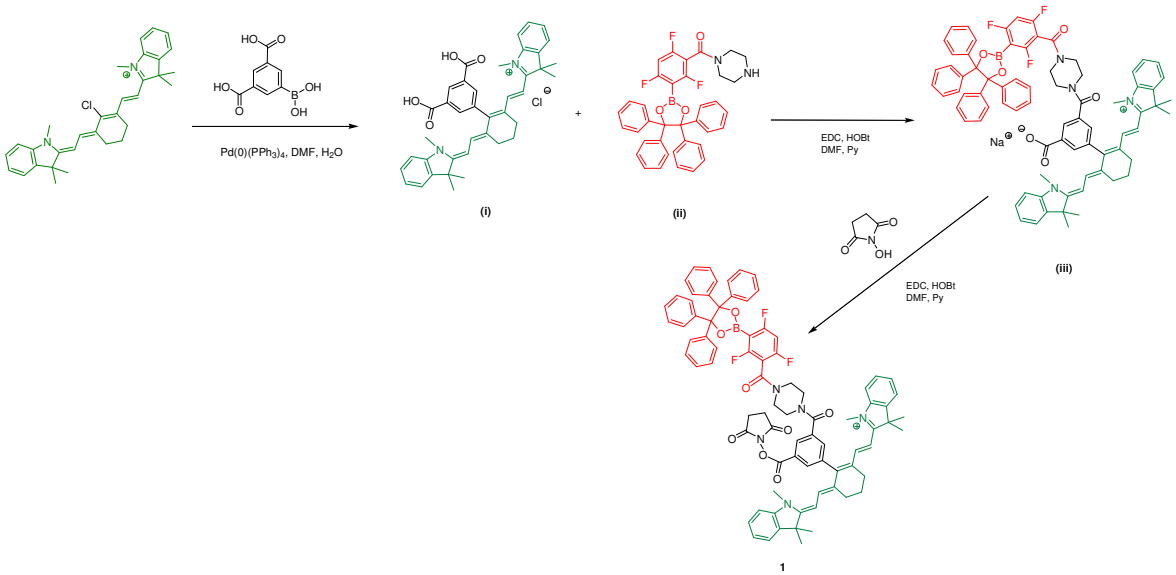
Fluoride containing compounds were purchased from Oakwood Products. Lymphoseek was received as a gift from Reliable Biopharmaceutical. Other chemicals were purchased from Aldrich, Strem, or Combi blocks. Deuterated solvents were purchased from Cambridge Isotope Laboratories.

All ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on Varian 300 or 400 MHz Mercury or 500 MHz Joel instruments. Chemical shifts are reported using the δ scale in ppm and all coupling constants (J) are reported in hertz (Hz). ^{1}H NMR spectra are referenced to the tetramethylsilane peak (δ = 0.00) and ^{19}F NMR spectra are referenced to NEAT CF_2Cl_2 (δ = 0.00). All thin layer chromatography experiments were run on Silica Gel 60 F_{254} Glass TLC plates from EMD chemicals. Fluorescence readings were made on a Fluorolog 3 spectrophotometer with Datamax v2.2 software. High resolution mass spectrometry was performed at the University of California San Diego (UCSD) Chemistry and Biochemistry Small Molecule/Chemical Mass Spectrometry Facility.

Dual Probe NHS ester synthesis:

The synthesis of the dual probe PET/NIR NHS ester **1**, was synthesized in 3 steps. The crystallization of **(i)** is the lowest yielding step in the reaction scheme (12%). **If higher product yields are desired, direct use of (i) following acidic extraction can be carried out in lieu of crystallization. In this case the final yield of 1 is 50.7 %.**

The scheme for the synthesis of the generally conjugatable PET/NIR-probe is as shown in Scheme 1:



Scheme 1. Synthetic scheme detailing the synthesis of an activated NHS ester of the ^{18}F/ heptamethine cyanine (C7-Cy) PET/NIR probe. Boron based ^{18}F trap technology is shown in red, while the NIR fluorophore is shown in green.

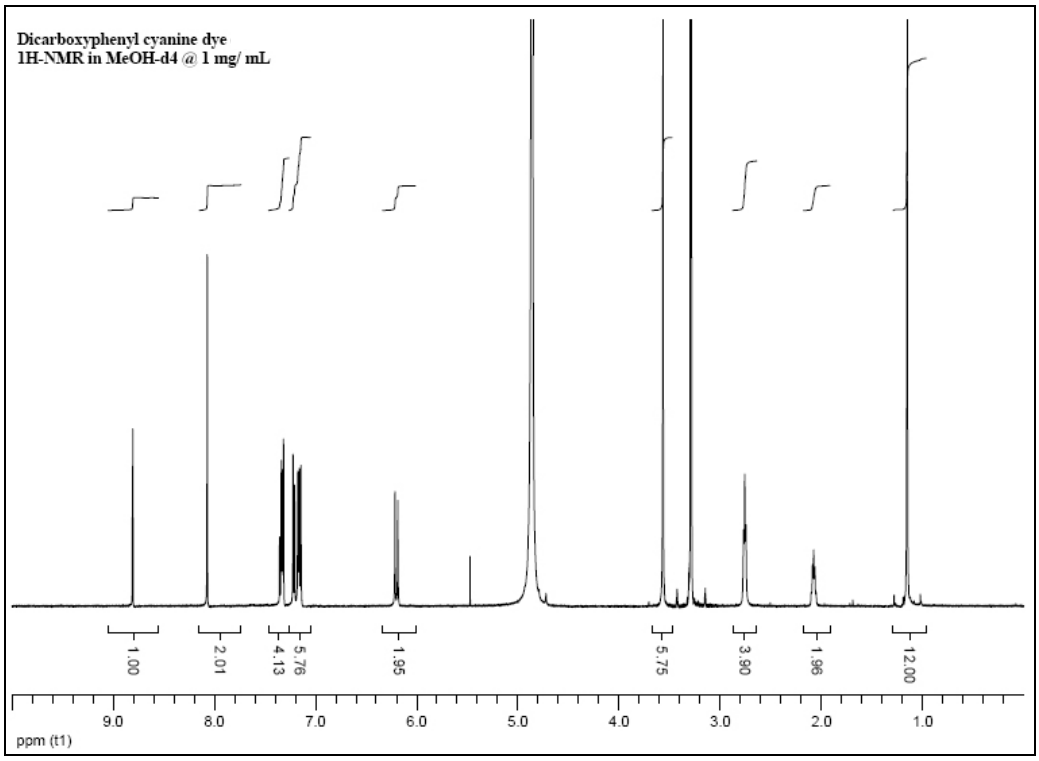
Synthesis of dicarboxyphenyl cyanine dye (i)

Reagents were added to a 25 mL round bottom flask charged with a magnetic stir bar in the following order: IR-775 (200 mg, 0.48 mmols, Sigma-Aldrich #544914), 3,5-Dicarboxyphenyl boronic Acid (200 mg, 0.97 mmols, Combi Blocks #BB-3936), Tetrakis(triphenylphosphine)palladium (0) (60 mg, 0.053 mmols, Strem #46-2150), 9 mL of MeOH, 1 mL of triethylamine, and 1 mL of H_2O. This reaction was stirred for 14 hours at room temperature and then concentrated to oil. **78% of all 775 nm absorbing material was converted to the product (i) as observed by LCMS.** The oil was resuspended in 100 mL of methylene chloride (CH_2Cl_2), and water soluble products were extracted with 50 mL of H_2O. The CH_2Cl_2 layer was washed with 100 mL of saturated ammonium chloride, 100 mL of 100 mM sodium bicarbonate, and then two washes of 100 mL of 1 M HCl. The product (i) could be converted to (ii) as is, but if pure product was desired for NMR, QY, or absorbance coefficient studies, (i) could be crystallized.

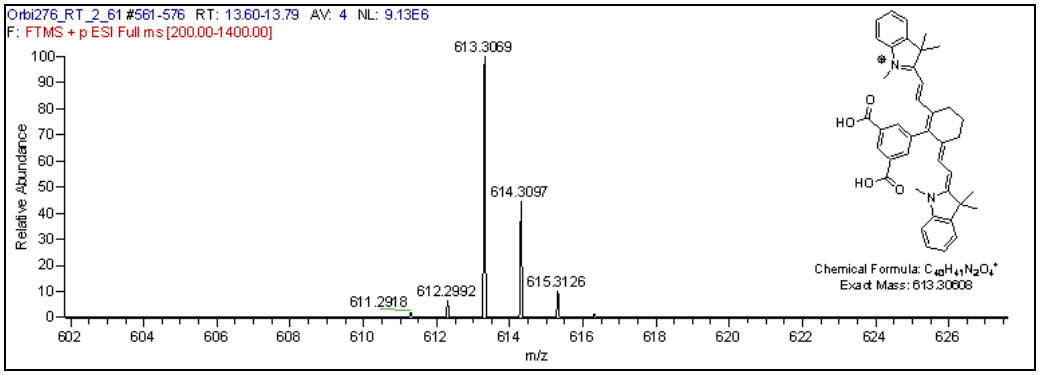
To crystallize (i), product contained in the CH_2Cl_2 layer was filtered through Whatman #5 filter paper and concentrated to ~ 5 mL at which point crystals of the **(i)** began to form. Filtration and collection of **(i)** through Whatman #5 filter paper resulted in a first crop containing 54.5 mg (0.08 mmols, 12 %) of crystalline product.

^{1}H NMR (MeOH-d4, 500 MHz, 21°C): 8.81 (*t*, J= 2 Hz, 1H), 8.07 (*d*, J= 1 Hz, 2H), 7.35-7.315 (*m*, 4H), 7.22-7.14 (*m*, 6H), 6.20 (*d*, J= 14 Hz, 2H), 3.57 (*s*, 6H), 2.76 (*t*, J= 6 Hz, 4H), 2.08 (*t*, J= 6 Hz, 2H), 1.15 (*s*, 12H). HRMS (ESI) calculated for $C_{40}H_{41}N_2O_4^+$ $(M)^+$: 613.3061, found 613.3069. Spectrophotometric constants on a 0.322 µM solution in water: λ_{max} = 749 nm, ε_{749} = 107 000 cm^{-1} M^{-1}, ex= 747 nm, em = 765 nm, and φ = 0.033.

^{1}H-NMR



HRMS-ESI^{+}



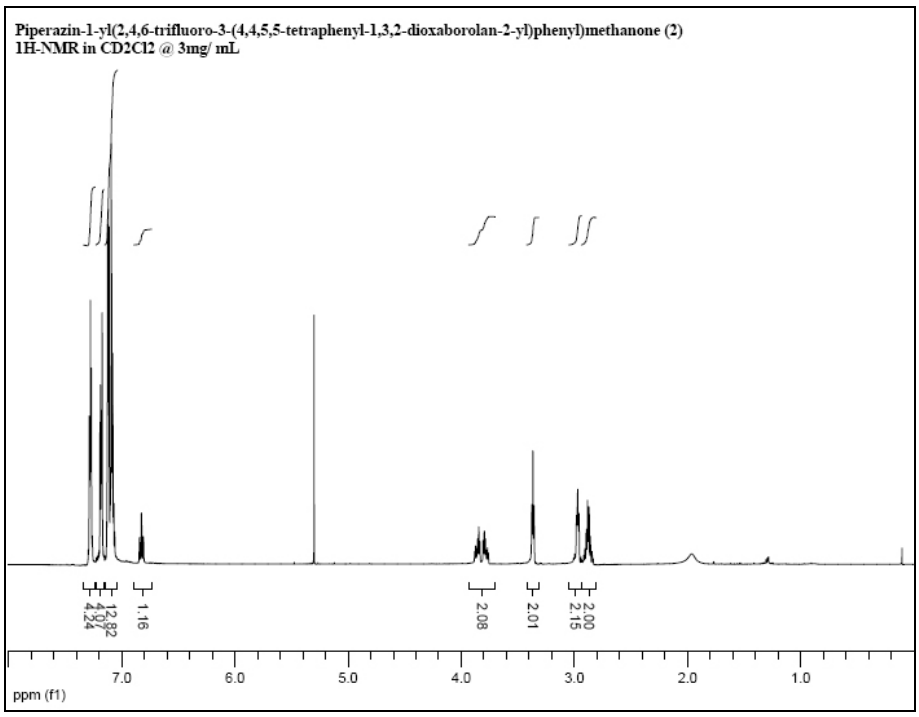
Piperazin-1-yl(2,4,6-trifluoro-3-(4,4,5,5-tetraphenyl-1,3,2-dioxaborolan-2-yl)phenyl)methanone (ii)

Reagents were added to a 25 mL vial containing 3.55 mL of DMF in the following order: piperazine (52 mg, 0.60 mmols), pyridine (133 µL, 1.56 mmols), HOBt monohydrate (61 mg, 0.40 mmols), 2,4,6-Trifluoro-3-(4,4,5,5-tetraphenyl-1,3,2-dioxaborolan-2-yl)benzoic acid (200 mg, 0.36 mmols, synthesis (1 step from commercially available reagents, synthesis and reagent sources previously described (*1*))), and N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC) (77 mg, 0.40 mmols). This reaction was left at room temperature for 24 hours after which it was concentrated to

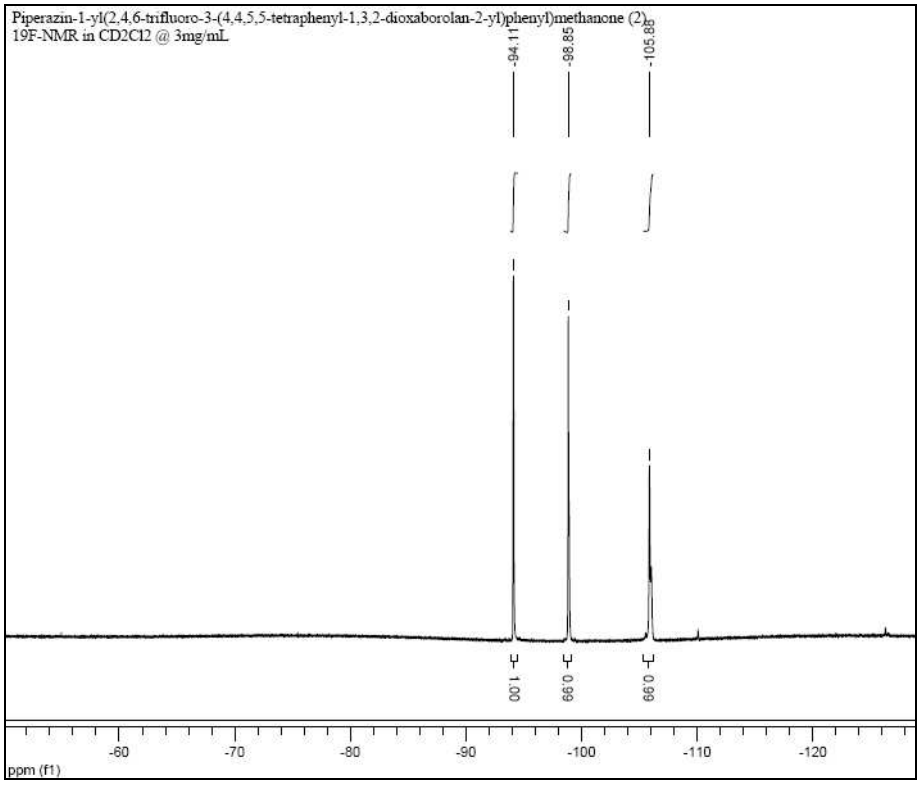
oil. A 100 mg amount of piperazine in 1 mL of CH_2Cl_2 was added to the oil to keep **(ii)** in its deprotonated state. The oil was loaded onto a 10 cm x 2.5 cm silica column where **(ii)** was eluted with 2 % MeOH/$CHCl_3$. Elution was monitored by TLC where **(ii)** displays an R_f of 0.30 in a 30 % MeOH/$CHCl_3$ v/v developing solution and gives a positive result in ninhydrin tests. The appropriate fractions were concentrated to give 136.8 mg of **(ii)** (a 61 % yield) as a white solid. The product, **(ii)**, is best stored as a 10 to 100 mM solution in CH_2Cl_2 at -78 °C.

^{1}H NMR (CD_2Cl_2, 500 MHz, 21 °C): 7.28-7.25 (*m*, 4H, CH), 7.22-7.19 (*m*, 4H, CH), 7.14-7.09 (*m*, 12H, CH), 6.87 (*t*, J= 9 Hz, 1H), 3.80-3.68 (*m*, 2H), 3.42-3.32 (*m*, 2H), 2.92-2.79 (*m*, 2H), 2.61-2.51 (*m*, 2H). ^{13}C NMR (MeOH-D4, 400 MHz): 164.9, 162.2, 145.2, 144.3, 130.2, 129.6, 129.1, 128.4, 128.1, 127.8, 125.6, 125.5, 118.8, 112.6, 96.8, 45.9, 45.3, 44.8, 40.8, 37.1, 31.8. ^{19}F NMR (CD_2Cl_2, 300 MHz): -94.11 (1F), -98.85 (1F), -105.88 (1F). HRMS (ESI) calculated for $C_{37}H_{31}BF_3N_2O_3^+$ $(M+H)^+$: 619.2374 m/z, found: 619.2394.

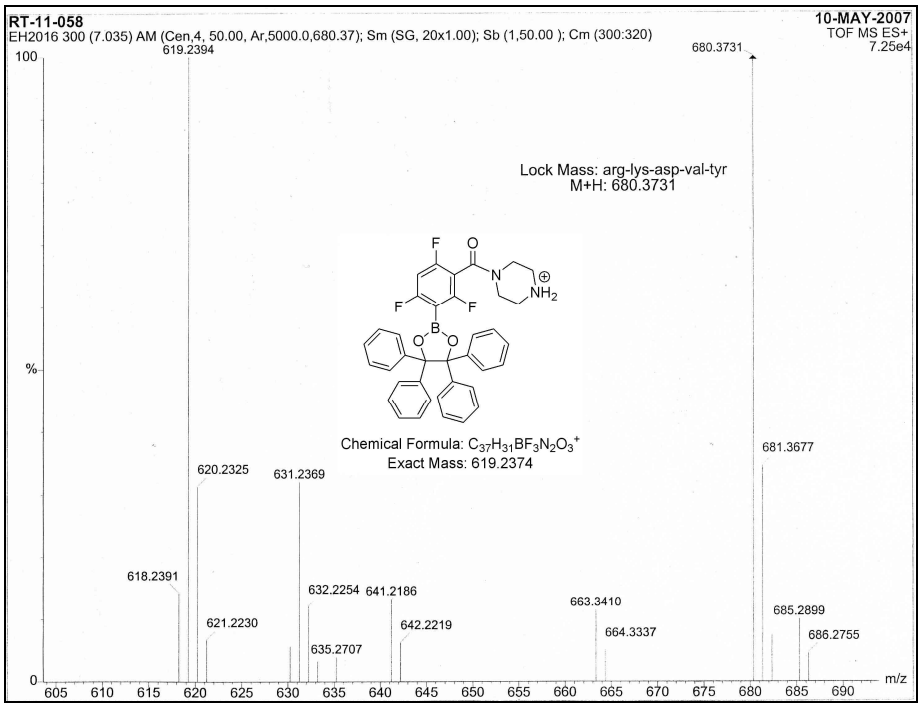
^{1}H- NMR



^{19}F- NMR



HRMS-ESI$^+$

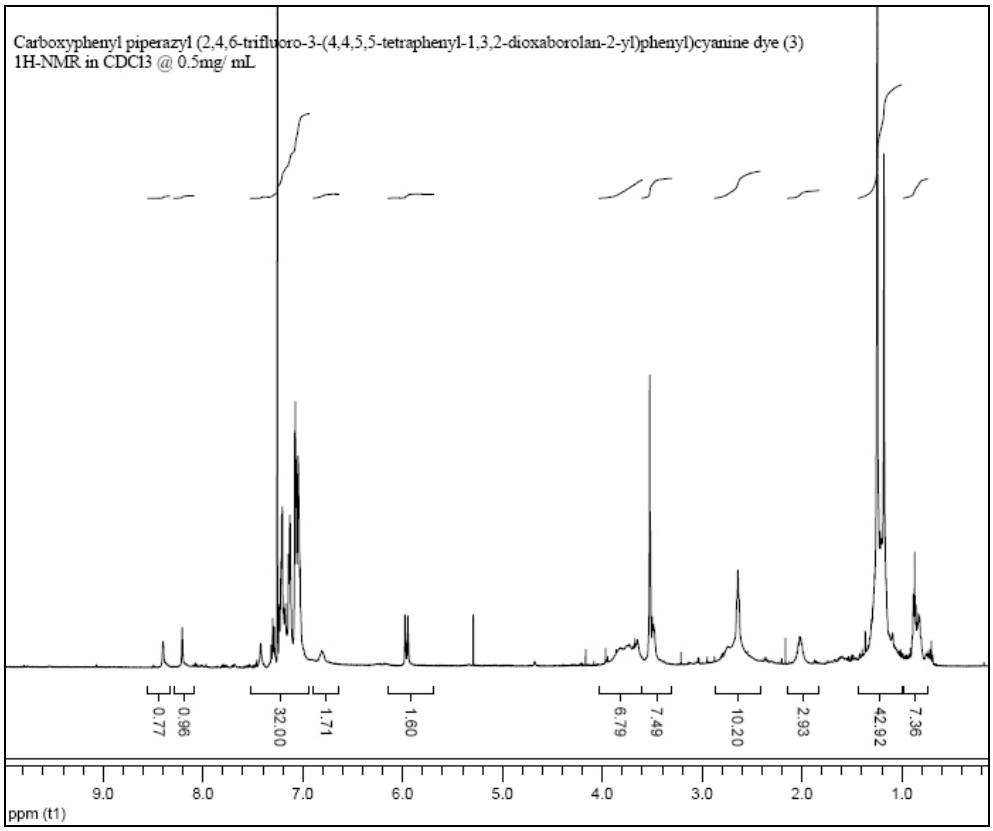


Synthesis of carboxyphenyl piperazyl (2,4,6-trifluoro-3-(4,4,5,5-tetraphenyl-1,3,2-dioxaborolan-2-yl)phenyl)cyanine dye (iii)

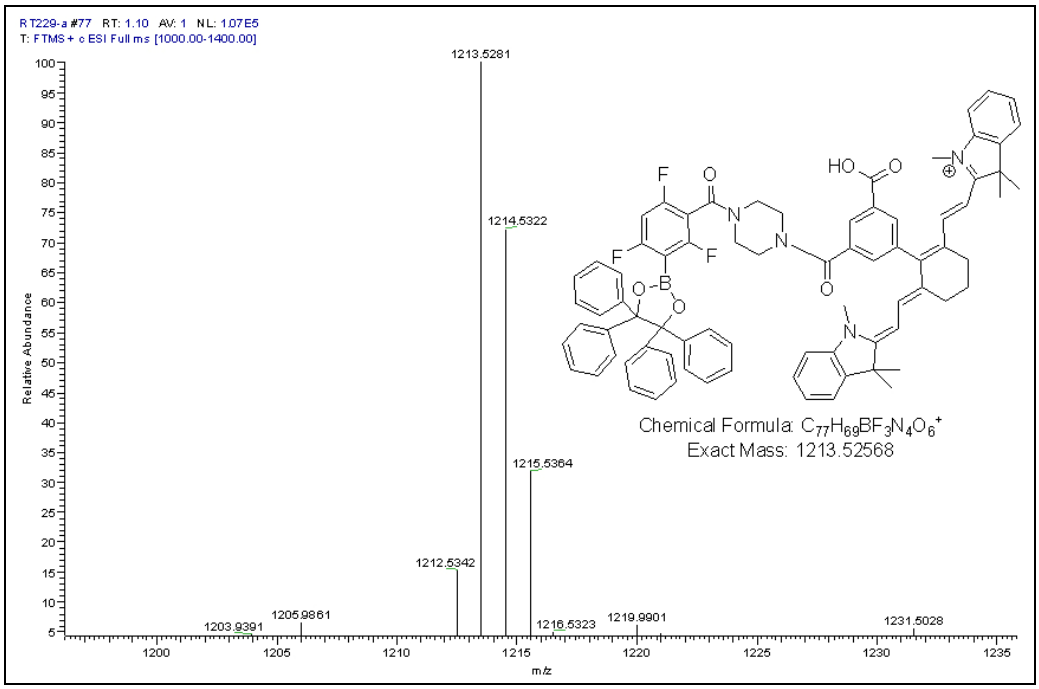
A 1.04 mL, 21.6 mM solution of **(ii)** (15 mg, 0.0225 mmols) in CH_2Cl_2 was added to **(i)** (15 mg, 0.0225 mmols). This solution was concentrated to dryness and the following reagents were added in the following order: DMF (0.9 mL), pyridine (11 µL, 0.132 mmols), HOBt monohydrate (3.45 mg, 0.0225 mmols), and N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC) (4.5 mg, 0.0225 mmols). This reaction was sonicated for 1 hour, left at room temperature for 2 hours and then quenched with 40 mL of chloroform. This volume of chloroform was washed once with saturated aqueous ammonium chloride and twice with 40 mL of deionized water before it was concentrated. The resulting solid was loaded onto a 10 cm x 2.5 cm silica column where **(iii)** was eluted with 2 litres of 8 % MeOH/$CHCl_3$. Elution was monitored by TLC where **(iii)** displays an R_f of 0.10 with a 15 % MeOH/$CHCl_3$ v/v developing solution. The appropriate fractions were concentrated to give 17.6 mg of **(ii)** (a 65 % yield) as a stable green residue.

^{1}H NMR ($CDCl_3$, 500 MHz, 21 °C): 8.40 (*s*, 1H), 8.21 (*s*, 2H), 7.42-7.03 (*m*, 30H), 6.81 (*broad*, 1H), 5.96 (*d*, J= 7 Hz, 2H), 3.90-3.65 (*m*, 4H), 3.52 (*s*, 6H), 2.50-2.48 (*m*, 2H), 2.75-2.61 (*m*, 6H), 2.02 (*m*, 2H), 1.24-1.18 (*m*, 12H). HRMS (ESI) calculated for $C_{77}H_{69}BF_3N_4O_6^+$ $(M)^+$: 1213.5257 m/z, found: 1213.5281.

^{1}H- NMR



HRMS-ESI^{+}



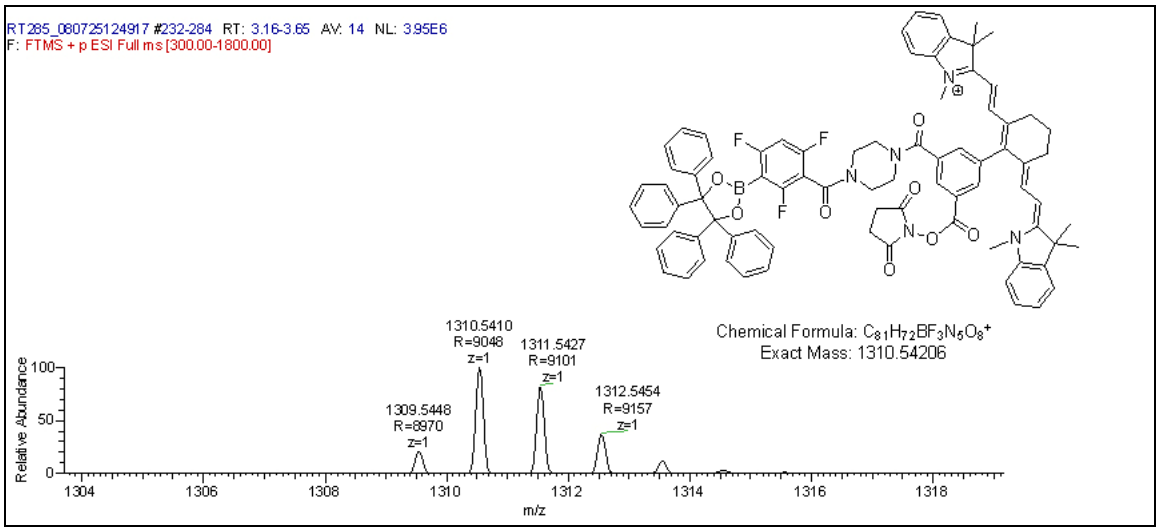
Synthesis of N-Hydroxysuccinimide phenyl piperazyl (2,4,6-trifluoro-3-(4,4,5,5-tetraphenyl-1,3,2-dioxaborolan-2-yl)phenyl)cyanine dye 1

Reagents were added to a 1.5 mL vial in the following order: **(iii)** (8.9 mg, 7.3 µmols), N-Hydroxysuccinimide (1.2 mg, 11 µmols), HOBt monohydrate (1.7 mg, 11 µmols), DMF (144 µL), pyridine (1.2 µL, 14 µmols), and N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC) (2.2 mg, 11 µmols). This reaction was left at room temperature for 3 hours after which it was directly diluted with 2 ml of CH_2Cl_2. The CH_2Cl_2 layer was washed 3 times with 2 mL of deionized water. The CH_2Cl_2 was decanted directly into a vial and concentrated to dryness.

As a dry, crude mixture following extraction, **1** is stable and can be used as is. Unfortunately, attempts to isolate **1** as a chloride salt for ^{1}H-NMR analysis via silica chromatography gave a disproporationation product. It is speculated that the highly acidic conditions required for transforming **1** into the chloride salt induced this reactivity.

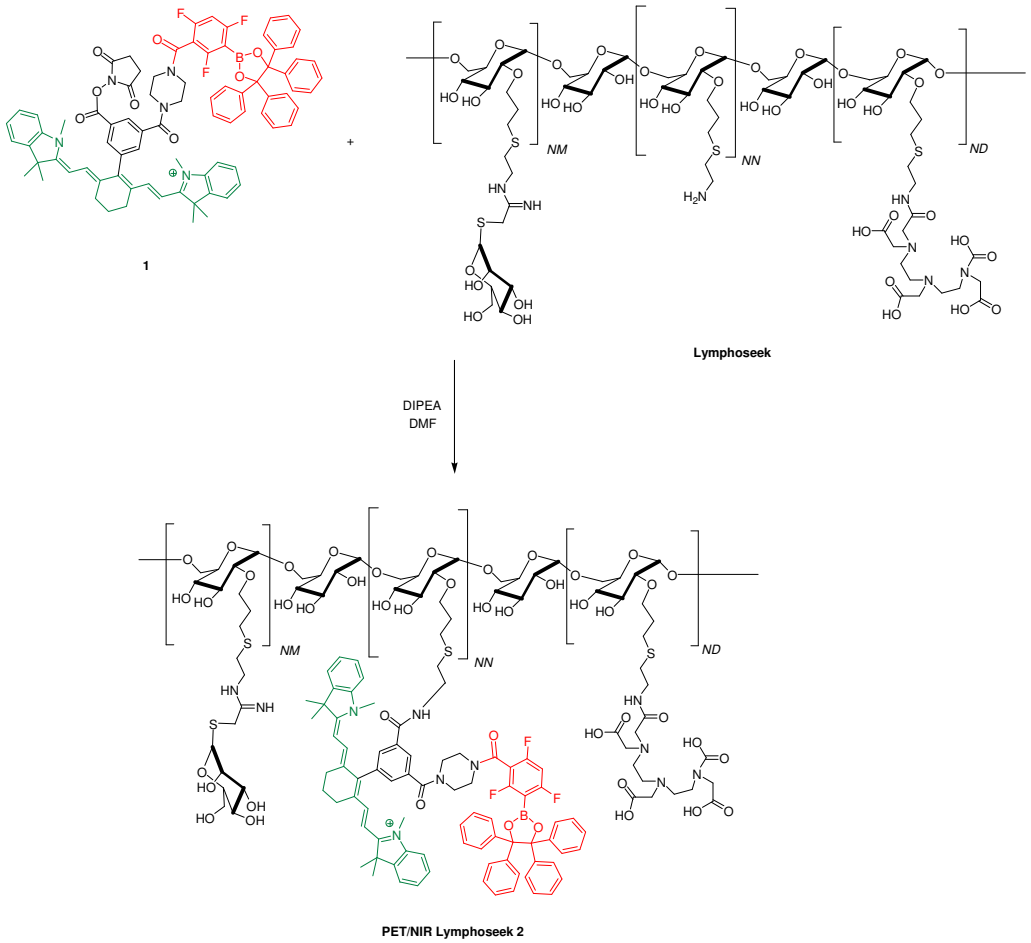
HRMS (ESI) calculated for $C_{81}H_{72}BF_3N_5O_8^+$ $(M+H)^+$: 1310.5421 m/z, found: 1310.5410.

HRMS-ESI$^+$



PET/NIR Dual Probe reaction with Lymphoseek:

The scheme for the conjugation of the PET/NIR-probe NHS ester to Lymphoseek is as shown in Scheme 2:



Scheme 2. Reaction of ^{18}F/ heptamethine cyanine (C7-Cy) PET/NIR NHS ester to DTPA-mannosyl-dextran amine (NN), mannose (NM), and DTPA (ND) (Lymphoseek). Densities were 5.8, 16.5, and 4.2 mol /dextran respectively. Exact densities were obtained from certificate of analysis (vide infra). Average molecular weight was 16122 g/mol. The boron based ^{18}F trap is shown in red, while the NIR fluorophore is shown in green.

Synthesis of PET/NIR- Lymphoseek probe 2

A 5.5 mg quantity of unconjugated Lymphoseek ligand (0.34 µmols, Reliable Biopharmaceutical # 70-2913, vide infra for Certificate of Analysis) bearing 5.8 amine groups per mol dextran was used per synthesis of Lymphoseek **2**. The generally conjugatable NHS ester of the PET/NIR probe, **1**, was added as a 4 µmol solution in CH_2Cl_2 directly into a 1.5 mL glass vial containing 5.5 mg (0.34 µmols) of Lymphoseek. This solution was concentrated to dryness, after which the resulting solid was resuspended in 100 µL of DMF. This solution was sonicated for 1 hour and left to react for 18 hours. The next day, a 10 µL volume of diisopropyl ethyl amine (DIPEA) was added and the reaction was left for another 2 hours. The reaction was then transferred to a polypropylene centrifuge tube containing 25 mL of CH_2Cl_2. On an Allegra X-22R centrifuge, a pellet of **2** was isolated following 20 min of centrifugation at 3 000 rcf. The CH_2Cl_2 supernatant was decanted away from the pellet and fresh CH_2Cl_2 was added to the centrifuge tube. The pellet was triturated into a suspension and the centrifuge process was repeated two more times. The resulting pellet was dried under high vacuum and resuspended in a 1 mL of deionized water. This solution was transferred to a 1.5 mL eppendorf that was centrifuged for 2 min at 18 000 rcf. Water soluble **2** was decanted from the pellet and divided into 4.25 x 10.6 nmol aliquots in 600 µL microcentrifuge tubes. These aliquots were lyophilized and stored at -78 °C. Based on fluorescence readings, 115.9 nmols of fluorophore conjugated Lymphoseek **2** was isolated. Based on a glucose quantization assay (*2*), 49.16 to 33.63 nmols of Lymphoseek was isolated as **2.** This corresponds to a 10-14 % yield of **2** from unconjugated Lymphoseek.

Characterization of Lymphoseek labeling

In order to determine the dextran yield of **2**, a sugar assay was carried out on one aliquot of **2**. To a 10 mL test tube containing a 0.500 mL solution of **2**, 0.300 mL of a 5% phenol solution followed by 1.800 mL of 18 M H_2SO_4 were added. The exothermic reaction was vortexed for one minute and allowed to cool to room temperature after which an absorbance reading at 450 nm was taken. The amount of Lymphoseek contained in an aliquot of **2** was determined from interpolation through a standardized curve prepared from glucose assays on samples with known quantities of Lymphoseek. The reactive nature of 18 M H_2SO_4 was such that no long wavelength fluorescence was observed from **2**, therefore spectroscopic interference by the fluorophore at A_{450} was not an issue. Based on this glucose assay, 49.16 to 33.63 nmols of Lymphoseek was isolated as **2.** This corresponds to a 10-14 % yield of **2** from unconjugated, commercial Lymphoseek. The estimated molar ratio of fluorophore to Lymphoseek is 2 to 3 fluorophores per molecule of Lymphoseek. Spectrophotometric constants of a 1.5 µM solution in water: λ_{max} = 757 nm, ε_{757} = 110 000 cm^{-1} M^{-1}, ex= 757 nm, em = 777 nm, and φ = 0.026.

The estimated molar ratio of fluorophore to Lymphoseek following NHS condensation is 2 to 3 fluorophores per molecule of Lymphoseek. Although there were 5.8 amines on Lymphoseek (vide infra for certificate of analysis) and **1** was added at a ratio of 4:1 of **1** per amine, the same ratio of fluorophore: Lymphoseek labeling was always achieved.

Attempts to label a greater ratio of amines by increasing the molar ratio of **1** to facilitate greater fluorophore transfer did not change the product outcome.

Lymphoseek Certificate of Analysis:

Reliable Biopharmaceutical

Certificate of Analysis

Product: 70-2913 Lymphoseek	**Manufacturing Date**: September 15, 2006 **QC Date**: September 27, 2006 ***Retested**: December 11, 2007
Lot Number: M60834	**Retest Date**: December 11, 2008

Specification		Theory	Found
*Appearance		Off-white to buff colored powder	Off-white powder
Bacterial Endotoxin		Report	<0.08 Eu/mg
Identification (IR)		Conforms to Reference Standard	Conforms
*Water Content		≤ 5.0%	2.0%
Specific Rotation		Report	+156.2°
Heavy Metals		≤ 0.002%	Conforms
Total Microbial Count		≤ 1000 cfu/g	<1000 cfu/g
*Assay		95.0 – 105.0%	104.1%
*Free DTPA Content		≤ 0.5% w/w	N.D.
*Free Mannose Content		≤ 0.5% w/w	N.D.
Determination of Substitutions and MWT	Amine Number	0 – 22 Avg amine groups / mol dextran	5.8
	DTPA Number	3 – 8 Avg DTPA groups / mol dextran	4.2
	Mannose Number	12 – 20 Avg mannose groups / mol dextran	16.5
	Calculated Molecular Wt	Report	16122 g/mol
Residual Solvents	Methanol	≤ 0.2%	N.D.
	Ethanol	≤ 0.5%	N.D.
	Acetone	≤ 0.5%	0.01%
	Total	≤ 1.0%	0.01%

Storage Condition: Store at 15° to 30°C

Remove 0.106 mg 4/22/8 to Richard Ting

Approval Signature/Date 3-18-08

Page 1 of 1

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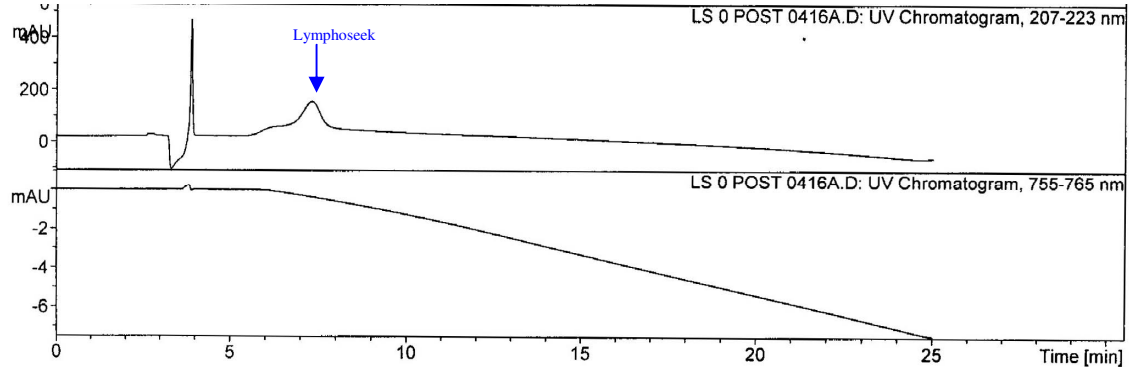
www.reliablebiopharm.com • Mailing Address: P.O. Box 140192 • St. Louis, MO 63114-0192

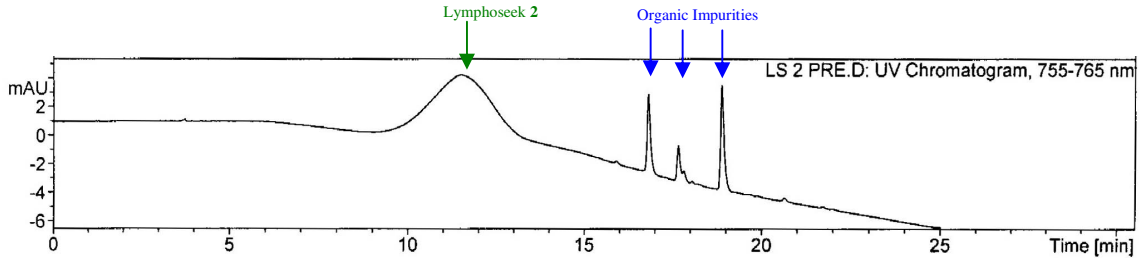
HPLC characterization of 2 and [^{19}F]-3

The HPLC characterization[†] of the reaction to give **2** and HPLC confirmation of the purity in both **2** and [^{19}F]-**3** following size exclusion chromatography are shown below.

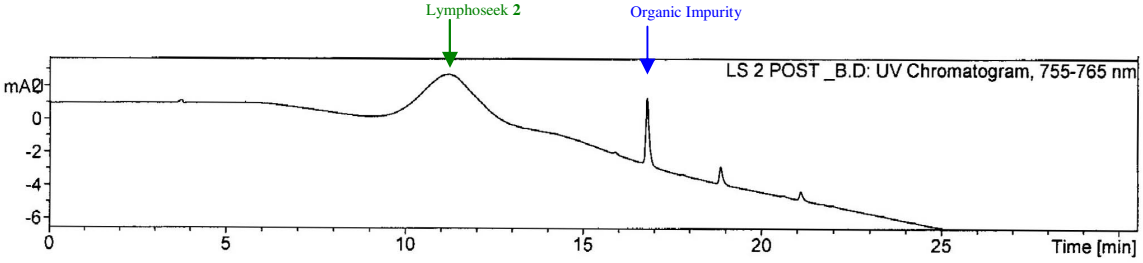
The following spectra are 215 and 780 nm absorbance HPLC elution profiles of unconjugated Lymphoseek alone. Note that Lymphoseek's elution is not sharp as it is a polymeric mixture and that Lymphoseek possesses no absorbance at 760 nm.



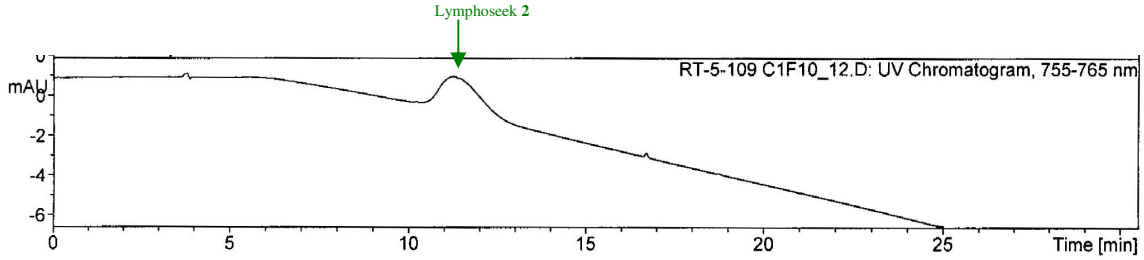
The following spectrum is the 780 nm absorbance HPLC elution profile of the crude, unpurified reaction mixture between **1** and Lymphoseek. The product, Lymphoseek **2**, absorbs at 760 nm due to labeling with **1**. Note the organic impurities at 17, 18, and 19 min from the crude, unpurified reaction mixture.



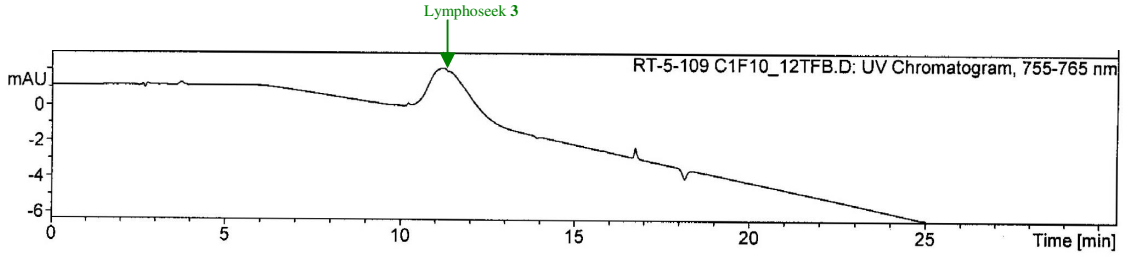
The following spectrum is the 760 nm absorbance HPLC elution profile of the crude reaction mixture containing **2** after it was extracted and passed through a single biorad-P6 size exclusion column. Note some removal of the mentioned organic impurities.



The next spectrum is the 760 nm absorbance HPLC elution profile of **2** after it was passed trough four subsequent Biorad P-6 size exclusion columns. Note the quantitative removal of organic impurities.



The final spectrum is the 760 nm absorbance HPLC elution profile of **2** after conversion to [^{19}F]-**3**, and passage trough four subsequent Biorad P-6 size exclusion columns. Again quantitative removal of organic impurities is achieved.



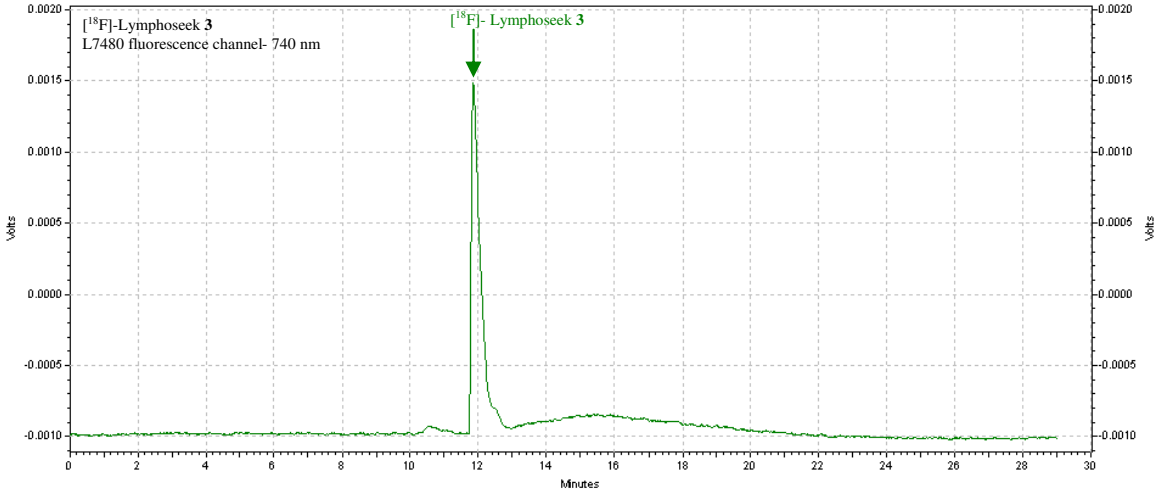
This section demonstrates by HPLC that Lymphoseek can be labeled with **1** to give **2**. It also demonstrates that **2** and **3** can be purified by size exclusion chromatography as described in the materials and methods. The transformation of **2** to **3** is difficult to characterize by MS due to the large mass of Lymphoseek and the fact that it is polymeric. Therefore, to demonstrate by MS that the trifluoroborate technology conveyed on **1** is generally applicable, a pure alterative target was labeled with **1**. MS characterization of this transformation follows the next section.

[†] HPLC Chromatography was carried out on an Agilent 1100 Series HPLC with a phenomenex Luna 5 µ C18(2) 100A 250 x 4.60 mm 5 µ column and a 1 mL/min flow rate with a 20 min, 10 to 90 % elution gradient followed by a 5 min isocratic elution with 90% acetonitrile and water where both solvents contained 0.05% trifluoroacetic acid.

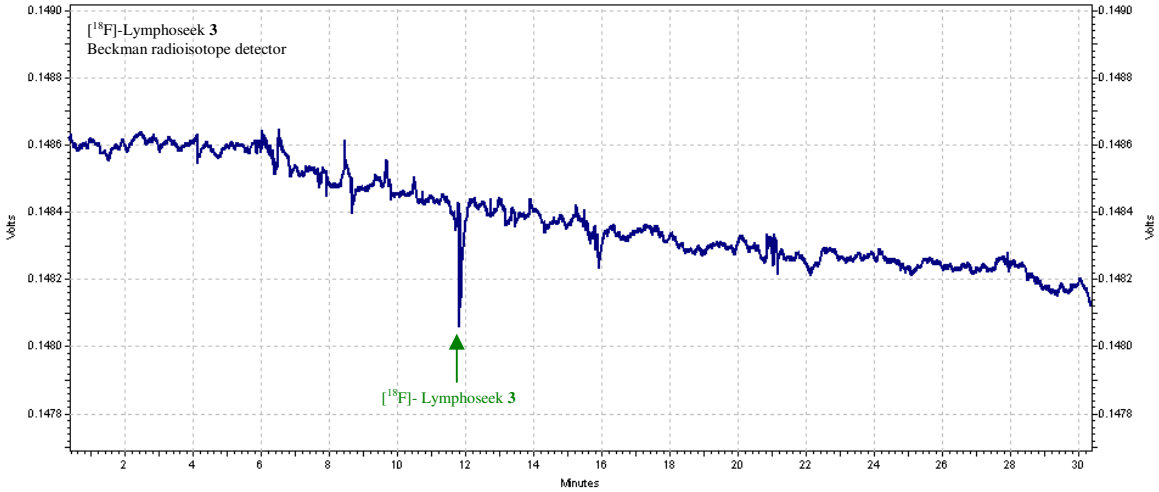
CHARACTERIZATION AND FURTHER APPLICATION

Final radiochemical analysis of [^{18}F]-3

Radiolabeled [^{18}F]-**3** was prepared as described in the materials and methods. The following HPLC analysis of [^{18}F]-**3** demonstrates its radiochemical purity. A 10 µCi amount of [^{18}F]-**3** was loaded on a Beckman Coulter System Gold HPLC equipped with a TOSOH Bioscience TSKgel G2000SW 7.8mm ID x 30 cm x 5 µ M size exclusion column, a L-7480 fluorescence detector, and a Beckman Radioisotope detector. Elution of [^{18}F]-**3** was achieved with an isocratic flow of 0.9% saline at 1 mL/min. The following figure is the elution profile of [^{18}F]-**3** as measured at 740 nm on the L7480 fluorescence detector. Note the single peak in the fluorescence channel that is indicative of a clean synthesis.

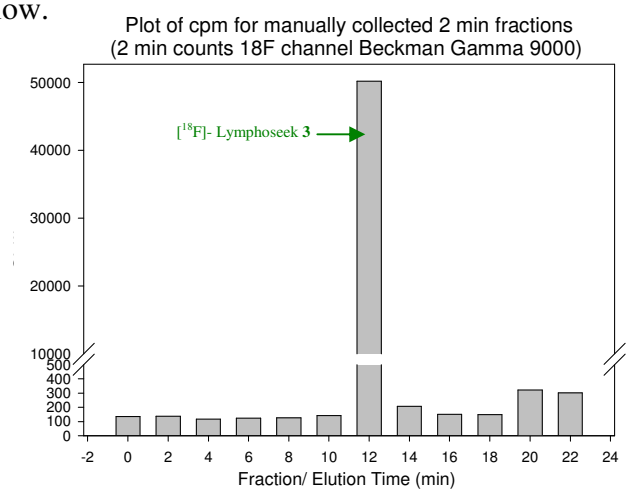


The following figure is the elution profile as observed in the Beckman radioisotope detector channel.

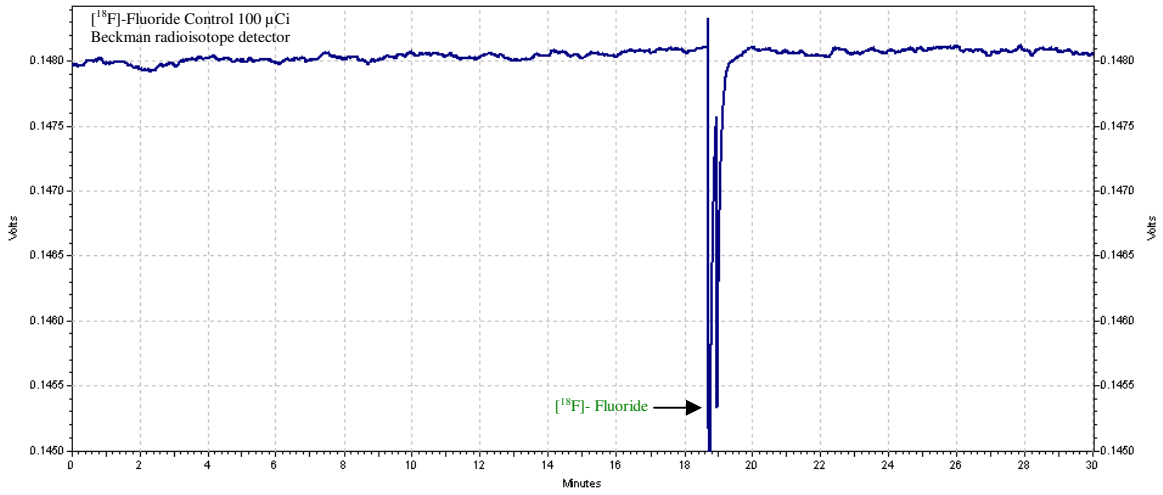


Unfortunately, the available Beckman radioisotope detector did not give a very good signal to noise ratio in the analysis of [^{18}F]-**3**. This detector is intended for use in high

activity syntheses and not for analytical preparations. To demonstrate that the fluorescent peak that is eluted at 12 min, corresponds to a pure sample of [^{18}F]-**3** with minimal radioactive contaminants**,** a 10 µCi sample of [^{18}F]-3 was injected and 2 min fractions were collected manually in eppendorf tubes. These samples were analyzed by scintillation counting on a Beckman Gamma 9000 scintillation counter. The elution profile is shown below.



Note that the majority of the radioactivity is contained within fractions 11-13. This is indicative of a radio-chemically pure species that possesses 740 nm fluorescence. As a control, a 100 µCi sample of [^{18}F]-fluoride was run and detected by the Beckman radioisotope detector. Minimal [^{18}F]-fluoride contamination is observed



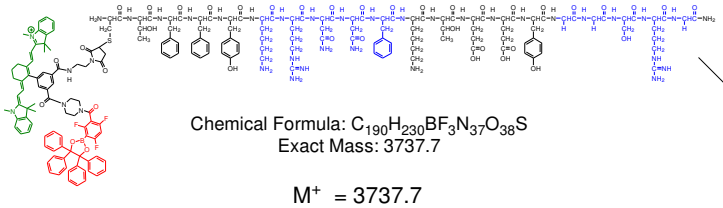
[^{18}F]-fluoride elutes at 19 min, this is consistent with the elution profile of a small molecule on a size exclusion column. The purification of [^{18}F]-**3** as described in the materials and methods gives a relatively pure compound that contains little contaminating [^{18}F]-fluoride.

General multimodality probe application and HPLC-MS analyses

The fluoridation of Lymphoseek **3**, is a perturbation that is difficult to characterize by mass spectrometry for two reasons: the large mass of the species being transformed and the polymeric nature of the species being labeled. In order to demonstrate that the trifluoroborate technology involved in transforming **2** to **3** is both clean and does not generate B-F intermediates, the following peptide was labeled with **1** and converted to a trifluoroborate under the conditions reported in this manuscript. This conversion was monitored by both MS and HPLC. The primary structure of the peptide amino acid sequence has been scrambled.

A.i

NH_2-C(FBOMB)TFFY KRNNF KTEEY GGSRG -$CONH_2$

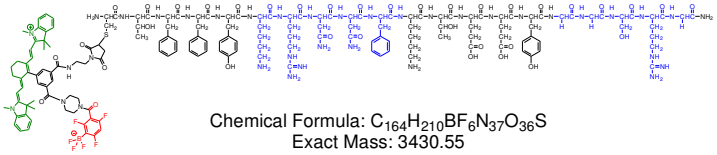


Chemical Formula: $C_{190}H_{230}BF_3N_{37}O_{38}S$
Exact Mass: 3737.7

M^+ = 3737.7
M^{2+} = 1868.8
M^{3+} = 1245.9
M^{4+} = 934.4
M^{5+} = 747.5

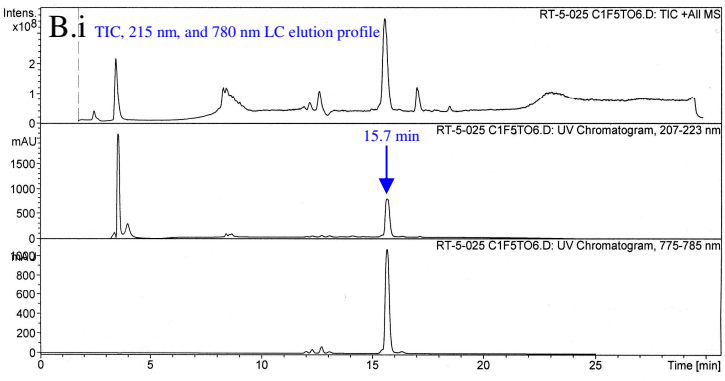
A.ii

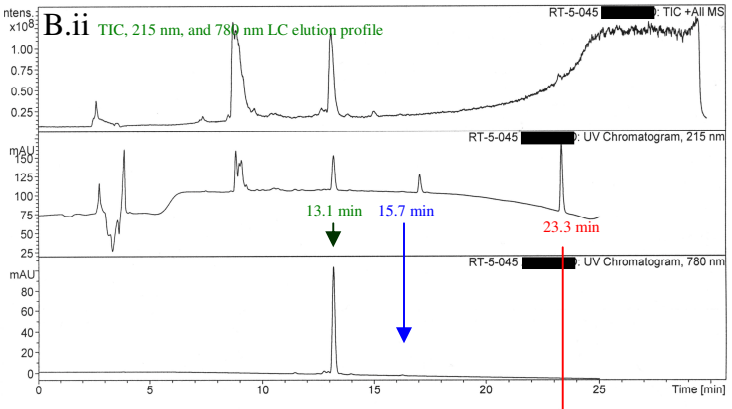
HF
HCl, H_2O
40 °C, 1h

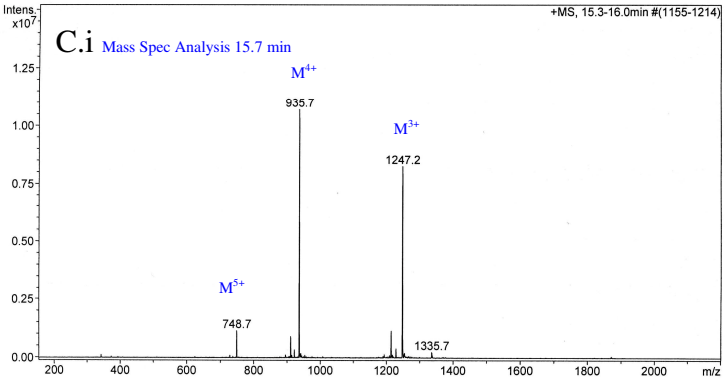


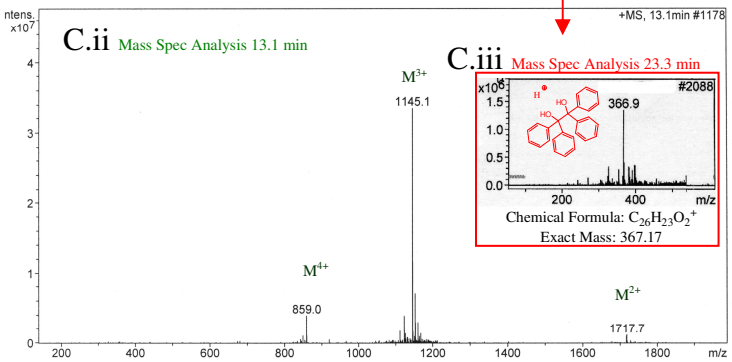
Chemical Formula: $C_{164}H_{210}BF_6N_{37}O_{36}S$
Exact Mass: 3430.55

M^+ = 3430.55
M^{2+} = 1715.3
M^{3+} = 1143.5
M^{4+} = 857.6









A peptide with the sequence, NH_2-(CTFFY KRNNF KTEEY GGSRG) -$CONH_2$ (Figure Ai, scrambled amino acid sequence), was labeled with **1** on its N-terminal cystine and was purified by preparative HPLC. The purified peptide-**1** is shown in figure B.i where the ionization current and the absorbance elution profiles at 780 nm and 215 nm are shown (vide infra for HPLC conditions)[†]. Note the characteristics indicative of purity including a single peak in both 780 nm (15.7 min) and 215 nm (15.7 min) absorbance spectra. The mass spectrometry profile of the peak eluted at 15.7 min is shown in figure Ci. Note a MS spectra indicative of relative purity, where M^{3+} (1247.2 M/z), M^{4+} (935.7 M/z), and M^{5+} (748.7 M/z) ions are observed, while unrelated masses are less abundant.

When the peptide is treated with aqueous fluoride, the trifluoroborate species shown in figure Aii is generated. HPLC analysis was carried out 10 min after fluoride treatment.[‡] This is clear in the HPLC absorbance elution profile at 215 nm (figure Bii), three peaks* in addition to the product (13.1 min) and the injection artifact 2-4 min are observed. Note the absence of a peak at 15.7 min indicating quantitative consumption of tetraphenyl pinacol peptide-**1** and production of [^{19}F]-trifluoroborate labeled peptide. This is observed in the absorbance elution profile at 780 nm, where the starting material at 15.7 min is completely converted to 780 nm absorbent material at 13.1 min. There is only a single peak at 13.1 min in the 780 nm spectra indicating that there are no B-^{19}F non-trifluoroborate intermediates produced (the boron on **1** is linked directly to a 780 nm fluorophore). The mass spectrometry profile of the peak eluted at 13.1 min is shown in figure Cii. Note a spectra indicative of relative purity, where M^{2+} (1717.7 M/z), M^{3+} (1145.1 M/z), and M^{5+} (859.0 M/z) ions are observed, while unrelated masses are not. The peak at 13.1 min can be purified by HPLC, stored at -20 °C, and reanalyzed by HPLC with no observed decomposition. All 215 nm absorbing peaks including peptide-**1** are removed when the reaction mixture Aii is passed through a Biorad-P6 spin column.

This HPLC-MS analysis demonstrates that the conditions for generating trifluoroborates reported in this manuscript: 1) produce trifluoroborates quantitatively, 2) do not produce B-F intermediates, and 3) are generally applicable to biomolecules other than polysaccharide polymers.

[†]Analysis was carried out on an Agilent 1100 Series HPLC equipped with a LC/MSD trap XCT. Chromatography was carried out on a phenomenex Luna 5 µ C18(2) 100A 250 x 4.60 mm 5 µ column with a 1 mL/min flow rate with a 20 min, 10 to 90 % elution gradient followed by a 5 min isocratic elution with 90% acetonitrile and water where both solvents contained 0.05% trifluoroacetic acid. Mass spectrometry was carried out through electrospray ionization in the positive mode with at trap focus at 1000 M/z. The traces shown were generated with Chemstation Rev.B.01.03[204].

[‡]The purification used for Lymphoseek **3** does not work when applied to the peptide in this analysis. This peptide has a molecular weight of 3432.51 g/mol, therefore it does not elute from the Biorad-P6 spin columns used to purify **3** in this manuscript (Biorad P6 MWCO, molecular weight cut-off,= 6000 g/mol). Preparative HPLC or the use of spin columns with a lower MWCO can be used instead in order to successfully purify low MW species.

* The new peak at 9 min in the 215 nm absorbance spectrum is not related to **1** or the fluoridation of **1** as it is not observed when other peptides of similar hydrophobicity and sequence are [^{19}F]-labeled (Data not shown). The peak at 23.3 min is attributed to tetraphenyl pinacol (figure Ciii) that is generated when the tetraphenylpinacol on peptide-**1** (15.7 min) is displaced by fluoride to form the trifluoroborate at 13.1 min.

Radiochemical Synthesis

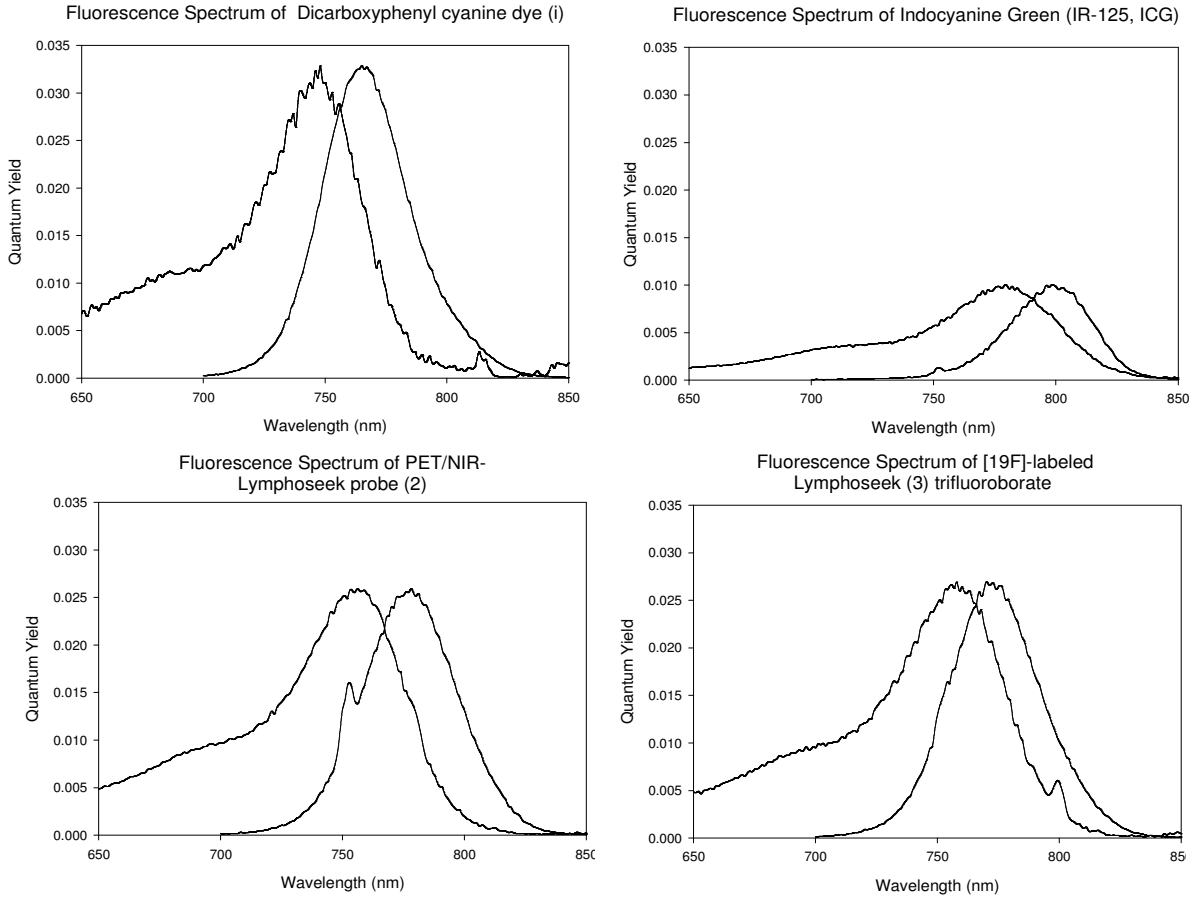
The table below summarizes the results of additional radiochemical labeling experiments that were carried out on **2** prior to the 12 mouse in vivo trial of ^{18}F labeled Lymphoseek **3** described in this manuscript. These ^{18}F labelings were carried out using protocol similar to that listed in the materials and methods unless indicated otherwise.

Experiment	Date	Reaction Volume	Activity of ^{18}F at time of addition to Lymphoseek 2	^{19}F carrier added	Moles of Lymphoseek 2	**Ratio of Fluoride/ Boron**	Activity Isolated as 3	Time of synthesis (Rxn and Purification)	Specific activity (assuming 60% yield of 3 by NIRF) (Ci/µmol)	**Decay Uncorrected Radiochemical Yield**
Section 1 : Replicate High Activity ^{18}F labeling trials @ ~4:1 Fluoride to acceptor ratio:										
RT-3-031A	Nov. 5 2008	5 µL	47.5 mCi	40 nmols	10 nmols	**4:1**	0.292 mCi	1h 10 min	0.048	**0.61 %**
RT-3-031B	Nov. 5 2008	5 µL	27.8 mCi	40 nmols	10 nmols	**4:1**	0.095 mCi	1h	0.016	**0.34 %**
RT-3-029	Sept. 3 2008	5 µL	49.6 mCi	25 nmols	5 nmols	**5:1**	0.347 mCi	1h 43 min	0.116	**0.70 %**
RT-3-027	Aug. 27 2008	5 µL	24.0 mCi	25 nmols	5 nmols	**5:1**	0.726 mCi*	1h 31 min	0.242	**3.02 %**[a]
Section 2: Low Activity Experimental ^{18}F labeling trials:										
RT-3-025A	Aug. 15 2008	5 µL	1.1 mCi[b]	800 nmols	0.8 nmols	**1000:1**	0.00024 mCi	1h 55 min	0.00005	**0.02%**
RT-3-025B	Aug. 15 2008	5 µL	1.1 mCi[b]	480 nmols	0.8 nmols	**600:1**	0.00028 mCi	1h 55 min	0.0006	**0.03 %**
RT-3-025C	Aug. 15 2008	5 µL	1.1 mCi[b]	160 nmols	0.8 nmols	**200:1**	0.00084 mCi	1h 55 min	0.002	**0.07 %**
RT-3-025D	Aug. 15 2008	5 µL	1.1 mCi[b]	40 nmols	0.8 nmols	**50:1**	0.00147 mCi	1h 55 min	0.003	**0.13 %**
RT-3-025E	Aug. 15 2008	5 µL	1.1 mCi[b]	10 nmols	0.8 nmols	**13:1**	0.00346 mCi	1h 55 min	0.007	**0.31 %**
Section 3: High Activity Experimental ^{18}F labeling trials:										
RT-3-021	Aug. 08 2008	5 µL	37.3 mCi	2 000 nmols	7.4 nmols	**270:1**	0.0058 mCi	3 h	0.0008	**0.02 %**
RT-3-047B	June 14 2010	8 µL	11.7 mCi	2.5 nmols	5 nmols	**0.5:1**[c]	0.169 mCi	29 min	0.056	**1.14 %**
RT-3-049	June 16 2010	5 µL	35 mCi	2.5 nmols	5 nmols	**0.5:1**[c]	0.386 mCi	34 min	0.127	**1.12 %**

[a]* [^{18}F]-Fluoride contamination verified
[b] Activity was used as is from PETNET and therefore activity was NOT dried down and resuspended at smaller volumes
[c] Stoichiometry of added fluoride to boron.

Fluorescence and Absorption data

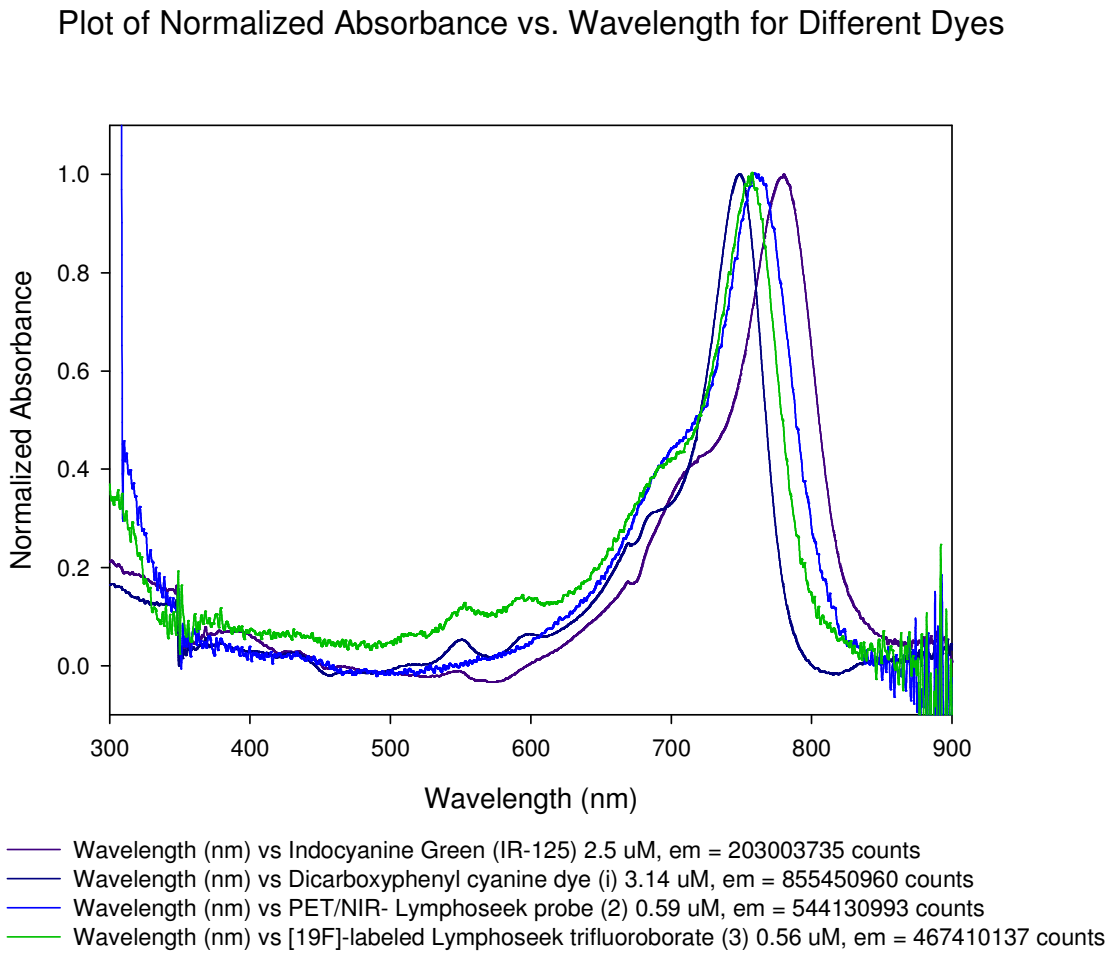
The excitation and emission spectra of (i), 2, the trifluoroborate 3, and a reference; indocyanine green (ICG, IR-125)(*3*) taken as 1-3 µM solutions in water are shown below:



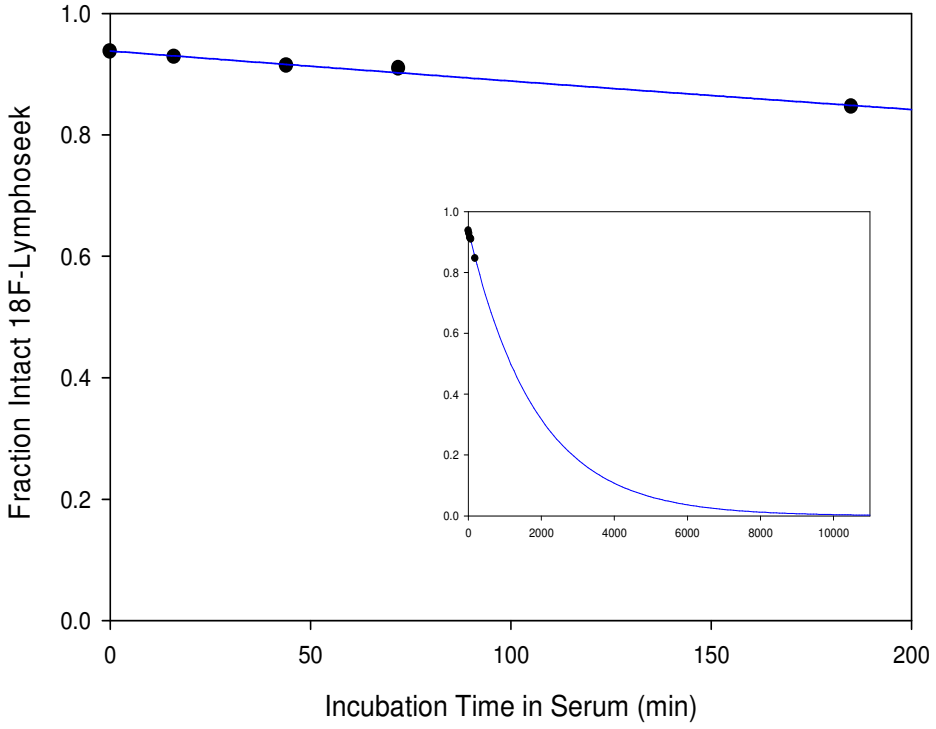
The table below details the constants associated with the described compounds (constants measured on 1-3 µM solutions in water).

Fluorophore	λ_{max} (nm) (1-3 µM solution)	ε ($cm^{-1}M^{-1}$) (1-3 µM solution)	Excitation (nm)	Emission (nm)	**Stokes Shift**	**Quantum Yield, φ**	**φ, relative to ICG**	**Ratio of Raw Counts em/ex**
Indocyanine Green (IR-125, ICG)	780	102 000	778	800	22	0.01(*3*)	1	702 (203003735 / 288896)
Dicarboxyphenyl cyanine dye **(i)**	749	107 000	747	765	18	0.033	3.2	490 (855450960 / 1743333)
PET/NIR- Lymphoseek probe **2**	758	110 000	757	777	20	0.026	2.6	544 (544130993 / 1000358)
[^{19}F]-labeled Lymphoseek trifluoroborate **3**	756	110 000	755	772	17	0.027	2.7	474 (467410137 / 985137)

Plot of normalized absorbance demonstrating the lack of self-quenching in Lymphoseek constructs **2** and **3**.



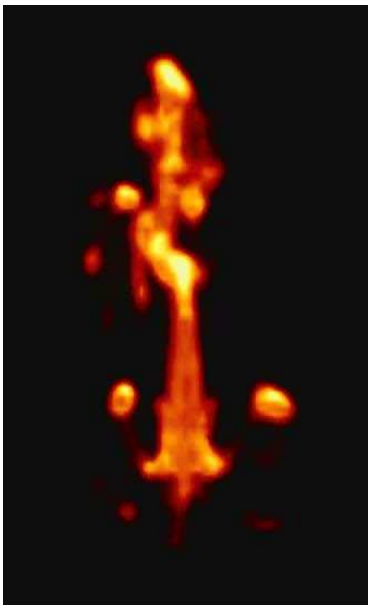
LYMPHOSEEK PLASMA STABILITY ASSAY



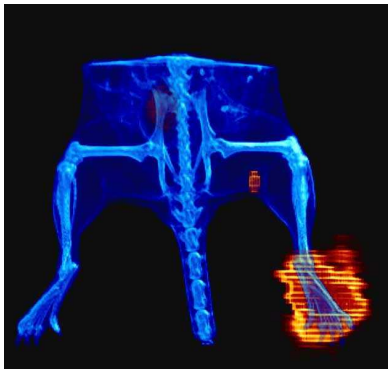
Stability plot of [^{18}F]-labeled of Lymphoseek 3. **The fraction of [^{18}F]-labeled Lymphoseek** 3 **that clears the matrix of a Bio Rad P6 size exclusion gel chromatography column (MWCO = 6000 MW) following incubation for different times in mouse serum.**

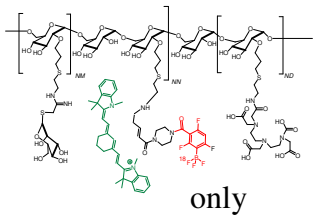
To determine the stability of [^{18}F]-labeled of Lymphoseek **3** in serum, approximately 3 nmols of labeled [^{18}F]-**3** (98 µCi @ 10:45 am) in 100 µCi of 10 mM Tris buffer pH 7.3 was added to 100 µL of mouse serum at 10:51 AM **(Start of Reaction (SOR)).** At time points corresponding to 1 min, 16 min, 44 min, 72 min, and 185 min vs. SOR, 40 µL aliquots were removed from the reaction and placed over Bio Rad Bio Gel P6 spin columns. Both the material retained in the spin column and the flow through were counted by scintillation counter. All counts were corrected for ^{18}F decay. The ratio of corrected counts obtained in the flow through divided by the sum of the counts in the flow through plus those retained in the spin column were plot against time. A non-linear regression analysis of this data was carried out using Sigma Plot 2001 v7.101. A first order decay constant of 0.00054 ± 0.00003 min^{-1} corresponding to a half-life of 1280 ± 80 min was obtained. Assuming that the material retained in the Bio Rad P6 spin column matrix represents the amount of [^{18}F]-labeled of Lymphoseek **3** that is degraded into products of molecular weight that are less than 6000 (the MWCO of the Bio Rad P6 spin columns), it can be assumed that the half-life of [^{18}F]-labeled of Lymphoseek **3** in mouse serum is 1280 ± 80 min, a value that corresponds to a very stable ^{18}F tracer considering that this value is 11.6 times that of ^{18}F nuclear decay (110 min).

EVIDENCE OF IN VIVO ^{18}F-BORON BOND STABILITY

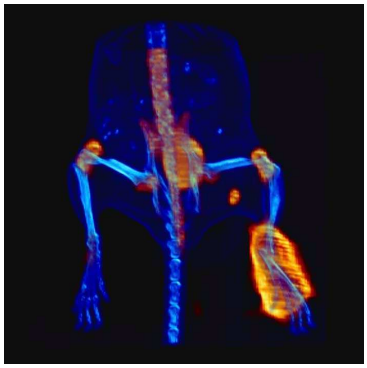


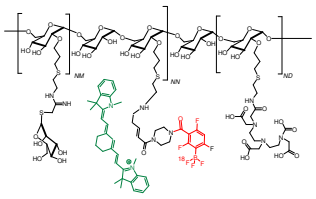
$^{18}F^-$ only





only





+

Deliberate $^{18}F^-$ contamination

The left image is of a mouse injected with ^{18}F-Fluoride after 1 hour. Note signal accumulation in bone structures and the bladder. [^{18}F]-fluoride does not accumulate in lumbar and popliteal lymph nodes. The middle image is a 1 h image of a mouse that has been injected with ^{18}F-3 in the right rear footpad. Note that this image is different from the previous image. There is specific signal accumulation in the sentinel lymph node but no signal accumulation in the knee caps or bone structures. Right image, a 1 h image of a mouse that has been injected with a sample of ^{18}F-3 that was deliberately contaminated with ^{18}F-Fluoride. Note accumulation in the sentinel lymph node as well as in the spine and kneecaps of the mouse. If ^{18}F-3 was experiencing ^{18}F-fluoride loss from F-boron bond dissociation during the in vivo PET experiment, accumulation in the spine and kneecaps of the mouse would be an expected consequence. Because the middle image, and not the right image, is typical of PET images that are acquired with ^{18}F-3, we can qualitatively conclude that the ^{18}F-boron bond is stable on ^{18}F-Lymphoseek 3 in vivo.

REFERENCES:

(1) Ting, R., Lo, J., Adam, M. J., Ruth, T. J., and Perrin, D. M. (2008) Capturing aqueous [F-18]-fluoride with an arylboronic ester for PET: Synthesis and aqueous stability of a fluorescent [F-18]-labeled aryltrifluoroborate. *Journal of Fluorine Chemistry 129*, 349-358.

(2) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry 28*, 350-356.

(3) Soper, S. A., and Mattingly, Q. L. (1994) Steady-State and Picosecond Laser Fluorescence Studies of Nonradiative Pathways in Tricarbocyanine Dyes - Implications to the Design of near-Ir Fluorochromes with High Fluorescence Efficiencies. *Journal of the American Chemical Society 116*, 3744-3752.