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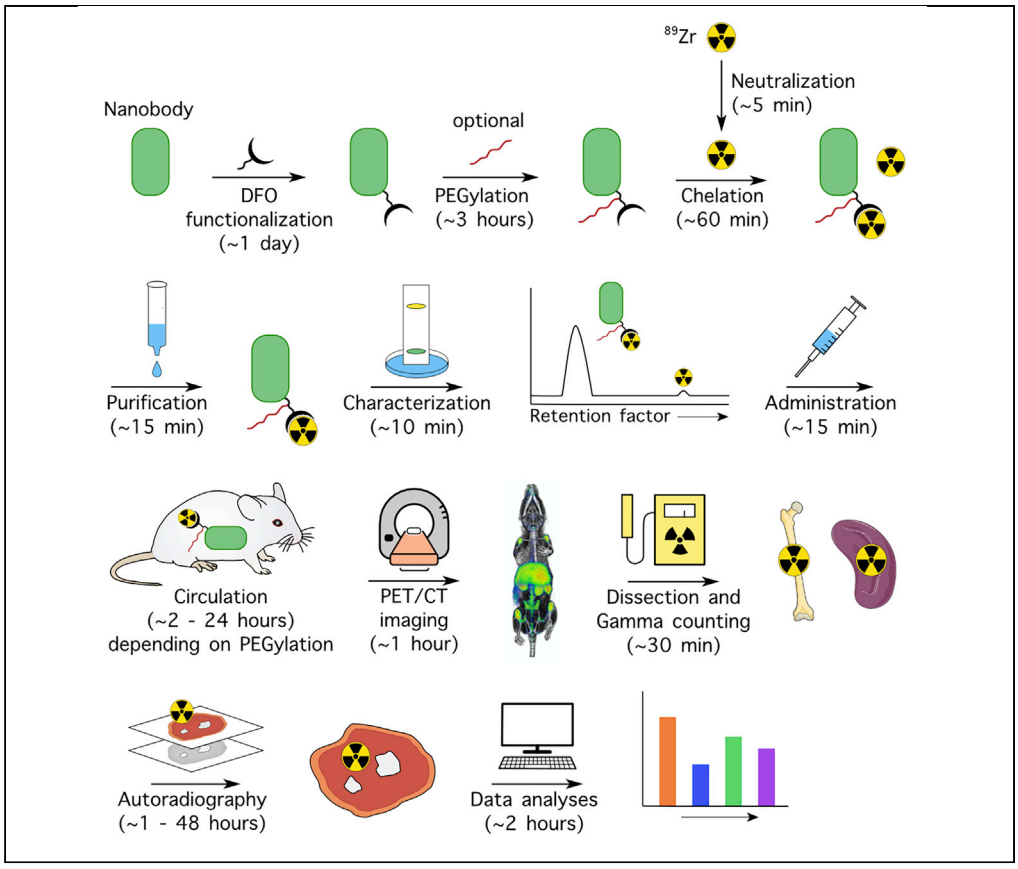
Protocol

Employing nanobodies for immune landscape profiling by PET imaging in mice



Noninvasive immunoimaging holds great potential for studying and stratifying disease, as well as therapeutic efficacy. Radiolabeled single-domain antibody fragments (i.e., nanobodies) are appealing probes for immune landscape profiling, as they display high stability, rapid targeting, and excellent specificity, while allowing extremely sensitive nuclear readouts. Here, we present a protocol for radiolabeling an anti-CD11b nanobody and studying its uptake in mice by a combination of positron emission tomography imaging, *ex vivo* gamma counting, and autoradiography. Our protocol is applicable to nanobodies against other antigens.

Abraham J.P. Teunissen, Omar B. Abousaway, Jazz Munitz, ..., Carlos Pérez-Medina, Willem J.M. Mulder, Mohammad Rashidian

bram.teunissen@mssm.edu (A.J.P.T.)
mohammad_rashidian@dfci.harvard.edu (M.R.)

Highlights

Expressing and purifying nanobodies and sortase

Site-specifically labeling nanobodies with a chelator

Radiolabeling nanobodies using deferoxamine and zirconium-89

In vivo PET imaging and *ex vivo* autoradiography

Teunissen et al., STAR Protocols *2*, 100434
June 18, 2021
https://doi.org/10.1016/j.xpro.2021.100434

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Employing nanobodies for immune landscape profiling by PET imaging in mice

Abraham J.P. Teunissen,[1,8,*] Omar B. Abousaway,[2] Jazz Munitz,[1] Mandy M.T. van Leent,[1] Yohana C. Toner,[1] Bram Priem,[1,3,4] Max L. Senders,[1] Carlos Pérez-Medina,[5] Willem J.M. Mulder,[1,6,7] and Mohammad Rashidian[2,9,*]

[1]Biomedical Engineering and Imaging Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[2]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215-5418, USA

[3]Department of Medical Biochemistry, Amsterdam UMC, Amsterdam 1105 AZ, the Netherlands

[4]Angiogenesis Laboratory, Amsterdam UMC, Cancer Center Amsterdam, Amsterdam 1105 AZ, the Netherlands

[5]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid 28029, Spain

[6]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[7]Department of Pharmacological Sciences and Mount Sinai Center for Therapeutics Discovery, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[8]Technical contact

[9]Lead contact

*Correspondence: bram.teunissen@mssm.edu (A.J.P.T.), mohammad_rashidian@dfci.harvard.edu (M.R.)
https://doi.org/10.1016/j.xpro.2021.100434

SUMMARY

Noninvasive immunoimaging holds great potential for studying and stratifying disease as well as therapeutic efficacy. Radiolabeled single-domain antibody fragments (i.e., nanobodies) are appealing probes for immune landscape profiling, as they display high stability, rapid targeting, and excellent specificity, while allowing extremely sensitive nuclear readouts. Here, we present a protocol for radiolabeling an anti-CD11b nanobody and studying its uptake in mice by a combination of positron emission tomography imaging, *ex vivo* gamma counting, and autoradiography. Our protocol is applicable to nanobodies against other antigens.

For complete details on the use and execution of this protocol, please see Priem et al. (2020), Senders et al. (2019), or Rashidian et al. (2017).

BEFORE YOU BEGIN

Obtaining nanobodies

Nanobodies (or VHHs: **V**ariable domain of the **H**eavy chain of **H**eavy chain-only antibodies) are single-domain antibody fragments derived from camelid antibodies. Their small size (~14 kDa) and high affinities (low nM range) for their targets affords them with characteristics ideal for *in vivo* imaging probes, including a high tissue penetration and short circulatory half-life (when not PEGylated) (Blykers et al., 2015; Keyaerts et al., 2016; Rashidian et al., 2015). Generating nanobodies against proteins of interest has been detailed elsewhere (Pardon et al., 2014). Camelids such as llamas or alpacas are immunized with (a) protein(s) of interest. Subsequently, peripheral blood mononuclear cells (PBMCs) are collected and used to generate a repertoire of immune VHHs. Next a phage or yeast display library is generated. Lead VHHs against antigens of interest are then recovered, for example by panning against the antigen of interest. Validated clones can then be bacterially expressed in different strains of *E. coli* such as WK6. Periplasmic expression of nanobodies in WK6 cells is described below.

Expressing a nanobody

Timing: 3 days

These steps describe how to periplasmically express an anti-CD11b-nanobody with a sortase recognition tag in WK6 cells.

1. Transform competent WK6 cells with a pHEN6 plasmid coding for the desired nanobody with an N-terminal pelB sequence and a C-terminal -LPETGHHHHHH (sortase recognition sequence followed by a polyhistidine-tag). For the sequence of anti-CD11b-nanobody please see (Rashidian et al., 2015). Spread the transformed cells onto Luria-Bertani (LB)-agar plates containing 100 μg/mL ampicillin and incubate for 16–24 h at 37°C.

   Note: The pelB sequence directs the nanobody to the periplasm of the bacteria, where it will be cleaved once the protein is translocated to the periplasm. LPETG is a recognition sequence for sortase, later used to label the nanobodies with a chelator (deferoxamine)-containing sortase substrate. The substrate can also contain an azide, which can be used to derivatize the nanobody with a second functionality such as polyethylene glycol (PEG). Here we use pHEN6 plasmid, but other plasmids could possibly be used as well.

2. Start a culture from a single colony in 25 mL LB-agar containing 100 μg/mL ampicillin.
   a. Shake the sample at 225 rpm for 16 h at 37 °C on an orbital shaker.
3. Add 10 mL of culture to 1 L of Terrific Broth (TB) containing 100 μg/mL ampicillin. Shake the culture at 225 rpm and 37°C.
4. Check the optical density at 600 nm (OD_{600}) of the culture to monitor bacterial growth.
   a. Pipette 1 mL of culture into a cuvette and measure its absorbance at 600 nm using a spectrophotometer. Use 1 mL of TB as a blank.
   b. Measure the OD_{600} after 1 h of culturing and every 10–30 min thereafter as needed.
5. When an OD_{600} between 0.5 and 0.7 is reached, induce expression by adding isopropyl-β-D-galactoside (IPTG) to a final concentration of 1 mM. Subsequently, shake the culture at 225 rpm for 16 h at 30°C.
6. Collect the cells by centrifuging the culture at 5000 × *g* for 10 min and discard the supernatant.

Purifying the nanobody

Timing: 1 day

These steps describe how to purify periplasmically expressed nanobodies from WK6 cells. An osmotic shock step releases the proteins from the periplasm, which are subsequently purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA agarose beads.

7. Resuspend the cells in ice-cold TES buffer and incubate at 4°C for 2 h while gently shaking. For cells obtained from 1 L of culture, use 15 mL of TES buffer pH 8.
8. Add 45 mL of 0.1 × TES buffer that has been cooled to 4°C (diluted with deionized water) to the resuspended cells and gently shake for 16 h at 4°C.
9. Centrifuge the resuspended cells at 7000 × *g* for 10 min before collecting the supernatant.
   a. If needed, repeat until the supernatant is clear.
10. Purify the His-tagged nanobody by Ni-NTA chromatography.
    a. Pass the periplasmic extract through a column containing Ni-NTA agarose resin that has been washed with the wash buffer. For extraction of protein obtained from 1 L use 3–4 mL of resin.
    b. Collect the flow-through and pass it through the column again.
    c. Wash the resin 3 times with 10 mL of the wash buffer.

d. Elute the nanobodies by adding the elution buffer.
   i. Collect 8 fractions of 2 mL each.
   ii. Measure the nanobody concentrations in each of the fractions by measuring the absorbance at 280 nm using a Nanodrop and pool any fractions that contain the nanobody.
e. Size-exclusion fast protein liquid chromatography (SEC-FPLC) can be used to purify the nanobody and remove imidazole. Alternatively, dialyze the pooled fractions against a 1 L solution of Tris buffer (Tris 50 mM, NaCl 150 mM, pH 8) using a 2 kDa or 5 kDa molecular weight cutoff (MWCO) membrane at 4°C for 16–24 h to remove any excess imidazole.
f. Add glycerol to reach a final concentration of 5% and aliquot in smaller fractions as desired. The glycerol stock is typically a Chelexed 50% glycerol in water solution. Store the nanobody at −80°C; nanobodies typically remain fully functional for at least 1 year under these conditions.

Note: Typical nanobody yields are approximately 10–40 mg per liter of expression culture.

Expressing sortase

Timing: 3 days

This section describes how to cytosolically express calcium-independent sortase (sortase 7M) from BL21(DE3) cells. The 7 mutations introduced in sortase A enable efficient nanobody labeling in the absence of calcium, which would otherwise be required to obtain catalytically active sortase. Sortase 7M will facilitate site-specific labeling of the nanobody with deferoxamine (DFO), which will subsequently be used to chelate the positron emission tomography (PET) imaging radioisotope ^{89}Zr.

11. Transform competent BL21(DE3) *E. coli* with pET30b plasmid coding for sortase 7M with a C-terminal hexahistidine tag. Add transformed cells to LB-agar plates containing 50 μg/mL Kanamycin and incubate for 16 h at 37°C.
12. Start a culture by inoculating 25 mL of LB containing 50 μg/mL Kanamycin with a single colony from the plate. Shake at 225 rpm at 37°C for 16 h.

⚠ CRITICAL: Use freshly transformed agar plates to ensure optimal expression.

13. Dilute 10 mL of the culture into 1 L of TB medium containing 50 μg/mL Kanamycin. Shake the culture at 225 rpm for 16 h at 37°C.
14. Check the OD_{600} of the culture to monitor bacterial growth.
    a. Measure the culture's OD_{600} after 1 h and every 10–30 min after that as needed. Pipette 1 mL of the culture into a cuvette and measure its absorbance at 600 nm using a spectrophotometer. Use 1 mL of TB as a blank.
15. When an OD_{600} between 0.5 and 0.7 is reached, induce expression by adding IPTG to a final concentration of 0.5 mM. Subsequently, shake the culture at 225 rpm for 16 h at 30°C.
16. Collect the cells by centrifuging the culture at 7000 × *g* for 10 min, discard the supernatant.
17. Resuspend the cells in 50 mL of lysis buffer on ice.

⚠ CRITICAL: Do not add protease inhibitors to the buffer as sortase is a protease and the inhibitors may interfere with its activity.

18. Sonicate the suspension using a sonicator equipped with a microtip to achieve complete lysis of all cells.
    a. Sonication should be performed on ice and for a total of 10 min, in cycles of 5 s on, 5 s off.
19. Centrifuge the lysed cells at 16,000 × *g* for 20 min and collect the supernatant.
20. Purify the His-tagged sortase by Ni-NTA chromatography.

a. Pass the soluble cell lysate through a column containing 5 mL of Ni-NTA agarose resin that has been washed with Tris NaCl buffer.
b. Collect the flow-through and pass it through the column again.
c. Wash the resin 3 times with 10 mL of wash buffer.
d. Elute sortase using elution buffer
   i. Collect 8 fractions of 2 mL each.
   ii. Measure the sortase 7M concentration in each fraction using a Nanodrop and pool any fractions that contain the protein. The sortase 7M's purity can be analyzed by SDS-PAGE.
e. SEC-FPLC, using a Tris NaCl buffer as eluent, can be used to purify the sortase and remove imidazole. Alternatively, dialyze the pooled fractions against a 1 L solution of Tris NaCl buffer using a 2 kDa or 5 kDa MWCO membrane at 4°C for 16–24 h, to remove any excess imidazole.
f. Add a 50% aqueous glycerol solution to reach a final concentration of 5% glycerol, aliquot the sample into smaller fractions as needed, and store at −80°C. The sortase 7M remains active for at least 1 year under these conditions.

Note: Typical sortase 7M yields are approximately 10–20 mg per liter of expression culture.

Note: Our protocol can also be used to conjugate other chelators, such as NOTA or DOTA, which chelate other radioisotopes such as ^{64}Cu.(Chakravarty et al., 2014; Senders et al., 2019)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Benzonase Nuclease	Millipore Sigma	E1014-5KU
DBCO-mPEG, 5, 10, or 20 kDa	Click Chemistry Tools	A118-25, A119-25, A120-25
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418-50ML
Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate >99.0%	Sigma-Aldrich	03677
Gly3-PEG3-Lys(azide)-PEG3-DFO	GenScript (custom synthesized)	N/A
Glycerol, for molecular biology, ≥99.0%	Sigma-Aldrich	G5516-100ML
HEPES powder	Thermo Fisher, Gibco	11344041
Imidazole, for molecular biology, ≥99% (titration)	Sigma-Aldrich	I5513-25G
100 mM IPTG solution	Sigma-Aldrich	70527-3
Lysozyme	Millipore Sigma	L6876-1G
$MgCl_2$	Sigma-Aldrich	M8266-100G
Na_2CO_3 powder >99.5%	Sigma-Aldrich	223530
$Na_2HPO_4 \cdot 7H_2O$	Sigma-Aldrich	S9390-500G
NaCl, cell culture reagent grade	MP Biomedicals, Inc	02194738.5
$NaH_2PO_4 \cdot H_2O$	Sigma-Aldrich	S3522-500G
Oxalic acid 98%	Sigma-Aldrich	194131-250G
Sucrose BioReagent, suitable for cell culture ≥99.5% (GC)	Sigma-Aldrich	S1888-500G
TraceSELECT HCl	Thermo Fisher	60-007-78
Tris base	Sigma-Aldrich	10708976001
Triton™ X-100	Sigma-Aldrich	T8787-100ML
^{89}Zr oxalate in 1M aqueous oxalic acidSpecific activity approximately 5–10 mCi/μg.	3D-imaging	N/A
Yeast extract, for use in microbial growth medium	Sigma-Aldrich	Y1625-250G
Tryptone, pancreatic digest of casein	Sigma-Aldrich	T9410-250G
Tissue-Tek OCT compound 4 OZ	Andwin Scientific	NC9806257
Kanamycin sulfate from *Streptomyces kanamyceticus*	Sigma-Aldrich	K1377-1G
ISOVUE®-370 (iopamidol injection 76%)	Bracco	0270-1316-95

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HisPur™ Ni-NTA Resin	Thermo Fisher	88222
Forane (isoflurane, USP) liquid for inhalation	Baxter	1001936040
1× Dulbecco's phosphate-buffered saline (DPBS)w/o $CaCl_2$ and $MgCl_2$	Thermo Fisher, Gibco	14190144
Chelex® 100 sodium form	Sigma-Aldrich	C7901-100G
Ampicillin	Sigma-Aldrich	A5354-10ML
Agar, for microbiology	Sigma-Aldrich	05040-250G
Bacterial and virus strains		
Escherichia coli BL21(DE3) competent cells - Novagen	Sigma-Aldrich	69450-3
Escherichia coli (Migula) Castellani and Chalmers (WK6) competent cells	ATCC	47078
Experimental models: Organisms/strains		
Mouse C57BL/6J, 8 to 12 weeks old	Jackson Laboratories	000664
Recombinant DNA		
Plasmid pET30b-7M SrtA	Addgene	51141
Software and algorithms		
OsiriX MD software	OsiriX Foundation	N/A
ImageJ	National Institutes of Health	N/A
Mediso Nucline Software	Mediso	N/A
Other		
3M™ Micropore™ surgical paper tape	3M	7000002543
Alcohol prep pads	Medique	22133
Autoradiography cassettes	Cytiva	45-000-758
BD Lo-Dose™ U-100 insulin syringes 28G 0.5 mL	BD	329461
BD Micro-fine™ IV insulin syringe 28G 1 mL	BD	329420
BD PrecisionGlide™ single-use needles: regular bevel and regular wall 27G $1^1/_4$ in	BD	305136
Caution radioactive materials pre-strung tags	Research Products International Corp	140050
Clear plastic wrap	Fisherbrand	22-305654
Disposable PD-10 desalting column	Sigma-Aldrich, GE	GE17-0851-01
Disposable base molds	Fisherbrand	22-363-554
Intravascular polyurethane tubing 0.005 ID 0.010 OD 25 ft catheter	SAI Infusion Technologies	PU-025-50
iTLC-SG chromatography paper	Agilent	SGI0001
Kimberly-Clark Professional™ Kimtech Science™ Kimwipes™ delicate task wipers	Kimberly-Clark Professional	34120
pH test strips pH 4.5–10, resolution: 0.5 pH unit	Sigma-Aldrich	P4536
Phosphor image plate	Fujifilm	BASMS-2325
Rotating tail injector restrainer	Braintree Scientific	RTI STD
Shielded syringe carrier, small	Biodex	001-182
NanoDrop™ 2000/2000c spectrophotometers*	Thermo Scientific	ND2000CUSCAN
Misionix Sonicator 3000*	Misonix Inc	S-3000
Atomlab™ 500 dose calibrator*	Biodex	086-330
Auto flow anesthesia system with regulator, induction chamber, beds, breathers, and charcoal filters*	Euthanex	EZAF9000
NanoScan PET/CT*	Mediso	n/a
Chemyx Fusion 100T precision lab infusion syringe pump*	Thermo Scientific	BZA649110
CM3050 S Research Cryostat*	Leica	14903050S01
1480 Wizard 3-inch gamma counter*	PerkinElmer	10071799
Storage phosphor screen image eraser*	GE Healthcare	29187190
Fiber optic illuminator with gooseneck*	Dolan-Jenner	660000391014
Lighting specialties TEL-AF-3191 infrared heating lamp, 50/60 Hz*	Cole-Palmer	0305700
Typhoon 7000IP plate reader*	GE Healthcare	28-9836-18
Radio-TLC scanner*	LabLogic	Dual Scan-Ram

Note: *Specialized equipment used in this protocol. The use of identical apparatus is not required, rather we provide this information to guide readers in assessing the suitability of their own equipment.

MATERIALS AND EQUIPMENT

LB Medium		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
NaCl	10 g/L	10 g
Yeast extract	5 g/L	5 g
ddH_20	n/a	Add to 1L
*Must be autoclaved before use. Can be stored at 4°C for at least one week.		

LB-agar Medium		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
NaCl	10 g/L	10 g
Yeast extract	5 g/L	5 g
Agar	15 g/L	15 g
ddH_20	n/a	950 mL
*Must be autoclaved before use. Can be stored at 4°C for up to at least 1 week.		

Lysis Buffer		
Reagent	Final concentration	Amount
Tris	50 mM	6.06 g
NaCl	150 mM	8.77 g
$MgCl_2$	5 mM	0.48 g
Lysozyme	1 mg/mL	1 g
Benzonase	n/a	10 uL
ddH_2O	n/a	950 mL
*Filter-sterilize the buffer and store for up to 3 months at 4°C		

TB Medium		
Reagent	Final concentration	Amount
Tryptone	20 g/L	20 g
Glycerol	4 mL/L	4 mL
Yeast extract	24 g/L	24 g
Phosphate buffer	17 mM KH_2PO_4, 72 mM K_2HPO_4	100 mL
ddH_2O	n/a	950 mL
*Must be autoclaved before use. Can be stored at 4°C for at least one week.		

Phosphate buffer (1 L)		
Reagent	Final concentration	Amount
Na_2HPO_4-$7H_2O$ (mw: 268.07 g/mol)	75.4 mM	20.21 g
$NaH_2PO_4H_2O$ (mw: 137.99 g/mol)	24.6 mM	3.40 g
ddH_2O	n/a	800 mL*
* Adjust solution to final desired pH using HCl or NaOH and add H_2O to obtain 1L.		
*Can be stored at 25°C for up at least one year.		

TES buffer (1 L)		
Reagent	Final concentration	Amount
Tris	50 mM, pH 8.0	6.06 g
EDTA	0.65 mM	190 mg
Sucrose	0.50 M	171.2 g
ddH_2O	n/a	950 mL
*Can be stored at 25°C for up at least one year.		

Tris NaCl buffer (1 L)		
Reagent	Final concentration	Amount
Tris	50 mM, pH 7.5	6.06 g
NaCl	150 mM	8.77 g
ddH_2O	n/a	800 mL*
* Adjust solution to final desired pH using HCl or NaOH and add H_2O to obtain 1L.		
*Can be stored at 25°C for up at least one year.		

Tris wash/elution buffer (1 L)		
Reagent	Final concentration (mM or μM)	Amount
Tris	50 mM, pH 8	6.06 g
NaCl	150 mM	8.77 g
For wash Imidazole	20 mM	1.36 g
For elution Imidazole	400 mM	27.2 g
ddH_2O	n/a	800 mL*
* Adjust solution to final desired pH using HCl or NaOH and add H_2O to obtain 1L.		
*Can be stored at 25°C for up at least one year.		

STEP-BY-STEP METHOD DETAILS

Functionalizing the nanobodies site specifically with the chelator DFO

Timing: 1 day

In this step, the nanobody will be labeled with the chelator deferoxamine (DFO), later used for binding radioactive ^{89}Zr. Since we intend to also functionalize the nanobody with a PEG moiety via a click reaction, the described DFO substrate also contains an azide group. Note that the functionalization with PEG is optional (see next section). A DFO substrate without an azide group should be used, if PEGylation is not planned.

Note: If the nanobodies will not be functionalized with a 10 kDa or 20 kDa PEG, their blood half-life will be short enough to allow imaging using a radioisotope with a shorter physical half-life than that of ^{89}Zr. In that case, exposure to the workers and animals' might be reduced. Chelators such as DOTA and NOTA, can be used to bind various radioisotopes, including ^{68}Ga and ^{64}Cu. Alternatively, nanobodies can be directly functionalized with ^{18}F ($t_{1/2}$ of ~110 min). (Rashidian et al., 2015; Senders et al., 2019)

Note: Trace amount of metals such as iron can efficiently bind DFO, hindering the chelation of ^{89}Zr. Therefore, these impurities of metals should be removed from all reagents and solutions used in these steps.

1. Remove metal impurities from the nanobodies using Chelex beads.

a. Chelex 500 mL of PBS pH 7.4 by adding 5 grams of Chelex beads and gently stir the suspensions using a magnetic stir bar for at least 8 h at 4°C. Chelex treatment may raise the pH of buffers, check the pH of the Chelexed PBS and acidify with TraceSELECT HCl if necessary.
b. Dialyze a solution containing at least 2 mg of nanobodies against 500 mL of PBS containing 5 grams of Chelex beads for 16 h while stirring at 4°C.

2. Remove metal impurities from the purified sortase 7M using Chelex beads.
   a. Dialyze a solution containing sortase 7M against 500 mL of PBS containing 5 grams of Chelex beads for at least 16 h while stirring.
3. Use sortase to functionalize the nanobodies with the Gly_3-azide-DFO substrate.
   a. To a tube containing the Chelexed nanobody at a concentration between 2–4 mg/mL, add the following sequentially:
      i. Gly_3-azide-DFO substrate to a final concentration of 1–2 mM.
      ii. Chelexed sortase 7M to a final concentration of 5 μM. Allow the reaction to proceed for 2 h at 4°C.

⚠ CRITICAL: The Gly_3-azide-DFO substrate must be dissolved in DMSO or Chelexed PBS to limit the presence of metals.

Note: Progress of the reaction can be monitored via either mass spectrometry or anti-His western blotting and looking for the loss of His-tag. The sortase reaction usually reaches >90% conversion within 2 h. If the reaction has not reached >80% conversion after 2 h, the Gly_3-azide-DFO and sortase concentrations can be increased an additional 1 mM and 5 μM, respectively.

4. Purify the DFO-labeled nanobody from sortase.
   a. Add 200 μL of Ni-NTA beads that have been washed 3 times with Chelexed PBS (100 μL of bead slurry for each 0.5 mL of reaction mixture).
      i. Add Chelexed PBS (1.5 mL, pH 7.4) containing 200 μL beads to a 2 mL Eppendorf tube.
      ii. Resuspend the beads by inverting the tube 5–10 times.
      iii. Centrifuge for at least 30 s at 300 × *g* and remove the supernatant.
      iv. Add fresh Chelexed PBS (1.5 mL) and repeat steps ii and iii.
   b. Incubate the beads in the reaction mixture for 5 min while agitating the tube at 4°C.
   c. Centrifuge the suspension for at least 30 s at 300 × *g* and collect the supernatant.
   d. Wash the beads 3 times with Chelexed wash buffer, 0.20 mL each time.
   e. Combine the supernatant and wash solvent and purify the product by PD-10 desalting column, which has been thoroughly equilibrated with Chelexed buffer prior to use (see steps 11–13 for general instructions on using PD-10 columns). To ensure efficient removal of the excess substrate, we do not recommend applying more than 0.5 mL of reaction mixture per PD-10 column. Elute the nanobody using Chelexed PBS and collect 0.5 mL fractions. Determine the nanobody concentration in each fraction by measuring their absorbance at 280 nm. The nanobody typically elutes in fractions 5–8 (when 2.5–4.5 mL of PBS has already been added on top of the column after the sample is loaded).

Optional: Instead of a PD-10 column, size-exclusion chromatography can be used to purify the nanobody in step 4e. The column should be thoroughly washed with Chelexed PBS buffer before use.

Functionalizing the DFO-labeled nanobody with PEG

Timing: 3 h

This step is optional.

Functionalizing nanobodies with PEG decreases their kidney uptake, which is likely due to their increased hydrophilicity. PEGylation also increases nanobodies' blood half-lives, which can enhance the signal-to-background ratio obtained during nanobody-PET imaging. However, it also prolongs the time required to reach a relatively stable nanobody biodistribution, at which meaningful PET images can be obtained. In this manner, nanobodies' pharmacokinetics can be tuned by employing PEG chains of different molecular weights. For examples on how PEG length influences PEGylated nanobodies' *in vivo* behavior please see (Rashidian et al., 2016, 2017)

5. Add DBCO-PEG (5 kDa, 10 kDa or 20 kDa) to the nanobody-functionalized with the azide-DFO substrate (approximately 1–2 mg/mL) to reach 1.0 molar equivalents compared to the nanobody. Let the reaction proceed for 1 h at 4°C. DBCO-PEG should be added as a solution in Chelexed PBS (1–5 mM stock). This reaction is typically quantitative, if desired any trace amounts of unreacted PEG can be removed using an FPLC thoroughly washed with chelexed buffer.

⚠ CRITICAL: All buffers that come into contact with the DFO-labeled nanobody must be Chelexed.

6. Perform SDS-PAGE to verify if PEGylation has been completed. As PEG increases the nanobodies' molecular weight, the product will move slower on the gel than the starting material. The DBCO-azide click reaction, when performed in the above concentration range, usually reaches conversions of >95% within an hour, after which it is considered complete. If necessary, the reaction time can be extended and more DBCO-PEG added.

Note: To ensure PEGylation does not affect the nanobody's cellular specificity, we recommend imaging animals that lack the expression of protein of interest (knockouts). Alternatively, a blocking experiment involving pre-injecting animals with non-radiolabeled nanobody before imaging with the radiolabeled PEGylated nanobody can be performed. In either case, a significant decrease in PET signal intensity will confirm that the PEGylated nanobody has retained its specificity. Binding analyses by flow cytometry or *in vitro* cell binding assays can also be performed.

Radiolabeling DFO-functionalized nanobodies with ^{89}Zr

Timing: 1.5 h

Radioactive ^{89}Zr is chelated by DFO-functionalized nanobodies, unchelated ^{89}Zr removed, and the radiochemical purity of the radiolabeled nanobodies determined. The procedure is identical regardless of nanobody PEGylation.

Note: Necessary precautions for working with high-energy gamma radiation should be taken. These include, but are not limited to, receiving appropriate training, shielding radioactive sources, and monitoring workers' exposure.

7. Prepare a solution of 0.5–2.0 mg DFO-functionalized nanobody in 200 μL HEPES buffer (0.5 M, pH 7.5).
8. Dilute an ^{89}Zr-oxalate solution in 1 M oxalic acid (1.0–1.5 miliCurie (mCi), <20 μL) with Chelexed PBS 300 μL and adjust its pH to 6.8–7.5 using a Chelexed aqueous Na_2CO_3 solution (2 M).

Note: Typically, a volume of aqueous Na_2CO_3 solution equal to approximately 45% of the volume of $^{89}Zr^{4+}$ oxalate solution needs to be added to reach the desired pH. The pH is best checked by pipetting 0.5 μL of the solution to pH paper with a minimum resolution of 0.5 pH units.

9. Add the neutralized ^{89}Zr solution to the nanobody-DFO solution, mix well, and check if the pH remained between 6.8 – 7.5. Troubleshooting 1
10. Incubate the mixture for 60 min at 25°C using a thermomixer shaking at 350 rpm.
11. Open a disposable PD-10 size-exclusion cartridge and wait until the storage buffer has eluted. Equilibrate the column by applying 5 times 3 mL of PBS and waiting until this has eluted.
12. Load the nanobody-DFO solution (approx. 0.55 mL) on the PD-10 cartridge and wait until it has entirely run into the column. Add 2 mL of PBS and again wait until this has entirely run into the column. The eluent at this stage should not contain any activity and can be discarded.
13. Add 0.5 mL PBS and collect the 0.5 mL eluent in an Eppendorf tube. Repeat 9 times.
14. Measure the activity in each fraction and the column using a dose calibrator. Typically, the radiolabeled nanobodies elute as a clear peak in fractions 2–6, followed by free ^{89}Zr in fractions 8–10. Some activity typically remains on the column.

Optional: Depending on the desired injected dose, some fractions that contain ^{89}Zr-labeled nanobody-DFO at a relatively low concentration can be discarded before combining the remaining fractions.

15. Cut thin-layer chromatography (TLC) paper into a 0.5 × 7.5 cm strip and spot approximately 1–2 μL of the radiolabeled nanobodies 1.0 cm from one end. Troubleshooting 2
16. Add 1 mL of an aqueous disodium EDTA (50 mM) to a 50 mL Falcon tube and gently place the TLC strip inside with only the tip of the spotted end submerged in the solution.
17. Remove the TLC paper after the disodium EDTA solution has run to 0.5 cm of the top end of the strip (~5 min) and let dry for several min.

Optional: To reduce the risk of contamination, wrap the developed TLC strip in cellophane before radio-TLC analyses.

18. Place the strip in a radio-TLC scanner and measure the activity over the length of the strip. Radiolabeled nanobodies should remain at the baseline (*i.e.*, have a retention factor (Rf) of 0), while any unbound ^{89}Zr elutes with the solvent front (*i.e.*, have a Rf of 1).
19. Integrate the peaks belonging to the radiolabeled nanobody and free ^{89}Zr and calculate the radiochemical purity by dividing the area of the nanobody peak by that of the combined nanobody and free ^{89}Zr signals. The radiochemical purity is typically >97%. The decay-corrected radiochemical yield of the entire radiolabeling procedure is typically >80% (0.8–1.3 mCi).

Optional: The radiochemical purity of the product can also be determined by radio size exclusion HPLC. In this case, we advise using PBS as eluent and injecting at least 50 μCi of sample.

Pause point: The radiolabeled nanobodies should be either directly injected or stored in the dark at 4 °C. Although the nanobodies remain stable for at least a week under these conditions, their specific activity will diminish due to ^{89}Zr's radioactive decay ($t_{1/2}$ = 78 h). As a result, higher nanobody doses will need to be administered to obtain the same PET signal intensity, which might negatively impact their specificity.

Intravenous nanobody administration and circulation

Timing: 15 min for injection followed by 2.0, 3.0, 6.0, or 24 h circulation for no-PEG, 5 kDa-PEG, 10 kDa-PEG or 20-kDa PEG-functionalized nanobodies, respectively.

^{89}Zr-labeled nanobodies are intravenously injected in mice and allowed to circulate for periods of time depending on their PEG functionalization.

20. Prepare a dose of ^{89}Zr-labeled nanobody.

a. Using a 0.5 mL insulin syringe, draw approximately 200 μCi (approx. 50 μL) of ^{89}Zr-labeled nanobody.
b. Record the exact amount of activity using a dose calibrator as well as the measurement time. Dilute the dose to 150 μL with PBS.
c. Place the dose in a lead-shielded syringe holder for transfer.
d. Cut approximately 6 inches of catheter tubing. Using a pair of scissors, remove the plastic tip of a 27G × 1 1/4 needle and insert this into one end of the catheter. Fill a 1 mL insulin syringe with PBS and connect the PBS-filled syringe to the catheter on the side opposite of the needle. Advance the syringe until contrast flows out of the needle to ensure removal of air from the catheter.

21. Inject the mouse with ^{89}Zr-labeled nanobody.
a. When housing multiple mice in the same cage, mark each mouse using a unique ear tag, ear punch, or pen stroke on its tail.
b. Place the mouse under a heat lamp or on a heating pad for at least 5 min.
c. Place the mouse in a restrainer and turn on the fiber optic illuminator.
d. Find the lateral tail vein and rub the area with an alcohol swab to induce vasodilation.
e. Place the catheter needle in the tail vein and inject the nanobody dose. Swap the empty dose syringe for a 1 mL insulin syringe filled with PBS. Use approximately 50 μL PBS to flush any remaining activity into the mouse.
f. Record the time of injection.
g. Remove the needle and wipe any pooling blood with a Kimwipe. Place the mouse back in its cage.
h. Collect the Kimwipe, catheter, needle, and empty nanobody syringe in a 50 mL Falcon tube (and any other items that might be contaminated with the nanobody dose). Place the Falcon tube in a dose calibrator and record the time and activity.

22. Allow the administered ^{89}Zr-labeled nanobodies to circulate for the desired time.

Note: Make sure to label the mouse cage with a radioactive tag. Fasting is not necessary for this radiotracer.

PET imaging of mice injected with ^{89}Zr-labeled nanobodies

Timing: 1 h

The biodistribution of the radiolabeled nanobodies is non-invasively monitored by PET imaging.

23. Weigh the mouse and record its weight.
24. Place a catheter to inject CT contrast agent.
a. Place the mouse under a heat lamp or on a heating pad for at least 5 min.
b. Draw Isovue-370 iodine contrast agent into a 1.0 mL insulin syringe. Cut 1.5 ft of a catheter, remove the plastic component of a separate 27G × 1 1/4 needle, insert this needle into one end of the catheter, and the contrast-containing syringe into the other. Advance the syringe until contrast flows out of the needle to ensure removal of air from the catheter.
c. Place the mouse in a restrainer and turn on the fiber optic illuminator. Find the lateral tail vein and rub the area with an alcohol swab to induce vasodilation.
d. Place the needle in the tail vein and check if it is appropriately placed. The syringe's plunger may need to be pulled outward to observe blood flow and confirm placement, as Isovue-370 is quite viscous. Secure the needle in the vein using tape.

25. Induce anesthesia by placing the animal in an induction chamber. Set the oxygen flow at 1 L/min and the isoflurane vol.% at 1.5–2.0.
26. Prepare the PET scanner.
a. Create a new file and use a protocol that includes a scout scan, CT, and PET.

b. Set the CT voxel size to the minimum available setting. Ensure the whole mouse gets covered by one CT planning bed and use a similar size PET bed.
c. Input details about the mouse and radioactive dose, including the initial nanobody dose, ^{89}Zr's physical half-life, remaining activity in the used syringe, time points of the various dose measurements, as well as the bodyweight of the mouse.

27. Place the animal in the scanner.
    a. Place the mouse in a prone and head first position. Ensure that the respiratory monitor is located on the abdomen and that the respiratory signal is appropriately monitored.
    b. For anesthesia during the scan, set the oxygen flow at 1 L/min and the isoflurane vol.% at 1.5. Monitor the animal's respiratory rate while it is in the scanner, approximately 80–100 bpm is ideal.
28. Place the Isovue-370 syringe in an automated syringe pump. Program the pump to inject at 0.05 mL/minute and ensure that the correct syringe size has been selected.
29. Perform a scout scan to aid in positioning the field of view for CT and PET imaging.
30. Start injecting contrast agent using the syringe pump and wait 15 s before starting the CT scan to allow the contrast to circulate. Make sure all investigators are behind a lead shield. Stop the syringe pump when the CT scan is finished. Troubleshooting 3
31. Initiate a 30-minute PET scan and continue monitoring the animal's respiratory rate. Take the mouse out of the scanner when the PET is finished. Troubleshooting 4
32. Reconstruct the obtained PET data as a static acquisition with the highest possible number of iterations and the smallest available voxel size.

Optional: If the PET scanner has a bed heater it is advised to use it during the scan.

Note: Depending on the organ of interest, ECG- or respiratory-gated PET scans might be preferred. If examining the bone marrow or spleen, ungated PET scans are usually sufficient.

Ex vivo gamma counting of radioactive organs

Timing: 30 min

The animals are sacrificed and their organs separately gamma counted to quantify nanobody uptake.

33. Label and weigh 1.5 mL Eppendorf tubes, one for each organ of interest per mouse. Use a scale that is accurate to at least 10^{-5} grams.
34. Place the mouse in an induction chamber, set the oxygen flow at 1 L/min and the isoflurane vol.% at 2.5–3. Confirm the depth of anesthesia by pinching the toes of the mouse with forceps. The absence of reflex retraction of the leg indicates that the animal is fully anesthetized. Subsequently, perform cervical dislocation to humanely euthanize the mouse.
35. Secure the mouse in a supine position on a dissection tray.
36. Open the thorax and abdomen, draw blood for gamma counting and potential other assays, and perfuse the animal with 20 mL PBS through the left ventricle.
37. Collect organs of interest and place these in the corresponding pre-weighed vials.
38. Weigh the vials containing the organs.
39. Put the vials in a gamma counter and measure their activity while using an open field filter and measuring counts per minute (CPM).

Note: We recommend weighing the empty tubes before adding the organs, as there might be a small but significant difference between their weight (weight full – weight empty = sample weight). This is especially important for light tissues, including lymph nodes.

Note: When interested in bone marrow activity, it is recommended to analyze the bone marrow separate from the bone. To do so, snap off the ends of the femur, perforate the bottom of a 0.5 mL Eppendorf polymerase chain reaction (PCR) tube, and feed the open end of the femur through the hole. Subsequently, place the PCR tube inside the pre-weighed 1.5 mL Eppendorf. Spin the vials for approximately 30 s in a small centrifuge using the quick spin function to extract the bone marrow.

Ex vivo autoradiography of radioactive organs

Timing: 20 min + several min to days of exposure

Autoradiography allows determining the regional distribution of radioisotope deposition in both tissue samples and histological sections with sub-millimeter resolution. Staining adjacent sections for relevant cellular and molecular markers enables directly comparing distribution patterns.

40. Prepare tissues of interest for autoradiography.
    a. Thoroughly rinse tissue samples to prevent a false signal from blood clots.
    b. Dry the tissue samples before placing them on plastic foil.
    c. Exposing samples with hugely varying amounts of activity in the same film cassette will complicate optimizing their exposure time. Therefore, sort the samples by the amount of activity they contain.

    Optional: Large tissues can be sectioned to facilitate both histological and autoradiographic analyses. To achieve this the following procedure can be followed:
    d. Cut the tissue in smaller samples around 5 millimeters thick.
    e. Embed the sections in Optimal Cutting Tissue (OCT) Compound in a mold and put them on dry ice for at least 10 min. Avoid air bubbles as much as possible during the embedding.
    f. Using a cryotome, cut one thick (>10 μm) section and as many adjacent sections as needed for staining with other markers. The thickness of the adjacent sections depends on the desired histological staining protocol. Clean the cryotome regularly to prevent cross-contaminating the samples with activity.
    g. The thicker sections should be used for autoradiography, thinner ones for histological or fluorescent analyses.

41. Prepare autoradiography cassette and plate
    a. Check that the cassettes used for tissue exposure are clean and free of any trace radioactivity. Before use, blank the phosphor imaging plates using the image eraser to remove remnants of previous exposures, an erasure time of 60 min is typically used.
    b. Line the bottom inner side of the cassette with plastic film.
    c. Place the tissue samples or slides in the desired order over the lined bottom side of the cassette. It might be necessary to take several autoradiographs to accommodate for differences in required exposure time.
    d. Take a picture of the sample/slide layout for future reference during analysis.
    e. Carefully cover the samples/slides with another layer of plastic film. This will prevent contamination of the phosphor imaging plate.

    Optional: Plates can be read after blanking to make sure previous images have been completely erased.

42. Expose the phosphor imaging plate to the samples
    a. Place the phosphor imaging plate on top of the samples, with the imaging side (white) facing the samples.
    b. Carefully lock the cassette and place it in a freezer (−20°C).

c. Make sure the freezer is free of any other radioactive material as this can cause background signal spill.
d. Expose the plates between 2 h and several days, depending on the ^{89}Zr-labeled nanobody dose injected and its accumulation in the tissue of interest.

43. Read the phosphor imaging plate.
    a. Carefully open the cassette and remove the phosphor imaging plate, avoiding excessive light exposure.
    b. Quickly secure the plate onto the magnetic holder, with the imaging side (white) facing down.
    c. Place the holder with the plate in the phosphor imager tray (Typhoon 7000IP plate reader GE Healthcare, Pittsburgh, PA) and close the lid.
    d. Set the reading parameters at the maximum attainable pixel resolution (25 μm for Typhoon 7000IP).
    e. Read the plate, save the file, and check the image quality. Troubleshooting 5

Analyzing data from PET imaging, gamma counting, and autoradiography

Timing: 2 h (varies depending on the number of animals)

Data analyses for comparing – and potentially correlating – the PET, gamma counting, and autoradiography results.

44. PET imaging data analyses.
    a. Import data to image analysis software (*e.g.*, OsirixMD or Horos).
    b. Open the CT and PET data in the 2D viewer and ensure that both PET and CT images are displayed in the same orientation.
    c. Convert the PET scan data to standardized uptake values (SUV) by selecting "convert to SUV" and inputting mouse body weight, scan time, and activity of the injected dose.
    d. On CT, use the pencil tool to draw regions of interest (ROIs) around the organ of interest every 3–4 slices and label each ROI set with the corresponding name (*e.g.*, bone marrow, spleen, liver, etc.). To interpolate ROIs between slices, select "generate missing ROIs".
    e. Drag the small icon atop the CT window over to the PET scan to copy ROIs. Scroll through the scan to ensure that the ROIs are properly aligned and export SUV values for every ROI.
    f. Open the produced .csv file in any spreadsheet program and average all SUVs for each respective organ. For organs with homogenous signal distribution, such as the spleen, liver, or bone marrow, utilize ROIMean. For organs with a more heterogeneous distribution of PET signal, such as the heart, utilize ROIMin or ROIMax.
45. Gamma counting data analyses.
    a. Calculate the injected ^{89}Zr-labeled nanobody dose at the time of injection (pre-injection activity minus the remaining activity in the empty syringe). Make sure to correct for decay to the time of injection.
    b. Convert the injected dose from μCi to CPM, by making a calibration curve between the dose calibrator (μCi) and gamma counter (CPM) results. This curve should be made anew after any maintenance or intervention on the gamma counter.
    c. Decay-correct the CPM values obtained by gamma counting of the tissues to the time of injection. This way both the injected activity and activity in the tissues are expressed as CPM and decay-corrected to the time of injection.
    d. Calculate the activity in a specific organ as a percentage of the total injected dose (%ID).
    e. Divide the %ID by the weight of each sample to calculate the percentage of the injected dose per gram of tissue (%ID/g). This unit resembles the SUVmean values obtained by PET imaging.
46. Autoradiography data analyses.

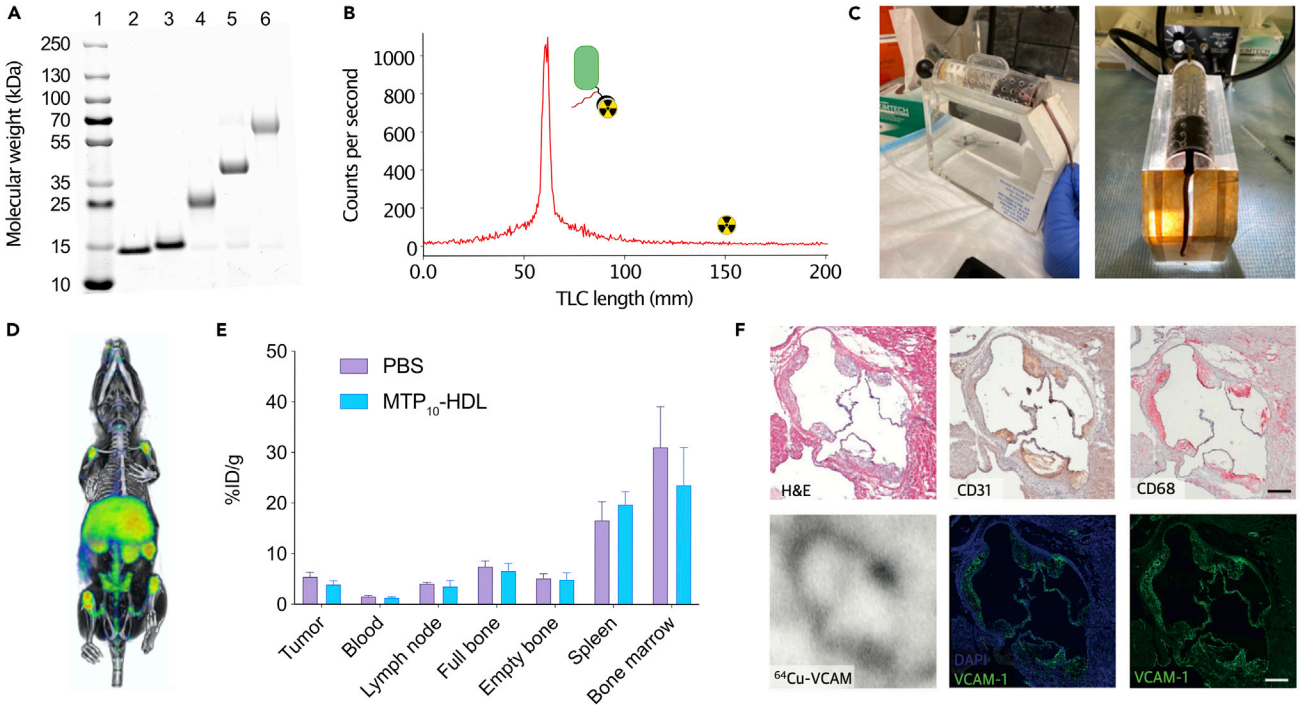

Figure 1. Expected outcomes

(A) SDS-PAGE results of the anti-CD11b nanobody and its derivatives. Lane 1 = mass ladder, lane 2 = anti-CD11b nanobody (NB), lane 3 = NB functionalized with the GGG-azide-DFO substrate (NB-azide-DFO), lane 4 = NB-DFO functionalized using DBCO-PEG 5 kDa, lane 5 = NB-DFO functionalized using DBCO-PEG 10 kDa, lane 6 = NB-DFO functionalized using DBCO-PEG 20 kDa.

(B) radio-TLC analyses of ^{89}Zr-labeled anti-CD11b nanobody. The base of the TLC strip was placed at 50 mm, the nanobody sample spotted at approximately 60 mm, and the solvent front lies at approximately 150 mm and indicates the absence of free ^{89}Zr.

(C) Photos of a mouse in a restrainer, used to facilitate tail vein injections.

(D and E) C57BL/6 mice were inoculated with B16F10 cells and 7 days later treated with PBS or MTP_{10}-HDL nanoimmunotherapy (for details on MTP_{10}-HDL please see (Priem et al., 2020)). Another 7 days later animals were injected intravenously with ^{89}Zr-labeled anti-CD11b-nanobody, which was allowed to circulate for 24 h. Subsequently, the mice were (D) imaged by PET/CT imaging and (E) sacrificed for gamma counting, n=5.

(F) Aortic root sections from Apoe$^{-/-}$ mice with atherosclerosis, showing in the **upper row** hematoxylin and eosin (H&E) staining (left), immunohistochemistry for CD31 (endothelial cells, middle), and CD68 (macrophages, right); in **lower row**, autoradiography showing distribution of a ^{64}Cu-labeled VCAM-1-targeting nanobody (left) and immunofluorescence for VCAM-1 in adjacent sections, with (middle) and without (right) 4,6-diamino-2-phenylindole (DAPI) stain. Scale Bar represents 200 μm. Panels D and F were adapted with permission from (Priem et al., 2020) and (Senders et al., 2019), respectively.

a. Quantification of the autoradiography images can be performed using ImageJ software, freely available from the National Institutes of Health.
b. Divide larger tissues into equal areas to facilitate comparison with similar areas analyzed by PET.
c. For histological correlation, create a mask of the autoradiography image and quantify the image intensity based on the grayscale.
d. Repeat the steps for the adjacent sections, which have been histologically or fluorescently stained.
e. Compare the intensity of the staining within the identical areas and calculate the correlation coefficient.

Note: For details on how to compare autoradiography results with PET or histological data, please see (Senders et al., 2019).

Optional: For improved accuracy in quantitative autoradiographic analyses, a calibration curve can be generated by exposing a ladder of standards of known activity, spotted on TLC paper, together with the samples. However, we recommend that all quantitative analyses should be based on gamma counting and PET imaging results.

EXPECTED OUTCOMES

This protocol typically yields approximately 10 - 40 mg nanobodies per liter of expression culture. The reactions used to functionalize the nanobodies with DFO and PEG usually reach near quantitative conversions (Figure 1A). Radiolabeling the DFO-functionalized nanobodies (with or without PEG chains) should yield ^{89}Zr-labeled nanobodies with a radiochemical purity of >97% and in a radiochemical yield of >80% (Figure 1B). Injecting the ^{89}Zr-labeled nanobodies in mice by tail vein injection takes practice and a dedicated set-up, but is otherwise well reproducible (Figure 1C). Administering ^{89}Zr-labeled (and PEGylated-)nanobody doses of approximately 200 μCi per animal generally yields excellent PET imaging results at 24 h post injection, with the optical imaging time varying with the type of PEG chain used (Figure 1D). Doses and imaging time points can be varied if desired, but might require adjusting the duration of the PET scan. A relatively high uptake in the spleen and bone marrow can be expected when using anti-CD11b-nanobody (Figure 1E). Any free ^{89}Zr that might inadvertently be administered will accumulate predominantly in bone. Autoradiographic results can be related to those of a variety of other techniques, including histological analyses and immunofluorescence (Figure 1F). The challenges associated with such comparisons will be case-specific, for inspiration please see (Senders et al., 2019).

LIMITATIONS

Our protocol's main limitation lies in producing the required nanobodies, as the reliance on camelids is time consuming and poses severe financial burdens and animal housing constraints. However, camelid-free nanobody production methods such as yeast surface display platforms might provide a future alternative. (McMahon et al., 2018) Although our use of ^{89}Zr limits nuclear imaging readouts to PET, others have radiolabeled nanobodies with single-photon emission computed tomography (SPECT)-compatible radionuclides.(Chakravarty et al., 2014) In addition, similar protocols as described here have been used to radiolabel nanobodies with radioisotopes possessing a broad range of physical half-lives (*e.g.*, ^{18}F, ^{64}Cu, ^{68}Ga)(Rashidian et al., 2015; Senders et al., 2019). We expect variations on our protocol to be applicable to a wide variety of nanobodies and animal species.

TROUBLESHOOTING

Problem 1

^{89}Zr does not (adequately) bind to the DFO-functionalized nanobody (step_9).

Potential solution

There could be two causes: 1) The reaction mixture's pH was not within the suggested 6.8–7.5 range. Check the pH of the Na_2CO_3 solution and HEPES buffer and repeat the radiolabeling. If the radiolabeling happens but in low yields, the reaction time can be extended or the reaction mixture incubated at 37°C rather than at 25°C. 2) The DFO group is blocked as a result of binding other metal ions, especially iron. Performing a mass spectrometric analysis on the DFO-functionalized nanobody can confirm whether this is the case. If so, repeat the procedure with freshly Chelexed solutions.

Problem 2

Inadequate separation between bound and unbound ^{89}Zr when performing PD-10 purification of the radiolabeled nanobodies (step_15).

Potential solution

This can happen when a large amount of unbound ^{89}Zr is present, either because too much ^{89}Zr has been used or it did not successfully bind to the DFO-nanobody (see Problem 1). Alternatively, the reaction mixture might have been loaded on the PD-10 in too large a volume (>1.0 mL rather than the advised 0.5 mL). Repeat the radiolabeling with less ^{89}Zr and in a smaller volume.

Problem 3

The CT scan indicates the contrast agent was not properly injected. Typically indicated by a lack of uptake in the kidneys and bladder (step_30).

Potential solution

This can happen if the catheter was placed incorrectly before positioning the mouse on the scanner, or if the catheter slipped in the process of moving the animal form the restrainer to the PET/CT scanner. To correct this, the mouse must again be restrained for tail vein placement of the catheter and the CT scans repeated. Always check the bottle with contrast agent before use to ensure there is no crystallization, as injecting crystalized contrast agent can be harmful to the animal and lead to insufficient contrast.

Problem 4

The signal-to-noise ratio of the PET data is low (step_31).

Potential solution

This can happen when looking at organs with a relatively low nanobody uptake. Try increasing the PET scan time and/or the injected ^{89}Zr-labeled nanobody dose. Decreasing the time between administration and PET scan initiation might also help. If the images are acquired using a nanobody with no PEG moiety, then try PEGylating the nanobody with a 5, 10 or 20 kDa PEG chain and repeating the imaging experiments; as the PEGylation can increase the signal-to-noise ratio for some nanobodies.

Problem 5

The autoradiographs seem under- or overexposed (step_43).

Potential solution

Autoradiographs can appear noisy or oversaturated when a suboptimal exposure time is used. A sample's optimal exposure time will predominantly depend on its radioactivity content, with "hotter" samples requiring shorter exposure times. One of the advantages of ^{89}Zr is its relatively long physical half-life, which allows repeating exposures if necessary. When poor results are obtained, the exposure can be repeated using a modified exposure time. When doing so, make sure to correct for the decay that took place during the initial exposure. For samples containing substantial amounts of activity, it is often best to wait several days before performing autoradiographic analyses.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mohammad Rashidian, mohammad_rashidian@dfci.harvard.edu.

Materials availability

This study did not generate new unique reagents. The sortase 7M is available in addgene (Plasmid #51141). The anti-CD11b nanobody sequence is published (Rashidian et al., 2015). The Gly_3-azide-DFO peptide synthesis has been reported (Rashidian et al., 2017) and can be made following standard solid-phase peptide synthesis.

Data and code availability

This study did not generate datasets.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants R01 CA220234, R01 HL144072, and P01 HL131478, NWO/ZonMW Vici 91818622 (all to W.J.M.M.), NIH 1K22CA226040 (to M.R.), and the Dana-Farber Cancer Institute Innovation Research Fund Basic Research Grant (to M.R). B.P. was supported by the AMC Ph.D. Scholarship. The anti-CD11b nanobody and sortase 7M plasmids were kind gifts from Dr. Hidde Ploegh's laboratory.

AUTHOR CONTRIBUTIONS

A.J.P.T., O.B.A., J.M., M.M.T.v.L., Y.C.T., B.P., M.L.S., and C.P.-M. wrote the manuscript. A.J.P.T. produced the figures. A.J.P.T., W.J.M.M., and M.R. edited the final draft. All authors approved the final draft. W.J.M.M. and M.R. provided funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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