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Heat-induced radiolabeling and fluorescence labeling of Feraheme nanoparticles for PET/SPECT imaging and flow cytometry

Hushan Yuan, **Moses Q Wilks**, **Marc D Normandin**, **Georges El Fakhri**, **Charalambos Kaittanis**, and **Lee Josephson**

Gordon Center for Medical Imaging, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA

Abstract

Feraheme (FH) nanoparticles (NPs) have been used extensively for treatment of iron anemia (due to their slow release of ionic iron in acidic environments). In addition, injected FH NPs are internalized by monocytes and function as MRI biomarkers for the pathological accumulation of monocytes in disease. We have recently expanded these applications by radiolabeling FH NPs for positron emission tomography (PET) or single-photon emission computed tomography (SPECT) imaging using a heat-induced radiolabeling (HIR) strategy. Imaging FH NPs using PET/SPECT has important advantages over MRI due to lower iron doses and improved quantitation of tissue NP concentrations. HIR of FH NPs leaves the physical and biological properties of the NPs unchanged and allows researchers to build on the extensive knowledge obtained about the pharmacokinetic and safety aspects of FH NPs. In this protocol, we present the step-by-step procedures for heat (120 °C)-induced bonding of three widely employed radiocations (89Zr4 + or 64Cu2 + for PET, and 111In3 + for SPECT) to FH NPs using a chelateless radiocation surface adsorption (RSA) approach. In addition, we describe the conversion of FH carboxyl groups into amines and their reaction with an N-hydroxysuccinimide (NHS) of a Cy5.5 fluorophore. This yields Cy5.5-FH, a fluorescent FH that enables the cells internalizing Cy5.5-FH to be examined using flow cytometry. Finally, we describe procedures for in vivo and ex vivo uptake of Cy5.5-FH by monocytes and for in vivo microPET/CT imaging of HIR-FH NPs. Synthesis of HIR-FH requires experience with working with radioactive cations and can be completed within < 4 h. Synthesis of Cy5.5-FH NPs takes ~17 h.

Correspondence should be addressed to L.J. (ljosephson@mgh.harvard.edu).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS: L.J. conceived of the HIR strategy and conducted synthetic and analytical experiments. H.Y. synthesized and analyzed all materials, and developed all analytical methods. M.Q.W., C.K., and M.D.N. performed in vivo imaging and flow cytometry studies. G.E.F. and L.J. analyzed data and wrote the manuscript.

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INTRODUCTION

FH (Fig. 1a) is an ultrasmall superparamagnetic iron oxide (USPIO) NP currently used for treating iron anemia (approved indication)[1,2] and as an MRI contrast agent (off-label)[3–6]. Here we describe the heat-induced radiolabeling of FH NPs, using a chelateless, RSA labeling method[7–10]. We provide step-by-step procedures for the synthesis of FH NPs for any of three cations widely employed in clinical imaging: $^{89}Zr^{4+}$ or $^{64}Cu^{2+}$ for PET, and $^{111}In^{3+}$ for SPECT imaging. The advantage of our approach is the ability to bond radiocations to an NP drug with heat while leaving the physical and biological properties of the NP drug unchanged. The considerable clinical experience obtained by using FH NPs as a treatment for iron anemia and as an MRI contrast agent allows researchers to build on previous knowledge with respect to predicting the likely pharmacokinetics, safety, and imaging applications of the new HIR-FHs.

Injected FH NPs are internalized by monocytes and function as MR imaging biomarkers for the pathological accumulation of monocytes in disease. FH NPs have been used to image monocyte infiltration in the pancreas in type 1 diabetes[11], in the heart during myocardial infarction[12], and in cerebral aneurysms[13,14]. USPIOs similar to FH NPs have been used to image monocyte infiltration in multiple sclerosis, even with an intact blood–brain barrier[15,16], and in carotid atherosclerotic plaques (for a review, see Usman et al.[17]).

In this protocol we describe our recently developed strategy for HIR of FH NPs, which allows them to be used for PET and SPECT imaging[18–21]. In addition, we present a strategy for labeling FH NPs with fluorescent Cy5.5 fluorophores, which enables researchers to examine NP-internalizing cells by flow cytometry[19].

FH nanoparticles are heat stable but acid labile

The heat stability and acid lability of FH are important fea-tures for labeling the NP with radiometals or fluorochromes. FH consists of a superparamagnetic iron oxide core and a shell of carboxymethyldextran (CMD) polymer (Fig. 1b, top)[22–24]. According to the manufacturer's insert, FH has a chemical for-mula of $(Fe_{5874}O_{8752})$core-$(C_{11719}H_{18682}O_{9933}Na_{414})$coating, which corresponds to a spherical iron oxide core with a diameter of 6.6 nm. FH has an overall hydrodynamic diameter (iron oxide core plus CMD) of 17–31 nm. There are multiple carboxyl groups on the CMD, which confer heat stability, an observation often noted in the patent literature[25–27]. The heat stability of FH is evident both from the use of terminal sterilization at 121 °C during manufacture[25] and from its storage for months at room temperature (20–25 °C) as a concentrated (30 mg of Fe/ml) fluid for intravenous injection. The chemical stability of CMD–iron oxide interaction permits the conversion of carboxyl groups to amines, as well as reaction with the NHS esters of fluorochromes (see below and refs.[28,29]).

However, the CMD is extended and porous (Fig. 1b, bottom), and this allows hydrogen ion access to the iron oxide. In vivo dis-solution of FH in phagolysosome results in the release of ionic iron and reversal of iron anemia. The acid lability of FH means that radiolabeling procedures should maintain a pH of 7 or above, as is accomplished with the HIR protocols

presented here. See also the 'Precautions' section of the FH product labeling. Radiolabeling protocols that use acid should not be adapted for use with FH.

HIR, an RSA method

HIR is an RSA method in which radiocations bind to the iron oxide surface of the FH NP (Fig. 2a). This conclusion is based on X-band electron spin resonance studies showing ^{63}Cu binding to iron oxide, as well as on the fact that HIR can use non-CMD-coated magnetic NPs[18]. Finally, iron oxides have active surfaces, evident by their surface doping with nonradioactive cations, including manganese[30], cerium[10], and gallium[10].

$^{89}Zr^{4+}$, $^{64}Cu^{2+}$, and $^{111}In^{3+}$ bind to FH NPs with the same heating protocol (2 h, 120 °C), one that does not alter NP physical properties; these include NP size and relaxivity (see Fig. 1 of ref. 19 and Fig. 3 of ref. 18), and NP blood clearance (Fig. 2 of ref. 19 and Fig. 3 of ref. 18). A typical HIR reaction requires a milligram of NP iron and several millicuries of radiometal, a situation that corresponds to a molar ratio of iron to radiocation of at least 100 for all radiocations employed here[19]. Hence, the over-all chemical composition of FH NPs is not substantially altered by radiocation addition. A further comparison with other NP radiolabeling methods is given below.

Overview of the procedure

The HIR reaction of FH consists of three main stages (Fig. 2a)[18,19]. In the first stage, the FH NP is 'loaded' with a radiocation ($^{111}In^{3+}$, $^{89}Zr^{4+}$, or $^{64}Cu^{2+}$) by heating (120 °C, 2 h, Steps 6–8). In the next stage, all unreacted radiocations are 'stripped' by addition of deferoxamine (DFO), which binds to radiocations loosely adsorbed to the NP (Steps 9–11). In the final stage, the HIR-FH NPs are 'purified' by size-exclusion chromatography (SEC) (Steps 21–25).

To analyze the fate of FH in biological systems, it is desirable to have fluorescently labeled NPs, which enable cells that internalize NPs to be analyzed by flow cytometry. We also provide detailed procedures for the synthesis of a fluorescent FH, Cy5.5-FH (Steps 79–129; Fig. 2b), which allows for the analysis of cells that have internalized FH NPs by flow cytometry (see below and Boxes 1 and 2). The three types of FH NPs described in this protocol (FH, HIR-FH (Fig. 2a), and Cy5.5-FH (Fig. 2b)), have the same physical properties (size, relaxivity) and the same blood half-life[19].

The role of heat in the RSA of $^{89}Zr^{4+}$, $^{111}In^{3+}$, and $^{64}Cu^{2+}$ is shown in Figure 3. Incubation of FH with these radiocations at 25 °C leaves radioactivity at the origin, where the radiometal–DFO chelates migrate (Fig. 3a,c,e). Incubation of any of these radiocations plus FH at 120 °C causes the cations to run at the solvent front, where FH migrates (Fig. 3b,d,f). Hence, we term our technique HIR.

We also provide detailed procedures for determining the radio-chemical stability of HIR-FHs using a chelation challenge/SEC assay (Steps 51–64) and a serum challenge/thin-layer chromatog-raphy (TLC) assay (Steps 65–78). In the chelation challenge assay, 89Zr-FH NPs are incubated with 10 μM DFO in Tris buffer (10 mM), followed by an analysis of low-molecular-weight $^{89}Zr^{4+}$: DFO by SEC (Fig. 4a). In the serum challenge assay, ^{89}Zr-FH

NPs are incubated with serum, followed by TLC. Here protein-bound $^{89}Zr^{4+}$, if present, is separated from the radioactive NP that moves at the solvent front (Fig. 4b).

Finally, we describe procedures for in vivo and ex vivo uptake of Cy5.5-FH by monocytes isolated from mice (Box 1; Supplementary Fig. 1) and rhesus macaque monkeys (Box 2), and for in vivo microPET/CT imaging of HIR-FH NPs in mice (Box 3).

Comparison of HIR with other NP radiolabeling methods

Strategies for radiolabeling NPs have been the subject of recent reviews[31–33]. Perhaps the most common is the synthesis of NPs with a surface chelate, followed by radiometal chelation[34–36]. A second strategy involves mixing radiocations and nonradioactive cations before NP synthesis, a strategy used with the synthesis of ^{59}Fe- and ^{64}Cu-doped iron oxide NPs[37–39]. However, the synthesis must be fast in comparison with the radiochemical decay of the radioisotopes that are used in clinical diagnosis, as these tend to have shorter radiochemical half-lives to limit radiation exposure. The final method is RSA, in which radioactive cations bind to surfaces, and which includes the HIR method we use. The RSA method has been used to bind radioactive arsenics to iron oxides[7], ^{64}Cu to graphene[8], ^{59}Fe to oleic acid-stabilized SPIOs[9], and ^{68}Ga to charge-stabilized iron oxide[10].

Flow cytometry to identify nanoparticle-bearing cells

After intravenous injections, Cy5.5-FH (or HIR-FH) NPs are internalized by blood-residing monocytes (Fig. 5a) that slowly traffic to lymph nodes or sites of inflammation (NPs are also internalized by hepatic Kupffer cells). To identify blood cells internalizing injected FH NPs, mice are injected with Cy5.5-FH, and whole blood from Cy5.5-FH-injected and control mice is collected. The leukocyte buffy coat is isolated, and the cells are submitted to dual-channel flow cytometry (Fig. 5b,c) using a fluorescein-labeled anti-CD11b to identify monocytes. To dem-onstrate that monocyte internalization is not limited to mice, Cy5.5-FH is intravenously injected into a Rhesus monkey as shown in Figure 5d. Both mice and the monkey internalize intravenously injected Cy5.5-FH by dual-wavelength flow cytometry.

Imaging inflammation with PET/CT and 89Zr-FH

After intravenous injection, ^{89}Zr-FH is internalized by monocytes that traffic to sites of mild irritation as shown in Figure 6a, in which a mouse that had a minor, self-inflicted irritation of its paw is shown (Fig. 6b). A 3D video of the animal shown in Figure 6a is available as Supplementary Video 1. PET imaging showed localization of radioactivity in the paw and the associated leg, extreme asymmetry in accumulation in the lymphatic system, and much greater activity in the lymph node ipsilateral to the injury, as shown in Figure 6a.

Limitations and considerations for HIR or fluorochrome labeling

There are three major limitations of the HIR approach. First, the radiochemical half-life of the isotope used must be long compared with the 3–4 h time for the HIR reaction. Second, because of the acid lability of FH and the fact that radiocations are typically supplied in acid, radiocation neutralization must be performed in a manner that does not induce formation of a nonmagnetic radiocation–oxide NP. See limitations and considerations in ‘Experimental design’. Third, if considering the use of an HIR with NPs other than FH, the possibility of

heat-induced NP aggregation must be considered. (As noted above, FH has an unusual temperature stability related to the use of its CMD coating.) The temperature stability of non-FH particles can be evaluated by determining the particle size, performing the HIR procedure without radioactivity, and again determining particle size.

The conversion of FH carboxyl groups to amines, followed by their reaction with NHS esters, has been used to attach a variety of fluorochromes and biomolecules (see Fig. 2b and refs.[28,29,40]). An advantage of Cy5.5 is that its absorption maxima do not over-lap with iron's absorption, facilitating determination of an accu-rate ratio of fluorochrome to iron (see Fig. 2b).

Experimental design

Considerations with HIR reactions—There are several limitations/considerations to bear in mind regarding HIR of FH NPs. First, as described in the limitations above, as acid can dissolve iron oxides (see above and Fig. 1b, bottom), the radiocation labeling must be carried out at neutral or basic pH. Use of good pH paper, with proper pH controls, is needed to assess the pH during the proce-dure with microliter quantities of radioactive liquids. Second, an appropriate stripping step must be used. After the heat-induced loading step, a small fraction of radiocations are weakly bound to the NP, so the addition of chelator before gel filtration purification serves to extract weakly bound cations from the NP. The rationale for 'stripping' in the HIR process is analogous to vigorous washing procedures used when synthesizing particles for column affinity purification. Here a tight, covalent coupling of a biomolecule (one involved in affinity purification) is accompanied by some weaker biomolecule-to-particle interactions. Weaker associations are resolved by wash steps (often at high ionic strength and/or acidic or basic pH values) analogous to our stripping step. Phosphate buffers, which can form insoluble radiocation-phosphate particu-lates, are not used in the steps in which radiocations are present (Steps 2–11). (Phosphate buffers can be used in steps after the radiocation has been incorporated into the iron oxide.) To assess the formation of unwanted, radioactive, high-molecular-weight species, HIR procedures can be performed in the absence of FH by repeating Steps 2–8. Radioactivity should in this case be present exclusively in the low-molecular-weight fraction as determined by SEC; this is achieved by repeating Steps 39–45.

Yield, purity, and scale—The radiochemical yield (RCY) of HIR can be readily obtained by the division of the radioactivity of the HIR-FH by the amount of activity used at the beginning, as described in Step 33. For ^{89}Zr-HIR-FH and ^{111}In-HIR-FH, decay correction is neglected because of their longer half-lives. However, in the case of ^{64}Cu-HIR-FH, decay correction should be taken into account for an accurate RCY because of its shorter half-life.

To obtain specific activity (Step 50), payload calculation (Step 127), and the yields for iron recovery (Steps 49, 90, 109, and 129), two standard curves must be generated. One is for iron and is generated by plotting the iron concentrations (M) of different dilutions of FH versus the UV absorbance of FH at 450 nm in 0.9% (wt/vol) saline (Step 46). The other is for Cy5.5 and is generated by plotting the concentration of Cy5.5 (M) versus the absorbance at 675 nm in DPBS (Step 123). As the UV absorbance of FH (< 500 nm) is quite different

from that of Cy5.5 (620–700 nm) (Fig. 2b), the contribution of Cy5.5 to FH can be ignored in the concentration range of this protocol. After the measurement of the UV absorbance at 450 nm and 675 nm, the concentrations of iron and Cy5.5 can be calculated according to the standard curves and dilution factors. The iron recovery yield will further be determined by dividing the iron amount in the purified FH products (FH, HIR-FH, FH-amine, and Cy5.5-FH) by the original iron amount (Steps 49, 90, 109, and 129). The payload of Cy5.5 on each FH NP can be obtained from the ratio of Cy5.5 concentration/FH NP concentration (Step 127). The specific activity of HIR-FH NPs can be calculated through the division of the activity in the purified HIR-FH NPs by the amount of NPs (Step 50).

Both TLC and SEC have been used to verify the radiochemi-cal purity (RCP) and labeling efficiency (RCY), as described in Steps 12–20 and 34–45. For the TLC method, a strong acidic cation exchange plate is used. When the sampled plate is developed in Chelex-treated water (CTW), either free metal cations or metal:DFO chelates will be held at the origin through the cation exchange mechanism. Even though they have been doped with trace amounts of radiocations, HIR-FH NPs retain their high density of negative charges. They will freely move with the solvent. Therefore, TLC is a rapid and reliable method of interpreting the labeling efficiency, or so-called analytical RCY (Steps 12–20) and RCP (Steps 34–38). A PD-10 column is used for SEC analysis of RCP (Steps 39–45). The separation relies upon the size difference between HIR-FH NPs and small radiometal:DFO chelates. We use SEC instead of labeling efficiency for RCP analysis because the time needed for this analysis is long. In general, TLC analysis has been informative enough to assess both RCY and RCP.

HIR allows the radiolabeling to be scaled up in regard to the amount of activity required for making HIR-FH NPs with higher specific activity. We have tested HIR under a range of activities from 0.5 to 6 mCi in the presence of 0.5 mg Fe. In all cases, the labeling efficiencies were > 90%. As more activity will be used in high-specific-activity labelings, a major concern will be prevention of the radio cations from forming colloids during neutralization (see advice for Step 3 in the ‘Troubleshooting’ section).

HIR also allows for scaling to increase the total amount of iron, which would be needed when HIR-FH NPs are used in larger ani-mals (0.05 mg Fe/kg) or for possible future human doses (5 mg Fe per patient). Thus far, we have performed the labeling using < 1 mg of Fe, as that has been sufficient for current animal studies. When more iron is needed, before the addition of FH (Step 6), we suggest taking an additional step to concentrate FH with centrifugation, by using a 50-kDa MWCO Amicon filter to a volume of 50 μl, and keeping the concentration of radiocations consistent with this protocol (1–5 mCi/200–300 μl) (Steps 4–6).

Radioactivity—All experiments involving radioactivity must be performed by persons qualified and trained to handle radio-isotopes and by following all relevant state, federal and institu-tional regulations and approvals. Approval for the radioactive experiments described in the protocol was obtained from Massachusetts General Hospital, Harvard Medical School. The person who conducts the radioactive experiments must wear personnel monitoring devices such as a clip-on dosimeter and ring badge bearing the individual’s name and the

date of the monitoring period. The radiolabeling should be performed in a certified, well-ventilated, lead-lined fume hood with a sliding lead glass shield. A plastic transparent bag (double-bagged) should be placed for collecting radioactive non-sharp solid wastes (e.g., gloves, used PD-10 columns, plastic tubes). A 500-ml plastic bottle should be prepared for containing liquid wastes (e.g., elution buffers, filtrates). A sharps container should be ready for disposal of glass vials, pipette tips, needles, syringes, and other sharp wastes. All of these waste containers should be kept behind a lead brick–shielded area for reducing the exposure of the operator during the labeling procedure. After the HIR labeling, all waste should be labeled according to institutional regulations and state/national laws, relocated to the designated decay area, and documented.

Clinical safety of FH nanoparticles—Based on the considerable clinical experience with FH for anemia treatment[1,2,41,42] or as MRI contrast agents[12,43,44], adverse reactions with HIR-FHs are likely to be exceedingly rare. With the initial, rapid bolus method of administration of FH used for anemia (a strategy used between 2009 and 2015), a total dose of ~510 mg of Fe was injected into patients in < 1 min, resulting in anaphylaxis in ~35 patients per 100,000 doses[42]. Since 2015, FH has been administered by dilution into saline or dextrose, and infusion over at least 15 min, a presumably safer method[45]. In addition, the amount of iron needed for PET/SPECT imaging (0.5–10 mg) is a small fraction of the iron dose needed for either anemia treatment or MRI contrast. Even with FH's history of clinical use and the iron dose reduction obtained with radiolabeling, a radiolabeled FH can be used clinically only after obtaining approval from appropriate institutional and governmental regulatory authorities.

Recommendations for running the HIR reaction with various radiocations—A key issue for the use of different radiocations is the need to neutralize the acid with Na_2CO_3 (before mixing radio-cations with FH) without inducing the formation of radiocation–oxide particulates. A stepwise addition of Na_2CO_3 with repeated pH evaluation is used (see CRITICAL STEP note for Step 3). Basic pH is avoided, as this may hasten radiocation–oxide particulate formation. The precise neutralization procedure will depend on the intensity of the radiation (in millicuries) of the radiocation used, the radiocation concentration, and the strength of the acid used. Lack of formation of radiocation–oxide particulates can be demonstrated by mixing a trace amount of neutralized radioca-tion solution (10 μCi) with a chelator and using SEC analysis as described in Steps 39–45. The combination of a neutral pH and low-molecular-weight chelate–radiocation upon SEC indicates a successful neutralization.

MATERIALS

REAGENTS

Common solvents, reagents, and buffers

- Millipore water (≥18.0 MΩ/cm)
- Sodium carbonate (Na2CO3; Fluka, cat. no. 71347) **! CAUTION** Na_2CO_3 is an irritant. Wear protective gloves/protective clothing/eye protection/face protection/no contact lenses. In the case of eye contact, rinse cautiously with

water for 15–20 min. If eye irritation persists, get medical advice/attention. In the case of skin contact, wash the affected area with soap and water. Rinse thoroughly. Seek medical attention if irritation, discomfort, or vomiting persists. For more precautions, see the Safety Data Sheet for Na_2CO_3.

- Deferoxamine mesylate (DFO; Sigma-Aldrich, cat. no. D9533-1G)
- Chelex 100 sodium form (200–400-mesh particle size; Fluka, cat. no. 95621-100G-F) **!CAUTION** Contact with acids may cause release of toxic gases. Wear protective gloves/protective clothing/eye protection/face protection/no contact lenses. For more precautions, see the Safety Data Sheet for Chelex.
- Feraheme, 510 mg of Fe/bottle at 30 mg/ml (FH; AMAG Pharmaceuticals)
- Argyle sterile saline (pH 5–6, 0.9%, Covidien, ref. no. 1020) or saline (0.9% (wt/vol), pH 5–6, Fisher Scientific, cat. no. NC0336901) CRITICAL The pH must be adjusted to pH ≥7 (Reagent Setup).
- HCl (Fluka, cat. no. 84415) **! CAUTION** HCl is corrosive and causes serious eye damage, skin corrosion, and respiratory irritation. Wear protective gloves/protective clothing/eye protection/face protection/no contact lenses. If HCl comes into contact with skin (or hair), immediately remove all contaminated clothing. Rinse the skin with water or shower. If in eyes, rinse cautiously with water for several minutes and immediately call a poison center or physician. For more precautions, see the Safety Data Sheet for HCl.
- Na_2HPO_4 (Fisher Scientific, cat. no. S374-3)
- DMSO (Sigma-Aldrich, cat. no. 276855) **! CAUTION** Wear protective gloves/eye protection/face protection/no contact lenses. DMSO is a flammable liquid: Keep away from heat/sparks/open flames/hot surfaces. Do not smoke near DMSO. DMSO is rapidly absorbed through the skin. In the case of skin contact, wash off with soap and plenty of water and consult a physician. For more precautions, see the Safety Data Sheet for DMSO.
- DPBS (Gibco, cat. no. 14190-144)
- Ice (from an ice maker)

HIR labeling of FH

- $^{89}Zr^{4+}$ oxalate (3D Imaging), which provides an ^{89}Zr-oxalate at 5 mCi/10 μl **! CAUTION** All experiments involving radioactivity must be performed by persons qualified and trained to handle radioisotopes and following all relevant state, federal, and institutional regulations and approvals. Approval for the radioactive experiments described in this protocol was obtained from Massachusetts General Hospital, Harvard Medical School. In addition to the general cautions for chemical safety above, the person who conducts the radioactive experiments must wear personnel monitoring devices such as a clip-on dosimeter and ring badge bearing the individual's name and date of the

monitoring period. The radiolabeling should be done in a certified well-ventilated, lead-lined fume hood with a sliding lead glass shield.

- Silicone oil (Alfa Aesar, cat. no. A12728)

Synthesis of Cy5.5-FH

- Ethylenediamine (Sigma-Aldrich, cat. no. E1521-250ML) **! CAUTION** Ethylenediamine is a flammable liquid; it can cause acute oral, inhalation and dermal toxicity; skin corrosion; serious eye damage; and respiratory sensitization. Wear protective gloves/eye protection/face protection/no contact lenses. If inhaled, move the person into fresh air. If not breathing, give artificial respiration and consult a physician. In the case of skin contact, remove contaminated clothing and shoes immediately. Wash off with soap and plenty of water. Take the victim to a hospital immediately. In the case of eye contact, rinse thoroughly with plenty of water for at least 15 min and consult a physician. Continue rinsing eyes during transport to the hospital. If swallowed, do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse the mouth with water and consult a physician. For more precautions, see the Safety Data Sheet for ethylenediamine.
- Hydroxybenzotriazole (HOBT; TCI, cat. no. H0468) **! CAUTION** HOBT is a flammable solid. Keep away from heat/sparks/open flames/hot surfaces. Do not smoke near HOBT. HOBT causes eye irritation. Wear protective gloves/eye protection/face protection/no contact lenses. For more precautions, see the Safety Data Sheet for hydroxybenzotriazole.
- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; EMD Millipore, cat. no. 01-62-0011) **! CAUTION** EDC causes skin irritation, serious eye damage, and respiratory irritation. Wear protective gloves/eye protection/face protection/no contact lenses. Avoid breathing its dust. If EDC comes into contact with skin, wash with plenty of soap and water. In the case of eye contact, rinse thoroughly with plenty of water for at least 15 min and consult a physician. For more precautions, see the Safety Data Sheet for EDC.
- MES (Sigma-Aldrich, cat. no. M5057-25G) **! CAUTION** Wear protective gloves/eye protection/face protection/no contact lenses. Avoid dust formation. For more precautions, see the Safety Data Sheet for MES.
- Sephadex G-25 (Sigma-Aldrich, cat. no. G2580-100g) **! CAUTION** Wear protective gloves/eye protection/face protection/no contact lenses. Avoid dust formation. For more precautions, see the Safety Data Sheet of Sepha-dex G-25.
- Vitamin B12 (Sigma-Aldrich, cat. no. V-2876)
- Blue dextran (Sigma-Aldrich, cat. no. D5751-1G)
- Drierite (Fisher Scientific, cat. no. 23-116582)
- Cy5.5-NHS ester (GE Healthcare, cat. no. PA15602) **! CAUTION** Harmful if swallowed. May cause allergy or asthma symptoms or breathing difficulties if

inhaled. May cause an allergic skin reaction. Wear protective gloves/eye protection/face protection/no contact lenses. For more precautions, see the Safety Data Sheet for Cy5.5-NHS.

NP radiochemical stability, chelation challenge

- Trizma base (Sigma-Aldrich, cat. no. T4661-100G) **! CAUTION** Wear protective gloves/eye protection/face protection/no contact lenses. Avoid dust formation. For more precautions, see the Safety Data Sheet for Trizma base.
- Acetic acid (Fluka, cat. no. 07692-250ML-F) **! CAUTION** Acetic acid is a flammable liquids and can cause skin corrosion and serious eye damage. Wear protective gloves/eye protection/face protection/no contact lenses. Beware of vapors accumulating to form explosive concentrations. Vapors can accumulate in low areas. For more precautions, see the Safety Data Sheet for acetic acid.

Radiochemical stability, serum challenge

- Normal mouse serum (Abcam, cat. no. ab7486)

In vivo procedures

- Mice, male BALB/c, 15–20 weeks old, 25–30 g **! CAUTION** All animals must be handled under institutionally and nationally approved protocols and procedures. Approval for the experiments involving animals described in this study was obtained from the Institutional Animal Care and Use Committee of Massachusetts General Hospital.
- Monkey (Rhesus), male, 10.8 kg, 5 years old **! CAUTION** All animals must be handled under institutionally and nationally approved protocols and procedures. Approval for the experiments involving animals described in this study was obtained from the Institutional Animal Care and Use Committee of Massachusetts General Hospital. **! CAUTION** Non-human primates, including rhesus macaques, may carry communicable blood-borne diseases. Proper personal protective equipment should be used when handling the animals or biological specimens obtained from them.
- Isoflurane (Henry Schein Animal Health, NDC code 11695-6776-2) **! CAUTION** Isoflurane is a controlled substance in many jurisdictions, including the United States. Always comply with applicable local and federal regulations regarding permitting, use, and storage of isoflurane for research purposes. Avoid inhalation of vapor or mist. Isoflurane may cause damage to organs (cardio-vascular system and CNS) through prolonged or repeated exposure if inhaled. Store in a locked cabinet.
- USP compressed oxygen (Airgas, part no. OX USP200)
- Heparin (Hospira, NDC code 0409-2720-02)
- Ficoll-Paque PLUS (GE Healthcare, cat. no. 17-1440-02)

- Rat anti-mouse anti-CD11b-FITC antibody (AbD Serotec MCA711F, clone 5C6)
- Mouse anti-human anti-CD11b-FITC antibody ((AbD Serotec, cat. no. MCA551F, clone ICRF44)
- Ketamine hydrochloride (Ketamed, 100 mg/ml; Vedco, cat. no. 05098916106) **! CAUTION** Ketamine is a controlled substance in many jurisdictions, including the United States, where it is listed as a Schedule III compound (notably including other countries such as the United Kingdom and Canada). Always comply with applicable local and federal regulations regarding permitting, use, and storage of ketamine for research purposes. Avoid inhalation of vapor or mist. Ketamine may cause damage to organs (cardiovascular system and CNS) through prolonged or repeated exposure if inhaled. Store in a locked cabinet.
- Dexmedetomidine hydrochloride (Dexdomitor, 0.5 mg/ml; Zoetis, cat. no. 10002752) **! CAUTION** Dexmedetomidine hydrochloride can be absorbed following direct exposure to skin, eyes, or mouth, and may cause irritation. In case of accidental eye exposure, flush with water for 15 min. In case of accidental skin exposure, wash with soap and water. Remove contaminated clothing. Appropriate precautions should be taken while handling and using filled syringes. Accidental topical (including ocular) exposure, oral exposure, or exposure by injection could cause adverse reactions, including sedation, hypotension, and bradycardia. Seek medical attention immediately. Users with cardiovascular disease (e.g., hypertension or ischemic heart disease) should take special precautions to avoid any exposure to this product. Caution should be exercised when handling sedated animals. Handling or any other sudden stimuli, including noise, may cause a defensive reaction in an animal that is heavily sedated.
- Atipamezole hydrochloride (Antesedan, 5 mg/ml; Zoetis, cat. no. 10000449) **! CAUTION** Not for human use. Keep out of reach of children. Atipamezole hydrochloride can be absorbed and may cause irritation following direct exposure to skin, eyes, or mouth. In the case of accidental eye exposure, flush with water for 15 min. In the case of accidental skin exposure, wash with soap and water. Remove contaminated clothing. If irritation or other adverse reaction occurs (e.g., increased heart rate, tremor, muscle cramps), seek medical attention. In the case of accidental oral exposure or injection, seek medical attention. Caution should be used while handling and using filled syringes. Users with cardiovascular disease (e.g., hypertension or ischemic heart disease) should take special precau-tions to avoid any exposure to this product. The material safety data sheet (MSDS) contains more detailed occupational safety information.
- Endotracheal tube (5.0 mm; Covidien Mallinckrodt, cat. no. 86446) **!CAUTION** As this device may have been subjected to handling, storage conditions, or preparation that compromised functional integrity, each tube's cuff, pilot balloon, and valve should be tested by inflation before use. If dysfunction is detected in any part of the inflation system, the tube should not be used. More information is

available in the product insert. Reading the product insert before using this device is suggested.

EQUIPMENT

HIR labeling of FH

- 0.3-ml V vials with magnetic stirrer and solid thread liner cap (Wheaton, cat. no. W986253NG)
- Model 3 survey meter (Ludlum Measurements)
- Dose calibrator (CRC-25R; Capintec, item no. 5130-3215)
- UV-visible spectrophotometer (Thermo Scientific, model no. Evolution 300)
- Centrifuge (Thermo Scientific, model no. Legend Micro 17)
- Amicon ultracentrifuge filters (0.5 ml; Millipore, cat. no. UFC505096)
- Radio-TLC scanner (Bioscan, Flow-count and Mini Scan models)
- Stir plate (Pierce Reacti-Therm I; Thermo Scientific, cat. no. 18821)
- PD-10 column (Fisher Scientific, cat. no. 45000148)
- Thermometer (Fisherbrand, cat. no. 15-041-4E)
- Whatman pH indicator papers (TC, cat. no.2611628)
- TLC plate (silica gel, strongly acidic cation exchange; Sorbent Technologies, cat. no. 1224026)
- Fisherbrand disposable cuvettes: (Fisher Scientific, cat. no. 14-955-127)
- UV-visible light spectrophotometer (Thermo Scientific, model no. Evolution 300)
- Nalgene disposable filter unit (0.2 μm; Thermo Scientific, cat. no. 450-0020)
- Invisible tape (Staples, cat. no. 901-487908)
- 5-ml Eppendorf tubes (Eppendorf, cat. no. 0030119401)
- 1.5-ml Fisherbrand microcentrifuge tubes (Fisher Scientific, cat. no. 05-408-129)

Synthesis of Cy5.5-FH

- Legend XTR centrifuge (Thermo Scientific)
- Amicon ultracentrifuge filters (50 ml; Millipore, cat. no. UFC905024)
- Amicon ultracentrifuge filters (15 ml; Millipore, cat. no. UFC805024)
- Labquake shaker (Barnstead/Thermolyne, model no. 400110)
- Beaker (glass, 1 liter)
- Bottle (glass, 2 liters)

- UV-visible light spectrophotometer (Thermo Scientific, model no. Evolution 300)
- Fisherbrand disposable cuvettes (Fisher Scientific, cat. no. 14-955-127)
- 50-ml Falcon centrifuge tubes, polypropylene (Fisher Scientific, cat. no. 14-959-49A)
- 15-ml Falcon tubes (Corning Science, cat. no. 352095)

NP radiochemical stability, chelation challenge

- Survey meter (Ludlum Measurements, model no. 3)
- Dose calibrator (CRC-25R; Capintec, item no. 5130-3215)
- PD-10 column (Fisher Scientific, cat. no. 45000148)
- Plastic gamma counter tubes (12 × 55 mm; PerkinElmer, cat. no. 1270-401)
- Caps for plastic gamma counter tubes (PerkinElmer, cat. no. 1270-402)
- Water bath (Fisher Scientific, model no. Isotemp 102)
- Thermometer (Fisherbrand, cat. no. 103414)
- Wizard automatic gamma counter (PerkinElmer)
- 1.5-ml Fisherbrand microcentrifuge tubes (Fisher Scientific, cat. no. 05-408-129)

Radiochemical stability, serum challenge

- Survey meter (Ludlum Measurements, model no. 3)
- Dose calibrator (CRC-25R; Capintec, item no. 5130-3215)
- TLC plate (silica gel strongly acidic cation exchange; Sorbent Technologies, cat. no. 1224026)
- Radio-TLC scanner (Bioscan, Flow-count and Mini Scan models)
- Water bath (Fisher Scientific, model no. Isotemp 102)
- Thermometer (Fisherbrand, cat. no. 103414)
- 50-ml Polypropylene conical tubes (Falcon, cat. no. 352098)
- Invisible tape (Staples, cat. no. 901-487908)
- 1.5-ml Fisherbrand microcentrifuge tubes (Fisher Scientific, cat. no. 05-408-129)

In vivo procedures

- AMIDE software (http://amide.sourceforge.net/index.html), which uses the Volpack library to generate maximum intensity projection images[46]
- Cyflogic flow software (http://www.cyflogic.com/index.php?link1=1&link2 =4)
- Oxygen tank with flow regulator

- Isoflurane vaporizer (Midmark, model no. VIP3000)
- Veterinary anesthesia induction chamber (Smiths Medical, model no. V711802)
- Absorbent bedding (underpads; VWR, cat. no. 82020-845)
- Needle (PrecisionGlide Needle, 30 gauge × 1/2; BD, ref. no. 305106)
- Needle (30 gauge 0.5-inch blunt needle; SAI Infusion Technologies, part no. B30-50)
- Tubing (Intramedic polyethylene (PE10) tubing, cat. no. 427401)
- Syringe (NORM-JECT; HSW, cat. no. 4010.200V0)
- Needle for cardiac puncture (PrecisionGlide, 25 gauge; BD, ref. no. 305122)
- Catheter for monkey i.v. injections and blood draws (Surflo ETFE i.v. cath-eter, 22 gauge × 1 inch; Terumo Medical Products, cat. no. SR-OX2225CA)
- Catheter for mouse i.v. injection (see 'Equipment Setup for in vivo procedures')
- MicroPET/CT instrument (General Electric eXplore Vista small animal PET/CT scanner)
- Flow cytometer (BD, model no. LSR II)
- Dose calibrator (CRC-25R; Capintec, item no. 5130-3215)
- Centrifuge (IEC, model no. Centra CL2)
- Water bath (Fisher Scientific, model no. Isotemp 102)
- 1.5-ml Fisherbrand microcentrifuge tubes (Fisher Scientific, cat. no. 05-408-129)
- 15-ml Falcon tubes (Corning Science, cat. no. 352095)
- Ice bucket

REAGENT SETUP

1M Na_2CO_3

Dissolve 1.06 g of Na_2CO_3 in 10 ml of CTW for neutralization of HIR radioisotopes. This can be stored in small fractions at −80 °C for > 3 years and used by freshly thawing a small fraction.

0.9% (wt/vol) Saline (pH 7)

A pH 7 solution of saline was made by adding 120 μl of 1 M Na_2CO_3 in CTW to saline (0.9% (wt/vol), pH 5–6, 100 ml). We advise making fresh saline solution for each experiment.

Chelex-treated water

Ion exchange–purified water is treated by room temperature incubation of 25 g of Chelex resin with 500 ml of Millipore water under stirring overnight. It is filtered through a 0.2-μm

Nalgene disposable filter unit and brought to pH 7–8 with dilute Na2CO3. Store it at 4 °C for up to 1 year. It can also be stored in small fractions at −80 °C for > 3 years and used by freshly thawing a small fraction.

20 mM DFO solution

Dissolve 131.36 mg of DFO in 10 ml of CTW. This solution can be prepared in small fractions, frozen at −80 °C for > 3 years, and used by freshly thawing a small fraction.

20 mM Tris-acetate buffer (pH 7–8)

The stock is made by mixing 1.21 g of Trizma base with 0.4 ml of acetic acid in 500 ml of Millipore water. This solution can be stored at 4 °C for up to 6 months. **CRITICAL** The pH can be adjusted by adding a small amount of Trizma base or acetic acid.

10 mM Tris—0.01 mM DFO buffer (pH 7–8)

The stock is made by dissolving 3.3 mg of DFO in a mixture of 250 ml of 20 mM Tris-acetate buffer with 250 ml of Millipore water. This solution can be stored at −80 °C for up to 2 years.

Phosphate buffer (1 mM, pH 7.5)

Dissolve 142 mg of Na2HPO4 in 1 liter of Millipore water. Adjust the pH by adding a trace amount of HCl. This solution can be stored at 4 °C for up to 6 months.

MES buffer (0.1 M, pH 6.0–6.4)

Dissolve 10.86 g of MES sodium salt in 500 ml of Millipore water. Adjust the pH to 6–6.4 by adding HCl. This solution can be stored at 4 °C for up to 6 months.

1 M ethylenediamine in MES buffer

Mix 1.004 ml of ethylenediamine with 0.1 M MES buffer to a final volume of 15 ml. This solution can be stored in small fractions at −80 °C for up to 2 years and used by freshly thawing a small fraction.

25 mM Cy5.5-NHS DMSO solution

Dissolve 10 mg of Cy5.5-NHS in 0.354 ml of anhydrous DMSO. We advise making this solution immediately before each experiment. However, the unused stock can be stored in small fractions within sealed Falcon tubes (50-ml size) at −80 °C (with Drierite in the tube as a drying agent) for > 2 years.

EQUIPMENT SETUP

Sephadex G-25 size-exclusion column

The Sephadex G-25 size-exclusion column (50 × 500 mm; slurry bed height: ~ 250 mm) is a self-packed open column. Swell the Sephadex G-25 powder (100 g) with 1 liter of Millipore water for 3 h at room temperature; pour the slurry into the glass column and equilibrate the column with MES buffer (1 liter, 0.1 M, pH 6.0–6.4); calibrate it with vitamin B12 and blue

dextran. When the column is not in use, it can be stored in 20% (vol/vol) ethanol at 4 °C and can be regenerated by washing with MES buffer at room temperature (1 liter, 0.1 M, pH 6.0–6.4)

Tail-vein catheter for mouse

Cut 4 inches of PE10 tubing. Guide the metal end of blunt needle into the tubing, keeping it against the interior wall, while twisting the tubing until the blunt needle is ~5 mm deep. Using a needle holder, grab the sharp needle along the metal shaft, and remove it from the plastic base by working it back and forth. Adjust the grip of the needle holder on the needle shaft such that 5 mm is free on the blunt end. Guide the blunt end of the sharp needle into the interior of the empty end of the tubing, twisting the tubing until the needle is 5 mm deep. Fill the tubing with saline to check for leaks, and then remove the saline from the catheter.

General Electric eXplore VISTA small animal PET/CT scanner

Wrap the imaging bed in Chux, and secure with medical tape. **! CAUTION** Due to the very long half-life of 89Zr, the scanner bed should be covered with absorbent material in case of spills or leaks. Secure the medical tubing with the nose cone from the isoflurane vaporizer to the imaging bed, on top of the absorbent bedding. For CT scans, set up the scanner to acquire images at 300 ∝A and 40 kV, with 360 projections. For PET acquisitions, the scanner should be set to acquire a dynamic acquisition in List-Mode.

BD LSR II flow cytometer

The cytometer should be set to acquire forward- and side-scatter on a linear scale (both area and height). The cytometer should also be set to acquire data on the FITC and Cy5.5 channels on a log-scale (both area and height).

Isoflurane vaporizer

The vaporizer should be attached to the flow-limited oxygen tank on the intake side, and the output side should be attached to the induction chamber or the nose cone in the microPET/CT scanner (in mouse studies) or to the endotracheal tube (in primate studies). In all cases, the vaporizer should be set to 1–2%, as needed to maintain anesthetic effect.

PROCEDURE

CRITICAL Steps 1–50 of the Procedure contain the step-by-step procedures for synthesis of heat-induced radiolabeling of FH with 89Zr. HIR-NP radiochemical stability assays are described in Steps 51–64 (DFO chelation challenge) and Steps 65–78 (serum challenge). Steps 79–129 describe the synthesis of Cy5.5-FH.

Synthesis of heat-induced radiolabeled ^{89}Zr-FH ● TIMING 3–4 h

!CAUTION Wear goggles, gloves, and a lab coat for chemical safety.

!CAUTION All experiments involving radioactivity must be performed by persons qualified and trained to handle radioisotopes, and by following all relevant state, federal, and institutional regulations and approvals. Approval for the radioactive experiments described

in the protocol was obtained from Massachusetts General Hospital, Harvard Medical School. In addition to the general cautions for chemical safety above, the person who conducts the radioactive experiments must wear personnel monitoring devices such as a clip-on dosimeter and ring badge bearing the individual's name and the date of the monitoring period. The radiolabeling should be done in a certified, well-ventilated, lead-lined fume hood with a sliding lead glass shield.

!CAUTION A double-bagged plastic transparent bag should be used for collecting radioactive nonsharp solid wastes (e.g., gloves, used PD-10 columns, plastic tubes). A 500-ml plastic bottle should be prepared to contain liquid wastes (e.g., elution buffers, filtrates). A sharps container should be ready for disposal of glass vials, pipette tips, needles, syringes, and other sharp waste. All these waste containers should be kept behind a lead brick–shielded area for reducing the exposure of the operator. After the HIR labeling, all the waste should be labeled according to the institutional regulations and state laws, relocated to the designated decay area, and documented.

1| Preheat a silicone oil bath with a depth of 10–15 mm to 120 °C.

2| Neutralization of ^{89}Zr-oxalate (Steps 2–5). Dilute 20 μl of ^{89}Zr-oxalate solution (12.26 mCi) with 120 μl of CTW in the original vial shipped from the vendor.

CRITICAL STEP The 89Zr-oxalate solution should be 4 mCi per 10 μl when high specific activity is expected. The activity of the solutions can be determined using a dose calibrator.

? TROUBLESHOOTING

3| Neutralize the diluted ^{89}Zr-oxalate solution by adding 5 μl of 1 M Na_2CO_3 and mix by pipetting. Repeat this step three more times.

CRITICAL STEP Prompt mixing after base addition is required.

CRITICAL STEP pH control during the HIR reaction is essential. FH (Fig. 1b, top) undergoes dissolution in acidic environments (Fig. 1b, bottom; FH switches from brownish/red iron oxide to a yellow mixture of low-molecular-weight chelates), but is highly stable at pH 7 or above. It is therefore essential to measure the pH with Whatman pH indicator papers during the neutralization reaction to verify a pH of 7.5–9. Pipette 1 μl of the solution onto the border between the yellow zone and the light-blue zone (pH 8–9).

? TROUBLESHOOTING

4| Pipette 79.5 μl of the neutralized ^{89}Zr solution (5.03 mCi) into a clean 0.3-ml glass V vial fitted with a magnetic stir bar.

5| Add 102.5 μl of CTW and mix by pipetting up and down three times.

6| *^{89}Zr-HIR 'loading' (Steps 6–8).* Add 18 μl of FH (0.54 mg of Fe) to the V vial from Step 5.

!CAUTION The HIR reactions can be scaled up to produce greater amounts of iron, depending on intended dose, size of the animals, and numbers of animals to

be injected. An increased amount of FH can be used to increase the RCY, if lower specific activity is expected.

7| Confirm that the pH is 8 with pH paper.

CRITICAL STEP The ideal pH is 8–9. If the pH is > 9, we advise adjusting the pH by adding a trace amount of 0.1 M oxalic acid in CTW. If the pH is < 8, adjust it to 8 by adding more 1 M Na_2CO_3 in CTW. Measure the pH with Whatman pH indicator papers to verify the pH.

8| Place the V vial in the preheated silicone oil bath (from Step 1) with constant magnetic stirring for 2 h.

9| *^{89}Zr-HIR 'stripping' (Steps 9–11).* Remove the V vial from the oil bath and place it in an ice-water bath for 15 min with stirring. During this incubation time, perform Steps 12–15.

CRITICAL STEP Use forceps to remove the V vial from the hot oil bath and wipe off adhering oil. The vial may be slippery. To minimize the time of cooling, never open the vial before the full 15 min in ice water. As a thick-walled vial is used, the cooling time will be much longer if cooled by ambient temperature.

10| Add 2.5 μl of DFO stock solution (20 mM) for stripping non-adsorbed radiocations.

CRITICAL STEP The molar ratio of Fe/DFO should be 180:1, a vast molar excess over the radiocation. A high DFO concentration ensures complete complexation of $^{89}Zr^{4+}$ (or $^{64}Cu^{2+}$ or $^{111}In^{3+}$ when making ^{64}Cu-FH or ^{111}In-FH, respectively).

11| Incubate for 15 min at room temperature with constant magnetic stirring. During this incubation time, perform Step 16.

12| ^{89}Zr-HIR labeling efficiency and RCY analysis by TLC (Steps 12–20). Turn on the TLC scanner during Step 9.

13| Pipette CTW into two 50-ml Falcon tubes (2 ml per tube) as developing chambers during Step 9.

14| Cut TLC plates as 5 mm × 100 mm strips during Step 9.

15| Mark a starting point on each plate with a pencil during Step 9.

CRITICAL STEP Use a blunt-head pencil to avoid scratching the TLC coating.

16| Presoak the origin section with CTW and let it dry for 5 min during Step 11.

17| Spot 0.5 μl of reaction mixture from Step 11 onto the TLC plates.

18| Develop the plates in the two separate chambers prepared in Step 13 for labeling efficiency analysis.

19| Take the TLC plates out of the chambers when the solvent reaches ~10 mm from the top.

CRITICAL STEP Cover the TLC strip with invisible tape to prevent contamination

20| Scan the plates with a radioactive TLC scanner at a scanning speed of 1 mm/s (Fig. 3b). The area under the curve (AUC) represents the labeling efficiency.

21| *^{89}Zr-HIR SEC purification (Steps 21–25).* Load the reaction solution from Step 11 onto a PD-10 column preconditioned with 20 ml of 0.9% (wt/vol) saline (pH 7) to remove low-molecular-weight ^{89}Zr:DFO.

CRITICAL STEP The sample should be evenly distributed on the top of the column. The loading volume should be limited to be < 0.4 ml to avoid dilution of the eluted sample. Measure the volume as pipetted volume.

22| Wash the V vial with 200 μl of 0.9% (wt/vol) saline (pH 7) and load it onto the PD-10 column.

23| Add 2.1 ml of 0.9% (wt/vol) saline to elute the column. This is the dead volume (2.5 ml), which contains no detectable radioactivity.

CRITICAL STEP Sometimes the dead volume is slightly different. Therefore, we strongly advise careful measurement of the dead volume by close observation of the motility of the brownish color of the band.

24| Add 1.8 ml of 0.9% (wt/vol) saline to elute the band of ^{89}Zr-FH off the column and collect the fraction containing the product, which can be identified by the brown color of FH, into a 5-ml Eppendorf plastic tube.

25| Measure the FH fraction activity using a dose calibrator; this is typically ~4.95 mCi.

26| *89Zr-HIR: concentration and reconstitution of ^{89}Zr-FH (Steps 26–33).* Add 300 μl of the solution from Step 24 to two separate Amicon filters (0.5-ml size, MWCO = 50 kDa).

27| Centrifuge the solutions in the filters from Step 26 with a Legend Micro 17 centrifuge at a speed of 5,000g for 5 min at room temperature.

28| Concentrate the 89Zr-FH fraction from Step 24 to a volume of 100 ∝l by repeating Steps 26 and 27 three times.

?TROUBLESHOOTING

29| Wash the 5-ml Eppendorf tube from Step 24 with 2 × 350 μl of 0.9% (wt/vol) saline.

30| Add the washing solutions from Step 29 to the two filters from Step 28 (350 μl for each filter) and concentrate them by centrifugation with a Legend Micro 17 centrifuge at a speed of 5,000g for 6 min at room temperature.

?TROUBLESHOOTING

31| Remove the concentrated ^{89}Zr-FH from Step 30 from the two filters and combine the solutions in a clean Eppendorf tube (1.5-ml size).

32| Wash the filters from Step 31 with 2 × 50 μl of 0.9% (wt/vol) saline (50 μl for each filter) and combine them in the Eppendorf tube from Step 31. The product ^{89}Zr-FH is reconstituted in 200 μl of 0.9% (wt/vol) saline.

CRITICAL STEP In addition to saline, 1 mM phosphate buffer (pH 7.5) can also be used for elution and reconstitution.

33| Measure the activity of the recovered ^{89}Zr-FH in Step 32 using a dose calibrator. This is usually ~4.78 mCi. The RCY is 95% (separation yield).

34| *Radiochemical purity by TLC analysis (Steps 34–38).* Prepare two TLC plates by repeating Steps 14–16.

35| Spot the solution from Step 32 onto two TLC plates (0.7 μl each).

36| Develop the plates by CTW in the chambers from Step 13.

37| Take the TLC plates out of the chambers when the solvent reaches ~10 mm from the top.

CRITICAL STEP Cover the TLC strip with invisible tape to prevent contamination.

38| Scan the plates with a radioactive TLC scanner at a scanning speed of 1 mm/s. The AUC represents the purity of the purified ^{89}Zr-FH (> 95%) (Fig. 4b at 0.5 h shows an example).

39| *Assessment of RCP by SEC method (Steps 39–45).* Label 40 gamma counter tubes from no. 1 to no. 40.

40| Load 0.5 μl of the solution from Step 33 (~10 μCi) onto a PD-10 column preconditioned with 20 ml of 0.9% (wt/vol) saline (pH 7).

CRITICAL STEP Ensure that the total activity used for analysis is between 5 and 12 μCi. Activity overloading will saturate the gamma counter.

41| Elute the column with 400 μl of 0.9% (wt/vol) saline (pH 7) first and then 2.1 ml of 0.9% (wt/vol) saline (pH 7). This is the dead volume (2.5 ml), which contains no detectable radioactivity. (Note that none of the SEC chromatograms show this fraction.)

CRITICAL STEP Sometimes the dead volume is a little different. Therefore, we advise careful measurement of the dead volume by close observation of the movement of the weak brownish color of the band.

42| After a 2.5-ml dead volume (Step 41), collect 35 × 200 μl fractions from 2.5 ml to 9.5 ml (by adding 200μl buffer volumes each time) to tube nos. 1–35, which were prelabeled in Step 39, and then cap the tubes. Total eluted volume = 2.5 + 7 ml = 9.5 ml.

43| Collect 5 × 500 μl fractions (adding 0.5-ml buffer volumes) from 9.5 ml to 12 ml (by adding 500 μl each time) to tube nos. 36–40, which were prelabeled in Step 39, and then cap the tubes. Total eluted volume = 2.5 + 9.5 ml = 12 ml.

44| Count and record the radioactivity in each tube using a gamma counter in the order from nos. 1–40. The counting time is 1 min per tube.

45| Export the data and reconstruct the SEC chromatogram (counts per min (CPM) versus elution volume, in milliliters) with Microsoft Excel. The major peak at 3.5 ml indicates that the majority of the activity (> 95%) is tightly binding to FH nanoparticles (FHNPs) (Fig. 4a at 0.5 h represents an example).

46| *Iron recovery yield and specific activity (Steps 46–50).* Make a standard curve (y = 0.0021×, r^2 = 0.9998) of iron concentration (y, M) as a function of UV absorbance (x) of FH at 450 nm by using diluted FH stock solutions in 0.9% (wt/vol) saline (pH 7). The absorbance value should be between 0.2 and 0.8.

47| Measure the UV absorbance of ^{89}Zr-FH at 450 nm by diluting 10 μl of the stock solution from Step 32 in 1 ml of 0.9% (wt/vol) saline (pH 7). (Note that the dilution does not need to be precise; it is acceptable as long as the absorbance is within the range of 0.2–0.8.)

48| Calculate the iron amount (0.008868 mmol) according to the standard curve from Step 46.

49| Determine the yield. We typically obtain a yield of 90.7% Fe (note that 5–20% iron loss has been found).

50| Determine the specific activity with the following equation: specific activity = activity from Step 33/amount of iron from Step 48 = 4.78 mCi/0.008868 mmol Fe = 539 mCi/mmol Fe (3,166 Ci/mmol NP). Specific activity is expressed as mCi per mmole of iron, in massive excess over moles of the radiocation (16 nmol $^{89}Zr^{4+}$ per mmol Fe, calculated according to the specific activity of $^{89}Zr^{4+}$: 33mCi/nmol, according to the manufacturer).

89Zr-NP radiochemical stability, DFO chelation challenge ● TIMING 1 d

51| Mix 32.5 μl of 89Zr-FH (110 μCi, 0.25 mg of Fe) with 5 ml of 10 mM Tris–0.01 mM DFO buffer (pH 7.6) in a 15-ml Falcon tube.

52| Incubate the mixture at 37 °C in a preheated water bath.

53| Label 40 plastic gamma counter tubes from nos. 1 to 40.

54| At 0.5 h, measure the activity in the incubated tube from Step 52 and calculate the volume containing ~5–10 μCi of activity.

55| Remove 10 μCi of solution with a pipette and place the Falcon tube back in the water bath for incubation at 37 °C for a complete 20-h time course.

56| Load the 10 μCi of solution onto a PD-10 column preconditioned with 20 ml of 0.9% (wt/vol) saline (pH 7).

CRITICAL STEP Ensure that the total activity used for each analysis is between 5 and 12 μCi. Activity overloading will saturate the gamma counter.

57| Add additional 0.9% (wt/vol) saline (pH 7) for a total of 2.5 ml (2.5 ml = loading volume of Step 56 + additional amount added at this step) and collect this 2.5 ml into a 5-ml Eppendorf tube. This is the dead volume that contains no detectable radioactivity. (Note that Fig. 4a does not show this fraction.)

CRITICAL STEP The loading volume (~0.4–0.5 ml) must be counted as the elution volume because the 89Zr-FH band starts to move while the sample is loading. Otherwise, most of the activity that is supposed to be fractionally collected in Step 58 will be missed and collected in the dead volume by mistake. In this case, the entire analysis fails. Sometimes the dead volume is a little different. Therefore, we strongly advise careful measurement of the dead volume by close observation of the movement of the weak brownish color of the band.

58| After a 2.5 ml dead volume (Step 41), collect 35 × 200 μl fractions from 2.5 ml to 9.5 ml (by adding 200 μl each time) to tube nos. 1–35, and then cap the tubes.

59| Collect 5 × 500 μl fractions from 9.5 ml to 12 ml (by adding 500 μl each time) to tube nos. 36–40, then cap the tubes.

60| Count and record the radioactivity in each tube in numerical order using a gamma counter. The counting time is 1 min per tube.

61| Export the data and reconstruct the SEC chromatogram (CPM versus elution volume, in milliliters) using Microsoft Excel (Fig. 4a). The peak at 3.5 ml is ^{89}Zr-FH and the peak at 7 ml represents the dissociated ^{89}Zr:DFO chelates.

62| Integrate the AUCs.

63| Determine the AUC ratio of the peak at 3.5 ml to the peak at 7 ml (> 90% in our case). This indicates that > 90% of 89Zr is still tightly bound to the FH NPs under DFO challenge.

64| Evaluate the radiochemical stability of ^{89}Zr-FH with a time course (at 4 and 20 h) according to Steps 53–63 (Fig. 4a).

HIR-NP radiochemical stability, serum challenge ● TIMING 2 d

65| Mix 30 μl of ^{89}Zr-FH (123 μCi, 0.078 mg of Fe in 1 mM phosphate buffer, pH 7.5) together with 60 μl of normal mouse serum in a 1.5-ml Eppendorf tube.

CRITICAL STEP The instability of HIR-FH NPs in serum generates radiocations that likely bind serum proteins and therefore have the molecular weight of those proteins. We provide a TLC separation system in which the radiocation/protein ratio remains at the origin, whereas HIR-FHs move at the solvent front, as shown in Figure 4b. EDTA or diethylenetriamine pentaacetic acid (DTPA) is avoided because of a lack of retention at the origin.

66| Incubate the mixture at 37 °C in a preheated water bath.

67| Turn on the TLC scanner.

68| Pipette 2 × 2 ml of CTW into two 50-ml Falcon tubes as developing chambers.

69| Cut TLC plates as 5 mm × 100 mm strips.

70| Mark a starting point with a pencil on each TLC plate.

CRITICAL STEP Use a blunt-head pencil to avoid scratching the TLC coating.

71| Presoak the origin section in CTW and let it dry for 5 min.

72| Spot 1–2 μl of solution right on the mark and place the tube back in the 37 °C water bath for a complete time course of 2 d.

73| Develop the plates in two separate chambers prepared in Step 68.

74| Take the TLC plates out of the chambers when the solvent reaches ~10 mm from the top.

!CAUTION Cover the TLC strip with invisible tape to prevent contamination.

75| Scan the plates with a radioactive TLC scanner at a scanning speed of 1 mm/s (Fig. 4b).

76| Integrate the AUC of peaks both at the origin and the solvent front.

77| Determine the AUC ratio of the peak at the front to the peak at the origin (> 90% in our case). This indicates that > 90% of 89Zr is still tightly bind to the FH NPs under serum challenge.

78| Evaluate the radiochemical stability of ^{89}Zr-FH under serum challenge with a time course (at 20 and 42 h), according to Steps 69–77 (Fig. 4b).

Synthesis of Cy5.5-FH ● TIMING 17 h

79| *Reconstitution of FH (Steps 79–90).* Draw 5 ml of FH (150 mg of Fe, 30 Fe/ml) from the FH bottle with a syringe.

CRITICAL STEP The procedure has been used for making the similar compounds Feraheme-Thiazole Orange (FH-TO) and FH-protamine-rhodamine as in reference, but at much smaller scale[28,29,40]. The current iron scale (150 mg) is 25 times higher than that of the previously reported procedure for FH-amine preparation.

80| Load FH solution onto the Sephadex G-25 column (containing 100 g Sephadex G-25) precoditioned by 1 liter of MES buffer (0.1 M, pH 6.0–6.4).

CRITICAL STEP The FH solution should be evenly distributed on the top surface of the column using a pipette. Close the bottom of the column until FH is fully loaded.

81| Drain the column until the FH solution reaches the top of the resin bed.

82| Use 183 ml of MES buffer (0.1 M, pH 6.0–6.4) to elute the FH of the column to remove the other adjuvant.

CRITICAL STEP Add the buffer carefully in order to avoid disturbing the top of the resin bed. Sometimes the dead volume is a little different from the original calibration. Therefore, we strongly advise careful measurement of the dead volume by close observation of the movement of the brownish color of the band.

83| Collect the 70-ml FH fraction into two 50-ml Falcon plastic tubes. The brown color indicates the FH band.

84| Concentrate the FH solution to 5 ml in each filter by centrifugation using two 50-ml Amicon filters (MWCO = 50 kDa) at 2,000g for 20 min at 4 °C.

?TROUBLESHOOTING

85| Use 20 ml of MES buffer (0.1 M, pH 6.0–6.4) to wash the FH in the Amicon filters (10 ml each) by centrifugation as in Step 84. Repeat this step once.

?TROUBLESHOOTING

86| Reconstitute the concentrated FH with 25 ml of MES buffer (0.1 M, pH 6.0–6.5) in a 50-ml Falcon tube.

PAUSE POINT The reconstituted FH can be stored at 4 °C for 2 months, but we suggest making it fresh before each experiment.

87| Rinse the Sephadex G-25 column with 2 liters of MES buffer (0.1 M, pH 6.0–6.4) to regenerate the column for the purification of FH-amine.

88| Measure the UV absorbance of FH at 450 nm by diluting 5 ∝l of the stock solution from Step 86 in 1 ml of 0.9% (wt/vol) saline (pH 7). (Note that the dilution does not need to be precise; it is acceptable if the absorbance is within the range of 0.2–0.8.)

89| Calculate the iron amount (140 mg of Fe) according to the standard curve from Step 46.

90| Determine the recovery yield. We typically obtain a yield of 93%. (Note that 5–20% iron loss has been observed.)

91| *Synthesis of FH-Amine (Steps 91–110).* Add 125 mg of hydroxybenzotriazole (HOBT, 0.816 mmol) and 625 mg of N-(3-dimethyaminopropyl)-N′-ethylcarbodiimide (EDC, 3.26 mmol) to the 25 ml of reconstituted FH in MES buffer from Step 86.

92| Incubate the mixture at room temperature for 20 min while mixing the solution with a Labquake shaker (a rotator).

93| Add 1 ml of ethylenediamine (1 M in 0.1 M MES buffer) to the mixture above.

94| Mix by shaking the tube several times.

95| Incubate the mixture at 50 °C for 90 min while shaking the tube every 15 min manually.

96| Decrease the temperature by placing the tube in an ice water bath for 5 min.

97| Transfer the solution to two 50-ml Amicon filters (MWCO = 50 kDa) by pipetting.

98| Spin the filters at 2,000g at 4 °C to reduce the total volume to 3 ml in order to remove most of the small molecular components. This takes ~15–20 min.

?TROUBLESHOOTING

99| Remove the 3 ml of crude FH-amine solution generated in Step 98 from the filters and load them onto the regenerated Sephadex G-25 column in Step 87.

100| Wash the two filters with 1 ml of MES buffer (0.1 M, pH 6.0–6.4) and load the washing solutions onto the column from Step 99. Repeat this step for four times.

CRITICAL STEP The FH-amine solution must be evenly distributed.

101| Drain the column until the FH-amine solution reaches the top of the resin bed.

102| Use 178 ml of MES buffer (0.1 M, pH 6.0–6.4) to elute the FH-amines from the column.

CRITICAL STEP Add the buffer with caution in order to avoid disturbing the top of the resin bed. Sometimes the dead volume is a little different from the original calibration. Therefore, we strongly advise careful measurement of the dead volume by close observation of the movement of the brownish color of the band.

103| Collect the 65-ml FH-amine fraction into two 50-ml Falcon plastic tubes (brown color band).

104| Use two 50-ml Amicon filters (MWCO = 50 kDa) to concentrate the FH-amine solution to 5 ml in each filter by centrifu-gation at 2,000g at 4 °C. This takes ~20 min.

?TROUBLESHOOTING

105| Use 20 ml of MES buffer (0.1 M, pH 6.0–6.4) to wash the FH-amine in the Amicon filters (10 ml for each) by centrifugation as in Step 104. Repeat this step once to obtain a volume of 8 ml (4 ml in each filter).

?TROUBLESHOOTING

106| Remove the 8 ml of concentrated FH-amine solutions from the filters and combine them in a clean 50-ml Falcon tube.

107| Wash the filters with 2 ml of MES buffer (0.1 M, pH 6.0–6.4) and combine the solutions with the FH-amine solution of Step 106. Repeat this step five times.

108| Measure the UV absorbance of FH-amine at 450 nm by diluting 5 μl of the stock solution from Step 107 in 1 ml of 0.9% (wt/vol) saline (pH 7). (Note that the dilution does not need to be precise; it is acceptable if the absorbance is within the range of 0.2–0.8.)

109| Calculate the iron amount (130 mg of Fe, 92.8% yield) according to the standard curve from Step 46. (Note that 5–20% iron loss has been observed.)

110| Reconstitute the FH-amine into a final volume of 26 ml in MES buffer (0.1 M MES buffer, pH 6.0–6.4) to create a concentration of 5 mg Fe/ml.

PAUSE POINT The reconstituted FH-amine can be stored at −80 °C for up to 2 years. Avoid frequent freeze–thaw cycles. We do, however, suggest making it fresh before use.

111| *Synthesis of Cy5.5-FH (Steps 111–129).* Pipette 20.66 ml of FH-amine solution in MES buffer (103.3 mg Fe) from Step 110 into a 50-ml Falcon tube.

CRITICAL STEP The iron scale is 103 mg for coupling Cy5.5, which is 206 times more than the previously reported procedure for FH-TO preparation28,29.

112| Add 0.31 ml (25 mM, 7.75 μmol) of Cy5.5-NHS ester in DMSO solution to the above FH-amine solution.

113| Incubate the mixture for 3 h at room temperature under constant mixing on a Labquake shaker.

114| Concentrate the mixture by centrifugation with two Amicon filters (MWCO = 50 kDa) at 2,000g under 4 °C until the volume is 10 ml. This takes ~20 min.

?TROUBLESHOOTING

115| Wash the Cy5.5-FH solution by applying 20 ml of DPBS to the filters (10 ml each) and centrifuge as in Step 114 until the volume is ~10 ml. This is done in order to partially remove the MES buffer and the unreacted and hydrolyzed Cy5.5 derivatives.

?TROUBLESHOOTING

116| Load the concentrated crude Cy5.5-FH solution of Step 115 onto a Sephadex G-25 column regenerated by washing with 1 liter of DPBS.

117| Separate the Cy5.5-FH from the unreacted and hydrolyzed Cy5.5 derivatives by eluting the column with 180 ml of DPBS.

CRITICAL STEP Most of the small-molecular dye will be removed by this step because of the nonspecific affinity of Cy5.5 to Sephadex. Add the buffer with caution in order to avoid disturbing the top of the resin bed. Sometimes the dead volume is a little different from the original calibration. Therefore, we strongly advise careful measurement of the dead volume by close observation of the movement of the pale darker green color of the band.

118| Collect 80 ml of Cy5.5-FH fraction (identified by a pale-green color) into two 50-ml Falcon tubes.

119| Concentrate the Cy5.5-FH solution by centrifugation with two 50-ml Amicon filters (MWCO = 50 kDa) at 2,000g at 4 °C until the volume is 10 ml. This takes ~20 min.

?TROUBLESHOOTING

120| Wash the concentrated Cy5.5-FH with DPBS by centrifugation at 2,000g at 4 °C until no color is seen in the filtrates down to a volume of ~4 ml (around 2 ml in each filter). The time for this step is dependent on the free dyes. In our experience it has been 30 min to 1 hr.

?TROUBLESHOOTING

121| Remove the concentrated solutions from the filters from Step 120 and place them into a 50-ml Falcon tube.

122| Wash the filters with 1 ml of DPBS and combine the solution with the solution from Step 121. Repeat this step once.

PAUSE POINT Cy5.5-FH can be stored at −80 °C for at least 1 year after being aliquoted into small fractions according to the injection needs. Avoid frequent freeze–thaw cycles.

123| Make a standard curve ($y = 5 \times 10^{-6}\times$, $r^2 = 0.9999$) of the concentration of Cy5.5 (y, M) as the function of absorbance of Cy5.5 at 675 nm (x) by using diluted solutions of Cy5.5-NHS ester in DPBS.

124| Measure the absorbance spectrum in DPBS between 350 and 750 nm (Fig. 2b) by diluting 3 μl of the stock from Step 122 with 1 ml of DPBS. The coherence of the FH iron oxide absorbance and the typical Cy5.5 absorbance proves the presence of reactive amines and the successful conjugation of Cy5.5 on the surface of FH NPs. (Note that the dilution factor does not need to be precise; it is acceptable if the absorbance is within the range of 0.2–0.8.)

125| Calculate the iron amount (92 mg of Fe) according to the iron standard curve from Step 46 and the absorbance of Cy5.5-FH at 450 nm.

126| Calculate the Cy5.5 concentration according to the Cy5.5 standard curve from Step 123 and the absorbance of Cy5.5-FH at 675 nm in Step 124.

127| The payload is 8.5. Calculate the molar ratio of Cy5.5/FHNP by dividing the Cy5.5 concentration (from Step 126) by the iron concentration (from Step 125).

CRITICAL STEP The payload of fluorochrome is more accurate because the absorbance (675 nm) is far away from FH iron absorbance (< 500 nm). The two absorbances do not interfere with each other. However, the common issue in determination of the payloads for NP surface modifications is that the overlay between the Cy5.5 dyes themselves indicated by a shorter wavelength absorbance peak at 625 nm (Fig. 2b) is still unresolved because of the proximity of the dye moieties when they are attached on the same surface.

128| Concentrate the solution from Step 122 using a 15-ml Amicon filter (MWCO = 50 kDa) for centrifugation at 2,000g for 30 min at 4 °C and reconstitute it in 4.7 ml of Cy5.5-FH solution in DPBS (19.5 mg of Fe/ml).

129| Calculate the iron recovery yield, which should be 92 mg of Fe, 89.1%. (Note that 5–20% iron loss has been observed.)

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● **TIMING—**Steps 1–50, synthesis of HIR-FH NPs, take ~5–6 h, but, in general, HIR can be completed in 3–4 h because many steps can be carried out simultaneously. Steps 79–129, synthesis of Cy5.5-FH, take ~17 h. Box 1, in vivo monocyte uptake assay in mice and ex vivo labeling of leukocytes, takes 32.5 h because many steps can be carried out simultaneously. Box 2, in vivo monocyte uptake assay in rhesus macaque, takes 6 h.

Step 1, preheating of silicone oil bath: 30 min

Steps 2–5, neutralization of 89Zr-oxalate: 15 min

Steps 6–8, 89Zr-HIR ‘loading’: 2 h 5 min

Steps 9–11, 89Zr-HIR ‘stripping’: 30 min

Steps 12–20, 89Zr-HIR labeling efficiency and RCY analysis by TLC: 15 min

Steps 21–25, 89Zr-HIR SEC purification: 30 min

Steps 26–33, 89Zr-HIR—concentration and reconstitution of 89Zr-FH: 20 min

Steps 34–38, determination of radiochemical purity by TLC analysis: 15 min

Steps 39–45, determination of radiochemical purity by SEC method: 1.5 h

Steps 46–50, iron recovery yield and specific activity: 10 min

Steps 51–64, HIR-NP radiochemical stability, DFO chelation challenge: 1 d

Steps 65–78, HIR-NP radiochemical stability, serum challenge: 2 d

Steps 79–90, reconstitution of FH: 4 h

Steps 91–110, synthesis of FH-amine: 6 h

Steps 111–129, synthesis of Cy5.5-FH: 7 h

Box 1, steps 1–15, in vivo monocyte uptake assay in mice: 26 h

Box 1, steps 16–20, ex vivo labeling of leukocytes with Cy5.5-FH: 4.5 h

Box 1, steps 21–26, labeling of leukocytes with anti-CD11b and flow cytometry: 2 h

Box 2, steps 1–18, injection of monkey and leukocyte preparation: 4 h

Box 2, steps 19–24, labeling of leukocytes with anti-CD11b and flow cytometry: 2 h

Box 3, in vivo microPET/CT imaging of HIR-FH nanoparticles: 61.5 h

ANTICIPATED RESULTS

Through HIR labeling of FH (Fig. 2a, Steps 1–50) by using the procedures described in this protocol, we typically recover 4.78 mCi, which results in an RCY of 95% (separation yield). The RCY may vary within a range of 80–95%, as 5–20% iron loss has been found. When analyzed by both TLC (Steps 34–38) and SEC (Steps 39–45) methods, we usually obtain an

RCP of > 98% (see Fig. 4 at 0.5 h) with a specific activity of 539 mCi/mmol Fe (3,118 Ci/mmol NP). $^{111}In^{3+}$ and $^{64}Cu^{2+}$ can be successfully doped on FHNPs by HIR using procedures very similar to that presented in this protocol (Fig. 3d,f)[18,19].

The synthesis of Cy5.5-FH (Fig. 2b) provided in Steps 79–129 involves a conversion of carboxylic groups on the CMD coating to amine groups and a conjugation between amine and Cy5.5-NHS ester. The protocol allows the reaction scale to start with 150 mg Fe. The overall yield of iron recovery is 77%, including three steps of SEC Sephadex G-25 gel filtration and ultracentrifugations with 50kDa MC Amicon filters. The average payload of Cy5.5/FHNP is 8.5; however, this protocol permits much payload flexibility by a simple variation of the ratio of Cy5.5-NHS to FH-amine.

HIR NP radiochemical stability has been tested with the DFO chelation challenge (Fig. 4a). With the procedures provided in Steps 51–64, chromatogram measurements can be obtained from serial elution fractions, yielding the time-course elution of radioactivity from the column (Fig. 4a). The integration of the AUC (Fig. 4a) indicates that > 90% of the 89Zr is tightly bound to FHNPs after 20 h of DFO challenge (Fig. 4a).

HIR NP radiochemical in vitro stability has also been evaluated with the mouse serum challenge (Fig. 4b). By following the procedures in Steps 65–78, a group of TLC chromatograms have been generated (Fig. 4b) at different time points. The AUC of the peak at the solvent front shows that > 90% of the ^{89}Zr is tightly bound to FHNPs after 42 h of mouse serum challenge (Fig. 4b, 42 h), which is in agreement with the results of the DFO chelation challenge above.

Internalization of Cy5.5-FH by monocytes (Fig. 5) has been verified by the procedures in Box 1 and Box 2. The abil-ity of monocytes present in crude buffy coats to internalize Cy5.5-FH (Fig. 5c, right panel) is notable because it suggests that HIR-generated FHs might be used for the ex vivo labeling of buffy coat monocytes in clinical settings, similar to the ex vivo labeling of leukocytes in white blood cell SPECT imaging[47,48]. Our results give solid evidence that Cy5.5-FH can label monocytes both in vivo and ex vivo, as Cy5.5-FH NPs were always found nearly exclusively in monocytes, as measured by flow cytometry. The difference in the extent of labeling between in vivo and ex vivo procedures most likely results from trafficking of in vivo-labeled monocytes out of the blood and into tissues and the lymphatic system[19,49] (Fig. 5c). This result extends to multiple species as well, as shown by the exclusive monocyte internalization of Cy5.5-FH after having been intravenously injected into a Rhesus monkey (Fig. 5d).

In vivo stability of HIR-FHs can be determined by comparing the biodistribution of HIR-FH (Figure. 5a of ref. 18) with the biodistributions of $^{89}Zr^{4+}$ or $^{64}Cu^{2+}$ cations. (To our knowledge, biodistribution studies with $^{111}InCl_3$ have not been report-ed.) Bone uptake is very limited for HIR-FH, as opposed to $^{89}Zr^{4+}$, injections. Furthermore, the in vivo stability of HIR-FH can be also evaluated by the fact that the bone PET signal is non-detectable in mouse imaging (Fig. 6, Figure. 2 of ref. 19, and Supplementary Video 1).

The PET-CT modality (Fig 6 and Supplementary Video 1) can be achieved using HIR-FHNPs by following the procedure de-scribed in Box 3. After i.v. injection in mice, HIR-

generated 89Zr-FH was internalized by monocytes that carried NP radioac-tivity to sites of normal immune activity (lymph nodes)[19] and sites of abnormal immune activity (inflamed paw and muscle), as revealed by PET imaging (Fig. 6). The 89Zr-FH distribution pattern indicates that the FH NPs are cleared from the blood by the liver (mouse, $t_{1/2}$ = 1 h)[19], with fast accumulation in the spleen and very low bone and kidney uptake. Following this initial phase, ^{89}Zr-FH shows a slow accumulation in lymph nodes and sites of inflammation. The uptake of radioactive FH seen with mouse lymph nodes and PET parallels that seen with FH and clinical MRI. FH NPs accumulate in normal lymph nodes, and the lack of NP accumulation (due to metastases) is diagnostically useful[50,51]. In this example, we injected a mouse that had a self-inflicted paw injury (Fig. 6b) and observed that ^{89}Zr-FH traffics to sites of irritation as in the classic turpentine model[18] (Fig. 6). In the mouse, the lymphatic system draining the rear extremity can pass through the central iliac and renal nodes or through a second lymphatic channel that runs from the rear extremity to the inguinal node and on to the axillary node. Thus, inflammation in the rear extremity activates the ipsilateral nodes near the site of inflammation, as well as the ipsilateral axillary node, as shown in Figure 6a. PET imaging showed localization of radioactivity in the paw and associated leg, as well as extreme asymmetry in accumulation in the lymphatic system, consistent with draining of ^{89}Zr-FH-labeled cells from the site of inflammation (Fig. 6a and Supplementary Video 1). A 3D video of Figure 6a is provided as Supplementary Video 1. Therefore, we anticipate that HIR-FH NPs represent a useful radioactive nanomaterial for PET imaging of lymph node mapping, and of inflammation and related inflammatory diseases, with a reduced iron dose and a need for increased quantitative analysis, as compared to MRI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1

In vivo monocyte uptake assay in mice and ex vivo labeling of leuko-cytes ● TIMING 32.5 h

!CAUTION All studies involving animals should be performed according to national and institutional regulations. Permission for the studies described here was obtained from the Massachusetts General Hospital Institutional Animal Care and Use Committee.

1. In vivo monocyte uptake assay in mice (steps 1–15). Anesthetize the mice by placing them in the induction chamber until they lose consciousness.

   CRITICAL STEP Check the depth of anesthesia by toe pinch before proceeding.

2. Insert a catheter into the tail vein under anesthesia and use it as the injection port for the Cy5.5-FH probe.

3. Through the injection port, inject mice from one cohort (experimental mice) with 125 μg Fe as Cy5.5-FH in 100 μl of PBS.

4. Through the injection port, inject mice from a second cohort (control mice) with 100 μl of PBS.

5. Return the mice to the housing room.

6. After 24 h, anesthetize experimental and control animals as described in step 1.

7. Collect 1 ml of blood from each mouse into a heparinized syringe by cardiac puncture.

8. Prepare one 15 ml Falcon tube for each sample by placing 1 ml of Ficoll-Paque into each tube.

9. Dilute each blood sample 1:1 (vol/vol) with DPBS and carefully layer this on top of the Ficoll-Paque layer in the prepared tubes from step 8.

   CRITICAL STEP The blood and DPBS must be layered slowly, such that the barrier between layers is maintained.

10. Centrifuge the samples at 900g for 30 min at room temperature.

11. For each tube, use a pipette to aspirate the top layer of plasma and transfer the buffy coat to a new 15-ml Falcon tube.

    ? TROUBLESHOOTING

12. Dilute the buffy coat sample with 1 ml of DPBS in the new tubes from the previous step.

13. Centrifuge the tubes from step 12 at 400g for 10 min at room temperature.

14. Obtain the leukocytes by using a pipette to remove and discard the supernatant carefully.

15. Resuspend the pellet in 100 μl of DPBS in the tube from step 14, and then transfer the suspension to a 1.5-ml Eppendorf tube.

16. Ex vivo labeling of leukocytes with Cy5.5-FH (steps 16–20). Preheat a water bath to 37 °C.

17. Repet step 1, for a single mouse.

18. Collect 1 ml of blood by cardiac puncture into a heparinized syringe, and transfer the sample to a 1.5-ml Eppendorf tube.

19. Add Cy5.5-FH to the sample to achieve final concentration of 50 μg Fe/ml, and place sample in a 37 °C water bath for 180 min.

20. Remove sample from water bath and repeat steps 8–15.

21. Labeling leukocytes with anti-CD11b and flow cytometry (steps 21–26). Place the samples from steps 15 and 20 on ice in an ice bucket.

22. Add 10 μl of rat anti-mouse CD11b-FITC antibody to each of the cell suspensions and incubate for 30 min in the covered ice bucket prepared in step 21.

23. Dilute each sample with 1 ml of DPBS, centrifuge at 300g for 5 min at room temperature, and remove the supernatant. Repeat this step three times.

24. Resuspend the final pellets in 500 μl of DPBS and submit the cells to flow cytometry.

25. Turn on the BD Biosciences LSR II or equivalent system and perform flow cytometry, measuring forward- and side-scatter on a linear scale (area and height), and measuring FITC and Cy5.5 channels on a log scale (area and height) (Supplementary Fig. 1).

26. Use forward- and side-scatter measurements to exclude small debris and doublets (Supplementary Fig. 1). A Beckman Dickenson LSR II was used for this measurement, and analysis for Figure 5d was done using Cyflogic software.

Box 2

In vivo monocyte uptake assay in rhesus macaque ● TIMING 6 h

!CAUTION All studies involving animals should be performed according to national and institutional regulations. Permission for the studies described here was obtained from the Massachusetts General Hospital Institutional Animal Care and Use Committee.

1. Injection of monkey and leukocyte preparation (steps 1–18). Administer an i.m. injection of ketamine (4 mg/kg) and Dexdomitor (0.015 mg/kg) to induce sedation of the animal.
2. After loss of consciousness (~5 min), transfer the animal from its home cage to the procedure room.
3. Intubate the animal with an endotracheal tube and connect it to the isoflurane vaporizer (1–2% (vol/vol) isoflurane in 100% O_2 gas). After the animal has been stabilized on isoflurane anesthesia, administer Antesedan (0.25 mg/kg i.m.) to reverse the effects of the Dexdomitor.
4. Insert a catheter into the lateral saphenous vein.
5. Before injection of the Cy5.5-FH, draw 3 ml of 'control' blood into a heparinized syringe.
6. Inject Cy5.5-FH to achieve an injection of 3 mg Fe/kg via the saphenous vein. Remove the venous catheter.
7. Immediately before the 3 h post-injection time point, insert a catheter by saphenous venipuncture.
8. At 3 h post injection, draw 3 ml of blood into a heparinized syringe,

   CRITICAL STEP Blood must be drawn slowly to prevent hemolysis or other damage to cells.
9. Remove the venous catheter and endotracheal tube, then immediately return the animal to its home cage and monitor until it is ambulatory.
10. Prepare one 15-ml Falcon tube for each of the samples from steps 5 and 8 by placing 4-ml of Ficoll-Paque into each tube.
11. Dilute the blood samples from steps 5 and 8 1:1 (vol/vol) with PBS and layer this on top of the Ficoll-Paque layer in the tubes prepared in the previous step.

    CRITICAL STEP The blood and DPBS must be layered slowly, such that the barrier between layers is maintained.
12. Centrifuge the samples at 900g for 30 min at room temperature.
13. For each tube, aspirate the top layer of plasma and transfer the buffy coat to a new 15-ml Falcon tube.

    ? TROUBLESHOOTING

14. Dilute each buffy coat sample with 3 ml of DPBS.
15. Centrifuge the tubes from step 14 at 400g for 10 min at room temperature.
16. Obtain the leukocytes by removing and discarding the supernatant carefully with a pipette.
17. Resuspend each pellet in 300 μl of DPBS in the tubes from step 16.
18. For each tube in step 17, make three aliquots of sample (100 μl each) in 1.5-ml Eppendorf tubes.
19. Labeling leukocytes with anti-CD11b and flow cytometry (steps 19–24). Place the samples from step 18 on ice in an ice bucket.
20. Add 10 μl of mouse anti-human CD11b antibody to each of the cell suspensions and incubate for 30 min in the covered ice bucket prepared in step 19.
21. Add 1 ml of DPBS to each sample and centrifuge at 300g for 5 min at room temperature and remove the supernatant. Repeat this step three times.
22. Resuspend the final pellets in 500 μl of DPBS and submit the cells for flow cytometry.
23. Turn on the BD Biosciences LSR II or equivalent equipment and perform flow cytometry, measuring forward- and side-scatter on a linear scale (area and height), and measuring FITC and Cy5.5 channels on a log scale (area and height) (Supplementary Fig. 1).
24. Use forward- and side-scatter measurements to exclude small debris and doublets (Supplementary Fig. 1). A Beckman Dickenson LSR II was used for this measurement, and analysis was done using Cyflogic software to produce Figure 5d

Box 3

In vivo microPET/CT imaging of HIR-FH nanoparticles in mice ● TIMING 61.5 h

!CAUTION All studies involving animals should be performed according to national and institutional regulations. Permission for the studies described here was obtained from the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Procedure

1. Anesthetize a BALB/c mouse (16 weeks old, 28 g) with a self-inflicted paw injury with 2% isoflurane in 2 l/min O_2 in the induction chamber.

   CRITICAL STEP Before proceeding, check depth of anesthesia by toe pinch.

2. Insert a catheter into the tail vein under anesthesia and use it as the injection port for the ^{89}Zr-FH probe.

3. Draw ^{89}Zr-FH solution into a syringe to a dose of 160 μCi (as measured by dose calibrator), and record the time of measurement.

4. Dilute the ^{89}Zr-FH solution with DPBS to a final volume of 200 μl.

5. Prepare a syringe with 200 μl of DPBS.

6. Inject the mouse through the catheter with the sample from step 4, and record time of injection.

   ? TROUBLESHOOTING

7. Flush the catheter using the DPBS syringe prepared in step 5.

   CRITICAL STEP Inject only enough DPBS to flush the catheter line. Injection of a large volume will result in death of the animal.

8. Remove the catheter from mouse, and measure residual activity in the syringe, in the catheter line, and on the gloves of the technician performing the injection (by dose calibrator).

9. Calculate the total injected dose by subtracting the residual measurement from step 8 from the recorded dose in step 3.

   ? TROUBLESHOOTING

10. Return the mouse to its cage and return it to the housing facility.

11. 60 h post injection, remove the mouse from the housing facility and place it in the induction chamber until unconscious (verify by toe pinch)

12. Prepare microPET/CT as described in the Equipment Setup section.

13. Remove the mouse from the induction chamber and place it face-down, head-first on the scanner bed, on top of absorbent bed-ding, placing the mouse such that the oxygen/isoflurane nose cone covers its face.

14. Acquire a scout X-ray image to define the field of view for the PET and CT acquisitions.

15. For the PET acquisition, define the field of view to keep all desired anatomy in the acquisition window.

    CRITICAL STEP Keep important tissues close to the center when defining the field of view, as the image resolution is poorer near the edges of the field of view.

16. Acquire a dynamic list-mode PET acquisition for 20 min.

17. Using the same scout image from step 14, define a CT field of view to cover the areas acquired in the PET scan.

18. Perform a CT acquisition at 300 μA and 40 kV, with 360 projections.

19. Remove the mouse from the scanner bed, return it to the cage, and allow the mouse to regain consciousness before returning it to the housing facility.

20. Analyze the images with the volume-rendering tool in AMIDE (Equipment) (Fig. 6).

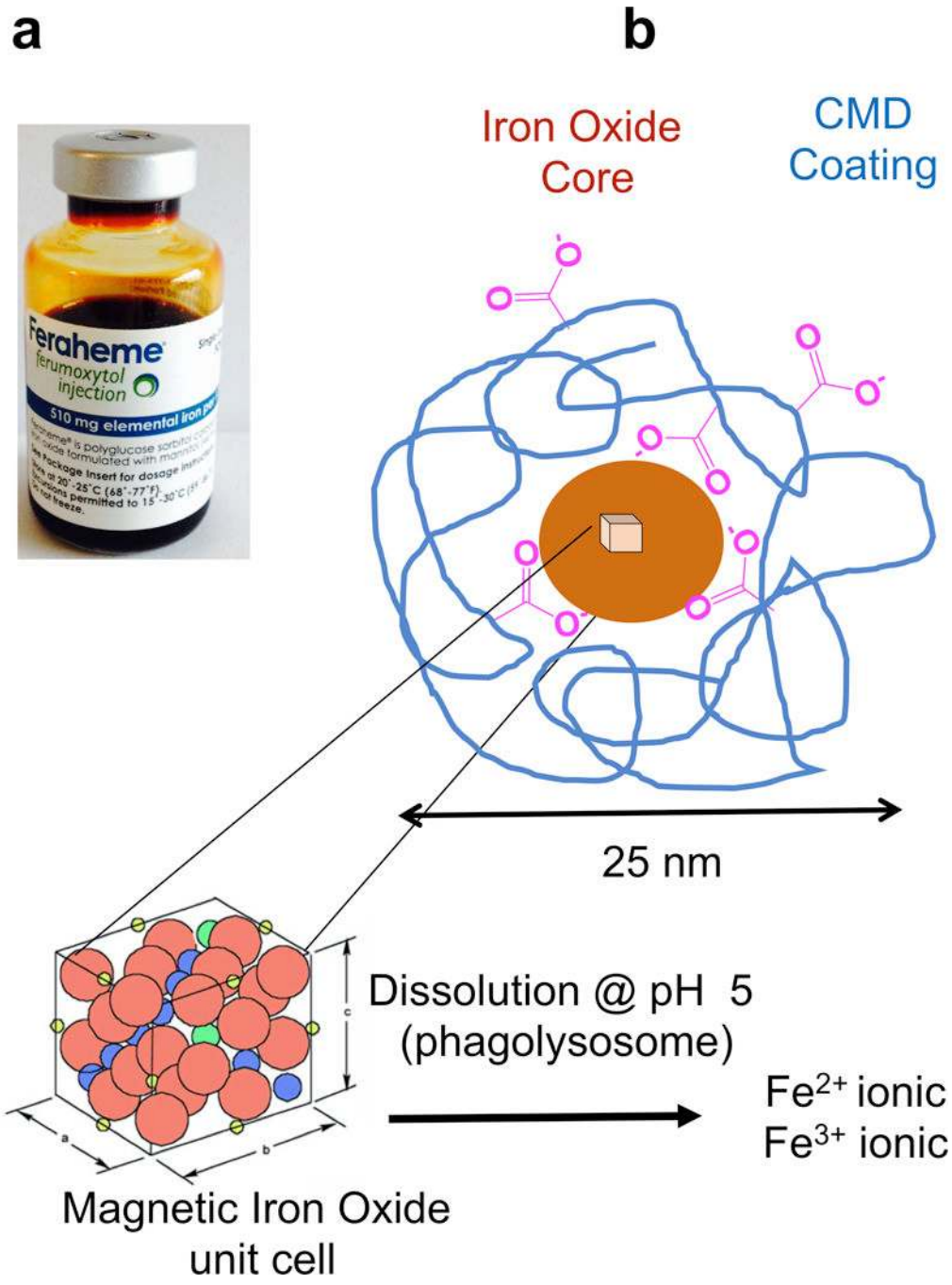

Figure 1.

Feraheme (FH) nanoparticles (NPs) are heat stable but acid labile. (**a**) FH is a liquid that contains 510 mg of Fe/bottle and can be stored at room temperature, reflecting very high stability. (**b**, Top) The FH NPs consist of a superparamagnetic iron oxide core (~6.6 nm) surrounded by a polymeric coating of carboxymethyldextran (CMD) with an overall diameter of 17–31 nm. Multiple carboxyl groups bind the CMD to the iron oxide surface, providing a heat-stable bond between the polymer and iron oxide. Other free carboxyl groups can be converted to amines and reacted with fluorochromes; see Figure 2b. (**b**,

Bottom) A magnetite unit cell is shown with large orange anionic oxygen molecules, and smaller ferric ions (blue) and ferrous ions (green). The iron oxide core of FH (central orange ball) has many unit cells. When FH is exposed to mild acid, protons diffuse through the extended and porous CMD layer, dissolving the iron oxide crystal and generating ionic iron species. Dissolution reduces the crystal-based superparamagnetism.

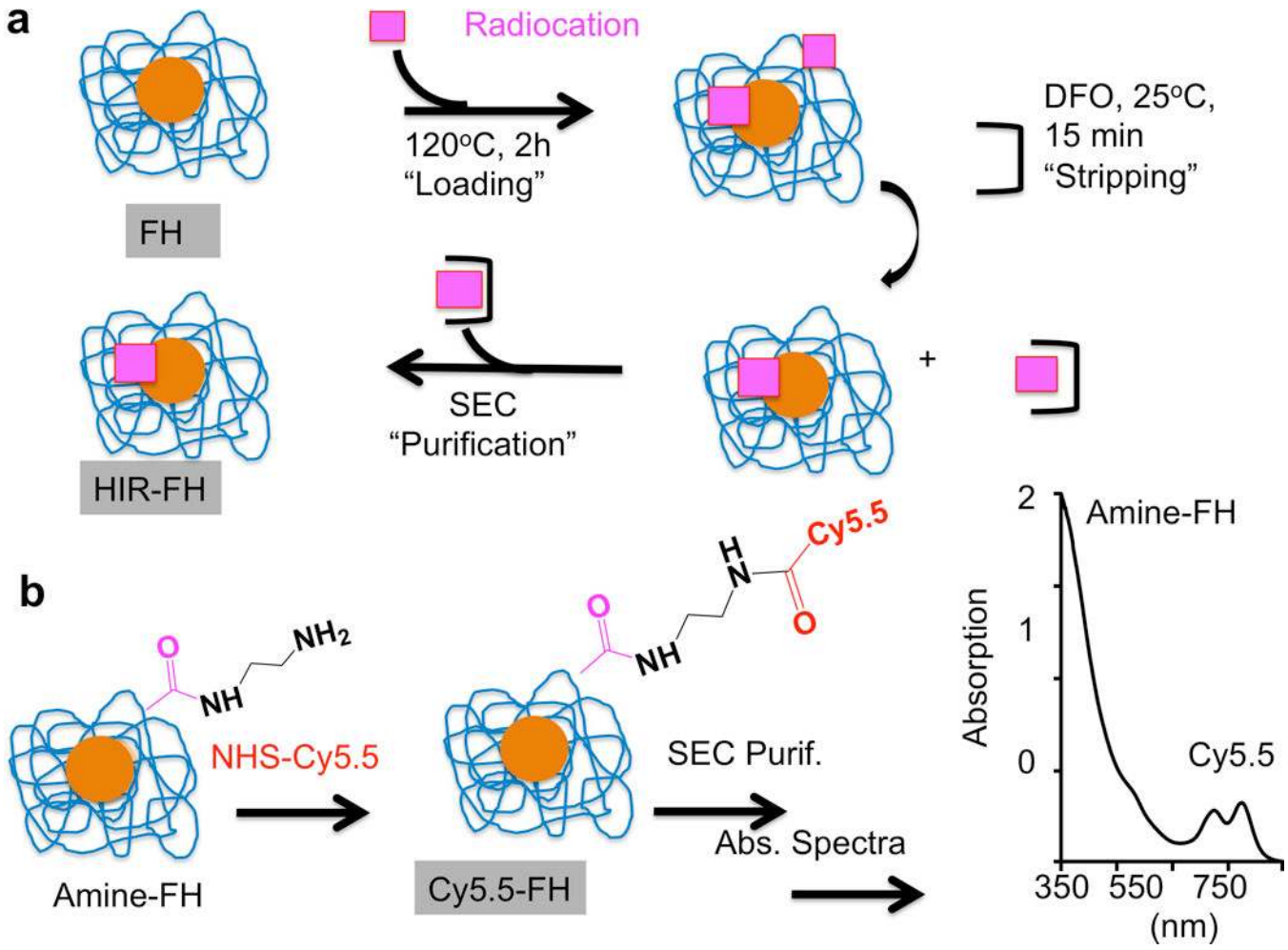

Figure 2.

Synthesis of HIR-FH and Cy5.5-FH nanoparticles. (**a**) Heat-induced radiolabeling (HIR) of FH (Steps 1–50). A radiocation ($^{64}Cu^{2+}$, $^{111}In^{3+}$, or $^{89}Zr^{4+}$, indicated with a magenta square) is heated in the presence of FH (120 °C, 2 h) during the 'loading' phase (Steps 6–8), followed by incubation with DFO ('stripping'; Steps 9–11) and then by SEC purification (Steps 21–25). (**b**) The carboxyl groups of FH react with ethylene diamine to generate amine-FH (Steps 91–110), followed by reaction with the NHS ester of Cy5.5 (Steps 111–113). After SEC purification (Steps 116–118), the number of Cy5.5 molecules per FH NP is determined spectrophotometrically (Steps 123–128), with Amine-FH distinct from Cy5.5. FH, HIR-FH, and Cy5.5-FH (gray highlighting) are indistinguishable from each other.

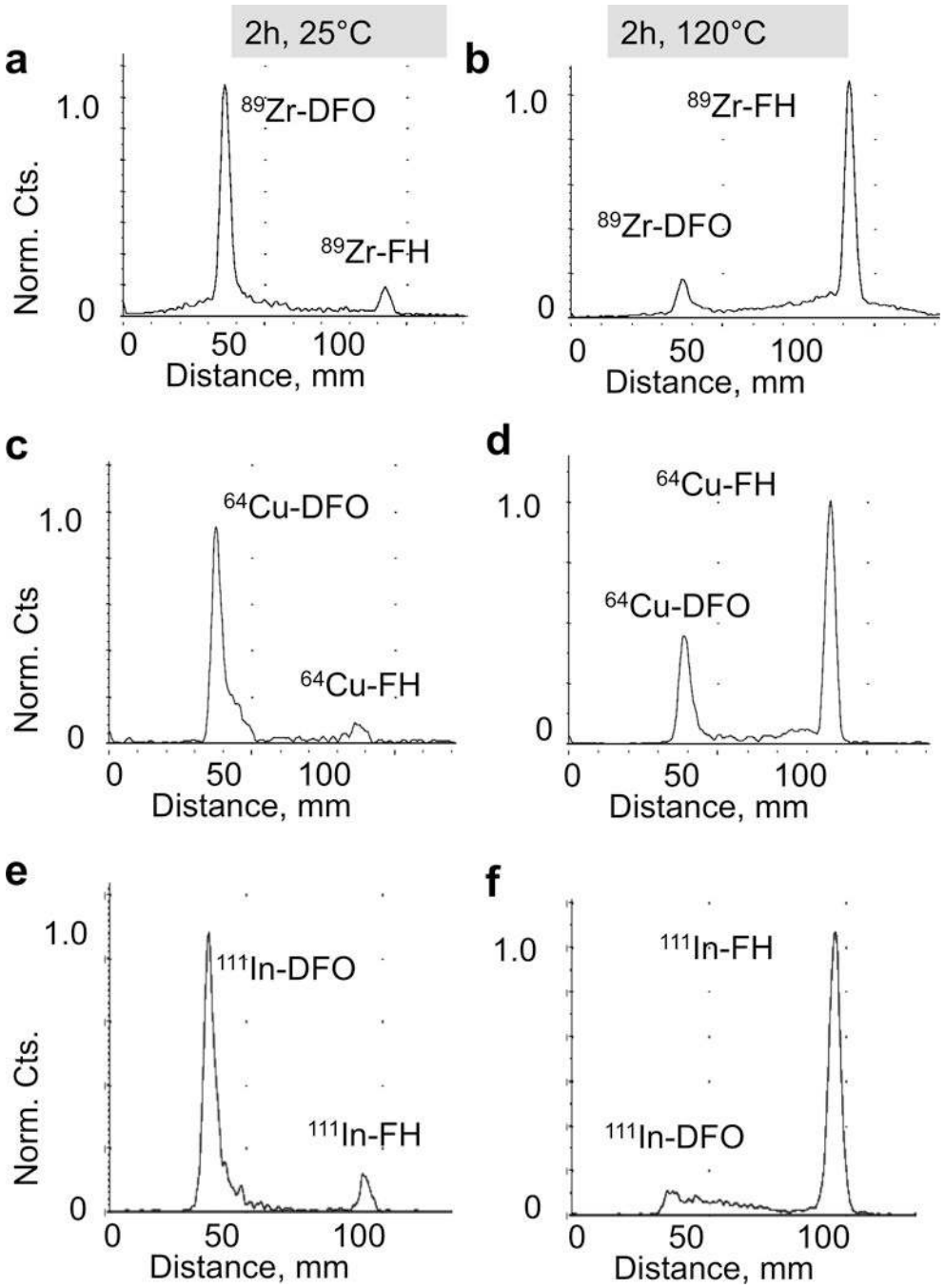

Figure 3.

Heat-induced radiolabeling of FH with $^{89}Zr^{4+}$, $^{64}Cu^{2+}$, or $^{111}In^{3+}$. TLC of reaction mixtures after incubating FH and the indicated cation, followed by DFO addition. (**a**) $^{89}Zr^{4+}$ and FH incubated for 2 h at 25 °C (**a**) or 2 h at 120 °C (**b**) (Steps 12–20). (**c,d**) TLC of reaction mixtures (DFO, $^{64}Cu^{2+}$, FH) either for 2 h at 25 °C (**c**) or heated for 2 h at 120 °C (**d**). (**e,f**) TLC of reaction mixtures (DFO, $^{111}In^{3+}$, FH) either incubated for 2 h at 25 °C (**e**) or heated for 2 h at 120 °C (**f**). Cts., counts; Norm., normalized.

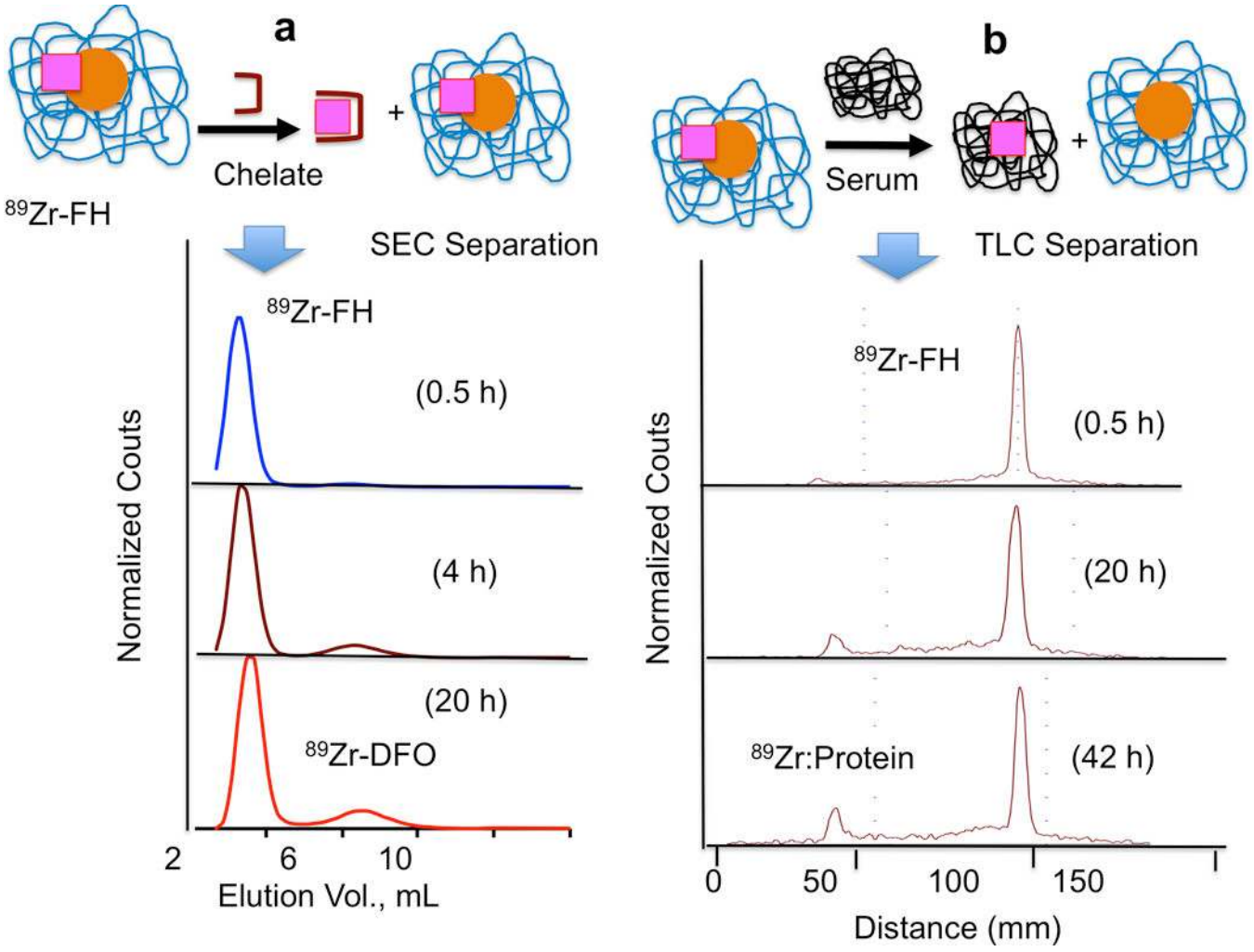

Figure 4.

Radiochemical stability of HIR ^{89}Zr-FH. (**a**) For chelation challenge stability, ^{89}Zr-FH was incubated with 10 μM deferoxamine (DFO) in 10 mM Tris-acetate, pH 7, at 37 °C and analyzed by size-exclusion chromatography (SEC) (Steps 51–64). (**b**) For serum challenge stability, ^{89}Zr-FH was incubated in 67% (vol/vol) mouse serum at 37 °C and analyzed by thin layer chromatography (TLC) (Steps 65–78). Released $^{89}Zr^{4+}$ (red) can bind to serum proteins (indicated in black in schematic at top).

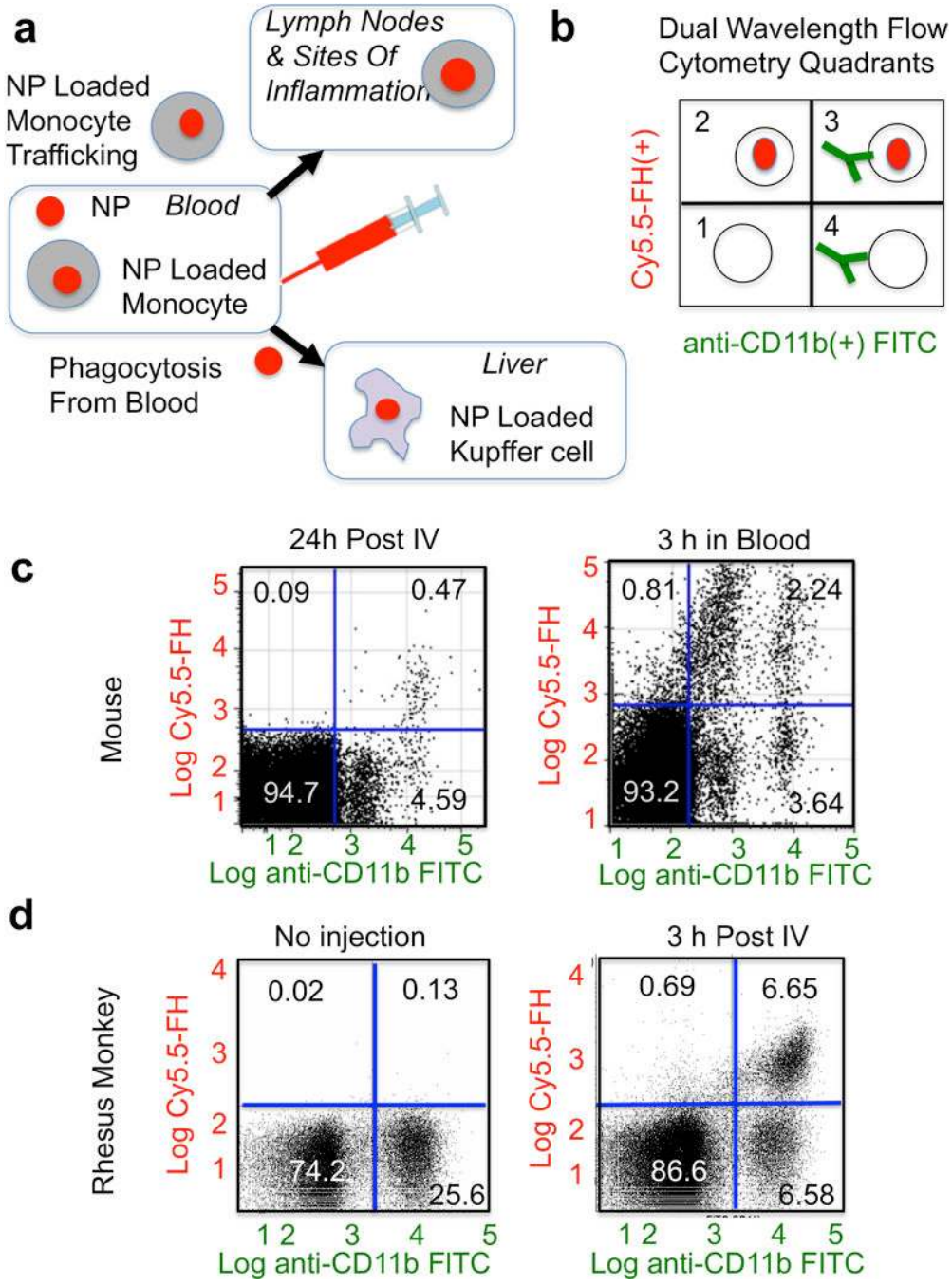

Figure 5.

Monocyte internalization of Cy5.5-FH by dual-wavelength flow cytometry (Box 1 and Box 2). (**a**) Schematic showing i.v.-injected Cy5.5-FH NPs (red spheres) are internalized by blood-residing monocytes (gray spheres) or phagocytized by hepatic Kupffer cells (purple) in the liver. (**b**) Four quadrants of dual-wavelength flow cytometry are (1) unlabeled cells, (2) Cy5.5-FH cells, (3) Cy5.5 and anti-CD11b cells (FITC), and (4) anti-CD11b cells (FITC). (**c**, left) At 24 h post Cy5.5-FH (0.125 mg Fe/25 g mouse) i.v. injection, isolated buffy coat leukocytes were reacted with a fluorescein-labeled anti-CD11b antibody and

submitted to flow cytometry (Box 1, steps 1–15 and 21–26). (Right) A blood sample was incubated with 50 μg of Fe/ml of Cy5.5-FH for 3 h, after which, the buffy coat was isolated and reacted with a FITC-labeled anti-CD11b antibody for flow cytometry (Box 1, steps 16–26). Numbers in quadrant boxes are the percentages of cells. Cell counts were 33,000 (IV) and 13,000 (blood). (**d**) Blood taken before (left) (Box 2, step 5) and 3 h after (right) Cy5.5-FH injection (3 mg Fe/kg, monkey) (Box 2, steps 6–8). Isolated buffy coat leukocytes (Box 2, steps 5–18) were reacted with fluorescein-anti-CD11b and submitted to flow cytometry (Box 2, steps 19–24). Cell counts were 33,000 (No injection) and 19,000 (3 h post i.v. injection). All animals must be handled under institutionally and nationally approved protocols and procedures. Approval for the experiments involving the animals described in this study was obtained from the Institutional Animal Care and Use Committee at Massachusetts General Hospital.

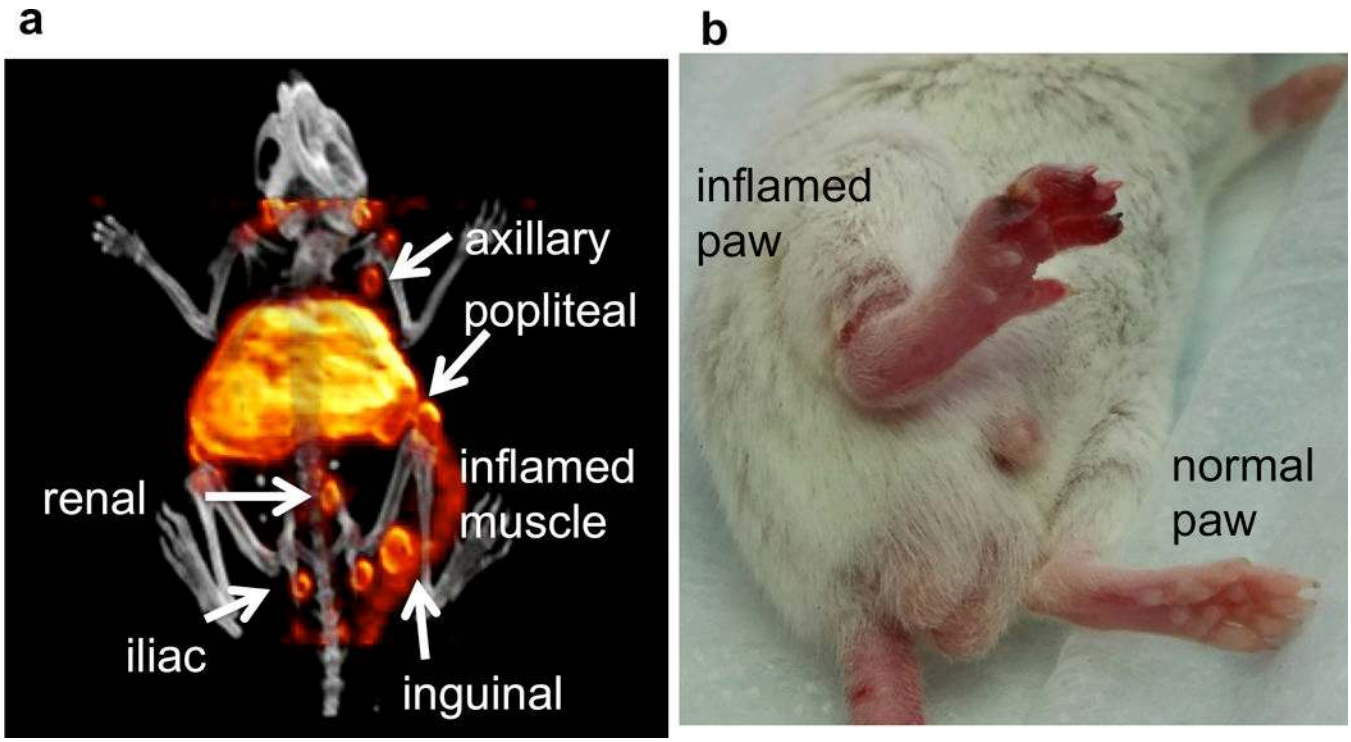

Figure 6.

Imaging of normal and abnormal monocyte trafficking with HIR ^{89}Zr-FH (Box 3). (**a**) PET/CT MIP image of mouse with self-inflicted paw injury 60 h post injection of 160 μCi/100 μg Fe of ^{89}Zr-FH. A 3D-rotational video of the mouse in this figure is provided as Supplementary Video 1 and shows detail that is not visible in the 2D image. (**b**) Photograph of inflamed paw shown in a. MIP, maximum-intensity projection. **! CAUTION** All animals must be handled under institutionally and nationally approved protocols and procedures. Approval for the experiments involving the animals described in this study was obtained from the Institutional Animal Care and Use Committee at Massachusetts General Hospital.

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
Step 2	Low yields (RCY) are obtained after size-exclusion purification	Oxalate complexation of ^{89}Zr	• Increase the amount of FH in the reaction when low specific activity is need. • Purchase more concentrated ^{89}Zr solutions (e.g., 4 mCi/10 μL from 3D Imaging, Inc) when high specific activity is expected.
Step 3	High-molecular weight radioactivity is observed in the absence of Feraheme (HIR control).	Inadequate pH control during HIR reaction	• Neutralize ^{89}Zr more slowly by adding 1M Na_2CO_3 in small fractions. • Perform more frequent and duplicate determinations of pH.
Steps 28, 30	A brown color is observed in the filtrate.	The membrane of Amicon filter is broken.	Switch to a new filter
Step 84, 85, 98, 104, 105	A brown color is observed in the filtrate.	The membrane of Amicon filter is broken	Switch to a new filter
Steps 119, 120	A blue color is observed in the filtrate.	Free Cy5.5 non-specifically binds to other dye fragments on the FH surface	Run another SEC separation with freshly packed Sephadex G-25 column to remove the free dye by repeating steps 116 - 118, which will cause a lower iron recovery.
Step 114, 115, 119, 120	A pale green color is observed in the filtrate.	The membrane of Amicon filter broken	Switch to a new filter
Box 1 Step 11, Box 2 Step 13	Distinct layers are not seen after centrifugation.	The blood was not layered carefully enough atop ficoll layer.	Dilute the contents of falcon tube 1:1 with DPBS and layer atop new ficoll layers in fresh falcon tubes.
Box 1 Step 11, Box 2 Step 13	Layers are seen after centrifugation, but no buffy coat layer is seen.	Buffy coat cells have been lysed due to sheer stress.	A larger-bore needle/catheter should be used to draw blood from the animal, using a slower draw. This will reduce shear stress experienced by cells being drawn
Box 3 Step 6	A bubble is seen under the skin at the injection site.	There has been extravasation at the injection site.	If a second animal is not available, the dose will eventually move from the extravasated site to the rest of the body, but the timing will be different. The animal can be scanned at multiple time points
box 3 Step 9	A large percentage of the original dose is in the residual activity in the syringe, in the catheter line, and on the gloves of the technician performing the injection	There was not a clean injection.	PET acquisitions can be extended for longer periods to compensate for low injected activity.