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Supplementary Information for:

Automated synthesis of [^{68}Ga]oxine, improved preparation of ^{68}Ga-labeled erythrocytes for blood-pool imaging, and preclinical evaluation in rats

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General Experimental Information

HPLC grade acetonitrile, sodium acetate and sodium chloride were obtained from Fisher Chemical. Ethanol was purchased from American Reagent. 8-Hydroxyquinoline was purchased from Sigma Aldrich, and [^{nat}Ga]oxine reference standard was purchased from Oakwood Chemical. Sterile 0.9% saline and sterile water for injection were purchased from Hospira. Ultrapure water was obtained from a Millipore MilliQ Gradient A10 system. HCl (0.05M, ultrapure) as purchased from iTG. Sterile vials were purchased from Hollister-Stier. Sep-Pak C18 1cc Vac and Sep-Pak C18 Light cartridges were purchased from Waters Corporation. Radioactivity was counted using a CRC-15 (Capintec) detector.

High performance liquid chromatography (HPLC) was performed using a Shimadzu LC-2010A HT system equipped with a Bioscan B-FC-1000 radiation detector in series. A 0.2 min offset was applied to all traces to account for the detectors being in series. HPLC conditions were as follows: column: Phenomenex Luna 5 µm C18(2) 100 Å 150 mm x 4.6 mm; flow rate: 1.5 $mL.min^{-1}$; solvent: 35% MeCN in H_2O.

Synthesis of [^{68}Ga]oxine

Reagent and hardware kit for synthesis of Ga-68 peptides (SC-01, ABX, Germany), tubing and reagents were attached to the Scintomics module (Scintomics GRP Cassette Module, Germany). 150 µL of oxine (8-hydroxyquinoline) stock solution (1 mg.mL^{-1} in EtOH) was dissolved in sodium acetate buffer (2 M, pH 5.5, 1.5 mL) in the reaction vessel. The ^{68}Ge/^{68}Ga-generator (iTG) was eluted with HCl (0.05 M, 4 mL) and the eluent was diluted with water for injection (9 mL). The eluent mixture was then transferred to a cation exchange cartridge (CHROMAfix PS-H^{+}, Machery Nagel) and [^{68}Ga]Ga^{3+} was eluted with 5 M NaCl solution (1.5 mL) into the reaction vessel. The complexation reaction proceeded for 10 min at room temperature. The resultant complex was processed as follows, dependent on the desired application:

Synthesis of [^{68}Ga]oxine for rat and human erythrocyte labeling and washing studies

The crude reaction mixture was removed from the reaction vessel and transferred to a C_{18} cartridge (Waters, SepPak 1cc vac, preconditioned with EtOH (10 mL) followed by water for injection (10 mL)). The reaction vessel was rinsed with water for injection (9 mL) and this too passed through the C_{18} cartridge. The final product was eluted from the C_{18} cartridge by hand, with EtOH (250 µL), sterile filtered (Millex GV, 0.22 µm, 13 mm) and diluted with saline solution (500 µL) to yield a solution of [^{68}Ga]oxine (9 ± 3 mCi (n = 6), 33% EtOH in saline) in 35 ± 9% (n = 6) non-decay corrected radiochemical yield in ~50 min, with a radiochemical purity of >99%, determined by HPLC.

Synthesis of [^{68}Ga]oxine for direct injection

The crude reaction mixture was removed from the reaction vessel and transferred to a C_{18} cartridge (Waters, SepPak 1cc vac, preconditioned with EtOH (10 mL) followed by water for injection (10 mL)). The reaction vessel was rinsed with water for injection (9 mL) and this too passed through the C_{18} cartridge. The final product was eluted from the C_{18} cartridge by hand,

with EtOH (250 µL), sterile filtered (Millex GV, 0.22 µm, 13 mm) and diluted with saline solution (3.2 mL) to yield a solution of [^{68}Ga]oxine (6.2 mCi in 7% EtOH in saline) in 26% RCY (n = 1) in >99% radiochemical purity, determined by HPLC.

Fully automated synthesis of [^{68}Ga]oxine

All steps conducted under automation on the Scintomics module. After synthesis of the complex under automation, the crude reaction mixture was removed from the reaction vessel and transferred to a C_{18} cartridge (Waters, SepPak C_{18} Light, preconditioned with EtOH (5 mL) followed by water for injection (38 mL)). The reaction vessel was rinsed with water for injection (9 mL) and this too passed through the C_{18} cartridge. The final product was eluted from the C_{18} cartridge with EtOH/H_2O (1:1 v/v, 2 mL), sterile filtered (Millex GV, 0.22 µm, 25 mm) and diluted with saline (14.5 mL) to yield a solution of [^{68}Ga]oxine (22 ± 11 mCi (n = 3), 12% EtOH in saline) in 50 ± 5% (n = 3) non-decay corrected radiochemical yield in ~42 min, with greater than 99& radiochemical purity, determined by HPLC.

Assessment of Purity and Stability

In all cases, the identity of the product was verified by comparison of the retention time of the radiolabeled product with that of an authentic [^{nat}Ga]oxine reference standard on HPLC. An example of the HPLC is shown below in Figure S1.

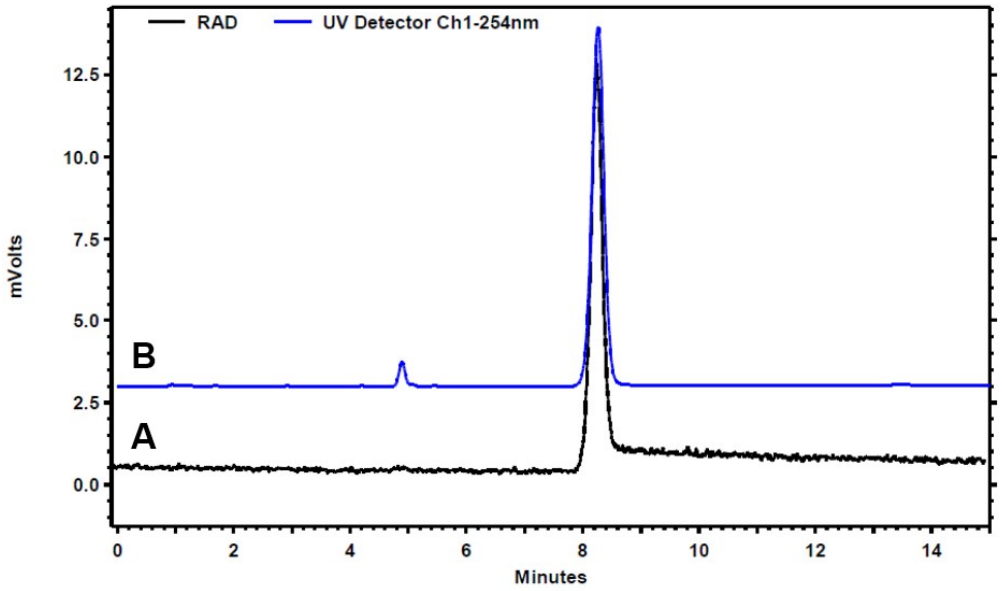

Figure S1A: Gamma HPLC trace of the isolated product showing a single peak at 8.3 minutes and >99% radiochemical purity of the product. **B:** UV HPLC trace (254nm) of the reformulated product spiked with an authentic sample of [^{nat}Ga]oxine, showing co-elution of the peaks at 8.3 minutes. The peak at 4.9 minutes is excess 8-hydroxyquinoline.

[^{68}Ga]$GaCl_3$ does not elute from the HPLC under these conditions. The final dose was determined to be free of [^{68}Ga]Ga^{3+} by running a C_{18} trapping experiment. A aliquot of [^{68}Ga]$GaCl_3$ of known activity was passed through a preconditioned C_{18} Light cartridge (Waters, preconditioned with EtOH (10 mL), H_2O (10 mL)), and the flow through collected in a vial. Under these conditions, >95% of the activity eluted from the cartridge, demonstrating that free [^{68}Ga]Ga^{3+} is not trapped on a C_{18} cartridge. The trapping experiment was repeated with aliquots of [^{68}Ga]oxine immediately following the synthesis, 1 hour post-synthesis and 2 hours post-synthesis. Briefly, 1-4 mL of the solution of [^{68}Ga]oxine was passed through a freshly preconditioned C_{18} cartridge, and the flow through collected and counted. The activity trapped on the C_{18} cartridge was also counted.

The decay corrected data for both free [^{68}Ga]$GaCl_3$ and for [^{68}Ga]oxine immediately following synthesis, 1 hour post synthesis, and 2 hours post synthesis are summarized below, in **Table S1**.

Table S1: Decay corrected activities for the C_{18} trapping experiment for [^{68}Ga]$GaCl_3$, and [^{68}Ga]oxine at various time points post-synthesis.

	[^{68}Ga]$GaCl_3$	*[^{68}Ga]oxine*		
		t = 0 h	t = 1 h	t = 2 h
total activity in syringe (μCi):	1230	662	738	894
activity left in syringe (μCi):	12	0	43	24
activity trapped on C_{18} (μCi):	52	616	686	804
activity in flow through (μCi):	1113	0	2	0
% activity in flow through	95.5%	0.0%	0.3%	0.0%

The data demonstrated that in each case, a negligible amount of radioactivity eluted from the C_{18} cartridge, confirming that there was a negligible quantity of free [^{68}Ga]Ga^{3+} in the final dose. The data also demonstrates that the final formulated dose of [^{68}Ga]oxine is stable for at least 2 hours post-synthesis, and that free [^{68}Ga]Ga^{3+} does not leach from the complex during this time.

Acquisition and Preparation of Blood and Erythrocytes

Acquisition of Blood Samples

Rat venous blood was drawn after rats were lightly anaesthetized with isoflurane (Fluriso, VetOne), before a catheter was inserted into the tail vein. Venous blood was collected in an Eppendorf tube containing heparin (5 μL, 0.5 units, Heparin Lock Flush, Fresenius Kabi USA). Human venous blood was drawn into an evacuated heparinized tube (BD Vacutainer with PST gel and lithium heparin, 83 units) from a healthy volunteer. Blood was stored at 4 °C until use, and was used within 24 hours.*

* The difference in tubes used to draw rat (Eppendorf tube) and human (Vacutainer with PST gel and lithium heparin) blood were due to procedural differences in how blood is taken from rodents (overseen by IACUC) and humans (overseen by IRB). Since the procedure worked in each case, we did not amend the protocols to standardize the tubes. As we move into clinical studies we will use Vacutainers since they are already used in our clinic to label RBCs with ^{99m}Tc using the Ultratag kit.

Erythrocyte preparation from whole blood

Heparinized whole blood (500 uL) was centrifuged (3000 rpm, 4 min) to sediment erythrocytes. The plasma and buffy coat were carefully removed and discarded, and the erythrocytes were gently resuspended in saline (500 µL). The mixture was centrifuged again (3000 rpm, 4 min) and the supernatant carefully removed from the erythrocyte pellet, and discarded. The washing procedure was repeated with a further aliquot of saline (500 µL), and the supernatant again discarded. The erythrocytes were resuspended in saline (1000 µL).

^{68}Ga-labeled Erythrocyte Washing Studies

[^{68}Ga]Oxine solution (100 - 200 µL, 0.054–1.73 mCi) was added to a suspension of erythrocytes prepared as described above, the tube was inverted a few times, then incubated for 15 minutes at room temperature, with occasional inversion. A cell washing study was performed with the [^{68}Ga]oxine radiolabeled human and rat erythrocytes, immediately after labeling.

After incubation with [^{68}Ga]oxine, the erythrocytes were centrifuged (3000 rpm, 4 min) and the supernatant carefully removed from the erythrocyte pellet, and radioactivity associated with the erythrocyte pellet and the supernatant were quantified (wash 0, represents initial labeling efficiency). The erythrocyte pellet was gently resuspended in saline (1 mL), and the mixture was again centrifuged (3000 rpm, 4 min). The supernatant and pellet were again separately counted. The wash-centrifuge procedure was repeated four times (wash 1-4), with the activity associated with the erythrocyte pellet and supernatant quantified after each wash. The washing was repeated four times, and the resultant data are shown in Figure S2, along with the reported data from Welch et al.[1] and Ballinger et al.[2]

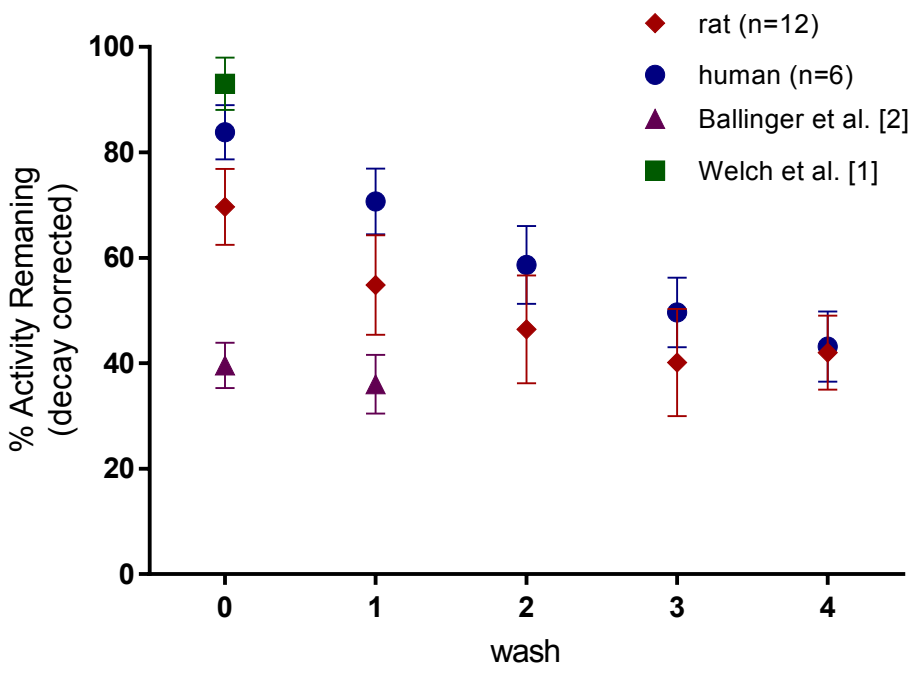

Figure S2: Decay corrected radioactivity (and standard deviation) associated with erythrocytes after each washing step for both rat and human erythrocytes. The initial labeling yield was 84% for human erythrocytes, and 75% for rat erythrocytes. After 4 washes, radioactivity associated with the erythrocytes plateaued at 42% for both human and rat erythrocytes

Labeling of rat erythrocytes with [^{68}Ga]oxine for microPET imaging

Rat erythrocytes were prepared as described above. [^{68}Ga]oxine solution (100 µL, 0.054–1.73 mCi) was added to the resuspended erythrocytes, the tube was inverted a few times, then incubated for 15 minutes at room temperature, with occasional inversion. Erythrocytes were centrifuged (3000 rpm, 4 min) and the supernatant carefully removed from the erythrocyte pellet. The erythrocyte pellet was gently resuspended in saline (1 mL), and the mixture was again centrifuged (3000 rpm, 4 min). The supernatant was carefully aspirated, and discarded, before the pellet was resuspended in saline (1 mL). The centrifuge-wash procedure was repeated two more times. After the final centrifugation (3000 rpm, 4 min), the erythrocyte pellet was resuspended in saline (500 µL) to give ^{68}Ga-labeled rat erythrocytes (0.157–0.545 mCi) ready for injection.

MicroPET imaging in rats

MicroPET imaging studies were conducted using a Concorde Microsystems P4 PET scanner. Anesthesia was induced in healthy, female Sprague-Dawley rats (290-419 g) using isoflurane/O_2, and anesthesia was maintained with 2-4% isoflurane/O_2 throughout the experiment. Body temperature was maintained by a heating pad. A transmission scan for attenuation correction was acquired prior to administration of the radiolabeled compound of interest.

Emission data were collected over 60 minutes. The emission data were corrected for decay, dead time and random coincidences before reconstruction using an iterative ordered subset expectation maximization–maximum a posteriori (MAP) method to generate the reconstructed images. The frames were summed, smoothed, and volumes of interest were defined over the heart and liver using isometric, circular regions-of-interest (ROIs) over five adjacent frames in the coronal projection. The volumetric ROIs were then applied to the full dynamic data sets to obtain the regional tissue time-radioactivity data. Standardized uptake values (SUVs) were calculated for each of the regions of interest,

MicroPET imaging unwashed ^{68}Ga-labeled erythrocytes in a rat

Erythrocytes were prepared, and labeled as described above. After the 15 minute incubation with [^{68}Ga]oxine, the erythrocytes were pelleted (3000 rpm, 4 min), the supernatant discarded, and the erythrocyte pellet resuspended in saline (500 uL). The donor rat was anesthetized as described above, before being administered unwashed ^{68}Ga-labeled erythrocytes (476 µCi, n = 1) via tail vein cannula, followed by a saline flush (150 uL). Emission data was collected and processed as described above. The images from the original Welch et al study, this study, and eh corresponding time-activity curves for the heart and liver ROIs are shown below in Figure S3.

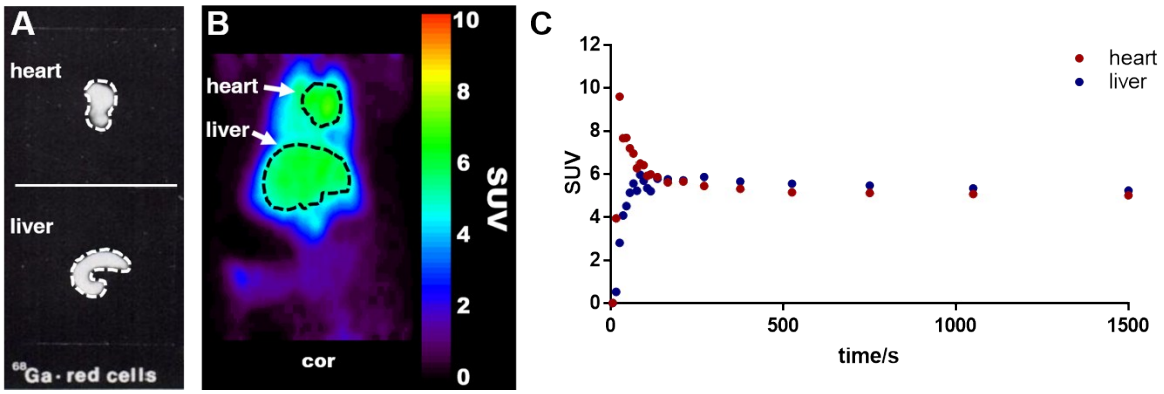

Figure S3A. PET image of ^{68}Ga-labeled erythrocytes in a dog, showing distribution in the heart (top frame) and liver (bottom frame) reported by Welch et al[1]. This research was originally published in JNM. © by the Society of Nuclear Medicine and Molecular Imaging, Inc **B.** Coronal (cor) summed (0-25 min) micro PET image of ^{68}Ga-labeled erythrocytes in a rat, showing similar heart and liver uptake **C.** Time activity curve (n=1) showing SUV_{max} of 10 in the heart and 6 in the liver, and equilibrium SUV of 5.5 in both.

MicroPET imaging of washed ^{68}Ga-labeled erythrocytes in a rat

Rats were anesthetized as described above, before being administered washed ^{68}Ga-labeled erythrocytes (213-279 µCi, n = 3) via tail vein cannula, followed by a saline flush (150 uL). Emission data was collected and processed as described above. Data is presented in the main body of the paper (Figure 1).

MicroPET imaging of [^{68}Ga]oxine in a rat

Rats were anesthetized as described above, before being administered [^{68}Ga]oxine (359-419 µCi, n = 2) via tail vein cannula, followed by a saline flush (150 uL). Emission data was collected and processed as described above. Data is presented in the main body of the paper (Figure 2).

^{68}Ga-labeled erythrocytes followed by [^{68}Ga]oxine microPET imaging in a rat

A healthy, female Sprague-Dawley rat (372 g, n=1) was anaesthetized as described above, and a transmission scan was obtained. Washed ^{68}Ga-labeled erythrocytes (173 µCi) were administered, and the rat scanned for 30 minutes, before [^{68}Ga]oxine (369 µCi) was

administered and scanning was continued for a further 60 minutes. The time activity curves of ROIs in the heart and liver are shown in Figure S4.

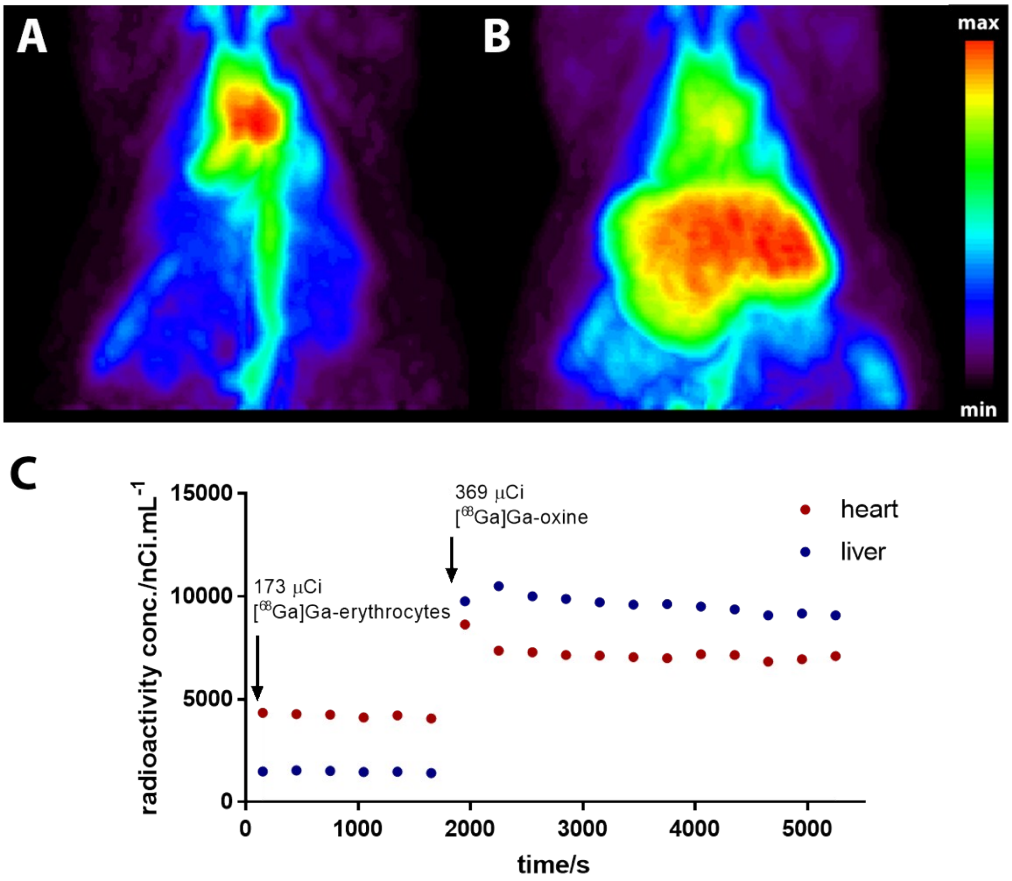

Figure S4A: Sagittal maximum intensity projection (MIP) summed (0-30 min) micro PET image ^{68}Ga-labeled erythrocytes in a rat, showing the heart and major vessels **B:** Sagittal maximum intensity projection (MIP) summed (30-90 min) micro PET image of the same rat administered [^{68}Ga]oxine 30 minutes into the scan. Uptake in the liver has increased relative to that of the heart. **C:** Time activity curve showing rapid equilibration of radioactivity in the heart, with minimal uptake in the liver for the early duration of the scan, while after administration of [^{68}Ga]oxine, liver uptake increases dramatically to greater than that observed in the blood pool.

Cell Viability Study

Heparinized whole blood (500 uL) from a human volunteer was centrifuged (3000 rpm, 4 min), and the plasma and buffy coat removed. The erythrocytes were and washed with saline according to the procedure described above, before being were resuspended in saline (1000 µL). An aliquot (150 µL) of these washed erythrocytes were stored at room temperature, and

used as a control sample. To the remaining erythrocyte suspension, [^{68}Ga]oxine solution (150 µL, 2.12-2.23 mCi) was added, the tube was inverted a few times, then incubated for 15 minutes at room temperature, with occasional inversion. The labeled erythrocytes were sedimented by centrifugation, and washed with saline (4 × 1000 µL) as described above, before being resuspended in saline (500 µL). Immediately after labeling, aliquots of both the control erythrocytes and the [^{68}Ga]oxine labeled erythrocytes (2 µL each) were diluted with saline (98 µL, 1:50 dilution). An aliquot (25 µL) of each dilution was then added to a solution of Trypan Blue (0.4%, Gibco, 25 µL) and incubated at room temperature for 5 minutes. Erythrocytes were then counted using a hemocytometer (Bright-Line). This procedure was repeated with both control and labeled aliquots of cells 1 h and 2 h post labeling. Data is shown in Figure S5 and Table S2.

Table S2: Erythrocyte viability study showing the number of viable erythrocytes and the number of lysed erythrocytes for both ^{68}Ga-labelled and unlabelled samples, at various time points post labelling.

	Unlabeled erythrocytes			*^{68}Ga-labeled erythrocytes*		
	t = 0 h	t = 1 h	t = 2 h	t = 0 h	t = 1 h	t = 2 h
number of viable cells	683	379	689	483	191	555
number of stained cells	0	5	0	28	3	3
% viable cells:	100	99	100	95	99	99

Undiluted, unstained samples (20 µL) were smeared onto poly-L-lysine coated slides, air dried for 5 mins and fixed in methanol for 10 minutes. After air drying for 5 minutes, slides were incubated in distilled water for 5 minutes, and stained with Giesma (Sigma), and visualized under a microscope at 400x. This procedure was repeated with both control and labeled aliquots of cells 1 h and 2 h post labeling.

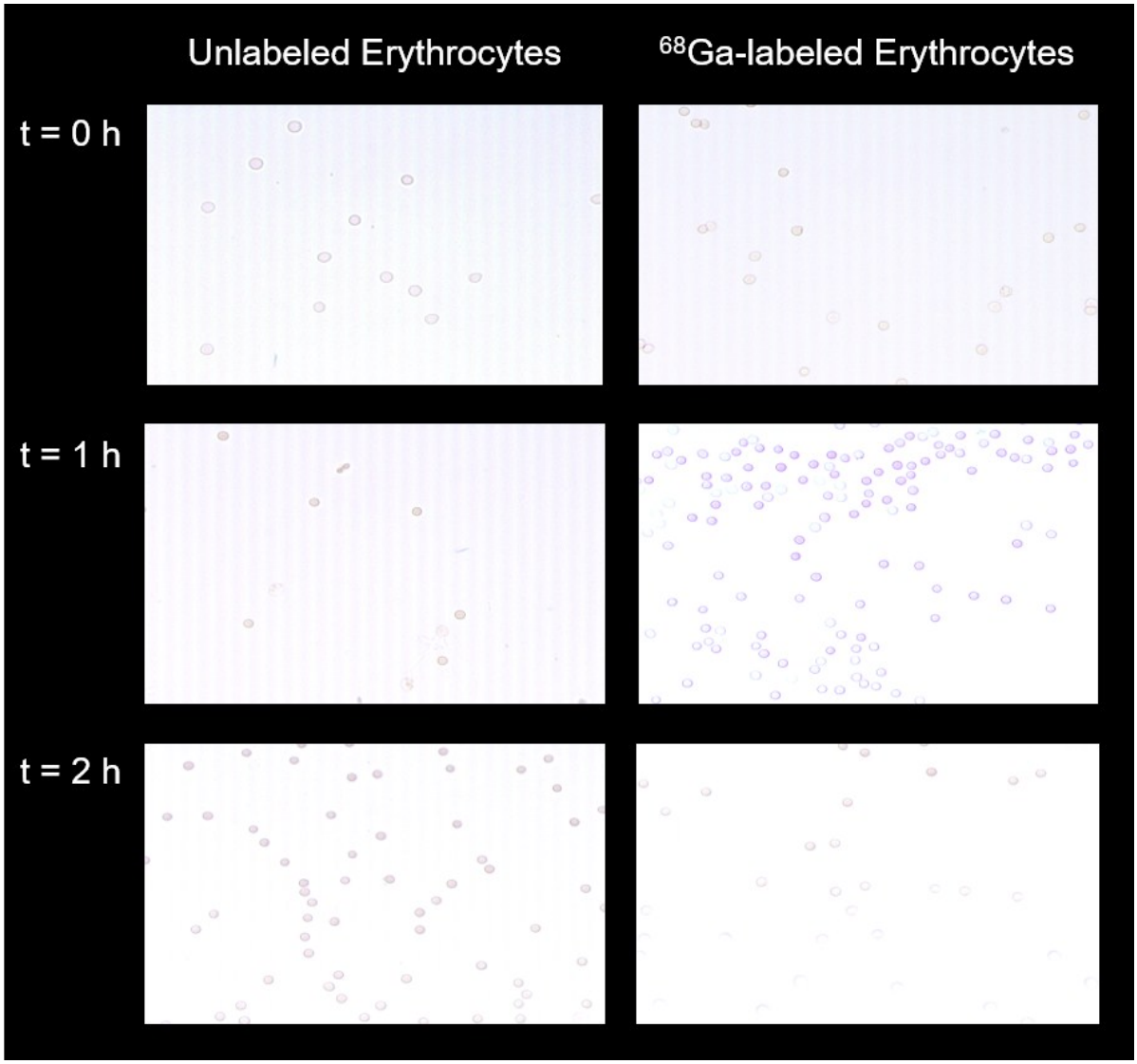

Figure S5: Visible light micrographs (400x) of unlabelled (left) and ^{68}Ga-labeled (right) erythrocytes, stained with Giesma, at three time points post-labeling. Erythrocytes have maintained their biconcave, discotic shape up to 2hours post-labeling.

[^{68}Ga]Oxine Washout Study

Erythrocytes were prepared, labeled, and washed as described above. Aliquots (n = 2) of the erythrocyte suspension (100 µL) was removed immediately post-labeling, counted, and centrifuged (1500 rpm,† 2 min) to sediment erythrocytes. The supernatant was carefully aspirated from the erythrocyte pellet, and placed in a clean Eppendorf tube. Radioactivity in

† We slowed down the speed for the washout studies to avoid any possible lysis (we did see some cell lysis of rat cells at 3000 rpm, but not human cells). Lysis was undesirable as it would result in release of ^{68}Ga into the supernatant by a different mechanism, and make it impossible to determine if there was any washout of the radiolabel occurring.

both the supernatant and pellet were counted, and this used to calculate the percentage of activity that was washed from the erythrocytes. This procedure was repeated for a second aliquot of erythrocytes (n = 2) at 1-hour post labeling to assess washout of the radioactivity from the cells upon standing at room temperature. The decay corrected data (n = 2) is summarized below, in **Table S3**.

Table S3: Washout study showing the percentage of total activity associated with the erythrocyte pellet and percentage of total activity which was released into the supernatant, immediately post-synthesis, and 1 hour post-synthesis.

	t = 0 h	*t = 1 h*
% Activity in erythrocyte pellet	96.4	96.2
% Activity in supernatant	3.6	3.8

References

(1) Welch, M. J., Thakur, M. L., Coleman, R. E., Patel, M., Siegel, B. a, and Ter-Pogossian, M. (1977) Gallium-68 labeled red cells and platelets: new agents for positron tomography. *J. Nucl. Med. 18*, 558–62.

(2) Ballinger, J. R., and Boxen, I. (1992) Gallium-67-labelled red blood cells as a blood-pool marker for dual-isotope imaging. *Int. J. Radiat. Appl. Instrumentation. Part B. Nucl. Med. Biol. 19*, 79–81.