Supporting Information

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SI Text

SI Materials and Methods

Synthesis of the Biotinylated CrA Modules. *Instrumentation and reagents.* All solvents were purchased from Carl Roth at peptide synthesis pure grade. Fmoc-Lys(5/6 FAM)-OH was purchased from Anaspec, and Fmoc-CH_2-CH_2-$(PEG)_3$-COOH (Fmoc-AEAAA) was purchased from ChemPep. N,*N*-Diisopropylethylamine (DIPEA) and H-Rink amide ChemMatrix resin was purchased from Sigma Aldrich. Fmoc-Gly-OH and [Ethyl cyano(hydroxyimino)acetato-O_2]tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyOxim), were purchased from Merck Novabiochem. Microwave (mw)-assisted synthesis was carried out using a Biotage SP wave Initiator+ microwave integrated system for organic synthesis and peptide synthesis (Biotage). Purification has been carried out with by RP-HPLC using a CBM10A HPLC equipped with a SPD-M10 PDA detector (Shimadzu) and a VariTide RPC column, 200 Å, 6 μm, 10 × 250 mm (Agilent). The masses were confirmed using a MALDI-TOF AB SCIEX 5800 mass spectrometer (Applied Biosystems).

Mw-assisted synthesis of cryptophane-A–biotin module. The cryptophane-A (CrA)–biotin module, more completely called CrA-$(PEG)_3$-biotin, was synthesized using a mw-assisted procedure. Briefly, 224 mg of CrA were dissolved in dimethyformammide (DMF) at the concentration of 0.15 M and activated for 2 min at room temperature with 1 eq of PyOxim (121 mg) and 2.5 eq of DIPEA (148 μL). The mixture was then transfer in a 5-mL mw vial containing 100 mg of Biotin-PEG_3-amine (0.5 eq) already dissolved in DMF. The acylation was carried out using microwave irradiation for 10 min at 50 °C (40). The reaction was then quenched, diluted 1:10 in HPLC mobile phase (30% CH_3CN in water, 0.1% of TFA) and purified. The calculated mass was m/z = 1338 for $C_{73}H_{86}N_{18}O_{18}S$. For CrA-$(PEG)_3$-biotin, MALDI-TOF analysis showed a m/z = 1339 (corresponding to m+1) and 1361 (corresponding to m+23, mass + sodium) (Fig. S8). The final yield was 48%.

Mw-assisted synthesis of 'branched' module, CrA–fluorescein–biotin. The branched construct consists of three parts connected together: (*ii*) biotin-$(PEG)_3$-amine, (*ii*) CrA diacid, and (*iii*) PEG-FAM-amide. This latter was synthesized as previously described (22) with the adaption of the synthesis for mw-assisted Fmoc-solid phase peptide synthesis (SPPS). Briefly, Fmoc-Gly-OH (5 eq), previously activated with PyOxim (5 eq)/DIPEA (10 eq) in DMF, was attached on H-Rink amide ChemMatrix resin (0.45 g/mmol loading) using microwave irradiation for 10 min at 50 °C. The degree of acylation was calculated with UV-Fmoc determination (41) and was around 92%. The Fmoc deprotection was achieved using 25% piperidine in DMF for 15 min, twice, at room temperature. The resin was then coupled with Fmoc-Lys(5/6 FAM)-OH using PyOxim(5 eq)/DIPEA (10 eq), deprotected from Fmoc and finally coupled with Fmoc-AEAAA, using the same protocol used for Fmoc-Gly-OH. Then the last Fmoc group was removed before cleavage from the resin. The peptide was cleaved from the resin with TFA/TIS/H_2O (95:2.5:2.5) for 2 h and then substrates were precipitated and washed six times with cold diethyl ether. The cleaved peptide was purified with RP-HPLC/UV, confirmed via MALDI/TOF and lyophilized. The yield was 53%. The synthesis of the CrA–fluorescein–biotin construct was performed with the mw using a "one pot" protocol (36) modified for the acylation. Briefly, 1 eq of CrA diacid has been activated for 5 min at room temperature with 2 eq of PyOxim and 5 eq of DIPEA. Then, 1 eq of biotin-$(PEG)_3$-amine and 1 eq of PEG-FAM-amide were added and the mixture irradiated with the mw for 10 min at 50 °C. After that, the reaction was quenched, diluted 1:10 in HPLC mobile phase (30% CH_3CN in water, 0.1% of TFA) and purified. The calculated mass was m/z = 2141 for $C_{114}H_{135}N_9O_{30}S$. For CrA–fluorescein–biotin, MALDI-TOF analysis showed a m/z = 2,164 (corresponding to m+23, mass + sodium) (Fig. S8). The fractions corresponding to CrA–fluorescein–biotin were lyophilized, and the final yield was 10%.

Flow Cytometry Analysis of Cell Viability After Xenon Imaging Protocols. Samples of both cell lines were prepared at the same cell density as for MRI studies [5 million cells per milliliter in DPBS with 0.2% Pluronic L-81 (Sigma Aldrich)]. The samples were then transferred to the NMR double phantom and MRI experiments performed as described in *Hyper-CEST spectroscopy and Imaging*. Samples of 1×10^6 cells were taken at the beginning of the experiment (control) and after a xenon imaging sequence. The cells were stained with 7-AAD (7-Aminoactinomycin D) (Life Technologies) viability dye before flow cytometry analysis. The median fluorescence intensity of 7-AAD was measured on the FL3 channel and compared for each cell line under identical acquisition parameters. Flow cytometry for 10,000 events was performed on a BD FACSCalibur flow cytometer using BD CellQuest Pro Acquisition software. Data were then analyzed with FlowJo analysis software. Scatter plots of the cell populations as well as histogram plots of 7-AAD staining (FL3) are shown in Fig. S4.

CEST Spectroscopy. ***Image CEST spectrum.*** Xenon images were taken for a range of different saturation frequencies. Regions of interest (ROIs) for the inner and outer compartment were calculated from proton images with higher resolution but otherwise matching geometry (see Fig. S7). Xenon images were then interpolated from 32 × 32–128 × 128 to match the resolution of the proton images, and the pixels in each of the two ROIs were averaged for each saturation frequency. The zero level was determined by averaging the pixels in a region that just contained noise. The xenon signal in each of the ROIs was then normalized to the signal with a saturation frequency of −140 ppm and plotted as a function of saturation frequency.

Data fitting. Data were fit to the function $f(x)$, a negative exponential of the sum of two Lorentzians

$$f(x) = e^{-(lor(A_1,\omega_1,x_1,x)+lor(A_2,\omega_2,x_2,x))}$$

where $lor(A,\omega,x_0,x)$ is a Lorentzian with maximum A, full width at half maximum ω, and location x_0, and x is the saturation frequency offset in parts per million.

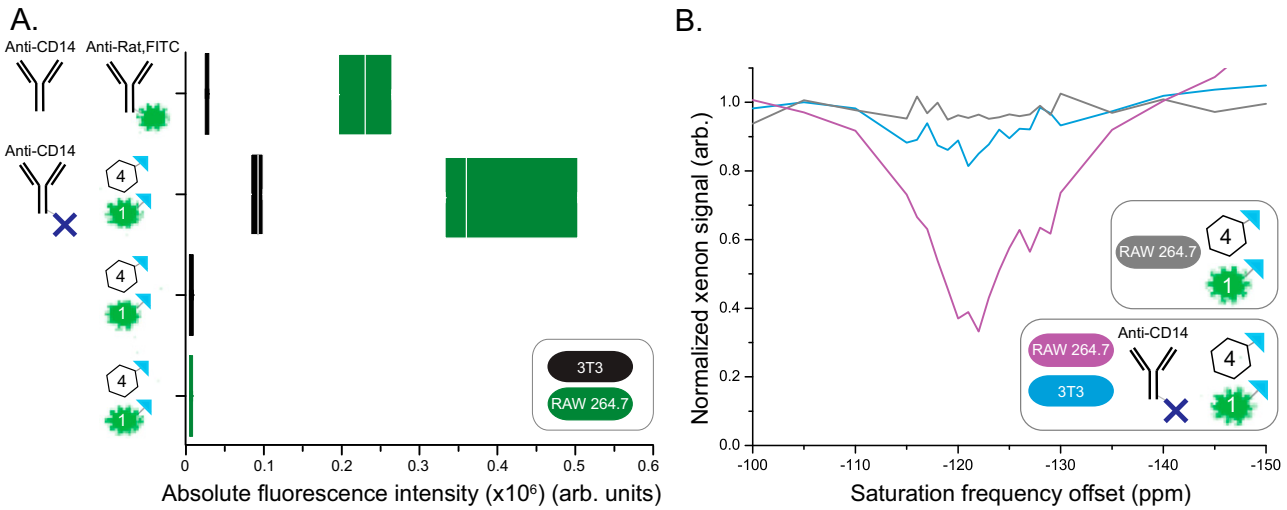
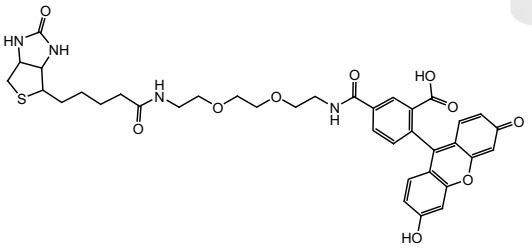


Fig. S1. Evaluation of specificity. (*A*) Comparison of specificity of anti-CD14 antibody before and after avidin conjugation by flow cytometry. Specific binding of the unconjugated anti-CD14 antibody to RAW 264.7 macrophage cells confirmed via the fluorescent anti-rat secondary antibody control experiment. Comparable specificity is seen with the avidin-conjugated anti-CD14 construct as determined by the fluorescein–biotin readout. Incubation of the cells with the readout modules alone shows no significant background binding of the fluorescein–biotin construct. Box plot shows the absolute fluorescence intensity of each cell population. Median of triplicate experiments indicated by white line; range denotes first and third quartile. (*B*) Biosensor specificity confirmation by xenon NMR. RAW 264.7 cells and control 3T3 cells were incubated sequentially with the targeting module and then readout modules. Xenon CEST spectroscopy (using a saturation pulse of 32.4 μT for 12 s) of cells shows a specific response at the resonance frequency for CrA in the sample containing the target RAW 264.7 cell line. No significant response is seen in the 3T3 control cell sample. Incubation of the RAW 264.7 cells with the readout modules alone shows no significant background binding of the CrA–biotin construct.

Commercial Products:

A. Fluorescein - Biotin (22030, Thermo Scientific)

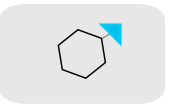


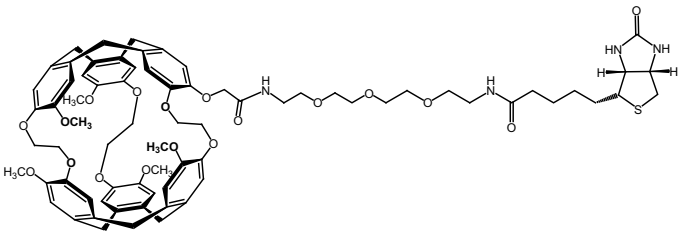
Custom Synthesized Molecules:

B. Cryptophane A - Biotin

$C_{73}H_{86}N_4O_{18}S$

Mol. Wt.: 1339,54

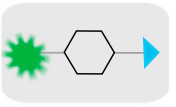




C. Cryptophane A (diacid)- Fluorescein - Biotin

$C_{114}H_{135}N_9O_{30}S$

Mol. Wt.: 1339,54



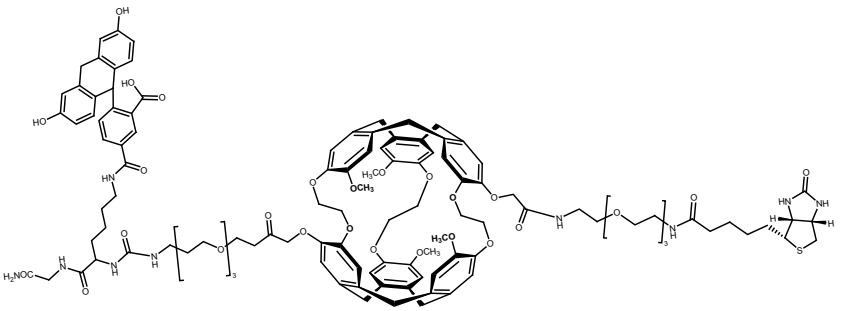


Fig. S2. Chemical details of the readout modules. (*A*) Commercially available fluorescein–biotin. (*B*) Custom synthesized CrA–biotin. (*C*) Custom synthesized CrA (diacid)–fluorescein–biotin with a defined 1:1:1 ratio of CrA:fluorescein:biotin. This was termed the "branched" construct as it uses the cryptophane diacid building block from which two separate PEG linkers can be conjugated.

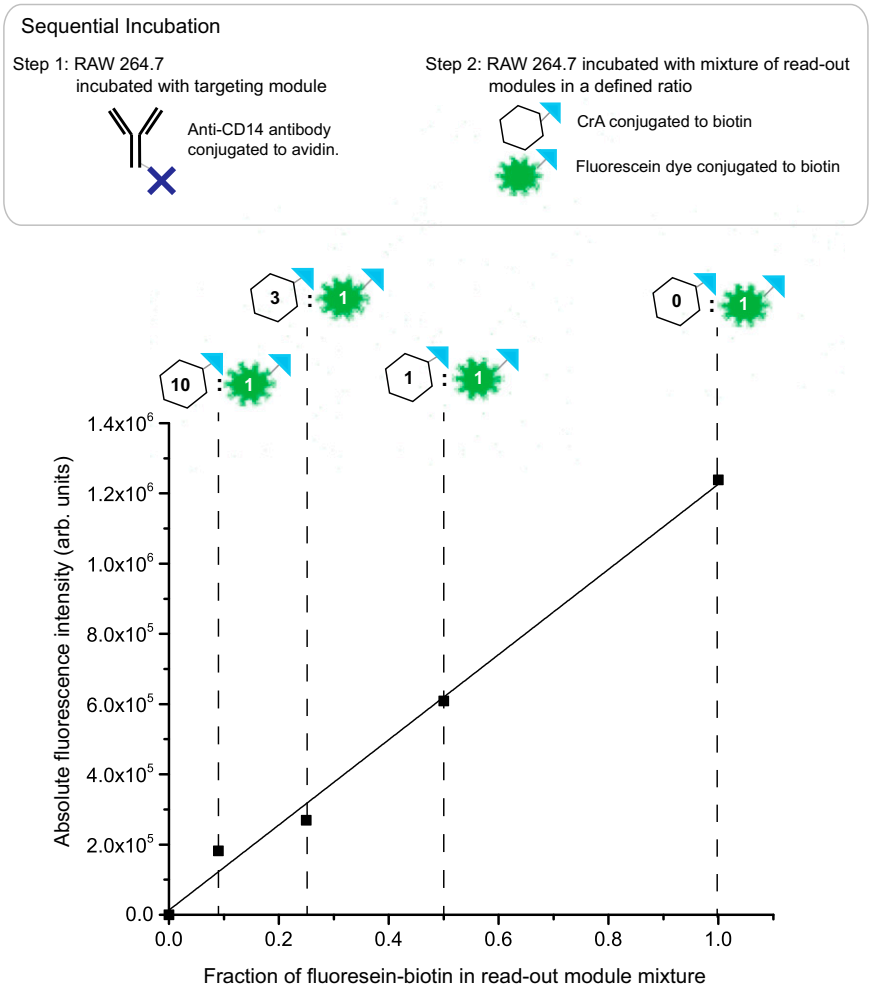


Fig. S3. Flow cytometry analysis of sequential incubation method. RAW 264.7 cells were incubated with the targeting module, washed, then incubated with the readout modules in a defined ratio of CrA–biotin:fluorescein–biotin. Analysis of the absolute fluorescence intensity of the cell population by flow cytometry shows that the ratio of fluorescein in the incubation mixture is retained in the labeled cells.

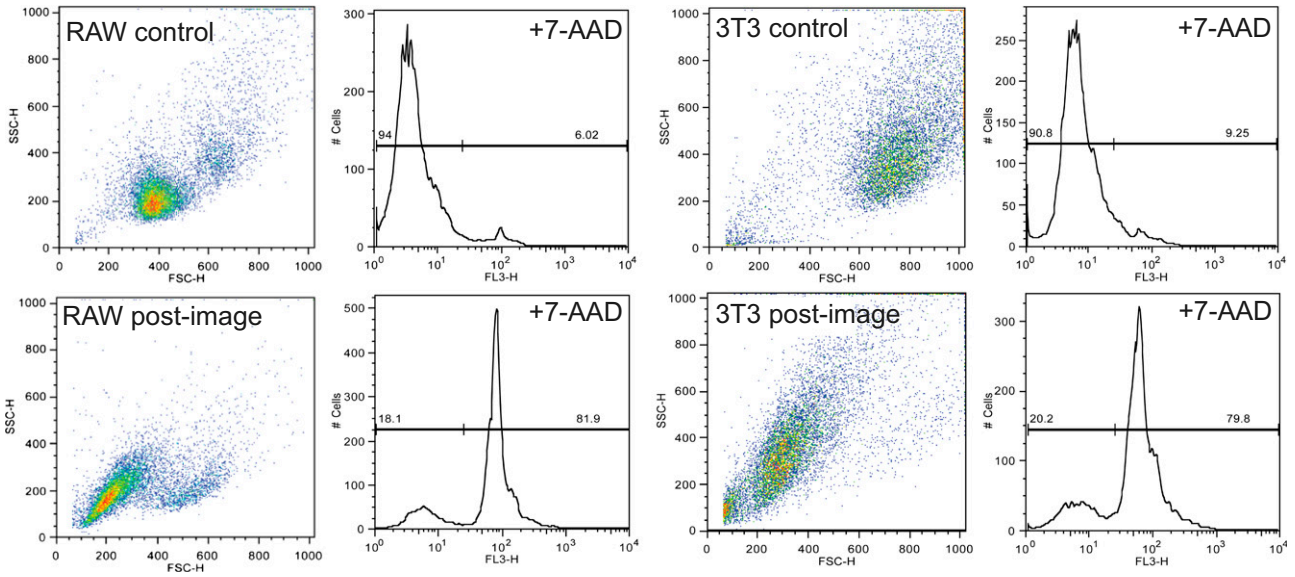
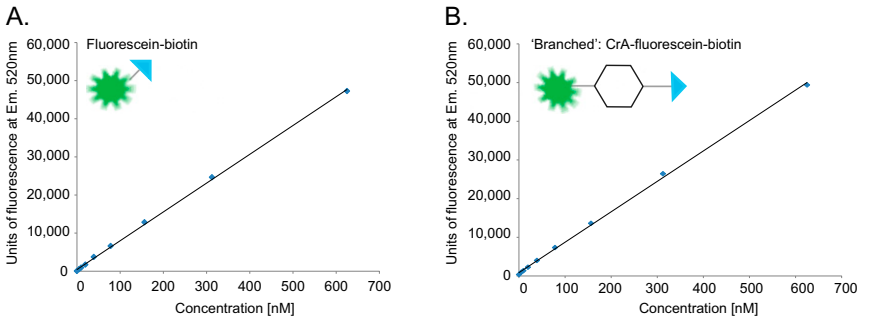


Fig. S4. Cell viability analysis after xenon MRI measurements. Left column for each cell line: Scatter plots showing the cell populations before and after xenon imaging experiments. Right column for each cell line: FL3 histograms show uptake of 7-AAD (7-Aminoactinomycin D) dye into nonviable cells before and after xenon imaging experiments. Marker indicates viable (left of marker) and nonviable (right of marker) cell populations.



C.

RAW 264.7 cells	Fluorescence	total fluorescein concentration/well [nM]	Well volume (l)	# cells/well	fluorescein per cell (nmol)	Effective NMR (Fluorescein) concentration [nM]		Effective NMR (Cryptophane A) concentration [nM]
Sequential	1558	15	0.15×10^{-6}	2×10^{6}	9.9×10^{-10}	5	x4	20
Branched	6300	69	0.15×10^{-6}	2×10^{6}	4.3×10^{-9}	22	x1	22
Complete	8955	112	0.15×10^{-6}	2×10^{6}	7.7×10^{-9}	38	x4	153

Fig. S5. Quantification of CrA bound to target cells. (*A* and *B*) Standard curves of the fluorescence of the fluorescein–biotin and the branched CrA–fluorescein–biotin readout modules. (*C*) Example calculations from the cell samples used for the xenon MRI images in Fig. 2. Just before MRI measurements, 2 million cells were removed from each sample and lysed. The fluorescence of the cell lysate was then quantified from the appropriate standard curve. Given the number of cells and that the total volume in the MRI sample is known, an estimate for the "effective NMR cryptophane concentration" could be calculated. In samples where the CrA:fluorescein ratio was 4:1, the calculation was adjusted accordingly from the fluorescein concentration determination. This was not necessary for the sample using the branched construct where a 1:1 ratio of CrA:fluorescein is present.

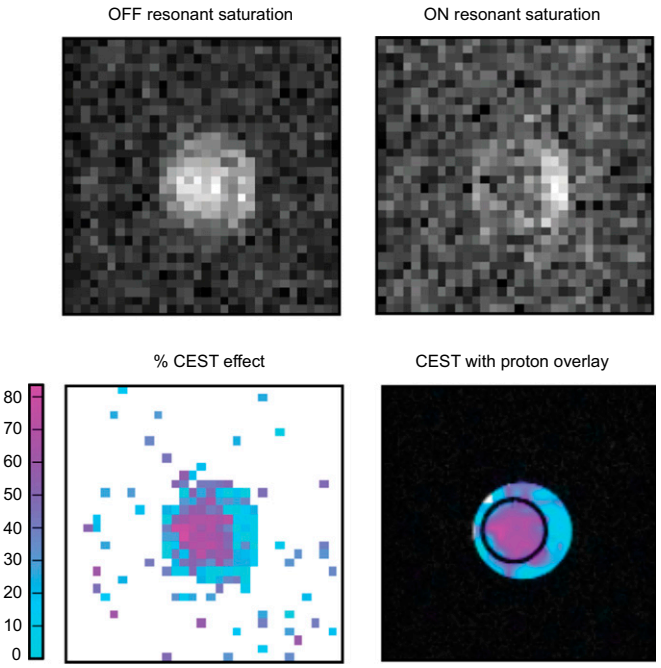


Fig. S6. Representative off- and on-resonant spectra from cells incubated with the complete biosensor construct, as shown in Fig. 2. The CEST effect (in percent) was calculated pixel wise from the off-resonant saturation image (offRes, *Upper Left*) and on-resonant saturation image (onRes, *Upper Right*) using the formula CEST effect = 100 × [(offRes−onRes)/offRes] and thresholded so only pixels with a xenon signal > 30% of the maximum pixel intensity in the off-resonant image were plotted. A color map was then applied to the CEST images (*Lower Left*). The CEST overlay (*Lower Right*) was created by (*i*) Interpolating the color CEST image (from 32 × 32–128 × 128) to match the resolution of the proton images, (*ii*) plotting the proton image in gray scale, (*iii*) converting both images from RGB to HSV color space, (*iv*) taking the hue and saturation channel from the CEST image while the value channel was taken from the proton image, and (*v*) converting the image back to RGB color space for display.

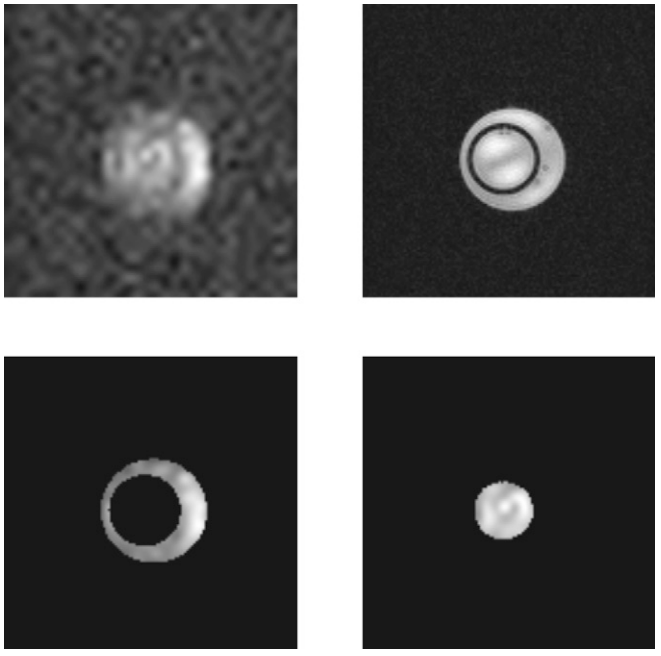
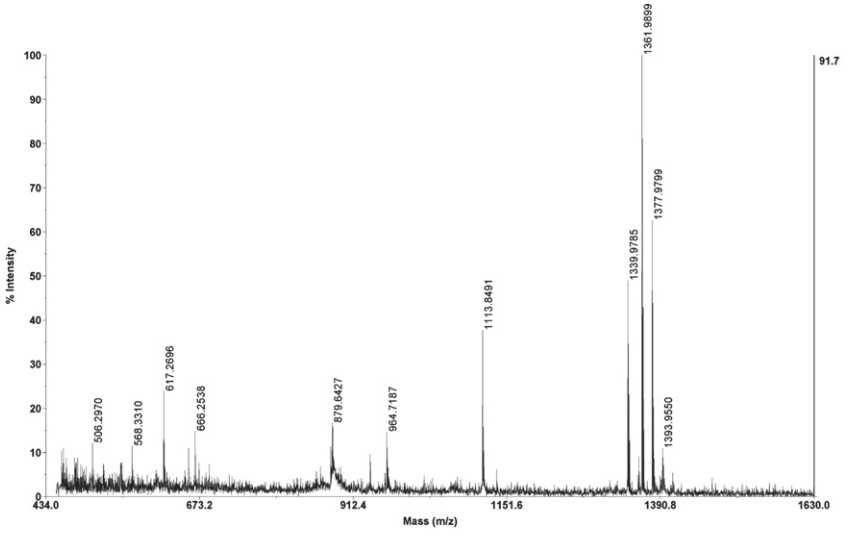


Fig. S7. Illustration of ROIs used for localized CEST spectroscopy, as shown in Fig. 2. (*Upper Left*) Average xenon signal across all saturation frequencies. (*Upper Right*) High-resolution proton image used to find ROIs. (*Lower Left*) Average xenon signal across all saturation frequencies in outer ROI. (*Lower Right*) Average xenon signal across all saturation frequencies in inner ROI.

A. Cryptophane A (CrA) - biotin



B. Cryptophane A (CrA) diacid - Fluorescein - biotin

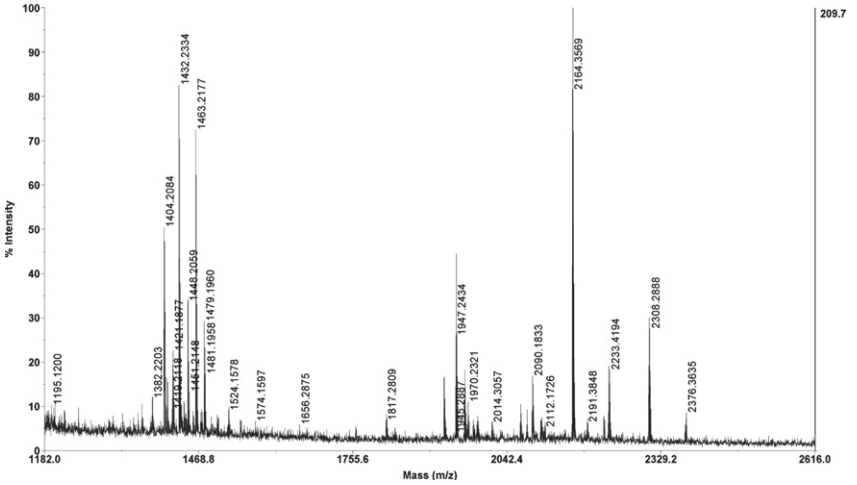


Fig. S8. MALDI-TOF mass spectrometry analysis of the two custom synthesized cryptophane readout modules: (*A*) CrA–biotin and (*B*) CrA–diacid–fluorescein–biotin.