SUPPLEMENTARY INFORMATION

Design of Irreversible Optical Nanothermometers for Thermal Ablations

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GENERAL INFORMATION

Materials: Solvents (methanol, DMF, DMSO, DCM, acetonitrile) and reagents for synthesis (CTAB, $HAuCl_4$, $AgNO_3$, $NaBH_4$, HOBT, HBTU) were purchased from Sigma-Aldrich Inc. and ThermoFisher Sci. and used without further purification. Protected amino acids Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH were purchased from Chem-Impex International Inc., $mPEG_{2kD}$-thiol was received from JenKem Technology. Millipore water (18.2 MΩ) was used throughout the study.

Analysis: Dye-peptide conjugates **1-3** were analyzed using LC/MS-ESI analysis in the positive mode conducted on a Shimadzu LCMS 2010A equipped with a UV/Vis detector at different wavelengths using a reversed-phase C-18 Vydac column (218TP, 4.6X50 mm) at a flow rate of 0.7 mL/min with a gradient 10-95% acetonitrile (ACN) in water (both solvent contained 0.1 % TFA).

Optical Measurements: UV/Vis spectra of samples were recorded on a DU 640 (Beckman Coulter) UV-visible spectrophotometer. Steady state fluorescence spectra were recorded on a Fluorolog-3 (Horiba Jobin Yvon) equipped with temperature controlled cuvette holder in equilibrium with water bath. Fluorescence anisotropy was conducted in L-format with automated Glan-Thompson polarizing prisms. The anisotropy values for each dye and dye-conjugate were determined at relatively low concentrations with absorption below 0.2 a.u. to avoid aggregation of the nanoconstruct. For single point measurements the excitation was set at 410 nm and the emission at 440 nm. Slits were set to 5 nm and integrating time to 0.5 sec. Each single point measurement was conducted in quadruplicates at T=20^{o}C in 1x1 cm^2 quartz cuvette using a temperature controlled cuvette holder in conjunction with a circulating water bath. The alignment of the polarizers was checked daily by recording the anisotropy of Ludox-40 (Sigma-Aldrich) suspension in water (ex/em. 440/440 nm).

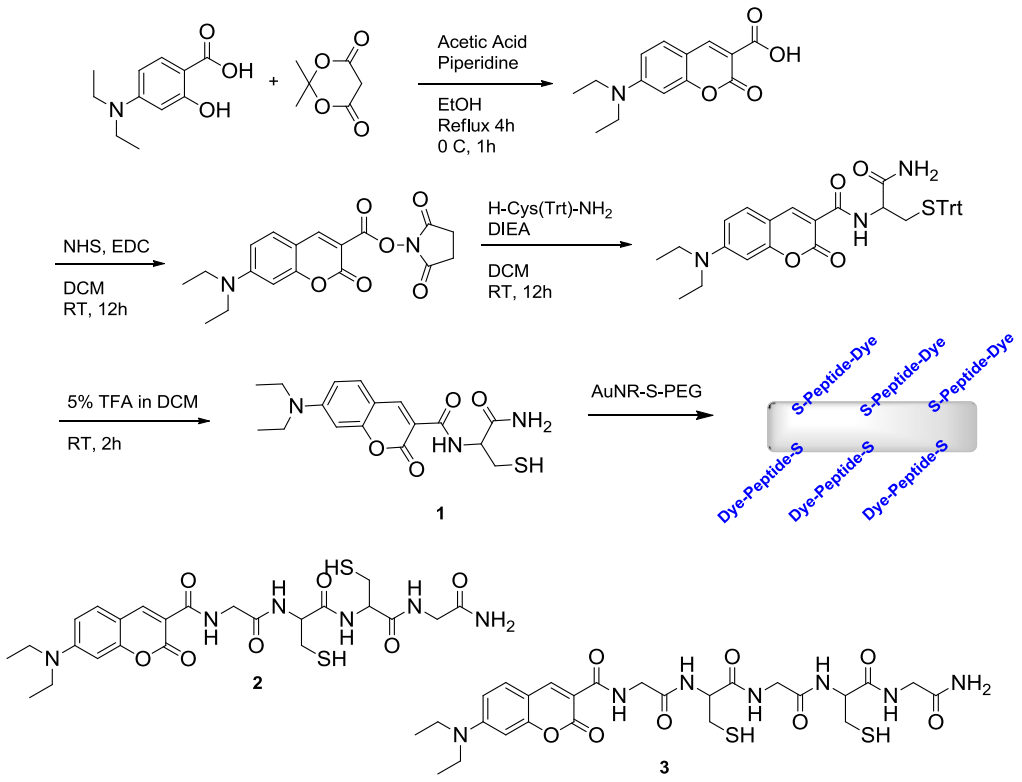
Sample preparations for optical measurements: Nanothermometers were dissolved in 1x PBS buffer (Fisher Scientific) and placed in 1x1 cm^2 quartz cuvettes prior to optical measurements. Dye-peptide conjugates were prepared as stock solution 1 mg/mL in DMSO and added to 1x PBS, at the final concentration 1 μg/mL.

Transmission Electron Microscopy: TEM images were acquired on a JEOL 2100F FE-(S)TEM system at room temperature using a Formvar/Carbon on 200 mesh TEM grid (Ted

Pella, Inc) under the following conditions: $1.5x10^{-5}$ Pa, emission current 150 uA, electron acceleration voltage 200 kV.

SYNTHESIS

A representative synthesis of nanothermometers with dye-peptide conjugates is shown in Scheme S1.



Scheme S1. Representative synthesis of nanothermometers with dye-peptide conjugate **1** and synthesized dye-peptide conjugates **1-3**

Synthesis of CTAB stabilized gold nanorods: CTAB stabilized gold nanorods were synthesized by seed-mediated growth as described by Chen et al.[1] CTAB(aq) solution (5 mL, 0.2M) at 30-35°C was first mixed with $HAuCl_4$(aq) solution (5 mL, 0.5 mM) with stirring. Then of ice-cold $NaBH_4$(aq) solution (0.60 mL, 0.01 M) was added to the mixture and vigorously stirred for 2 min at 30°C, which resulted in the formation of a brownish yellow solution known as "seed solution". The growth solution was prepared by adding $AgNO_3$(aq) (0.15–0.2 mL, 4 mM) and then $HAuCl_4$(aq) (5 mL, 1 mM) solutions to a CTAB(aq) solution (5 mL, 0.2 M) at 30-35 °C, under gentle mixing, followed by ascorbic acid solution (70 µL, 0.0788 M). To grow nanorods, 12 µL of the seed solution was added to the growth solution at 30°C under gentle stirring for 30 seconds. The transparency of the solution changed to burgundy red within 10–20 min. The solution then aged for another 12-18 hours at 27–30°C before being centrifuged at

13,000 rpm for 10 min. The collected CTAB gold nanorods were re-dispersed in water, and stored at room temperature. Absorption spectra are shown in Fig. S1a.

PEGylation of gold nanorods: The stabilization agent, CTAB, on the surface of the gold nanorods was replaced by mPEG-thiol through ligand exchange according to a reported procedure[1]. Briefly, the CTAB-stabilized gold nanorod dispersion was added to an equal volume of mPEG-thiol (0.2 mM) aqueous solution under vigorous stirring. The mixture was sonicated for 5 minutes and left to react for 2 hours. Excess mPEG-thiol molecules were removed by centrifugation filtration (Amicon 50,000 D cutoff, Millipore Inc.) at 3,300 rpm for 10 min. Resulting PEGylated gold nanorods residue were re-suspended in water. Centrifugation filtration was repeated twice. Absorption spectra of the Au-NS-R-PEG are shown in Fig. S1b.

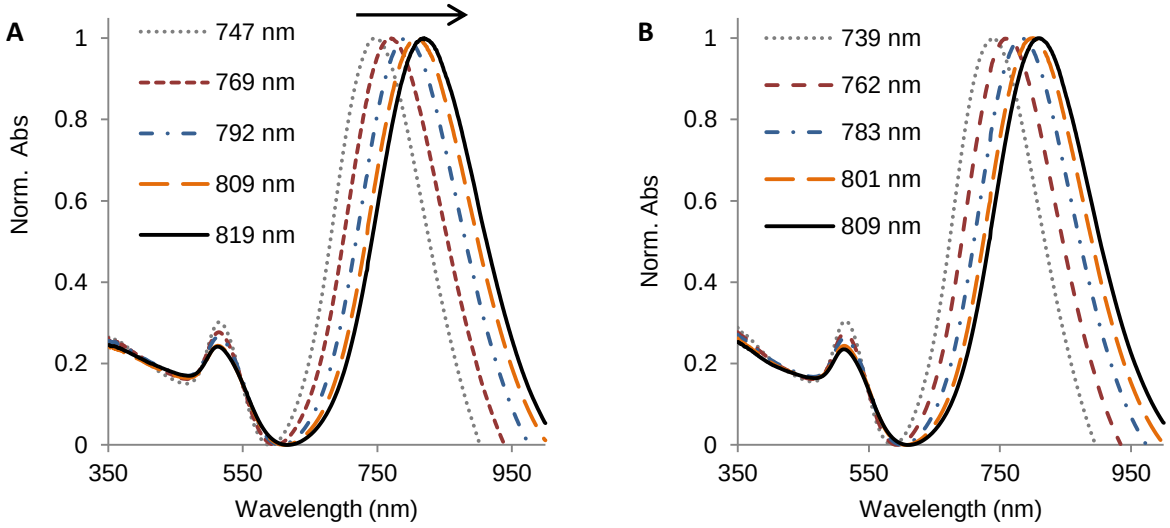
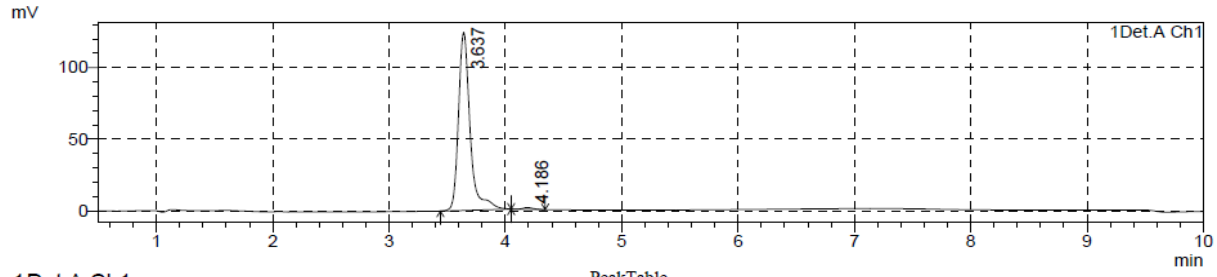


Fig. S1 A: Representative absorption spectra of synthesized gold nanorods protected with CTAB with varied amount of silver nitrate. An arrow shows the increase of silver nitrate amount.
B: Same gold nanorods after a ligand exchange with $mPEG_{2kD}$-thiol (Au-NS-R-PEG).

Dye-peptide conjugate synthesis: The dye-peptide conjugates **1-3** were prepared using a standard solid state peptide synthesis protocol. A representative synthesis of nanothermometers with dye-peptide conjugate **1:** Briefly, Fmoc was removed from Rink amide resin with 20% piperidine in DMF with shaking and washed with DMF and DCM. After filtration DMF, Fmoc protected amino acid, DIEA, a solution of coupling reagents HOBT and HBTU in DMF were added. After the reaction was completed, the solvent was removed, and Fmoc was deprotected. The resin was washed, filtered and the next amino acid was added and the procedure repeated. After addition of the final amino acid, coumarin-NHS was added to the chain. The product was cleaved from the resin filtered and purified by reverse phase flash chromatography.

Representative synthesis of AuNR-Gly-Cys-Cys-Gly-NH_2 (2). Fmoc was removed from 0.58 mmol Rink amide resin (0.10 g, 0.058 mmol loading) with 20% piperidine in DMF, 3x10 min each with shaking. Deprotected resin was washed with DMF (3x10 min) and DCM (3x10 min) with shaking. After filtration following reagents were added in this order: DMF (0.3 mL), Fmoc protected amino acid 3 eq. (0.17 mmol) in DMF (0.30 mL), DIEA (0.05 mL), and a solution of HOBT (0.17 mmol) and HBTU (0.17 mmol) in DMF (0.30 mL). The reaction was shaken vigorously for 2 hours at room temperature, the solvent was removed, and Fmoc was deprotected with 20% piperidine in DMF (3x10 min) with shaking. The resin was washed with DMF (3x10 min) and DCM (3x10 min) with shaking. After filtration, the next amino acid was added as previously described.

After addition of the final amino acid, coumarin-NHS (1.12 mmol)[2] was combined with DIC (0.12 mmol) in DMF (1 mL) for 15 min. HOBt (0.044 mmol) was added and the solution was left for 5 min with stirring. The resin was wet with DMF (0.5 mL) and the dye solution was added and reacted for 4 h with shaking. The resin was collected by filtration and washed with DMF and DCM (3x 10 min each). The resin was dried by filtration and placed into a glass vial. To the resin was added a solution containing 94% TFA, 2.5% 1,2-ethanedithol (EDT), 2.5% H_2O, and 4 mL of 1% triisopropylsilane (TIS). The reaction was shaken for 2 h at room temperature, then filtered through glass wool to remove the beads, solvents and volatiles were removed under vacuum. The product was purified by reverse phase (C18) flash column eluted with 50 mL of ACN:water solvent mixture starting with 10% ACN and increasing to 60% ACN in 10% increments. Fractions with the product (by LCMS) were combined and the solvent was evaporated under vacuum. The final product was lyophilized. For compound **2,** 30 mg of orange product was collected (>98 area % pure by LC (UV detectors 300 nm and 370 nm), Fig S2). MH^+=581, dimer: $2MH^+$=1183.



1Det.A Ch1

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.637	868398	124493	99.114	98.979
2	4.186	7760	1284	0.886	1.021
Total		876158	125777	100.000	100.000

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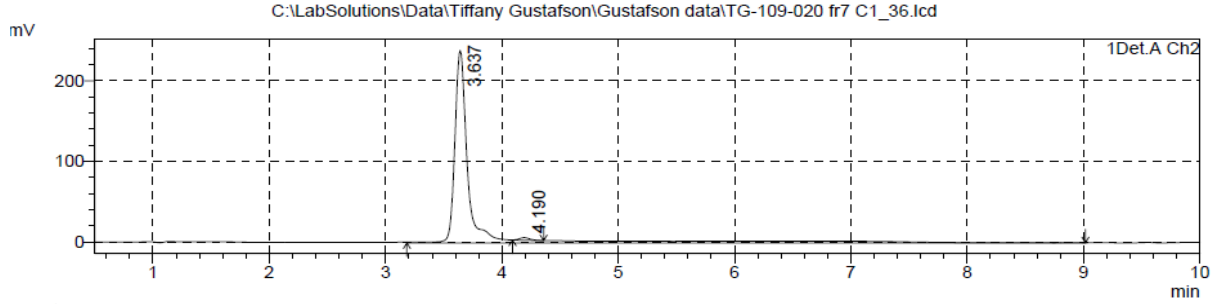

1Det.A Ch2/370nm

PeakTable

Detector A Ch2 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.637	2099922	238724	99.093	98.711
2	4.190	19221	3117	0.907	1.289
Total		2119143	241841	100.000	100.000

MS Spectrum Graph

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BG Mode:?
Mass Peaks:1789 Base Peak:580.90(32008) Polarity:Pos Segment1 - Event1

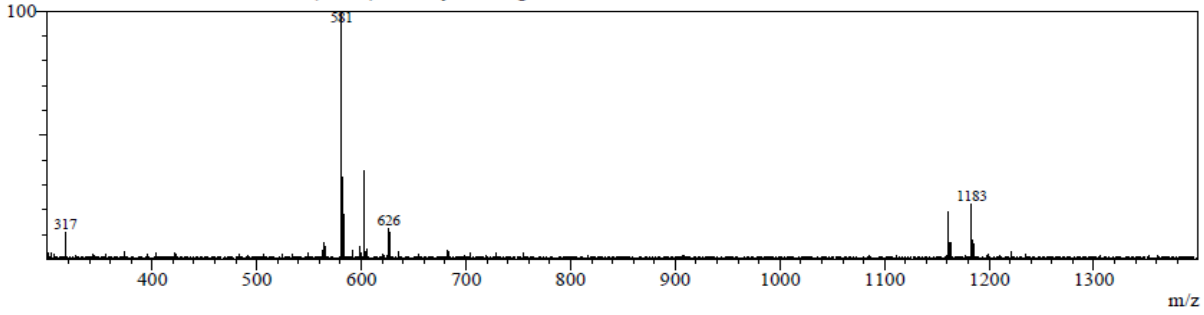

Fig. S2 HPLC and MS spectra of dye-peptide conjugate **NH_2-Gly-Cys-Cys-Gly-NH_2 (2)**

Nanothermometers assembly: $mPEG_{2kD}$-thiol on the surface of the gold nanorods was replaced by dye-peptide conjugates through ligand exchange. For that, the PEG coated gold nanorod dispersion was added to an equal volume of coumarin-peptide conjugates **1-3** dissolved in 0.5% DMSO in water (0.2 mM) under vigorous stirring. The mixture was sonicated for 10 minutes and left to react overnight with gentle stirring. mPEG-thiol and excess of the dye-conjugate were removed by centrifugation filtration (Amicon 50,000 kD cutoff, Millipore, Inc.) at 3,300 rpm for 10 min. The eluent was decanted and the dye-peptide protected gold nanorods were re-suspended

in water. Centrifugation filtration was repeated until minimal coumarin absorption was present in the eluent. The formed nanorods covered with dye-peptide conjugates (nanothermometers) were then dispersed in water and stored at room temperature.

NANOTHERMOMETER EVALUATION

Thermal measurements of nanothermometers and dye-peptide conjugates: *Slow temperature ramp*. Gold nanorods coated in coumarin-peptide conjugates **1-3** were dispersed into PBS, pH 7.0 at a concentration of 5 μL/mL. For a free dye-peptide conjugate, a stock solution 1 mg/mL in DMSO was added to PBS, pH 7.0, and final concentration was 1 μg/mL. Temperature dependent fluorescence was monitored by fluorescence spectrophotometer from 25 to 85°C and back to 25°C in 15°C increments, holding for 5 min at each temperature. Heating 0.9°C/min, cooling 0.8°C/min. Full emission spectra (excitation 395 nm) were recorded for each temperature point. Overall temperature heating-cooling cycle is shown in Fig. S3a. *Fast temperature ramp.* Gold nanorods coated in coumarin-peptide conjugates **2** were dispersed into PBS, pH 7.0 at a concentration of 10 μL/mL and placed in a 1.5 mL glass vial. Temperature based release of dye-conjugate was monitored by a CCD camera under continuous heating from 25 to 85°C from a heat gun in "hot" mode and subsequent air in a "cold" mode. Overall temperature heating-cooling cycle is shown in Fig. S3b. Images were collected every 20 seconds and analyzed with ImageJ software.

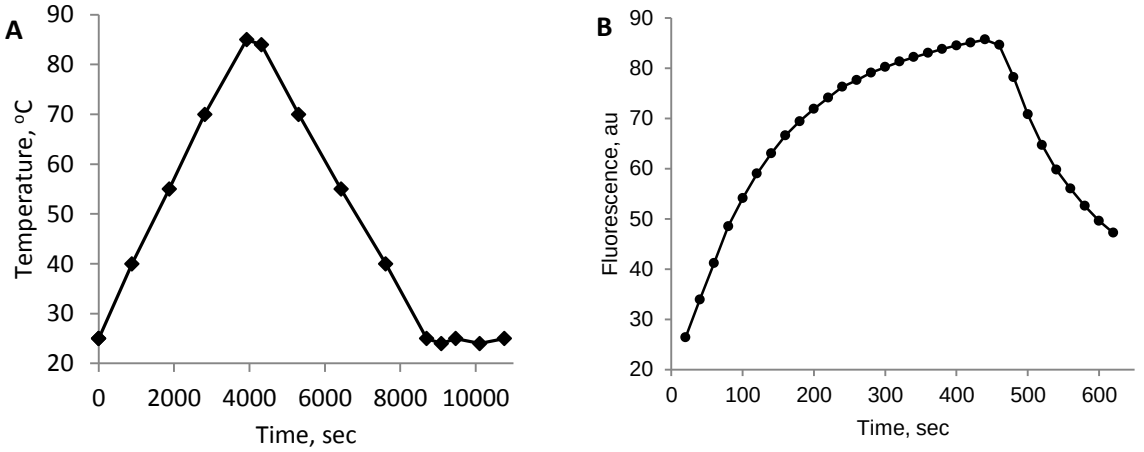

Fig. S3 A: Slow temperature ramp: heating 0.9°C/min, cooling 0.8°C/min. Temperature controlled cuvette holder was connected to a water circulating bath. **B**: Fast temperature ramp (heating and cooling cycle) using thermal imager described below.

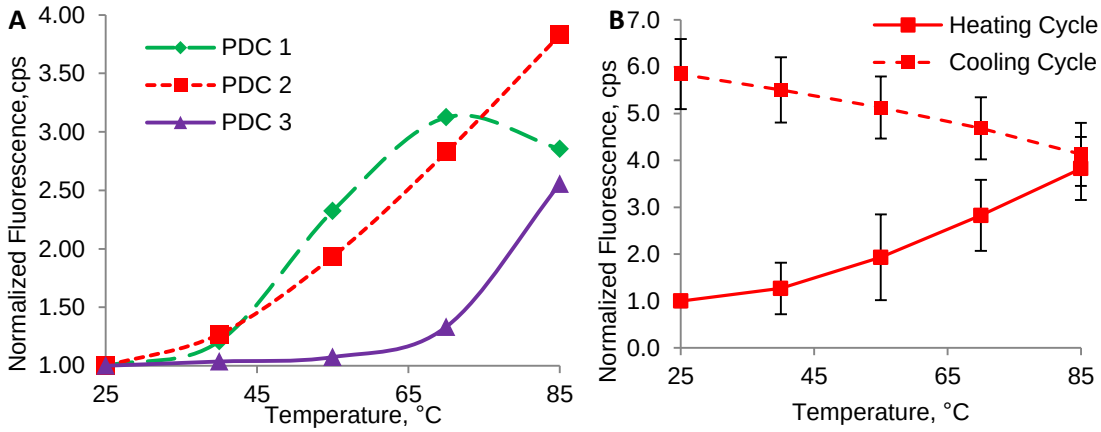

Fig. S4 A: Temperature release profiles for dye-peptide conjugates **1-3** in PBS with slow temperature ramp from 25° to 85°C. Excitation/emission 395/462 nm, points were normalized to starting fluorescence intensity. **B**: Representative temperature release profile of nanothermometer with dye-peptide conjugate **2** during a slow heating-cooling cycle from 25° to 85° to 25°C. Error bars indicate standard deviation from four samples.).

Thermal ablation imager: A home-made thermal ablation imager featured a light source made from a mounted high power LED 405 nm (410 mW nominal power, Thorlabs) equipped with a collimating lens, a bandpass filter 410 nm, 10 nm bandwidth, Ø25.4 mm, ED1-S50-MD – and 50º Square Engineered Diffuser with SM1-threaded mount, Ø1" (Thorlabs). The emission was measured with INFINITY3-1C — 1.4 Megapixel Color Cooled CCD Camera (Lumenera Corp.) with APO-Xenoplan 1.4/23mm SWIRON C-Mount Lens (Schneider Inc.) and a broadband emission filter 500 nm, 80 nm bandwidth, Ø25.4mm (Edmund Optics, Inc) placed in front of the lens. Images were made every 20 sec, and analyzed by ImageJ software (National Institutes of Health). A region of interest was drawn over the vial (as shown in Fig. S6), and the intensity over the same ROI was evaluated for every image. Simultaneous temperature measurements were conducted using a T-type hypodermic needle Teflon coated thermocouple (17 gauge) (J-Kem Scientific. Inc) connected to a high resolution (0.1^{o}C) temperature controller (Model 210, J-Kem Scientific. Inc). Fast heating of the solution in the glass vial was achieved with a variable temperature heat gun. This gun produces a stable output.

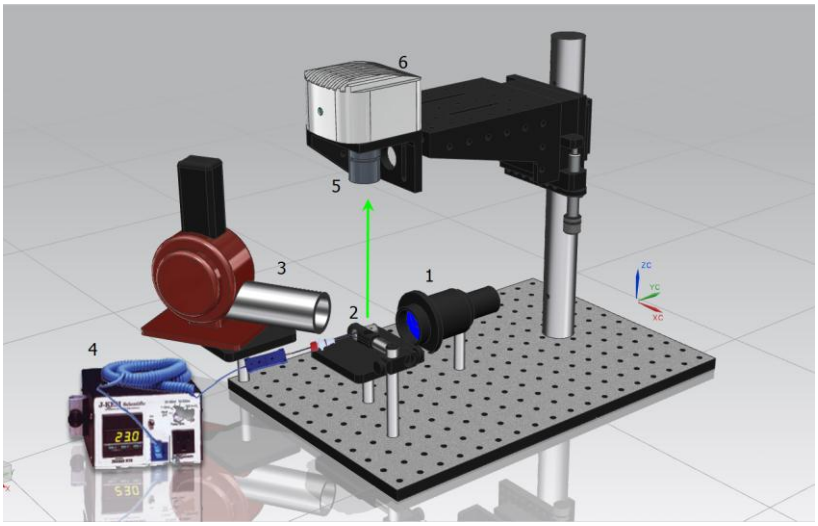

Fig. S5 Schematics of a thermal ablation imager: **1** - mounted high power LED 405 nm (410 mW nominal power) (Thorlabs) equipped with a collimating lens, a bandpass filter 410 nm, 10 nm bandwidth, Ø25.4 mm, ED1-S50-MD – and 50º Square Engineered Diffuser with SM1-threaded mount, Ø1" (Thorlabs). **2** - Sample holder for 1.5 mL glass vial. **3** – variable temperature heat gun VT-750C (Master Appliance Corp), **4** - T-type hypodermic needle Teflon coated thermocouple (17 gauge) (J-Kem Scientific. Inc) connected to a high resolution (0.1^{o}C) temperature controller (Model 210, J-Kem Scientific. Inc). **5** - INFINITY3-1C — 1.4 Megapixel Color Cooled CCD Camera (Lumenera Corp.) with APO-Xenoplan 1.4/23mm SWIRON C-Mount Lens (Schneider Inc) and a broadband emission filter 500 nm, 80 nm bandwidth, Ø25.4mm (Edmund Optics, Inc) placed in front of the lens, **6** – focusing movable platform (Thorlabs).

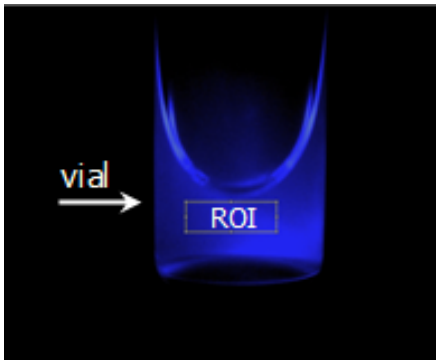

Fig. S6 Image of a vial (total volume 2 mL, solution volume 1 mL) using a thermal ablation imager. Shown: a region of interest (ROI) utilized for calculations with ImageJ software.

Quantitative thermal sensitivity of nanothermometers under rapid heating: Two parameters, critical temperature sensitivity (T_s) and the rate of fluorescence response (K_F) were evaluated using fast thermal ramp. Both parameters were calculated from the fluorescence

change at any given temperature relative to the body temperature 37°C using the following set of equations:

$$K_f(T,t) = \frac{L(T)}{dt},$$

where

$$L(T) = \frac{\Delta F(T)}{dT} \times 100\%, \quad \Delta F = \frac{F_T - F_{37}}{F_{37}}, \qquad dT = T_n - T_{n-1}$$

L(T) - is the fluorescence change as a function of temperature, F_T- is a fluorescence intensity measured at temperature *T*, F_{37} – fluorescence intensity at 37°C –body temperature, *dT* – is the difference between two adjacent temperature measurements, (temperature increment, °C). Second, the plot of *K* vs. reaction temperature (Fig. S7a) and *K* vs. heating time were constructed (Fig. S7b). The intersection of the two slopes provided critical temperature sensitivity parameter (T_s), the slope of the curve provided the rate of fluorescence response (K_F).

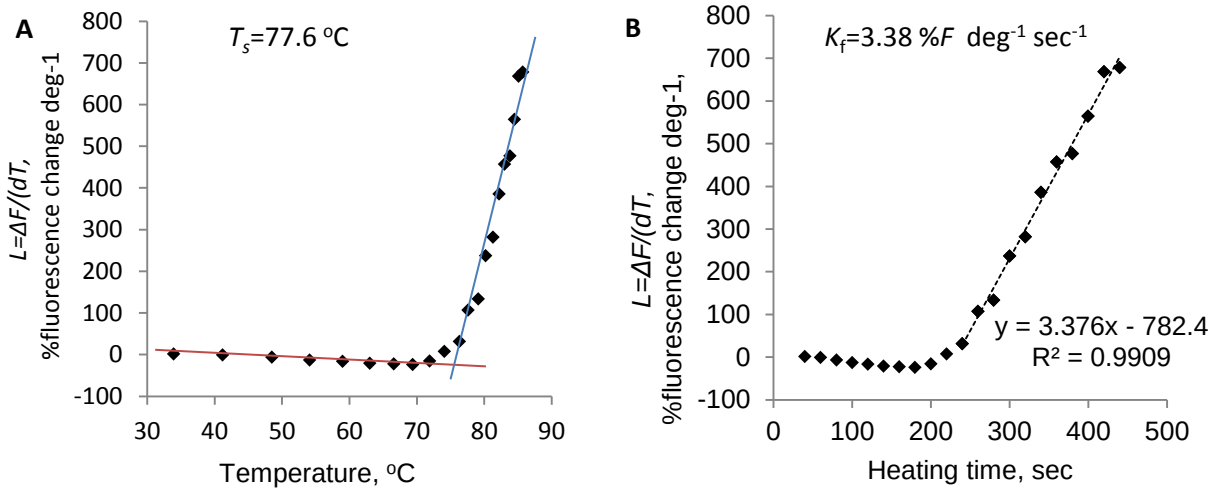

Fig. S7 Temperature response of the nanothermometers carrying peptide-dye conjugate **2 A**: Measurement of critical temperature sensitivity parameter (T_s=77.6 °C, B: rate of fluorescence response (K_F =3.38 % fluorescence increase/(deg•sec), R^2=0.99.

REFERENCES

1. Chen, Y. S.; Frey, W.; Kim, S.; Kruizinga, P.; Homan, K.; Emelianov, S., Silica-coated gold nanorods as photoacoustic signal nanoamplifiers. *Nano Lett.*, 2011, 11, 348-54.

2. Gustafson, T. P.; Metzel, G. A.; Kutateladze, A. G., Photochemically amplified detection of molecular recognition events: an ultra-sensitive fluorescence turn-off binding assay. *Org Biomolec. Chem* 2011, 9, 4752-5.