Supporting Information

Ultrasensitive Triple-Mode Nile Red-L64 Niosome Nanothermometers for Real-Time Cellular Thermogenesis Mapping

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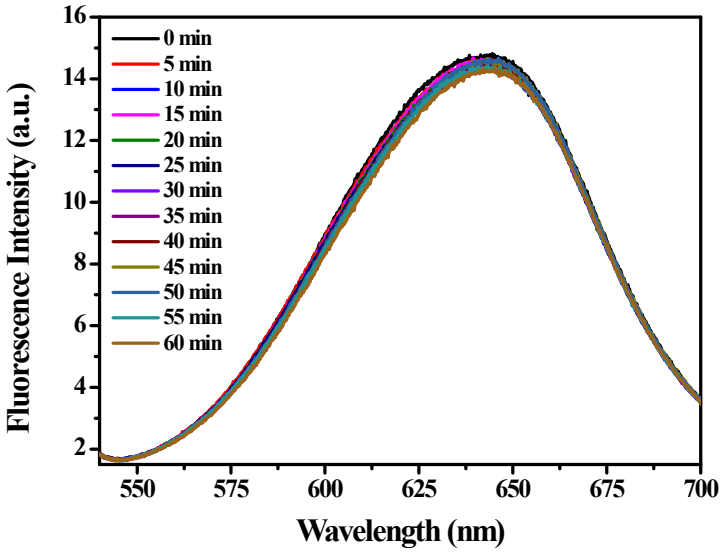


Figure S1. Fluorescence spectra of the Interaction of Nile Red and glucose in cell media at 37°C over a period of one hour.

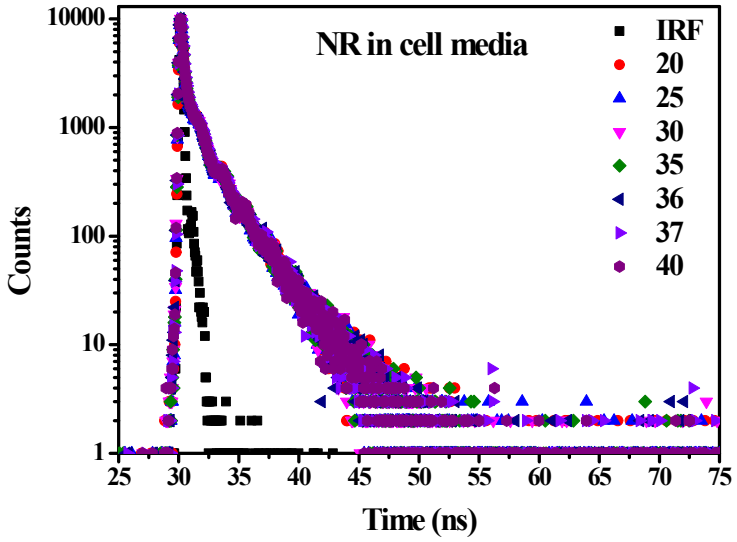


Figure S2. The lifetime of only Nile Red in cell media at different temperatures.

Table S1: The lifetime decay of only Nile Red in cell media at different temperatures.

Temperature	λ_{ex} (nm)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	α_1	α_2	χ^2	Average lifetime

								(ns)
20	**510**	**618**	**0.101**	**2.189**	**0.955**	**0.044**	**1.013**	**0.194**
25	**510**	**618**	**0.110**	**2.176**	**0.953**	**0.046**	**0.957**	**0.207**
30	**510**	**618**	**0.126**	**2.208**	**0.946**	**0.053**	**0.925**	**0.238**
35	**510**	**618**	**0.138**	**2.207**	**0.941**	**0.058**	**0.955**	**0.260**
36	**510**	**618**	**0.138**	**2.203**	**0.942**	**0.057**	**0.901**	**0.257**
37	**510**	**618**	**0.140**	**2.206**	**0.940**	**0.059**	**0.908**	**0.263**
40	**510**	**618**	**0.151**	**2.199**	**0.938**	**0.061**	**0.966**	**0.278**

Table S2: the lifetime decay values of NRL64 in cell media at different temperatures

Temperature (°C)	**λ_{ex} (nm)**	**λ_{em} (nm)**	**τ_1 (ns)**	**τ_2 (ns)**	**α_1**	**α_2**	**χ^2**	**Average lifetime (ns)**
37	**510**	**618**	**0.122**	**1.920**	**0.940**	**0.059**	**0.961**	**0.229**
38	**510**	**618**	**0.165**	**2.107**	**0.911**	**0.088**	**1.200**	**0.336**
39	**510**	**618**	**0.177**	**2.210**	**0.889**	**0.110**	**1.085**	**0.402**
40	**510**	**618**	**0.249**	**2.570**	**0.779**	**0.220**	**1.185**	**0.760**

Table S3: The lifetime decay values of NR-L64 in live cells at different temperatures.

Temperature (°C)	**λ_{ex} (nm)**	**λ_{em} (nm)**	**τ_1 (ns)**	**τ_2 (ns)**	**α_1**	**α_2**	**χ^2**	**Average lifetime (ns)**
37	**510**	**618**	**0.263**	**4.260**	**0.485**	**0.514**	**1.07**	**2.318**
37.4	**510**	**618**	**0.247**	**4.269**	**0.500**	**0.499**	**0.996**	**2.256**
37.8	**510**	**618**	**0.248**	**4.250**	**0.509**	**0.490**	**0.992**	**2.211**
38.2	**510**	**618**	**0.231**	**4.257**	**0.502**	**0.479**	**1.092**	**2.162**
38.6	**510**	**618**	**0.231**	**4.181**	**0.521**	**0.478**	**1.059**	**2.119**
39	**510**	**618**	**0.230**	**4.181**	**0.558**	**0.441**	**1.075**	**1.972**
39.4	**510**	**618**	**0.234**	**4.143**	**0.550**	**0.449**	**1.120**	**1.990**
39.8	**510**	**618**	**0.255**	**4.266**	**0.569**	**0.430**	**0.939**	**1.980**

Table S4: the lifetime decay values of NR with glucose at different time intervals

Temperature (°C)	**λ_{ex} (nm)**	**λ_{em} (nm)**	**τ_1 (ns)**	**τ_2 (ns)**	**α_1**	**α_2**	**χ^2**	**Average lifetime (ns)**
5	**510**	**618**	**0.1**	**2.119**	**0.969**	**0.030**	**0.859**	**0.16**

10	510	618	0.122	2.189	0.962	0.037	0.853	0.199
15	510	618	0.118	2.097	0.964	0.035	0.938	0.188
20	510	618	0.126	2.214	0.965	0.034	0.866	0.199
25	510	618	0.115	2.196	0.964	0.035	0.864	0.188
30	510	618	0.140	2.247	0.960	0.039	0.833	0.222
35	510	618	0.127	2.145	0.963	0.036	1.124	0.200
40	510	618	0.128	2.301	0.966	0.033	0.990	0.200
45	510	618	0.142	2.236	0.959	0.040	0.925	0.226
50	510	618	0.125	2.216	0.963	0.036	0.973	0.202
55	510	618	0.126	2.144	0.963	0.036	1.008	0.199
60	510	618	0.114	2.126	0.965	0.034	0.928	0.183

Method S1. Two-photon power-dependent intensity of NR-L64 loaded in cells.

To confirm the nonlinear (quadratic) nature of two-photon excitation, the relationship between fluorescence intensity and excitation laser power was investigated. NR-L64 was loaded in cells and their fluorescence images were acquired at varying laser powers, and the total fluorescence intensity in each image was measured by taking the region of interest (ROI) of the probe in the cells at different laser powers. Both the fluorescence intensity and laser power values were plotted on a log-log scale ($\log_{10}$ of fluorescence intensity versus $\log_{10}$ of laser power). Linear regression of the data yielded a slope of approximately 1.74, consistent with the expected near-quadratic dependence for two-photon excitation. This provides strong evidence that the observed fluorescence of NR-L64 in FaDu cells arises predominantly from a two-photon absorption process.

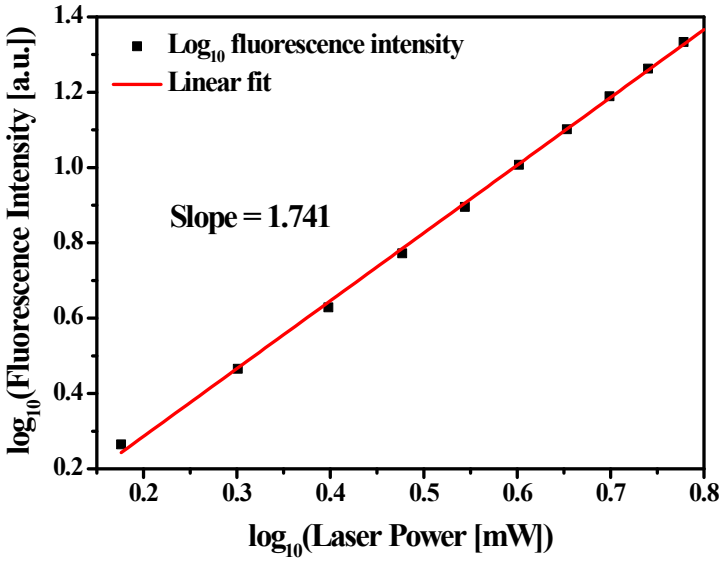


Figure S3. Two-photon power-dependent intensity of NR-L64 loaded in cells.

Table S5: Mean fluorescence intensity of NR-L64 niosomes loaded in FaDu cells irradiated under a two-photon fluorescence microscope at 37 °C and 39 °C for 70 min.

Time (min)	Average fluorescence	Average fluorescence

	intensity at 37 °C	intensity at 39 °C
0	18.65	54.58
5	18.7	54.96
10	18.95	53.99
15	19.03	55.16
20	18.59	54.73
25	18.49	55.52
30	18.71	55.4
35	18.72	54.56
40	18.43	54.63
45	18.5	53.98
50	18.57	53.97
55	18.84	54.05
60	18.59	54.96
65	19.1	55.18
70	19.05	55.07

Method S2. Thermal monitoring of cell metabolism using confocal and multiphoton imaging

FaDu cells were seeded in confocal petri dishes (50×10^3 cells/dish) and cultured for 24 h at 37 °C under 95% relative humidity and 5% CO_2. The cells were then incubated for 2 h with NR-L64 (1.25×10^{-5} M NR), suspended in EMEM supplemented with 10% FBS, based on our previous cell viability studies. After incubation, the cells were washed and resuspended in fresh 10% FBS-containing EMEM.

Cellular metabolism was stimulated by adding 1 mL of glucose solution (5 mg mL^{-1}) to the culture medium, mimicking hyperglycemic conditions in the cell. Once taken up by the cells, glucose undergoes glycolysis to generate pyruvate, which either enters the mitochondria to fuel oxidative phosphorylation or is converted into lactate, with both pathways contributing to energy production[1]. A parallel group containing only NR-L64 was used to obtain the calibration curve. Fluorescence intensity changes of NR-L64 in response to cellular metabolism were monitored using confocal laser scanning microscopy (LSCM) and two-photon fluorescence and lifetime microscopy. For confocal imaging, NR-l64 in live FaDu was excited at 514 nm, and emission was collected between 610–670 nm. For two-photon imaging, 825 nm excitation was used, and emission was recorded using a 580–632 nm band pass filter. Calibration was achieved by recording fluorescence and lifetime images between 37–39 °C in 0.2 °C increments. For experimental measurements, glucose-treated cells were observed for 60 min, with fluorescence images captured every 5 min at 20× magnification. The fluorescence

intensity and lifetime values were obtained from the images by taking a region of interest (ROI) from the cells (as described in methods S3 and S4). Temperature was precisely maintained using a controller (H301-EC-M, OKO Laboratories) with an accuracy of ±0.1 °C.

Table S6: Calculation of relative sensitivity from average fluorescence intensity obtained from the confocal calibration experiment.

Temperature (°C)	Average Fluorescence Intensity (Q)	d(Q)	Relative sensitivity (Sr)
37.2	0.05035	0.00185	18.37
37.4	0.05251	0.00216	20.56
37.6	0.06252	0.01001	80.05
37.8	0.06739	0.01001	74.26
38	0.07685	0.01446	94.07
38.2	0.09639	0.01954	101.35
38.4	0.10658	0.01019	47.80
38.6	0.11148	0.0049	21.97
38.8	0.12171	0.01023	42.02
39	0.14171	0.02	70.56

Table S7: Calculation of relative sensitivity from average fluorescence intensity obtained from the Two-photon calibration experiment.

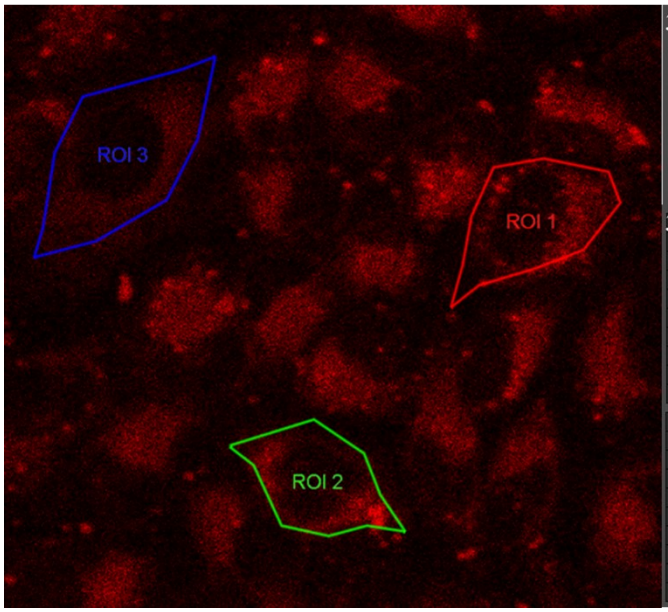
Temperature (°C)	Average Fluorescence Intensity (Q)	d(Q)	Relative sensitivity (Sr)
37.2	0.15506	0.00891	28.73
37.4	0.15685	0.00179	5.70
37.6	0.16566	0.00881	26.59
37.8	0.17081	0.00515	15.07
38	0.1847	0.01389	37.60
38.2	0.23525	0.05055	107.43
38.4	0.27959	0.04434	79.29
38.6	0.30678	0.02719	44.31
38.8	0.32419	0.01741	26.85
39	0.35419	0.03	42.35

Table S8: Calculation of relative sensitivity from the average fluorescence lifetime obtained from the fluorescence lifetime calibration experiment.

Temperature (°C)	Average Fluorescence Lifetime (Q)	d(Q)	Relative sensitivity (Sr)
37.2	3.278689	0.02	3.278689
37.4	3.30033	0.02	3.30033
37.6	5	0.03	5
37.8	1.672241	0.01	1.672241
38	1.677852	0.01	1.677852
38.2	6.802721	0.04	6.802721
38.4	3.424658	0.02	3.424658
38.6	1.706485	0.01	1.70648
38.8	1.70068	0.01	1.70068

Method S3. Average Fluorescence intensity calculation from confocal and Two- photon microscope images using LAS X software

The confocal images of NR-L64 in cells were taken using leica confocal microscope (TCS SP8, Leica Microsystems, Germany) and the Two-photon images in cells were taken using Leica SP8 microscope coupled with a Chameleon Vision II femtosecond Ti: Sapphire oscillator. To calculate the average fluorescence intensity from the images, LAS X software from Leica was used. The images were opened in the quantification window of the software and from the tools tab “Histogram” was selected which determines the distribution of gray-scale values in images and in the individual images of image series based on regions of interest (ROI). The region of interest (ROI) was drawn around different cells present in the image using the “draw polygon” feature present in the tool bar. The mean intensity of each ROI was obtained from the statistics window of the software which corresponds to the fluorescence intensity obtained from that particular cell as highlighted in figures (S6, S7, S8 and S9), to calculate the average fluorescence intensity the mean intensity of each cell was divided by the ROI area of that particular cell to get the average fluorescence intensity per area. The average fluorescence intensity per area of multiple cells were taken in a image and their mean value was used as the average fluorescence intensity of that particular image as given in table S5 and S6.

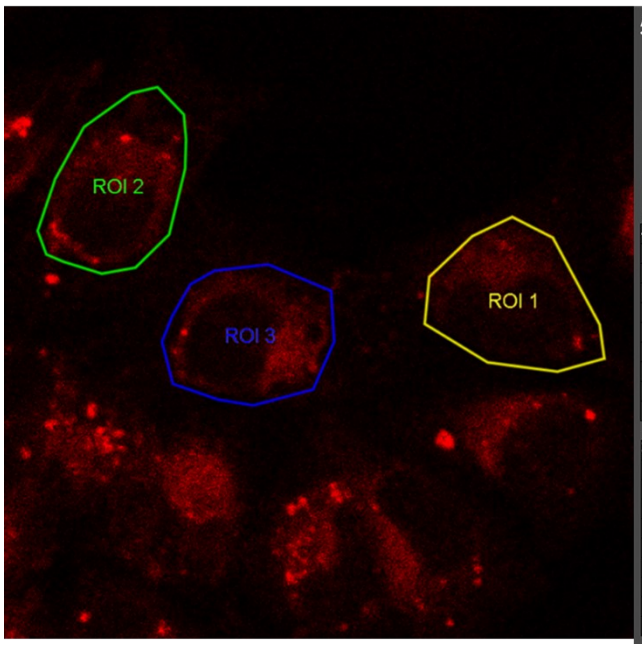


37 °C Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	21 kpixel	17 kpixel	30 kpixel
ROI Area	392.67 µm²	326.07 µm²	572.10 µm²
Intensity Sum	478400	349940	500420
Mean Intensity	23.15	20.39	16.62
Intensity Variance	613.59	503.85	373.80
Intensity Standard Deviation	24.77	22.45	19.33
Intensity Average Deviation	19.12	16.78	15.27
Intensity Maximum	160.00	180.00	140.00
Intensity Minimum	0.00	0.00	0.00

38 °C Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	21 kpixel	17 kpixel	29 kpixel
ROI Area	392.67 µm²	324.46 µm²	557.42 µm²
Intensity Sum	750927	625371	710780
Mean Intensity	36.33	36.62	24.23
Intensity Variance	1,251.89	1,447.75	614.06
Intensity Standard Deviation	35.38	38.05	24.78
Intensity Average Deviation	27.31	27.98	19.40
Intensity Maximum	255.00	255.00	200.00
Intensity Minimum	0.00	0.00	0.00

39 °C Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	16 kpixel	14 kpixel	29 kpixel
ROI Area	310.65 µm²	267.08 µm²	557.42 µm²
Intensity Sum	886821	760160	1160210
Mean Intensity	54.24	54.08	39.55
Intensity Variance	2,725.96	3,023.25	1,308.77
Intensity Standard Deviation	52.21	54.98	36.18
Intensity Average Deviation	40.40	41.34	27.56
Intensity Maximum	255.00	255.00	255.00
Intensity Minimum	0.00	0.00	0.00

Figure S4: Confocal image (only at 37°C is shown) showing the ROI of cells taken for calibration calculations and their corresponding fluorescence mean intensity values at different temperatures.

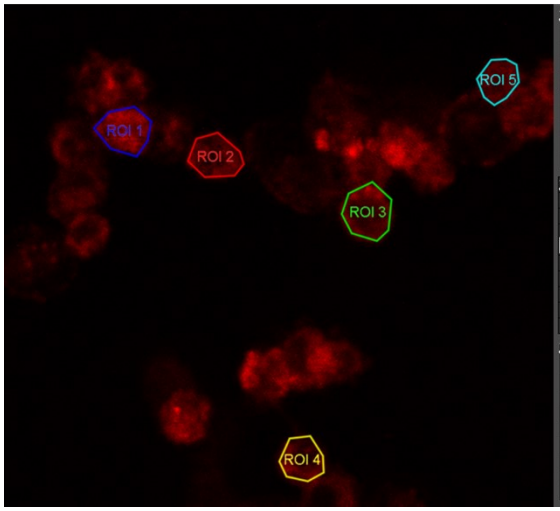


5 min Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	29 kpixel	28 kpixel	24 kpixel
ROI Area	552.23 µm²	535.17 µm²	464.20 µm²
Intensity Sum	617060	667740	555160
Mean Intensity	21.23	23.71	22.72
Intensity Variance	548.06	610.73	580.76
Intensity Standard Deviation	23.41	24.71	24.10
Intensity Average Deviation	17.33	19.08	18.25
Intensity Maximum	200.00	200.00	200.00
Intensity Minimum	0.00	0.00	0.00

30 min Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	29 kpixel	27 kpixel	24 kpixel
ROI Area	546.21 µm²	518.75 µm²	464.20 µm²
Intensity Sum	740615	828150	735098
Mean Intensity	25.76	30.33	30.09
Intensity Variance	804.05	940.64	917.21
Intensity Standard Deviation	28.36	30.67	30.29
Intensity Average Deviation	21.53	23.93	23.64
Intensity Maximum	255.00	255.00	255.00
Intensity Minimum	0.00	0.00	0.00

55 min Ch1	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	19 kpixel	25 kpixel	23 kpixel
ROI Area	363.56 µm²	468.04 µm²	428.03 µm²
Intensity Sum	765171	898607	804735
Mean Intensity	39.99	36.48	35.72
Intensity Variance	2,119.62	1,330.12	1,367.91
Intensity Standard Deviation	46.04	36.47	36.99
Intensity Average Deviation	32.81	27.53	28.00
Intensity Maximum	255.00	255.00	255.00
Intensity Minimum	0.00	0.00	0.00

Figure S5: Confocal image (after 55 min is shown) showing the ROI of the cells after glucose addition and their corresponding fluorescence mean intensity values at different time intervals.

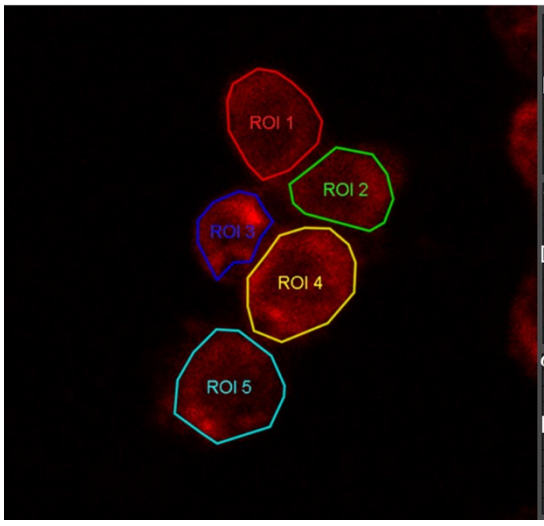


37 °C Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	3 kpixel	4 kpixel	3 kpixel	3 kpixel	3 kpixel
ROI Area	108.80 µm²	166.44 µm²	115.99 µm²	128.63 µm²	137.81 µm²
Intensity Sum	56402	72287	49683	77422	54667
Mean Intensity	21.92	18.37	18.11	25.45	16.77
Intensity Variance	112.34	109.50	78.59	192.93	76.08
Intensity Standard Deviation	10.60	10.46	8.87	13.89	8.72
Intensity Average Deviation	8.84	8.14	7.05	10.95	6.90
Intensity Maximum	56.00	76.00	60.00	75.00	84.00
Intensity Minimum	1.00	0.00	0.00	0.00	0.00

38 °C Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	3 kpixel	3 kpixel	3 kpixel	3 kpixel	2 kpixel
ROI Area	106.18 µm²	142.29 µm²	110.83 µm²	132.78 µm²	104.95 µm²
Intensity Sum	54963	52215	47753	71910	51246
Mean Intensity	21.89	15.52	18.22	22.90	20.65
Intensity Variance	144.90	75.17	73.99	138.98	68.37
Intensity Standard Deviation	12.04	8.67	8.60	11.79	8.27
Intensity Average Deviation	9.87	6.40	7.02	9.70	6.71
Intensity Maximum	70.00	79.00	50.00	63.00	53.00
Intensity Minimum	1.00	0.00	0.00	0.00	2.00

39 °C Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	2 kpixel	3 kpixel	2 kpixel	2 kpixel	2 kpixel
ROI Area	87.78 µm²	117.26 µm²	88.72 µm²	101.65 µm²	103.22 µm²
Intensity Sum	71234	76270	49380	100648	68461
Mean Intensity	34.31	27.50	23.54	41.87	28.05
Intensity Variance	320.03	132.60	122.14	330.44	136.38
Intensity Standard Deviation	17.89	11.52	11.05	18.18	11.68
Intensity Average Deviation	14.62	8.66	8.54	14.92	9.57
Intensity Maximum	92.00	98.00	69.00	103.00	76.00
Intensity Minimum	3.00	3.00	2.00	1.00	3.00

Figure S6: TPFM image (only at 37°C is shown) showing the ROI of cells taken for calibration calculations and their corresponding fluorescence mean intensity values at different temperatures.



5 min Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	3 kpixel	4 kpixel	5 kpixel	6 kpixel	3 kpixel
ROI Area	141.84 µm²	149.00 µm²	192.58 µm²	240.52 µm²	128.19 µm²
Intensity Sum	85736	117355	157494	150292	80840
Mean Intensity	25.62	33.39	34.67	26.49	26.73
Intensity Variance	71.81	227.25	256.49	145.04	210.63
Intensity Standard Deviation	8.47	15.07	16.02	12.04	14.51
Intensity Average Deviation	6.71	12.31	13.27	9.88	11.70
Intensity Maximum	66.00	84.00	90.00	73.00	79.00
Intensity Minimum	4.00	0.00	4.00	3.00	2.00

30 min Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	3 kpixel	3 kpixel	4 kpixel	5 kpixel	3 kpixel
ROI Area	128.91 µm²	118.82 µm²	176.34 µm²	216.57 µm²	129.84 µm²
Intensity Sum	86644	107088	153494	160892	87733
Mean Intensity	28.49	38.20	36.90	31.49	28.64
Intensity Variance	76.17	210.79	381.60	218.34	201.52
Intensity Standard Deviation	8.73	14.52	19.53	14.78	14.20
Intensity Average Deviation	6.99	11.88	16.58	12.00	11.72
Intensity Maximum	69.00	92.00	98.00	91.00	79.00
Intensity Minimum	7.00	6.00	2.00	3.00	1.00

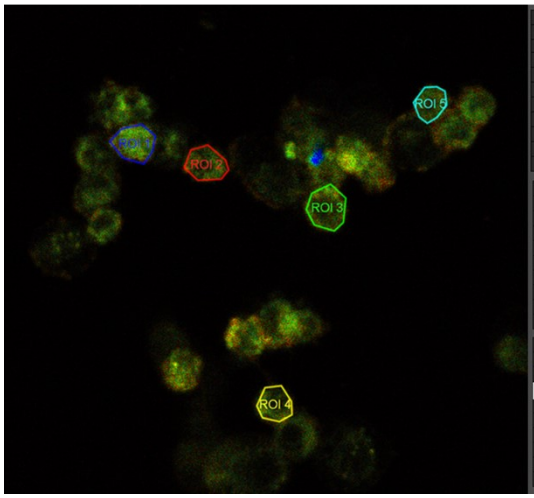
60 min Ch2	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	3 kpixel	3 kpixel	4 kpixel	5 kpixel	3 kpixel
ROI Area	133.36 µm²	111.36 µm²	149.55 µm²	209.28 µm²	120.64 µm²
Intensity Sum	101969	130811	174869	180885	93912
Mean Intensity	32.41	49.79	49.57	36.64	33.00
Intensity Variance	94.20	324.88	642.21	275.71	263.15
Intensity Standard Deviation	9.71	18.02	25.34	16.60	16.22
Intensity Average Deviation	7.75	15.00	21.98	13.52	13.54
Intensity Maximum	75.00	106.00	117.00	101.00	92.00
Intensity Minimum	6.00	5.00	3.00	3.00	4.00

Figure S7: TPFM image (after 60 min is shown) showing the ROI of the cells after glucose addition and their corresponding fluorescence mean intensity values at different time intervals.

Method S4: TauModes for the quantification of the average fluorescence lifetime from the Two- photon microscope images using LAS X software

The Fluorescence lifetime images in cells were taken using a Leica SP8 microscope coupled with a Chameleon Vision II femtosecond Ti: Sapphire oscillator. To visualize the fluorescence lifetime in images taken with the Leica microscope, the Leica system provides different

TauModes and a TauContrast function. The TauContrast provides the average photon arrival time per pixel in the detector and displays it as a color-lifetime image (figures S10 and S11). In the TauModes feature, the Tau Intensity feature was selected, which gives the full photon arrival time intensity image. To calculate the average fluorescence lifetime from the images, a process similar to that described in Method S3 was used. The region of interest (ROI) was drawn around different cells present in the image using the "draw polygon" feature present in the toolbar. The "mean weighted arrival time" of each ROI was obtained from the statistics window of the software which corresponds to the average fluorescence lifetime obtained from that particular cell as highlighted in figures (S10, and S11), to calculate the average fluorescence lifetime the "mean weighted arrival time" of multiple cells were taken in an image and their mean value was calculated as the average fluorescence lifetime of that particular image as given in Table S7.

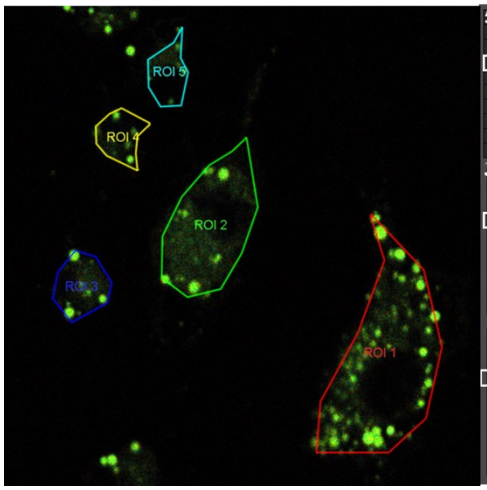


37 °C Ch3	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	2 kpixel	2 kpixel	3 kpixel	2 kpixel	2 kpixel
ROI Area	100.81 µm²	93.20 µm²	111.08 µm²	79.54 µm²	70.74 µm²
Intensity Minimum	2.00	2.00	1.00	0.00	4.00
Mean Weighted Arrival Time	3.00 ns	3.10 ns	3.16 ns	3.08 ns	3.06 ns
Most Prominent Arrival Time	2.88 ns	2.88 ns	3.36 ns	3.36 ns	2.78 ns
Weighted Arrival Time Variance	2,96,448.77 ps²	4,88,150.82 ps²	4,37,253.28 ps²	4,85,235.22 ps²	4,28,823.44 ps²
Weighted Arrival Time Standard Deviation	544.47 ps	698.68 ps	661.25 ps	696.59 ps	654.85 ps
Weighted Arrival Time Average Deviation	424.98 ps	547.99 ps	522.46 ps	540.90 ps	520.57 ps
Max Arrival Time	5.88 ns	6.95 ns	7.24 ns	6.85 ns	5.50 ns
Min Arrival Time	1.23 ns	745.45 ps	745.45 ps	-1.00 ns	1.04 ns

38 °C Ch3	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	2 kpixel	1 kpixel	2 kpixel	1 kpixel	967 pixel
ROI Area	64.74 µm²	53.41 µm²	75.44 µm²	54.97 µm²	40.89 µm²
Mean Weighted Arrival Time	2.92 ns	3.03 ns	3.02 ns	2.97 ns	3.01 ns
Most Prominent Arrival Time	2.78 ns	2.88 ns	2.98 ns	2.88 ns	2.98 ns
Weighted Arrival Time Variance	2,30,078.24 ps²	3,69,062.99 ps²	4,02,796.38 ps²	2,70,643.72 ps²	3,80,692.55 ps²
Weighted Arrival Time Standard Deviation	479.66 ps	607.51 ps	634.66 ps	520.23 ps	617.00 ps
Weighted Arrival Time Average Deviation	380.45 ps	480.64 ps	502.26 ps	410.34 ps	486.82 ps
Max Arrival Time	4.53 ns	6.76 ns	5.69 ns	5.40 ns	5.59 ns
Min Arrival Time	1.33 ns	939.39 ps	939.39 ps	1.04 ns	1.04 ns

39 °C Ch3	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	2 kpixel	1 kpixel	2 kpixel	1 kpixel	949 pixel
ROI Area	70.53 µm²	61.95 µm²	102.33 µm²	57.80 µm²	40.13 µm²
Mean Weighted Arrival Time	2.87 ns	2.97 ns	3.00 ns	2.93 ns	2.97 ns
Most Prominent Arrival Time	2.78 ns	2.88 ns	2.88 ns	2.98 ns	2.88 ns
Weighted Arrival Time Variance	2,27,158.43 ps²	2,83,130.91 ps²	2,74,417.64 ps²	1,97,636.80 ps²	2,90,856.69 ps²
Weighted Arrival Time Standard Deviation	476.61 ps	532.10 ps	523.85 ps	444.56 ps	539.31 ps
Weighted Arrival Time Average Deviation	375.12 ps	419.35 ps	413.53 ps	348.47 ps	426.48 ps
Max Arrival Time	5.69 ns	5.21 ns	5.21 ns	5.21 ns	5.21 ns
Min Arrival Time	1.33 ns	1.13 ns	1.42 ns	1.42 ns	1.23 ns

Figure S8: Two-photon Fluorescence lifetime image (only at 37°C is shown) showing the ROI of cells taken for calibration calculations and their corresponding fluorescence lifetime values at different temperatures.



5 min Ch3

	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	54 kpixel	38 kpixel	11 kpixel	6 kpixel	5 kpixel
ROI Area	1,409.49 µm²	1,004.36 µm²	278.51 µm²	167.37 µm²	134.19 µm²
Mean Weighted Arrival Time	3.01 ns	3.06 ns	3.04 ns	3.14 ns	3.15 ns
Most Prominent Arrival Time	2.68 ns	2.01 ns	2.20 ns	2.59 ns	1.52 ns
Weighted Arrival Time Variance	3.43 ns²	4.60 ns²	4.17 ns²	5.18 ns²	5.52 ns²
Weighted Arrival Time Standard Deviation	1.85 ns	2.14 ns	2.04 ns	2.28 ns	2.35 ns
Weighted Arrival Time Average Deviation	1.41 ns	1.66 ns	1.55 ns	1.78 ns	1.87 ns
Max Arrival Time	11.41 ns	11.41 ns	11.41 ns	11.41 ns	11.41 ns
Min Arrival Time	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns

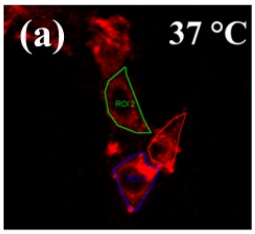
30 min Ch3

	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	58 kpixel	34 kpixel	10 kpixel	7 kpixel	7 kpixel
ROI Area	1,528.33 µm²	882.77 µm²	257.38 µm²	182.14 µm²	182.48 µm²
Mean Weighted Arrival Time	2.95 ns	2.92 ns	2.99 ns	3.03 ns	3.07 ns
Most Prominent Arrival Time	2.88 ns	2.59 ns	2.39 ns	2.68 ns	2.30 ns
Weighted Arrival Time Variance	2.53 ns²	3.29 ns²	2.99 ns²	3.84 ns²	4.66 ns²
Weighted Arrival Time Standard Deviation	1.59 ns	1.81 ns	1.73 ns	1.96 ns	2.16 ns
Weighted Arrival Time Average Deviation	1.18 ns	1.38 ns	1.30 ns	1.50 ns	1.67 ns
Max Arrival Time	11.41 ns	11.41 ns	11.51 ns	11.41 ns	11.41 ns
Min Arrival Time	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns

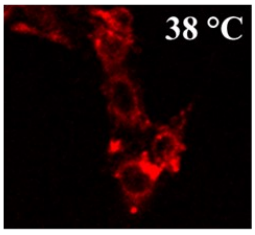
60 min Ch3

	ROI 1	ROI 2	ROI 3	ROI 4	ROI 5
Number of Pixels in Area	94 kpixel	60 kpixel	24 kpixel	10 kpixel	12 kpixel
ROI Area	2,470.86 µm²	1,570.28 µm²	616.70 µm²	253.74 µm²	306.79 µm²
Mean Weighted Arrival Time	2.94 ns	2.96 ns	2.97 ns	2.96 ns	2.99 ns
Most Prominent Arrival Time	2.98 ns	2.59 ns	2.78 ns	2.78 ns	2.49 ns
Weighted Arrival Time Variance	2.37 ns²	3.16 ns²	3.10 ns²	3.29 ns²	3.98 ns²
Weighted Arrival Time Standard Deviation	1.54 ns	1.78 ns	1.76 ns	1.81 ns	2.00 ns
Weighted Arrival Time Average Deviation	1.11 ns	1.32 ns	1.27 ns	1.36 ns	1.52 ns
Max Arrival Time	11.41 ns	11.41 ns	11.41 ns	11.41 ns	11.41 ns
Min Arrival Time	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns	-1.00 ns

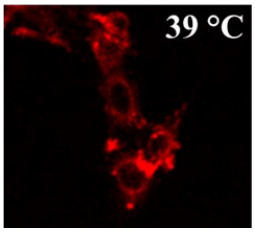
Figure S9: Two-photon Fluorescence lifetime image (after 60 min is shown) showing the ROI of the cells after glucose addition and their corresponding fluorescence lifetime values at different time intervals.



Ch2	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	634 pixel	771 pixel	758 pixel
ROI Area	168.78 µm²	205.25 µm²	201.79 µm²
Intensity Sum	32535	29324	46123
Mean Intensity	51.32	38.03	60.85
Intensity Variance	744.13	234.85	1,737.24
Intensity Standard Deviation	27.28	15.32	41.68
Intensity Average Deviation	21.47	12.42	29.71
Intensity Maximum	174.00	92.00	255.00
Intensity Minimum	10.00	5.00	13.00



Ch2	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	634 pixel	771 pixel	758 pixel
ROI Area	168.78 µm²	205.25 µm²	201.79 µm²
Intensity Sum	32493	30110	47078
Mean Intensity	51.25	39.05	62.11
Intensity Variance	802.33	227.03	1,870.59
Intensity Standard Deviation	28.33	15.07	43.25
Intensity Average Deviation	21.84	12.16	30.76
Intensity Maximum	193.00	102.00	255.00
Intensity Minimum	8.00	10.00	13.00



Ch2	ROI 1	ROI 2	ROI 3
Number of Pixels in Area	634 pixel	771 pixel	758 pixel
ROI Area	168.78 µm²	205.25 µm²	201.79 µm²
Intensity Sum	32771	30388	47197
Mean Intensity	51.69	39.41	62.27
Intensity Variance	762.54	235.81	1,784.50
Intensity Standard Deviation	27.61	15.36	42.24
Intensity Average Deviation	21.83	12.52	30.14
Intensity Maximum	158.00	106.00	255.00
Intensity Minimum	7.00	8.00	16.00

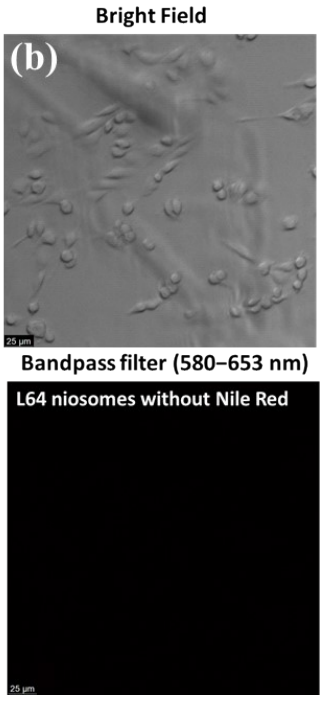


Figure S10: TPFM images of (a) only Nile red in cells at different temperatures, and (b) only L64 niosomes in cells at 37°C.

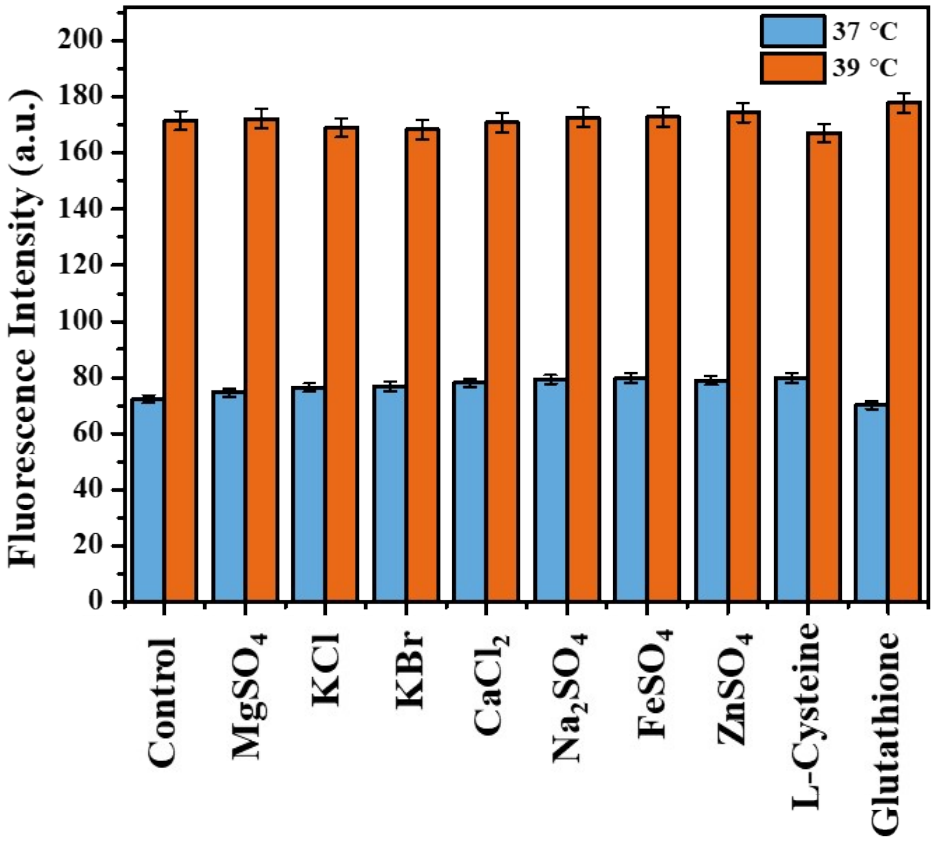


Figure S11. Fluorescence response of Nile Red–L64 (at 37 °C and 39 °C) in the presence of common intracellular interferents (metal ions, anions, and biothiols), each at 10 mM. Control: Without any interferents.

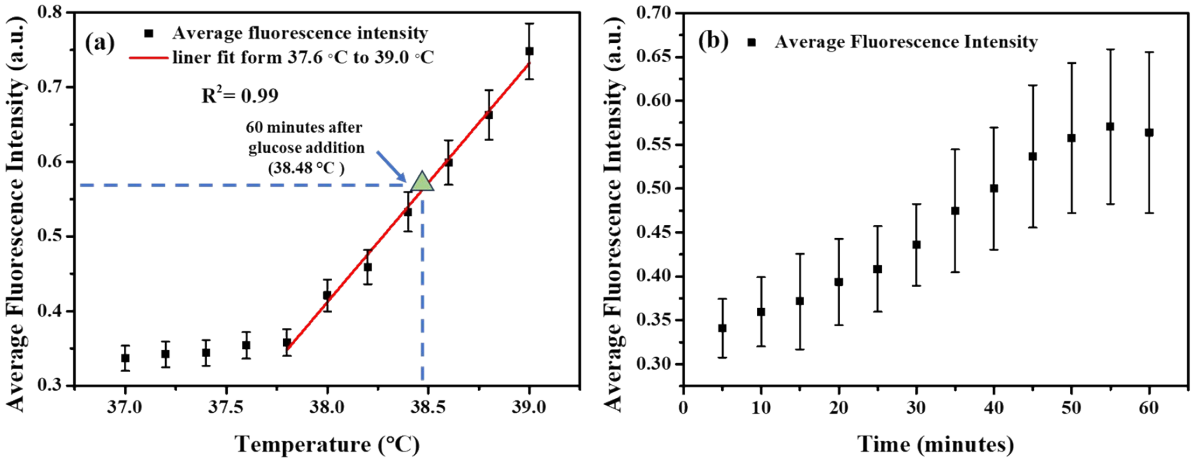


Figure S12: (a) Two-photon fluorescence calibration curve obtained from NR-L64 in live FaDu cells, and (b) Two-photon average fluorescence intensity obtained after glucose addition in live FaDu cells at lower laser power (1 mW).

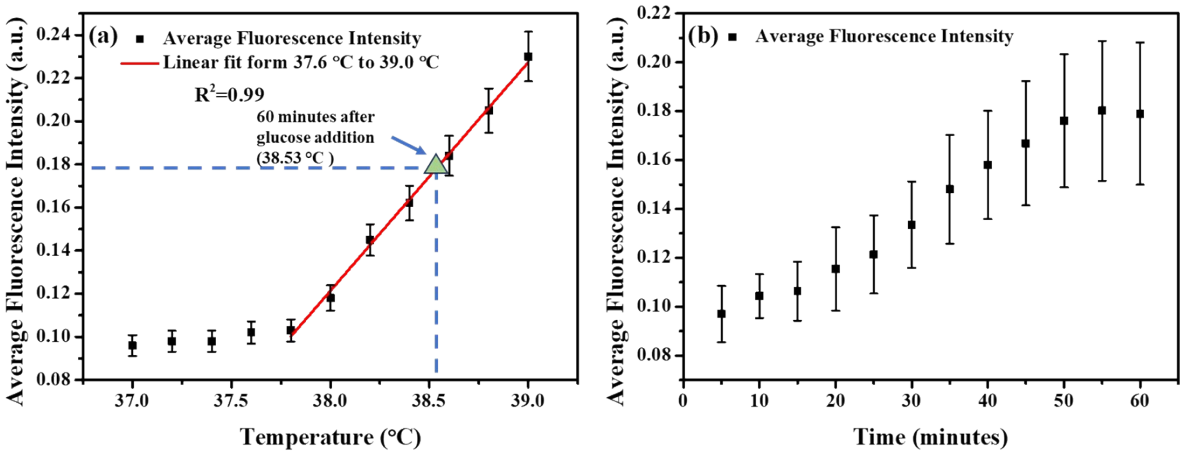


Figure S13: (a) Two-photon fluorescence calibration curve obtained from NR-L64 in live FaDu cells, and (b) Two-photon average fluorescence intensity obtained after glucose addition in live FaDu cells at higher laser power (4 mW).

References:

1. S. J. Kierans and C. T Taylor, *J. Biol. Chem*., 2024**, 300**, 107906.