A general method for the development of multicolor biosensors with large dynamic ranges
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a. Schematic representation of the chemogenetic FRET pair based on EGFP and HaloTag7 (HT7) labeled with a synthetic rhodamine fluorophore. Shown are cartoons of the fusion of HT7 to the N- (HT7-EGFP) or C-terminus of EGFP (EGFP-HT7) b. Fluorescence intensity (FI) emission spectra of HT7-EGFP and EGFP-HT7 (=ChemoG1) labeled with SiR or not labeled. Represented are the means of 3 technical replicates. c. Normalized excitation (Ex) and emission (Em) spectra of EGFP and SiR. Represented are the means of 3 technical replicates.
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g. FI emission spectra of static ChemoRuby2 labeled with SiR. The FRET efficiency and FRET ratio can be found in Supplementary Table 4.

h. FI emission spectra of ChemoR-CaM labeled with JF\textsubscript{525} in absence and presence of free Ca\textsuperscript{2+}. JF\textsubscript{525} served as FRET donor and mRuby2 as FRET acceptor.
**Supplementary Figure 6 | Impact of calmodulin-based calcium sensors on intracellular calcium oscillations.**

**a.** Time course measurement of free intracellular calcium fluctuations using yellow cameleon 3.6 (YC 3.6). Represented are the FRET/ECFP ratios for 8 representative single cell traces from 3 biological replicates. HeLa Kyoto cells were treated with 10 μM histamine at the time point indicated with an arrow. Most of the cells show no or reduced calcium oscillations as observed for ChemoX-CaM (Fig. 2f).

**b-c.** Time course measurements of free intracellular calcium fluctuations using the synthetic calcium indicator Cal520. HeLa Kyoto cells were transiently transfected to express ChemoR-CaM. ChemoR-CaM has been chosen to not interfere spectrally with Cal520. Ten representative single cell traces from 3 biological replicates not expressing (b) and expressing (c) ChemoR-CaM were analyzed for calcium oscillations upon treatment with 10 μM histamine at the time point indicated with an arrow. Represented are the fluorescence intensity changes (ΔF/ΔF0) of Cal520. Cells that did not express ChemoR-CaM mostly highlight calcium oscillations while the expression of ChemoR-CaM seem to repress this behavior as for YC 3.6, which is as well a calmodulin-based calcium sensor. This phenomenon was already reported in the literature and seems to occur through calcium buffering due to the sensor over-expression\(^\text{21}\).
Supplementary Figure 7 | Emission spectra of ChemoB-NAD and ChemoR-NAD.

**a, b.** Fluorescence intensity (FI) emission spectra of SiR-labeled ChemoB-NAD (a) and ChemoR-NAD (b) in presence or absence of 1 mM NAD⁺. Shown are the means of 3 technical replicates.
Supplementary Figure 8| Performance of ChemoB-NAD and ChemoR-NAD in U-2 OS cells.

a, c. Confocal images of U-2 OS cells expressing ChemoB-NAD (a) or ChemoR-NAD (c) in the cytosol labeled with SiR. Shown are the respective FP channel, FRET channel and ratio image (FRET/FP) in pseudocolor (LUT = mpl-viridis). Cells were treated for 24 h either with DMSO (Ctrl), 100 nM FK866 or 1 mM NR. All scale bars = 25 μm. b, d. FRET/FP ratios of U-2 OS cells corresponding to panels a and c, respectively. Shown are the FRET/FP values of single cells (circles) and the mean ± s.d. (black line) (ChemoB-NAD: n = 107 (ctrl), 127 (NR), 113 (FK866) cells; ChemoR-NAD, n = 66 (ctrl), 59 NR), 62 (FK866) cells; from 3 independent experiments). p-values are given based on unpaired two-tailed t-test with Welch’s correction (**** p<0.0001, ** p = 0.0021).
Supplementary Figure 9| Structural comparison of ChemoG and HaloTag7<sub>P174W</sub>. Zoom-in of the X-ray structure of ChemoG<sub>5TMR</sub> (PDB ID: 8B6T) overlayed with the X-ray structure of HaloTag<sub>7P174WTMR</sub> (PDB ID: 6ZVV). The EGFP chromophore (green), EGFP surface residue T225R (green), HaloTag7 residues (grey or slate) and TMR (orange or cyan) are shown as sticks. The atoms of the surface residue W174 of HaloTag<sub>P174W</sub> are additionally shown as spheres to visualize the steric clash with T225R of EGFP in the current conformation, suggesting the necessity of a conformational change in the closed form of the ChemoD-NAD sensor.
Supplementary Figure 10 | ChemoL sensor performances in U-2 OS cells.

a-f. Time course measurements of ChemoL sensors expressed in U-2 OS cells (ChemoL-NAD, a, d) or HeLa Kyoto cells (ChemoL-ATP, b, e) and ChemoL-CaM (c, f) upon drug treatments. Sensors were labeled with CPY. Represented are the BRET-FRET/EGFP ratios normalized to 1 at \( t = 0 \) min. Cells were untreated (+ medium) or treated with different reagents indicated by an arrow (\( n = 3 \) wells for each condition of each experiment). Represented are the mean (solid line) and the standard deviation (shade areas). The treatments are identical to time courses in Fig. 6e-g.

g-i. Luminescent intensity (LI) spectra of ChemoL-NAD (g), ChemoL-ATP (h) or ChemoL-CaM (i) expressed in U-2 OS cells (ChemoL-NAD) or HeLa Kyoto cells (ChemoL-ATP and ChemoL-CaM). Sensors were labeled with CPY. The treatments are identical to time courses in Fig. 6e-g. Spectra were acquired immediately after the duration of the time courses (ChemoL-NAD = 40 min, ChemoL-ATP = 60 min, ChemoL-CaM = 10 min).
Supplementary Figure 11 | Development of ChemoG biosensors.
See Supplementary Note 1 for explanations.
Supplementary Figure 12] Tuning the spectral properties of the optimized ChemoG sensor. See Supplementary Note 2 for explanations.

Supplementary Figure 13] Tuning the readout mode of the optimized ChemoG sensor. See Supplementary Note 3 for explanations.
Supplementary Tables

Supplementary Table 1 | FRET efficiencies of the ChemoG interface variants.

| Construct | Interface mutations | FRET ratio  | FRET efficiency [%] |
|-----------|---------------------|-------------|---------------------|
|           | EGFP                | HaloTag7    |                     |
| ChemoG1   | -                   | 2.2 ±0.1    | 74.8 ±0.4           |
| ChemoG2   | A206K               | 4.0 ±0.1    | 84.1 ±0.6           |
| ChemoG3   | A206K               | L271E       | 8.9 ±0.1            | 90.9 ±0.1           |
| ChemoG4   | A206K               | L271E-E143R-E147R | 11.6 ±0.3   | 93.2 ±0.1           |
| ChemoG5   | A206K-T225R         | L271E-E143R-E147R | 20.3 ±0.8   | 95.8 ±0.1           |

FRET ratios (FRET/EGFP) and FRET efficiencies were determined for purified constructs labeled with SiR. Shown are the means ±s.d. (n = 3 technical replicates).
**Supplementary Table 2 | FRET ratios of ChemoX constructs expressed in U-2 OS cells.**

| Construct     | Subcellular localization | Localization tag | FRET ratio | Number of cells |
|---------------|--------------------------|------------------|------------|-----------------|
| HT7-EGFP      | -                        | -                | 0.1 ±0.05  | 9               |
| ChemoG1       | -                        | -                | 3.7 ±0.3   | 18              |
| ChemoG2       | -                        | -                | 5.7 ±1.1   | 15              |
| ChemoG3       | -                        | -                | 8.7 ±1.4   | 20              |
| ChemoG4       | -                        | -                | 13.6 ±2.2  | 29              |
| ChemoG5       | -                        | -                | 16.4 ±2.7  | 32              |
| ChemoG5       | Cytosol                  | NES              | 21.5 ±5.3  | 60              |
| ChemoG5       | Outer plasma membrane    | PDGFR\textsubscript{tm} | 26.5 ±8.7  | 59              |
| ChemoG5       | Nucleus                  | NLS              | 17.8 ±3.4  | 51              |
| ChemoG5       | Mitochondria             | Cox8             | 16.3 ±7.0  | 126             |
| ChemoG5       | Nuclear envelope         | Lamin B1         | 15.9 ±6.5  | 29              |
| ChemoB        | -                        | -                | 14.6 ±3.0  | 20              |
| ChemoC        | -                        | -                | 14.5 ±2.6  | 18              |
| ChemoY        | -                        | -                | 17.5 ±5.6  | 24              |
| ChemoR        | -                        | -                | 14.2 ±2.5  | 27              |

FRET ratios (FRET/FP) were determined for each construct expressed in U-2 OS cells labeled with SiR. Shown are the means ±s.d.
Supplementary Table 3 | FRET efficiencies of ChemoG5 labeled with different rhodamine fluorophores.

| Construct | Fluorophore | Max emission [nm] | FRET ratio  | FRET efficiency [%] |
|-----------|-------------|-------------------|-------------|---------------------|
| ChemoG5   | JF525       | 556 nm            | 18.0 ±1.4   | 94.9 ±0.3           |
| ChemoG5   | TMR         | 580 nm            | 23.6 ±2.7   | 96.6 ±0.3           |
| ChemoG5   | 580CP       | 606 nm            | 23.8 ±2.6   | 96.1 ±0.5           |
| ChemoG5   | CPY         | 628 nm            | 15.8 ±1.3   | 94.9 ±0.4           |
| ChemoG5   | SiR         | 668 nm            | 20.2 ±0.8   | 95.6 ±0.1           |
| ChemoG5   | JF669       | 686 nm            | 14.2 ±0.1   | 94.7 ±0.4           |

FRET/EGFP ratios and FRET efficiencies were determined for purified ChemoG5 labeled with different rhodamine fluorophores. Shown are the means ±s.d. (n = 3 technical replicates).
## Supplementary Table 4 | FRET efficiencies of ChemoX FRET pairs.

| Construct | FP       | Interface mutations | FRET ratio  | FRET efficiency [%] |
|-----------|----------|---------------------|-------------|---------------------|
|           | XFP      | HaloTag7            |             |                     |
| ChemoB    | EBFP2    | N39Y-V206K-T225R    | 36.2 ±0.3   | 96.6 ±0.1           |
| ChemoC*   | mCerulean3 | T225R              | 22.3 ±0.7   | 94.6 ±0.3           |
| ChemoG5   | EGFP     | A206K-T225R         | 20.3 ±0.8   | 95.8 ±0.1           |
| ChemoY    | Venus    | A206K-T225R         | 22.4 ±1.9   | 96.6 ±0.1           |
| ChemoR    | mScarlet | D201K               | 8.4 ±0.2    | 91.3 ±0.3           |
| ChemoRuby2| mRuby2   | -                   | 15.0 ±0.1   | 91.7 ±0.2           |

FRET/FP ratios of purified ChemoX constructs were determined upon labeling with SiR. Shown are the means ±s.d. (n = 3 technical replicates). *mCerulean3 contains already K206, thus additional mutation at this position was not needed.
### Supplementary Table 5 | Summarizing characteristics of the calcium sensors.

| Construct | FP | # of mut. | Interface mutations | C50  | Max ΔR/R₀ | Hill slope |
|-----------|----|-----------|---------------------|------|-----------|------------|
| 1         | EGFP | 0         | -                    | 189 nM | 22.8 ±0.3 | 2.2        |
| 2         | EGFP | 1         | A206K                | 203 nM | 33.3 ±0.8 | 1.8        |
| 3 (ChemoG-CaM) | EGFP | 2         | A206K, L271E         | 179 nM | 36.1 ±1.0 | 2.2        |
| 4         | EGFP | 3         | A206K, L271E-E143R-E147R | 121 nM | 5.2 ±0.2 | 1.5        |
| 5         | EGFP | 4         | A206K-T225R, L271E-E143R-E147R | 207 nM | 0.8 ±0.1 | 1.1        |
| ChemoB-CaM | EBFP2 | 2         | N39Y-V206K          | 206 nM | 12.7 ±0.2 | 1.8        |
| ChemoC-CaM | mCerulean3 | 1         | A206K                | 158 nM | 2.3 ±0.1 | 3.2        |
| ChemoY-CaM | Venus | 1         | A206K                | 226 nM | 21.7 ±0.6 | 2.0        |
| ChemoR-CaM0.1 | mScarlet | 1         | -                    | n.d. | 2.6 ±0.1 | n.d.       |
| ChemoR-CaM | mRuby2 | 0         | -                    | n.d. | 3.4 ±0.1 | 2.7        |
| ChemoR-CaM0.2 | mRuby3 | 0         | -                    | n.d. | 2.5 ±0.1 | n.d.       |
| ChemoR-CaM0.3 | mCherry | 0         | -                    | n.d. | 2.1 ±0.1 | n.d.       |
| ChemoR-CaM0.4 | mKO2 | 0         | -                    | n.d. | 1.9 ±0.1 | n.d.       |
| ChemoR-CaM0.4 | TagRFP | 0         | -                    | n.d. | 2.0 ±0.1 | n.d.       |
| YC 3.6 | ECFP/Venus | -         | -                    | 243 nM | 5.7 ±0.1 | 1.6        |

Maximum FRET/FP ratio changes ($\text{MaxΔR/R₀}$), C50 and Hill slope were determined for purified constructs. ChemoX-based calcium sensors were labeled with SiR. Values are based on titrations performed at 37 °C. Shown are the means and for ΔR/R₀ also the standard deviations (n = 3-4 technical replicates).
**Supplementary Table 6** | Summarizing characteristics of ChemoG-CaM labeled with different FRET acceptors.

| Construct     | Fluorophore | Max emission [nm] | C50   | Max ΔR/R₀ | Hill slope |
|---------------|-------------|-------------------|-------|-----------|------------|
| ChemoG-CaM    | TMR         | 580 nm            | 66 nM | 3.9 ±0.1  | 1.4        |
| ChemoG-CaM    | JF₅₈₅       | 610 nm            | 100 nM| 10.5 ±0.4 | 1.5        |
| ChemoG-CaM    | CPY         | 628 nm            | 76 nM | 8.6.0 ±0.1| 2.2        |
| ChemoG-CaM    | JF₆₃₅       | 656 nm            | 114 nM| 24.4 ±0.3 | 2.5        |
| ChemoG-CaM    | SiR         | 668 nm            | 179 nM| 36.8 ±0.2 | 2.2        |

Maximum FRET/EGFP ratio changes (MaxΔR/R₀), C50 and Hill slope were determined for purified ChemoG-CaM labeled with different fluorophores. Values are based on titrations performed at 37 °C. Shown are the mean and for ΔR/R₀ also the standard deviations (n = 3 technical replicates).
Supplementary Table 7 | Summarizing characteristics of ATP sensors.

| Construct | FP           | Interface mutations       | C50  | Max ΔR/R₀ | Hill slope |
|-----------|--------------|---------------------------|------|-----------|------------|
|           |              | XFP | HaloTag7 |
| 1         | EGFP         | A206K | -   | N.D  | 9.9 ±0.1 | N.D        |
| 2a (ChemoG-ATP) | EGFP       | A206K | L271E | 2.3 mM | 12.1 ±0.4 | 1.4        |
| 2b        | EGFP         | A206K-T225R | -   | N.D  | 6.0 ±0.1 | N.D        |
| 3         | EGFP         | A206K-T225R | L271E | N.D. | 1.9 ±0.0 | N.D        |
| ChemoB-ATP | EBFP2       | N39Y-V206K | L271E | 2.8 mM | 5.0 ±0.1 | 1.6        |
| ChemoR-ATP | mRuby2      | -   | -   | 3.2 mM | 0.8 ±0.1 | 2.0        |
| ATeam 1.03 | mseCFP/cpVenus | -   | -   | 1.8 mM | 1.4 ±0.1 | 1.8        |

Maximum FRET/FP ratio changes (Max ΔR/R₀), C50 and Hill slope were determined for purified constructs. ChemoX-based ATP sensors were labeled with SiR. Values are based on titrations performed at 37 °C. Shown are the mean and for ΔR/R₀ also the standard deviations (n = 3 technical replicates).
## Supplementary Table 8: Summarizing characteristics of NAD⁺ sensors.

| Construct                  | FP       | Affinity mutation | Interface mutations | Fluo  | C50  | Max ΔR/R₀ | Hill slope |
|----------------------------|----------|-------------------|---------------------|-------|------|-----------|------------|
|                            | XFP      | tLigA             | Halotag             |       |      |           |            |
| 1                          | EGFP     |                   | A206K               | TMR   | 38 µM| 10.1 ±0.1 | 1.6        |
| 2                          | EGFP     | V292A             | A206K               | TMR   | 75 µM| 6.2 ±0.1  | 1.2        |
| 3                          | EGFP     | Y226W             | A206K               | TMR   | 129 µM| 6.3 ±0.1  | 1.2        |
| 4                          | EGFP     | Y226W-V292A       | A206K               | SiR   | 205 µM| 2.0 ±0.1  | 0.9        |
| 5                          | EGFP     | Y226W-V292A       | A206K-T225R         | SiR   | 167 µM| 18.1 ±0.3 | 1.0        |
| 6 (ChemoG-NAD)             | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 200 µM| 34.7 ±0.4 | 0.8        |
| 7                          | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 136 µM| 7.5 ±0.1  | 1.0        |
| 8                          | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 36 µM | 18.5 ±0.1 | 0.9        |
| 9                          | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 117 µM| 20.4 ±0.1 | 0.8        |
| 10                         | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 22.5 µM| 22.5 ±0.1 | 0.9        |
| 11                         | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 25 µM | 32.5 ±0.3 | 0.8        |
| 12                         | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 103 µM| 11.2 ±0.1 | 0.9        |
| 13                         | EGFP     | Y226W-V292A       | A206K-T225R         | L271E | 78 µM | 3.0 ±0.1  | 1.0        |

Maximum FRET/FP ratio changes (\(\text{Max}\Delta R/R₀\)), C50 and Hill slope were determined for purified constructs labeled with indicated fluorophore substrates. Values are based on titrations performed at 37 °C. Shown are the mean and for ΔR/R₀ also the standard deviations (n = 3 technical replicates).
Supplementary Table 9 | Summarizing characteristics of intensiometric NAD⁺ sensors.

| Construct | Fluorophore | Max emission [nm] | C50  | Max $\Delta F/F_0$  | Hill slope |
|-----------|-------------|-------------------|------|---------------------|------------|
| ChemoG-NAD | SiR         | 666 nm            | 21.0 μM | 28.0 ±1.9 %  | 0.89       |
| ChemoD-NAD | SiR         | 666 nm            | 32.7 μM | 161.1 ±5.0 % | 0.84       |
| ChemoD-NAD | CPY         | 628 nm            | 36.8 μM | 104.7 ±1.2 % | 0.83       |
| ChemoD-NAD | JF<sub>635</sub> | 662 nm       | 47.5 μM | 226.6 ±4.3 % | 0.59       |

Maximum fluorescence intensity changes ($^{\text{Max}}\Delta F/F_0$), C50 and Hill slopes were determined for purified constructs labeled with the indicated fluorophores. Values are based on titrations performed at 37 °C. Shown are the means and for $\Delta F/F_0$ also the standard deviations (n = 3 technical replicates).
### Supplementary Table 10: Summarizing characteristics of fluorescence lifetime-based NAD⁺ sensors.

| Construct     | Fluorophore | Max emission [nm] | C50     | MaxΔτ         | Hill slope |
|---------------|-------------|-------------------|---------|---------------|------------|
| ChemoG-NAD    | SiR         | 666 nm            | 14.2 μM | 0.53 ±0.03 ns | 1.34       |
| ChemoD-NAD    | SiR         | 666 nm            | 22.4 μM | 1.16 ±0.01 ns | 0.99       |
| ChemoD-NAD    | CPY         | 628 nm            | 44.6 μM | 1.18 ±0.01 ns | 0.91       |
| ChemoD-NAD    | JF635       | 662 nm            | 32.3 μM | 0.77 ±0.01 ns | 0.68       |

Maximum intensity-weighted average fluorescence lifetime changes (MaxΔτ), C50 and Hill slopes were determined for purified constructs labeled with the indicated fluorophores. Values are based on titrations performed at 37 °C. Shown are the means and for MaxΔτ also the standard deviations (n = 3 technical replicates).
Supplementary Table 11 | ChemoG FRET pairs recommended for the development of ChemoG FRET biosensors.

| Construct | Interface mutations | Addgene# |
|-----------|---------------------|----------|
|           | EGFP                | HaloTag7 |
| ChemoG1   | -                   | -        | 193799   |
| ChemoG2   | A206K               | -        | 193800   |
| ChemoG3   | A206K               | L271E    | 193801   |
| ChemoG3.1 | A206K-T225R         | -        | 193802   |
| ChemoG3.2 | A206K-T225R         | L271E    | 193803   |
| ChemoG5   | A206K-T225R         | L271E-E143R-E147R | 193805   |
**Supplementary Table 12 | Chemicals and reagents used in this study.**

| Chemical/Reagent                                                                 | Manufacturer                     | Catalogue number       |
|---------------------------------------------------------------------------------|----------------------------------|------------------------|
| KOD Hot Start Master Mix                                                        | Sigma-Aldrich                    | 71842                  |
| Q5® Site-Directed Mutagenesis Kit                                               | NEB                              | E0554S                 |
| QIAprep Spin Miniprep Kit                                                       | Qiagen                           | 27106                  |
| GeneJET Endo-Free Plasmid-Maxiprep-Kit                                          | ThermoFisher                     | K0861                  |
| Isopropyl-β-D-thiogalactopyranoside (IPTG)                                      | Roth                             | CN084                  |
| Phenylmethylsulfonyl fluoride (PMSF)                                             | ThermoScientific                 | 36978                  |
| Lysozyme                                                                        | ThermoScientific                 | 89833                  |
| HisPur™ Ni-NTA Superflow Agarose                                                 | ThermoScientific                 | 25217                  |
| 4-20% Mini Protean TGX stain-free gel                                            | Bio-Rad                          | 568094                 |
| Amicon® Ultra 4 mL Centrifugal Filters                                           | Merck                            | UFC803024 (30 kDa)     |
|                                                                                 |                                  | UFC805024 (50 kDa)     |
| Glycerol                                                                        | Merck                            | 356350                 |
| Bovine serum albumin (BSA)                                                       | Roth                             | 01634                  |
| 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)                       | Sigma-Aldrich                    | H4034                  |
| Sodium chloride (NaCl)                                                           | Merck                            | 106404                 |
| Dimethyl sulfoxide (DMSO)                                                        | Applichem                        | A36720100              |
| Calcium chloride                                                                 | Roth                             | A1191                  |
| ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)       | Sigma-Aldrich                    | E4378                  |
| Calcium Calibration buffer Kit #1                                               | Life technologies               | C3008MP                |
| SPG pH 4.0 - 1 M buffer                                                          | Jena Bioscience                 | CSS-389                |
| SPG pH 10.0 - 1 M buffer                                                         | Jena Bioscience                 | CSS-390                |
| Histamine                                                                       | Sigma-Aldrich                    | H7250                  |
| Ionomycin                                                                       | Sigma-Aldrich                    | I9657                  |
| cOmplete™ Protease inhibitor cocktail                                           | Roche                            | 11836153001            |
| CelLytic™ M                                                                     | Sigma-Aldrich                    | C2978                  |
| Cal520-AM                                                                       | Abcam                           | ab171868               |
| Digitonin 5%                                                                    | ThermoScientific                 | BN2006                 |
| 1x PBS pH 7.4                                                                   | Gibco                            | 10010015               |
| 1x HBSS with calcium and magnesium                                              | Corning                          | 21-023-CMR             |
| TrypLE™ Express                                                                 | Gibco                            | 12604013               |
| DMEM high glucose +GlutaMAX™                                                    | Gibco                            | 31966021               |
| DMEM high glucose, phenol red-free                                              | Gibco                            | 31053028               |
| DMEM no glucose, phenol red-free                                                 | Gibco                            | A1443001               |
| Sodium pyruvate (100X)                                                          | Gibco                            | 11360070               |
| GlutaMAX™ Supplement (100x)                                                     | Gibco                            | 35050038               |
| Fetal bovine serum (FBS, heat-inactivated)                                      | Gibco                            | 10500064               |
| Opti-MEM™ reduced serum                                                         | Gibco                            | 31985047               |
| Lipofectamine 3000 Transfection Reagent                                         | Invitrogen                       | L3000001               |
| Adenosine-5′-triposphate (ATP) magnesium salt                                    | Sigma-Aldrich                    | A9187                  |
| Adenosine-5′-diphosphate (ADP) disodium salt                                    | Sigma-Aldrich                    | 1897                   |
| Adenosine-5′-monophosphate (AMP) sodium salt                                    | Sigma-Aldrich                    | A1752                  |
| Guanosine-5′-triphosphate (GTP) sodium salt                                     | Sigma-Aldrich                    | 10106399001            |
| 2-Deoxy-D-glucose (2DG)                                                         | TCI Chemicals                    | D0051                  |
| D-glucose monohydrate                                                           | Roth                             | 6780                   |
| Nicotinamide (NAM)                                                              | Sigma-Aldrich                    | 72340                  |
| Nicotinamide riboside (NR)                                                      | Combi-Blocks                     | HB-5832                |
| Nicotinamide mononucleotide (NMN)                                               | Sigma-Aldrich                    | N3501                  |
| Nicotinamide adenine dinucleotide (NAD+)                                        | Roche                            | 10127965001            |
| Nicotinamide adenine dinucleotide phosphate (NADP+)                             | Roth                             | AE13.3                 |
| Chemical Name                                                                 | Supplier       | Catalog Number |
|------------------------------------------------------------------------------|----------------|----------------|
| Nicotinamide adenine nucleotide, reduced (NADH)                              | Roth           | AE12.2         |
| Nicotinic acid adenine dinucleotide (NAAD+)                                  | Sigma-Aldrich  | N4256          |
| FK866                                                                        | Selleckchem    | S2799          |
| N-methyl-N-nitro-N-nitrosoguanidine (MNNG)                                   | Biozol         | N529925        |
| MitoTracker™ Red FM                                                           | Invitrogen     | M22425         |
| Hoechst 33342                                                                | Invitrogen     | H3570          |
| Penicillin-Streptomycin (Pen/Strep)                                          | Gibco          | 15140122       |
| NanoBRET™ Nano-Glo Substrate                                                 | Promega        | N157C          |
| Extracellular NanoLuc® Inhibitor                                             | Promega        | N235A          |
| Nano-Glo™ Substrate                                                          | Promega        | N113B          |
| DL-2-Amino-5-phosphonovaleric acid (APV)                                     | SantaCruz      | sc-201503      |
| NBQX disodium salt                                                           | Sigma-Aldrich  | N183           |
| Black non-binding flat bottom 96 well plates                                 | Perkin Elmer   | 6005720        |
| Black low volume flat bottom 384 well plates                                 | Corning        | 3820           |
| White non-binding flat bottom 96 well plates                                 | Perkin Elmer   | 6005290        |
| White 96 well plate, cell culture treated                                    | BrandTech      | 782090         |
| White low volume flat bottom 384 well plates                                 | Corning        | 3824           |
| Black 96 well glass bottom imaging plate                                     | IBL, Cellvis   | P96-1.5H-N     |
| Black 24 well glass bottom imaging plate                                     | IBL, Cellvis   | P24-1.5H-N     |
### Supplementary Table 13 | Fluorophores used in this study.

| Number | Structure | Name       | Ex\text{max}/Em\text{max} [nm] | Source, reference |
|--------|-----------|------------|-------------------------------|-------------------|
| 1      | ![Structure 1](image) | JF\textsubscript{525}-CA | 525/549                      | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA\textsuperscript{1} |
| 2      | ![Structure 2](image) | TMR-CA     | 548/572                      | Purchased from Promega, Madison, WI, USA\textsuperscript{2} |
| 3      | ![Structure 3](image) | 580CP-CA | 582/607                      | Gift from Dr. Alexey N. Butkevich, MPI-MF, Heidelberg, Germany\textsuperscript{3} |
| 4      | ![Structure 4](image) | JF\textsubscript{585}-CA | 585/609                      | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA\textsuperscript{1} |
| 5      | ![Structure 5](image) | CPY-CA     | 606/626                      | Butkevich et al.\textsuperscript{4} |
| 6      | ![Structure 6](image) | JF\textsubscript{635}-CA | 635/652                      | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA\textsuperscript{1} |
| 7      | ![Structure 7](image) | SiR-halo (=SiR-CA) | 643/662                      | Lukinavicius et al.\textsuperscript{5} |
| 8      | ![Structure 8](image) | JF\textsubscript{669}-CA | 669/682                      | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA\textsuperscript{1} |
| 9      | ![Structure 9](image) | Cy3-CA     | 554/568                      | Wilhelm and Kuehn et al.\textsuperscript{6} |
$R = \text{(Chloroalkane (CA))}$

$JF = \text{Janelia Fluor}$
### Supplementary Table 14 | Plasmids and stable cell lines used in this study.

| Construct | Plasmid | Gene | Entry plasmids (Addgene#) | Addgene# | Stable cell line |
|-----------|---------|------|---------------------------|----------|------------------|
| pET-51b(+) HaloTag7-EGFP | pET-51b(+) | HaloTag7-EGFP | 167266<sup>a</sup>, 130706<sup>b</sup> | n.a. | n.a. |
| pET-51b(+) ChemoG1 | pET-51b(+) | ChemoG1 | 167266<sup>a</sup>, 193799 | n.a. | n.a. |
| pET-51b(+) ChemoG1<sup>Y38A</sup> | pET-51b(+) | ChemoG1<sup>Y38A</sup> | ChemoG1 | n.a. | n.a. |
| pET-51b(+) ChemoG1<sup>R41A</sup> | pET-51b(+) | ChemoG1<sup>R41A</sup> | ChemoG1 | n.a. | n.a. |
| pET-51b(+) ChemoG1<sup>222R</sup> | pET-51b(+) | ChemoG1<sup>222R</sup> | ChemoG1 | n.a. | n.a. |
| pET-51b(+) ChemoG2 | pET-51b(+) | ChemoG2 | 193800 | n.a. | n.a. |
| pET-51b(+) ChemoG3 | pET-51b(+) | ChemoG3 | 193801 | n.a. | n.a. |
| pET-51b(+) ChemoG3.1 | pET-51b(+) | ChemoG3 | 193802 | n.a. | n.a. |
| pET-51b(+) ChemoG3.2 | pET-51b(+) | ChemoG3 | 193803 | n.a. | n.a. |
| pET-51b(+) ChemoG4 | pET-51b(+) | ChemoG4 | 193804 | n.a. | n.a. |
| pET-51b(+) ChemoG5 | pET-51b(+) | ChemoG5 | 193805 | n.a. | n.a. |
| pET-51b(+) ChemoB | pET-51b(+) | ChemoB | 54572<sup>a</sup> | n.a. | n.a. |
| pET-51b(+) ChemoC | pET-51b(+) | ChemoC | 48203<sup>4</sup> | n.a. | n.a. |
| pET-51b(+) ChemoY | pET-51b(+) | ChemoY | 39813<sup>10</sup> | n.a. | n.a. |
| pET-51b(+) ChemoR | pET-51b(+) | ChemoR | 85042<sup>21</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoG1 | pCDNA5/FRT | ChemoG1 | 167266<sup>a</sup>, 193806 | n.a. | n.a. |
| pCDNA5/FRT-ChemoG2 | pCDNA5/FRT | ChemoG2 | ChemoG1 | n.a. | n.a. |
| pCDNA5/FRT-ChemoG3 | pCDNA5/FRT | ChemoG3 | ChemoG2 | n.a. | n.a. |
| pCDNA5/FRT-ChemoG4 | pCDNA5/FRT | ChemoG4 | ChemoG3 | n.a. | n.a. |
| pCDNA5/FRT-ChemoG5 | pCDNA5/FRT | ChemoG5 | ChemoG4 | n.a. | n.a. |
| pCDNA5/FRT-ChemoB | pCDNA5/FRT | ChemoB | 54572<sup>a</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoC | pCDNA5/FRT | ChemoC | 48203<sup>4</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoY | pCDNA5/FRT | ChemoY | 39813<sup>10</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoR | pCDNA5/FRT | ChemoR | 85042<sup>21</sup> | n.a. | n.a. |
| pCDNA5/FRT-NES-ChemoG5 | pCDNA5/FRT | NES-ChemoG5 | ChemoG5 | n.a. | n.a. |
| pCDNA5/FRT-ChemoG5-PDGFR<sub>tm</sub> | pCDNA5/FRT | ChemoG5-PDGFR<sub>tm</sub> | ChemoG5, in-house plasmid<sup>12</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoG5-NLS3x | pCDNA5/FRT | ChemoG5-NLS3x | ChemoG5 | n.a. | n.a. |
| pCDNA5/FRT-[2xCox8]-ChemoG5 | pCDNA5/FRT | [2xCox8]-ChemoG5 | ChemoG5, 113916<sup>13</sup> | n.a. | n.a. |
| pCDNA5/FRT-ChemoG5-LaminB1 | pCDNA5/FRT | ChemoG5-LaminB1 | 55069 | n.a. | n.a. |
| pET-51b(+) EGFP-CaM-P30-M13-HaloTag7 | pET-51b(+) | EGFP-CaM-P30-M13-HaloTag7 | 40755<sup>14</sup> | n.a. | n.a. |
| pET-51b(+) EGFP<sup>PA206K</sup>,CaM-P30-M13-HaloTag7 | pET-51b(+) | EGFP<sup>PA206K</sup>,CaM-P30-M13-HaloTag7 | 40755<sup>14</sup> | n.a. | n.a. |
| pET-51b(+) ChemoG-CaM | pET-51b(+) | ChemoG-CaM | 40755<sup>14</sup> | n.a. | n.a. |
| pET-51b(+) EGFP<sup>PA206K,225R</sup>,CaM-P30-M13-HaloTag7<sup>PA143R,E147R,L271E</sup> | pET-51b(+) | EGFP<sup>PA206K,225R</sup>,CaM-P30-M13-HaloTag7<sup>PA143R,E147R,L271E</sup> | 40755<sup>14</sup> | n.a. | n.a. |
| pET-51b(+) EGFP<sup>PA206K,225R</sup>,CaM-P30-M13-HaloTag7<sup>PA143R,E147R,L271E</sup> | pET-51b(+) | EGFP<sup>PA206K,225R</sup>,CaM-P30-M13-HaloTag7<sup>PA143R,E147R,L271E</sup> | 40755<sup>14</sup> | n.a. | n.a. |
| pET-51b(+) ChemoB-CaM | pET-51b(+) | ChemoB-CaM | 40755<sup>14</sup> | 193812 | n.a. |
| pET-51b(+) ChemoC-CaM | pET-51b(+) | ChemoC-CaM | 40755<sup>14</sup> | 193813 | n.a. |
| pET-51b(+) ChemoY-CaM | pET-51b(+) | ChemoY-CaM | 40755<sup>14</sup> | 193814 | n.a. |
| pET-51b(+) ChemoR-CaM | pET-51b(+) ChemoR-CaM | 4075514 | 193815 | n.a. |
|------------------------|------------------------|---------|--------|------|
| pET-51b(+) YC 3.6      | pET-51b(+) YC 3.6      | 5196615 | n.a.   | n.a. |
| pCDNAS5/FRT-ChemoG-CaM| pCDNAS5/FRT-ChemoG-CaM| 4075514 | 193816 | n.a. |
| pGP-AAV2-HSyn1-NES-ChemoG-CaM| pGP-AAV2-NES-ChemoG-CaM| 10106116 | 193817 | n.a. |
| pET-51b(+) EGFP<sub>pA205K</sub>F<sub>pF</sub>-HT7 | pET-51b(+) EGFP<sub>pA205K</sub>F<sub>pF</sub>-HT7 | 5195817 | n.a.   | n.a. |
| pET-51b(+) ChemoATP   | pET-51b(+) ChemoATP   | 5195817 | n.a.   | U-2 OS Flp-In T-Rex |
| pET-51b(+) EGFP<sub>pA205K</sub>T225R,F<sub>pF</sub>-HT7 | pET-51b(+) EGFP<sub>pA205K</sub>T225R,F<sub>pF</sub>-HT7 | 5195817 | n.a.   | n.a. |
| pET-51b(+) ChemoATP   | pET-51b(+) ChemoATP   | 5195817 | n.a.   | n.a. |
| pET-51b(+) ChemoR-ATP | pET-51b(+) ChemoR-ATP | 5195817 | n.a.   | n.a. |
| pET-51b(+) pCDNAS5/FRT | pET-51b(+) pCDNAS5/FRT | ATeam 1.03 | 5195817 | n.a. |
| pCDNAS5/FRT-ChemoG-ATP| pCDNAS5/FRT-ChemoG-ATP| 5195817 | 193818 | n.a. |
| pCDNAS5/FRT-ChemoB-ATP| pCDNAS5/FRT-ChemoB-ATP| 5195817 | 193819 | n.a. |
| pCDNAS5/FRT-ChemoB-ATP| pCDNAS5/FRT-ChemoB-ATP| 5195817 | 193820 | n.a. |
| pCDNAS5/FRT-TO-Team 1.03 | pCDNAS5/FRT-TO-Team 1.03 | ATeam 1.03 | 5195817 | U-2 OS Flp-In T-Rex |
| pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-D289N</sup>,HT7 | pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-D289N</sup>,HT7 | - | n.a.   | n.a. |
| pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-D289N-V220A</sup>,HT7 | pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-D289N-V220A</sup>,HT7 | - | n.a.   | n.a. |
| pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-V220W-D289N-V250A</sup>,HT7 | pET-51b(+) EGFP<sub>pA205K</sub>ttLig<sub>A</sub><sup>118L-V220W-D289N-V250A</sup>,HT7 | - | n.a.   | n.a. |
| pET-51b(+) ChemoG-NAD  | pET-51b(+) ChemoG-NAD  | - | n.a.   | n.a. |
| pET-51b(+) EGFP<sub>pA205K</sub>T225R,ttLig<sub>A</sub><sup>K118L-V220W-D289N-V250A</sup>-HT7 | pET-51b(+) EGFP<sub>pA205K</sub>T225R,ttLig<sub>A</sub><sup>K118L-V220W-D289N-V250A</sup>-HT7 | - | n.a.   | n.a. |
| pET-51b(+) ChemoB-NAD  | pET-51b(+) ChemoB-NAD  | - | n.a.   | n.a. |
| pET-51b(+) ChemoR-NAD  | pET-51b(+) ChemoR-NAD  | - | n.a.   | n.a. |
| pCDNAS5/FRT-ChemoG-NAD | pCDNAS5/FRT-ChemoG-NAD | - | 193821 | U-2 OS Flp-In T-Rex |
| pCDNAS5/FRT-ChemoG-NAD-NLS3x | pCDNAS5/FRT-ChemoG-NAD-NLS3x | - | 193822 | U-2 OS Flp-In T-Rex |
| pCDNAS5/FRT-TO-[4xCox8]-ChemoG-NAD | pCDNAS5/FRT-TO-[4xCox8]-ChemoG-NAD | - | 193823 | U-2 OS Flp-In T-Rex |
| pCDNAS5/FRT-ChemoB-NAD | pCDNAS5/FRT-ChemoB-NAD | - | 193824 | U-2 OS Flp-In T-Rex |
| pCDNAS5/FRT-ChemoB-NAD-NLS3x | pCDNAS5/FRT-ChemoB-NAD-NLS3x | - | n.a.   | n.a. |
| pCDNAS5/FRT-ChemoR-NAD | pCDNAS5/FRT-ChemoR-NAD | - | 193825 | U-2 OS Flp-In T-Rex |
| pCDNAS5/FRT-ChemoB-NAD-NLS3x-[hsOpti]-NLS3x-T2A-[4xCox8]-ChemoG-NAD-[opti] | pCDNAS5/FRT-ChemoB-NAD-NLS3x-[hsOpti]-NLS3x-T2A-[4xCox8]-ChemoG-NAD-[opti] | - | n.a.   | n.a. |
| pET-51b(+) ChemoO-NAD  | pET-51b(+) ChemoO-NAD  | - | n.a.   | n.a. |
| pCDNAS5/FRT-ChemoD-NAD | pCDNAS5/FRT-ChemoD-NAD | - | 193826 | U-2 OS Flp-In T-Rex |
| pET-51b(+) ChemoL-NAD | pET-51b(+) ChemoL-NAD | - | 193826 | U-2 OS Flp-In T-Rex |
| pET-51b(+) ChemoL-CaM | pET-51b(+) ChemoL-CaM | 11790919 | n.a.   | n.a. |
| pET-51b(+) ChemoL-ATP | pET-51b(+) ChemoL-ATP | 11790919 | n.a.   | n.a. |
| pCDNAS5/FRT-ChemoL-NAD | pCDNAS5/FRT-ChemoL-NAD | - | 193826 | n.a. |
| pCDNAS5/FRT-[4xCox8]-ChemoL-NAD | pCDNAS5/FRT-[4xCox8]-ChemoL-NAD | - | 193829 | n.a. |
| pCDNAS5/FRT-ChemoL-CaM | pCDNAS5/FRT-ChemoL-CaM | - | 193830 | n.a. |
| pCDNAS5/FRT-ChemoL-ATP | pCDNAS5/FRT-ChemoL-ATP | - | 193831 | n.a. |
| pET-51b(+) His-TEV-ChemoG1 | pET-51b(+) His-TEV-ChemoG1 | 1672666 | n.a.   | n.a. |
| pET-51b(+) His-TEV-ChemoG5 | pET-51b(+) His-TEV-ChemoG5 | 1672666 | n.a.   | n.a. |
| pET-51b(+) His-TEV-HaloTag7 | pET-51b(+) His-TEV-HaloTag7 | HT7 | 1672666 | n.a. |

n.a. = not available.
## Supplementary Table 15 | Data collection and refinement statistics.

| Data collection                  | HaloTag7-Cy3 8B6R | ChemoG1-TMR 8B6S | ChemoG5-TMR 8B6T |
|----------------------------------|------------------|-----------------|-----------------|
| **Space group**                  | P4₁2₁2           | P1              | P12₁1           |
| **Unit-cell parameters**         |                  |                 |                 |
| a, b, c (Å)                      | 112.56, 112.56, 44.33 | 46.19, 63.71, 89.42 | 46.60, 64.04, 172.95 |
| α, β, γ (°)                      | 90.00, 90.00, 90.00 | 93.56, 91.02, 90.85 | 90.00, 97.67, 90.00 |
| **Radiation source**             | PXII-X10SA, SLS  | PXII-X10SA, SLS | PXII-X10SA, SLS |
| **Wavelength (Å)**               | 0.99988          | 0.99996         | 0.99992         |
| **Temperature (K)**              | 100              | 100             | 100             |
| **Resolution range (Å)**         | 50.150 (1.60-1.50) | 50-1.80 (1.90-1.80) | 50-2.00 (2.10-2.00) |
| **No. of observed reflections**  | 341056 (60343)   | 182229 (26711)  | 216345 (30251)  |
| **No. of unique reflections**    | 46121 (7965)     | 89852 (13310)   | 66470 (9089)    |
| **Multiplicity**                 | 7.4 (7.6)        | 2.0 (2.0)       | 3.3 (3.3)       |
| **Completeness (%)**             | 99.9 (99.9)      | 95.3 (94.3)     | 97.0 (97.8)     |
| **R_{merge} (%)**                | 6.8 (65.7)       | 4.1 (40.0)      | 8.6 (41.0)      |
| **<I/σ(I)>**                     | 18.2 (3.4)       | 12.0 (2.1)      | 8.5 (3.4)       |
| **CC₁/₂ (%)**                    | 99.9 (90.2)      | 99.8 (75.2)     | 99.5 (87.4)     |
| **Refinement**                   |                  |                 |                 |
| **Molecules per a.u.**           | 1                | 2               | 2               |
| **No. of reflections**           | 46120            | 89842           | 66470           |
| **No. of reflections in test set** | 2306           | 4492            | 3399            |
| **Resolution range (Å)**         | 41.25-1.50       | 46.18-1.80      | 46.18-2.00      |
| **No. of non-hydrogen atoms**    |                  |                 |                 |
| Protein                          | 2365             | 8273            | 8276            |
| Ligand/ion                       | 72               | 146             | 134             |
| Water                            | 297              | 460             | 308             |
| Total                            | 2734             | 8879            | 8718            |
| R (%)                            | 16.20            | 17.28           | 22.06           |
| R_{free} (%)                     | 19.19            | 20.08           | 24.51           |
| **RMS deviations from ideal**    |                  |                 |                 |
| bonds (Å)                        | 0.013            | 0.007           | 0.002           |
| angles (°)                       | 1.229            | 1.094           | 0.779           |
| **B-factors (Å²)**               |                  |                 |                 |
| Protein                          | 14.80            | 26.03           | 20.62           |
| Ligand/ion                       | 23.87            | 22.14           | 17.73           |
| Water                            | 24.40            | 29.14           | 19.67           |
| Average                          | 16.08            | 26.13           | 20.54           |
| **Wilson B (Å²)**                | 14.42            | 24.95           | 22.88           |
| **Ramachandran statistics (%)**  |                  |                 |                 |
| favored regions                  | 95.9             | 97.5            | 96.8            |
| allowed regions                  | 4.1              | 2.5             | 3.2             |
| disallowed regions               | 0                | 0               | 0               |
| Clashscore                       | 1.04             | 1.33            | 3.03            |

*as implemented in XDS. Values in parentheses are for the highest resolution shell.*
Supplementary Table 16 | Spectral settings for fluorescence spectroscopy measurements.

| Chromophore/fluorophore | Max. emission wavelength | Excitation wavelength used | Emission wavelength range measured |
|-------------------------|-------------------------|---------------------------|-----------------------------------|
| EBFP2                   | 446 nm                  | 360 nm                    | 400-800 nm                        |
| mCerulean3              | 474 nm                  | 400 nm                    | 440-800 nm                        |
| EGFP                    | 510 nm                  | 440 nm                    | 480-800 nm                        |
| Venus                   | 528 nm                  | 460 nm                    | 494-800 nm                        |
| mKO2                    | 566 nm                  | 510 nm                    | 550-800 nm                        |
| TagRFP                  | 584 nm                  | 510 nm                    | 550-800 nm                        |
| mRuby2                  | 594 nm                  | 510 nm                    | 550-800 nm                        |
| mRuby3                  | 594 nm                  | 510 nm                    | 550-800 nm                        |
| mScarlet                | 594 nm                  | 520 nm                    | 560-800 nm                        |
| mCherry                 | 610 nm                  | 530 nm                    | 570-800 nm                        |
| JF525                   | 554 nm                  | -                         | -                                 |
| TMR                     | 576 nm                  | -                         | -                                 |
| Cy3                     | 576 nm                  | -                         | -                                 |
| 580CP                   | 606 nm                  | -                         | -                                 |
| JF585                   | 612 nm                  | -                         | -                                 |
| CPY                     | 628 nm                  | 580 nm                    | 610-750 nm                        |
| JF635                   | 662 nm                  | 610 nm                    | 640-750 nm                        |
| Cy5                     | 664 nm                  | -                         | -                                 |
| SiR                     | 666 nm                  | 610 nm                    | 640-750 nm                        |
| JF669                   | 688 nm                  | -                         | -                                 |
| YC 3.6                  | 474/528 nm              | 400 nm                    | 440-650 nm                        |
| ATeam 1.03              | 474/528 nm              | 400 nm                    | 440-650 nm                        |
**Supplementary Table 17| Analyte concentration ranges used for sensor titrations.**

| Experiment                                                                 | Analyte                        | 10x concentration (range) | Final 1x concentration (range) |
|----------------------------------------------------------------------------|--------------------------------|----------------------------|--------------------------------|
| ChemoX-CaM titration with free Ca\(^{2+}\) (Fig. 2c, d, ED3d, g, j)       | Free Ca\(^{2+}\)              | -                         | 10 nM – 39 μM*                  |
| ChemoX-CaM response to free Ca\(^{2+}\) at different pH (ED3k)           | CaCl\(_2\)                     | 20 mM                     | 2 mM                           |
|                                                                            | EGTA                           | 20 mM                     | 2 mM                           |
| RFP-based calcium sensor responses to free Ca\(^{2+}\) (Fig. S5)           | CaCl\(_2\)                     | 20 mM                     | 2 mM                           |
|                                                                            | EGTA                           | 20 mM                     | 2 mM                           |
| ChemoX-ATP titration with ATP or structurally similar analytes (Fig. 3c, ED5c) | ATP                            | 0.1 - 100 mM              | 0.01 - 10 mM                   |
|                                                                            | ADP                            | 0.1 - 100 mM              | 0.01 - 10 mM                   |
|                                                                            | AMP                            | 0.1 - 100 mM              | 0.01 - 10 mM                   |
|                                                                            | GTP                            | 0.1 - 100 mM              | 0.01 - 10 mM                   |
| ChemoX-NAD titration with NAD\(^{+}\) or structurally similar analytes (Fig. 4c, d, ED6e) | NAD\(^{+}\) (titration)       | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | NAM (constant)                 | 10 mM                     | 1 mM                           |
|                                                                            | NR (constant)                  | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | NMN                            | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | NADH                           | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | NADP\(^{+}\) (constant)        | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | NAA\(^{+}\) (constant)         | 10 nM – 100 mM            | 1 nM – 10 mM                   |
|                                                                            | ATP (constant)                 | 10 mM                     | 1 mM                           |
|                                                                            | ADP (constant)                 | 10 mM                     | 1 mM                           |
|                                                                            | AMP (constant)                 | 10 mM                     | 1 mM                           |
| ChemoD-NAD titration with NAD\(^{+}\) (intensiometric, Fig. 5c, ED9b, d, e) | NAD\(^{+}\) (intensiometric)  | 100 nM – 100 mM           | 10 nM – 10 mM                  |
| ChemoD-NAD titration with NAD\(^{+}\) (fluorescence lifetime, Fig. 5f, ED9f-i) | NAD\(^{+}\)                    | 1 μM – 100 mM             | 100 nM – 10 mM                 |
| ChemoL-NAD titration with NAD\(^{+}\) (Fig. 6c)                           | NAD\(^{+}\)                    | 100 nM – 100 mM           | 10 nM – 10 mM                  |
| ChemoL-CaM titration with free Ca\(^{2+}\) (ED10d)                        | Free Ca\(^{2+}\)              | -                         | 50 nM – 39 μM*                  |
| ChemoL-ATP titration with ATP (ED10f)                                      | NAD\(^{+}\)                    | 100 nM – 100 mM           | 10 nM – 10 mM                  |

*For titrations of calcium sensors, special calcium buffers with defined concentrations of free Ca\(^{2+}\) were prepared (see Analyte titrations of biosensors below for details).
### Supplementary Table 18 | Settings for confocal and widefield fluorescence microscopy.

| Figure | Construct | Label | Microscope | Objective | Excitation [nm] | Emission [nm] | Pixel dwell time [μs] | Size [pixels] | Z size [μm] |
|--------|-----------|-------|------------|-----------|----------------|--------------|----------------------|--------------|-------------|
| 1f     | ChemoG5-NLS | -     | Confocal   | 40x/1.10 water | 480 | 490-540 | 3.16 | 512x512 | 5 |
|        | ChemoG5-NLS | TMR   | Confocal   | 40x/1.10 water | 480 | 490-540/550-600 | 3.16 | 512x512 | 5 |
|        | ChemoG5-NLS | CPY   | Confocal   | 40x/1.10 water | 480 | 490-540/620-670 | 3.16 | 512x512 | 5 |
|        | ChemoG5-NLS | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
| 1h     | ChemoB     | SiR   | Confocal   | 40x/1.10 water | 405 | 420-470/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoC     | SiR   | Confocal   | 40x/1.10 water | 405 | 460-500/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG5    | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoY     | SiR   | Confocal   | 40x/1.10 water | 505 | 515-565/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoR     | SiR   | Confocal   | 40x/1.10 water | 550 | 570-620/650-700 | 3.16 | 512x512 | 5 |
| 2e     | ChemoG-CaM | SiR   | Widefield  | 20x/0.80 dry | 470* | 525/50, 700/75** | n.d. | 512x512 | 2 |
| 2g     | ChemoG-CaM | SiR   | Widefield  | 20x/0.80 dry | 470* | 525/50, 700/75** | n.d. | 256x256 | 0 |
| 3d     | ChemoG-ATP | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
| 3f     | ChemoB-ATP | SiR   | Confocal   | 40x/1.10 water | 405 | 420-470/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG-ATP | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG-ATP | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoR-ATP | SiR   | Confocal   | 40x/1.10 water | 550 | 570-620/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoY     | SiR    | Confocal   | 40x/1.10 water | 550 | 570-620/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoR     | SiR    | Confocal   | 40x/1.10 water | 550 | 570-620/650-700 | 3.16 | 512x512 | 5 |
| 4e     | ChemoG-NAD | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 0 |
| 4g     | ChemoB-NAD-cyto | SiR | Confocal   | 40x/1.10 water | 405 | 420-460/650-700 | 3.84 | 2048x2048 | 0 |
|        | ChemoG-NAD-mito | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.84 | 2048x2048 | 0 |
| 4h     | ChemoB-NAD-cyto | SiR | Confocal   | 40x/1.10 water | 405 | 420-460/650-700 | 3.16 | 512x512 | 4 |
|        | ChemoG-NAD-mito | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 4 |
| 5f     | ChemoD-NAD | CPY   | Confocal   | 40x/1.10 water | 610 | 620-670 | 1.75 | 512x512 | 0 |
|        | ChemoD-NAD | JF635  | Confocal   | 40x/1.10 water | 640 | 650-700 | 1.75 | 512x512 | 0 |
|        | ChemoD-NAD | SiR    | Confocal   | 40x/1.10 water | 625 | 635-685 | 1.75 | 512x512 | 0 |
| 5g/h   | ChemoD-NAD | CPY   | Confocal   | 40x/1.10 water | 610 | 620-670 | 7.69 | 512x512 | 0 |
| S4a    | HT-EGFP   | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG1-5 | SiR   | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG5-NES | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG5-PDGFR | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 0 |
|        | ChemoG5-NLS | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 5 |
|        | ChemoG5-Cox8 | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 0 |
|        | ChemoG5-LaminB1 | SiR | Confocal   | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 0 |
| ED7a   | ChemoG-NAD-NLS | SiR | Confocal   | 40x/1.10 water | 480 | 490/540/650-700 | 3.16 | 512x512 | 0 |
| ED7b   | ChemoG-NAD-mito | SiR | Confocal   | 40x/1.10 water | 480 | 490/540/650-700 | 3.16 | 512x512 | 0 |
| S8a    | ChemoB-NAD | SiR   | Confocal   | 40x/1.10 water | 405 | 420-470/650/700 | 3.16 | 512x512 | 0 |
|   | ChemoR-NAD | SiR | Confocal | 40x/1.10 water | 550 | 570-620/650-700 | 3.16 | 512x512 | 0 |
|---|------------|-----|----------|----------------|-----|-----------------|------|---------|---|
| ED8a | ChemoB-NAD-cyto | SiR | Confocal | 40x/1.10 water | 405 | 420-460/650-700 | 3.16 | 512x512 | 4 |
|    | ChemoG-NAD-mito | SiR | Confocal | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 4 |
| ED8b | ChemoB-NAD-NLS | SiR | Confocal | 40x/1.10 water | 405 | 420-460/650-700 | 3.16 | 512x512 | 0 |
|    | ChemoG-NAD-mito | SiR | Confocal | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 0 |
| ED8c | ChemoB-NAD-NLS | SiR | Confocal | 40x/1.10 water | 405 | 420-460/650-700 | 3.16 | 512x512 | 4 |
|    | ChemoG-NAD-mito | SiR | Confocal | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 4 |
| ED8d | ChemoB-NAD-NLS | SiR | Confocal | 40x/1.10 water | 405 | 420-460/650-700 | 3.16 | 512x512 | 4 |
|    | ChemoG-NAD-mito | SiR | Confocal | 40x/1.10 water | 480 | 490-540/650-700 | 3.16 | 512x512 | 4 |
| ED9i/g | ChemoG-NAD | SiR | Confocal | 40x/1.10 water | 640 | 650-700 | 1.75 | 512x512 | 0 |
|    | ChemoD-NAD | SiR | Confocal | 40x/1.10 water | 640 | 650-700 | 1.75 | 512x512 | 0 |
| ED9h | ChemoD-NAD | CPY | Confocal | 40x/1.10 water | 610 | 620-670 | 1.75 | 512x512 | 0 |
| ED9i | ChemoD-NAD | JF<sub>635</sub> | Confocal | 40x/1.10 water | 620 | 635-685 | 1.75 | 512x512 | 0 |
| ED9j/k | ChemoD-NAD | SiR | Confocal | 40x/1.10 water | 640 | 650-700 | 7.69 | 512x512 | 0 |
| ED9l/m | ChemoD-NAD | JF<sub>635</sub> | Confocal | 40x/1.10 water | 620 | 635-685 | 7.69 | 512x512 | 0 |

*470 nm LED was used together with a 474/24 nm bandpass filter. **525/50 nm and 700/75 nm bandpass filters were used for acquisition of EGFP and FRET(SiR) fluorescence, respectively
Supplementary Notes

Supplementary Note 1 – Development of ChemoG biosensors.

**Generation of sensor variants.** Certain ChemoG interface mutations increase FRET to a larger extent than others. For example, the interface mutation T225R\(_{\text{EGFP}}\) usually leads to a stronger FRET increase than the interface mutation L271E\(_{\text{HT7}}\). This feature revealed useful to fine-tune the dynamic range of ChemoG-based sensors. For the generation of new sensors (Fig. S21), we recommend to try a palette of ChemoG FRET pairs with different interface mutations (**Supplementary Table 11**, available on Addgene). The sensing domain can be derived from an existing biosensor as *e.g.* ChemoG-CaM that was derived from YC 3.6\(^{22}\) or a new sensing domain, preferentially exhibiting a large conformational change. To create ChemoG sensor variants, the sensing domain should be cloned between the EGFP and HaloTag7 variants (*i.e.* ChemoG FRET pairs). Using ChemoG-encoding plasmids and DNA encoding the sensing domain of interest, 6 plasmids encoding sensor variants can simply be obtained through PCR and molecular cloning (*e.g.* by Gibson assembly\(^{23}\)). We recommend to use single GGS linkers connecting the ChemoG FRET pairs with the sensing domain but these can also be further engineered in a second step if necessary. The linkers can be created during the design of the primers used for the PCR amplification of the fragments. We deposited plasmids encoding ChemoG variants for protein production in *E. coli*. In case the sensor variants should be tested in mammalian cells, the vector backbone should first be exchanged.

**Testing sensor variants in vitro or in cells.** Two options are available:

- produce the sensor variants in *E. coli*, purify them and test them *in vitro*, or
- express and test the sensor variants in mammalian cells (require extra sub-cloning, see above).

For the first option, the purified sensor variants should be labeled with an orange/red fluorophore substrate. We recommend SiR-halo or a rhodamine substrate with similar spectral properties to minimize direct excitation of the synthetic fluorophore. The labeled sensors should then be titrated with different concentrations of an analyte of interest (AOI). The sensor variant with the largest dynamic range can be identified from fluorescence emission spectra. An ideal sensor exhibits low FRET in absence and high FRET in presence of the AOI (*or vice versa*), showing a large peak inversion in each emission channel. Some noticeable fluorescence should remain in both channels for precise measurements.

For the second option, mammalian cells should be transfected with plasmids encoding the sensor variants. The transfected cells should be labeled with cell-permeable fluorophore substrates. As previously, we recommend SiR-halo. Labeled cells can subsequently be treated with reagents known to act on the biological activity of interest (*e.g.* AOI concentration change). Via fluorescence microscopy or flow cytometry, the fluorescence profile of treated and untreated cells can be compared to identify sensor variants with the largest dynamic range. Sensors presenting noticeable fluorescence signal in both channels in presence and absence of treatment should be chosen in order to ensure precise measurement.

Technical details on how to conduct the different experiments can be found in the method section of the manuscript.
Supplementary Note 2 – Tuning the spectral properties of the optimized ChemoG sensor.
The spectral properties of the optimized ChemoG sensor can be tuned by exchanging the FRET donor EGFP with other fluorescent proteins and/or by using different fluorophore substrates as FRET acceptor (Supplementary Fig. 12). For exchanging the FRET donor, EGFP is substituted with an alternative fluorescent protein (e.g. EBFP2 = ChemoB) with the same interface mutations (e.g. EGFP\textsuperscript{T225R} $\rightarrow$ EBFP2\textsuperscript{T225R}) via molecular cloning. As FRET donor, we recommend EGFP-derived fluorescent proteins such as EBFP2, mCerulean3 or Venus to ensure a good transferability of the interface mutations. We recommend to use FP constructs we deposited on Addgene to ensure that the adequate FP mutations are used. For red fluorescent proteins, we recommend using mRuby2 without additional mutations for biosensor design. The FRET acceptor can be readily chosen by simply labeling the ChemoX sensors with different rhodamine-based HaloTag substrates (e.g. JF\textsubscript{525}, CPY or JF\textsubscript{669}). The ChemoX sensors performance can be evaluated as explained in Supplementary Note 1 and in the methods.

Supplementary Note 3 – Tuning the readout mode of the optimized ChemoG sensor.
The readout of ChemoG FRET sensors can be tuned by small modifications (Supplementary Fig. 13). Single channel fluorescence intensity and fluorescence lifetime-based ChemoD sensors are obtained by substituting EGFP with its non-fluorescent variant ShadowG\textsuperscript{18} carrying the same interface mutation(s). Additionally, the fluorescence quenching mutation P174W should be introduced into HaloTag7. For intensiometric sensors, we recommend labeling with JF\textsubscript{635} while for fluorescence lifetime imaging, CPY worked best in our hands so far. The performance of the sensors can be evaluated analogously as explained in Supplementary Note 1 and in the methods.

To convert ChemoG FRET sensors into a bioluminescent ChemoL sensor, a circularly permuted variant of NanoLuc is fused to the N-terminus of EGFP. We recommend labeling the sensor with rhodamine fluorophore substrates whose spectral properties are compatible with the available equipment. In our case, CPY was the most red-shifted fluorophore compatible with our plate reader but we foresee no conceptual hurdle in using any rhodamine fluorophore substrate proven functional for FRET biosensing. The sensors performance can be evaluated analogously as explained in Supplementary Note 1 and in the methods. It should be noted, that the expression of ChemoL sensors in mammalian cells, assessed by direct excitation of EGFP, was found to be substantially lower than the corresponding ChemoG FRET sensors. Since bioluminescent signals can be detected with high sensitivity, i.e. also for sensors with low expression levels, it is possible to acquire robust emission spectra for ChemoL biosensors. Due to the dim EGFP signal, however, it is not advisable to use ChemoL sensors for FRET applications even if this is conceptually possible.
Supplementary Note 4 – Protein sequences

Static FRET constructs

>ChemoG5
MVSKGEEFGVTVVPGVLDGVDN3GKFSVSGSECEGADAYGKTLTKICTGKLVPVWPTLTTTLTYGVQCFSPYD
HKMQHDFKSSAMPEGYQVRTEIFKKDDGNYKTRAEVKFEQDOKTLVNRIELKGDIFKEDGNLHKGLEYNYSNHVNTYIM
ADKQXNGIKVFRKIRHLNEDGSVLADHQQNTPIDGPVLVLPDNYLSTQSLKGDPEKRDMVLPVDXYFLAAGIT
LMGDELYKIGTGFFPDHYVEVLGERMHYDVGPRDGTVPVFLHGNTPSSYVRNNIIIHVAPTHCRAPDLIGMGKSDPKDLY
FKDDHMRDMDAFIEALGEEVVLHIDWDSAGLFCXKPRNVKXKAFMEFIRPIPTTWDFEFKAPATF
QAFRTDVGKLRIDQNVFIEGTLMVPMGVVPRLTEVMDHYREPLFNPQDREPLWRFPFNELPAINEPANIVALVEEYM
DLWQSPVPPKLLFWGTPVGLAPPAEALKLAPLCKVAKAVDIPGGLNLLQEDNPDLIGSEIARWSTLEISG

>ChemoB
MVSKGEEFGVTVVPGVLDGVDN3GKFSVSGSECEGADAYGKTLTKICTGKLVPVWPTLTTTLTYGVQCFSPYD
HKMQHDFKSSAMPEGYQVRTEIFKKDDGNYKTRAEVKFEQDOKTLVNRIELKGDIFKEDGNLHKGLEYNYSNHVNTYIM
AVKQXNGIKVFRKIRHLNEDGSVLADHQQNTPIDGPVLVLPDNYLSTQSLKGDPEKRDMVLPVDXYFLAAGIT
LMGDELYKIGTGFFPDHYVEVLGERMHYDVGPRDGTVPVFLHGNTPSSYVRNNIIIHVAPTHCRAPDLIGMGKSDPKDLY
FKDDHMRDMDAFIEALGEEVVLHIDWDSAGLFCXKPRNVKXKAFMEFIRPIPTTWDFEFKAPATF
QAFRTDVGKLRIDQNVFIEGTLMVPMGVVPRLTEVMDHYREPLFNPQDREPLWRFPFNELPAINEPANIVALVEEYM
DLWQSPVPPKLLFWGTPVGLAPPAEALKLAPLCKVAKAVDIPGGLNLLQEDNPDLIGSEIARWSTLEISG

>ChemoC
MVSKGEEFGVTVVPGVLDGVDN3GKFSVSGSECEGADAYGKTLTKICTGKLVPVWPTLTTTLTYGVQCFSPYD
HKMQHDFKSSAMPEGYQVRTEIFKKDDGNYKTRAEVKFEQDOKTLVNRIELKGDIFKEDGNLHKGLEYNYSNHVNTYIM
ADKQXNGIKVFRKIRHLNEDGSVLADHQQNTPIDGPVLVLPDNYLSTQSLKGDPEKRDMVLPVDXYFLAAGIT
LMGDELYKIGTGFFPDHYVEVLGERMHYDVGPRDGTVPVFLHGNTPSSYVRNNIIIHVAPTHCRAPDLIGMGKSDPKDLY
FKDDHMRDMDAFIEALGEEVVLHIDWDSAGLFCXKPRNVKXKAFMEFIRPIPTTWDFEFKAPATF
QAFRTDVGKLRIDQNVFIEGTLMVPMGVVPRLTEVMDHYREPLFNPQDREPLWRFPFNELPAINEPANIVALVEEYM
DLWQSPVPPKLLFWGTPVGLAPPAEALKLAPLCKVAKAVDIPGGLNLLQEDNPDLIGSEIARWSTLEISG

>ChemoY
MVSKGEEFGVTVVPGVLDGVDN3GKFSVSGSECEGADAYGKTLTKICTGKLVPVWPTLTTTLTYGVQCFSPYD
HKMQHDFKSSAMPEGYQVRTEIFKKDDGNYKTRAEVKFEQDOKTLVNRIELKGDIFKEDGNLHKGLEYNYSNHVNTYIM
ADKQXNGIKVFRKIRHLNEDGSVLADHQQNTPIDGPVLVLPDNYLSTQSLKGDPEKRDMVLPVDXYFLAAGIT
LMGDELYKIGTGFFPDHYVEVLGERMHYDVGPRDGTVPVFLHGNTPSSYVRNNIIIHVAPTHCRAPDLIGMGKSDPKDLY
FKDDHMRDMDAFIEALGEEVVLHIDWDSAGLFCXKPRNVKXKAFMEFIRPIPTTWDFEFKAPATF
QAFRTDVGKLRIDQNVFIEGTLMVPMGVVPRLTEVMDHYREPLFNPQDREPLWRFPFNELPAINEPANIVALVEEYM
DLWQSPVPPKLLFWGTPVGLAPPAEALKLAPLCKVAKAVDIPGGLNLLQEDNPDLIGSEIARWSTLEISG

>ChemoR
MVSKGEEFGVTVVPGVLDGVDN3GKFSVSGSECEGADAYGKTLTKICTGKLVPVWPTLTTTLTYGVQCFSPYD
HKMQHDFKSSAMPEGYQVRTEIFKKDDGNYKTRAEVKFEQDOKTLVNRIELKGDIFKEDGNLHKGLEYNYSNHVNTYIM
ADKQXNGIKVFRKIRHLNEDGSVLADHQQNTPIDGPVLVLPDNYLSTQSLKGDPEKRDMVLPVDXYFLAAGIT
LMGDELYKIGTGFFPDHYVEVLGERMHYDVGPRDGTVPVFLHGNTPSSYVRNNIIIHVAPTHCRAPDLIGMGKSDPKDLY
FKDDHMRDMDAFIEALGEEVVLHIDWDSAGLFCXKPRNVKXKAFMEFIRPIPTTWDFEFKAPATF
QAFRTDVGKLRIDQNVFIEGTLMVPMGVVPRLTEVMDHYREPLFNPQDREPLWRFPFNELPAINEPANIVALVEEYM
DLWQSPVPPKLLFWGTPVGLAPPAEALKLAPLCKVAKAVDIPGGLNLLQEDNPDLIGSEIARWSTLEISG

EGFP, EBFP2, mCerulean3, Venus, mScarlet

HaloTag7
Interface mutations (XFP^{A206K}, XFP^{T225R}, HT7^{E143R}, HT7^{E147R}, HT7^{L271E}, mScarlet^{D201K}, EBFP2^{N39Y})
Calcium sensors

>ChemoG-CaM

MV5GKEElFTGVPVILPELGDVNGHKSFSVSEEGEDATYGLKLTFICTTTGLPVPWPTLVTTLTYGVCFSRYPD
HMKQHDFKKSAMPEGYVQERTIFKFDDGNYKTRAEVKFEQGDTLVNRIELKGIDFKEDGNILGHKLEYNSHNYIT
ADKQNGIKVFKRHNIEDGSVQLADHYQNTPIGDGPVPLPDDHYLSTQSLKDPEKRDMVLLVEFVTAAGIT
GGTLLPDQLTEEQIAEKEAFSLFDKGDGTTITTELETGMTRSLQGQNTEAEQLDMINEVDADGDGTDIFPEFLTMMA
RKMKTDDOTEETEAREFVFDKDGNGYISAAELHVMTNLGKEKLTDDEEVEEDMIREDIDGDGVQYNEEFVVMTAKEF
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMSVDSRKRKFNKTKGKALRAIGRLSSLSSGIGTGFPFDPHYVEVL
GEMHYVDPGRDTPVPLHLHGMPTSSYVWNR1IPHAVAPTHRCAPDLIGMDKSDKPDLGYFFDDHRVMDAIEAL
GLEEVLVILHDWSALGFHWAKRNPERVKQGIAMEFIRIPPTEDWEPEFARETFQAFRTTDVGKLIIDQNVFIEGT
LMGVVVRPLTEVEMHDYREFRPLFPNFLPELPIAGENIVALVEEYMWDLHQSPFKLLFWGTPGVLIAPP
AEAAARLKLSPNCVKADIGPPGELLQEDNFDLIGSEIARWLSTLEISG

>ChemoB-CaM

MV5GKEElFTGVPVILPELGDVNGHKSFSVSEEGEDATYGLKLTFICTTGTLKLVWPTLVTTLSGWVVCFAFYPD
HMKQHDFKKSAMPEGYVQERTIFKFDDGNYKTRAEVKFEQGDTLVNRIELKGIDFKEDGNILGHKLEYNSHNYIT
ADKQNGIKVFKRHNIEDGSVQLADHYQNTPIGDGPVPLPDDHYLSTQSLKDPEKRDMVLLVEFVTAAGIT
GGTLLPDQLTEEQIAEKEAFSLFDKGDGTTITTELETGMTRSLQGQNTEAEQLDMINEVDADGDGTDIFPEFLTMMA
RKMKTDDOTEETEAREFVFDKDGNGYISAAELHVMTNLGKEKLTDDEEVEEDMIREDIDGDGVQYNEEFVVMTAKEF
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMSVDSRKRKFNKTKGKALRAIGRLSSLSSGIGTGFPFDPHYVEVL
GEMHYVDPGRDTPVPLHLHGMPTSSYVWNR1IPHAVAPTHRCAPDLIGMDKSDKPDLGYFFDDHRVMDAIEAL
GLEEVLVILHDWSALGFHWAKRNPERVKQGIAMEFIRIPPTEDWEPEFARETFQAFRTTDVGKLIIDQNVFIEGT
LMGVVVRPLTEVEMHYREFRPFLPNVDFRELPNFLPLELPIAGENIVALVEEYMWDLHQSPFKLLFWGTPGVLIAPP
AEAAARLKLSPNCVKADIGPPGELLQEDNFDLIGSEIARWLSTLEISG

>ChemoC-CaM

MV5GKEElFTGVPVILPELGDVNGHKSFSVSEEGEDATYGLKLTFICTTGTLKLVWPTLVTTLSGWVVCFAFYPD
HMKQHDFKKSAMPEGYVQERTIFKFDDGNYKTRAEVKFEQGDTLVNRIELKGIDFKEDGNILGHKLEYNSHNYIT
ADKQNGIKVFKRHNIEDGSVQLADHYQNTPIGDGPVPLPDDHYLSTQSLKDPEKRDMVLLVEFVTAAGIT
GGTLLPDQLTEEQIAEKEAFSLFDKGDGTTITTELETGMTRSLQGQNTEAEQLDMINEVDADGDGTDIFPEFLTMMA
RKMKTDDOTEETEAREFVFDKDGNGYISAAELHVMTNLGKEKLTDDEEVEEDMIREDIDGDGVQYNEEFVVMTAKEF
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMSVDSRKRKFNKTKGKALRAIGRLSSLSSGIGTGFPFDPHYVEVL
GEMHYVDPGRDTPVPLHLHGMPTSSYVWNR1IPHAVAPTHRCAPDLIGMDKSDKPDLGYFFDDHRVMDAIEAL
GLEEVLVILHDWSALGFHWAKRNPERVKQGIAMEFIRIPPTEDWEPEFARETFQAFRTTDVGKLIIDQNVFIEGT
LMGVVVRPLTEVEMHYREFRPFLPNVDFRELPNFLPLELPIAGENIVALVEEYMWDLHQSPFKLLFWGTPGVLIAPP
AEAAARLKLSPNCVKADIGPPGELLQEDNFDLIGSEIARWLSTLEISG

>ChemoY-CaM

MV5GKEElFTGVPVILPELGDVNGHKSFSVSEEGEDATYGLKLTFICTTGTLKLVWPTLVTTLSGWVVCFAFYPD
HMKQHDFKKSAMPEGYVQERTIFKFDDGNYKTRAEVKFEQGDTLVNRIELKGIDFKEDGNILGHKLEYNSHNYIT
ADKQNGIKVFKRHNIEDGSVQLADHYQNTPIGDGPVPLPDDHYLSTQSLKDPEKRDMVLLVEFVTAAGIT
GGTLLPDQLTEEQIAEKEAFSLFDKGDGTTITTELETGMTRSLQGQNTEAEQLDMINEVDADGDGTDIFPEFLTMMA
RKMKTDDOTEETEAREFVFDKDGNGYISAAELHVMTNLGKEKLTDDEEVEEDMIREDIDGDGVQYNEEFVVMTAKEF
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMSVDSRKRKFNKTKGKALRAIGRLSSLSSGIGTGFPFDPHYVEVL
GEMHYVDPGRDTPVPLHLHGMPTSSYVWNR1IPHAVAPTHRCAPDLIGMDKSDKPDLGYFFDDHRVMDAIEAL
GLEEVLVILHDWSALGFHWAKRNPERVKQGIAMEFIRIPPTEDWEPEFARETFQAFRTTDVGKLIIDQNVFIEGT
LMGVVVRPLTEVEMHYREFRPFLPNVDFRELPNFLPLELPIAGENIVALVEEYMWDLHQSPFKLLFWGTPGVLIAPP
AEAAARLKLSPNCVKADIGPPGELLQEDNFDLIGSEIARWLSTLEISG

>ChemoR-CaM

MV5GKEElFTGVPVILPELGDVNGHKSFSVSEEGEDATYGLKLTFICTTGTLKLVWPTLVTTLTYGVCFSRYPD
HMKQHDFKKSAMPEGYVQERTIFKFDDGNYKTRAEVKFEQGDTLVNRIELKGIDFKEDGNILGHKLEYNSHNYIT
ADKQNGIKVFKRHNIEDGSVQLADHYQNTPIGDGPVPLPDDHYLSTQSLKDPEKRDMVLLVEFVTAAGIT
GGTLLPDQLTEEQIAEKEAFSLFDKGDGTTITTELETGMTRSLQGQNTEAEQLDMINEVDADGDGTDIFPEFLTMMA
RKMKTDDOTEETEAREFVFDKDGNGYISAAELHVMTNLGKEKLTDDEEVEEDMIREDIDGDGVQYNEEFVVMTAKEF
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMSVDSRKRKFNKTKGKALRAIGRLSSLSSGIGTGFPFDPHYVEVL
GEMHYVDPGRDTPVPLHLHGMPTSSYVWNR1IPHAVAPTHRCAPDLIGMDKSDKPDLGYFFDDHRVMDAIEAL
GLEEVLVILHDWSALGFHWAKRNPERVKQGIAMEFIRIPPTEDWEPEFARETFQAFRTTDVGKLIIDQNVFIEGT
LMGVVVRPLTEVEMHYREFRPFLPNVDFRELPNFLPLELPIAGENIVALVEEYMWDLHQSPFKLLFWGTPGVLIAPP
AEAAARLKLSPNCVKADIGPPGELLQEDNFDLIGSEIARWLSTLEISG
MDELYKGGTLPDLTEEQIAEFKEAFSFLDKDGDGTITTKELGTVMRSGLQNPTEAELQDMINEVDADGDGTIDFPEFLTMARKMKDTSDEEEIREAHEFRVFVDDKDGNFQSGSYIAYAELRVMNLGKLTDDEEVDDEMIREADIDGDQVNYEEFVVMMTAKHEFPQPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMVDSSRRAFKNTGKALRAIGRSSLSEGGIGTFGFDPHYVEVLGERHMGYVDGVPRDGTPVFLHLGHNPSTSSYNVRNNIPVHAPTHRCAPDLGMGSKDPLGELYFFDDHHVFMDAFIAALGEEVVLVHIWDGSLALGHWAKRNPERVKGIAFMENEFAIPFIPFWDEPEFAEFQARFTTDVRGKLIIDQNVFIEGTLPMGVFRPLTEVEMDHYREPLNPVDREPLWFRPNELPIAGEPANIVALVEEYMDDLHQPVPKLLLFWGTPGVLIPPAEAARLASSFLPNACKAVDIGPGENLLQEDNFDLIGSEIRWLSLEIGS

>ChemoL-CaM
MGLSGDQMQQIEKIFKVYYPVDDHHRKFVILHYGTLVIDGVPNMDYFGRPYEIGAVFDGKIKTVGTLWNGNKBDFERLINPDGSLLEDRTINGVTRGLCRELIGAGTTGSGGTGSSMVFTLEDVFVGDWRQTAGYNLDQVEQQGSTFQNLGVVSTPIQRIVLSENGLKIDIHVIIPYEVSKEEELFTGVPILVEVLDGDVGKHFSVSGEGPDATYGBKLFKICTGKLVPVFPTLVTTLTYGVQCFRYPDHMQHDFFKSMAPWGVQVRITFFKDDGNKTRAPEVKFEGETGVNLRIELKIDKEDGNIIGHLKLEYNSHNYVIMADQKNGKVNFKIRHNLDEDGPSQALDHYQONTFINGDPVIIIPDHYLSTGSLSKDPNEKRDMVLLLEFVTAGITGGTLPDLTEEQIAEFKEAFSFLDKDGDGTITTKELGTVMRSGLQNPTEAELQDMINEVDADGDGTIDFPEFLTMARKMKDTSDEEEIREAHEFRVFVDDKDGNFQSGSYIAYAELRVMNLGKLTDDEEVDDEMIREADIDGDQVNYEEFVVMMTAKHEFPQPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPGMVDSSRRAFKNTGKALRAIGRSSLSEGGIGTFGFDPHYVEVLGERHMGYVDGVPRDGTPVFLHLGHNPSTSSYNVRNNIPVHAPTHRCAPDLGMGSKDPLGELYFFDDHHVFMDAFIAALGEEVVLVHIWDGSLALGHWAKRNPERVKGIAFMENEFAIPFIPFWDEPEFAEFQARFTTDVRGKLIIDQNVFIEGTLPMGVFRPLTEVEMDHYREPLNPVDREPLWFRPNELPIAGEPANIVALVEEYMDDLHQPVPKLLLFWGTPGVLIPPAEAARLASSFLPNACKAVDIGPGENLLQEDNFDLIGSEIRWLSLEIGS

EGFP, EBFP2, mCerulean3, Venus, mRuby2, cpNanoLuc
HaloTag7
Calmodulin
M13 peptide
Linker
Interface mutations (XFP\textsuperscript{A206K}, HT7\textsuperscript{L271E}, EBFP2\textsuperscript{N39Y})
ATP sensors

>ChemoG-ATP

MG5KGELEGTFVGPLVILEDGDVGKHSVSSEGEGDATYGBKTLKFTCTTGKLPVIPWPTLVTTLYGVTQCGFSYDP

_HMKQHDFKFSAMPEYGTVQERTIFFKDDGNYKTRAEVRFEGDVLNVRIELKDIFGKEDNIGLHKGKLEYNSHNVYM

_ADQKXNKIFKNFKIRHIEDGSQVQHADHYQONNTPIGDGFVPLIDPHNYLSTQSLSKDPNKRHDVMVLEFVTAAGIT

GGG4KTVKVNITTPDPGVYADIDEMSVRASESDGLILPGHIPTKAPLIGAVRLKKGQTAVAVGTVTVHINAQAAETADIGK

ERAINQARQERLNGQSDDTRIDIRAELALQRNRLDVGKANEGGGG4GTFDFFPDHYVEVLGERMYXVDFPRGDPVTFLVLNG

_QTPSSYWRNIIIPHAPTHRCIAPDLGMSKDPDLYFVFDDHRMFAFI

EALGLEDVVLHIDWGSALGFWAKRNPERVKIGAMEFIRIPITDWEFEPARETFQARTTDVKGRLIIDQNFIVE

EGMPGMVQRLTEVMDHYREPLFNPVLWRFNPHELPIAGEPANIVALVEEYMMLQHSVPPKLLFWGTPGVG

IPPAAARLAKSLPNCKAVDIPGGGLNQLQEDNPDLIGSEIAWRSTLEISG

>ChemoB-ATP

MG5KGELEGTFVGPLVILEDGDVGKHSVSSEGEGDATYGBKTLKFTCTTGKLPVIPWPTLVTTLYGVTQCGFSYDP

_HMKQHDFKFSAMPEYGTVQERTIFFKDDGNYKTRAEVRFEGDVLNVRIELKDIFGKEDNIGLHKGKLEYNSHNVYM

_ADQKXNKIFKNFKIRHIEDGSQVQHADHYQONNTPIGDGFVPLIDPHNYLSTQSLSKDPNKRHDVMVLEFVTAAGIT

GGG4KTVKVNITTPDPGVYADIDEMSVRASESDGLILPGHIPTKAPLIGAVRLKKGQTAVAVGTVTVHINAQAAETADIGK

ERAINQARQERLNGQSDDTRIDIRAELALQRNRLDVGKANEGGGG4GTFDFFPDHYVEVLGERMYXVDFPRGDPVTFLVLNG

_QTPSSYWRNIIIPHAPTHRCIAPDLGMSKDPDLYFVFDDHRMFAFI

EALGLEDVVLHIDWGSALGFWAKRNPERVKIGAMEFIRIPITDWEFEPARETFQARTTDVKGRLIIDQNFIVE

EGMPGMVQRLTEVMDHYREPLFNPVLWRFNPHELPIAGEPANIVALVEEYMMLQHSVPPKLLFWGTPGVG

IPPAAARLAKSLPNCKAVDIPGGGLNQLQEDNPDLIGSEIAWRSTLEISG

>ChemoR-ATP

MG5KGELEGTFVGPLVILEDGDVGKHSVSSEGEGDATYGBKTLKFTCTTGKLPVIPWPTLVTTLYGVTQCGFSYDP

_HMKQHDFKFSAMPEYGTVQERTIFFKDDGNYKTRAEVRFEGDVLNVRIELKDIFGKEDNIGLHKGKLEYNSHNVYM

_ADQKXNKIFKNFKIRHIEDGSQVQHADHYQONNTPIGDGFVPLIDPHNYLSTQSLSKDPNKRHDVMVLEFVTAAGIT

GGG4KTVKVNITTPDPGVYADIDEMSVRASESDGLILPGHIPTKAPLIGAVRLKKGQTAVAVGTVTVHINAQAAETADIGK

ERAINQARQERLNGQSDDTRIDIRAELALQRNRLDVGKANEGGGG4GTFDFFPDHYVEVLGERMYXVDFPRGDPVTFLVLNG

_QTPSSYWRNIIIPHAPTHRCIAPDLGMSKDPDLYFVFDDHRMFAFI

EALGLEDVVLHIDWGSALGFWAKRNPERVKIGAMEFIRIPITDWEFEPARETFQARTTDVKGRLIIDQNFIVE

EGMPGMVQRLTEVMDHYREPLFNPVLWRFNPHELPIAGEPANIVALVEEYMMLQHSVPPKLLFWGTPGVG

IPPAAARLAKSLPNCKAVDIPGGGLNQLQEDNPDLIGSEIAWRSTLEISG

>ChemoL-ATP

MG5KGELEGTFVGPLVILEDGDVGKHSVSSEGEGDATYGBKTLKFTCTTGKLPVIPWPTLVTTLYGVTQCGFSYDP

_HMKQHDFKFSAMPEYGTVQERTIFFKDDGNYKTRAEVRFEGDVLNVRIELKDIFGKEDNIGLHKGKLEYNSHNVYM

_ADQKXNKIFKNFKIRHIEDGSQVQHADHYQONNTPIGDGFVPLIDPHNYLSTQSLSKDPNKRHDVMVLEFVTAAGIT

GGG4KTVKVNITTPDPGVYADIDEMSVRASESDGLILPGHIPTKAPLIGAVRLKKGQTAVAVGTVTVHINAQAAETADIGK

ERAINQARQERLNGQSDDTRIDIRAELALQRNRLDVGKANEGGGG4GTFDFFPDHYVEVLGERMYXVDFPRGDPVTFLVLNG

_QTPSSYWRNIIIPHAPTHRCIAPDLGMSKDPDLYFVFDDHRMFAFI

EALGLEDVVLHIDWGSALGFWAKRNPERVKIGAMEFIRIPITDWEFEPARETFQARTTDVKGRLIIDQNFIVE

EGMPGMVQRLTEVMDHYREPLFNPVLWRFNPHELPIAGEPANIVALVEEYMMLQHSVPPKLLFWGTPGVG

IPPAAARLAKSLPNCKAVDIPGGGLNQLQEDNPDLIGSEIAWRSTLEISG

EGFP, EBFP2, mRuby2, cpNanoLuc

HaloTag7

Fo2-F1 subunit

Linker

_Interface mutation\((\text{EGFP}^{206\text{K}}, \text{HT7}^{271\text{E}}, \text{EBFP2}^{390\text{Y}})\)
NAD⁺ sensors

>ChemoG-NAD

MVSKGEELFTGVPVILVLELDGVDGVDHGFCKSVSVGECEGADYTAGKLTLKICTTGTGKLPVWPTLVTTLTYGYGQCSRFYPD

HMKHQHDFKKFMPAEGYGVEQRTIKFEDGYKXRAWKEFEGDTLVR1ELKGIDFEDQGILHGLKLYNNSHYVIM

>ChemoB-NAD

MVSKGEELFTGVPVILVLELDGVDGVDHGFCKSVSVGECEGADYTAGKLTLKICTTGTGKLPVWPTLVTTLTYGYGQCSRFYPD

HMKHQHDFKKFMPAEGYGVEQRTIKFEDGYKXRAWKEFEGDTLVR1ELKGIDFEDQGILHGLKLYNNSHYVIM

>ChemoR-NAD

MVSKGEELFTGVPVILVLELDGVDGVDHGFCKSVSVGECEGADYTAGKLTLKICTTGTGKLPVWPTLVTTLTYGYGQCSRFYPD

HMKHQHDFKKFMPAEGYGVEQRTIKFEDGYKXRAWKEFEGDTLVR1ELKGIDFEDQGILHGLKLYNNSHYVIM

>ChemoD-NAD

MVSKGEELFTGVPVILVLELDGVDGVDHGFCKSVSVGECEGADYTAGKLTLKICTTGTGKLPVWPTLVTTLTYGYGQCSRFYPD

HMKHQHDFKKFMPAEGYGVEQRTIKFEDGYKXRAWKEFEGDTLVR1ELKGIDFEDQGILHGLKLYNNSHYVIM

43
EWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLGVRPRLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPA
IVALVEEYMWDLHQSPVFKLLFWGTFGVLIPPAEAILAKSLPNCACKAVDIGPGNLLQEDNFDLIGSEIARWLSTLEISG

>ChemoL-NAD
MGSGDMQIEKIFIKKVVPVDHHFHVILHYGTVDGTPNMYDFGRYPYEIGAVFDGKKITTVGTGLNWNGKIID
ERLIPDGSLLFVTINGVTGRWLECERILAGGTGSSSGTGSMVFTLEDVFDRVQTAQYNLDQVLEQGVSSLFQN
LGVSVTPIQRIVLSENGENLKIDIHVIIYEVSKGEELFTGVPVPIVELGDVNGHKFVSVSLEGEGDATYGKLTLLKFI
CTTGKLPFPVPTLTTTTYGQCFSRYPHDKMQHDFKFSAMPEGYZQERTIFFKDDGNYKTRAERVKFGDITLVRNIE
LKGIDFKEDGNILGHKLEYNYNSHNYVIMADCKRNK_INVALIDGSSVQADHYQPFGVNDPNAVSTQISSLKDPNFKEKDMVLEFV
EGFP, EBFP2, mRuby2, ShadowG, cpNanoLuc

HaloTag7

Linker

Interface mutations (EGFP^{A206K}, EGFP^{T225R}, HT7^{L271E})

Catalytic mutations #LigA (K117L, D289N)

Affinity mutations #LigA (Y226W, V292A)

HT7^{P174W}
Supplementary Note 5 – Purification sequences

>Strep-tag®II + enterokinase cleavage sequence (N-terminal)
WSHPQFEKGAADDDKVPH[...] (pET-51b(+) plasmids)

>Poly-histidine tag sequence (C-terminal)
[...]APGFSISAHHHHHHHHH

>Poly-histidine tag + TEV cleavage sequence (N-terminal)
HHHHHHHHHHHENLYFQGGG[...] (pET-51b(+) plasmids for crystallography)

Supplementary Note 6 – Localization sequences

>Nuclear exit signal (NES) (N-terminal or C-terminal)
[...]LPPLERLTL (pCDNA5 plasmids)
LQNELALKAGLDINKTGS[...] (pAAV plasmids)

>Nuclear localization sequence (NLS) (C-terminal, 3 copies)
[...]KSGLRSPADPKKKR KVDPKKKRKVKPGSTSGR

>Exterior plasma membrane localization sequence (IgKchL[...]PDGFRm) (N-terminal and C-terminal)
METDTLLLWVLLLVVPGSTGDPYDVPDYA[...]EQLISEEDLNAVQDTQEVIVPHSLPFKVVISAILALVLT
IISLIIILWQKPKR

>Nuclear envelope (LaminB1) localization sequence (C-terminal)
[...]MATATPVPPRMSRAGPTTPLSPTRLSRLQKEEELRELDDLAVYIDKVRSLTENSAQLQVTIEREEVGR
LTGLKALYETELADARRALDDTARERAKLQIELGCKAEHDOQLLNYAKKESDLNGAQLRYEALNSKDAALAT
ALGDKKSLGEDLDLKDQIAQLESLLAAAQQQLADETLKLKVDELNCQSLTEDLEFRKSMYELEDINETRKRHKHEL
LVEDSGRQIEYKLAQLAHREQMQRHDAYRLEYELEQTYHAKLENARLSSEMTSTVNSAREELMERSMRIESLSS
QLSNLQKESSRACLLETQLEDLLAKEDNSRMLTDKEREMAERDQMQQQLNDYEQLLDVKLALDMEISAYRKLLE
GEEERLKSFSRVSRRASSRSRSVTTRGRKRKDVEESEASSSVSHSASATGNVIEEIDVDKFIILKNT
SEQQPMGGWEMIRKIGDTSVSYKTSYVLKAGQTVTIAANAGVTASSPTDLIWKQNQSNGTGEDVKVILKNSQG
EEVATVRSTFQKTTIPFEEEEEEEAAGVVVEELELFHQGTPRASNRSCAIM

>Mitochondrial localization sequence (Cox8) (N-terminal, 4 copies)
4x[MSVLTPLLLRGLTGSARRLPVPRAKHSLVSLTLPLLRLGLTGSARRLPVPRAKHSL] [...]

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