Supporting information for

Intracellular click reaction with a fluorescent chemical Ca$^{2+}$ indicator to prolong its cytosolic retention

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Supplementary Materials and Methods

1. Synthesis and characterization of N$_3$-fura-2 AM

**Materials**

$N,N$-diisopropylethylamine (DIPEA), $p$-hydroquinone, benzyl chloride, 1,2-epoxy-5-hexene, trityl chloride, tosyl chloride, $N,N$-dimethyl-4-aminopyridine (DMAP), 4-chloro-2-nitrophenol, ethyl bromoacetate, 30% HBr in acetic acid, ethyl oxalyl chloride, chloroacetonitrile, Cu(II) acetylacetonate, 2-chloromethyloxagole-5-carboxrate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Magnesium bromide, trimethyl silyl diazomethane and bromo methyl acetate were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan). Phosphorus oxychloride was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other reagents and solvents were purchased from Kanto Chemical Industry Co. (Tokyo, Japan). All reagents were used as received.

**Synthesis and characterization of N$_3$-fura-2 AM**

$N_3$-fura-2 16 and $N_3$-fura-2 AM 17 was synthesized from $p$-hydroquinone 1 with 14 and 15 steps$^{1,2}$ (Scheme S1). Full descriptions of each reaction step are described below. All compounds were characterized by $^1$H NMR, $^{13}$C NMR, ESI-MS, and IR. The NMR spectra of compound 1 – 17 were obtained using samples prepared in a deuterated solvent and were recorded using OXFORD NMR AS 400(400 MHz) spectrometer. For $N_3$-fura-2 AM 17, The NMR spectrum was recorded with AVANCE600 (600 MHz, Bruker BioSpin K.K., Karlsruhe, Germany). Electrospray ionization mass spectra (ESI-MS) were recorded using a LCQ Fleet (Thermo Fisher Scientific, USA) mass spectrometer. Infrared (IR) spectra were obtained using FT-IR4100 (JASCO, Tokyo, Japan) and samples were prepared on NaCl crystal plate. Plastic sheets coated with 0.2 mm silica gel 60 (Merck & Co., Germany) were used for Thin Layer chromatography (TLC) to monitor all reactions. For silica gel column chromatography, silica gel 60 (Kanto Chemical Industry Co.) were used with appropriate solvents indicated in each step.
Scheme S1  Synthesis route of N3-fura-2 and N3-fura-2 AM
**Step 1: \( p \)-hydroquinone 1 to 2**

\( p \)-Hydroquinone \( 1 \) (5.55 g, 0.050 mol) and potassium hydroxide (6.80 g, 0.121 mol) were dissolved in EtOH (100 ml) and gently added benzyl chloride (16.50 g, 0.116 mol), immediately after black precipitate appeared. After being rotated over night (12 hrs, r.t.), the suspension was poured into water and the precipitate was filtered off, then recrystallized with EtOH, to yield white crystal \( 2 \) (10.60 g, 0.037 mol, yield 72%). **TLC: \( R_f = 0.7 \) (CHCl\( _3 \)).**

**\( ^1H \) NMR (\( \delta \) ppm in CDCl\( _3 \)):** 7.43-7.23(m, 10H, ArH), 6.90(s, 4H, ArH), 5.00(s, 4H, CH\( _2 \)). **\( ^{13}C \) NMR (\( \delta \) ppm in CDCl\( _3 \)):** 153.4, 137.5, 128.8, 128.1, 127.7, 116.0(ArC), 70.88(CH\( _2 \)). **ESI-MS (m/z):** 291.08([M+H\(^+\)]\(^\ddagger\), found), 290.1(calcd). **IR (cm\(^{-1}\)):** 1020(\( \nu_{C-O} \)).

**Fig. S1** (left) \( ^1H \) NMR and (right) \( ^{13}C \) NMR spectra of 2.
Step 2: 2 to 3

70% nitric acid (1 ml, 0.016 mol) was added to the acetic acid suspension (20 ml) of 2 (4.29 g, 0.015 mol) on ice. The mixture was gradually warmed to 50°C and stirred over night (12 hrs). After cooling and being poured into water, the precipitate was filtered off and purified by recrystallization from MeOH to yield yellow crystal 3 (4.30 g, 0.012 mol, yield 87%). TLC: Rf = 0.6 (CHCl3). $^1$H NMR (δ ppm in CDCl3): 7.48-7.32 (m, 11H, ArH), 7.10 (dd, $J = 3.2$ Hz, 9.2 Hz, 1H, ArH), 7.05 (d, $J = 9.2$ Hz, 1H, ArH), 5.16 (s, 2H, CH2), 5.03 (s, 2H, CH2). $^{13}$C NMR (δ ppm in CDCl3): 152.5, 146.6, 140.5, 136.2, 128.9, 128.9, 128.5, 128.4, 127.8, 127.4, 121.7, 117.4, 111.4 (ArC), 72.3, 71.1 (CH2). ESI-MS (m/z): 357.92 ([M+Na]$^+$, found), 335.1 (calcld). IR (cm$^{-1}$): 1529 (νN–O), 1223 (νN–O), 1024 (νC=O).

Fig. S2 (left) $^1$H NMR and (right) $^{13}$C NMR spectra of 3.
To a solution of 3 (4.30 g, 12.85 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10 ml) TFA (1.09 ml, 16.70 mmol) was added and then stirred for 2 days (48 hrs, r.t.). The acidic solution was evaporated under reduced pressure. The organic phase was repeatedly extracted with 1M NaOH. Then the water phase was neutralized with 1N HCl and extracted with chloroform. Finally, the organic phase was concentrated and purified by silica gel chromatography (chloroform/hexane=1/1, v/v) to yield 4 (1.83 g, 7.47 mmol, yield 58\%). TLC: \( R_f = 0.4 \) (Chloroform/Hexane=1/1).

\( ^1 H \) NMR (δ ppm in CDCl\textsubscript{3}): 10.34(s, 1H, OH), 7.59(d, \( J = 3.2 \) Hz, 1H, ArH), 7.44-7.33(m, 5H, ArH), 7.27(dd, \( J = 3.2 \) Hz, 9.2 Hz, 1H, ArH), 7.10(d, \( J = 9.2 \) Hz, 1H, ArH), 5.06(s, 2H, CH\textsubscript{2}). \( ^{13}C \) NMR (δ ppm in CDCl\textsubscript{3}): 151.8, 150.4, 128.9, 128.6, 128.1, 127.8, 121.1, 107.4(ArC), 71.1(CH\textsubscript{2}). ESI-MS (m/z): 244.0([M-H\textsuperscript{+}], found), 245.1(calcd). IR (cm\textsuperscript{-1}): 3230(\( \nu \)O-H), 1538(\( \nu \)N-O), 1317(\( \nu \)N-O), 1019(\( \nu \)C-O).

Fig. S3 (left) \( ^1 H \) NMR and (right) \( ^{13}C \) NMR spectra of 4.
Step 4: 4 to 5

A solution of 1,2-epoxy-5-hexene (1.70 ml, 14.94 mmol) and completely dried 4 (1.83 g, 7.47 mmol) in dry DMF with DIPEA (2.5 ml, 14.94 mmol) was stirred until disappearance of 4 (3 days, 100°C, under N₂) and then concentrated. The residue was dissolved in chloroform and purified with silica gel column chromatography (chloroform) to yield a mixture of two regioisomers (2.40 g, 7.00 mmol). From estimation with ¹H NMR spectra, the percentages of 5 and regioisomer in this mixture were 80% and 20%, respectively. The required compound 5 was secondary alcohol and the regioisomer was a primary alcohol. The regioisomer was selectively tritylated to allow chromatographic separation: The mixture of regioisomers and trityl chloride (1.56 g, 5.60 mmol) in CH₂Cl₂ (8 ml) with pyridine (1.5 ml, 18.58 mmol) was stirred overnight (12 hrs, r.t.) and the residue after concentration was purified by column chromatography on silica gel (Chloroform) to yield a mixture of two regioisomers (2.40 g, 7.00 mmol, yield 64%, 2 steps). TLC:

Rₜ = 0.4 (CHCl₃). ¹H NMR (δ ppm in CDCl₃): 7.45(d, J = 3.2 Hz, 1H, ArH), 7.41-7.29(m, 5H, ArH), 7.14(dd, J = 9.2 Hz, 9.2 Hz, 1H, ArH), 6.99(d, J = 9.2 Hz, 1H, ArH), 5.83(m, 1H, -CH=CH₂), 5.09-4.96(m, 4H, -CH=C₃H₂, -CH₂Ar), 4.08(dd, J = 2.8 Hz, 8.8 Hz, 1H, -OCH₂CHOH), 4.00(m, 1H, -CHOH), 3.88(dd, J = 2.8 Hz, 8.8 Hz, 1H, -OCH₂CHOH), 2.97(br, 1H, OH), 2.30-2.13(m, 2H, -CH₂CH₂CHOH), 1.72-1.59(m, 2H, -CH₂CH₂CHOH). ¹³C NMR (δ ppm in CDCl₃): 152.51, 147.00, 139.80, 138.22, 136.18, 128.93, 128.55, 127.78, 122.19, 117.13, 115.39, 111.47(ArC, -CH=CH₂), 77.74, 77.62, 77.43, 74.69, 71.11, 69.39(-CH₂Ar, -OCH₂CHOH, -CHOH), 32.14, 29.86(-CH₂CH₂CHOH). ESI-MS (m/z): 366.3([M+Na⁺]⁺, found), 343.1(calcd). IR (cm⁻¹): 3552(v_o-H), 1641(v_c-O), 1531(v_N,O), 1349(v_N,O), 1019(v_c-O).

Fig. S4 (left) ¹H NMR and (right) ¹³C NMR spectra of 5.
Step 5: 5 to 6

5 (699.60 mg, 1.48 mmol) and tosyl chloride (422.9 mg, 2.23 mmol) dissolved in CH$_2$Cl$_2$ (8 ml) with DMAP (8.6 mg, 0.74 mmol) at 0°C. The mixture was stirred overnight (12 hrs, r.t.) to completely dissolve 5. The solution was carefully neutralized and washed with 1M HCl and 1M NaCl aq, dried with Na$_2$SO$_4$ and evaporated. The residue was recrystallized with MeOH to 6 (607.1 mg, 1.22 mmol, yield 82%). TLC: $R_f = 0.6$ (CHCl$_3$).

$^1$H NMR (δ ppm in CDCl$_3$): 7.75(s, 1H, ArH), 7.41-7.25(m, 8H, ArH), 7.10(dd, $J = 3.2$ Hz, 9.2 Hz, 1H, ArH), 6.91(d, $J = 9.2$ Hz, 1H, ArH), 5.68(m, 1H, -CH=CH$_2$), 5.02-4.92(m, 4H, -CH=CH$_2$, -CH$_2$Ar), 4.76(m, 1H, -CHOTs), 4.19-4.10(m, 2H, -OCH$_2$CHOH), 2.38(s, 3H, CH$_3$), 2.11-1.86(m, 4H, -CH$_2$CH$_2$CHOTs).

$^{13}$C NMR (δ ppm in CDCl$_3$): 152.87, 146.06, 145.20, 140.35, 136.94, 136.15, 133.83, 130.03, 128.95, 128.57, 128.01, 127.77, 121.65, 117.51, 115.98, 111.43(ArC, -CH=CH$_2$), 79.69, 71.20, 71.10(-CH$_2$Ar, -OCH$_2$CHOH, -CHOTs), 30.63, 28.89, 21.83(-CH$_2$CH$_2$CHOTs, Ar-CH$_3$).

ESI-MS (m/z): 520.3([M+Na]$^+$, found), 497.2(calcd). IR (cm$^{-1}$): 1530(ν$_{N-O}$), 1454(ν$_{S=O}$), 1354(ν$_{N,O}$), 1020(ν$_{C,O}$), 1175(ν$_{S=O}$).

Fig. S5 (left) $^1$H NMR and (right) $^{13}$C NMR spectra of 6.
Step 6: 6 to 7

A solution of KOH (80.36 mg, 1.41 mmol) in water (2 ml) was added to a solution of 4-chloro-2-nitrophenol (215.86 mg, 1.41 mmol) in hot EtOH (6 ml). After complete dissolution, an orange solution of the potassium salt of 4-chloro-2-nitrophenol was obtained. The potassium salt of nitrophenol and 6 (539.40 mg, 1.08 mmol) in dry DMF was heated overnight (12 hrs, 120°C, under N₂) and concentrated. The solution was concentrated and purified with silica gel column chromatography (chloroform/hexane=1/1) to 7 (360.2 mg, 0.75 mmol, yield 69%).

TLC: Rf = 0.5 (AcOEt/Hexane = 1/3).

1H NMR (δ ppm in CDCl₃): 7.69 (d, J = 8.4 Hz, 1H, ArH), 7.42-7.30 (m, 6H, ArH), 7.14-7.11 (m, 2H, ArH), 7.02 (d, J = 9.2 Hz, 1H, ArH), 6.81 (m, 1H, ArH), 5.04-4.98 (m, 4H, -CH=CH₂, -CH₂Ar), 4.84 (m, 1H, -CHO-CH₂), 4.30 (dd, J = 6.8 Hz, 10.0 Hz, 2H, -OC₃H₂CHOH), 4.15 (dd, J = 4.0 Hz, 10.0 Hz, 2H, -OCH₂CH₂OH), 2.41 (s, 3H, -CH₃), 2.31-2.23 (m, 2H, -CH₂CH₂CHO), 2.00-1.89 (m, 2H, -CH₂CHO).

13C NMR (δ ppm in CDCl₃): 152.71, 152.17, 146.39, 146.08, 140.17, 138.66, 137.45, 136.15, 128.92, 128.54, 127.76, 125.67, 122.07, 121.76, 117.80, 117.08, 116.08, 111.47 (ArC, -CH=CH₂), 78.62, 72.82, 71.12 (-CH₂Ar, -OCH₂CHOH, -CH₂CH₂CH), 31.09, 29.25, 22.04 (-CH₂CH₂CH, Ar-CH₃). ESI-MS (m/z): 501.3 ([M+Na⁺]+, found), 478.2 (calcd). IR (cm⁻¹): 1529 (vN=O), 1348 (vN=O), 1026 (vC=O).

**Fig. S6 (left) ¹H NMR and (right) ¹³C NMR spectra of 7.**
Step 7: 7 to 8

A solution of 7 (406.5 mg, 0.85 mmol), SnCl$_2$·2H$_2$O (1.15 g, 5.10 mmol) and 12N HCl (1 ml, 1 mmol) dissolved in EtOH (6 ml) was refluxed (10 min 90 °C), cooled and brought to neutral with 1M NaOH aq. The resulting suspension was extracted with chloroform. The organic layers were dried with Na$_2$SO$_4$, concentrated and the residue was purified with silica gel column chromatography (AcOEt/hexane=1/3(v/v)) to yield 8 (193.4 mg, 0.46 mmol, yield 54%).

**TLC:** $R_f$ =0.7 (chloroform/acetone=20/1) .

**$^1$H NMR** ($\delta$ ppm in CDCl$_3$): 7.39-7.19(m, 5H, ArH), 6.72(s, 1H, ArH), 6.64(d, $J$ = 8.8 Hz, 1H, ArH), 6.60(d, $J$ = 8.8 Hz, 2H, ArH), 6.33(d, $J$ = 3.2 Hz, 1H, ArH), 6.25(dd, $J$ = 3.2 Hz, 8.8 Hz, 1H, ArH), 5.89-5.79(m, 1H, -CH=CH$_2$), 5.08-4.99(m, 2H, -OCH$_2$CHOC-), 4.93(s, 2H, -CH$_2$Ar), 4.54(m, 1H, -OCH$_2$CHOCH-), 4.11-3.98(m, 2H, CH=CH$_2$), 3.73(s, 4H, -NH$_2$), 2.35-2.22(m, 5H, -CH$_2$CH$_2$CH=CH$_2$, CH$_3$), 1.99-1.86(m, 2H, -CH$_2$CH$_2$CH=CH$_2$).

**$^{13}$C NMR** ($\delta$ ppm in CDCl$_3$): 154.36, 146.05, 141.15, 138.10, 137.72, 135.17, 128.76, 128.29, 128.05, 127.69, 122.70, 116.26, 115.65, 113.61, 113.61, 103.47, 103.30(ArC, -CH=CH$_3$), 77.52, 77.39, 77.07(-CH$_2$Ar, -OCH$_2$CHOH, -CH$_2$CH$_2$CH), 31.20, 29.83, 21.15(-CH$_2$CH$_2$CH, Ar-CH$_3$).

**ESI-MS** (m/z): 419.3([M+H$^+$]$^+$, found), 418.5(calcd).

**IR** (cm$^{-1}$): 3367(\(v\)N-H), 1213(\(v\)N-H), 1027(\(v\)C-O).

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**Fig. S7** (left) $^1$H NMR and (right) $^{13}$C NMR spectra of 8.
Step 8: 8 to 9

A solution of 8 (193.4 mg, 0.46 mmol) was dissolved in acetonitrile (10 ml) and then ethyl bromo acetate (403.6 µl, 3.70 mmol) and DIPEA (785.8 µl, 4.62 mmol) were added to it. The mixed solution was refluxed (12 hrs, 90°C). After confirmation that reaction was completed, the residue dissolved in chloroform was washed with NaCl aq (×2), dried with Na₂SO₄ and concentrated, the residue was purified with silica gel column chromatography (AcOEt/hexane=1/3 (v/v)) to yield 9 (239 mg, 0.31 mmol yield 68%). TLC: Rᵣ = 0.8 (Chloroform/Acetone = 20/1).

H NMR (δ ppm in CDCl₃): 7.41-7.28(m, 5H, ArH), 6.79-6.74(m, 3H, ArH), 6.66(dd, J = 3.2 Hz, 8.8 Hz, 1H, ArH), 6.55(d, J = 3.2 Hz, 1H, ArH), 6.46(d, J = 8.8 Hz, 1H, ArH), 5.88-5.81(m, 1H, -C=CH₂), 5.07-4.96(m, 4H, -OCH₂CHOC₂, -CH₂=CH₂), 4.62(m, 1H, -OCH₂CHOC₂), 4.20-3.01(m, 18H, -CH₂Ar), 2.30-2.20(m, 5H, -CH₂CH₂CH=CH₂, CH₃), 1.93-1.83(m, 2H, -CH₂CH≡CH₂), 1.38-1.19(m, 12H, CH₂CH₃).

C NMR (δ ppm in CDCl₃): 171.71, 171.30(-CH₂COOEt), 153.94, 149.87, 145.39, 140.67, 138.29, 138.03, 137.47, 132.37, 128.70, 128.05, 127.70, 122.26, 120.15, 117.13, 115.93, 115.21, 108.15, 107.26(ArC, -CH=CH₂), 76.82, 71.19, 70.63(-CH₂Ar, -OCH₂CH₂OH), 60.83, 60.71, 54.05, 53.69(-CH₂NCOOCH₂CH₃), 31.18, 29.61, 21.09(-CH₂CH₂CH₂CH₂CH₃), 14.38(-CH₂CH₃). ESI-MS (m/z): 763.2([M+H⁺]⁺, found), 762.2(calcd).

IR (cm⁻¹): 1745(νC=O), 1027(νC-O).

Fig. S8 (left) H NMR and (right) C NMR spectra of 9.
Step 9: 9 to 10

30% HBr in acetic acid (77 μl, 0.38 mmol) was added to a solution of 9 (150 mg, 0.19 mmol) in CH₂Cl₂ and stirred over night (12 hrs, r.t.). Since the Rf of compound 10 was almost similar to 9’s Rf, the reaction progress was confirmed as disappearance of 9 with ESI-MS. The crude solution was neutralized with NaHCO₃ aq and washed NaClaq (×2) dried with Na₂SO₄ and concentrated to yield 10. At this step, further purification was not needed. TLC: Rf = 0.8 (Chloroform/Acetone = 20/1). ESI-MS (m/z): 865.2([M+Na¹⁺], found), 842.3(calcd).

Fig. S9 ESI-MS spectrum of 10.
Step 10: 10 to 11

A solution of the residue 10 and sodium azide (38.36 mg, 0.59 mmol) in dry DMF was heated (2 hrs, 100°C) and concentrated. A solution of the residue in CH₂Cl₂ was washed with NaClaq (x2) and confirmed as disappearance of 10 by ESI-MS. The organic layers were pooled, dried with Na₂SO₄, concentrated and the residue was purified with silica gel column chromatography (AcOEt/hexane=1/3(v/v)) to yield brown residue 11 (47.5 mg, 0.058 mmol, yield 30%, 2 steps). TLC: Rf = 0.8 (Chloroform/Acetone = 20/1). ¹H NMR (δ ppm in CDCl₃):

7.41-7.29 (m, 5H, ArH), 6.90-6.68 (m, 4H, ArH), 6.56 (d, J = 3.2 Hz, 1H, ArH), 6.47 (d, J = 3.2 Hz, 8.0 Hz, 1H, ArH), 4.96 (s, 2H, -OCH₂CHOC-), 4.61 (m, 1H, -OCH₂CHOC-), 4.17-4.00 (m, 18H, -CH₂NCOOCH₂CH₃, -CH₂Ar), 3.49 (m, 1H, -CHN₃CH₃), 2.23 (s, 3H, Ar-CH₃), 1.88-1.62 (m, 4H, -CH₂CH₂CH(N₃)CH₃), 130-1.20 (m, 15H, CH₂CH₃, CHN₃CH₃). ¹³C NMR (δ ppm in CDCl₃): 172.85, 171.68 (-CH₂C(OEt)), 154.36, 149.98, 145.33, 140.89, 138.13, 132.43, 129.88, 128.91, 128.11, 127.77, 122.63, 120.43, 117.75, 116.21, 108.31, 107.32 (ArC), 72.12, 71.11, 70.70 (-CH₂Ar, -OCH₂CH₂CH₂CH₂), 61.04, 60.67, 58.11, 53.95, 53.82 (-CHN₃CH₃, -CH₂NCOOCH₂CH₃), 43.36, 36.84, 28.04 (-CH₂CH₂CH(N₃)CH₃, -Ar-CH₃), 14.38, 29.89 (-CH₂CH₃, CHN₃CH₃). ESI-MS (m/z): 806.3 ([M+H]⁺, found), 705.3 (calcld). IR (cm⁻¹): 2102(νN=N), 1743(νC=O), 1608(νC=O), 1027(νC-O).

Fig. S10 (left) ¹H NMR and (right) ¹³C NMR spectra of 11.
Step 11: 11 to 12

Phosphorus oxychloride (50 µl, 0.55 mmol) was slowly added to dry DMF (1 ml) and rotated (15 min, r.t., under N₂) to prepare vilsmeier reagent. The resulting red vilsmeier reagent (1 ml) was carefully mixed with 11 (47.5 mg, 0.55 mmol) in dry DMF (1 ml). The solution was stirred overnight (12 hrs, r.t.), then neutralized with NaHCO₃ aq and gently concentrated. The residue was washed with NaCl aq (×2), dried with Na₂SO₄, concentrated and purified with silica gel column chromatography (AcOEt/hexane=1/3(v/v)) to yield 12 (20 mg, 0.025 mmol, yield 43%).

**TLC:** \( R_f = 0.75 \) (chloroform/aceton = 20/1). ^1^H NMR (δ ppm in CDCl₃): 10.30(s, 1H, -CHO), 7.41-7.32(m, 5H, ArH), 7.29(s, 1H, ArH), 6.82(d, \( J = 8.4 \) Hz, 1H, ArH), 6.70(m, 2H, ArH), 6.38(s, 1H, ArH), 5.01(s, 2H, -OC₂H₃), 4.62(m, 1H, -OCH₂CH₂CH₂CH₂-), 4.19-4.03(m, 18H, -C₂H₄NCOOC₂H₃, -CH₂Ar), 3.49(m, 1H, -C₂H₃N₃-CH₃), 2.24(s, 3H, Ar-CH₃), 1.86-1.60(m, 4H, -CH₂CH₂CH₂=CH₂), 1.32-1.19(m, 15H, CH₂C₃H₃, -CHN₃-CH₃). ^1^C NMR (δ ppm in CDCl₃): 187.74(CHO), 171.46, 170.58(-CH₂C₂OEt), 157.52, 149.64, 146.55, 144.30, 138.30, 136.52, 128.40, 127.51, 122.89, 120.64, 118.48, 117.89, 112.01, 103.57(ArC), 76.77, 71.25, 70.61(-CH₂Ar, -OCH₂CH₂CH₂-), 61.24, 60.72, 58.14, 54.07, 53.93(-CH₂NCOOCH₂CH₃, -CHN₃-CH₃), 31.80, 29.88, 28.35(-CH₂CH₂CH₂N₃-CH₃, Ar-CH₃), 14.38(-CH₃CH₃). **ESI-MS** (m/z): 834.3([M+H]^+) found, 833.3(calcd).

**IR** (cm⁻¹): 2100(νN=O), 1739(νC=O), 1668(νC=O), 1026(νC-O).

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**Fig. S11 (left) ^1^H NMR and (right) ^1^C NMR spectra of 12.**
Step 12: 12 to 13

12 (103.6 mg, 0.124 mmol) and magnesium bromide (34.9 mg, 0.186 mmol) were dissolved in benzene (8 ml). The reaction mixture was stirred overnight (12 hrs, r.t.) to precipitate yellow precipitate. The reaction mixture was gently refluxed at 90°C overnight (12 hrs, 90°C). When the spot of 12 was disappeared on TLC, the cooled residue was filtered and washed with benzene. The yellow magnesium complex was transferred to an aqueous 1M HCl solution. The mixture was extracted with chloroform and 1M HCl solution (×1), NaCl aq (×1), then the combined extracts were dried with Na₂SO₄ and concentrated. The crude products were purified with silica gel column chromatography (chloroform/acetone=20/1(v/v)), yielding yellow solid 13 (21.9 mg, 0.029 mmol, 23%).

TLC: R_f = 0.75 (Chloroform/Acetone = 20/1). ¹H NMR (d ppm in CDCl₃): 11.15(s, 1H, -OH), 9.54(s, 1H, -CHO), 6.96(s, 1H, ArH), 6.74(s, 1H, ArH), 6.15(s, 1H, ArH), 4.54(m, 1H, -OCH₂CH₂CH₂CH₂-), 4.24-4.03(m, 18H, -CH₂CH₂NCOOCH₂CH₃, -OCH₂CH₂CH₂CH₂-), 3.51(m, 1H, -CH₂CH₂N₃-C₃H₃), 2.24(s, 3H, Ar-CH₃), 1.88-1.63(H, -CH₂CH₂CH₃-C₃H₃, -CH₂CH₂CH₃-C₃H₃), 1.30-1.21(m, 15H, CH₂C₃H₃, -CH₂CH₂CH₂CH₂-), 1.24(s, 3H, Ar-CH₃). ¹³C NMR (d ppm in CDCl₃): 193.67(CHO), 171.40, 170.32(-CH₂COOEt), 158.87, 149.89, 148.35, 142.91, 138.53, 132.72, 123.21, 120.40, 118.59, 117.79, 113.59, 104.71 (ArC), 71.54, 71.45(-OCH₂CH₂CH₂CH₂-), 61.36, 60.77, 58.25, 58.00, 54.13, 53.99(-CH₂NCOOCH₂CH₃, -CH₂CH₂CH₃-C₃H₃), 32.17, 31.85, 29.88, 28.98, 28.66(-CH₂CH₂CH₃-C₃H₃, Ar-CH₃), 21.02, 19.66, 14.38(-CH₂CH₃).

**Fig. S12 (left)** ¹H NMR and (right) ¹³C NMR spectra of 13.
Step 13: **13** to **N₂-fura-2 Et 15**

Ethyl chloro glyoxylate (250 µl, 2.25 mmol) was dissolved in THF (10 ml) and trimethyl silyl diazomethane (10 ml, 6.75 mmol) was dropwisely added to it. After over night reaction (12hrs, r.t.), evaporated the solvent at 50°C under reduced pressure and purified with silica gel column chromatography (AcOEt/hexane=1/5 (v/v)) to yield ethyl diazo pyruvate **18** (274 mg, 1.93 mmol, yield **88%**). **TLC**: \( R_f = 0.6 \) (chloroform/acetonitrile=20/1).

\(^1\)H NMR (δ ppm in CDCl₃): 6.20 (s, 1H, CH), 4.34 (q, \( J = 7.2 \text{ Hz} \), 2H, -CH₂CH₃), 1.38 (t, \( J = 7.2 \text{ Hz} \), 3H, -CH₂CH₃).

\(^13\)C NMR (δ ppm in CDCl₃): 160.54 (-COO), 63.14 (-CH₂CH₃), 35.59 (CH), 14.41 (-CH₂CH₃).

ESI-MS (m/z): 165.00 ([M+Na⁺]⁺, found), 142.04 (calcd).

**IR** (cm⁻¹): 3081 (νC-H), 2103 (νC-N), 1735 (νC=O), 1022 (νC-O).

[Diagram: Synthesis route of 14]

**Scheme S2.** Synthesis route of 14

**Fig. S13 (left)** \(^1\)H NMR and **(right)** \(^13\)C NMR spectra of 18.
Ethyl diazo pyruvate 15 (274 mg, 1.93 mmol) and Cu(II) acetylacetonate (a few mg) were dissolved in benzene (5 ml). Chloroacetonitrile (1.1 ml, 17.36 mmol) were dropwisely added into the benzene solution and rotated over night (12 hrs, 60°C). After the mixture was cooled and concentrated, a solution of the residue in chloroform was washed with NaHCO₃ (x1) and NaCl aq (x1), dried with NaSO₄, and concentrated. The residue was purified with silica gel column chromatography on (AcOEt/Hexane = 1/5) to obtain 2-chloromethyloxagole-5-carboxrate 14 (84.9 mg, 0.38 mmol, yield 20%). Evaporation or drying of 14 should be carefully performed not to evaporate 14 itself. **TLC: Rf = 0.6 (Chloroform/Acetone = 20/1) .**

**¹H NMR (δ ppm in CDCl₃):** 7.74(s, 1H, ArH), 4.65(s, 2H, -CH₂Cl), 4.41(q, J = 7.2 Hz, 2H, -CH₂CH₃), 1.40(t, J = 7.2 Hz, 3H, -CH₂CH₃). **¹³C NMR (δ ppm in CDCl₃):** 161.93(COO), 157.52, 143.85, 134.34(ArC), 61.97(-CH₂Cl), 35.57(-CH₂CH₃), 14.38(-CH₂CH₃).

**IR (cm⁻¹):** 1735(νC=O), 1022(νC-O).

**Fig. S14 (left) **¹H NMR and (right) **¹³C NMR spectra of 14.
13 (100 mg, 0.134 mmol), ethyl 2-chloromethyloxagole-5-carboxrate 14 (45 mg, 0.238 mmol), and K$_2$CO$_3$ (125 mg, 0.906 mmol) were stirred in dry DMF (1.5 hrs, 100°C under N$_2$). The reaction mixture was cooled, diluted with 1M HCl and extracted with chloroform ($\times$2). The combined extracts were dried with Na$_2$SO$_4$, and concentrated with vacuum drying. The residue was purified by silica gel flash chromatography (AcOEt/Hexane=1/3(v/v) to yield yellow residue 17 (87.8 mg, 0.100 mmol, yield 75%). TLC: $R_f$ = 0.7 (Chloroform/Acetone = 20/1). $^1$H NMR (d ppm in CDCl$_3$): 7.87 (s, 1H, -ArH), 7.41 (s, 1H, -ArH), 7.10 (s, 2H, -ArH), 6.82 (d, $J$ = 8.0 Hz, 1H, -ArH), 6.77 (s, 1H, -ArH), 6.72 (d, $J$ = 8.0 Hz, 1H, -ArH), 4.70 (m, 1H, -OCH$_2$CH$_2$CH$_2$-) 4.43 (q, $J$ = 7.2 Hz, 2H, ArCOOCH$_2$CH$_3$), 4.21-4.08 (m, 1H, -OC$_2$H$_2$C$_2$H$_2$-), 3.52 (m, 1H, -CH$_2$NCOOCH$_2$CH$_3$), 2.24 (s, 3H, Ar-CH$_3$), 2.16 (s, 3H, Ar-COOCH$_2$CH$_3$), 2.04-1.63 (m, 4H, -CH$_2$CH$_2$N$_2$CH$_2$-), 1.43-1.40 (m, 3H, -CHN$_3$-CH$_3$), 1.30-1.21 (m, 15H, N-COOCH$_2$CH$_3$). $^{13}$C NMR (d ppm in CDCl$_3$): 171.50, 170.93 (COOCH$_2$CH$_3$), 157.82, 157.03, 151.90, 149.85, 149.45, 142.23, 140.90, 138.33, 135.64, 132.64, 122.87, 121.60, 120.43, 118.67, 110.54, 106.09, 102.99 (ArC), 71.07, 70.97 (OCH$_2$CHCH$_2$CH$_2$-), 61.80, 61.01, 60.73, 58.25, 58.07 (-COOCH$_2$CH$_3$, -CHN$_3$-CH$_3$, -OCH$_2$CHCH$_2$CH$_2$-), 54.04 (-CH$_2$COOCH$_2$CH$_3$), 32.04, 31.75, 28.74, 28.48 (-CH$_2$CH$_2$CHN$_3$-CH$_3$), 21.05, 19.61, (Ar-COOCH$_2$CH$_3$, Ar-CH$_3$), 14.43 (-CH$_2$COOCH$_2$CH$_3$). ESI-MS (m/z): 901.4 ([M+Na$^+$]$^+$, found), 878.3 (calcd).

**Fig. S15** (left) $^1$H NMR and (right) $^{13}$C NMR spectra of 15.
Step 14: N$_3$-fura-2 Et 15 to N$_3$-fura-2 16

Ethyl protected N$_3$-fura-2 17 (13.1 mg, 14.9 µmol) was dissolved in 500µl of MeOH and 500µl of KOH aq (20 mg/ml) was added to it. After stirring over night (12 hrs, r.t.), deprotection of ethyl groups were confirmed by reverse phase TLC (RP-19 F$_{254}$S, Merck & Co.) and ESI-MS. The solution was diluted to MeOH/water=1/1(v/v) and purified with column chromatography on reverse phase silica gel (YMC Co. Ltd., Kyoto, Japan) (MeOH/water=1/1) to yield N$_3$-fura-2 18 (10.2 mg 13.8 µmol, yield 93%). TLC (reverse phase): $R_f$ = 0.6 (MeOH/water=1/1). ESI-MS (m/z): 737.4([M-H$^+$]+, found), 738.2(calcld).

Fig. S16 (left) $^1$H NMR and (right) ESI-MS spectra of 16.
Step 15: N$_3$-fura-2 16 to N$_3$-fura-2 AM 17

N$_3$-fura-2 17 (10.2 mg, 13.8 μmol) and DIPEA (50 μl, 287 μmol) were dissolved in dry DMF and bromomethyl acetate (50 μl, 510 μmol) was added dropwise. The mixture was stirred overnight at room temperature. When 17 disappeared on reverse-phase TLC (MeOH/water=1/1 (v/v)) and 18 was confirmed by TLC (Chloroform/Acetone = 20/1), the mixture was diluted with toluene and washed with 1M HCl (×2) and saturated NaCl aq (×2). After organic layers were combined and concentrated, the crude compound was purified with silica gel column chromatography (chloroform/acetone=20/1) to yield N$_3$-fura-2 AM 19 (13.5 mg, 12.3 μmol, yield 89%).

TLC: $R_f$ = 0.4 (chloroform/acetone=20/1). $^1$H NMR (d ppm in CDCl$_3$): 7.96 (s, 1H, -ArH), 7.47 (s, 1H, -ArH), 7.12 (d, $J = 6.6$ Hz, 2H, -ArH), 6.83 (d, $J = 7.8$ Hz, 2H, -ArH), 6.78 (d, $J = 7.8$ Hz, 1H, -ArH), 7.72 (d, $J = 7.8$ Hz, 1H, -ArH), 5.99 (s, 2H, Ar-COOCH$_2$OCOCH$_3$), 5.73-5.68 (m, 8H, NCH$_2$COOCH$_2$OCO), 4.68 (m, 1H, -OCH$_2$CHCH$_2$CH$_2$-), 4.24 (s, 2H, -Ar-COOCH$_2$OCO), 4.21-4.03 (m, 10H, -OCH$_2$CHCH$_2$CH$_2$-, -CH$_2$NCOOCH$_2$OCO), 3.52 (m, 1H, -CHN$_3$-CH$_3$), 2.31 (s, 3H, Ar-CH$_3$), 2.16 (s, 3H, Ar-COOCH$_2$OCOCH$_3$), 2.03-1.63 (m, 4H, -CH$_2$CH$_2$CHN$_3$-CH$_3$, -CH$_2$CH$_2$CHN$_3$-CH$_3$), 1.30-1.25 (m, 3H, -CHN$_3$-CH$_3$), $^{13}$C NMR (d ppm in CDCl$_3$): 169.98, 169.48 (-COOCH$_2$OCO-), 157.43, 156.07, 151.62, 149.91, 149.42, 142.33, 140.84, 140.06, 137.23, 133.274, 122.78, 122.02, 120.67, 117.61, 110.91, 106.00, 103.54 (ArC), 79.30 (-CH$_2$COOCH$_2$OCO-), 70.86 (OCH$_2$CHCH$_2$CH$_2$-), 58.06 (-CHN$_3$-CH$_3$), 53.55 (-CH$_2$COOCH$_2$OCO-), 31.90, 31.56, 28.49, 28.19 (-CH$_2$CH$_2$CHN$_3$-CH$_3$), 20.92, 20.70, 19.40 (COOCH$_2$OCOCH$_3$, Ar-CH$_3$). ESI-MS (m/z): 1121.3([M+Na$^+$]$^+$, found), 1198.3 (calcd).
Fig. S17 (left) $^1$H NMR and (right) $^{13}$C NMR spectra of N$_3$-fura-2 AM 17.

Fig. S18 ESI-MS spectra of N$_3$-fura-2 AM 17.
To determine the purity of N\textsubscript{3}-fura-2 AM, analytical high performance liquid chromatography (HPLC) was performed on LC-10AD VP (Simadzu Corporation) at 285 nm equipped with RP-18 GP column (f = 5 μm, Kanto Chemical Co.) A 20 μl sample solution in MeOH (100 μM) was injected and then chromatography was performed at 25°C with an initial flow rate of 50 ml/min of MeOH/water = 1/1 (v/v) as a moving phase.

**Fig. S19** HPLC analytical spectrum of N\textsubscript{3}-fura-2 AM. The purity of N\textsubscript{3}-fura-2 AM more than 95%. 


2. Evaluation of the fluorescent properties of N\textsubscript{3}-fura-2

The concentration of N\textsubscript{3}-fura-2 and N\textsubscript{3}-fura-2 AM solution were determined from the maximum UV absorbance (370 nm) in an aqueous solution. N\textsubscript{3}-fura-2 and N\textsubscript{3}-fura-2 AM were dissolved in deionized water and DMSO, respectively. Absorbance was recorded with a V660 spectrophotometer (JASCO, Tokyo, Japan) using a quartz cell with a 10-mm pathlength. Concentrations of N\textsubscript{3}-fura-2 and N\textsubscript{3}-fura-2 AM were determined by comparison with the absorbance of fura-2 (Dojindo, Tokyo, Japan) and fura-2 AM (Dojindo), respectively. The fluorescence emission spectra and Ca\textsuperscript{2+} sensitive excitation spectra of N\textsubscript{3}-fura-2 were recorded on a RF-5300 spectrofluorophotometer (SHIMAZU, Tokyo, Japan) at 25 ± 2°C. N\textsubscript{3}-fura-2 solution (50 \mu M) was diluted with Ca\textsuperscript{2+}/EGTA buffers from a calcium calibration buffer kit (Invitrogen, Eugene, OR). The zero calcium buffer (10 mM K\textsubscript{2}EGTA, 100 mM KCl, 10 mM MOPS, at pH 7.20 r.t.) and high calcium buffer (10 mM CaEGTA, 100 mM KCl, 10 mM MOPS, at pH 7.20, r.t.) were mixed at an appropriate ratio to prepare buffers with different concentrations of free Ca\textsuperscript{2+} (0 \mu M, 0.017 \mu M, 0.038 \mu M, 0.065 \mu M, 0.100 \mu M, 0.150 \mu M, 0.225 \mu M, 0.351 \mu M, 0.602 \mu M, 1.350 \mu M, 39.800 \mu M). Using these buffers, excitation spectra (260 nm - 440 nm, \lambda_{Em}: 510 nm) of N\textsubscript{3}-fura-2, BSA-fura-2 and fura-2 were recorded (Figure S2).
3. Evaluation of click reaction using DBCO–modified BSA

Preparation of DBCO-modified BSA

DBCO-modified BSA was prepared with DBCO-NHS ester (Click chemistry tools, Arizona, USA) and bovine serum albumin (Sigma Aldrich, St Louis, MO). Reaction compounds were mixed as 10 mg/ml BSA (150 μM), DBCO-NHS ester (1.5 mM) as a final concentration in PBS 500 μl (PB 10 mM, pH 7.4, NaCl 150 mM). The mixture was incubated at 37°C for 4 h and quenched with 500 μl of Tris buffer (pH 8.0). The crude mixture was then purified using a Sephadex PD10 column (GE Healthcare, Tokyo, Japan). The concentration of obtained DBCO-modified BSA solution (DBCO-BSA) was estimated to be 4.89 mg/ml by the Bradford protein assay method calibrated with BSA standard (dye reagent and BSA standards were purchased from BIO-RAD, Hercules, CA). The concentration of DBCO was also estimated by UV absorbance at 311 nm to be 0.71 mM (which is equivalent to 10 times that of BSA).

Click reaction between N\textsubscript{3}-fura-2 and DBCO-modified BSA in a cuvette

To demonstrate the clickability of N\textsubscript{3}-fura-2 and N\textsubscript{3}-fura-2 AM, both compounds were individually reacted with DBCO-modified BSA \textit{via} click chemistry in a sample tube. DBCO-modified BSA and N\textsubscript{3}-fura-2 AM were mixed in HEPES buffer at a final concentration of 0.7 mg/ml DBCO-modified BSA and 10 μM N\textsubscript{3}-fura-2 AM. The reaction was allowed to proceed for 30 min, 1 h, 2 h and 4 h at 37°C. As controls, a solution of N\textsubscript{3}-fura-2 AM only, and DBCO-modified BSA only were also prepared.

Electrophoresis assay of click reaction in cuvette

After reaction, the coupling of N\textsubscript{3}-fura-2 AM and DBCO-BSA was confirmed by electrophoresis. Mini-PROTEAN® precast gels were purchased from BIO-RAD. 5 μl of samples were mixed with 10 μl of sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and heated at 95°C for 4 min to denature the protein. Samples and markers were added to the gel wells (13 μl/well) and electrophoresis was performed at 200 V for 30 min in running buffer (25 mM Tris, 192 mM glycine, 0.01% SDS). After electrophoresis, the gel was immersed in fixing solution (10% AcOH, 40% MeOH) overnight and
fluorescence was observed under UV irradiation ($\lambda_{ex} = 365$ nm). The gel was then stained with Coomassie Brilliant

Blue solution (1 mg/ml) in 40% MeOH, 10% AcOH for 30 min and washed with a fixing solution overnight. The

stained gel was observed and photographed. N$_3$-fura-2 AM and fura-2 AM after the click reaction procedure were

also analyzed by electrophoresis using the same procedure as described above.
4. Evaluation of the click reaction using DBCO-modified cells

Cell culture

HeLa cells were cultured on glass-bottom dishes (Iwaki, Tokyo, Japan) in culture medium (89% DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen)) at 37°C in the presence of 5% CO₂. Cells were cultured at a concentration of \(2 \times 10^5\) cells / 60 mm glass-bottom dish at the time of the experiment.

Preparation of DBCO-modified HeLa cells

To prepare a DBCO-NHS loading solution, 10 mM DBCO-NHS in DMSO was dispersed in DPBS (Invitrogen, pH 7.4, NaCl 140 mM) at a final concentration of 10 \(\mu\)M. A 1 ml aliquot of the loading solution was added to HeLa cells and the cells were incubated for 15 min at 37°C. The loading solution was then replaced with fresh, pre-warmed culture medium and the cells were incubated for a further 30 min at 37°C in order to completely wash away the free DBCO-NHS ester and hydrolyzed DBCO-NHS ester.

Dye loading

Cells were loaded with a Ca\(^{2+}\) indicator (N\(_2\)-fura-2 AM, fura-2 AM or fura-PE3 AM) at a concentration of 1 \(\mu\)M in DMEM supplemented with 0.02 % Pluronic F127 (AnaSpec Inc., San Jose, CA). Culture medium was replaced with pre-warmed loading solution, incubated for 30 min at 37°C and washed with the culture medium before imaging. In the case of fura-PE3 AM, cells were incubated for 45 min in loading solution according to the manufacturer’s instructions.

Electrophoresis assay using total cell lysate of DBCO-modified HeLa cells

For the electrophoresis assay using total cell lysate, cellular proteins were extracted from dye-loaded cells by repeated freeze/thaw cycles. Protein concentrations of the obtained lysates were estimated by a Bradford protein assay. Electrophoresis and observation was carried out according to “Evaluation of click reaction using DBCO–modified BSA”.
Confocal laser scanning microscopy (CLSM) observation and organelle staining

Cells were loaded with fura-2 AM and N3-fura-2 AM, and then observed under the confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan) at 37°C. To determine the localization of N3-fura-2, N3-fura-2 loaded cells were stained with organelle tracking dyes (Fig. S21). Mitochondria and endoplasmic reticulum of were stained with 50 nM of Mito Fluor far red (Invitrogen) or 500 nM of ER tracker green (Invitrogen) for 30 min in L-15 medium at 37°C. Golgi apparatus of RFNT pre-loaded HeLa cells was stained with NBD C6 ceramide solution (according to the protocol) for 30 min.

Optical setup of epi illumination microscopy

Fura-2 and its derivatives were excited using a 75 W xenon lamp (Ushio UXL-75XE) with a band-pass excitation filter FF01-340/26-25 (Semrock, Rochester, NY), MBP380 (Olympus, Tokyo, Japan) or MBP360 (Olympus). Cells were kept at 37°C in an atmosphere containing 5% CO2 using a stage top incubator (INU series; Tokai Hit, Shizuoka, Japan). Microscopic observations were performed on the cells whilst in the incubator.

Time course observations of cytosolic retention

Temporal decay of each fluorescent molecule in the cell was evaluated as follows. N3-fura-2 AM, fura-2-AM or fura-PE3/AM loaded cells were placed on the microscope immediately after wash and the fluorescence intensity within the cells were monitored using an excitation of 360 nm, which corresponds to the isosbestic point of the fura derivatives. Fluorescence images were taken every 15 min starting 5 min after the wash and continued for 3 hours (Fig. S22-24).

Ni2+ (2 mM) was added to the culture medium to quench extracellular Ca2+ indicator molecules3 (Fig. S25), thereby the presence of intracellular Ca2+ indicator was confirmed. The same experimental procedure was also performed in the absence of Ni2+. The function of each Ca2+ indicator was confirmed by applying histamine (Sigma-Aldrich) at a final concentration of 10 µM to the Ca2+ dye-loaded cells. Histamine was prepared as a 1 mM stock solution in DPBS, and 10 µl of which was added to 1 ml culture medium. Time-lapse recording of
fluorescence image pairs excited at 340 and 340 nm by alternating excitation filters were performed at 3 sec

intervals for 3 min. Histamine was added several seconds after the initial frame.

Data acquisition and analysis

TI Workbench software developed by T.I. (Inoue, J. Neurosci. 18:5366-5373 (1998)) was used for

fluorescence image acquisition and analyses.
Supplementary Results

5. Ca^{2+} sensitivity of N_{3}-fura-2 and fura-2

![Graphs showing calcium sensitivity of indicators](image)

**Fig. S20** Excitation wavelength of (a) fura-2, (b) N_{3}-fura-2 and (c) N_{3}-fura-2 conjugated with DBCO-modified BSA. K_d of each Ca^{2+} indicator was calculated by the relation of fluorescence ratio and Ca^{2+} concentration. (d, e, f) is for fura-2, N_{3}-fura-2 and N_{3}-fura-2 conjugated with DBCO-modified BSA, respectively.

Electronic Supplementary Material (ESI) for Chemical Communications

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6. Localization of clicked N$_3$-fura-2 in HeLa cells

![Localization of clicked N$_3$-fura-2 in HeLa cells compared with organelles. Mitochondria (upper), endoplasmic reticulum (middle) and golgi apparatus (bottom) was stained with organelle tracking dyes. N$_3$-fura-2 conjugated with biomacromolecules was dispersed in cytosol and there were no specific pattern which stained with organelle tracking dyes. This result imply that N$_3$-fura-2 was not localized in specific organelle and mainly exist in cytosol.](image-url)
7. Time course observation of cytosolic retention (N$_3$-fura-2, fura-2 and fura-PE3)

**Fig. S22** A series of time-course images of N$_3$-fura-2 AM loaded HeLa cells. HeLa cells were loaded with 1 μM N$_3$-fura-2 AM solution and washed at 0 min. The fluorescence images (excited at 360 nm) were taken every 15 min for 3 h.
Fig. S23 A series of time-course images of fura-2 AM loaded HeLa cells. HeLa cells were loaded with 1 μM fura-2 AM solution and washed at 0 min. The fluorescence images (excited at 360 nm) were taken every 15 min for 3 h.
A series of time-course images of fura-PE3 AM loaded HeLa cells. HeLa cells were loaded with 1 µM fura-PE3 AM solution and washed at 0 min. The fluorescence images (excited at 360 nm) were taken every 15 min for 3 h.

Actual working concentration of Ca$^{2+}$ indicators were estimated by quenching extracellular indicators using excess Ni$^{2+}$ ions. Fluorescence intensity of (a) fura-2 and (b) N$_3$-fura-2 loaded cells before and after Ni$^{2+}$ quenching every 30 min for 2 h.
References


