Supplementary Information

A General Approach for Development of Fluorogenic Probes Suitable for No-Wash Imaging of Kinases in Live Cells

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Abbreviations

Boc: tert-butyl dicarbonate
Cbz: carboxybenzyl
DCM: dichloromethane
DIPEA: N,N’-diisopropylethylamine
DMF: N,N’-dimethylformamide
EDCI: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
Et$_3$N: triethylamine
EtOAc: ethyl ester
HATU: (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazole[4,5-b]pyridinium 3-oxidhexafluorophosphate)
HOBT: N-hydroxylbenzotriazole
MeOH: methanol
TFA: trifluoroacetic acid
THF: tetrahydrofuran

BCR: B-cell receptor
BSA: Bovine serum albumin
Btk: Bruton’s tyrosine kinase
Btk-1: kinase domain of Bruton’s tyrosine kinase (382-659)
Btk-1*: kinase domain of Bruton’s tyrosine kinase (382-659, C481A)
CBB: coomassie brilliant blue staining
EGFR: epidermal growth factor receptor
FGS: fluorescence gel scanning
GSH: glutathione
IP: immunoprecipitation
Itk: IL-2-inducible T-cell kinase
Lck: lymphocyte-specific protein tyrosine kinase
PBS: phosphate-buffered saline
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Chemistry

General Information

All reagents were purchased from commercial vendors and used without further purification. Anhydrous DMF was distilled from calcium hydride. Anhydrous DCM was distilled from calcium hydride.

Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.2 mm Jiangyou silica gel plates (HSGF$_{254}$) using UV light as visualizing agent or ninhydrin as developing agent. Flash column chromatography was carried out using Puke silica (ZCX-II, 200-300 mesh).

$^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker Advance 400 and 500 spectrometers at ambient temperature. Chemical shifts are reported in ppm relative to residual chloroform ($\delta_H$ 7.26 and $\delta_C$ 77.16) and dimethylsulfoxide ($\delta_H$ 2.50 and $\delta_C$ 39.52) as standards. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants and number of protons. Mass spectrometric data were obtained using an AB Q-Star mass spectrometer.

Structures of ibrutinib and PCI-33380

![Ibrutinib](image1.png)

![PCI-33380](image2.png)
Experimental Procedures and Spectroscopic Data

**Supporting Scheme 1.** Synthetic Route of Probes 1 and 2.

**Compound 3.1**

Compound 51 (4.03 g, 0.009 mol) dissolved in 5ml DMF was added to a solution of compound 32 (2.35 g, 0.008 mol) in 45 ml DMF with K2CO3 (2.14 g, 0.016 mol). The reaction mixture was heated to 80 °C and stirred overnight under nitrogen. The reaction mixture was concentrated and extracted with ethyl acetate and water three times. The ethyl acetate layers were combined, washed with saturated NaCl, dried over anhydrous Na2SO4, concentrated in vacuo and purified by flash column chromatography (Hexanes:EtOAc = 1:3) to give compound 3.1 as white solids (3.71 g, yield 85%).

1H NMR(500 MHz, CDCl3): δ= 8.33 (d, J = 1.2 Hz, 1H), 7.63 (d, J = 6.8 Hz, 2H), 7.39-7.30 (m, 7H), 7.18-7.08 (m, 5H), 6.01 (brs, 2H), 5.51-5.47 (m, 1H), 5.22-5.06 (m, 2H), 4.62-4.54 (m, 1H), 4.27-4.18 (m, 1H), 4.02-3.96 (m, 1H), 3.71 and 3.53 (s, 3H), 2.90-2.84 (m, 2H)
Compound 4

\[
\text{HOBT (320.27 mg, 2.37 mmol), EDCI (1.02 g, 5.32 mmol), and DIPEA (1.55 ml, 8.9 mmol) were added to a solution of the acid, followed by addition of N-(2-aminoethyl)carbamic acid tert-butyl ester (850 mg, 5.31 mmol) in 50 ml DCM at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. The reaction mixture was then concentrated in vacuo, and extracted with ethyl acetate and water three times. The organic layers were collected, washed subsequently with 1N HCl, saturated NaHCO₃, saturated NaCl, dried over anhydrous Na₂SO₄, concentrated in vacuo and purified by flash column chromatography (EtOAc with 0.5% Et₃N) to give compound 4 (858 mg, yield 70%) as white solids.}
\]

\[\text{1H NMR (400 MHz, CDCl}_3\text{: } \delta = 8.29 \text{ (s, 1H), 7.59 (d, J = 8.4 Hz, 2H), 7.34-7.23 (m, 7H), 7.17-7.05 (m, 5H), 6.01 (brs, 2H), 5.46-5.43 (m, 1H), 5.15 (m, 2H), 4.47-4.43(m, 1H), 4.12-4.08 (m, 2H), 3.31-2.77 (m, 6H), 1.35 (s, 9H)}\]

\[\text{13C NMR (100 MHz, CDCl}_3\text{: } \delta = 158.56, 157.96, 156.25, 155.75, 154.46, 144.08, 135.90, 129.88, 129.82, 128.43, 128.12, 127.89, 127.28, 124.02, 119.49, 118.85, 98.60, 79.17, 67.58, 60.14, 54.12, 53.34, 50.53, 40.00, 28.24} \]

HRMS (m/z): [M+H]⁺ calcd. for C₃₇H₄₁N₈O₆⁺: 693.3149; found: 693.3128
Compound 6

Ethyl glycolate (21 g, 0.202 mol) was added to a suspended solution of Na₂CO₃ (42 g, 0.396 mol) in 200 ml THF. The mixture was stirred for 1 h and then 1-bromo-2,4-dinitrobenzene (5 g, 0.020 mol) was added to the solution. The reaction was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo, and partitioned with DCM and H₂O. The DCM layers were collected, washed with saturated NaCl, dried over anhydrous Na₂SO₄, concentrated in vacuo, and purified by flash column chromatography (Hexanes: EtOAc = 3:1) to give the intermediate ester as yellow solids (1.08 g, yield 67% brsm).

¹H NMR (400 MHz, CDCl₃): δ = 8.74 (d, J = 2.4 Hz, 1H), 8.40 (dd, J = 9.2, 2.8 Hz, 1H), 7.01 (d, J = 9.2 Hz, 1H), 4.91 (s, 2H), 4.27 (q, J = 7.2 Hz, 2H), 1.29 (t, J = 7.2 Hz, 3H)

¹³C NMR (100 MHz, CDCl₃): δ = 166.46, 155.55, 140.85, 139.20, 128.76, 121.95, 114.74, 66.31, 62.20, 14.00

HRMS (m/z): [M+Na]⁺ calcd. for C₁₀H₁₀N₂O₇Na⁺, 293.0386; found: 293.0370

40 ml concentrated hydrochloric acid was added to the ester (1 g, 0.004 mol) and then the reaction mixture was heated to 100°C. The reaction was monitored by TLC, after 8 h, the reaction mixture was cooled to room temperature, poured into 300 ml water, and extracted with ethyl acetate three times. The ethyl acetate layers were collected, washed with saturated NaCl, dried over anhydrous Na₂SO₄, and concentrated in vacuo to give intermediate 6 as yellow solid and directly used in future step.

Compound 7

Methyl glycinate hydrochloride (5.1 g, 0.041 mol) was added to a suspended solution of Na₂CO₃ (10 g, 0.094 mol) in 200 ml THF. The mixture was stirred for 1 h and then 1-bromo-2,4-dinitrobenzene (5 g, 0.020 mol) was added to the solution. The reaction was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo, and partitioned with DCM and H₂O. The DCM layers were collected, washed with saturated NaCl, dried over anhydrous Na₂SO₄, concentrated in vacuo, and purified by flash column chromatography (Hexanes: EtOAc = 1:1) to give the intermediate as yellow solids (4.84 g, yield 95%).
1H NMR (400 MHz, CDCl3): δ= 9.15 (d, J = 2.4 Hz, 1H), 8.94 (brs, 1H), 8.30 (dd, J = 9.2, 2.4 Hz, 1H), 6.79 (d, J = 9.2 Hz, 1H), 4.21 (d, J = 2.4 Hz, 2H), 3.87 (s, 3H)
13C NMR (100 MHz, CDCl3): δ= 168.49, 147.25, 136.89, 131.16, 130.44, 124.11, 113.92, 53.04, 44.73
HRMS (m/z): [M-H] calcd. for C9H8N3O6, 254.0413; found: 254.0420

39 ml 1N LiOH was added slowly to a solution of the methyl ester intermediate (1 g, 0.004 mol) in methanol. The reaction was monitored by TLC. After 2 h, the reaction mixture was concentrated, extracted with ethyl acetate and water three times. The aqueous layers were collected and acidized by 1N HCl to pH 3 at 0°C. The mixture was then extracted with dichloromethane and H2O, washed with saturated NaCl, dried over anhydrous Na2SO4, and concentrated in vacuo to give intermediate 7 and directly used in future step.

Compound 8

Compound 3.1 (130 mg, 0.23 mmol) was dissolved in ethyl ester (2 ml), catalytic Pd(OH)2/C was added to the solution. After three cycles of vacuum/H2, the reaction mixture was stirred under hydrogen atmosphere at ordinary pressure (balloon) at room temperature for 3 hrs. The reaction mixture was filtered by Celatom, the filtrate was concentrated in vacuo to get the crude intermediate and used directly in the next step.

HOBT (32.4 mg, 0.24mmol), HATU (152 mg, 0.4mmol), and Et3N (140ul, 1mmol) were added to a solution of compound 6 (48.4 mg, 0.2mmol) and the crude intermediate from previous step in DMF (2 ml) at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated in vacuo, extracted with ethyl acetate and water three times, the organic layers were collected, washed with 1N HCl, saturated NaHCO3, saturated NaCl, and dried over anhydrous Na2SO4, concentrated in vacuo and purified by flash column chromatography (EtOAc) to yield compound 8 (65 mg, yield 50% ) as yellow solid.

1H NMR (400 MHz, CDCl3): δ= 8.70(s, 1H), 8.36 (m, 1H), 8.28 (s, 1H), 7.61 (dd, J = 4.4, 8.4 Hz, 2H), 7.40-7.35 (m, 2H), 7.25 (d, J = 4.8 Hz, 1H), 7.19-7.06 (m, 5H), 5.60-
5.52 (m, 1H), 5.03 (s, 2H), 4.84-4.71 (m, 1H), 4.33-3.97 (m, 2H), 3.66 and 3.58 (s, 3H), 3.11-2.88 (m, 2H)

$^1$H NMR (100 MHz, CDCl$_3$): $\delta=$ 170.87, 170.44, 165.05, 164.61, 158.69, 158.63, 157.94, 155.96, 155.87, 155.65, 155.57, 154.61, 154.22, 144.53, 144.11, 140.58, 140.47, 138.64, 138.53, 129.91, 129.79, 129.06, 128.83, 127.18, 127.02, 124.12, 121.80, 121.72, 119.57, 119.54, 118.86, 118.71, 115.55, 98.65, 98.54, 68.20, 68.02, 58.02, 57.82, 54.40, 52.85, 52.52, 52.25, 52.07, 49.79

HRMS (m/z): [M+Na]$^+$ calcd. for C$_{31}$H$_{26}$N$_8$O$_9$Na$^+$, 677.1721; found: 677.1720

Compound 9

![Chemical structure of Compound 9](image)

Compound 4 (200 mg, 0.29 mmol) was dissolved in ethyl ester (3 ml), catalytic amount of Pd(OH)$_2$/C was added to the solution. After three cycles of vacuum/H$_2$, the reaction mixture was stirred under hydrogen atmosphere (balloon) at room temperature for 3 hrs. The reaction mixture was filtered by Celatom, the filtrate was concentrated in vacuo to get the crude intermediate and used in the next step.

HOBT (47 mg, 0.35 mmol), HATU (331 mg, 0.87 mmol), and DIPEA (252 $\mu$l, 1.45 mmol) were added to a solution of compound 6 (70 mg, 0.29 mmol) and the crude intermediate from previous step in DMF (3 ml) at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated in vacuo, and extracted with ethyl acetate and water three times; the organic layers were collected, washed with 1N HCl, saturated NaHCO$_3$, saturated NaCl, and dried over anhydrous Na$_2$SO$_4$, concentrated in vacuo and purified by flash column chromatography (EtOAc with 0.5% Et$_3$N, 2% MeOH) to yield compound 9 (147 mg, yield 65% over two steps) as brown solids.

HRMS (m/z): [M-H]$^-$ calcd. for C$_{37}$H$_{37}$N$_{10}$O$_{10}$, 781.2694; found: 781.2714
0.25 ml trifluoroacetic acid was added slowly to a solution of intermediate 9 (80 mg, 0.10 mmol) in 1 ml DCM at 0°C. The reaction was stirred at room temperature and monitored by TLC. After 3 hrs, the reaction mixture was concentrated, ethyl acetate was added, and washed with saturated NaHCO₃, saturated NaCl, dried over anhydrous Na₂SO₄, and concentrated in vacuo to give the amine as brown solids.

The coupling reagents HOBT (5 mg, 0.037 mmol), HATU (34.2 mg, 0.09 mmol), and DIPEA (26 μl, 0.15 mmol) were added to a solution of the amine intermediate and BODIPY-FL (8.6 mg, 0.029 mmol) acid in DMF at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated in vacuo, extracted with ethyl acetate and water three times; the organic layers were collected, washed with 1N HCl, saturated NaHCO₃, saturated NaCl, and dried over anhydrous Na₂SO₄, concentrated in vacuo and purified by flash column chromatography (EtOAc with 0.5% Et₃N, 5% MeOH) to yield probe 1 (5.54mg, yield 20% of two steps) as dark red solids.

**1H NMR (400 MHz, CDCl₃):** δ = 8.72(d, J = 2.8 Hz, 1H), 8.40 (dd, J = 9.6, 3.2 Hz, 1H), 8.28 (d, J = 10.8 Hz, 1H), 8.15 (s, 1H), 7.82 (s, 1H), 7.68-7.61 (m, 3H), 7.56 (d, J = 9.6 Hz, 1H), 7.43 (t, J = 7.6 Hz, 2H), 7.20-7.11 (m, 5H), 7.01 (d, J = 3.6 Hz, 1H), 6.26 (t, J = 7.2 Hz, 2H), 5.54 (m, 1H), 5.39-5.18 (m, 2H), 4.39-4.35 (m, 1H), 4.19 (m, 1H), 3.97 (m, 1H), 3.19-3.00 (m, 6H), 2.76-2.67 (m, 1H), 2.59-2.60 (m, 1H), 2.42 (s, 3H), 2.40-2.38 (m, 2H), 2.24 (s, 3H).

**13C NMR (100 MHz, CDCl₃):** δ = 171.50, 159.56, 158.63, 157.66, 156.78, 156.28, 144.08, 137.15, 134.92, 133.45, 130.58, 129.34, 128.83, 128.72, 128.25, 127.95, 127.60, 125.76, 124.98, 124.24, 120.72, 119.46, 119.42, 117.03, 98.16, 67.86, 66.73, 53.93, 53.39, 38.89, 38.73, 34.26, 28.93, 24.38, 22.72, 14.95, 14.40, 11.45, 11.27.

**HRMS (m/z):** [M-H] calcd. for C₄₆H₂₄BF₂N₁₂O₉, 955.3259; found: 955.3234.

Probe 1R and 1S were synthesized using similar procedures as probe 1.
Probe 1S

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$= 8.69(d, J = 3.2 Hz, 1H), 8.37 (dd, J = 9.3, 2.8 Hz, 1H), 8.28 (s, 1H), 8.14-8.11 (m, 1H), 7.84-7.78 (m, 1H), 7.66-7.63 (m, 2H), 7.59 (s, 1H), 7.53 (d, J = 9.5 Hz, 1H), 7.44-7.40 (m, 2H), 7.19-7.09 (m, 5H), 6.99 (d, J = 4 Hz, 1H), 6.27 (s, 1H), 6.23 (d, J = 3.96, 1H), 5.57 - 5.48 (m, 1H), 5.39-5.17 (m, 2H), 4.38-4.34 (m, 1H), 4.20-4.15 (m, 1H), 3.99-3.94 (m, 1H), 3.09-2.97 (m, 6H), 2.59-2.71(m, 2H), 2.43-2.40 (m, 5H), 2.24 (s, 3H).

HRMS (m/z): [M+Na]$^+$ calcd. for C$_{46}$H$_{43}$BF$_2$N$_{12}$O$_9$Na$^+$, 979.3235; found: 979.3230.

Probe 1R

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$= 8.70(d, J = 2.8 Hz, 1H), 8.26-8.22 (m, 2H), 8.19 (brs, 1H), 7.91 (brs, 1H), 7.67-7.64 (m, 3H), 7.45-7.38 (m, 3H), 7.20-7.10 (m, 5H), 7.04 (d, J = 4 Hz, 1H), 6.30-6.27 (m, 2H), 5.62-5.59 (m, 1H), 5.31-5.24 (m, 2H), 4.58-4.56 (m, 1H), 4.14-3.99 (m, 2H), 3.22-3.03(m, 6H), 2.78-2.68 (m, 1H), 2.48-2.41(m, 6H),2.40 (s, 3H).

HRMS (m/z): [M+Na]$^+$calcd. for C$_{46}$H$_{43}$BF$_2$N$_{12}$O$_9$Na$^+$, 979.3235; found: 979.3232.
Compound 10

![Compound 10](image)

Compound 4 (62 mg, 0.089 mmol) was dissolved in ethyl ester (1 ml), catalytic Pd(OH)$_2$/C was added to the solution. After three cycles of vacuum/H$_2$, the reaction mixture was stirred under hydrogen atmosphere (balloon) at room temperature for 3 hrs. The reaction mixture was filtered by Celatom, the filtrate was concentrated in vacuo to get the crude intermediate and used directly in the next step.

The coupling reagents HOBT (14.8 mg, 0.11 mmol), HATU (102 mg, 0.27 mmol), and DIPEA (78 μl, 0.45 mmol) were added to a solution of compound 7 (70 mg, 0.29 mmol) and the crude intermediate from the previous step in 1 ml DMF at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated in vacuo, extracted with ethyl acetate and water three times; the ethyl acetate layers were collected, washed with 1N HCl, saturated NaHCO$_3$, saturated NaCl, dried over anhydrous Na$_2$SO$_4$, concentrated in vacuo and purified by flash column chromatography (EtOAc with 0.5% Et$_3$N, 2% MeOH) to yield compound 10 (45 mg, yield 65%) as brown solids.

HRMS (m/z): [M-H]$^-$ calcd. for C$_{37}$H$_{38}$N$_{11}$O$_9$, 780.2854; found: 780.2871.

Probe 2

![Probe 2](image)

0.5 ml trifluoroacetic acid was added slowly to a solution of intermediate 10 (45 mg, 0.058 mmol) in 2 ml DCM at 0°C. The reaction was stirred at room temperature and
monitored by TLC. After 3 hrs, the reaction mixture was concentrated, washed with saturated NaHCO$_3$, saturated NaCl, dried over anhydrous Na$_2$SO$_4$, and concentrated to give the amino intermediate as brown solids.

The coupling reagents HOBT (5 mg, 0.037 mmol), HATU (35 mg, 0.092 mmol), and DIPEA (27 μl, 0.16 mmol) were added to a solution of the amino intermediate and BODIPY-FL (8.76 mg, 0.03 mmol) in DMF at 0°C under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was concentrated in vacuo, extracted with ethyl ester and water three times, the ethyl acetate layers were collected, washed with 1N HCl, saturated NaHCO$_3$, saturated NaCl, dried over anhydrous Na$_2$SO$_4$, concentrated in vacuo and purified by flash column chromatography (EtOAc with 0.5% Et$_3$N, 5% MeOH) to yield probe 2 (5.73 mg, yield 20% of two steps) as red solids.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 8.26 (d, $J = 3.6$ Hz, 1H), 8.16 (s, 1H), 7.68-7.64 (m, 3H), 7.42 (t, $J = 7.6$ Hz, 2H), 7.35-7.29 (m, 5H), 7.19-7.10 (m, 5H), 7.04 (d, $J = 4$ Hz, 1H), 6.29 (t, $J = 5.6$ Hz, 2H), 5.46-5.40 (m, 1H), 5.12-5.01 (m, 2H), 4.40-4.29 (m, 1H), 4.14-4.11 (m, 1H), 3.86-3.78 (m, 1H), 3.35-3.04 (m, 6H), 2.76-2.56 (m, 2H), 2.51-2.44 (m, 2H), 2.44 (s, 3H), 2.24 (s, 3H).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 173.59, 171.64, 170.86, 166.42, 165.60, 159.33, 158.52, 157.96, 157.56, 156.64, 156.24, 154.99, 154.01, 147.54, 144.22, 135.52, 134.72, 133.27, 131.98, 130.47, 130.13, 130.04, 129.14, 129.00, 128.03, 125.42, 124.14, 120.50, 119.37, 119.29, 116.78, 116.69, 97.97, 59.54, 53.95, 49.72, 43.03, 38.82, 38.42, 34.03, 33.87, 24.09, 23.59, 14.76, 11.26.

HRMS (m/z): [M+H]$^+$ calcd. for C$_{46}$H$_{45}$BF$_2$N$_{13}$O$_8$+, 956.3575; found: 956.3565.
$^{1}$H NMR, $^{13}$C NMR Spectra of New Compounds

Compound 3.1
Intermediate of compound 6
Intermediate of compound 7
Compound 8
Probe 1R
References


Biology

General Information

Regular reagents and reduced L-Glutathione were purchased from Sigma-Aldrich. Albumin Bovin V was purchased from Scientific Research Special. Protein marker (10022139), and other SDS-polyacrylamide gel preparing reagents were purchased from Bio-Rad. ECL Plus A + B (P0018) and other Tris-HCl buffers were purchased from Beyotime. Rmp Protein ASepharose Fast Flow (17-5138-01) was purchased from GE Healthcare.

Btk-1(kinase domain, 382-659) and Btk-1* (C481A, 382-659) were provided by Crown Bioscience Inc. Recombinant kinase EGFR (PV3872) was purchased from Life Invitrogen™. Recombinant kinases Itk (08-181), Btk (08-080), Lck (08-170) were purchased from Carna Biosciences. Anti-Btk (3533S, 8547S) and anti-Itk (2380S) antibodies were purchased from Cell Signaling Technology. Anti-Btk (558527) antibody was purchased from BD Pharmingen. The secondary antibody against rabbit(sc-2004) and mouse(sc-2005) were purchased from Santa Cruz Biotechnology. NorthernLights™ Anti-mouse IgG-NL557(NL007) were purchased from R&D Systems, Inc.

Cell lines(Namalwa, Raji, Jurkat) were purchased from Type Culture Collection of the Chinese Academy of Science, Shanghai, China and K562 was a gift from Prof. Shuo Lin’s lab at Peking University Shenzhen Graduate School. All cell lines were cultured in RPMI 1640 medium (A10491, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (10099-141, Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (C0222, Beyotime) and cultured in a humidified 37°C incubator with 5% CO₂.

CellTiter-Glo® Luminescent Cell Viability Assay Kit(G7572) was purchased from Promega.

All the fluorescence intensity analysis were recorded by Perkin Elmer Envision 2104 Multilabel Reader (Ex = 485 nm, Em = 520 nm) using OptiPlate™–384F. Luminescence analysis was recorded by Tecan Microplate Reader Infinite™ F200 using i-control™ software.

All the fluorescence gel scanning was conducted with PharosFX™ Plus Molecular Imager (Bio-Rad, Ex = 480 nm and Em = 530 nm) using Quantity One™ software.

Fluorescence microscopic images were acquired using a YOKOGAWA CV1000 confocal laser scanning microscope equipped with a X40 lens (Ex = 480 nm, Em =
520 nm) or a Zeiss Axio Observer A1 microscope equipped with charged coupled device.

**Fluorescent Properties Measurements**

Probes were dissolved in DMSO at 10 mM as stock solutions. For the fluorescence quenching efficiency analysis, probe solutions were diluted to 1 µM in D-PBS and the fluorescence intensity was recorded by Perkin Elmer Envision 2104 Multilabel Reader (Ex = 485 nm, Em = 520 nm) using OptiPlate™ – 384F. For the fluorescence "turn-on" assays, purified recombinant Btk-1( kinase domain 382-659) or other surrogates (BSA or GSH) for free thiols was diluted to the final concentrations in reaction buffer (50 mM HEPES, 250 mM NaCl, 5% glycerol, 10 mM MgCl₂, 2 mM DTT, pH 8); probes 1 and 2 were diluted to the desired concentrations by the reaction buffer. The fluorescence recovery was monitored and recorded by Perkin Elmer Envision 2104 Multilabel Reader (Ex = 485 nm, Em = 520 nm) using OptiPlate™ – 384F at 25°C. In the competition experiments, Btk inhibitor ibrutinib was diluted to desired concentrations in reaction buffer and pre-incubated with Btk-1 at room temperature for 45 min, probe 1 or 2 was then added subsequently. The fluorescence intensity was calculated as $I_F/I_F'$, $I_F$: fluorescence intensity of Btk-1 and probes 1 or 2, $I_F'$: fluorescence intensity of probe 1 or 2 alone in reaction buffer.

Probe 1 in DMSO was added to Btk-1 solution at the final concentration of 0.5 µM (probe 1) and 25 µM (Btk-1) and the emission spectrum of the reaction system was recorded by BioTekR Synergy H1 Hybrid Reader at different time points (0.5h, 1h, 3h, 6h, 10h).

**Supporting Figure 1.** Fluorescent properties of probe 1 and 2. a) Time curve of fluorescence increase of 0.5 µM probe 1 with 25 µM Btk-1 with different concentrations of ibrutinib (12.5 µM, 25 µM, 37.5 µM, 50 µM). b) Time curve of fluorescence increase of 0.5 µM probe 2 with 25 µM Btk-1 with different concentrations of ibrutinib (12.5 µM, 25 µM, 37.5 µM, 50 µM). c) Time curve of
fluorescence increase of 0.5 μM probe 1 and 2 with 25 μM BSA. d) Time curve of fluorescence increase of 0.5 μM probe 1 and 2 with 15 mM GSH. e) Emission spectrum of 0.5 μM probe 1 with 25 μM Btk-1 at different time.

Mass Spectrometry Study

Btk kinase domain (382-659) was labelled with probe 1 under the following conditions: 2 μl of 9 mg/ml Btk kinase domain in Btk kinase domain buffer (50 mM HEPES, pH 8.0, 250 mM NaCl, 5% glycerol, 10 mM MgCl₂, 2 mM DTT) and 5.6 μl of 1 mM probe 1 in DMSO were added to 92.4 μl of 50 mM pH 8.0 HEPES buffer, and incubated at room temperature for 15 hrs. After the labelling, 0.6 μg trypsin was added to the labelling system and incubated at 37°C for 8 hrs. The digested peptides were loaded onto a LC-MS/MS system (Waters Acquity UPLC- Waters XEVO G2 QTOF). ESI-MS spectra were processed using the software Waters Biopharma Lynx 1.3.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Calculated Mass (Da)</th>
<th>m/z</th>
<th>Charge State</th>
<th>Observed Mass (Da)</th>
<th>Mass Error (ppm)</th>
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Supporting Figure 2. Mass spectrum of the peptide fragment modified by probe 1.

In Vitro and Live Cell Probe Labelling Experiments

Recombinant kinases were diluted to the final concentrations in reaction buffer (50 mM HEPES, 250 mM NaCl, 5% glycerol, 10 mM MgCl₂, 2 mM DTT, pH 8) and incubated with various concentrations of probe 1 for 3 hrs at room temperature, unless indicated otherwise. Labelling was stopped by the addition of LDS Sample Buffer (NP0007, Invitrogen) and Sample Reducing Agent (NP0004, Invitrogen).

Cells (at a density of 1,500,000 cells/ml) in standard growth media were incubated with 2 μM probe 1 at 37°C in 5% CO₂ for 1h. Cells were washed with about 8X...
volumes of PBS three times to remove serum proteins and excess probes, and the pellets were lysed directly in LDS Sample Buffer containing Sample Reducing Agent (NP0007 and NP0004, Invitrogen).

In competition experiments, purified proteins or live cells were incubated with inhibitors ibrutinib or compound 3 at 2 μM for 45 min. Subsequently, the samples were labelled with probe 1 as mentioned above.

Samples from *in vitro* protein and live cell labelling experiments were heated at 75°C for 10 minutes and 100°C for 20 minutes separately without removing of excess probes. Then the denatured samples were resolved by SDS-polyacrylamide gels (12% or 10%), and the gels were visualized by fluorescence gel scanning with PharasFX™ Plus Molecular Imager (Bio-Rad, Ex = 480 nm and Em = 530 nm). The gels after scanning were transferred to poly(vinylidenedifluoride) membrane using Trans-Blot Turbo™ Transfer system (Bio-Rad). Total Btk was detected using anti-Btk antibody and standard western blotting techniques.

**Supporting Figure 3.** Probe 1 labelling of endogenous Btk in the lymphoma cell lines. a) Raji cells incubated with increasing concentrations of probe 1 for 1 hour. b) K562 cells incubated with increasing concentrations of probe 1 for 1 hour. c) Jurkat cells incubated with increasing concentrations of probe 1 for 1 hour. In Raji and K562 cells, two types of B cell lines, the predominant bands labelled by the probe were at the expected MW of Btk, which was confirmed by Btk western blotting. In the Jurkat cell samples, no significant band was detected. Immunoblotting showed the presence of Itk in Jurkat cells, but not Btk.

**Cell Viability Assays**

Namalwa cells were seeded in 96-well plates (6,000 cells/well), then incubated with probe 1 for 48h; Lymphoma cells (Raji, K562, Jurkat cells) were seeded in 96-well plates (20,000 cells/well), then incubated with probe 1 for 12 h. After the planned
incubation time, cell viability was measured by the standard protocol of CellTiter-Glo® Luminescent Cell Viability Assay by Promega.

Supporting Figure 4. Probe 1 has minimal impact on tested cells’ viability as detected by CellTiter-Glo® Luminescent Cell Viability Assay. a) Cell viability of Namalwa cells treated by probe 1 for 48h. b) Cell viability of Raji cells treated by probe 1 for 12h. c) Cell viability of K562 cells treated by probe 1 for 12h. d) Cell viability of Jurkat treated by probe 1 for 12h.

Probe 1, 1R and 1S Have Similar Labelling Effects

Probe 1 is a diastereomeric mixture containing chiral probes 1R and 1S. These probes were tested in labelling experiments with Btk protein and live Namalwa cells with similar procedures described above. Clearly, probes 1, 1R and 1S labelled Btk bands with similar intensities.
Supporting Figure 5. Comparison of Btk labelling by probe 1, 1R, 1S. a) Full length Btk labelling by probe 1, 1R, 1S. b) Endogenous Btk labelling by probe 1, 1R, 1S in live Namalwa cells.

No-wash Imaging of Btk in Live Cells

Namalwa cells were incubated with anti-human IgM at a final concentration of 4-5 mg/ml for 30 min to stimulate BCR signal transduction. Subsequently the stimulated cells were incubated with 1 µM probe 1 and the images of Btk labelling by probe 1 in live cells were captured every 1 min immediately after the adding of probe 1 without extrawashing steps.

Supporting Figure 6. No-wash imaging of live Namalwa cells by probe 1 at different time points (0 min, 5 min, 10 min, 15 min, 20 min, 25 min). BF: Bright Field.

Brief Video

A minute-by-minute short video is available.

Competition Study

Namalwa cells at 1,000,000 cells per ml density were incubated with anti-human IgM at a final concentration of 4-5 mg/ml for 30 minutes to stimulate BCR signal transduction. The stimulated cells were incubated with 5 µM competitor compound (compound 8 [IC\textsubscript{50}=357nM] or ibrutinib [IC\textsubscript{50}=22nM]) at 37°C 5% CO\textsubscript{2} for 1 hour. Subsequently the cells were incubated in competitor free medium and then incubated with 1 µM probe 1 at 37°C 5% CO\textsubscript{2} for 1 hour. The cells were harvested, washed with PBS and then resuspended in PBS. The imaging data were captured at the same exposure time by fluorescent microscopy and processed with the same setting.
Supporting Figure 7. No-wash imaging of live Namalwa cells by probe 1 could be effectively competed out by either ibrutinib or compound 8. Scale bar: 10 μM.

Jurkat cells at 1,000,000 cells per ml density were incubated with 1 μM probe 1 at 37°C 5% CO₂ for 1 hour. The cells were harvested, washed with PBS and then resuspended in PBS. The imaging data were captured at the same exposure time by fluorescent microscopy and processed with the same setting.

Supporting Figure 8. No-wash imaging of live Jurkat cells (no Btk was expressed) by probe 1. Scale bar: 10 μM.

Co-localization Study

Namalwa cells at 1,000,000 cells per ml density were incubated with anti-human IgM at a final concentration of 4-5 mg/ml for 30 minutes to stimulate BCR signal transduction. Subsequently the stimulated cells were incubated with 1 μM probe 1 at 37°C 5% CO₂ for 1 hour. The cells were harvested, washed with PBS and then resuspended in PBS. The cell suspension was then smeared to a gelatin-coated slide and fixed with 3.7% formaldehyde for 30 minutes. After washed with PBS, the cells were permeabilized with 0.5% Triton X-100 for 5 minutes, followed by blocking with 10% FBS. The cells were when incubated with anti-Btk antibody at 4°C overnight, washed with PBS, labelled with Northern Lights™ Anti-mouse IgG-NL557, and washed with PBS. Pearson’s correlation coefficient was calculated by ImageJ™ as 0.90.