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Supplementary Data

A novel FRET-based biosensor for the measurement of BCR–ABL activity and its response to drugs in living cells

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Supplementary Materials and Methods

Expression Plasmids. Pickles was generated using the pCAGGS eukaryotic expression

vector (1); it encodes a chimeric protein that consists of Venus, a variant of a yellow-emitting

mutant of green fluorescent protein (YFP) (2), human CrkL, and an enhanced cyan-emitting

mutant of green fluorescent protein (ECFP) from the amino terminus (see Fig. 1A). Pickles-1.00

and -2.00 harbor the full length and a carboxyl terminus-truncated form (corresponding to amino

acids 1-222) of CrkL, respectively. Circular permutated ECFP was constructed as described

previously (2) with slight modifications. The cDNA of ECFP was amplified by PCR using a

sense primer containing a *Not*I site and a reverse primer containing a *Bam*HI site and a sequence

encoding the linker (Gly-Gly-Ser-Gly-Gly). The cDNA of ECFP was also extended by PCR at

the 5′ end with the sequence encoding the linker and at the 3′ end with another sequence

containing a *Bgl*II site. The former PCR product was simultaneously digested with *Not*I and

*Bam*HI, and cloned into pBluescript (Stratagene) to generate pBluescript-ECFP. The latter PCR

product was digested with *Bam*HI and *Bgl*II, and cloned into the *Bam*HI site of pBluescipt-

ECFP. The resulting plasmid was named pCAGGS-ECFP×2. cDNAs of circularly permutated

ECFPs were amplified by PCR with primers containing restriction enzyme recognition sites,

cleaved with restriction enzymes, and ligated into the *Not*I/*Bgl*II sites of Pickles-2.00. Each

mutated ECFP harbored Thr-49, Gln-157, Asp-173, Leu-195, and Ile-229 as the new N-terminus,

and constituted Pickles-2.10, 2.20, 2.30, 2.40, 2.50, respectively (see Fig. 1B). cDNA for monomeric Venus-L222K/F224R was generated by PCR-based mutagenesis, and substituted Venus in Pickles-2.30 to obtain Pickles-2.31.

pGD210 (3), an expression vector for BCR–ABL, was kindly provided by Dr D. Baltimore (Massachusetts Institute of Technology, Cambridge, MA). The kinase domain-mutants of BCR–ABL were generated by a two-step PCR. The cDNA of the 5′ portion of Abl was amplified by PCR using a forward primer containing a *Hind*III site and reverse primers containing the sequence encoding the mutated amino acids. The 3′ portion was also amplified with forward primers containing the mutated sequence and a reverse primer containing a *Spe*I site. All cDNAs of the Abl variants were amplified using a mixture of the two PCR products together with the *Hind*III and *Spe*I-containing primers. The resulting 3.6 kb products were sub-cloned into the *Hind*III/*Spe*I sites of pCMV-3Myc (Stratagene) to generate pCMV-3Myc-Abl. The cDNA of BCR was cut out from pGD210 by *Eco*RI and *Hind*III, and ligated into pCMV-3Myc-Abl to obtain pCMV-3Myc-BCR–ABL.

cDNAs for JAK (4) and for other non-receptor tyrosine kinases (5, 6) were kindly provided by T. Hirano (Osaka University, Japan) and H. Hanafusa (Osaka Bioscience Institute, Japan), respectively. Expression plasmids for the CrkII-based biosensor Picchu (5) and human CrkII (7) were gifts from Dr. M. Matsuda. The coding region of CrkII and ECFP were amplified by PCR. The resulting fragment of ECFP was subcloned into the EcoRI/NotI sites of pCAGGS-Flag-YFPC (8), followed by subcloning of CrkII into XhoI/NotI sites, to obtain an expression vector for the ABL indicator (9).

Conditions for fluorescence image acquisition. Cells were imaged with an IX71 inverted microscope (Olympus) equipped with a chamber box at 37°C, and a Cool SNAP-HQ cooled charge-coupled device camera (Roper Scientific) controlled with MetaMorph software

(Universal Imaging). Filters used for the dual-emission ratio imaging studies were obtained from Omega Optical Inc.: an XF1071 (440AF21) excitation filter, an XF2034 (455DRLP) dichroic mirror, and two emission filters, XF3075 (480AF30) for CFP and XF3079 (535AF26) for FRET. Cells were illuminated with a 75 W Xenon lamp through a 20% ND filter and viewed through a $60\times$ immersion objective lens (numerical aperture, 1.40). Exposure times for 4×4 binning were 200 ms for CFP and FRET images, and 20 ms for differential interference contrast images.

Immunofluorescence. Peripheral blood mononuclear cells (PBMCs) obtained from CML patients were collected in 1.5 mL tubes, fixed in 3% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 4 min at room temperature, and incubated with 1% bovine serum albumin for 30 min at room temperature to block non-specific binding of antibodies. The cells were then incubated with antibodies against phospho-CrkL and c-Abl, followed by further incubation with a mixture of AlexaFluor488-conjugated goat anti-rabbit (1:2500 dilution) and AlexaFluor488-conjugated goat anti-mouse (1:2500 dilution) antibodies (Molecular Probes). Images are acquired using a Fluoview confocal microscope (Olympus) and analyzed as described previously (10).

Supplementary References

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Supplementary Figure Legends

Supplementary Fig. S1. Development of FRET-based sensors to monitor BCR–ABL activity. (A) Emission spectra of Pickles–1.00, 2.00, 2.10, 2.20, 2.30, 2.40, 2.50, and 2.31 (excited at 420 nm) expressed with (solid line) or without (dashed line) BCR–ABL in 293F cells. The increase in FRET efficiency is indicated in the upper right corner of each panel. (B) Emission ratios of Pickles–2.31, Picchu, and the Abl indicator expressed with or without BCR–ABL or c-Abl in 293F cells. Values are expressed as the mean emission ratio ± S.D. of pooled data obtained from three separate experiments. (C–D) 293F cells were transfected with indicated amounts of expression plasmid for BCR–ABL together with that for Pickles–2.31. After 24 h, the cells were analyzed with fluorescence spectrometry (C), followed by immunoblotting analysis (D). (E) Emission ratio of Pickles–2.31 expressed in K562, KU812, HL60, U937, and 293F cells for 24 h. Fluorescence emission is expressed as the mean emission ratio ± S.D. of pooled data obtained from three separate experiments. The asterisk (*) represents statistically significant differences ($P < 0.05$); values are determined by comparing 293F cells to other cells. (F) Emission ratios of Pickles–2.31 (WT), –2.31 R39V mutant (R39V), –2.31 W160L mutant (W160L), and –2.31 Y207F mutant (Y207F) expressed with or without BCR–ABL in 293F cells. Fluorescence emission is expressed as the mean emission ratio ± S.D. of pooled data obtained from three separate experiments. The asterisk (*) represents statistically significant differences ($P < 0.05$); values are determined by comparing control (□) to BCR–ABL-expressing (■) cells. (G) The cell lysate prepared as in (F) was analyzed by immunoblotting. Anti-c-Abl antibody was used for detecting BCR–ABL, and anti-phospho-CrkL (pCrkL) or anti-CrkL antibody was used for detecting Pickles (†) and endogenous CrkL (‡); representative results for at least three independent experiments are shown. (H) Emission spectra of Pickles–2.31 expressed with (solid line) or without (dashed line) BCR–ABL in 293F cells. One sample was treated with proteinase K (gray line) for 30 min before spectral analysis.

Supplementary Fig. S2. Dynamic range of Pickles in relation to IM treatment in K562 cells**.** BCR–ABL activities in K562 cells transfected with Pickles were measured by FRET analysis (A) and immunoblotting (B). Alternatively, phosphorylation levels of endogenous CrkL were evaluated by flow cytometry using an anti-phospho-CrkL antibody (C). Representative results for at least three independent experiments are shown.

Supplementary Fig. S3. Assessment of drug efficacy in cells expressing mutant BCR–ABL. (A–B) 293F cells expressing native or mutant BCR–ABL (G250E or T315I) were treated with either 10 µM imatinib (IM), 10 µM nilotinib (NL), or 1 µM dasatinib (DS) for 24 h, or left untreated, and then analyzed by immunoblotting analysis, where anti-c-Abl antibody was used for detecting BCR–ABL, and anti-phospho-CrkL or anti-CrkL antibody was used for detecting endogenous CrkL. Representative results are shown (A), and the relative phospho-CrkL levels were determined by quantitative densitometry (B). (C) Pickles was introduced into 293F cells expressing mutant BCR–ABL (G250E). After 24 h, the cells were subjected to time-lapse fluorescence microscopy. Starting at 1 to 8 h of the observation, the cells were initially treated with 2 µM IM, followed by further incubation with 4 µM NL (●), 1 µM DS (■), or vehicle alone (○). Average emission ratios of the cells were plotted with standard deviations.
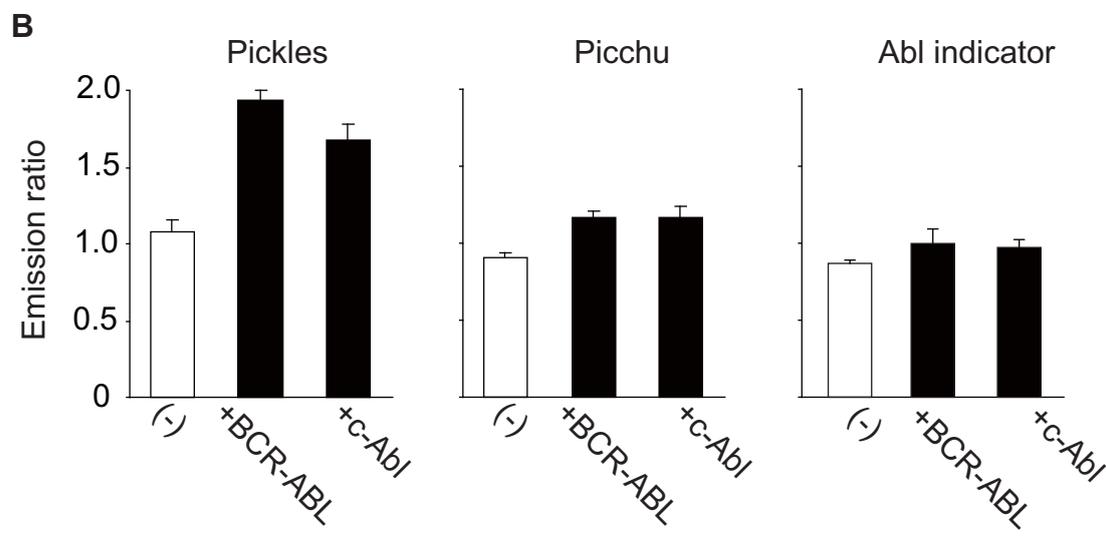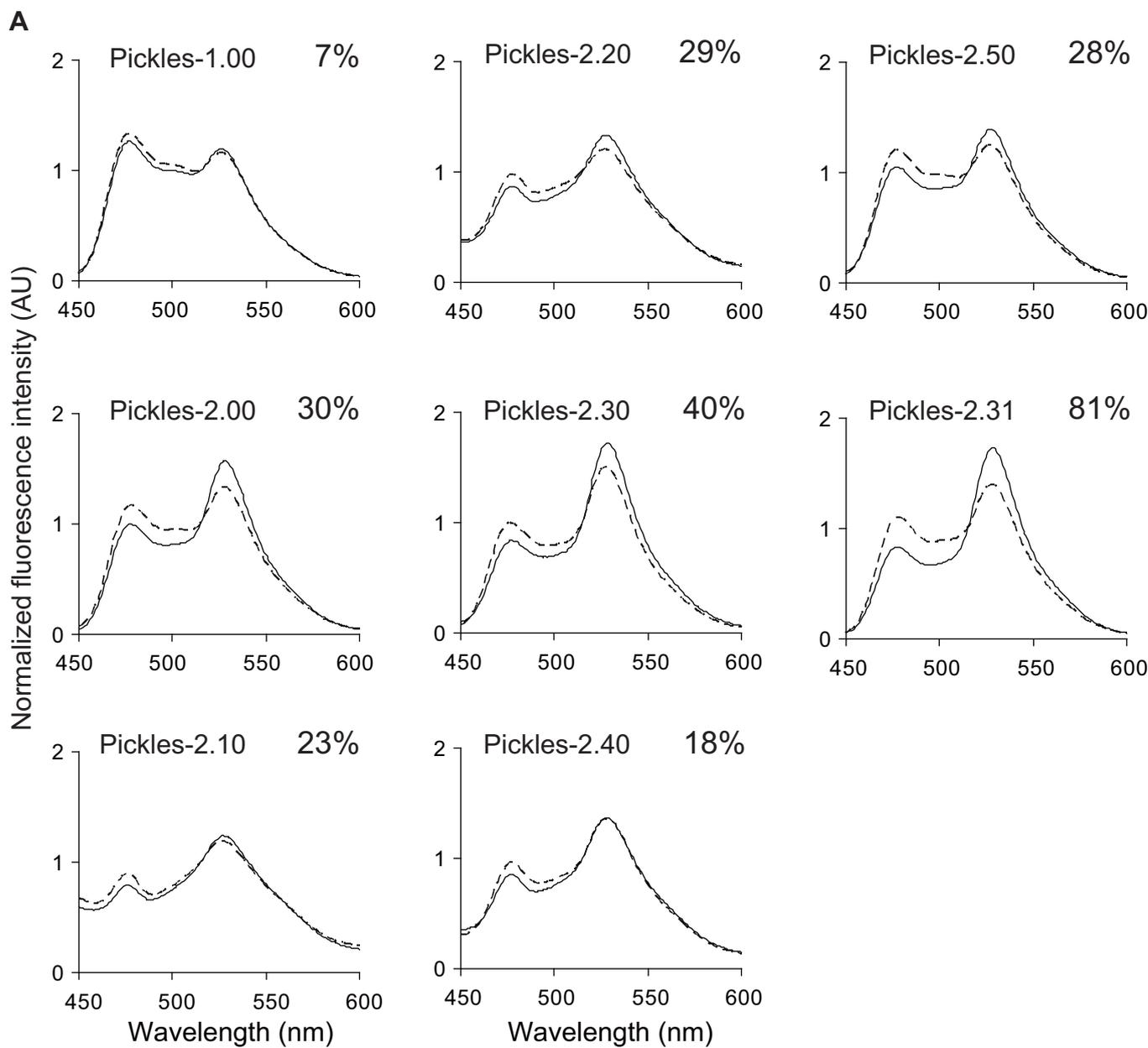
Supplementary Fig. S4. Distinguishing drug-resistant cells in heterogenous samples using Pickles. (A) Pickles was introduced into 293F cells expressing either native or mutated BCR–ABL (T315I). The cells were mixed at the indicated ratios and treated with 20 µM IM or left untreated for 24 h. Fluorescence emission is expressed as the mean emission ratio ± S.D. of pooled data obtained from three separate experiments. *, $P < 0.05$; when compared to untreated (□) cells. (B) Emission ratios of 293F cells expressing Pickles with (right panel) or without (left panel) IM were calculated and plotted. (C, D) Pickles was introduced into 293F cells expressing
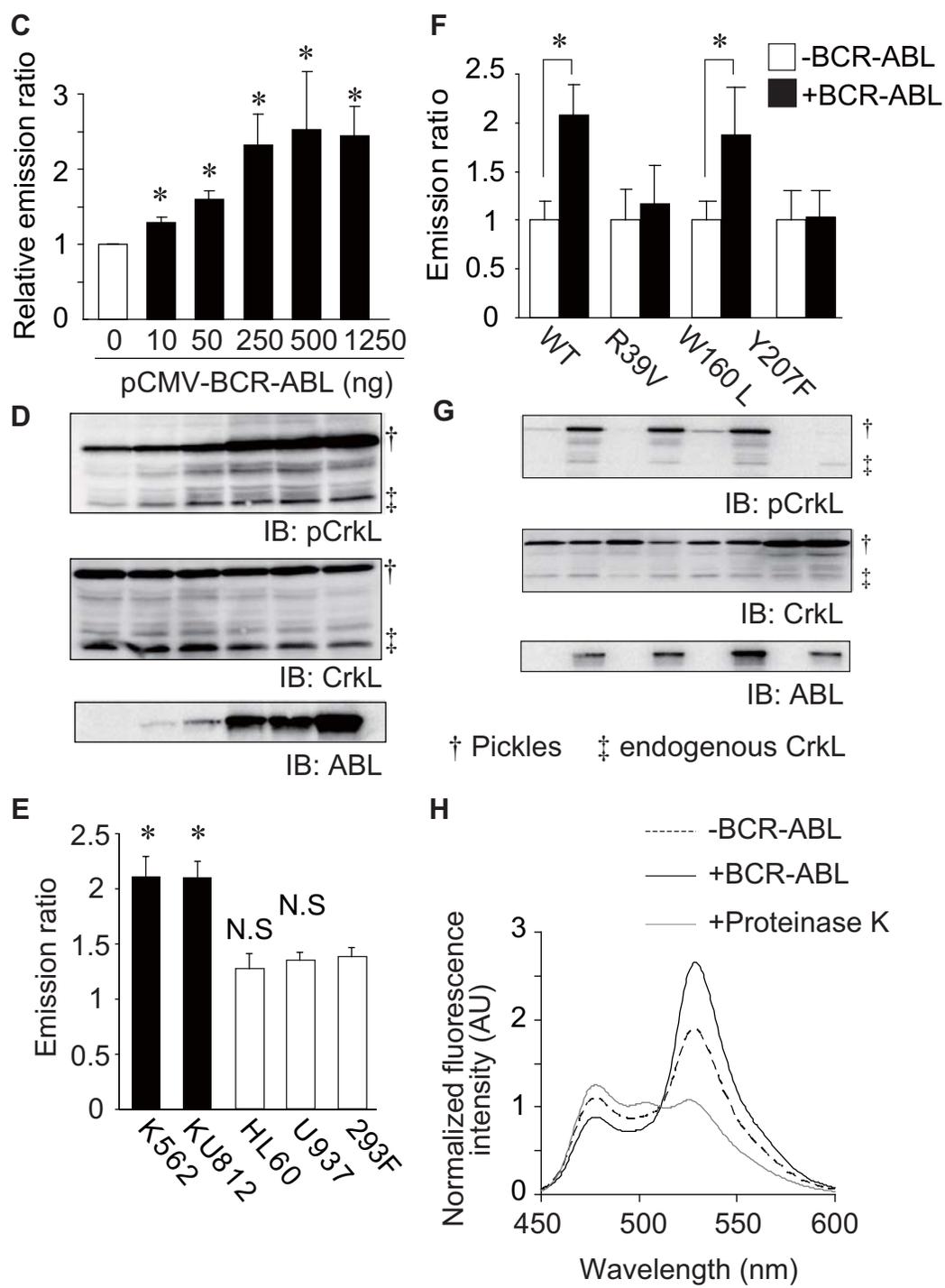
native BCR–ABL. Cells were treated with 20 µM IM, 10 µM NL, 1 µM DS, or left untreated for 24 h. Emission ratios for each individual cell were plotted in scatter diagrams (C) and box-and-whisker plots (D). Values at the top right of each scatter diagram indicate the percentage of cells above D-FRET (or FRET^{hi}). In (D), lowest and highest boundaries of the box indicate the 25th and 75th percentiles, respectively; the whiskers above and below the box designate the maximum and minimum values, respectively; the solid line within the box represents the median value. In the scatter diagrams, the *x*-axes indicate the cell index, and the dashed lines indicate the dividing value of FRET efficiency (D-FRET), which is defined by the following formula: D-FRET = Average + (3 × S.D.) of the FRET efficiency of cells without BCR–ABL expression (= 2.04, see Materials and methods).
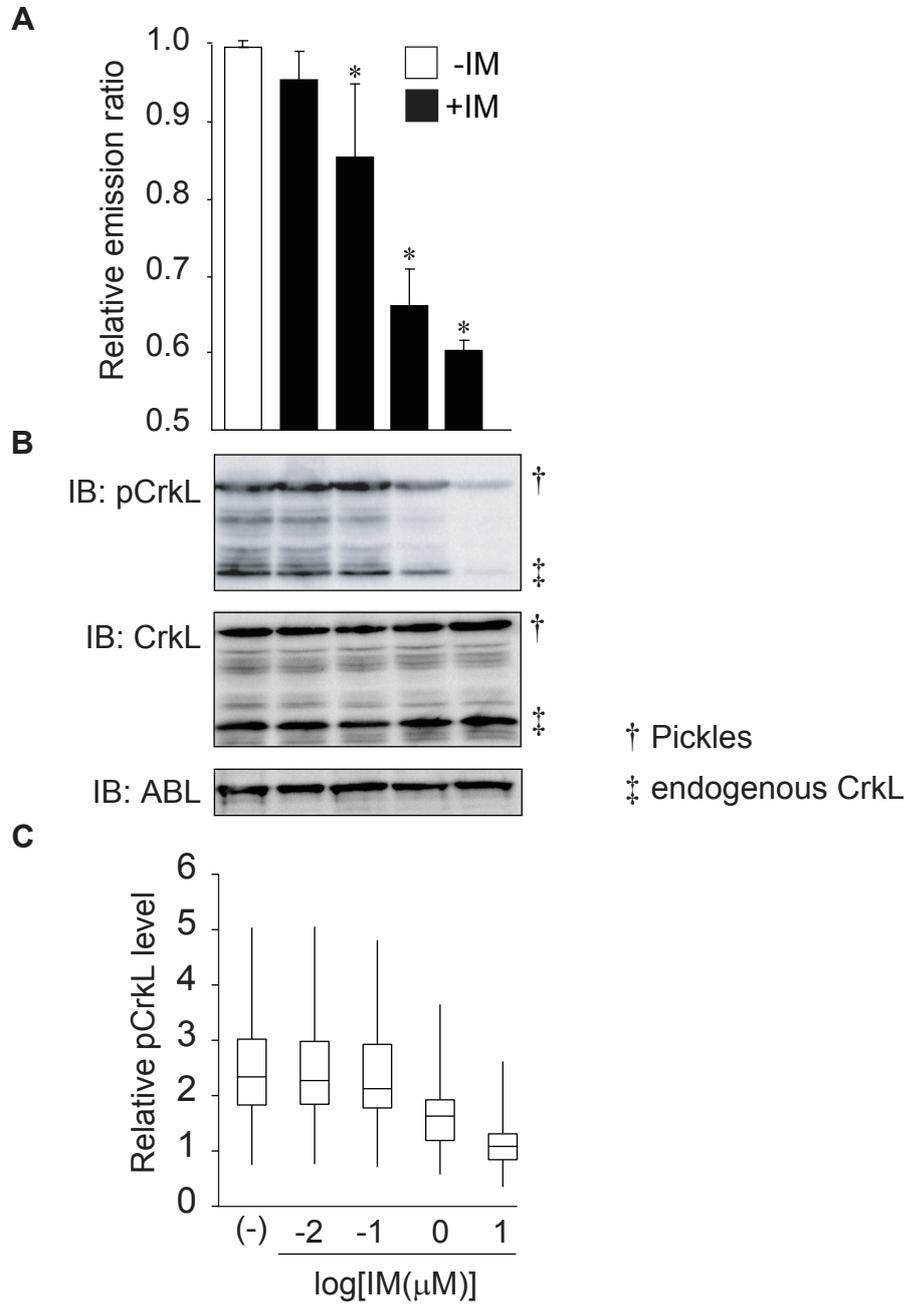
Supplementary Fig. S5. Assessment of drug efficacy by Pickles in cells expressing mutant BCR–ABL. (A) Cells were prepared as in Fig. 4A, and emission ratios of the cells are plotted in scatter diagrams. (B) Cells were prepared as in (A) except for being left untransfected with pPickles. The cells were then analyzed by flow cytometry using an anti-phospho-CrkL antibody. (C) Pickles was introduced into 293F cells expressing either native or mutated BCR–ABL (G250E). These cells were then mixed at the indicated ratios and treated with 10 µM IM,10 µM NL, and 1 µM DS for 24 h. Emission ratios of the cells are plotted in scatter diagrams. The dashed line represents D-FRET; the *x*-axis indicates the cell index. Results in the scatter diagrams are representative of at least three independent experiments. Values at the top right indicate the percentage of cells above D-FRET in the Figure, while those given in parentheses are the mean obtained from three independent experiments.

Supplementary Fig. S6. Pickles in primary CML cells. (A) Immunofluorescence analysis of primary CML cells. PBMCs were isolated from CML patients and double-stained with anti-phospho CrkL (pCrkL; green) and anti-c-Abl antibodies (Abl; red). The right panel is a merged image. Representative images are shown. (B–C) PBMCs (B) and BMCs (C) were purified from peripheral blood or bone marrow of healthy volunteers (HV) (n=3) and CML patients (CML) (n = 6). Emission ratios of single cells were plotted in scatter diagrams. The *x*-axes indicate the cell index and dashed lines indicate D-FRET. (D–I) PBMCs (D–F) and BMCs (G–I) were obtained from three CML patients (cases *#1–3*), transfected with Pickles, and incubated in the presence or absence of 2 μM IM. After 24 h, the cells were subjected to dual-emission fluorescence microscopy to determine the FRET efficiency. Scatter diagrams are shown. Scatter diagrams show data for 150 randomly chosen cells, which are representative of the total cell population analyzed.
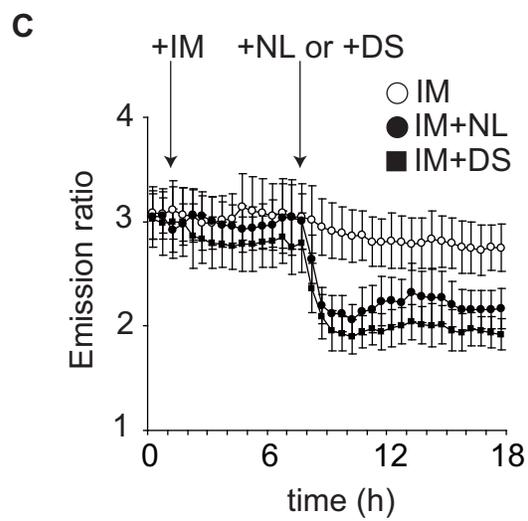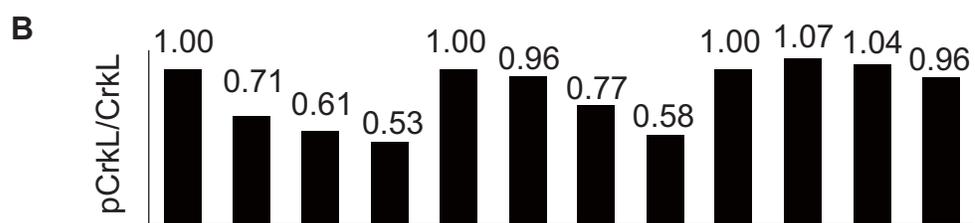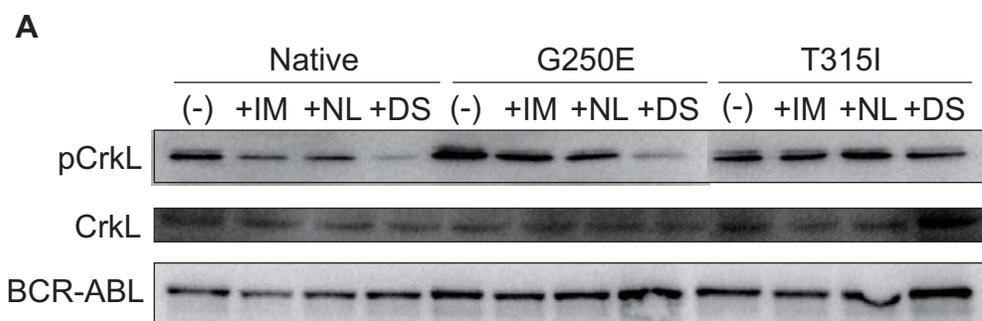
Supplementary Movie S1. A Quick Time movie file pertains to Fig. 2D.

A

B

Supplementary Fig S1. Mizutani, T., *et al.*

C Relative emission ratio vs pCMV-BCR-ABL (ng): 0, 10, 50, 250, 500, 1250. * at 10, 50, 250, 500, 1250.

D IB: pCrkL, IB: CrkL, IB: ABL. † ‡

E Emission ratio: K562 *, KU812 *, HL60 N.S, U937 N.S, 293F

F Emission ratio: WT *, R39V, W160 L *, Y207F. □ -BCR-ABL ■ +BCR-ABL

G IB: pCrkL, IB: CrkL, IB: ABL. † ‡
† Pickles   ‡ endogenous CrkL

H Normalized fluorescence intensity (AU) vs Wavelength (nm): 450–600. ------- -BCR-ABL, —— +BCR-ABL, —— +Proteinase K

A

B

IB: pCrkL † ‡

IB: CrkL † ‡

IB: ABL

† Pickles

‡ endogenous CrkL

C

A

B

C
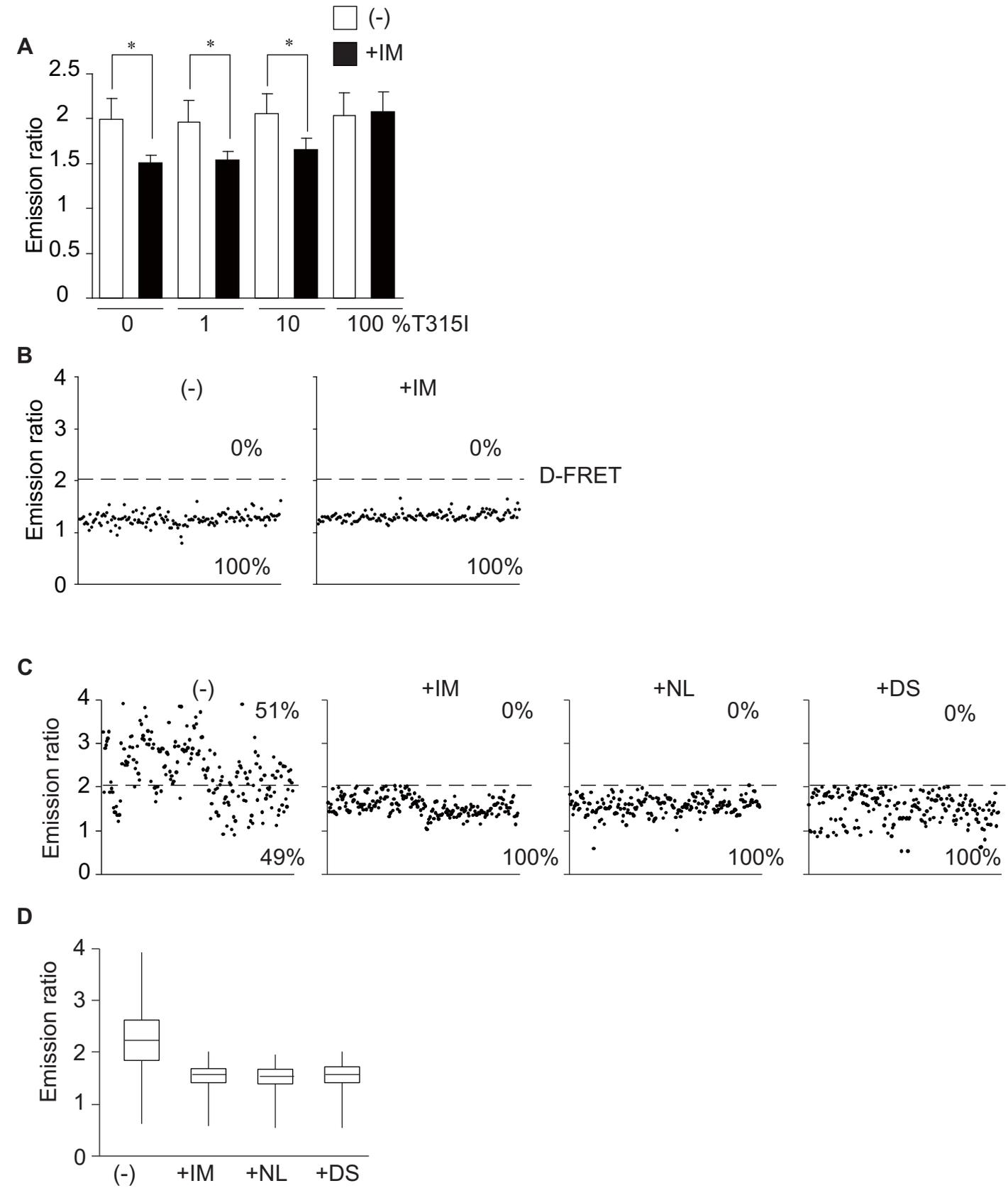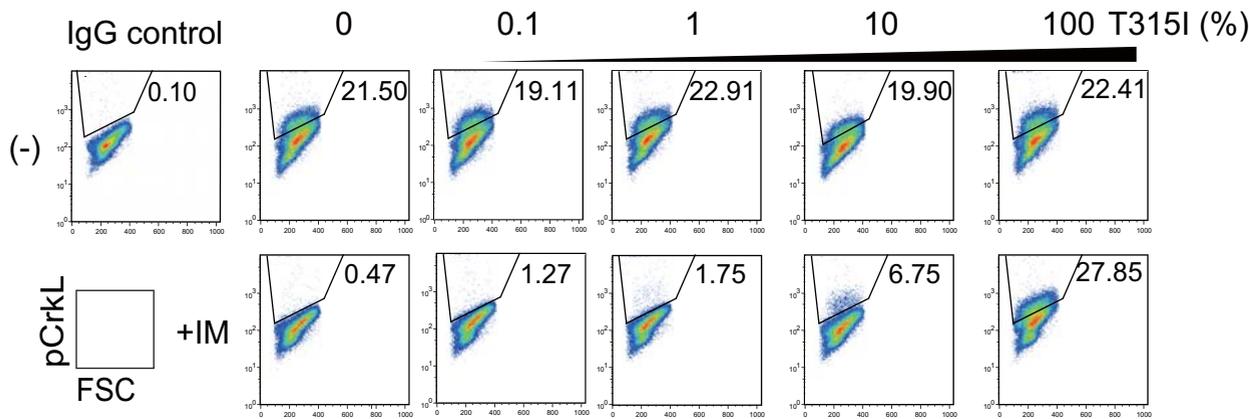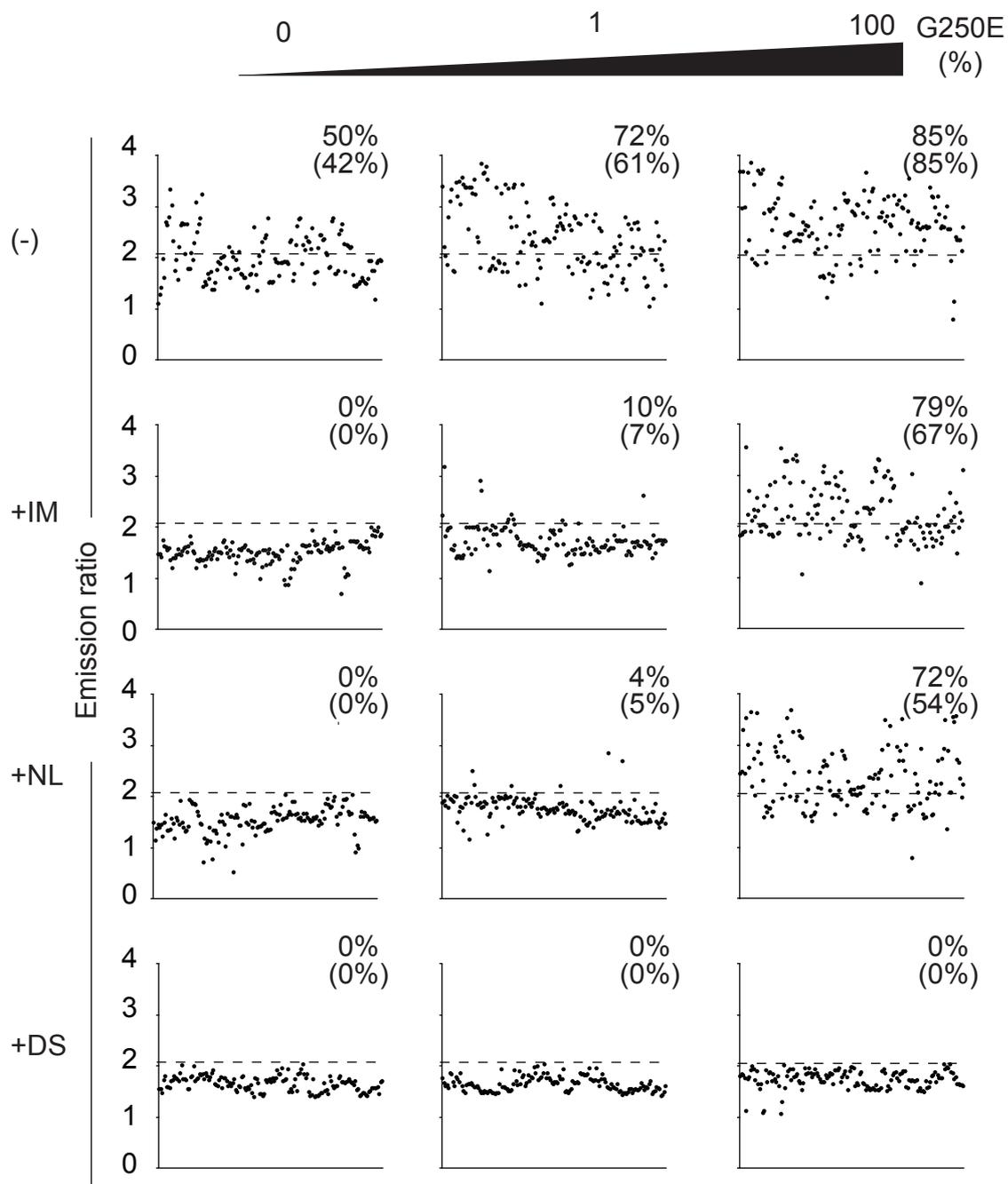
A Pickles-based microscopic analysis
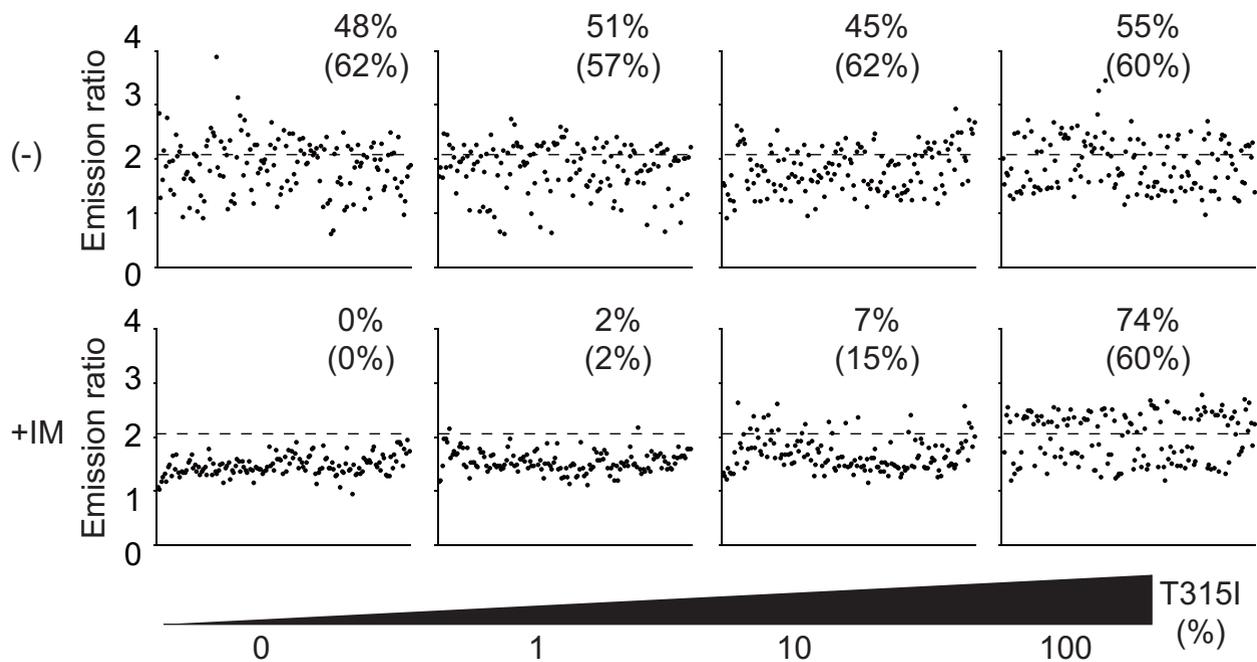
B Anti-pCrkL antibody-based flow cytometric analysis

C Pickles-based microscopic analysis