

Supplementary Information for

Single-molecule tracking-based drug screening

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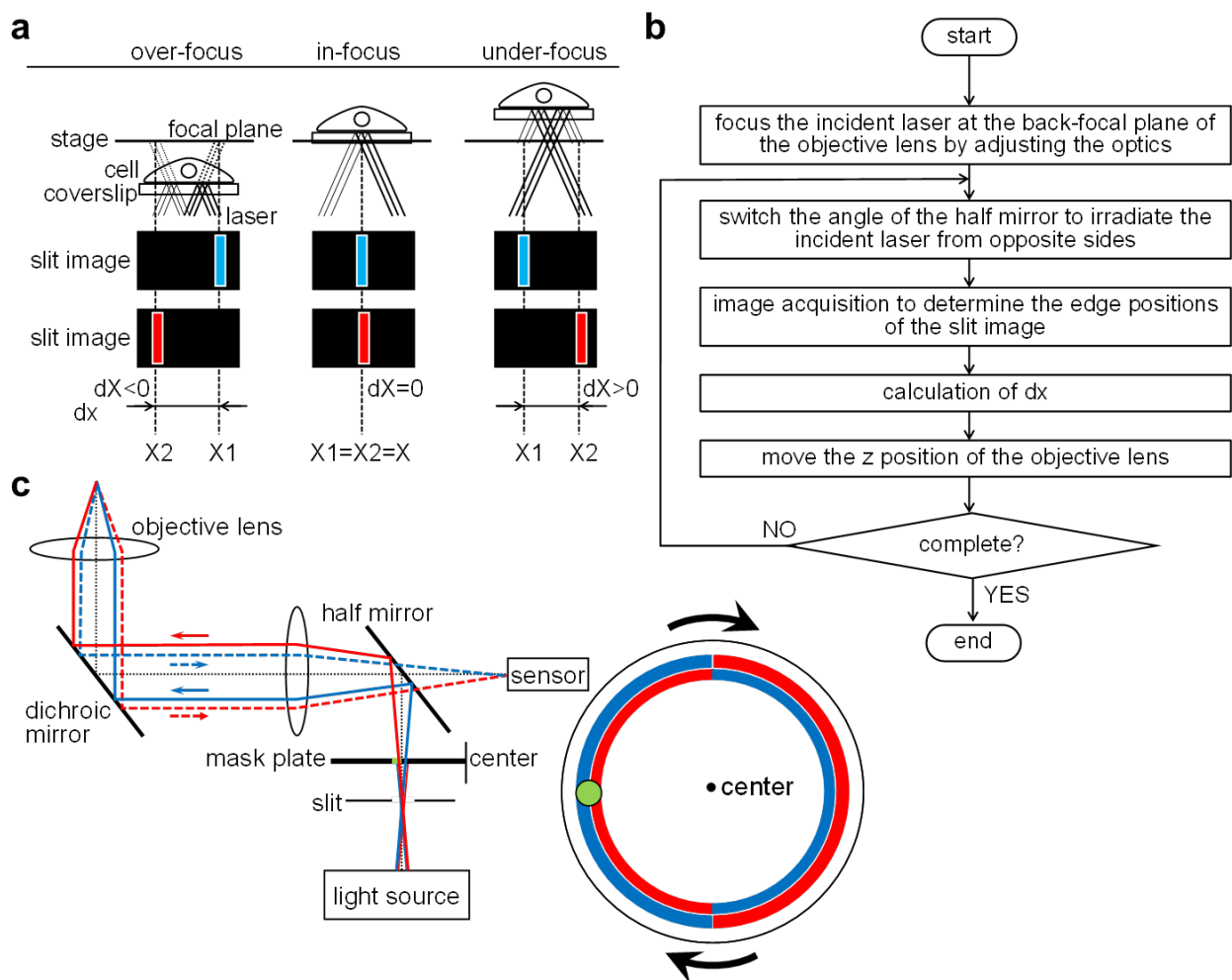
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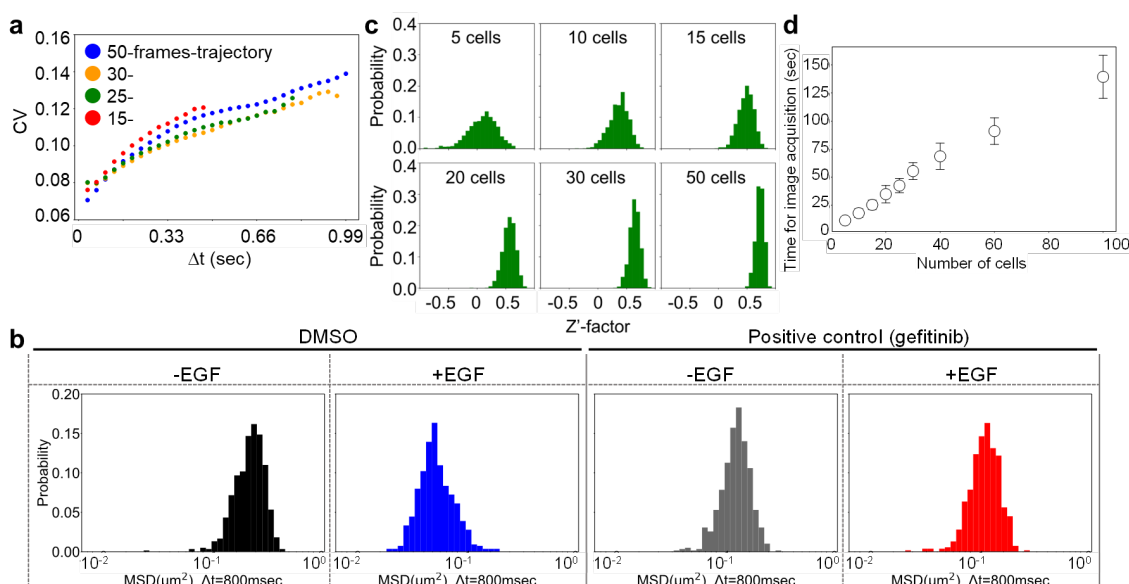
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Supplementary Figures



Supplementary Fig. 1. Details of the autofocus device in AiSIS.

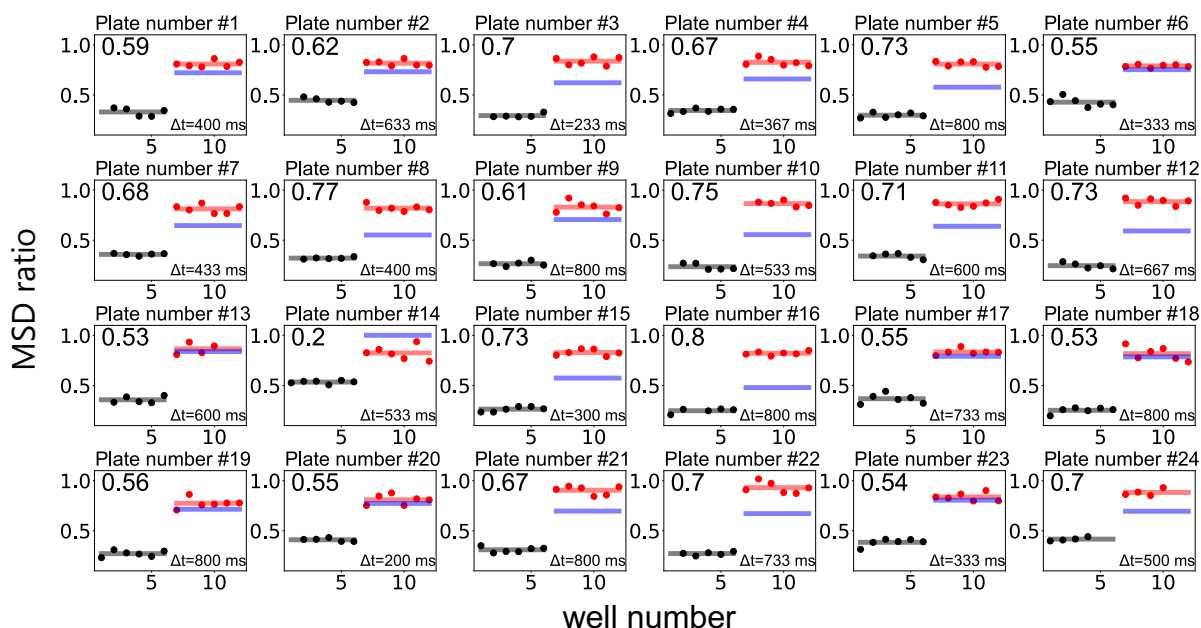
a, Correspondence between the slit images generated with laser light in opposite directions and the focal positions. From under- to over-focus, these slit images (red and blue bars) exchange their locations through the in-focus position where the images are overlapped. **b**, A flowchart of the autofocus algorithm. **c**, Instead of switching the illumination, rotation of a mask-plate (right), in which light is transmitted at the green region but not at the blue or red regions, can be used. The blue region in the mask-plate indicates the red region rotated 180° . The red and blue optical paths correspond to light affected by the same-colored regions in the mask-plate. Solid and dotted lines in the paths denote incident and reflected light, respectively.



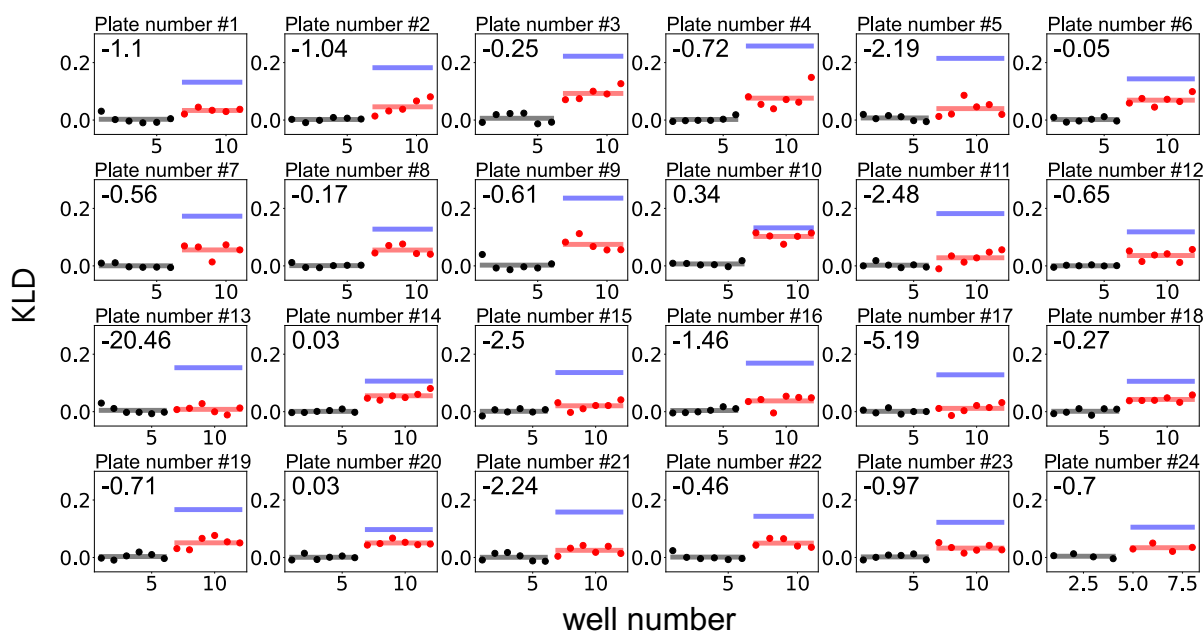
Supplementary Fig. 2. Optimized conditions for image acquisition.

a, The required number of frames in single-molecule imaging to obtain results with low deviation and high reproducibility. Single-molecule images were acquired for 50 frames at 30 frames/sec, and the first 15, 25, 30, and 50 frames were extracted from the same images to obtain the trajectories of single molecules. The MSD ratio at Δt before and after EGF addition was calculated for each trajectory group and obtained for each well where images from 10 cells both before and after EGF addition were acquired. The coefficients of variation (CV) of the MSD ratios were calculated from 48 wells. The MSD ratio from the trajectories of 15 frames showed a larger CV than the ratio from other imaging conditions due to the smaller number of frames, but the trajectories of 50 frames exhibited more GFP fluorescence bleaching. Therefore, movies of 25-30 frames (0.8 - 1.0 sec) were determined as suitable for the analysis. **b**, The MSD distribution at $\Delta t = 800$ ms obtained from 600 cells with and without EGF stimulation under gefitinib or DMSO (control) treatment. **c**, The required number of cells for single-molecule imaging to obtain a high Z'-factor. The Z'-factor at the indicated cell number was calculated by using MSD data selected randomly from the cell populations shown in b, and the statistical distribution of the Z'-factor was obtained. MSD ratios with and without gefitinib treatment, respectively, were used as positive and negative controls. For each graph, the calculation was done for 1000 different combinations of cells. **d**, The relationship between the number of measured cells and the elapsed time. Based on the assessment, 20 cells were measured before and after EGF addition in each well to obtain a Z'-factor satisfying the required screening accuracy (≥ 0.50) in as minimum time as possible. Data are acquired from 5 independent experiments and presented as mean $\pm$ SD.

a



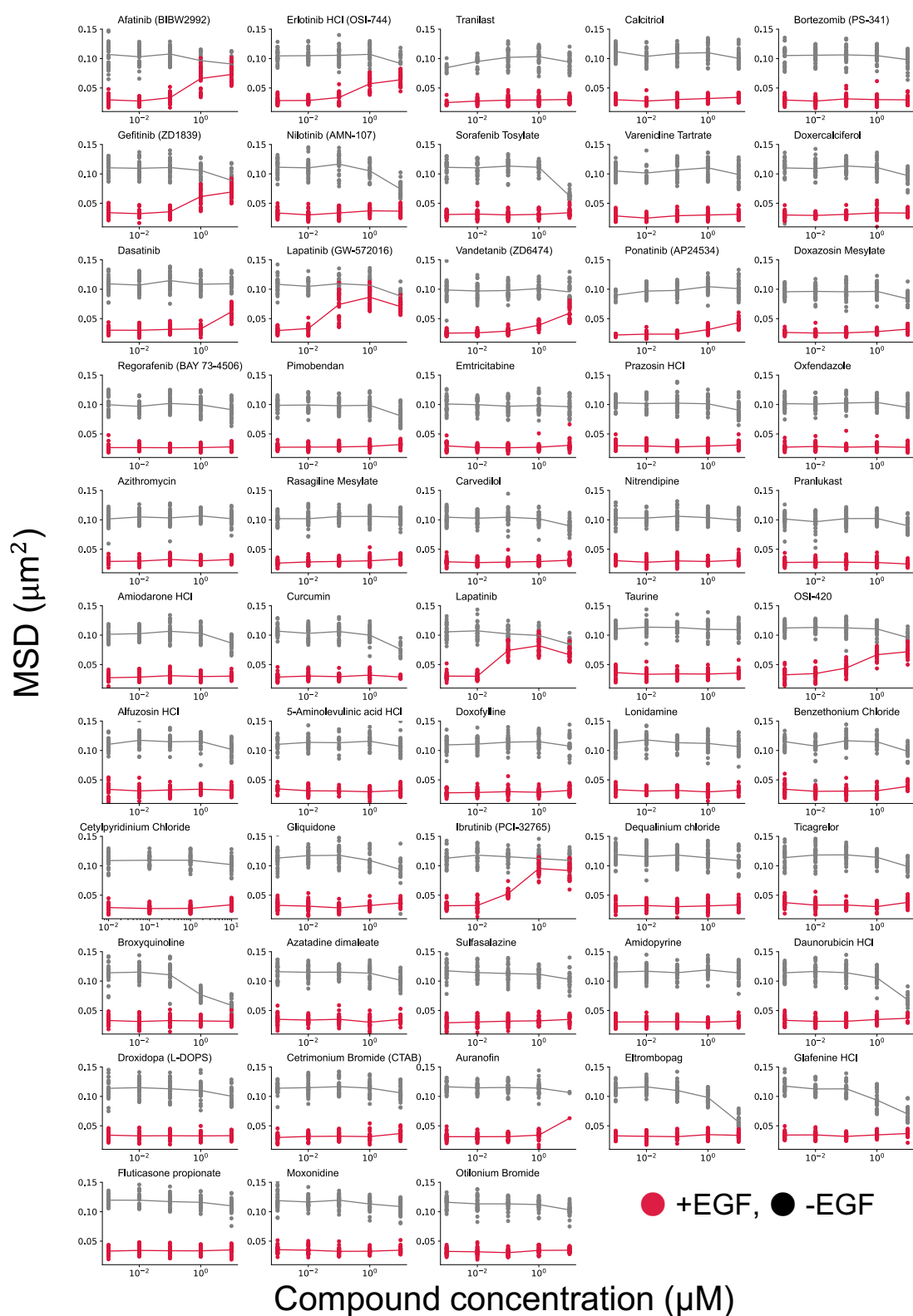
b



Supplementary Fig. 3. Z'-factor values of each well plate for EGFR single-molecule screening.

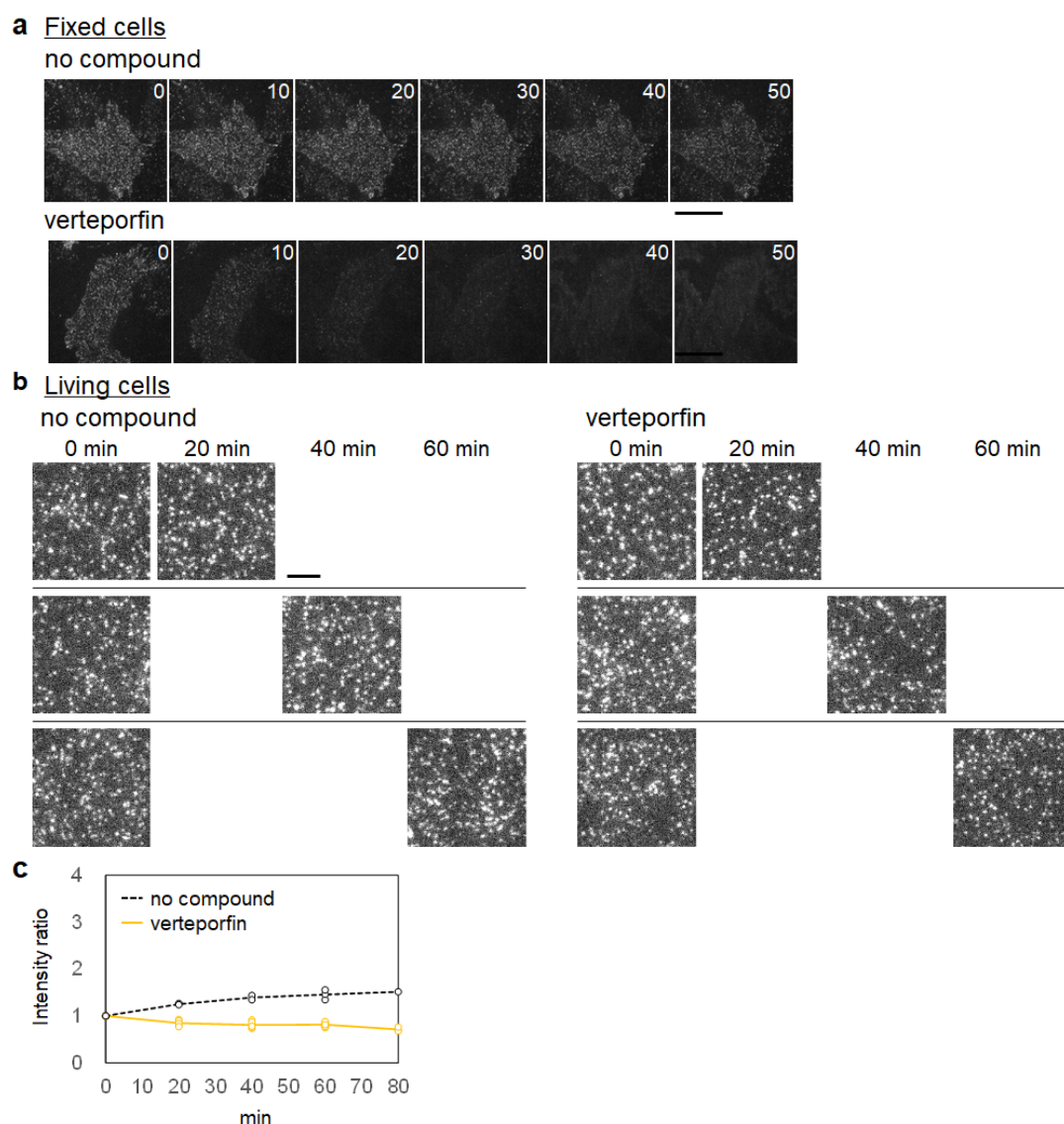
Z'-factor values of every well plate (upper left) obtained for mobility-based and clustering-based screenings, which were calculated from the MSD ratio and KLD, respectively. **a**, The MSD ratio is plotted without (black) and with (red) gefitinib treatment, which represent the negative control (cells treated only with EGF) and positive control (cells treated with gefitinib and EGF),

respectively. Δt (bottom right) indicates the duration of the MSD calculation that provided the best Z' -factor for each well plate used in the screening. **b**, KLD before (black) and after (red) EGF addition. Each point was calculated from images of 10 cells in the same well. Red and black lines indicate the average of the plots. Blue lines show the required average of the positive control to provide a Z' -factor (≥ 0.50) sufficient for screening, which was calculated using the deviation of the positive/negative controls and the average of the negative control. Only well-plates with red lines above the blue line were used for all subsequent analysis. For example, plate number #14 was excluded from the mobility-based screening.



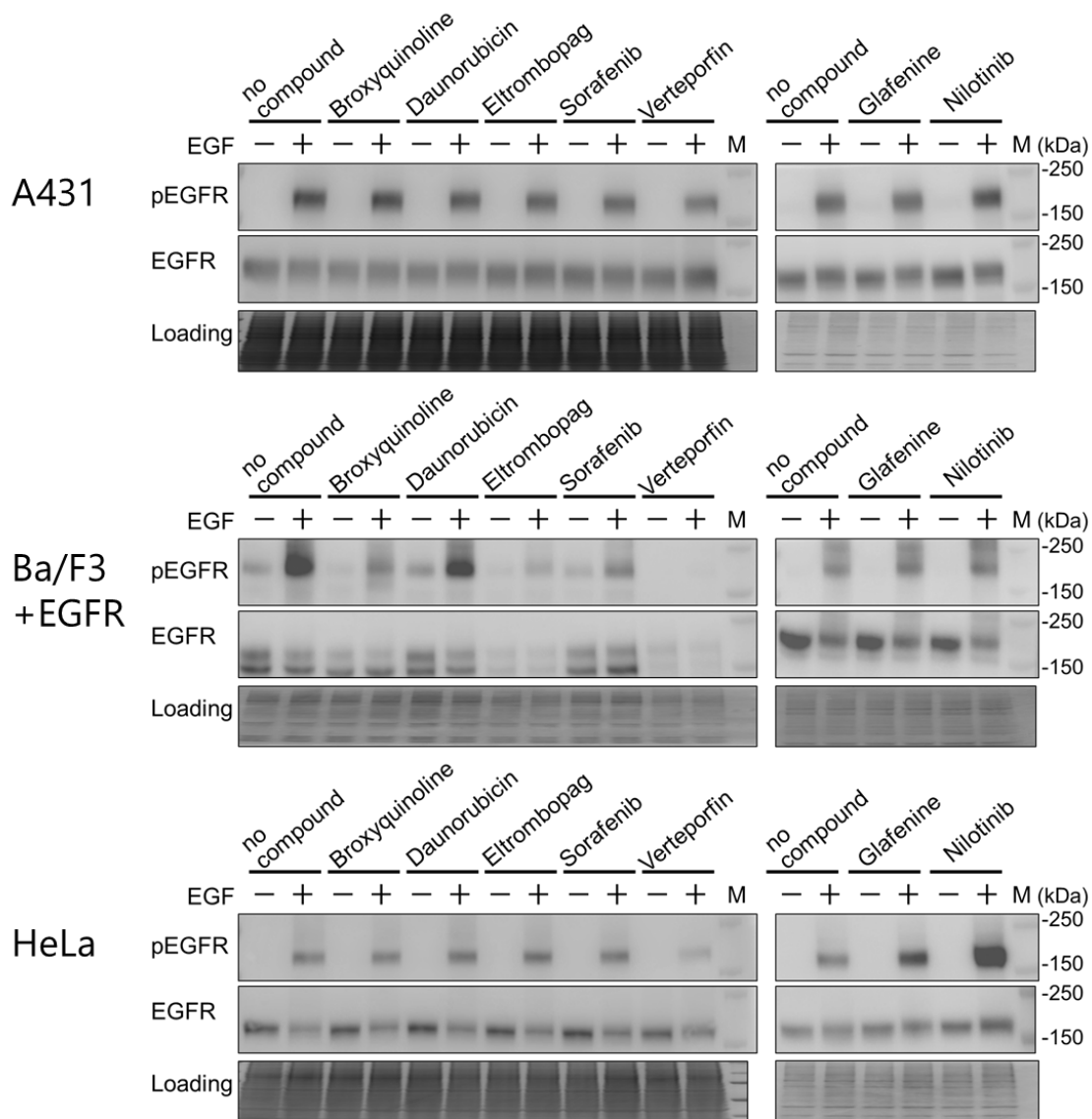
Supplementary Fig. 4. Compound dose-dependent mobility of EGFR.

MSDs at $\Delta t=167$ ms were obtained for cells treated with various concentrations of the 53 hit compounds. Red and black circles denote values with and without EGF, respectively.



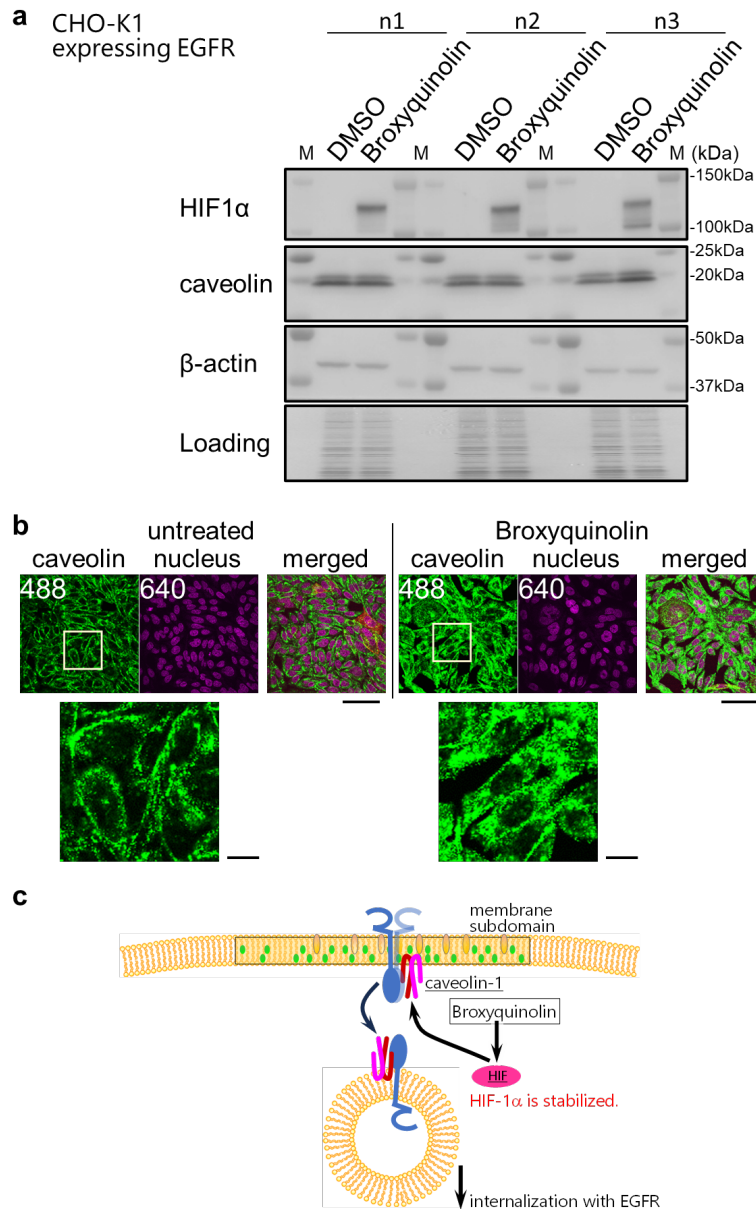
Supplementary Fig. 5. EGFR internalization and photophysical effects by verteporfin.

a, Timelapse single-molecule images of EGFR-mEGFP in fixed CHO-K1 cells with and without verteporfin treatment. The images were acquired every 10 min after treatment in the same cell. A 488-nm laser repeatedly irradiated the cells, resulting in faster GFP photobleaching due to verteporfin. Scale bar, 5 μ m. **b**, Single-molecule images of EGFR-mEGFP in the same living cell with and without verteporfin treatment. The images were acquired at two time points (0 min and 20/40/60 min) after the treatment. Scale bar, 3 μ m. **c**, Quantification of the brightness of EGFR-mEGFP on the plasma membrane in the same living cell. The measured intensity at each timepoint was normalized by that at time 0. EGFR-mEGFP on the plasma membrane increased slightly over time under control condition but decreased upon verteporfin treatment, suggesting EGFR internalization by verteporfin. Data were acquired from 9, 6, 6, 9, and 3 independent experiments including 215, 63, 60, 92, and 32 cells for 0, 20, 40, 60, and 80 min, respectively.



Supplementary Fig. 6. Effects of non-EGFR-TKI compounds on EGF-induced phosphorylation of EGFR in various cell lines.

The phosphorylation of EGFR was analyzed in A431, EGFR-expressing Ba/F3, and HeLa cells. After treatment with the indicated compounds for one hour, the cells were stimulated with 60 nM EGF for 2 minutes. EGF stimulation induced the phosphorylation of EGFR in these cell lines even with the application of non-EGFR-TKI compounds, although the extent of the phosphorylation varied with the cell type and compound. In particular, verteporfin-treated cells exhibited defective EGF-induced EGFR phosphorylation, consistent with the inhibition of cluster formation because the EGFR dimer is important for autophosphorylation. Representative images from 3 independent experiments are displayed. M indicates molecular weight marker.



Supplementary Fig. 7. HIF-1 α increase by the broxyquinoline treatment.

a, Western blotting of HIF-1 α and caveolin in broxyquinoline-treated and -untreated cells.

Results obtained from three replicated experiments are shown. M indicates molecular weight

marker. **b**, Immuno-fluorescent images of caveolin in cells with (right panels) and without (left

panels) broxyquinoline treatment. Caveolin was stained with Alexa 488-labeled antibody. Fluorescently labeled nuclei were observed with a 640-nm laser. Scale bar, 100 μ m. Lower

panels represent magnified images of the boxed regions in the upper panels. Scale bars, 20 μ m. Representative images from 2 independent experiments are displayed. **c**, A model of the broxyquinoline effect, including HIF-1 α production, which induces caveolin internalization with EGFR, based on the current and previous studies^{42, 43}.

Supplementary Table 1. Suitability of our method for drug screening.

All values obtained with our system satisfied the allowance.

Evaluation	Formula	Our system	Allowance
CV value	SD/Avg	6%	$\leq 10\%$
S/B ratio	$Avg_{100\%}/Avg_{0\%}$	6.2	≥ 2
Z'-factor	$Z' = 1 - \frac{(3 \times SD_{positive}) + (3 \times SD_{negative})}{Avg_{positive} - Avg_{negative}}$	0.69	≥ 0.50

Supplementary Table 2. Small molecule screening data.

Category	Parameter	Description
Assay	Type of assay	Cell-based single-molecule analysis
	Target	Epidermal growth factor receptor
	Primary measurement	Diffusion mobility and fluorescence intensity of EGFR-GFP molecules
	Key reagents	
	Assay protocol	Described in "Single-molecule screening" in the Method section
	Additional comments	
Library	Library size	1134 compounds
	Library composition	FDA approved drugs
	Source	Compound Library Screening Center, Graduated School of Pharmaceutical Sciences, Osaka University
	Additional comments	
Screen	Format	96-well
	Concentration(s) tested	10 μ M in 1% DMSO
	Plate controls	Positive control: gefitinib, Negative control: DMSO
	Reagent/ compound dispensing system	N/A
	Detection instrument and software	Automated in-cell single-molecule imaging system (AiSIS, laboratory development) operated with Auto Imaging System software (2.4, ZIDO corp., Japan)
	Assay validation/QC	Z' factor
	Correction factors	
	Normalization	Normalized by the mean value of negative controls in the same plate (see Methods in details)
	Additional comments	
Post-HTS analysis	Hit criteria	(Average + 3 SD) of the MSD ratio of the negative control
	Hit rate	53 compounds of 1134 compounds
	Additional assay(s)	Dose-dependent assay using single molecule imaging and cell viability assay
	Confirmation of hit purity and structure	
	Additional comments	

Supplementary Table 3. Number of cells or experiments included in the analysis.

	measurement	condition			number		
Fig. 1	d	mobility	—	—	EGF	0 and 60 nM	900 cells for each concentration
		clustering	—	—	EGF	0 and 60 nM	900 cells for each concentration
	e	mobility	—	—	EGF	0 and 300 nM	60 cells for each concentration
			gefitinib	10 μ M	EGF	0 and 300 nM	60 cells for each concentration
	f	mobility	—	—	EGF	0 - 300 nM	140 cells for each concentration
	phosphorylation	—	—	EGF	0 - 300 nM	7 experiments	
Fig. 2	g	mobility	gefitinib	0.001 – 10 μ M	EGF	0 - 300 nM	33 cells for each concentration
	a	mobility	gefitinib	10 μ M	EGF	60 nM	10 cells
	c	mobility	each compound	0.001 – 10 μ M	—	—	20 cells for each concentration
				0.001 – 10 μ M	EGF	60 nM	20 cells for each concentration
	d	mobility	afatinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
			elrotinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
			OSI-420	0.001 μ M	EGF	0 and 60 nM	28 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	27 cells (-EGF), 28 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				1 μ M		0 and 60 nM	29 cells (-EGF), 29 cells (+EGF)
				10 μ M		0 and 60 nM	23 cells (-EGF), 23 cells (+EGF)
			lapatinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	24 cells (-EGF), 25 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			lapatinib ditosylate	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 28 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 28 cells (+EGF)
				1 μ M		0 and 60 nM	29 cells (-EGF), 30 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			ponatinib	0.001 μ M	EGF	0 and 60 nM	10 cells (-EGF), 10 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 28 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			vandetanib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			dasatinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			iburtinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
				0.1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
				10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)

e	mobility	broxyquinoline	0.001 μ M	EGF	0 and 60 nM	29 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	28 cells (-EGF), 29 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	24 cells (-EGF), 24 cells (+EGF)
			10 μ M		0 and 60 nM	29 cells (-EGF), 30 cells (+EGF)
		daunorubicin	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	29 cells (-EGF), 29 cells (+EGF)
			0.1 μ M		0 and 60 nM	29 cells (-EGF), 29 cells (+EGF)
			1 μ M		0 and 60 nM	27 cells (-EGF), 20 cells (+EGF)
			10 μ M		0 and 60 nM	12 cells (-EGF), 12 cells (+EGF)
		eltrombopag	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	30 cells (-EGF), 20 cells (+EGF)
			10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
		sorafenib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			10 μ M		0 and 60 nM	29 cells (-EGF), 29 cells (+EGF)
		glafenine	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
		nilotinib	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	30 cells (-EGF), 29 cells (+EGF)
			10 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
		curcumin	0.001 μ M	EGF	0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.01 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			0.1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			1 μ M		0 and 60 nM	30 cells (-EGF), 30 cells (+EGF)
			10 μ M		0 and 60 nM	10 cells (-EGF), 10 cells (+EGF)
Fig. 3 b	clustering	—	—	EGF	0.03 nM	116 cells
			—		0.3 nM	116 cells
			—		3 nM	118 cells
			—		30 nM	116 cells
			—		300 nM	116 cells
c	clustering	each compound	0 μ M, 10 μ M	—	—	20 cells for each concentration
e	clustering	verteporfin	0.001 μ M	—	—	195 cells
			0.01 μ M	—	—	191 cells
			0.1 μ M	—	—	169 cells
			1 μ M	—	—	115 cells
			10 μ M	—	—	102 cells
f	clustering	—	—	EGF	0 - 300 nM	115 cells for each concentration
		verteporfin	0 - 10 μ M		—	115 cells for each concentration
g	mobility	verteporfin	0.001 μ M	—	—	132 cells
			0.01 μ M	—	—	147 cells
			0.1 μ M	—	—	189 cells
			1 μ M	—	—	192 cells
			10 μ M	—	—	195 cells
Fig. 4 e	brightness	—	—	—	—	46 cells
		broxyquinoline	10 μ M	—	—	56 cells
		daunorubicin	10 μ M	—	—	61 cells
		eltrombopag	5 μ M	—	—	72 cells
		sorafenib	10 μ M	—	—	47 cells
		verteporfin	1 μ M	—	—	63 cells
		glafenine	10 μ M	—	—	42 cells
		nilotinib	10 μ M	—	—	46 cells

Supplementary Table 4. p-values in Fig. 5a-c.

cell viability (Fig. 5a)								
cells	compounds							
	afatinib	erlotinib	gefitinib	lapatinib	ponatinib	vandetanib	dasatinib	ibrutinib
A431	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.002
BaF3 (+EGFR)	0.001	0.004	0.002	0.000	0.006	0.013	0.034	0.003
HeLa	0.004	0.912	0.117	0.000	0.000	0.001	0.000	0.000
CHO-K1	0.008	0.039	0.157	0.196	0.002	0.143	0.001	0.275
BaF3	0.070	0.104	0.674	0.105	0.245	0.030	0.011	0.011

cells	compounds						
	broxyquinolin	daunorubicin	eltrombopag	solafenib	verteporfin	glafenine	nilotinib
A431	0.000	0.002	0.001	0.000	0.000	0.589	0.001
BaF3 (+EGFR)	0.000	0.003	0.017	0.005	0.002	0.745	0.439
HeLa	0.000	0.000	0.027	0.000	0.000	0.037	0.015
CHO-K1	0.015	0.446	0.064	0.014	0.004	0.118	0.012
BaF3	0.120	0.019	0.008	0.000	0.007	0.065	0.030

EGFR phosphorylation (Fig. 5b)							
cells	compounds						
	broxyquinolin	daunorubicin	eltrombopag	solafenib	verteporfin	glafenine	nilotinib
A431	0.012	0.809	0.049	0.053	0.040	0.455	0.269
BaF3 (+EGFR)	0.009	0.919	0.274	0.016	0.002	0.479	0.481
HeLa	0.094	0.485	0.243	0.213	0.013	0.861	0.822

EGFR expression (Fig. 5c)							
cells	compounds						
	broxyquinolin	daunorubicin	eltrombopag	solafenib	verteporfin	glafenine	nilotinib
A431	0.005	0.013	0.122	0.461	0.547	0.676	0.626
BaF3 (+EGFR)	0.038	0.064	0.006	0.708	0.006	0.849	0.609
HeLa	0.379	0.487	0.048	0.474	0.296	0.591	0.194