Supporting information

Single-cell Encoded Gene Silencing for High-throughput Combinatorial siRNA Screening

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Supplementary notes

Supplementary Note 1. Characterization of zeta potential

The overall increase in zeta potential after this crosslinking step lies in the changes in the surface charge composition of the final micro/nano assembly. Initially, the iron oxide (Fe_3O_4) particles were positively charged due to the existence of a significant number of primary amine ($-NH_2$) groups on their surface. When the glutaraldehyde crosslinking occurs, these amine groups are transformed into imine (-N=CH-) groups, reducing the overall positive charge on the Fe_3O_4 surface (from + 6.34 mV to +3.99 mV). Next, the linkage of the gold nanorods to the Fe_3O_4 particles introduces additional primary amine ($-NH_2$) groups, which overwhelm the effects of the glutaraldehyde crosslinking and caused the increase in zeta potential as observed in **Figure S1.**

Supplementary Note 2. Methods for evaluating protein knockdown

At the protein level, we used western blot to show successful knockdown of actin in A549 cells by using the Knock-beads. A549 cells were seeded in 6-well dish at 1×10^6 cell/well (35 mm diameter). 24 hours later, Knock-beads (loaded with siRNA for actin) were applied at 1 $\times10^6$ beads/well. Then photoporation was perfomed. 24 hours later, the cells were collected and lysed by Cell lysis buffer (Beyotime) with addition of 1 mM Phenylmethanesulfonyl fluoride (PMSF). The proteins were extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Before electrophoresis, the concentration of total protein was determined by BCA Protein Assay Kit (Beyotime). All samples were diluted to the same protein concentration followed by 10 min heating at 100 ℃. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Beyotime 0.2 μm), using a constant current at 200 mA for 45 min. The membrane was blocked at room temperature for 2 h with a blocking solution containing 50 mM Tris-buffered saline (TBS), 5% skimmed milk powder and 0.05% Triton-X-100 (TBS-T, Thermo). Primary antibody (mouse monoclonal anti-actin 1:1000, from Beyotime) was diluted in the blocking solution and applied at 4 ℃. After overnight incubation, the membrane was washed with TBS-T solution and then probed with secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-mouse, Beyotime). The blots were further washed three times with TBS-T. Immunoreactivity was detected using enhanced chemiluminescence (ECL) reagent (BeyoECL Plus from Beyotime).

For a direct observation with single cell resolution, we also used the Knock-beads to specifically knock down GFP in each individual EGFP-Hela cells that were in contact with the Knock-beads. For an established stable line, EGFP-Hela cells were seeded in a culture dish at 1×10^6 cells/dish (35 mm diameter). 24 hours later, 1 $\times10^6$ Knock-beads (loaded with siRNA for GFP) were applied. Then the photoporation was performed. 24 hours after photoporation, the cells were examined with live cell imaging using confocal microscopy (Leica Sp8). The acquired images were analyzed by Cellpose and Image J software.

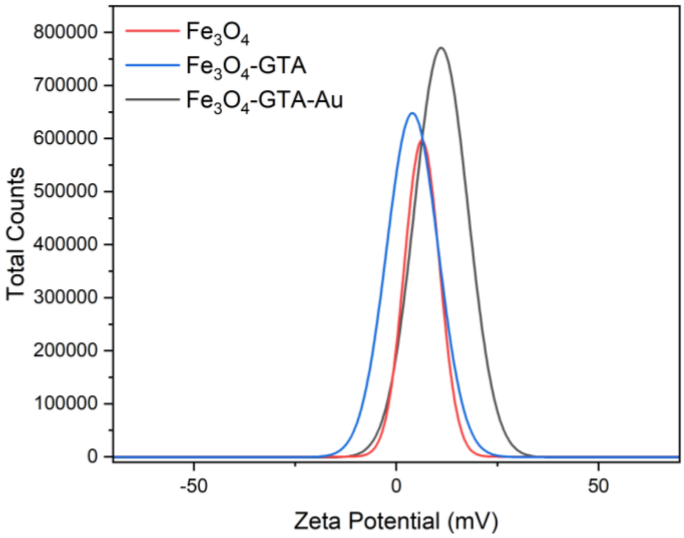
Supplementary Note 3. Control of the number of Knock-beads per cell

We specifically optimized the system and controlled the dose of Knock-beads to ensure ~3 beads per cell on average, as shown in **Figure 5e**. The number of beads interacting with the cells follows a Gaussian-like distribution, and the center peak of the distribution could be shifted to lower or higher number per cell by controlling the bead-to-cell ratio (**Figure S12**). Certainly, the pattern of Knock-bead distribution is crucial for the effectiveness of the combinatorial screening, as it allows for the capture of a diverse range of cell populations that have been randomly exposed to different combinations of siRNAs (Knock-combos). Increasing the bead dose would shift the distribution toward a higher bead number per cell, potentially reducing the population of cells exposed to the desired 2-3 component siRNA cocktails. Conversely, decreasing the bead dose would result in a lower proportion of cells encountering more complex siRNA combinations, which could potentially limit the capability to uncover some effective siRNA combinations. In an experiment, the Knock-beads of different types (Knock-combos) were applied to the cells with equal concentration (3.6×10^6 beads/mL), therefore, the chances of one cell receives multiple Knock-beads of the same type (Knock-combo) are rare, especially when the screen is targeting more genes (e.g. 20 genes in **Figure 7**). In most cases the number of Knock-beads was equal to the number of types of Knock-beads for a cell. Additionally, over-dosed Knock-beads causes reduced cell viability at the photoporation stage (**Figure S13**), which further require an optimization of the bead-to-cell ratio to construct an effective and efficient screening.

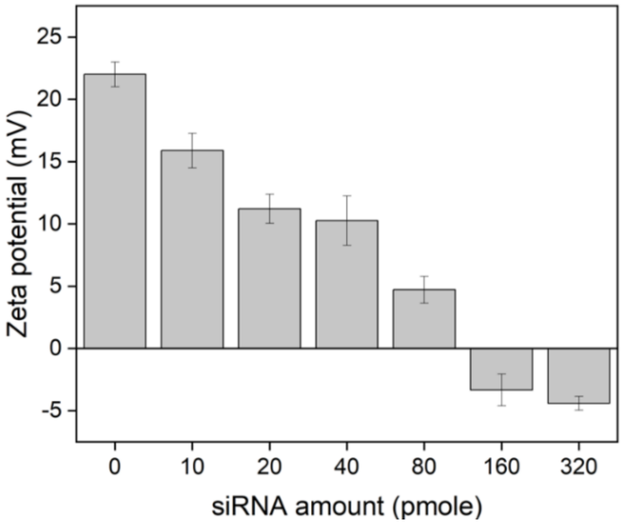
Supplementary Note 4. Explanation on missing Knock-combos out of the theoretical screening space

Regarding the number of Knock-combos be investigated, for a system involving 6 different Knock-bead variants (6 siRNA gene targets), theoretically, there are 62 possible Knock-combos. In the practical execution, we were not able to collect data from sufficient occurrence of cells (>20 per replicate) for some Knock-combos (5 or 6 Knock-beads) for statistical analysis, and therefore presented the screening for the observed 54 siRNA combinations. Similar situation was also met in the expanded largescale screening, which involved 20 siRNA gene targets. In theory, the screening space contains 1350 Knock-combos of 1 to 3 siRNAs. In our practical experiment, we were able to test and collect sufficient data for 1315 Knock-combos (**Figure 7**), but the missing of a few possible siRNA formulations does not compromise the extremely high-throughput nature of the scCode-fection system.

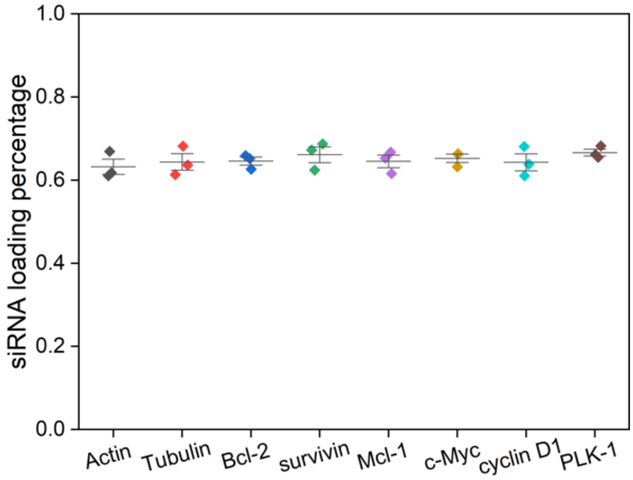
Supplementary Figures



Supplementary Figure 1. Change of zeta potential over the functional preparation of the Knock-beads. the iron oxide (Fe_3O_4) particles were positively charged due to the existence of primary amine ($-NH_2$) groups on their surface. When the glutaraldehyde (GTA) crosslinking occurs, these amine groups are transformed into imine (-N=CH-) groups, reducing the overall positive charge on the Fe_3O_4 bead surface (zeta potential reduces from + 6.34 mV to +3.99 mV). Next, the linkage of the gold nanorods to the Fe_3O_4 particles introduces additional primary amine ($-NH_2$) groups, which overwhelm the effects of the glutaraldehyde crosslinking and caused the increase in zeta potential to +18.16 mV.



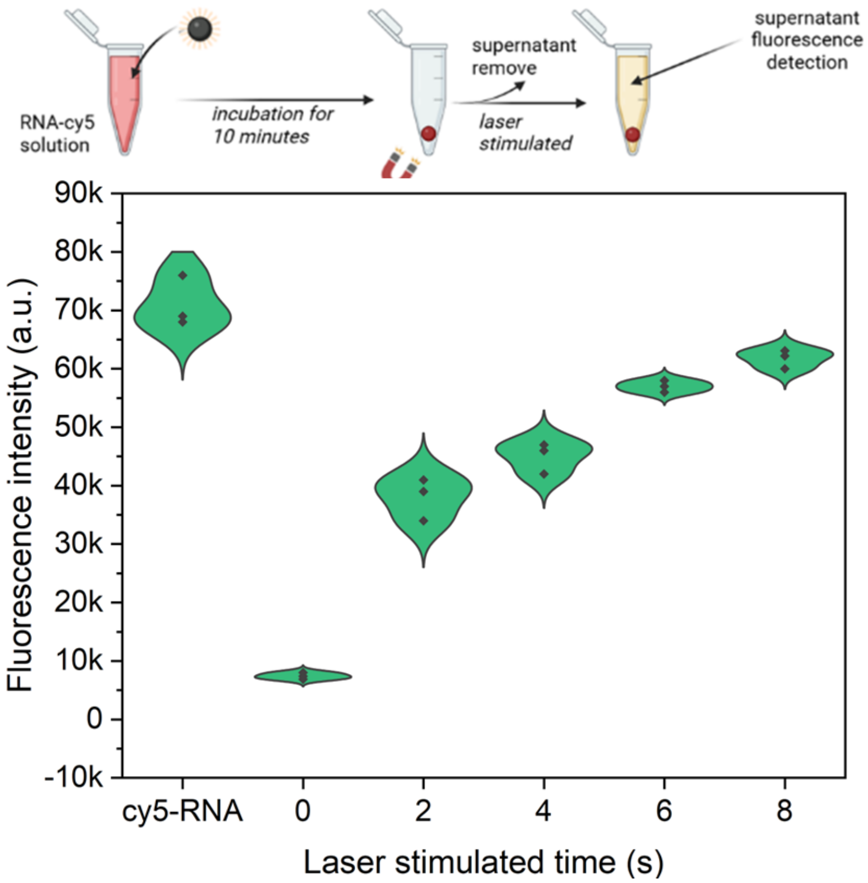
Supplementary Figure 2. Change of Zeta potential for fixed number of Knock-beads (8 x 10^5) with different amount of siRNA input ranging from 0 pmol to 320 pmol.



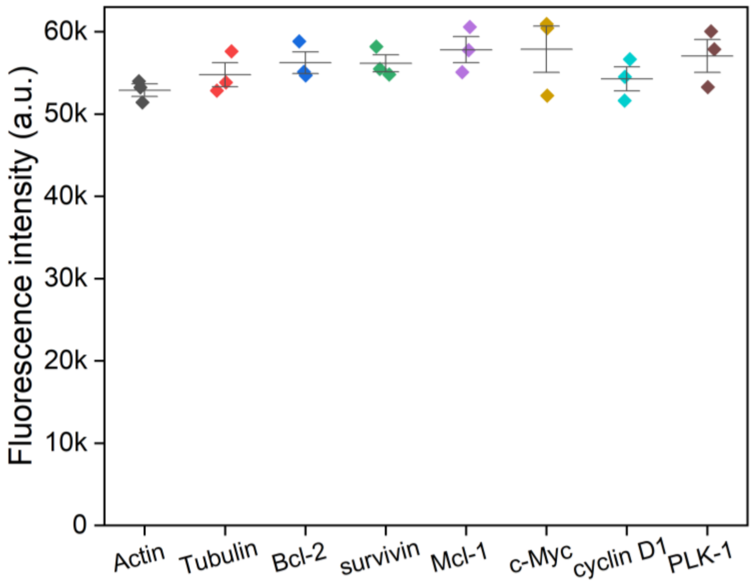
Supplementary Figure 3. Sequence-specific variation of siRNA loading on the Knock-beads. siRNA loading efficiency was tested by measuring the fluorescence depletion (by 8×10^5 Knock-beads) of the supernatant solution originally containing 20 pmol RNAs (cy5-labeled). Cy5-labelled siRNA was absorbed and pulled down by a magnet. Then the fluorescence of the supernatant before and after Knock-bead pulling-down was measured. The loading percentage was calculated by:

$$(Fluorescence_{before} - Fluorescence_{after})/Fluorescence_{before}$$

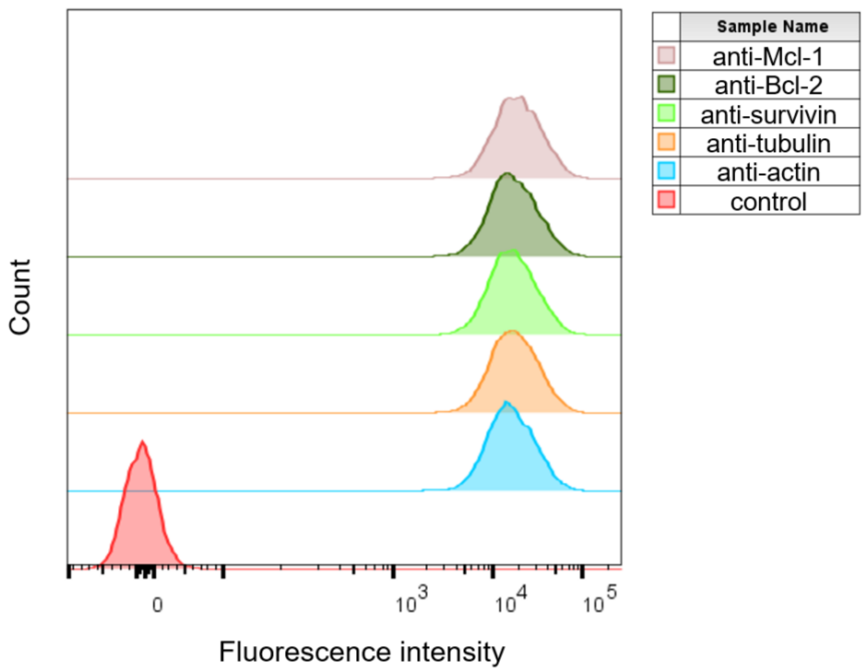
No significant differences in the loading efficiency across different siRNA sequences, n = 3, statistics by two-tailed unpaired t-test.



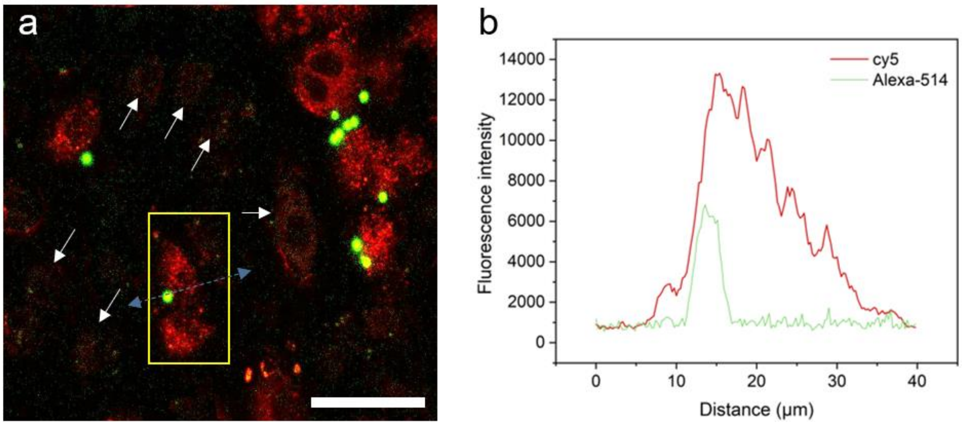
Supplementary Figure 4. Violin plot showing the characterization of siRNA release from the Knock-beads upon NIR irradiation. Fluorescence intensity of the supernatant solution was measured to reflect the RNA capture by the Knock-beads or RNA release from the Knock-beads upon NIR irritation of different duration from 2 to 8 seconds. "0" indicates the depletion of fluorescence by ~8×10^5 Knock-beads to absorb 20 pmol siRNA-cy5 in the solution through electrostatic interaction. The siRNA loaded Knock-beads were pulled down to the bottom of the reaction tube by an external magnet. Upon NIR irradiation for 2, 4, 6, 8 seconds, the siRNAs were released to the solution by 53.5%, 63.4%, 80.3% and 87.0% of the total load, as reflected by the recovery of the fluorescence of the supernatant solution. The dots in the violin plots indicate the data points for individual experiments. Created in BioRender. Guo, F. (2024) BioRender.com/q22o513



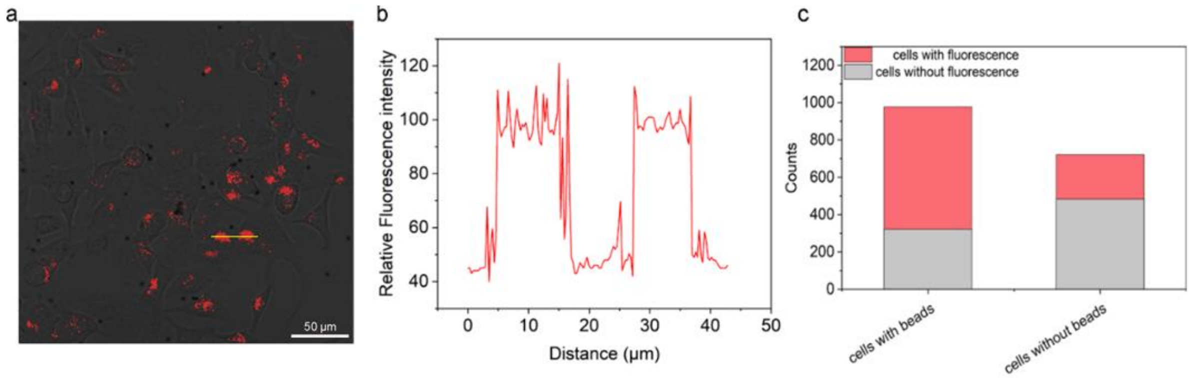
Supplementary Figure 5. Sequence-specific variation of siRNA release from the Knock-beads. Fluorescence intensity of supernatant solution was measured after siRNAs (cy5 labeled) release from the Knock-beads upon 6 second laser irradiation. 8 different types of siRNAs (cy5-labeled) were tested. They were designed with different sequences targeting actin, tubulin, Bcl-2, survivin, Mcl-1, c-Myc, cyclin D1 and PLK-1, respectively. No significant change of releasing efficiency was observed among these siRNAs, n = 3, statistics by two-tailed unpaired t-test.



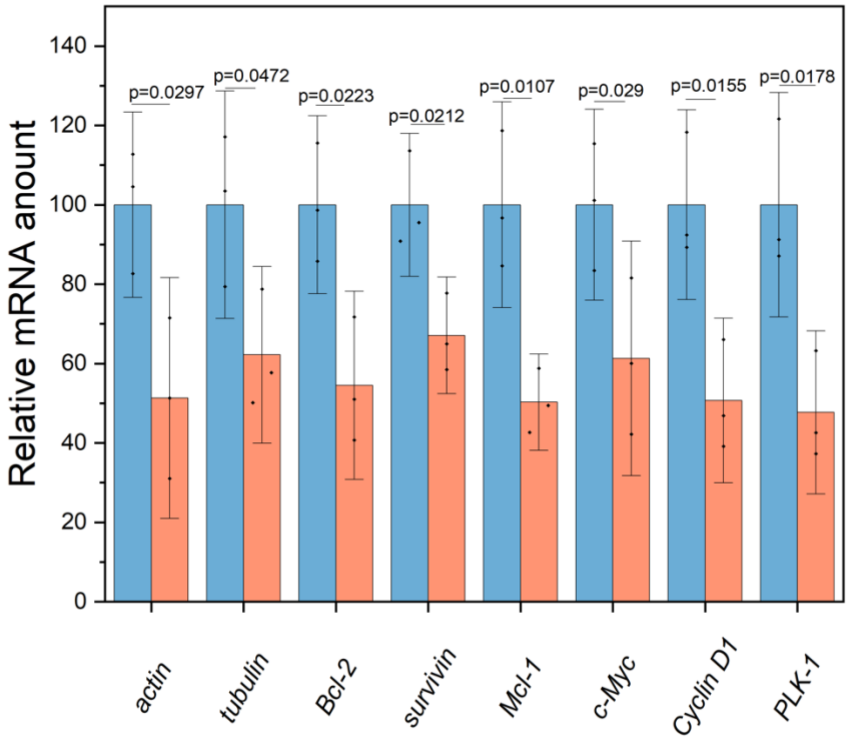
Supplementary Figure 6. Sequence-specific variation of siRNA intracellular translocation upon photoporation. This figure shows the intracellular translocation of 5 different siRNAs 4 hours after photoporation treatment. The siRNA was loaded on the Knock-beads and then applied the cells followed by a photoporation treatment. After 4 hours, the cells were analyzed using flow cytometry to assess the uptake of the fluorescence labeled siRNA. The negative control group refers to a sample that did not receive any photoporation.



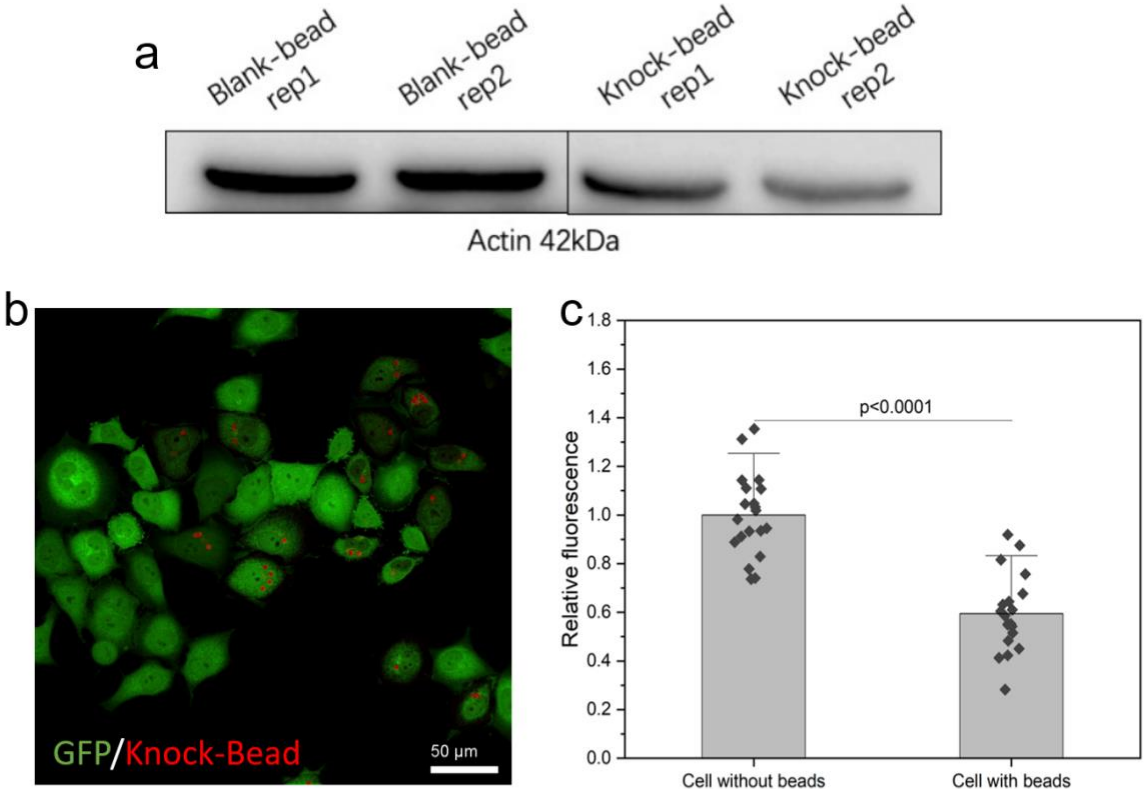
Supplementary Figure 7. Evaluation of siRNA intracellular translocation of siRNA (cy5-Labeled) 5 hours after photoporation. (**a**) Confocal microscopy image scanning across a horizonal plane sectioning the cells (A549) 5 hours after photoporation treatment. The cells were transfected with cy5-labeled siRNA by using the Knock-bead system. The white arrows indicate the cells without Knock-beads and siRNA delivery. Scale bar, 30 μm. (**b**) Fluorescence profile along the blue line across the cell in the yellow rectangle in panel (a).



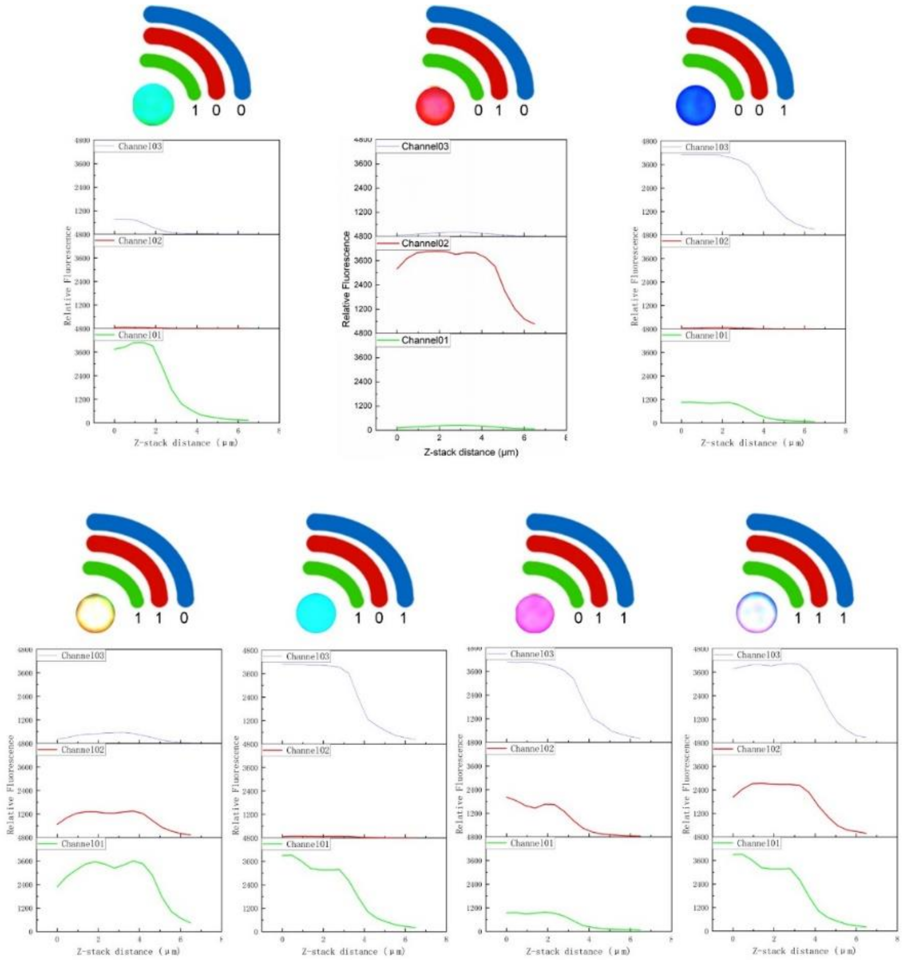
Supplementary Figure 8. (a) Intracellular delivery without a magnetic field, where RNAs are labeled by cy5 (red). **(b)** The relative fluorescence intensity along the yellow line in (a). **(c)** Number of cells categorized differently.



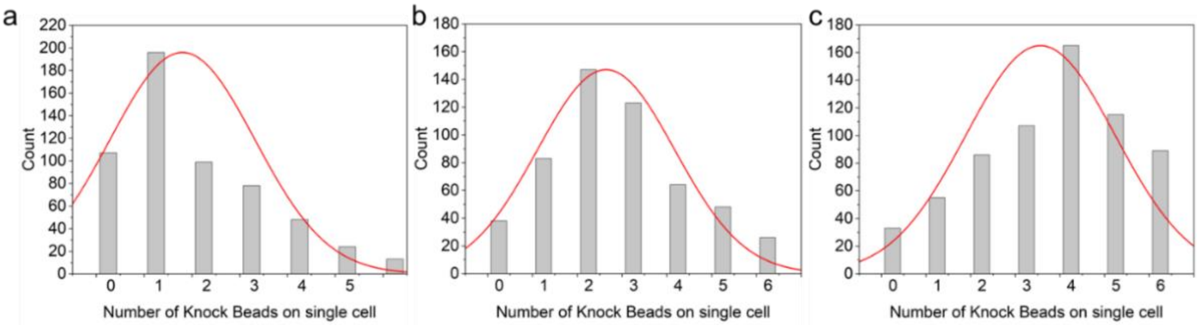
Supplementary Figure 9. Quantitative PCR analysis of knock down efficacy of specific siRNA targeting different genes. Each siRNA was delivered using the Knock-bead mediated photoporation. 24 hours later, the cells were lysed to extract and analyze the mRNA levels by using quantitative real-time PCR. The control group refers to cells that were treated with blank Knock-beads (without any siRNA loaded). The results shows that Knock-bead loaded with specific siRNAs can effectively knock down targeted genes, n=3, by tow-tailed unpaired t-test.



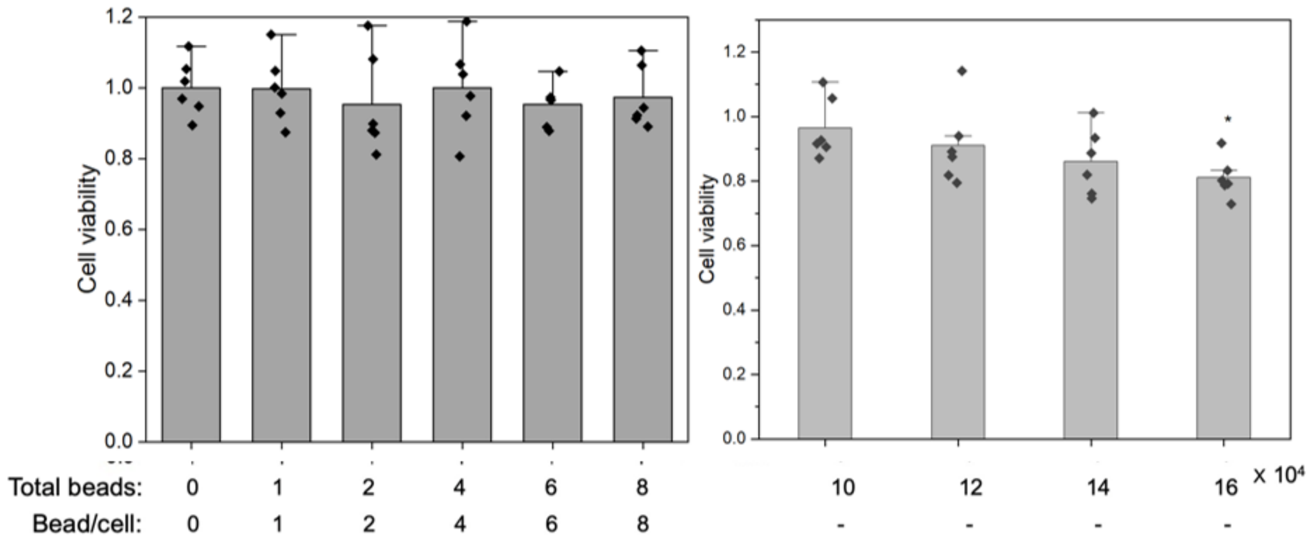
182
183
184 **Supplementary Figure 10.** Knock down protein expression by Knock-bead mediated intracellular
185 delivery of siRNA. (**a**) Western blot result showing successful knockdown of actin expression in A549
186 cells by using the Knock-beads (loaded with siRNA targeting actin). (**b**) Individual EGFP-Hela cells
187 in contact with the Knock-beads (loaded with siRNA targeting EGFP, labeled with Alexa-647) showed
188 a significant reduction in EGFP fluorescence. (**c**) Quantification of EGFP fluorescence in Hela cells
189 with or without Knock-beads. N = 20, by two-tailed unpaired t-test.
190



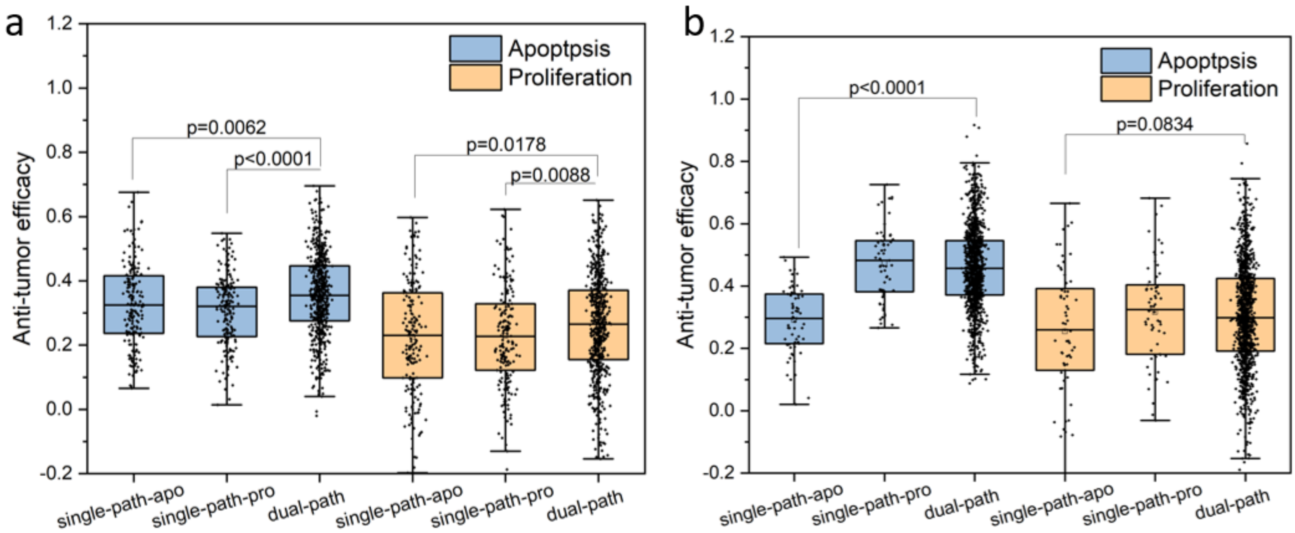
Supplementary Figure 11. Seven types of Knock-beads encoded by a rainbow labelling using three fluorophores: Alexa-514 (Channel 01), Alexa-568 (Channel 02), Alexa-647 (Channel 03). Created in BioRender. Guo, F. (2024) BioRender.com/t97h065



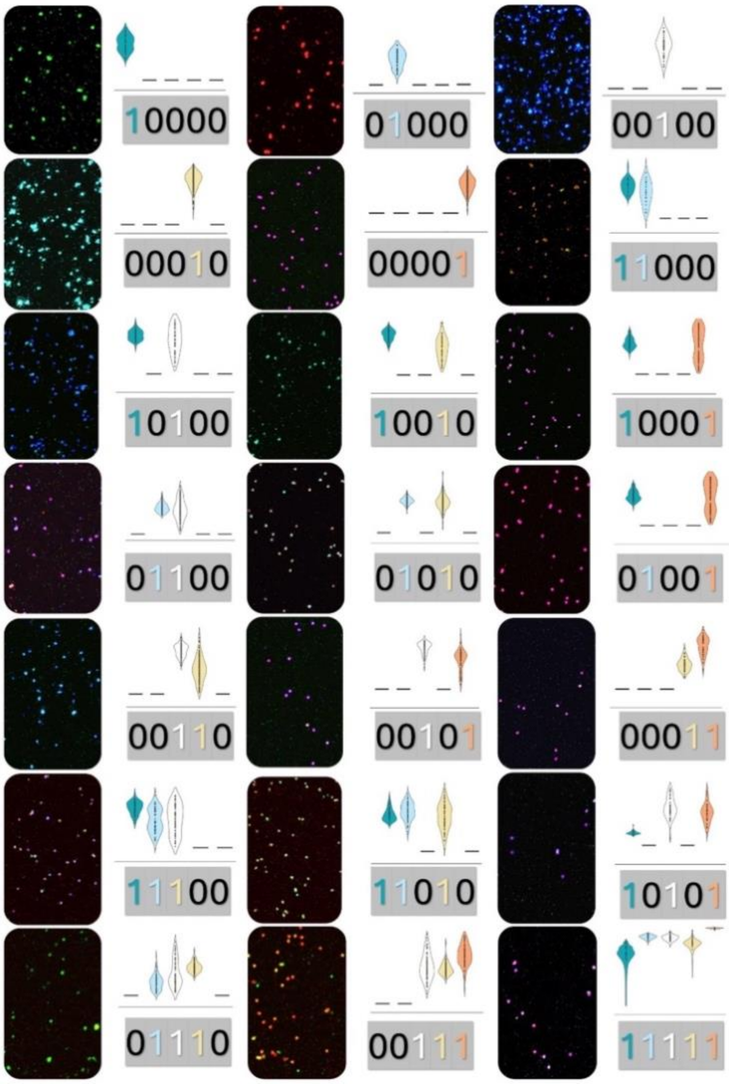
Supplementary Figure 12. Characterization of the Knock-bead distribution per single cell. This figure shows the distribution of the number of Knock-beads per single cell, at different bead-to-cell ratios: 1:1 (a), 2:1 (b), and 4:1 (c). The number of Knock-beads was calculated based on the total number of cells. By controlling the bead-to-cell ratio, we were able to obtain a Gaussian-like distribution of the number of beads interacting with each cell, ranging from 1 to 4 beads per cell.



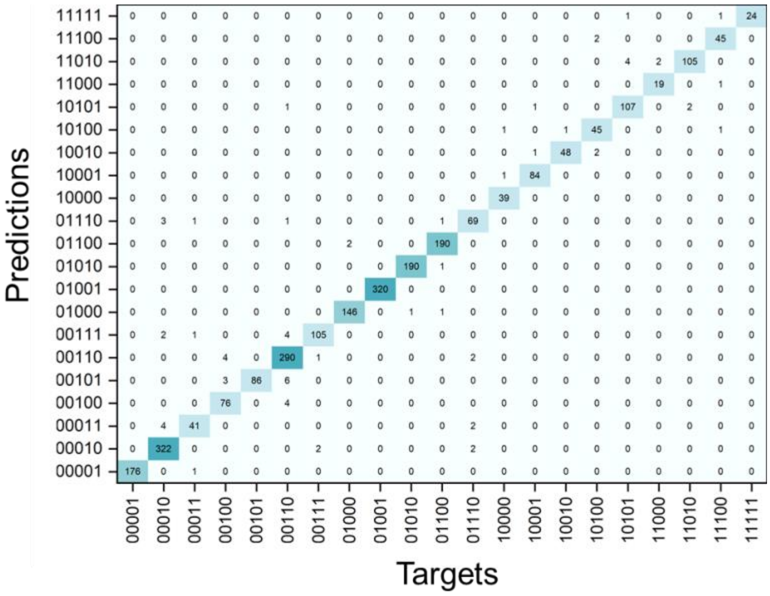
Supplementary Figure 13. Evaluation of cell viability at different Knock-bead to cell ratios. The cell viability was assessed using MTT assay following photoporation treatment at various Knock-bead density. The cells were incubated with Knock-bead at a concentration ranging from 0 to 16 × 10^4. The cell viability was evaluated 24 hours after photoporation. In most screening conditions, the number of Knock-bead per cell would not exceed 8, the photoporation process did not cause any damage in the cells. Only when the Knock-bead density reaches an extremely large level (e.g. 16× 10^4), significant cytotoxicity was observed. N = 6, * indicates P=0.012 (group 16 compared with group 0) by two-tailed unpaired t-test.



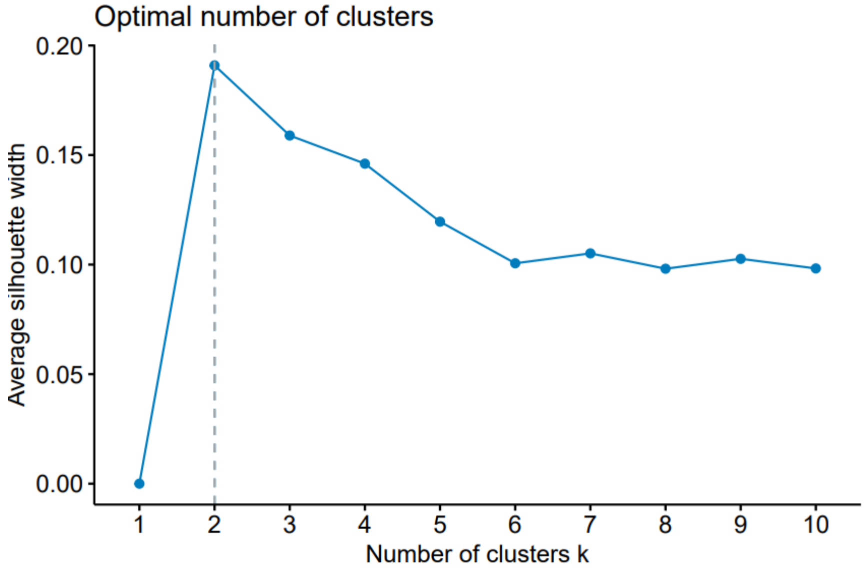
Supplementary Figure 14. Analysis of combinatorial knock down by siRNAs targeting multiple signal pathways by using (**a**) Two siRNA Knock-combos, (**b**) Three siRNA Knock-combos. "Single-path-apo" refers to combinations containing siRNAs exclusively targeting the apoptosis pathway. "Single-path-pro" refers to combinations containing siRNAs exclusively targeting the proliferation pathway. "Dual path" refers to combinations containing siRNAs targeting both the apoptosis and proliferation pathways. For each knock-combo n=21, for screening round n=3, by non-parametric Mann–Whitney test.



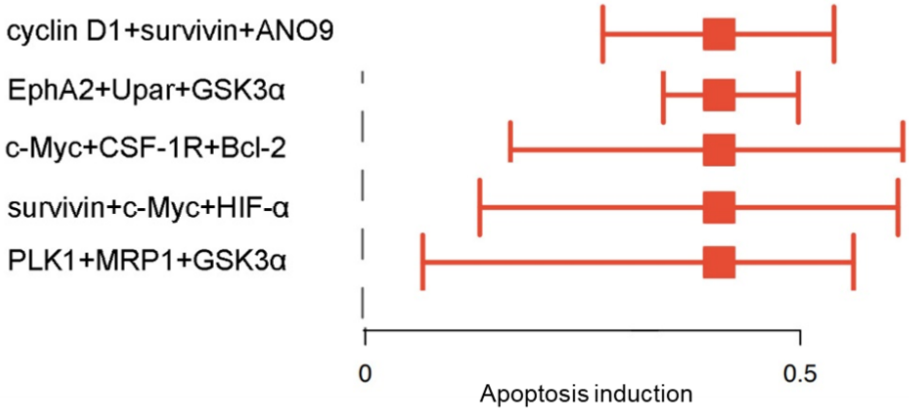
Supplementary Figure 15. Representative images showing the encoded fluorescence signal of 21 Knock-bead variants. A combinatory fluorescence labeling of the Knock-beads was used to substantially increase the multiplexing throughput by using 5 fluorescent channels. Each Knock-bead was made with a programmed rainbow fluorescence composed of 5 different fluorophores (quantum dot 547, 572, 604, 641, and Alexa fluor 647). For spectrum digitization, we used "1" to denote the inclusion and "0" to denote the exclusion of a fluorophore and to compose a 5-digit spectral code for each Knock-bead variant.



Supplementary Figure 16. Confusion matrix of machine learning-based target prediction for decoding the Knock-beads by using a 5-chanel fluoresce imaging system. The confusion matrix provides an intuitive assessment of the accuracy and performance of the predictive model. The results show that the majority of the targets were predicted correctly, as indicated by the distribution of data points along the diagonal line of the confusion matrix. This suggests a high level of accuracy in the machine learning model's capability to correctly decode different types of Knock-beads.



Supplementary Figure 17. Silhouette evaluation of optimal clustering for the 1315 Knock-combo associated phenotypic screening data. The maximum silhouette score was observed when the number of clusters was set to 2, indicating that a two-cluster solution represents the optimal partitioning of the data.



Supplementary Figure 18. Top 5 combinations that induced the most apoptosis among the 1315 Knock-combos.

Supplementary Tables

Supplementary Table 1. List of siRNA sequence targeting different genes.

Gene Target	Sense (5'-3')	Anti-sense (5'-3')
actin	UGCAUUUGGCGGAAAGUGGdTdT	CCACUUUCCGCCAAAUGCAdTdT
tubulin	AGACAAUUUGGCUAAUGAGdTdT	CUCAUUAGCCAAAUUGUCUdTdT
Bcl-2	GUACAUCCAUUAUAAGCUGdTdT	CAGCUUAUAAUGGAUGUACdTdT
Mcl-1	AAGUAUCACAGACGUUCUCdTdT	GAGAACGUCUGUGAUACUUdTdT
survivin	CACCGCAUCUCUACAUUCAdTdT	UGAAUGUAGAGAUGCGGUGdTdT
c-Myc	AACGUUAGCUUCACCAACAdTdT	UGUUGGUGAAGCUAACGUUdTdT
cyclin D1	ACACCAAUCUCCUCAACGAdTdT	UCGUUGAGGAGAUUGGUGUdTdT
PLK-1	CCUUGAUGAAGAAGAUCACdTdT	GUGAUCUUCUUCAUCAAGGdTdT
KRAS	GUAUUUGCCAUAAAUAAUACU	AGUAUUAUUUAUGGCAAAUAC
HIF-α	GGCGAAGUAAAGAAUCUGAdTdT	UCAGAUUCUUUACUUCGCCdTdT
CSF-1R	CUACUCAACUUUCUCCGAAdTdT	UUCGGAGAAAGUUGAGUAGdTdT
Hsp 27 gene	GCUGCAAAAUCCGAUGAGACdTdT	GUCUCAUCGGAUUUUGCAGCdTdT
EphA2	UUACUGUACGGCUAGAUGUAC	GUACAUCUAGCCGUACAGUAA
Upar	AGUCAAUGAGGAAAGUCUCUU	GAGACUUUCCUCAUUGACUGC
HER-2	UCACAGGGGCCUCCCCAGGAG	CCUGGGGAGGCCCCUGUGACA
Cbl-b	UGAGAUGCCCUGAUAUUAAdTdT	UUAAUAUCAGGGCAUCUCAdTdT
EpCAM	UCACAAAGCAGUUUACGGCCA	GCCGUAAACUGCUUUGUGAAU
ANO9	AGUAGUUCCUGAUUUCAUCAA	GAUGAAAUCAGGAACUACUUU
MRP1	GGCUACAUUCAGAUGACACdTdT	GUGUCAUCUGAAUGUAGCCdTdT
RRM2	GCGAUUUAGCCAAGAAGUUCA	UGAACUUCUUGGCUAAAUCGC
DNA-PKcs	GGGCGCUAAUCGUACUGAAdTdT	UUCAGUACGAUUAGCGCCCdTdT
GSK3α	ACUUUGAUGUCAGUGUAAGCC	CUUACACUGACAUCAAAGUGA

Supplementary Table 2. List of encoded Knock-Beads and the corresponding siRNA targets using a 3-channel fluorescence system.

Knock-bead Code	siRNA target for apoptosis	siRNA target for cell cycle
100	Bcl-2	
010	survivin	
001	Mcl-1	
110		c-Myc
101		cyclin-D1
011		PLK-1

Supplementary Table 3. Spectrum encoding of different Knock-beads for a 3-channel system.

Knock-bead index	Alexa-514	Alexa-568	Alexa-647	Code
1	100%	0	0	100
2	0	100%	0	010
3	0	0	100%	001
4	100%	100%	0	110
5	100%	0	100%	101
6	0	100%	100%	011
7	100%	100%	100%	111

Supplementary Table 4. List of Knock-combos (KC) classified by targeted signaling pathways.

siRNA # of a KC	Pathway coverage	Targeted apoptosis gene			Targeted cell-cycle gene			# of KC
		survivin	Bcl-2	Mcl-1	c-Myc	cyclin D1	PLK-1	
2	Single	√	√					6
		√		√				
			√	√				
					√	√		
					√		√	
						√	√	
2	Dual	√			√			9
		√				√		
		√					√	
			√		√			
			√			√		
			√				√	
				√	√			
				√		√		
				√			√	
3	Single	√	√	√				2
					√	√	√	
3	Dual	√	√		√			18
		√	√			√		
		√	√				√	
		√		√	√			
		√		√		√		
		√		√			√	
			√	√	√			
			√	√		√		
			√	√			√	
		√			√	√		
			√		√	√		
				√	√	√		
		√			√		√	
			√		√		√	
				√	√		√	
		√				√	√	
			√			√	√	
				√		√	√	

Supplementary Table 5. Spectrum encoding of different Knock-beads using a 5-channel fluorescent system (quantum dot 547, 572, 604, 641, and Alexa fluor 647). The shaded region indicates the extra codes not used in the expanded large-scale screening.

Knock-bead index	**Quantum dot-547**	**Quantum dot-572**	**Quantum dot-604**	**Quantum dot-641**	**Alexa-647**	**Code**
1	100%	0	0	0	0	10000
2	0	100%	0	0	0	01000
3	0	0	100%	0	0	00100
4	0	0	0	100%	0	00010
5	0	0	0	0	100%	00001
6	100%	100%	0	0	0	11000
7	100%	0	100%	0	0	10100
8	100%	0	0	100%	0	10010
9	100%	0	0	0	100%	10001
10	0	100%	100%	0	0	01100
11	0	100%	0	100%	0	01010
12	0	100%	0	0	100%	01001
13	0	0	100%	100%	0	00110
14	0	0	100%	0	100%	00101
15	0	0	0	100%	100%	00011
16	100%	100%	100%	0	0	11100
17	100%	100%	0	100%	0	11010
18	0	100%	100%	100%	0	01110
19	0	0	100%	100%	100%	00111
20	100%	0	100%	100%	100%	10111
21	100%	100%	100%	100%	100%	11111
22	100%	100%	0	0	100%	11001
23	100%	0	100%	100%	0	10110
24	100%	0	100%	0	100%	10101
25	100%	0	0	100%	100%	10011
26	0	100%	100%	0	100%	01101
27	0	100%	0	100%	100%	01011
28	100%	100%	100%	100%	0	11110
29	100%	100%	100%	0	100%	11101
30	100%	100%	0	100%	100%	11011
31	0	100%	100%	100%	100%	01111

Supplementary Table 6. Knock-bead decoding performance for a 5-channel encoding system, analyzed using non-parametric permutation test.

Statistics	Results
Accuracy	0.9748
95% CI	(0.9595,0.9856)
P-value	<0.0001
Kappa	0.9728
Multiclass area under the curve	0.9982

Supplementary Table 7. Summary of statistical significance for data analysis presented in Figure 5f.

Knock-combo	Apoptosis induction*	Proliferation inhibition*
1	<0.0001	0.4858
2	0.0030	0.0633
3	0.0003	0.0068
4	<0.0001	0.0396
5	<0.0001	0.1086
6	0.0117	0.2249
12	<0.0001	0.0032
13	0.0005	0.0142
14	<0.0001	<0.0001
15	<0.0001	<0.0001
16	<0.0001	<0.0001
23	<0.0001	<0.0001
24	<0.0001	<0.0001
25	<0.0001	<0.0001
26	<0.0001	0.0002
34	<0.0001	<0.0001
35	<0.0001	<0.0001
36	0.0013	<0.0001
45	<0.0001	0.0003
46	<0.0001	<0.0001
56	<0.0001	0.0002
123	<0.0001	<0.0001
124	<0.0001	<0.0001
125	<0.0001	<0.0001
126	<0.0001	<0.0001
134	<0.0001	<0.0001
135	<0.0001	<0.0001
136	<0.0001	<0.0001
145	<0.0001	<0.0001
146	<0.0001	<0.0001
156	<0.0001	<0.0001
234	<0.0001	<0.0001
235	<0.0001	<0.0001
236	<0.0001	<0.0001
245	<0.0001	<0.0001
246	<0.0001	<0.0001
256	<0.0001	<0.0001
345	<0.0001	<0.0001
346	<0.0001	<0.0001
356	<0.0001	<0.0001
456	<0.0001	<0.0001
1234	<0.0001	<0.0001
1345	<0.0001	<0.0001
2345	<0.0001	<0.0001
2456	<0.0001	<0.0001
1345	<0.0001	<0.0001
1245	<0.0001	<0.0001
1235	<0.0001	<0.0001
1236	<0.0001	<0.0001
2456	<0.0001	<0.0001
3456	<0.0001	<0.0001
12345	<0.0001	<0.0001
13456	<0.0001	<0.0001
23456	<0.0001	<0.0001

* Analyzed by unpaired one-way ANOVA. All groups are compared with the control group. Labeling of siRNA: 1, *Bcl-2*; 2, *surviving*; 3, *Mcl-1*; 4, *c-Myc*; 5, *Cyclin D1*; 6, *PLK-1*.