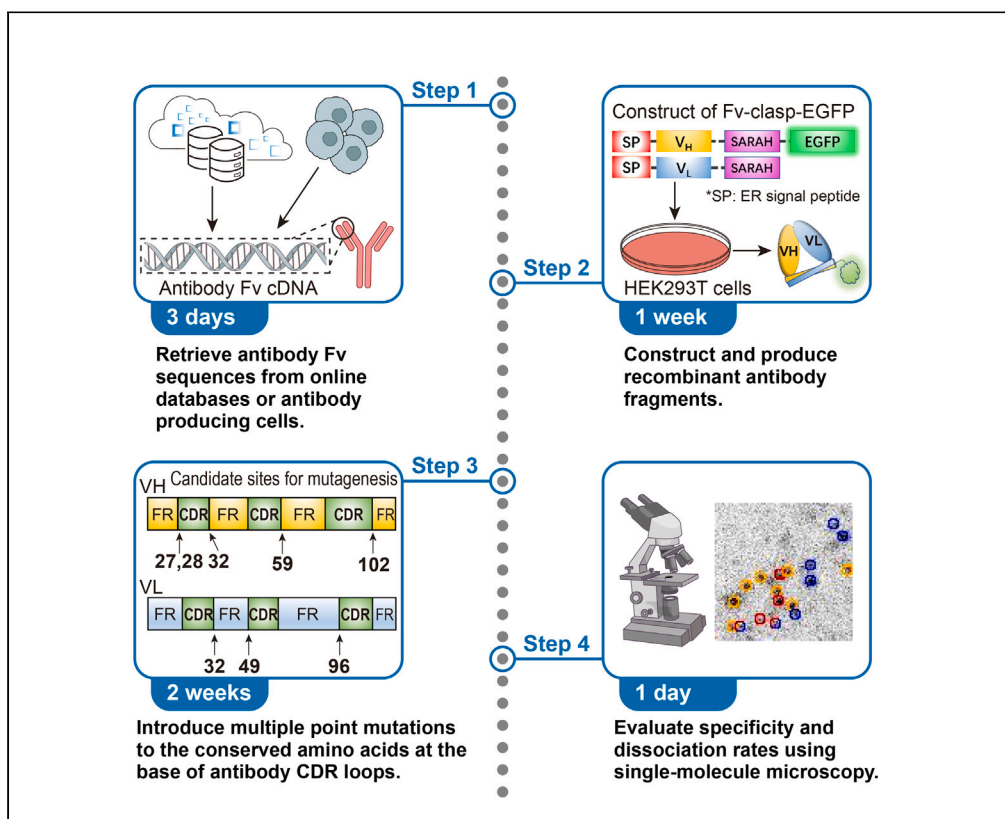


Protocol

Protocol to generate fast-dissociating recombinant antibody fragments for multiplexed super-resolution microscopy



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Highlights
Acceleration of
antibody-dissociation
rates by mutagenesis
at the base of CDR
loops

Production of
recombinant
antibody fragments
from cell culture
supernatant

k_{off} determination
and specificity
validation using a
single-molecule
approach

The protocol can be
widely applied to
existing antibodies
and nanobodies

Multiplexed high-density label super-resolution microscopy image reconstruction by integrating exchangeable single-molecule localization (IRIS) enables elucidating fine structures and molecular distribution in cells and tissues. However, fast-dissociating binders are required for individual targets. Here, we present a protocol for generating antibody-based IRIS probes from existing antibody sequences. We describe steps for retrieving antibody sequences from databases. We then detail the construction, purification, and evaluation of recombinant probes after site-directed mutagenesis at the base of complementarity-determining region loops. The protocol accelerates dissociation rates without compromising the binding specificity.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Protocol to generate fast-dissociating recombinant antibody fragments for multiplexed super-resolution microscopy

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SUMMARY

Multiplexed high-density label super-resolution microscopy image reconstruction by integrating exchangeable single-molecule localization (IRIS) enables elucidating fine structures and molecular distribution in cells and tissues. However, fast-dissociating binders are required for individual targets. Here, we present a protocol for generating antibody-based IRIS probes from existing antibody sequences. We describe steps for retrieving antibody sequences from databases. We then detail the construction, purification, and evaluation of recombinant probes after site-directed mutagenesis at the base of complementarity-determining region loops. The protocol accelerates dissociation rates without compromising the binding specificity.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022).¹

BEFORE YOU BEGIN

The repository of antibodies, developed in biological and medical research, is expanding at a rapid pace. The protocol below outlines a mutagenesis strategy for accelerating the dissociation rate (k_{off}) of existing antibodies in the format of Fv-clasp,² or nanobodies. Fv-clasp is a small, artificially designed antibody fragment with higher stability and productibility than conventional single-chain Fv (scFv). It is constructed by fusing a coiled-coiled SARAH domain of human Mst1 kinase to each chain of antibody variable domains. Fv-clasp well preserves the conformation of Fv region of original antibodies.² A fluorescent protein is conjugated at the C-terminus of heavy chain of Fv (V_H) for rapid verification of antibody fragments. The fast-dissociating antibody fragments with site-specific fluorescent labeling are ideal for multiplexed high-density label super-resolution microscopy IRIS.^{1,3,4} The sample preparation of IRIS is simpler than DNA-PAINT and STORM because IRIS does not require the step of antibody incubation. IRIS does not suffer from photobleaching. IRIS probes could be removed by gentle washing. Importantly, IRIS can overcome the interference between multiple antibodies in a confined area and the resultant scarce labeling,¹ which has been an intrinsic problem of super-resolution microscopy. As we also showed in previous publication,¹ the IRIS images show more continuous labelling pattern and higher labelling densities than DNA-PAINT and STORM. In addition, compared with our previous methods, this protocol provides a more efficiency approach to generate IRIS probes within several weeks.

So far, more than 30 fast-dissociating IRIS probes have been generated in our laboratory using the protocol described in this paper. We also provide methods for purifying recombinant antibody fragments from the culture supernatant of HEK293T cells and evaluating the k_{off} of the fluorescent antibody fragments using single-molecule imaging.



Retrieve the antibody sequences from antibody databases

⌚ Timing: 1 h

1. Find the available antibody sequence from database such as ABCD Database (web.expasy.org/abcd), NeuroMabSeq (neuromabseq.ucdavis.edu), Addgene (www.addgene.org/antibodies/all) and Protein Data Bank (PDB).

Note: Use IgBlast tool (www.ncbi.nlm.nih.gov/igblast) or IMGT/V-QUEST (www.imgt.org/IMGT_vquest/input)⁵ to identify the variable fragment (V_H or V_L) of an antibody and verify the integrity of its sequence. If the antibody sequence is complete and free of internal frame shift and stop codons, the IgBlast or IMGT/V-QUEST will classify it as a “productive” sequence.

2. Align the variable fragment by Chothia numbering scheme using ANARCI web server (opig.stats.ox.ac.uk/webapps/newsabdab/sabpred/anarci).

Note: CDRs of antibodies are identified using the Chothia Numbering Scheme as follows: HCDR1, H26-H32; HCDR2, H52-H56; HCDR3, H96-H101; LCDR1, L26-L32; LCDR2, L50-L52; LCDR3, L91-L96.⁶ Nanobody CDRs are defined according to the previously described rule based on Chothia Scheme: CDR1, Nb26-Nb35; CDR2, Nb50-Nb56; CDR3, Nb95-Nb102.⁷

3. Retrieve the DNA sequences that correspond to amino acid number 1–107 for the V_L and 1–113 for the V_H .

⚠ **CRITICAL:** The 112th amino acid in V_H (usually Ser) has to be changed to Cys to enhance the stability of Fv-clasp.² This manipulation is not required for nanobody sequence.

4. If the plasmid containing antibody cDNA is not deposited at repositories such as Addgene, use the artificial gene synthesis service to generate the cDNA of V_H and V_L (~350 bps).

Preparation of cell culture for purification and validation of antibody fragments

⌚ Timing: 2–3 days

5. Culture HEK293T cells in 37°C, 5% CO₂ incubator using Dulbecco’s modified Eagle medium (DMEM, Nacalai Tesque, 0845935) containing 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, 10270106).

Note: The cell should be about 80% confluent at the time of transfection.

6. Culture *Xenopus laevis* XTC cells in 22°C incubators using 70% Leibovitz’s L-15 medium (Thermo Fisher Scientific, 11415064) supplemented with 10% FBS (Thermo Fisher Scientific, 10270106).⁸

Note: The L-15 medium is diluted to 70% by sterilized deionized distilled water (DDW). See [materials and equipment](#) for the detailed composition of the medium.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5 α	Toyobo	Cat#DNA-913

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Continued		
Chemicals, peptides, and recombinant proteins		
Imidazole	Nacalai Tesque	Cat#19004-35
2-Morpholinoethanesulfonic acid (MES)	Nacalai Tesque	Cat#21623-26
4% Paraformaldehyde (PFA) phosphate buffer solution	Nacalai Tesque	Cat#09154-85
PFA, powder	Nacalai Tesque	Cat#26126-54
ATP	Nacalai Tesque	Cat#01072-11
Poly-L-lysine (PLL) hydrobromide	Sigma-Aldrich	Cat#25988-63-0
EGTA	Nacalai Tesque	Cat#15214-34
PIPES	Nacalai Tesque	Cat#28104-74
Pyranose oxidase	Sigma-Aldrich	Cat#P4234-1KU
Catalase	SERVA	Cat#26910.01
Mercaptoethylamine	Sigma-Aldrich	Cat#M6500-25G
Experimental models: Cell lines		
<i>Xenopus laevis</i> XTC cells	Kerafast	Cat#EMU039
HEK293T	ATCC	Cat#CRL-1573; RRID: CVCL_0063
Oligonucleotides		
Primers for Fv cloning, see the Note of major step 1	Zhang et al. ¹	N/A
Recombinant DNA		
pcDNA3.1 vector	Thermo Fisher Scientific	Cat#V79020
pEGFP-C1	Clontech Laboratories	Cat#6084-1
pEGFP-actin	Clontech Laboratories	Cat#6116-1
Mammalian expression plasmid of anti-HA (12CA5) and anti-target (P20.1) Fv-clasp (v2)	Arimori et al. ²	Arimori et al., 2017
T-vector pMD20	TaKaRa	Cat#3270
Software and algorithms		
GraphPad Prism 9	Dotmatics	https://www.graphpad.com/
tanitracer	Miyoshi et al. ⁴	https://github.com/takushim/tanitracer
ANARCI	Dunbar and Deane, 2016. ⁹	http://opig.stats.ox.ac.uk/webapps/newsabdab/sabpred/anarci
IgBlast tool	U.S. National Library of Medicine	RRID: SCR_002873
SnapGene	Dotmatics	https://www.snapgene.com/
Other		
Leibovitz's L-15 medium (with phenol red)	Thermo Fisher Scientific	Cat#11415-064
Leibovitz's L-15 medium (phenol red-free)	Thermo Fisher Scientific	Cat#21083027
FBS	Thermo Fisher Scientific	Cat#10270-106
DMEM	Nacalai Tesque	Cat#08459-35
Opti-MEM, reduced serum medium, no phenol red	Thermo Fisher Scientific	Cat#11058021
2.5 g/L-trypsin/1 mmol/L-EDTA solution	Nacalai Tesque	Cat#35554-64
Ni-NTA agarose	QIAGEN	Cat#30210
Neon Transfection System	Thermo Fisher Scientific	Cat#MPK5000
Neon Transfection System 10 mL Kit	Thermo Fisher Scientific	Cat#MPK1096
Envision 2105	PerkinElmer	Cat#2105
BSA	Nacalai Tesque	Cat#01860-65
Lipofectamine 3000 Transfection Reagent	Thermo Fisher Scientific	Cat#L3000015
Bovine fibronectin	Sigma-Aldrich	Cat#F1141
KOD One PCR Master Mix	Toyobo	Cat#KMM-101
KOD Plus Neo	Toyobo	Cat#KOD-401
T4 DNA ligase	New England Biolabs	Cat#M0202
Ex Taq	TaKaRa	Cat#RR001A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TRIzol RNA isolation reagents	Thermo Fisher Scientific	Cat#15596018
Alkaline phosphatase (calf intestine) (CIAP)	TaKaRa	Cat#2250A
T4 polynucleotide kinase	TaKaRa	Cat#2021S
ReverTra Ace qPCR RT Master Mix	Toyobo	Cat#FSQ-201
Ligation high Ver.2	Toyobo	Cat#LGK-201
DpnI	New England Biolabs	Cat#R0176S
Spectra/Por membrane (MWCO: 6–8 kD)	Spectrum Laboratories	Cat#132645
IX83 inverted microscope	Olympus	IX83
Evolve 512 EMCCD camera	Photometrics	Evolve 512
Z-drift compensator IX3-ZDC2	Olympus	IX3-ZDC2

MATERIALS AND EQUIPMENT

70% L-15 (10% FBS) Medium (L-15 [+]) for XTC cell culture

Reagent	Final concentration	Amount
L-15 medium (phenol red +)	N/A	350 mL
DDW	N/A	150 mL
FBS	10% (v/v)	50 mL
Total	N/A	600 mL

Note: Store at 4°C for up to 6 months.

70% L-15 (Serum free) Medium (L-15 [-]) for XTC cell plating

Reagent	Final concentration	Amount
L-15 medium (phenol red -)	N/A	350 mL
DDW	N/A	150 mL
Total	N/A	500 mL

Note: Store at 4°C for up to 6 months.

TBS buffer (10×) for the purification of recombinant antibody

Reagent	Final concentration	Amount
Tris	200 mM	24.23 g
NaCl	1.5 M	87.66 g
DDW	N/A	N/A
Total	N/A	1 L

Note: Adjust the pH to 7.5 by high concentration HCl and mess up to 1 L.

Note: Store at 15°C–25°C.

Cytoskeleton buffer (10×)

Reagent	Final concentration	Amount
MES (powder)	100 mM	2.13 g

(Continued on next page)

Continued

Reagent	Final concentration	Amount
KCl (3 M)	900 mM	30 mL
MgCl ₂ (1 M)	30 mM	3 mL
EGTA (0.5 M, pH 8.0)	20 mM	4 mL
DDW	N/A	N/A
Total	N/A	100 mL

Note: The pH of 0.5 M EGTA solution should be adjusted to 8.0 by KOH.

Note: Adjusted the pH of 10× cytoskeleton buffer to 6.1 and mess up to 100 mL.

Note: Store at 4°C.

3.7% PFA buffer for XTC fixation

Reagent	Final concentration	Amount
PFA (powder)	3.7% (w/v)	3.7 g
10 M KOH	N/A	About 10 µL for titration
10× cytoskeleton buffer	N/A	10 mL
DDW	N/A	N/A
Total	N/A	100 mL

Note: To prepare the solution, add 3.7 g PFA powder to 80 mL DDW and heat the solution to 60°C. Add about 10 µL of 10 M KOH to dissolve the PFA completely and titrate the pH to 6.1–6.5. Adjust the volume to 100 mL. Sterilize the buffer through a 0.22 µm filter. Dispense the solution into 10 mL aliquots and store at –20°C.

Catalase stock solution

Reagent	Final concentration	Amount
PIPES (0.1 M, pH 7.1)	12 mM	1.2 mL
MgCl ₂ (1 M)	2 mM	20 µL
EGTA (0.5 M, pH 8.0)	1 mM	20 µL
Catalase	3.5 mg/mL	35 mg
DDW	N/A	N/A
Total	N/A	10 mL

Note: Store at –80°C and use within three months.¹⁰

Imaging buffer

Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 8.0)	50 mM	100 µL
KCl (3 M)	90 mM	60 µL
MgCl ₂ (1 M)	3 mM	6 µL
20% Glucose (w/v)	10% (w/v)	1 mL
MEA (1 M)	10 mM	20 µL
Catalase stock solution	60 µg/mL	34.2 µL
Pyranose oxidase (250 U/ml)	5 U/mL	40 µL
DDW	N/A	739.8 µL
Total	N/A	2 mL

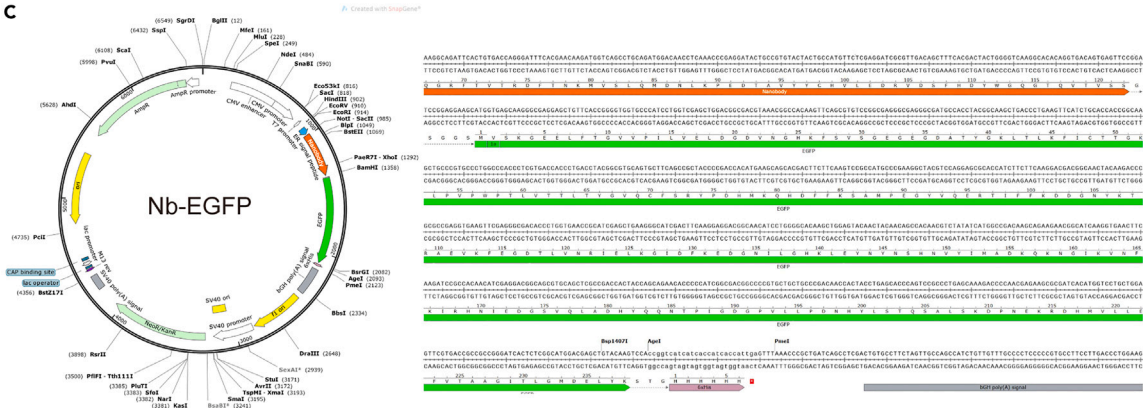
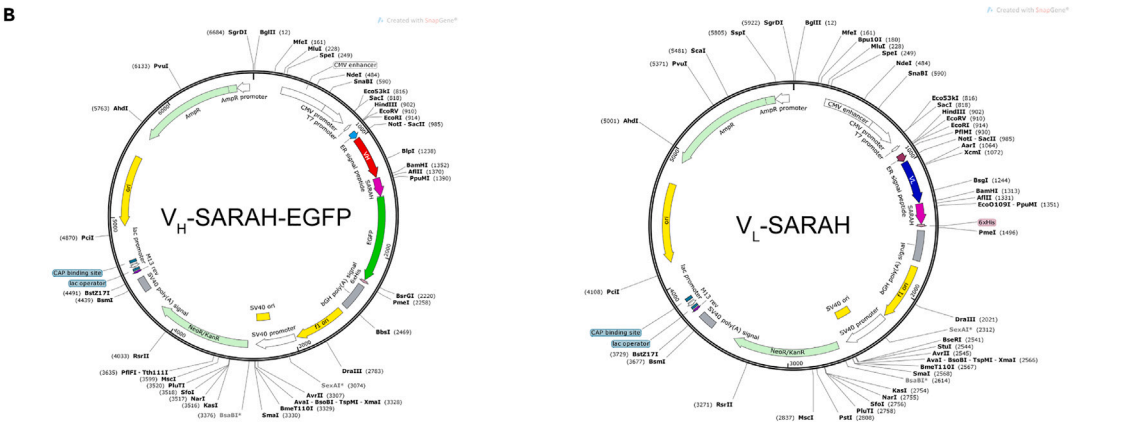


Figure 1. Illustration of expression vectors for recombinant antibodies

- (A) Sequence information of Fv-clasp V_H expression vector between CMV promoter and bGH poly A signal. Note that the 37th amino acid in SARAH domain of V_L expression vector should be changed to Cys.
- (B) Plasmid maps of EGFP fused Fv-clasp, including separately constructed V_H -SARAH-EGFP and V_L -SARAH. EGFP is incorporated into the V_H vector using AgeI site in (A), which is at the end of SARAH domain.
- (C) Sequence information and plasmid map of EGFP fused nanobody expression vector.

Note: Prepare the solution on ice and use immediately.

STEP-BY-STEP METHOD DETAILS

Construction of recombinant antibody

⌚ Timing: 4 days

This part is for the construction of the mammalian expression vector for fluorescently labeled Fv-clasp and nanobodies. The mammalian expression plasmid for Fv-clasp (Figure 1A) is constructed using a pcDNA3-based vector, containing a signal sequence from mouse IgH,¹¹ and a C-terminal 6xHis-tag.² The V_H and V_L are constructed in separate vectors. We fused an EGFP to the C-terminus of SARAH in the V_H vector using AgeI site (Figure 1B). For single domain antibody (nanobody or VHH), we directly fused the EGFP to the C-terminus of nanobody (Figure 1C).

1. Subclone the V_H and V_L cDNA fragments of the antibody (see [before you begin](#) for the identification and alignment of Fv).

Note: If you want to obtain the antibody sequence from mouse-derived antibody producing cells, use the following protocol for PCR cloning.

- a. Order the primer set below for PCR cloning. Premix and dissolve the primers to 10 μ M in DDW for V_H and V_L , respectively.

Primers for V_H cloning (5' - 3')	Primers for V_L cloning (5' - 3')
Rv primers	Rv primers
GATGTGAAGCTTCAGGAGTC	GATGTTTTGATGACCCAAACT
CAGGTGCAGCTGAAGSAGTC	GATATTGTGATRACSCAG
CAGGTTACTCTGAAAGAGTC	GACATTGTGMTGACCCARTCT
GAGGTCCAGCTGCARCAGTC	GATATTGTGCTAACTCAGTCT
CAGGTCCAAGCTGCAGCAGCCT	GAYATCCAGMTGACWCAGWCT
GAGGTGAAGCTGGTGGARTC	CAAATTGTTCTCACCCAGTCT
GATGTGAAGCTTGAAGTGTC	Fw primers
Fw primers	CCGTTTCAGTCCAGCTTG
TGCAGAGACAGTGACCAGAGT	CCGTTTTATTCCARCTTTG
TGAGGAGACKGTGASHGWGGT	GGATACAGTTGGTGCAGCATC

- b. Extract the total mRNA of antibody producing cells using TRIzol Reagent (Thermo Fisher Scientific, 15596026).
- c. Reverse-transcript 0.5 μ g of mRNA to synthesize cDNA (Total volume, 10 μ L) using ReverTra Ace qPCR RT Master Mix kit (Toyobo, FSQ-201).
- d. Amplify the antibody cDNA by PCR with ExTaq polymerase (TaKaRa Ex Taq, RR001A).

PCR reaction master mix

Reagent	Amount
Reverse transcription product	0.5 μ L
Primer master mix (10 μ M)	4 μ L
10 \times Ex Taq Buffer	5 μ L
dNTP Mixture (2.5 mM each)	4 μ L
TaKaRa Ex Taq (5 U/ μ L)	0.5 μ L
DDW	36 μ L
Total	50 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	10 s	30 cycles
Annealing	54°C	30 s	
Extension	72°C	40 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

- e. Subclone the antibody cDNA into pMD20 Vector (TaKaRa, 3270).
 - f. Obtain the antibody sequence using M13 Fw and Rv primer.
 - g. Align the sequencing result using IgBlast tool and ANARCI web server.
2. Insert the antibody Fv cDNAs into the expression vectors (Figures 1 B and 1C) using NotI and BamHI sites.

Note: Expression vectors for V_H , V_L and nanobodies are different (Figures 1B and 1C).

Purification of recombinant antibodies

⌚ **Timing:** 5 days

It is necessary to evaluate the specificity and the dissociation rate of the antibodies that are obtained from databases. In this part, we describe a method for the production and purification of recombinant antibodies.

3. Transfect HEK293T cells with the expression vector of recombinant antibodies.

Note: Antibody V_H -SARAH-EGFP and V_L -SARAH (Figure 1B) are co-transfected at a 1:1 molar ratio following the manufacture's instruction of Lipofectamine 3000 Transfection Reagent Kit (Thermo Fisher Scientific, L3000015). The protocol is also used for the transfection of Nanobody-EGFP (Figure 1C).

- a. Grow HEK293T cells to be 80% confluent in two 10 cm petri dishes for the production of one antibody. Culture media, 10 mL.
- b. Dilute 30 μ L Lipofectamine 3000 Reagent in 1.5 mL Opti-MEM (Thermo Fisher Scientific, 11058021) and vortex for 2–3 s (tube 1).
- c. Dilute 30 μ g (15 μ g V_H -SARAH-EGFP + 15 μ g V_L -SARAH or 30 μ g Nb-EGFP) DNA in 1.5 mL Opti-MEM separately, then add Opti-MEM containing 30 μ L P3000 Reagent and mix well (tube 2).

Note: We found that 30 μ L P3000 Reagent is enough.

- d. Add the solution in tube 2 to tube 1 (DNA-lipid complex, ~3 mL) and mix gently.
- e. Incubate for 10 min at 15°C–25°C.
- f. Add 1.5 mL DNA-lipid complex to each 10 cm dish containing 10 mL culture medium.

▮▮ **Pause Point:** 3 days

4. Harvest the supernatant of the transfected cells 3–4 days after the transfection. Use one 15 mL falcon tube to collect the supernatant from one dish.

Note: Centrifuge the culture supernatant at 200 × g for 3 min to spin down and remove the cell debris.

5. Collect the 6 × His tag fused recombinant antibodies with Ni-NTA agarose (QIAGEN, 30210).
 - a. Prepare the agarose beads by washing 3 times with 1 mL TBS (repeat following steps ii to iii for 3 times).
 - i. Thoroughly suspend Ni-NTA agarose beads in stock and immediately transfer an appropriate amount of Ni-NTA resin to a 1.5 mL tube. Fifty μL of agarose beads are used for the purification of one antibody.
 - ii. Add 1 mL TBS buffer.
 - iii. Centrifuge at 500 × g, 4°C for 5 min.

Note: The maximum resin pressure resistance is 2.8 psi.

- iv. Remove the supernatant. Be careful not to aspirate the beads.
 - b. Equilibrate the Ni-NTA beads by adding 5 bead volumes of TBS.
 - c. Adjust the pH of harvested culture supernatant to 8.0 by adding 1 M Tris-HCL pH 8.0 (1%, v/v).
 - d. Add 150 μL of equilibrated beads to the culture supernatant in each 15 mL tube.
 - e. Incubate with gentle rotation at 4°C for 2 h.

△ **CRITICAL:** Protect the fluorescent antibodies from the exposure to light.

▮▮ **Pause Point:** 2 h.

6. Wash the beads twice with TBS and once with TBS containing 10 mM imidazole.
7. Elute the recombinant antibodies with 200 μL of 200 mM imidazole diluted in TBS buffer (pH 7.5) for 3 min on ice. Then, centrifuge at 500 × g, 4°C for 5 min to spin down the beads. Collect 180 μL supernatant for dialysis.
8. Dialyze the purified antibodies with 1 L TBS using 6–8 kD Spectra/Por membrane (Spectrum Laboratories, Inc., 132645) 20 h at 4°C. The TBS buffer is exchanged once 4 h after the first dialysis.

▮▮ **Pause Point:** 1 day.

9. Measure the concentration of recombinant antibody by fluorescent intensity or Bradford protein assay (see [expected outcomes](#) for the typical yield).

Note: Dispense, snap-freeze in liquid nitrogen and store the antibody solution at –80°C.

Evaluation of recombinant antibodies

⌚ **Timing:** 2 days

The dissociation rate and specificity of purified antibody probes are evaluated using single-molecule approach as follows. *Compared with conventional SPR, the single-molecule approach requires only*

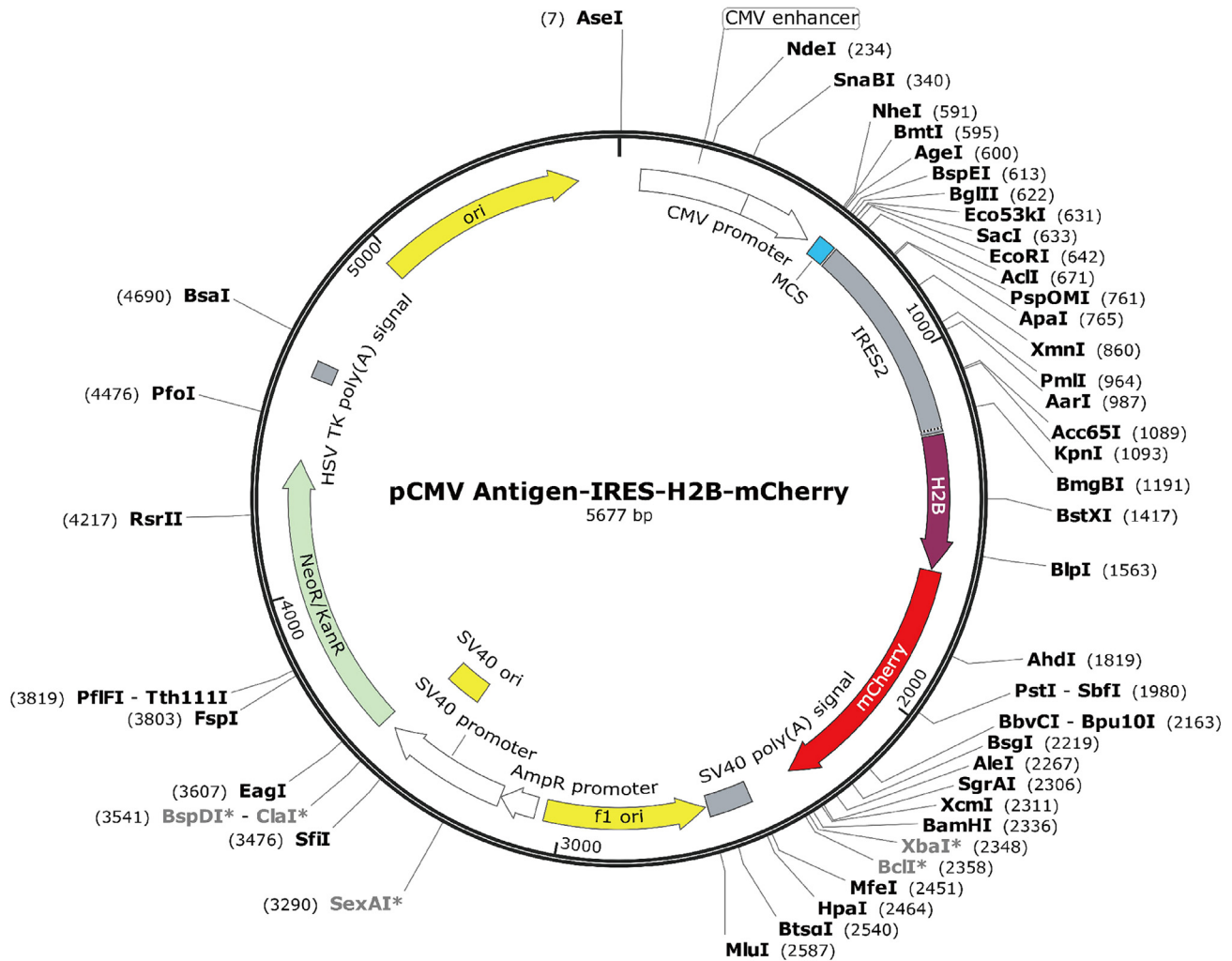


Figure 2. Map of vector for co-expressing antigen with H2b-mCherry in eukaryotic cells

Antigen sequence is cloned into the MCS of the plasmid. The vector was constructed based on pEGFP-C1 (Clontech Laboratories, 6084-1).

a small amount of probes (~ 1 pmol). Moreover, it enables to detect a minor fraction of probes with a wide range of detection limits (k_{off} , 10^{-4} – 10^2 s $^{-1}$). *Xenopus* XTC cells are utilized to express exogenous antigens because it is expected that endogenous proteins in XTC cells have limited cross-reactivity with antibody probes.

10. Expression of corresponding antigens of antibodies in XTC cells (see [before you begin](#) for the culture of XTC cells).
 - a. Subclone the antigen cDNA in the MCS of the expression vector as shown in [Figure 2](#). The vector was constructed based on pEGFP-C1 (Clontech Laboratories, 6084-1). H2b-mCherry is co-expressed to identify the transfected cell.
 - b. Transfect the XTC cells with antigens following manufacture's protocol of Neon transfection system (Thermo Fisher Scientific). The parameters for electroporation are described below.¹²

Amount of reagents

Plate format	Cell type	DNA	Neon tip	Plating medium	Cell number	Buffer
6-well plate	Adherent XTC cells	2–3 μ g	10 μ L tip	2 mL L-15(+)	2×10^5 cells	R buffer

Neon protocol		
Voltage	Width	Pulse
1005 V	35 ms	2 pulses

- c. Culture the transfected XTC cells in 22°C incubators for ~24 h.

Note: Subculture into a 25 cm² flask if the cells are used after >24 h.

▣ **Pause Point:** 1–2 days.

11. Coat the coverslip with 0.1 mg/mL PLL (Sigma-Aldrich, 25988630, diluted in DDW) at 4°C for 16 h.
12. Prepare the cell sample for single-molecule imaging.
 - a. Trypsinize the transfected XTC cells and resuspend in L-15 (+) buffer.
 - b. Centrifuge at 200 × g for 3 min.
 - c. Remove the supernatant and resuspend the cell using L-15 (-) buffer.
 - d. Seed the cells on PLL-coated coverslips in 6-well plates. Cell number, 2 × 10⁴ per Φ24 mm coverslip.

△ **CRITICAL:** PLL-coated coverslips should be rinsed at least 6 times by DDW before seeding the cells.

- e. Culture the cells in 22°C incubators for 1 h.
- f. Fix the cells using 3.7% PFA buffer in Cytoskeleton buffer (see [materials and equipment](#) for the composition) at 15°C–25°C for 20 min.

Note: Add 0.5% Tx-100 in case the fraction of the antigens in the cytoplasm needs to be removed.

Note: The Cytoskeleton buffer is used to preserve the structure and organization of the actin cytoskeleton especially when cells are fixed in the presence of detergents. It should be used with caution to study other cellular structures.

- g. Wash 3 times with PBS and permeabilize the cells using PBS containing 0.2% Tx-100 (v/v).
- h. Block the sample for 1 h at 15°C–25°C using 3% BSA (w/v) in PBS buffer.
- i. Wash the sample 3 times with PBS before imaging.
13. Acquire the single-molecule time-lapse images of bound antibodies in transfected cells.
 - a. Dilute the recombinant antibodies to 0.1–0.5 nM in imaging buffer (see [materials and equipment](#) for the composition).

△ **CRITICAL:** The concentration of antibody probes should be determined by the density of single-molecule speckles so that target proteins in the cells can be sparsely labeled (typically, 0.1–0.5 speckles/μm², see [Figure 3A](#)). The density of the speckles is proportional to the concentration of the fluorescent antibodies bound to the targets.³

- b. Find a transfected cell expressing H2B-mCherry ([Figure 3B](#)).
- c. Switch to the GFP channel and check if the density of speckles in the cell is suitable for single-molecule imaging ([Figure 3C](#)).

Note: If there is no binding probe or the density of binding probes are not substantially higher than in non-transfected XTC cells, the probe is not specific enough to recognize the antigen.¹

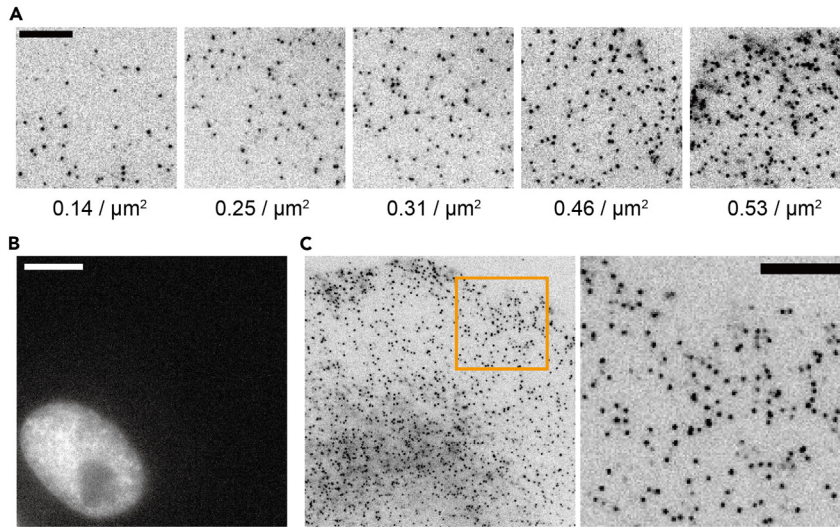


Figure 3. Demonstration for single-molecule imaging

(A) Demonstration for the speckle densities in single-molecule imaging (unit, speckles per μm^2). Many speckles are overlapped with each other and cannot be distinguished when the density is higher than $0.5/\mu\text{m}^2$. Scale bar, $5\ \mu\text{m}$.
 (B) A transfected XTC cell co-expressing antigen with H2b-mCherry. Scale bar, $10\ \mu\text{m}$.
 (C) Single-molecule imaging of the antigen expressed in (B) using $250\ \text{pM}$ Fv-clasp-EGFP. The left panel in (C) is the same imaging region with (B). The right panel in (C) is the enlarged image of boxed region in left panel. Scale bar, $5\ \mu\text{m}$.

Note: As mentioned above, the typical density is $0.1\text{--}0.5$ speckles/ μm^2 . For the analysis of dissociation rate, the speckle number in the imaging field should be more than 100.

d. Acquire time-lapse image series of bound single-molecules in the cell.

Note: Total Internal Reflection Fluorescence (TIRF) illumination can be used to reduce the background signals.

△ CRITICAL: Acquisition speed of time-lapse imaging should match with the antibody kinetics. Generally, time intervals should be no more than $1/2$ of the antibody half-lives ($T_{1/2} = \ln 2 / k_{\text{off}}$). Typically, we acquire $60\text{--}100$ frames with $5\text{--}30\ \text{s}$ time interval to measure the dissociation rate of wild-type antibodies with k_{off} of $10^{-2}\text{--}10^{-4}\ \text{s}^{-1}$.

Optional: In the time-lapse imaging with time interval longer than $20\ \text{s}$, a BF image should be recorded following each exposure for the compensation of stage drift.

14. Track the single-molecule using software such as tanitracer^{4,10} (<https://github.com/takushim/tanitracer>).

Optional: Use tanipoc.py in Tanitracer to correct the stage drift during the acquisition using BF images described in Step 13d.

15. Calculate the dissociation rate (k_{off}) using a one-phase-decay model.
 a. Output the regression of speckles detected in the first frame using tanitime.py in Tanitracer. The result is summarized in a file named as File-name_regression.txt, see Table 1 for the example of a raw data set.

Note: Type the following command to output the list of regression:

```
> tanitime.py -x time-interval File-name.txt
```

- b. Copy the “Lifetime” and “Regression” column to GraphPad Prism 9 and analyze the data using “Nonlinear Regression” > “one-phase-decay” model (www.graphpad.com/guides/prism/latest/curve-fitting/reg_exponential_decay_1phase.htm). The best-fit K (hereafter called K_R) and Half-life values are shown in the results. The black “WT” curve in Figure 4 is the fitting to the data in Table 1.

△ **CRITICAL:** Set the Plateau of curve constant equal to 0 in the “Constrain” tab.

- c. Measure the photobleaching rate (K_p) of fixed EGFP in XTC cells.
 - i. Express EGFP-actin in XTC cells and fix the cells with 3.7% PFA buffer containing 0.2% Tx-100. Then, wash the cell 3 times with PBS buffer.
 - ii. Acquire the time-lapse images for EGFP regression.

Note: The time-interval, exposure time and laser power for acquiring EGFP regression should be kept the same as those used for fluorescent antibodies.

- iii. Calculate the intensity of EGFP in each frame.

Note: Background should be subtracted from the fluorescent intensity.

- iv. Fit the regression curve of EGFP fluorescent intensity using a one-phase-decay model. The best fitted K value represents the photobleaching rate (K_p).
- d. Calculate the dissociation rate (k_{off}) by subtracting K_p from K_R .

Site-directed mutagenesis

⌚ **Timing:** 4 days – 2 weeks

To accelerate the dissociation rate of the recombinant antibodies, several conserved amino acids located at the base of antibody CDR loops are identified as candidate sites for Ala (A) or Gly (G) substitution.¹ This section outlines the principles for selecting the combination of substitution sites and includes the method for site-directed mutagenesis.

16. Align the antibody sequence and identify the candidate sites for mutagenesis (see [before you begin](#) for the alignment of antibody sequence).

Note: Candidate sites are: H27, H28, H32, H59 and H102 for antibody V_H ; L32, L49 and L96 for antibody V_L ; Nb27, Nb28, Nb32, Nb37, Nb59 and Nb102 for nanobodies. These candidate sites are highly conserved in antibodies and nanobodies.¹

17. Generate 3–5 antibody mutants with a single amino acid substitution at candidate sites.

Note: As a starting point, we recommend trying the following substitutions: H/Nb32A, H/Nb59A, Nb37A, L32A, L49A and L96A. Mutations at these sites could effectively accelerate the dissociation rate but occasionally lead to the loss of antigen recognition.¹ A concise protocol for DpnI-mediated site-directed mutagenesis is described as follows.

- a. Design the forward and reverse primer for the reaction.

Table 1. Example of raw data of speckle regression from $t = 0$

Life count (frame)	Lifetime (s)	Regression (speckle)
0	0	699
1	20	521
2	40	443
3	60	381
4	80	334
5	100	301
6	120	266
7	140	228
8	160	203
9	180	177
10	200	150
11	220	136
12	240	127
13	260	109
14	280	99
15	300	91
16	320	85
17	340	79
18	360	72
19	380	65
20	400	62
21	420	57
22	440	56
23	460	55
24	480	52
25	500	49
26	520	46
27	540	44
28	560	43
29	580	41
30	600	39
31	620	37
32	640	36
33	660	35
34	680	34
35	700	33
36	720	32
37	740	29
38	760	29
39	780	29
40	800	27
41	820	26
42	840	26
43	860	24
44	880	24
45	900	22
46	920	21
47	940	20
48	960	20
49	980	20
50	1000	20
51	1020	19
52	1040	19
53	1060	18

(Continued on next page)

Table 1. Continued

Life count (frame)	Lifetime (s)	Regression (speckle)
54	1080	17
55	1100	16
56	1120	14
57	1140	13
58	1160	13
59	1180	13

Note: The primer sequences should be complementary to each other. The primer should be 14–18 base pairs (bp) long on both sides of the mutation site, and terminate with a G or C. The melting temperature (T_m) should be between 55°C–65°C.

b. Introduce the point mutation by PCR reaction:

PCR reaction master mix (for single point mutation)

Reagent	Amount
Plasmid template (10 ng/μL)	1 μL
Forward primer (10 μM)	2 μL
Reverse primer (10 μM)	2 μL
DDW	5 μL
KOD One Master Mix	10 μL
Total	20 μL

PCR cycling conditions (for single point mutation)

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	10 s	20–25 cycles
Annealing	$T_m - 5^\circ\text{C}$	15 s	
Extension	68°C	2 min	
Final extension	68°C	5 min	1
Hold	4°C	forever	

c. Digest methylated plasmid template with DpnI (New England Biolabs, R0176S) for 90 min.

d. Transform the mutated plasmid into competent *E. coli* DH5α.

18. Purify the antibody mutants and measure the dissociation rates using the procedures described in steps 3–15.

Note: As an example, single point mutations HY32A and LY49A in 12CA5 antibody (anti-hemagglutinin [HA] tag) fragments increase the dissociation rate by 36 folds and 2.4 folds, respectively (Figure 4).¹

Note: Some antibodies may lose the antigen recognition after the mutagenesis. The substitution sites in those antibody mutants should be avoided in the following steps which combine multiple point mutations.

19. Combine two point mutations in V_H and V_L to further increase the dissociation rate (e.g., H32A + L49A in Figure 4).

20. Introducing an additional H/Nb27G, H/Nb28A or H/Nb102A substitution could further increase the dissociation rate with a high probability (e.g., H28A + H32A + L49A in Figure 4).

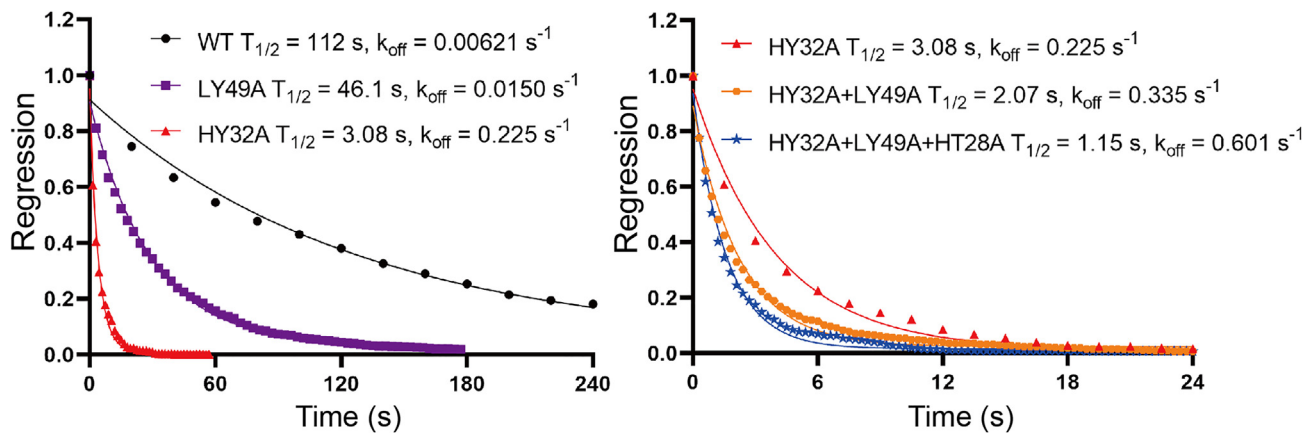


Figure 4. Demonstration for amino acid substitutions at candidate sites in 12CA5 antibody, which increases the dissociation rate by orders of magnitude

See [Table 1](#) for the source data of WT curve. The regression of speckles was fitted using one phase decay model in Prism 9.

Note: The desired half-life for antibody probes in IRIS super-resolution imaging is typically between 1–10 s. In general, it is recommended to only introduce 1–5 point mutations to the antibody as introducing too many mutations sometimes results in increased noise levels.

EXPECTED OUTCOMES

The expected yield of antibody probes purified from two dishes ($\Phi 10$ cm) of transiently transfected HEK293T cells is 0.34–4.02 μ g (5–60 pmol) for Fv-clasp-EGFP and 0.41–4.92 μ g (10–120 pmol) for Nb-EGFP, which are sufficient for 20–60 IRIS imaging experiments. SDS-PAGE analysis should show the Fv-clasp-EGFP dimer band in non-reducing conditions, while the V_H-EGFP-SARAH and V_L-SARAH bands are detected under reducing conditions (as shown in Zhang et al., 2022, [Figure 1B](#)).

The dissociation rate of antibody is expected to increase by 2–100 folds ([Figure 4C](#)) without compromising the binding specificity. Multiple point mutations can be introduced collectively to improve the efficiency. The process of generating a new fast-dissociating probe from an existing antibody sequence typically takes 2–4 weeks. These antibody fragments also have potential applications in multiplexed immunostaining, cell typing/sorting, western blotting, etc.

QUANTIFICATION AND STATISTICAL ANALYSIS

See [Table 1](#) for examples of speckle regression data.

LIMITATIONS

While we established a generalized strategy to accelerate the dissociation rate of existing antibodies by site-directed mutagenesis, the dissociation rate of mutants increased by only ~ 100 -fold in our previous study.¹ For high-affinity antibodies with k_{off} smaller than 10^{-4} s⁻¹, such as NbALFA,¹³ it might be necessary to modify additional antigen-binding sites in the middle of CDR loops to further increase the dissociation rate,¹ which would require the information of cocrystal structures.

Although the database for antibodies is expanding rapidly, the sequence information of existing antibodies is not always disclosed, making it difficult to develop fast-dissociating probes for the targets without available antibody sequences.

TROUBLESHOOTING

Problem 1

No available antibody sequence in a database (related to [before you begin](#), Step 1).

Potential solution

- Search if there is available antibody producing cells, the antibody sequence can be cloned from the cells (Major step 1).
- Develop new recombinant antibodies or hybridoma cells against the target.
- The repository of sequenced antibody is expanding rapidly. The sequences might be disclosed in the near future.

Problem 2

Selection appropriate antibodies among multiple sequenced antibody entries in the database (related to Before you begin, Step 1).

Potential solution

There may exist multiple entries of monoclonal antibodies in the databases such as Addgene and NeuroMab. However, not all of these entries are validated for use. To ensure that you are using a reliable antibody, it's important to choose one that has been validated for antigen recognition and specificity in immunofluorescence (IF) application. Before beginning, it is also recommended to check the productivity and sequence integrity of the antibody using the IgBlast tool. This will help to ensure that the sequence does not contain any stop codons or missing information in the variable fragment.

Problem 3

The yield of purified antibody probes is very low or no product (related to Major Step 9).

Potential solution

- Double check the integrity and productivity of the antibody sequence.
- Make sure the 112th amino acid in V_H (usually Ser) has been changed to Cys (see [before you begin](#), Step 3).
- Check the fluorescence of EGFP in transfected HEK293T cells before harvesting the supernatant. If the cells are not fluorescent, ensure that vectors are correct and properly transfected into the cells.
- Make sure that all Ni-NTA beads are thoroughly removed from the purified product.

Problem 4

The cell samples do not spread well on the coverslip after incubation (related to Major step 12e).

Potential solution

- If the cell sample does not spread out and looks very small, make sure the PLL-coated coverslips are thoroughly rinsed (Major Step 12d).
- If the cells spread in an anisotropic manner with many elongated edges, make sure serum-free L-15 medium (L-15[-]) are used to incubate the cells. In serum-free medium, the cell edges appear circular and flat.

Problem 5

The wild-type recombinant antibodies obtained from online databases do not recognize the targets in cells (related to Major Step 13c).

Potential solution

Not all antibodies in the databases are validated. Select a monoclonal antibody clone that has been used for IF in published papers or on antibody sellers' webpage. On the other hand, the expression efficiency of antigen might be low. In this case, try expressing the epitope fragment of the antigen in

the cell. The epitope sequence of corresponding antibodies could be found in the ABCD database. In addition, it is possible to use other cell lines which express the antigen endogenously.

Problem 6

The dissociation is still slow even after multiple rounds of site-directed mutagenesis (related to Major Step 19).

Potential solution

As written in the Limitations section, our method may accelerate the dissociation rate by 2–3 orders of magnitude. For antibody probes whose k_{off} is below 10^{-5} s^{-1} (e.g., NbALFA), it may be necessary to modify the antigen binding sites to further increase the dissociation rate.¹ If the co-crystal structure is not available in the PDB, try Ala substitution in center of HCDR1, HCDR2, LCDR1 and LCDR3 loops, where the residues are mostly in contact with antigens.¹⁴ The co-crystallization information may be available at PDB, which can help to identify the interaction sites.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Naoki Watanabe (watanabe.naoki.4v@kyoto-u.ac.jp).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

Q.Z., A.M., and N.W. developed the mutagenesis strategy and wrote the manuscript. Q.Z. and A.M. conducted the experiments and analyzed the data.

DECLARATION OF INTERESTS

Q.Z., A.M., and N.W. are inventors of a pending patent related to the antibody engineering technology submitted by Kyoto University to the Japan Patent Office. Q.Z. is an employee of Takeda Pharmaceutical Company Limited.

REFERENCES

- Zhang, Q., Miyamoto, A., Watanabe, S., Arimori, T., Sakai, M., Tomisaki, M., Kiuchi, T., Takagi, J., and Watanabe, N. (2022). Engineered fast-dissociating antibody fragments for multiplexed super-resolution microscopy. *Cell Rep. Methods* 2, 100301. <https://doi.org/10.1016/j.crmeth.2022.100301>.
- Arimori, T., Kitago, Y., Umitsu, M., Fujii, Y., Asaki, R., Tamura-Kawakami, K., and Takagi, J. (2017). Fv-clasp: An Artificially Designed Small Antibody Fragment with Improved Production Compatibility, Stability, and Crystallizability. *Structure* 25, 1611–1622.e4. <https://doi.org/10.1016/j.str.2017.08.011>.
- Kiuchi, T., Higuchi, M., Takamura, A., Maruoka, M., and Watanabe, N. (2015). Multitarget super-resolution microscopy with high-density labeling by exchangeable probes. *Nat. Methods* 12, 743–746. <https://doi.org/10.1038/nmeth.3466>.
- Miyoshi, T., Zhang, Q., Miyake, T., Watanabe, S., Ohnishi, H., Chen, J., Vishwasrao, H.D., Chakraborty, O., Belyantseva, I.A., Perrin, B.J., et al. (2021). Semi-automated single-molecule microscopy screening of fast-dissociating specific antibodies directly from hybridoma

- cultures. *Cell Rep.* 34, 108708. <https://doi.org/10.1016/j.celrep.2021.108708>.
5. Brochet, X., Lefranc, M.P., and Giudicelli, V. (2008). IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* 36, W503–W508. <https://doi.org/10.1093/nar/gkn316>.
 6. Al-Lazikani, B., Lesk, A.M., and Chothia, C. (1997). Standard conformations for the canonical structures of immunoglobulins. *J. Mol. Biol.* 273, 927–948. <https://doi.org/10.1006/jmbi.1997.1354>.
 7. Sircar, A., Sanni, K.A., Shi, J., and Gray, J.J. (2011). Analysis and modeling of the variable region of camelid single-domain antibodies. *J. Immunol.* 186, 6357–6367. <https://doi.org/10.4049/jimmunol.1100116>.
 8. Watanabe, N., and Mitchison, T.J. (2002). Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science* 295, 1083–1086. <https://doi.org/10.1126/science.1067470>.
 9. Dunbar, J., and Deane, C.M. (2016). ANARCI: antigen receptor numbering and receptor classification. *Bioinformatics* 32, 298–300. <https://doi.org/10.1093/bioinformatics/btv552>.
 10. Miyoshi, T., Friedman, T.B., and Watanabe, N. (2021). Fast-dissociating but highly specific antibodies are novel tools in biology, especially useful for multiplex super-resolution microscopy. *STAR Protoc.* 2, 100967. <https://doi.org/10.1016/j.xpro.2021.100967>.
 11. Suzuki, J., Kanemaru, K., Ishii, K., Ohkura, M., Okubo, Y., and Iino, M. (2014). Imaging intraorganellar Ca²⁺ at subcellular resolution using CEPIA. *Nat. Commun.* 5, 4153. <https://doi.org/10.1038/ncomms5153>.
 12. Yamashiro, S., Mizuno, H., Smith, M.B., Ryan, G.L., Kiuchi, T., Vavylonis, D., and Watanabe, N. (2014). New single-molecule speckle microscopy reveals modification of the retrograde actin flow by focal adhesions at nanometer scales. *Mol. Biol. Cell* 25, 1010–1024. <https://doi.org/10.1091/mbc.E13-03-0162>.
 13. Götzke, H., Kilisch, M., Martínez-Carranza, M., Sograte-Idrissi, S., Rajavel, A., Schlichthaerle, T., Engels, N., Jungmann, R., Stenmark, P., Opazo, F., and Frey, S. (2019). The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. *Nat. Commun.* 10, 4403. <https://doi.org/10.1038/s41467-019-12301-7>.
 14. Sela-Culang, I., Kunik, V., and Ofran, Y. (2013). The structural basis of antibody-antigen recognition. *Front. Immunol.* 4, 302. <https://doi.org/10.3389/fimmu.2013.00302>.