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Application of ALFA-tag and tyramide-based fluorescence signal amplification to expand the CRISPR-based DNA imaging toolkit

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We use this protocol and it's working

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Abstract

Understanding the spatial organization of genomes within chromatin is crucial for deciphering gene regulation. In this protocol we describe application of an ALFA-tagged dCas9 protein in combination with a *A. thaliana* centromere-specific gRNA and a fluorescence-labeled NbALFA nanobody to label centromere-specific sequences in fixed *Arabidopsis thaliana* nuclei. The dCas9 protein possessing multiple copies of the ALFA-tag, in combination with a minibody and fluorescence-labelled anti-rabbit secondary antibody, resulted in enhanced target-specific signals. Furthermore, we describe the combination of the highly sensitive Tyramide Signal Amplification (TSA) method with CRISPR-FISH.

Materials

1. Young leaf tissue (10 mg) of Arabidopsis thaliana
2. *Escherichia coli* Rosetta 2 (DE3): (71400 Sigma-Aldrich)
3. Constructs: pET22b+_dCas9-6xHis, pET22b+_dCas9-ALFA-tag-6xHis;
4. LB media: (tryptone 10 g/l, yeast extract 5 g/l and NaCl 10 g/l, pH 7.0)
5. 2x TY media: (tryptone 16 g/l, yeast extract 10 g/l and NaCl 5 g/l, pH 7.5)
6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) – 0.5 M
7. Ampicillin 100 mg/L
8. Chloramphenicol 30 mg/L
9. Lysis buffer: (50 mM NaH2PO4, 500 mM NaCl, 10% glycerol, 10 mM imidazole, pH 8.0)
10. Lysozyme (Roche, 45-10837059001)
11. cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche, 4693132001)
12. Benzonase (Merck Millipore, 70746)
13. PureCube 100 Ni-NTA Agarose (Cube Biotech, 31103).
14. Wash buffer (50 mM NaH2PO4, 500 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.0)
15. Elution buffer (50 mM NaH2PO4, 500 mM NaCl, 10% glycerol, 250 mM imidazole, pH 8.0)
16. SDS-PAGE gel
17. ROTI® Quant, Bradford assay reagents (Roth, Art. No. K015.1)
18. tracrRNA-ATTO550 (IDT, USA)
19. tracrRNA-biotin (IDT, USA)
20. crRNA (IDT, USA)
21. Nuclease-free duplex buffer (30 mM Hepes, pH 7.5; 100 mM CH3CO2K) (IDT, USA)
22. dCas9-ALFA-tag
23. 10x Cas9 buffer: (200 mM Hepes, pH 7.5, 1M 50 mM MgCl2, 50% glycerol, 10% BSA, and 1% Tween 20)
24. 10 mM dithiothreitol (DTT)
25. Double-distilled water (ddH2O) (aliquot and store at-20 °C
26. 37% formaldehyde (Roth, Art. No. CP10.1)
27. Ice-cold Tris buffer: (10 mM Tris-HCl (pH 7.5), 10 mM Na2-EDTA, 100 mM NaCl, 0.1% Triton X-100, and adjusted pH 7.5 with NaOH)
28. LB01 buffer: (15 mM Tris-HCl (pH 7.5), 2 mM Na2-EDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol and 0.1% Triton X-100)
29. 1x PBS: (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4)
30. Ethanol (70%, 85%, and 96%)
31. 4',6-diamidino-phenylindole (DAPI) in anti-fade (VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200-10))
32. 4% bovine serum albumin (BSA) buffer (dissolve fatty acid free BSA with 1× PBS buffer, 0.1% Triton X-100. Store small aliquots at-20 °C until use).
33. Triton-X100
34. 1 M Tris–HCl (dissolve 12.1 g TRIS in 80 mL of dH2O and adjust the pH to 9.0 with HCl. Add dH2O until a total volume of 100 mL. Autoclave and store at 4 °C)
35. NbALFA conjugated with ATTO488 (FluoTag®-X2 anti-ALFA, Cat No: N1502, NanoTag Biotechnologies GmbH)
36. Minibody: Recombinant sdAb anti-ALFA rabbit Fc-fusion, Cat No: N1583, NanoTag Biotechnologies GmbH
37. Goat anti-rabbit Alexa 488 antibody

38. Biotin XX Tyramide SuperBoost™ Kit, Streptavidin (Cat no: B40931, ThermoFisher)
39. Streptavidin-conjugated FITC (S3762, Sigma-Aldrich)

Equipment

1. Shaker
2. Centrifuge
3. OD checker
4. Vibra-Cell Model VC60, Sonics & Materials
5. Disposable polypropylene columns (Qiagen, 34924)
6. Glass rods
7. 50 mL Falcon tube
8. 1.5 mL Eppendorf tubes
9. Thermocycler
10. Vortex
11. speedy vac
12. Pre-cooled plastic Petri dish
13. Razor blade
14. Mesh with 30 or 50 µm pore size (e.g., Partec CellTrics Cat. No. BP486257 or BM786910).
15. Cytospin
16. Coplin jar
17. Parafilm
18. Moisture chamber
19. Microscopic slides and coverslips
20. Coplin glass jars with lids (e.g., #900470 or 900630, Wheaton)
21. Pasteur pipets
22. Fluorescence microscope (e.g., Olympus BX61 microscope equipped with an ORCA-ERCCD camera (e.g., Hamamatsu))

Safety warnings

Notes

1. Efficient cloning of the construct should be confirmed using sequencing approaches, in addition to restriction digestion
2. Ensure all steps of the protein purification process are performed either on ice or at 4°C, up until storing the eluted proteins at -20°C.
3. Collect small aliquots at every step of the protein purification process, from initial clarification to elution fractions, to monitor for any potential protein losses during purification.
4. The crRNA and tracrRNA are commercially produced and supplied by Integrated DNA Technologies (**https://eu.idtdna.com/**). It's important to note that results may vary when using gRNAs from other companies.
5. Storing sgRNA at -20 °C is crucial for maintaining stability. A 10 µM sgRNA solution is less stable than a 100 µM solution. When preparing more than 20 µL of gRNA at once, it is recommended to aliquot it into smaller portions (5–10 µL) before storing at -20 °C.
6. After this step, slides can be safely stored in 1x PBS at 4 °C for up to 24 hours.
7. For ethanol-acetic acid fixed chromosomes, incubate the slides in a Coplin jar with 0.2% Triton X-100 in 40 mM Tris–HCl (pH 9) at 37°C for 30 minutes to enhance chromatin permeability. For species with large chromosomes, extending the washing time to 60 minutes may improve labeling results. After incubation, wash the slides twice in 1x PBS.
8. These procedures can also be carried out by overnight incubation at 4 °C.
9. To mitigate a strong background, extend the washing time in 1x PBS. This helps reduce non-specific signals while preserving specific CRISPR-FISH signals.
10. Empirically optimize the incubation time of the tyramide working solution based on the target sequences. Ensure that the incubation time does not exceed 10 minutes for optimal results.

Construction of ALFA-Tagged dCas9 Vectors

1 (a) Cloning of ALFA-Tag Sequences:

- Clone the ALFA-tag sequences either at the N- or C-terminus of the dCas9 protein present in the pET22b-dCas9-6xHis vector using restriction-based cloning (see Note 1).

(b) Transformation and Cultivation:

1. Transform plasmids into *Escherichia coli* Rosetta 2(DE3) using heat shock transformation and plate on LB agar supplemented with 100 mg/L ampicillin and 30 mg/L chloramphenicol.
2. Select single colonies and inoculate them into liquid LB medium. Incubate overnight at 37 °C with shaking at 200 rpm.
3. Dilute the overnight cultures 1:50 into fresh 2x TY medium and grow at 37 °C until reaching an OD600 of 0.5.
4. Shift the cultures to 18 °C and incubate for 1 hour with shaking at 180 rpm.
5. Induce protein expression by adding IPTG to a final concentration of 0.5 mM. Grow the cells at 18 °C with shaking at 180 rpm for 16 hours.
6. Harvest the cells by centrifugation at 6500 rpm for 20 minutes at 4 °C. Store the resulting pellet at -80 °C or use immediately for protein purification.

(c) Purification of Recombinant Protein:

1. Thaw the cell pellet on ice for 20 minutes and resuspend in lysis buffer containing 1 mg/mL lysozyme and an EDTA-free Protease Inhibitor Cocktail tablet.
2. Transfer the suspension to a new 50 mL Falcon tube and lyse for 30 minutes on ice, stirring every 5 minutes.
3. Flash-freeze the cells in liquid nitrogen and immediately thaw in water at room temperature.
4. Sonicate on ice for four cycles of 30 seconds each at 50% intensity.
5. Incubate the lysate on ice after adding Benzonase to a final concentration of 1 µL/mL for 30 minutes, stirring every 5 minutes.
6. Centrifuge the lysate at 7000 rpm for 20 minutes at 4 °C. Transfer the supernatant to a new 50 mL Falcon tube containing 1 mL of PureCube 100 Ni-NTA Agarose. Rotate at 4 °C for 90 minutes.
7. Purify His-tagged proteins by gravity flow chromatography using disposable polypropylene columns. Discard the flow-through (see Note 2).
8. Wash the column twice with wash buffer and elute the protein with elution buffer in 10 x 1 mL fractions collected in 1.5 mL Eppendorf tubes (see Note 3).

(d) Protein Analysis and Storage:

1. Run the purified fractions on an SDS-PAGE gel and pool the fractions with higher protein concentration (see Note 3).
2. Determine the concentration of the purified proteins using the Bradford assay and store the proteins at -20 °C for further use.

Guide RNA and RNP complex preparation

2 CRISPR-FISH employs the two-part guide RNA (crRNA and trans-activating crRNA (tracrRNA)) system to produce functional guide RNA. This system is commercially prepared by Integrated DNA Technologies (IDT, USA) (see Note 4). The preparation of the ribonucleoprotein involves two steps: (a) sgRNA formation and (b) RNP complex assembly (Fig. 1).

(a) sgRNA Formation:

1. Add 20 µL of nuclease-free duplex buffer to lyophilized crRNA (2 nmol) on ice to produce 100 µM crRNA.
2. Add 50 µL of nuclease-free duplex buffer to lyophilized tracrRNA-ATTO550/biotin (5 nmol) on ice to produce 100 µM tracrRNA. Store the dissolved crRNA and tracrRNA separately at -20 °C.
3. To assemble 10 µM sgRNA, add 8 µL of nuclease-free duplex buffer, 1 µL of 100 µM crRNA, and 1 µL of 100 µM tracrRNA-ATTO550/ATTO488 in a 0.2 mL PCR tube on ice.
4. Incubate the sgRNA mix for 5 minutes at 95 °C using a Thermocycler. Turn off the machine and keep the PCR tube inside for about 10 minutes to cool down.
5. Store the labeled sgRNA at -20 °C (see Note 5).

(b) RNP Complex Assembly:

1. Add 80 µL of double-distilled water, 10 µL of 10x Cas9 buffer, and 10 µL of 10 mM DTT in a 1.5 mL Eppendorf tube. Mix by vortexing.
2. Add 1 µL of 10 µM sgRNA and 1 µL of 6.25 µM dCas9-ALFA-tag protein. Mix by vortexing.
3. Incubate the RNP complex at 26 °C for 10 minutes. Keep at 4 °C until use. Note that 100 µL of RNP complex is sufficient for 3-4 slides.

Preparation of leaf nuclei

3
1. Fix 10 mg of young *Arabidopsis thaliana* leaf tissue in 10 mL ice-cold 4% formaldehyde in Tris buffer for 5 minutes under vacuum using a speedy vac or similar device.
2. Continue fixation for an additional 25 minutes in ice-cold fixative without vacuum.
3. Rinse the tissue twice for 5 minutes each in ice-cold Tris buffer.
4. Chop the tissue in 400–500 µL ice-cold LB01 buffer in a pre-cooled plastic Petri dish using a new razor blade.
5. Filter the suspension through a mesh with a 30 or 50 µm pore size.
6. Dilute the extracted nuclei with LB01 buffer in a 1:10 ratio.
7. Use a cytospin to spin 100 µL of the diluted nuclei extraction per slide at 700 rpm for 5 minutes.
8. Transfer the slides into a Coplin jar and keep them in 1x PBS on ice until use (see Note 6).

CRISPR-FISH with ALFA tag system and NbALFA

4

1. Transfer the slides into a Coplin jar containing 100 mL of 1x PBS at room temperature and wash twice for 5 minutes each (see Note 7).
2. Remove the excess buffer by tilting the slide. Apply 100 µL of 1x Cas9 buffer with 1 mM DTT per slide and incubate for 5 minutes at room temperature. Remove the excess buffer by tilting the slide.
3. Add 20–30 µL of the RNP complex per slide and cover with a piece of parafilm to avoid drying. Transfer the slides into a moisture chamber and incubate at 37 °C for 1 hour (see Note 8).
4. Remove the parafilm by rinsing the slides in a Coplin jar with 1x PBS for 5 minutes on ice.
5. For post-fixation, place the slides in a Coplin jar containing 4% formaldehyde in 1x PBS for 5 minutes on ice.
6. Remove the formaldehyde by rinsing the slides in a Coplin jar with 1x PBS for 5 minutes on ice.
7. Block the slides with 100 µL of 4% BSA in 1x PBS for 1 hour at room temperature (RT) in a humid chamber.
8. Wash the slides with 1x PBS for 5 minutes at RT in the dark.
9. Add 50 µL of NbALFA conjugated with ATTO488 (diluted 1:500 in 2% BSA in 1x PBS) or 50 µL of eGFP-NbALFA (1 µM in 2% BSA in 1x PBS) per slide. Cover the slides carefully with parafilm tape.
10. Incubate the slides at 37 °C for 1 hour in a humid chamber (see Note 8).
11. Wash the slides twice with 1x PBS for 5 minutes each at RT in the dark.
12. Dehydrate the slides in a Coplin jar for 2 minutes in a series of 70%, 85%, and 96% ethanol, respectively. Dry the slides in a vertical position in a slide holder in the dark.
13. Counterstain the slides with approximately 8 µL of DAPI in anti-fade and cover with a coverslip, avoiding intense light.

Detection with a Minibody

1. After blocking and washing the slides with 1x PBS in the dark, add 50 µL of minibody (diluted 1:500 in 2% BSA in 1x PBS) per slide. Cover the slides carefully with parafilm tape.
2. Incubate the slides at 37 °C for 1 hour in a humid chamber (see Note 8).
3. Wash the slides twice with 1x PBS for 5 minutes each at RT in the dark.
4. Add 50 µL of anti-rabbit Alexa 488 antibody (diluted 1:100 in 2% BSA in 1x PBS) per slide. Cover the slides carefully with parafilm tape.
5. Incubate the slides at 37 °C for 1 hour in a humid chamber (see Note 8).
6. Wash the slides twice with 1x PBS for 5 minutes each at RT in the dark (see Note 9).
7. Follow subsequent steps of dehydration and counterstaining as described in the standard CRISPR-FISH protocol.

Tyramide Signal Amplification

5

1. Incubate the slides containing nuclei and chromosomes in 0.2% Triton-X100 in 40 mM Tris-HCl (pH 9) at 37 °C for 30 minutes to enhance permeability.
2. Wash the slides twice in 1x PBS for 5 minutes each at room temperature (RT).
3. Add 100 µL of 1x Cas9 buffer with 1 mM DTT in ddH2O to each slide and incubate for 5 minutes at RT.
4. Add 25 µL of the RNP complex prepared using 3' biotin-labeled tracrRNAs per slide and carefully cover with parafilm tape. Incubate the slides at 37 °C for 1 hour or overnight at 4 °C in a humid chamber.
5. Wash the slides in 1x PBS for 5 minutes at RT. Post-fix the slides with 4% formaldehyde in 1x PBS for 5 minutes at RT.
6. Wash the slides again with 1x PBS at RT.
7. Block the slides with 100 µL of 4% BSA in 1x PBS for 1 hour at RT in a humid chamber.
8. Wash the slides in 1x PBS for 5 minutes at RT in the dark.
9. Conduct tyramide signal amplification using the Biotin XX Tyramide SuperBoost™ Kit, Streptavidin (Cat No: B40931, ThermoFisher), following the manufacturer's instructions.
10. Block the slides with 2-3 drops of blocking buffer for 30 minutes at RT in a humid chamber, followed by washing in 1x PBS for 5 minutes.
11. Apply 2-3 drops of HRP-conjugated streptavidin per slide and carefully cover with parafilm. Incubate the slides for 1 hour at
    RT in a humid chamber.
12. Prepare the tyramide working solution by mixing 5 µL of 100x tyramide stock solution, 5 µL of 100x H2O2 solution, and 500 µL of 1x reaction buffer. Mix by vortexing and store on ice until used. This amount is sufficient for 5 slides.
13. Post-incubation with HRP-conjugated streptavidin, wash the slides thrice in 1x PBS for 5 minutes each at RT.
14. Add 100 µL of tyramide working solution per slide and incubate in a humid chamber for 10 minutes at RT in the dark (see Note 10).
15. Terminate the reaction by adding 100 µL of reaction stop reagent for 1 minute at RT.
16. Wash the slides thrice in 1x PBS for 5 minutes each at RT.
17. Incubate the slides with 50 µL of streptavidin-conjugated FITC (diluted 1:50 in 1% BSA/1x PBS) per slide at 37 °C for 1 hour in a humid chamber.
18. Wash the slides thrice in 1x PBS for 5 minutes each in the dark.
19. Dehydrate and counterstain the slides as described above.