Chapter 20

Live-Cell CRISPR Imaging in Plant Cells with a Telomere-Specific Guide RNA

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Abstract

Chromatin organization is highly dynamic in living cells. Therefore, it might have a regulatory role over biological mechanisms like transcription, replication, and DNA repair. To elucidate how these mechanisms are regulated, it is required to establish imaging methods to visualize the chromatin dynamic in living cells. Here, we provide a protocol for a live plant cell imaging technique based on application of two orthologs of the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) from *Streptococcus pyogenes* and *Staphylococcus aureus*. This technique uses the inactive variants of Cas9 combined with different fluorescent proteins (GFP and mRuby) and telomere-specific guide RNA to target telomeric repeats in *Nicotiana benthamiana*. Our immuno-FISH data revealed that signals arising from the CRISPR/dCas9 method are specifically belonging to telomeric regions.

Key words Chromatin organization, Live-cell imaging, CRISPR/dCas9, Telomere, *Nicotiana benthamiana*, Guide RNA

1 Introduction

Structural and spatial organization of chromatin affects gene regulation and pivotal processes like recombination and DNA repair mechanisms. Eventually, studying chromatin structural changes in different tissues over time has always been of interest to decipher these regulatory mechanisms [1]. Our knowledge about the 3D organization of chromatin is mainly based on fixed specimens. Although imaging methods including fluorescence in situ hybridization (FISH) have been well applied to study subnuclear dynamics, harsh treatment of cells during FISH like heat-based denaturation could provide data only from perturbed chromatin structure which is not indicative of its structure in living cells [2]. To overcome this problem, live chromatin imaging techniques were developed to enable studying of biological compartments in their native context [3].

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Live-cell imaging was boosted with the application of GFP-fused chromatin proteins including histones, condensins, and others [4]. Nonetheless, this method is unable to detect specific genomic loci. Later, fluorescent repressor/operator systems were used to allow specific labeling of genomic regions in comparison to chromatin proteins [5]. In plants, this technique was applied to *A. thaliana* to compare the ploidy level of guard cells and elongated epidermal root cells [6]. However, this method is not able to label predefined genomic regions as a random insertion of the tandem operator repeats into the genome occurs after plant transformation. In addition, methylation at operator insertion sites and subsequent alteration of the chromatin dynamics have been reported [7].

Subsequently, new live-cell imaging techniques were established that are based on the use of programmable DNA-binding proteins, including zing finger protein (ZFP), transcription activator-like effector (TALE), and, more recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Fig. 1). The major advantage of programmable DNA-binding proteins is that target regions like RNA or genomic DNA are recognized and marked in a sequence-specific way.

ZFP fused with GFP under the control of ribosomal protein 5S A promoter (*RPS5A*p) was first used to label the 180 bp tandem repeats of the centromere in the root meristems of *A. thaliana* [8]. However, the ZFP system could not be used for imaging of specific loci, such as 5S rDNA or *HPT* gene. Since the genomic and chromatin content around the target region along with the context-dependent interactions with neighboring zinc fingers affects the DNA sequence recognition ability of ZFP for different genomic targets [9, 10], the further application of ZFP for live-cell imaging was substituted with TALEs. Similar to ZFPs, TALEs can be programed to detect specific DNA sequences [11, 12]. Using this feature, TALEs were successfully fused to the GFP to visualize repetitive sequences in *A. thaliana* including telomeric sequences and centromeric 180 bp repeats [13]. However, being a time-consuming and laborious method due to reengineering of TALE proteins for the targeting of each new genomic region, the live-cell imaging techniques were improved to use a more user-friendly method called CRISPR/Cas9.

The application of CRISPR/Cas9 for chromatin imaging was first reported for the dynamic imaging of genomic loci in human cell cultures [14]. The CRISPR/Cas9 consists of a Cas9 protein and guide RNA (gRNA) scaffold. The gRNA is a fusion of crRNA (CRISPR RNAs) and tracrRNA (trans-activating crRNA). Part of crRNA which binds as a complementary strand to the foreign target sequence is called protospacer. tracrRNA has a stem-loop structure and supports the stability of Cas protein. The Cas9 protein contains

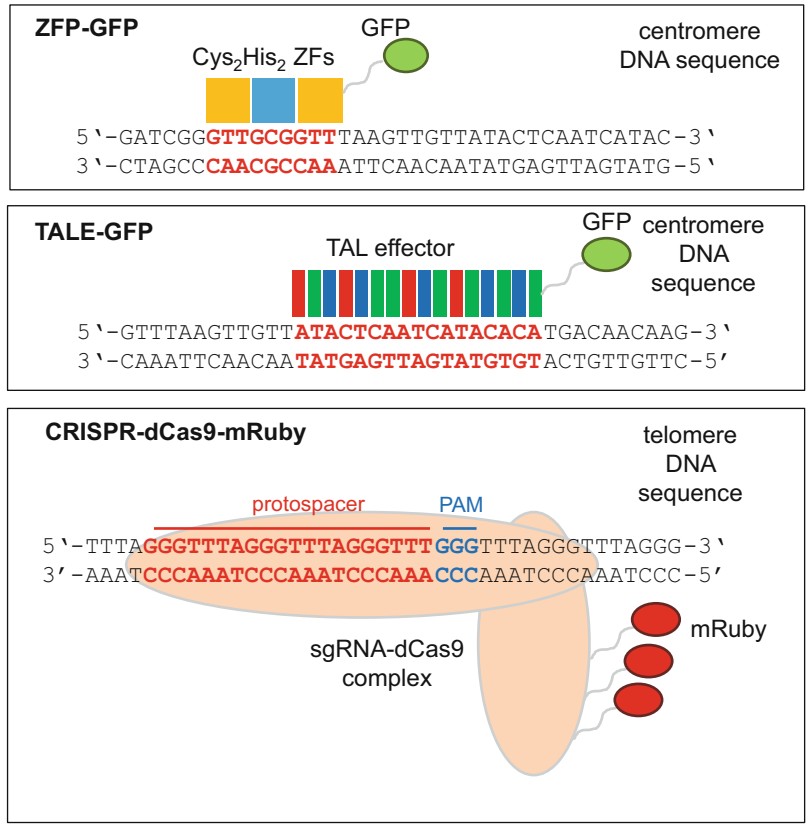

Fig. 1 Programmable DNA-binding proteins for live-cell imaging. (**a**) Zinc finger protein attached to GFP. Each protein can recognize just three base pairs in DNA. To recognize 9 bp, three ZFPs should be fused. (**b**) TALE protein fused to GFP. Each RVD region in TALE can recognize one base pair in DNA. (**c**) CRISPR/dCas9. A DNA cleavage-defective variant of Cas9 (dCAS9) fused to GFP can be easily programed for recognizing 20 base pairs in DNA

two HNH and RucV-like domains and recognizes specific sequences within the genome called protospacer adjacent motif (PAM). Each Cas protein has specificity for a specific PAM sequence. When Cas protein recognizes the specific PAM and attaches to the genome, the protospacer base pairs with the target sequence and allows the HNH domain to cut the DNA strand complementary to crRNA, and the RucV-like domain cleaves the other DNA strand [15]. Nevertheless, the nuclease activity of the Cas9 protein is not necessary for live-cell imaging. Therefore, in a deactivated Cas9 protein (dCas9) variant, this activity has been removed by the induction of two-point mutations in the HNH and RucV-like domains [16]. CRISPR/dCas9 was successfully used for visualizing of telomere repeats in human cells, pericentric and centric sequences in mouse cells, 45S rDNA in yeast, or even a single chromosomal locus in *Xenopus* egg extracts [14, 17–19]. The application of this method for studying the dynamic of

telomeres in living plant cells has been accomplished in transiently transformed *Nicotiana benthamiana* and *N. tabacum* plants [20, 21]. For *N. benthamiana*, dCas9 from two different bacterial sources, *Streptococcus pyogenes* and *Staphylococcus aureus*, was employed. Accordingly, both orthologs of Cas9 could visualize telomeric regions with equal efficiency [20].

In this chapter, the protocol of using different dCas9 orthologs in combination with target-specific gRNA for live-cell imaging of telomeres in *N. benthamiana* leaves is explained in detail. To start live-cell imaging of telomeres with CRISPR/Cas, pChimera and pEn-Sa-Chimera vectors are used to express gRNA from *S. pyogenes* and *S. aureus*, respectively. The dCas9-eGFP/mRuby2 vector is used for the expression of dCas9 from *S. pyogenes* (pCAS9-TP-Sp-dCAS9-eGFP/mRuby2) or *S. aureus* (pCAS9-TP-Sa-dCAS9-eGFP/mRuby2) fused with a fluorescent protein, either GFP or mRuby2 (*see* **Note 1**).

The protocol starts by designing the protospacers for labeling of telomers and annealing them to the pChimera vectors. Next, the gRNA from pChimera vectors is cloned next to dCas9-eGFP/mRuby2 with the help of conventional cloning. The resulting vector is agro-infiltrated into *N. benthamiana* leaves for the visualization of telomeres by confocal laser scanning microscopy. Subsequently, the specificity of observed signals is confirmed by immunostaining and FISH.

2 Materials

2.1 Material Used to Prepare CRISPR/Cas9 Constructs

1. gRNA expression vectors (here pChimera and pEn-Sa-Chimera) which express gRNA from *S. pyogenes* and *S. aureus*, respectively (*see* **Note 1**).
2. dCas9-eGFP/mRuby2 expression vectors (*see* **Note 1**):
   (a) pCAS9-TP-Sp-dCAS9-eGFP/mRuby2, referred to as pSpCas in this chapter.
   (b) pCAS9-TP-Sa-dCAS9-eGFP/mRuby2, referred to as pSaCas in this chapter.
3. *E. coli* NEB5α, DH5α derivative.
4. LB medium pH 7: 10 g Tryptone, 10 g sodium chloride (NaCl), 5 g yeast extract dissolved in 1 /L distilled water, supplement with 100 mg/L ampicillin or 100 mg/L spectinomycin.
5. Restriction enzymes *Bbs*I, *Mlu*I, *Avr*II, and respective restriction enzyme buffers.
6. T4 DNA ligase and T4 DNA ligase buffer.
7. Plasmid DNA isolation kit.
8. PCR purification kit.

Fig. 2 Final CRISPR/dCas9 live-cell imaging construct. LB: left border, Ubi-4 p: ubiquitin promoter for constitutive expression of Cas 9 protein, NLS: nuclear localization signal importing the expressed Cas9 protein to nucleus, 3× GFP/mRuby: fluorescent proteins, Pea3A T: terminator, AtU26-P: U6 promoter from *A. thaliana* for expression of gRNA, sgRNA telomere: sgRNA-containing telomere-targeting protospacer

2.2 Material Used for Agroinfiltration of N. benthamiana

1. Healthy 2–4-week-old *N. benthamiana* plants grown in a greenhouse under 16/8-h light/dark conditions and 22 °C temperature.
2. *Agrobacterium* strain harboring the imaging expression construct (Fig. 2).
3. LB medium with 100 mg/L spectinomycin and 50 mg/L rifampicin.
4. 100 mM Acetosyringone stock solution in ethanol, stored at −20 °C.
5. 0.5 M 2-(N-morpholino) ethanesulfonic acid buffer (MES-K), adjusted to pH 5.6 with KOH.
6. Resuspension solution: 10 mM $MgCl_2$, 10 mM MES-K.
7. Centrifuge for 50 mL tubes.
8. Spectrometer.
9. 5 mL Syringe.

2.3 Material Necessary for Microscopy of Plant Leaves

1. Confocal laser scanning microscope (e.g., LSM780, Carl Zeiss).
2. Phosphate buffer: 20 mM KH2PO4/Na2PO4, 0.01% Triton X100, pH 7.0.
3. Caulking gun.
4. 50 mL Syringe.
5. 76 × 26 mm Glass slides.
6. 40 × 24 mm Coverslips.
7. Sticking tape.
8. Fixogum rubber cement, Marabu GmbH, Germany.

2.4 Material Required for Immunostaining and FISH

1. Epifluorescence microscope (e.g., BX61, Olympus) equipped with a camera (e.g., Orca ER, Hamamatsu).
2. Cytocentrifuge (Shandon CytoSpin3, *see* **Note 2**).
3. Cytology funnels for cytocentrifuge.
4. 5 mL Polystyrene round-bottom tube with cell strainer cap (*see* **Note 3**).
5. Razor blade.

6. 4% (vol/vol) Paraformaldehyde solution (*see* **Note 4**).
7. LB01 lysis buffer: 15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCL, 20 mM NaCl, 15 mM β-mercaptoethanol, 0.1% (vol/vol) Triton X-100. Adjust pH to 7.5 with 1 M NaOH. Filtrate the buffer through 0.22 μm filter to sterilize it for longer storage.
8. 4% (vol/vol) Bovine serum albumin (BSA) solution.
9. PBS pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4.
10. 4′,6-Diamidino-2′-phenylindole dihydrochloride (DAPI) solution (*see* **Note 5**).
11. VECTASHIELD antifade mounting medium (Vector Laboratories) (*see* **Note 6**).
12. Ethanol solutions: 70, 90, 100%.
13. Fixation solution: (1:3) glacial acetic acid: 100% Ethanol.
14. Denaturation solution: 0.2 M NaOH in 70% ethanol.
15. FISH hybridization solution: 50% (vol/vol) Formamide, 10% (vol/vol) dextran sulfate in 2× SSC.
16. 2× Saline sodium citrate (SSC): 0.30 M Sodium citrate, 0.030 M NaCl, pH 7.0.
17. GFP antibody (directly labeled GFP mouse antibody Dylight 488; Rockland).
18. 5′-Cy5-labeled oligonucleotide probe (5′-Cy5-GGGTTTAGGGTTTAGGGTTT-3′).
19. Sucrose buffer: 100 mM Tris, 50 mM KCl, 2 mM MgCl2, 0.05% Tween, 5% sucrose.

3 Methods

3.1 Preparation of the Protospacer and Cloning of the CRISPR/Cas9-gRNA Vectors

1. Select a 20 nt protospacer for the telomere sequence upstream of 5′-NGG-3′ and 5′-NNGRRT-3′ PAM sequences for Cas9 from *S. pyogenesis* and *S. aureus*, respectively (*see* **Note 7**).
2. Synthesize the protospacer as oligonucleotides with appropriate overhangs for cloning into the gRNA expression vector (5′-ATTG-protospacer-3′ and 5′-AAAC-rev-com_protospacer-3′) (*see* **Note 8**) (Fig. 3c).
3. Mix the oligonucleotides with a final concentration of 2 μM each in a final volume of 50 μL and anneal them by incubation at 95 °C for 5 min and subsequent incubation at room temperature for 20 min (*see* **Note 9**).

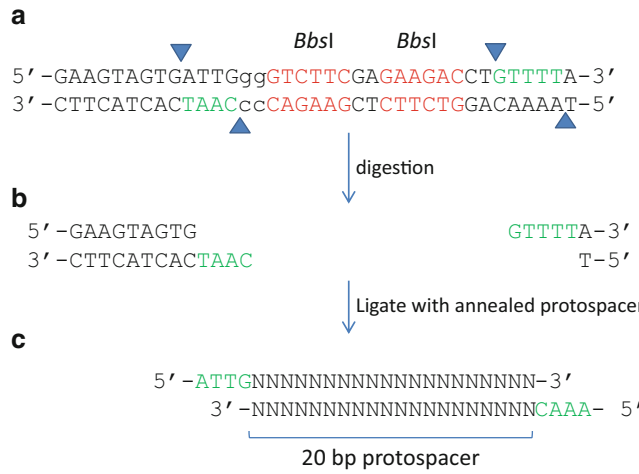

Fig. 3 Ligation of a telomere protospacer into the sgRNA expression vector. (**a**) *Bbs*I restriction site in pChimera/pEn-Sa-Chimera vectors. (**b**) *Bbs*I-linearized sgRNA expression vector DNA has TAAC and GTTT sticky ends. (**c**) Protospacer sequence which carries ATTG and CAAA sticky ends that are complementary to the sticky ends of the linearized sgRNA expression vector

4. Linearize the gRNA expression vector by digestion with *Bbs*I and purify the linearized vector. Adjust the concentration to 5 ng/μL (*see* **Note 10**) (Fig. 3a and b).
5. For the subsequent ligation, mix 2 μL of the linearized gRNA expression vector with 3 μL of the annealed oligonucleotides and proceed according to the manufacturer's protocol of the respective T4 DNA ligase (*see* **Note 11**).
6. Transform 5 μL of the ligation in 100 μL competent DH5α cells via the heat-shock method, plate 100 μL on LB medium with 100 mg/L ampicillin, and grow overnight at 37 °C.
7. Verify successful transformation via colony PCR, inoculate a single colony in 5 mL LB medium with 100 mg/L ampicillin, and grow overnight at 37 °C.
8. Isolate the plasmid DNA and verify successful cloning via sequencing. Adjust the concentration to 100 ng/μL.
9. Digest the specified gRNA expression vector with *Mlu*I (for pEn-Sa-Chimera) or *Avr*II (for pChimera) and purify the excised gRNA expression cassette.
10. Linearize the dCas9-eGFP/mRuby2 expression vectors via digestion with *Mlu*I (for SaCas9) or *Avr*II (for SpCas9) and purify the linearized vector.
11. Ligate the specified gRNA expression cassette into the linearized dCas9-eGFP/mRuby2 expression vector according to the manufacturer's protocol of the respective T4 DNA ligase.

12. Transform 5 μL of the ligation in 100 μL competent *E. coli* DH5α cells via the heat-shock method, plate 100 μL on LB medium supplemented with 100 mg/L spectinomycin, and grow overnight at 37 °C.
13. Verify successful transformation via colony PCR, inoculate a single colony in 5 mL LB medium supplemented with the 100 mg/L spectinomycin, and grow overnight at 37 °C.
14. Isolate the plasmid DNA and verify successful cloning via sequencing. Adjust the DNA concentration to 100 ng/μL.
15. The vectors are ready for transformation into *Agrobacterium* strain GV3101.

3.2 Plant Material Preparation and Agrobacterium-Mediated Transformation

1. Grow *N. benthamiana* seeds in a greenhouse under 16/8-h light/dark conditions and 22 °C temperature for 2–4 weeks.
2. Use a single colony of *Agrobacterium* (**step 15** from Subheading 3.1) to inoculate 5 mL LB with 100 mg/L spectinomycin and 50 mg/L rifampicin and grow overnight at 28 °C.
3. Use 1 mL of the overnight culture to inoculate 25 mL LB medium with 100 mg/L spectinomycin and 50 mg/L rifampicin and grow overnight.
4. Measure the optical density (OD600) of the overnight culture with a spectrometer.
5. Precipitate the bacteria in 50 mL tubes by centrifugation at 5000 × *g* for 15 min.
6. Meanwhile, prepare the resuspension solution by adding 100 μM acetosyringone. Acetosyringone should always be added to the resuspension solution after autoclaving and before injection of leaves.
7. Resuspend the bacterial pellet in resuspension solution and adjust the final A_{600} to 0.4.
8. Leave the bacterial suspension solution on the bench at room temperature for 2–3 h (or overnight) before infiltration.
9. Perform the infiltration with syringe. Simply press the syringe (without needle) on the abaxial surface of the leaf and exert a counter-pressure with finger on the other side (Fig. 4). Press the plunger of the syringe and allow the liquid to enter the leaf tissue. Successful infiltration is often observed as a spreading "wetting" area in the leaf.
10. Keep the injected plants in the greenhouse at 16/8-h light/dark conditions and 22 °C temperature for 48 h.
11. Look for the occurrence of fluorescent signals with a microscope 2–4 days after injection (Fig. 5) (*see* **Note 12**).

Fig. 4 Agroinfiltration of a *N. benthamiana* leaf. The infiltrated bacterial solution causes the spreading of a wet area in the leaf

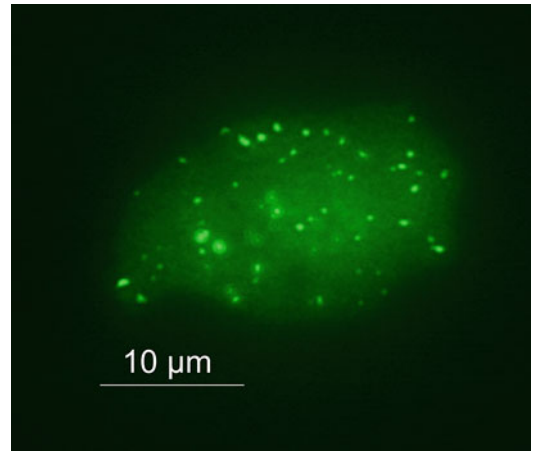

Fig. 5 Signal observation in living leaf tissue of *N. benthamiana* by fluorescence microscopy. The GFP signals emitted from telomeres can be detected 48 h after infiltration

3.3 Imaging of Telomeric Fluorescence Signals

3.3.1 Degassing of Leaf Samples

1. Remove the plunger from the 50 mL plastic syringe and seal the adaptor end of the funnel in a flame.
2. Fill the funnel with 15 mL 20 mM phosphate buffer/0.01% Triton X100.
3. While avoiding large veins, cut out a 15 × 8 mm rectangular piece of leaf tissue with a razor blade. Position the sample dorsal side up and remove part of the right upper corner to distinguish dorsal from ventral side.
4. Transfer the leaf sample into the funnel and install the plunger again.
5. Place the 50 mL syringe into the caulking gun and increase the pressure until the leaf sample is no longer afloat. The presence of 0.01% Triton X100 reduces surface tension and facilitates buffer infiltration.

6. Leave the syringe for 15 min in the caulking gun to ensure that residual air is replaced by buffer solution. Repeat if necessary.
7. Place degassed leaf sample ventral side up (position of cutoff corner on the left upper side) on a glass slide.
8. Add a droplet of buffer solution before covering the sample with a 40 × 24 mm coverslip.
9. Fix the short ends of the coverslip with sticking tape.
10. Remove air inclusions between sample and coverslip by careful tapping with the blunt end of a tweezer.
11. Fill empty space between coverslip and glass slide with buffer, and then seal the long ends with fast drying and easily removable fixogum.

3.3.2 Analysis of Telomeric GFP Signals

1. Place the slide in the microscope and examine with a 40× NA 1.2 water objective.
2. Select appropriate locations without air inclusions since the latter interfere with signal recording.
3. Select optimal resolution settings to avoid over- or undersampling.
4. GFP is excited with a 488 nm laser line and emission is detected over a range of 490–540 nm.
5. Collect telomeric GFP distribution as Z-stacks.

3.4 Combination of Immunostaining and FISH for Confirming the Specificity of CRISPR/dCas9-Caused Signals

1. Cut a 1 cm^3 piece of leaf from the infiltrated area, transfer into 4% paraformaldehyde solution, and fix on ice for 5 min under vacuum followed by 30 min under atmospheric pressure (*see* **Note 13**).
2. Remove the paraformaldehyde solution and wash twice in PBS.
3. In a drop of LB01 lysis buffer in a petri dish, finely chop the leaf using a sharp razor blade (*see* **Note 14**).
4. Add 500 μL LB01 lysis buffer to the homogenate and transfer into 35 μm cell suspension filter tube.
5. Apply 200 μL of the homogenate to a cytology funnel and centrifuge for 5 min at 450 rpm in a cytocentrifuge (*see* **Note 2**).
6. Wash slides two times in PBS for 5 min and add 60 μL of 4% BSA followed by incubation at room temperature for 45 min in a high-humidity plastic box. Carefully cover slides with parafilm tape.
7. Wash slides two times in PBS for 5 min and add 60 μL of GFP antibody solution (2% (vol/vol) BSA in PBS, GFP antibody in 1:2500 dilution) followed by incubation at room temperature for 1 h in a high-humidity plastic box. Carefully cover slides with parafilm tape.

8. Wash slides two times in PBS for 5 min and fix in fixation solution for 24 h in darkness.
9. Perform sequential dehydration in 70, 90, and 100% ethanol for 2 min each and leave to dry for 30 min.
10. Afterwards, perform pre-hybridization at 37 °C for 1 night by adding 15 μL FISH hybridization solution and cover slides with coverslip. Store slides in a high-humidity plastic box.
11. Wash slides twice in 2× SSC for 5 min and perform sequential dehydration in 70, 90, and 100% ethanol for 2 min each. Leave slides to dry for 30 min.
12. Perform DNA denaturation in denaturation solution at room temperature for 10 min followed by sequential dehydration in 70, 90, and 100% ethanol for 2 min each. Leave slides to dry for 15 min.
13. Meanwhile, prepare the FISH hybridization solution by mixing 0.5 μL of the 5′-labeled oligonucleotide probe (10 μM) with 14.5 μL of FISH hybridization solution per slide.
14. Incubate the mixture at 95 °C for 5 min followed by rapid transfer onto ice for 5 min.
15. Apply 15 μL of the mixture per slide and hybridize at 37 °C for 1 night. Cover slides with coverslips and store in a high-humidity plastic box.
16. Wash slides two times in 2× SSC for 5 min followed by sequential dehydration in 70, 90, and 100% ethanol for 2 min each. Leave slides to dry for 30 min.
17. Apply 10 μL VECTASHIELD solution containing DAPI (1:1000) and analyze slides by fluorescence microscopy (Fig. 6).

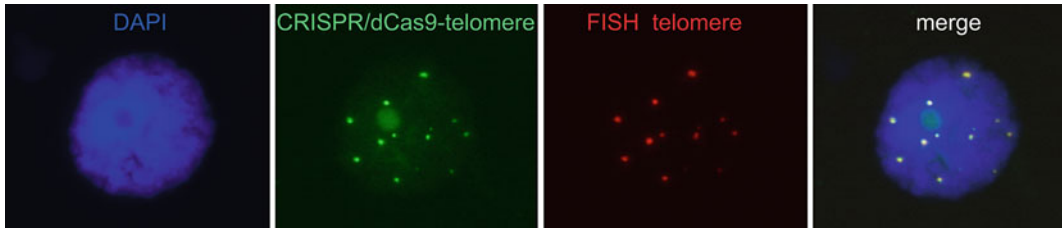

Fig. 6 *N. benthamiana* nuclei after CRISPR/dCas9-based staining of telomeres and subsequent FISH with a telomere-specific probe. From left to right: DAPI staining of nuclei (blue); GFP signals coming from CRISPR imaging vector targeting telomeres (green); FISH signals from telomere probe (red); co-localization of CRISPR and FISH signals demonstrating the specificity of CRISPR signals (merge)

4 Notes

1. All Cas9 expression vectors are based on the vector pCAS9-TPC [22, 23]. The vectors mentioned in this protocol are available from the Botanical Institute, Karlsruhe Institute of Technology, POB 6980, 76049 Karlsruhe, Germany. Vector information can be found on the respective webpage (https://www.botanik.kit.edu/molbio/1057.php).
2. This centrifuge is used to precipitate leaf nuclei onto a microscopic slide. It enables to obtain purified leaf nuclei at high density without any remaining cytoplasm. It is crucial to remove any cytoplasm for a successful immunostaining and FISH. If a cytocentrifuge is not available, the following alternative method could be used to prepare microscopic slides carrying nuclei. Drop 12 μL sucrose buffer on a clean glass slide, add 12 μL of nuclei suspension obtained after filtration of chopped leaf, and gently mix both types of drops with the pipette tip. Let slides dry overnight.
3. This tube contains a 35 μm nylon mesh to filtrate the extracted nuclei from leave debris.
4. Prepare 4% paraformaldehyde solution by diluting 37% ready-to-use paraformaldehyde solution.
5. DAPI solution is usually prepared by diluting 1 μL DAPI (stock) in 999 μL VECTASHIELD antifade mounting medium. Mix well by vortexing.
6. VECTASHIELD is used to prevent immediate fading of fluorescent signals during microscopy.
7. The PAM sequences which are recognized by the specific Cas9 proteins of *S. pyogenes* and *S. aureus* are 5′-NGG-3′ and 5′ of 5′-NNGRRT-3′, respectively. The protospacers have to be selected 20 bp upstream of the respective PAM sequence. There are web pages like DeskGen (https://www.deskgen.com/landing/#/), WU-CRIPSR (http://crispr.wustl.edu/), and CRISPOR (http://crispor.tefor.net/) available for selecting a suitable protospacer for the target telomere sequence of interest (Fig. 7).

Fig. 7 Protospacer design for pCAS9-TP-Sp-dCAS9-eGFP/mRuby2 and pCAS9-TP-Sa-dCAS9-eGFP/mRuby2 to target telomere DNA sequence. The protospacer has to be selected 20 bp upstream of the respective PAM sequence. Target sequence is shown in red. The NGG protospacer adjacent motif (PAM) for Sp-cCas9 is indicated in blue, whereas *NNGRRT* PAM for Sa-dCas9 is indicated in green

8. The appropriate *overhangs* are added to the 5′ end of selected **protospacer** for targeting telomeres in *N. benthamiana*:
   (a) Forward sequence: 5′-*attg***GGGTTTAGGGTTTAGGGTTT**-3′
   (b) Reverse sequence: 5′-*aaac***AAACCCTAAACCCTAAACCC**-3′
9. To guarantee a proper annealing of the oligonucleotides, do not decrease the incubation time at room temperature.
10. Mere heat inactivation of the digestion reaction to linearize the gRNA expression vector is not recommended to prevent religation of the excised fragment. However, due to the small size of the excised fragment, a PCR purification kit is sufficient for the purification of the linearized gRNA expression vector.
11. To guarantee a proper ligation, an incubation time of at least 1 h is recommended.
12. The signals can be detected even up to 4 days after injection; however, the number of nuclei showing signals decreases over time.
13. Optimal fixation conditions may vary between species or tissue types. The durations described here are working well for *N. benthamiana* leaves. However, if the quality of the immunostaining is not as desired, an increase or decrease of fixation time might have an impact on how well the structure of the nuclei is preserved.
14. Chop by quickly moving the razor blade up and down in a steady rhythm. It is important not to squeeze the leaf, as this does not release nuclei. The LB01 buffer should only turn green as a result of homogenization, not squeezing the leaf.

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