CRISPR/Cas9-Based RGEN-ISL Allows the Simultaneous and Specific Visualization of Proteins, DNA Repeats, and Sites of DNA Replication

Alžběta Němečková^{a,b} Christina Wäsch^a Veit Schubert^a Takayoshi Ishii^c
Eva Hřibová^b Andreas Houben^a

^aLeibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Seeland, Germany; ^bInstitute of Experimental Botany, Czech Academy of Sciences, Center of the Region Haná for Biotechnological and Agricultural Research, Olomouc, Czech Republic; ^cArid Land Research Center (ALRC), Tottori University, Tottori, Japan

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CRISPR/Cas9 · DNA replication · FISH · Immunostaining · RGEN-ISL · Super-resolution microscopy

Abstract

Visualizing the spatiotemporal organization of the genome will improve our understanding of how chromatin structure and function are intertwined. Here, we describe a further development of the CRISPR/Cas9-based RNA-guided endonuclease-in situ labeling (RGEN-ISL) method. RGEN-ISL allowed the differentiation between vertebrate-type (TTAGGG)_n and *Arabidopsis*-type (TTTAGGG)_n telomere repeats. Using maize as an example, we established a combination of RGEN-ISL, immunostaining, and EdU labeling to visualize in situ specific repeats, histone marks, and DNA replication sites, respectively. The effects of the non-denaturing RGEN-ISL and standard denaturing FISH on the chromatin structure were compared using super-resolution microscopy. 3D structured illumination microscopy revealed that denaturation and acetic acid fixation impaired and flattened the chromatin. The broad range of adaptability of RGEN-ISL to different combinations of methods has the potential to advance the field of chromosome biology.

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Since the 1990s, fluorescence in situ hybridization (FISH) has been widely used for the visualization of specific DNA sequences in fixed nuclei and chromosomes. In that process, a DNA denaturation step using heat, formamide, or sodium hydroxide is necessary to allow probe hybridization. However, these treatments may affect the native structure of chromatin. Nowadays, with the improved ultrastructural investigation possibilities via super-resolution microscopy and a high interest in analyzing the real native chromatin structure, its arrangement, and modifications during the cell cycle, more sensitive techniques for chromatin labeling are required.

The discovery and application of the type II clustered regulatory interspaced short palindromic repeat (CRISPR)-associated caspase 9 (Cas9) system for genome editing was the starting point to employ this system also for the fluorescence detection of genomic loci in living [Chen et al., 2013; Anton et al., 2014; Dreissig et al., 2017] and fixed non-denatured animal and plant cells [Deng et al., 2015; Ishii et al., 2019].

The principle of fluorescence labeling using the CRISPR/Cas9 system is based on the property of designed CRISPR RNA (crRNA), which contains a spacer complementary to a DNA sequence and provides the target specificity of the Cas9 system (Fig. 1). To find the target

Fig. 1. The principle of fluorescence labeling of genomic DNA using the CRISPR-Cas9-based RGEN-ISL method. The crRNA: tracrRNA complex uses optimized Alt-R crRNA and ATTO 550-labeled tracrRNA sequences that hybridize and then form a complex with Cas9 endonuclease to guide targeted binding to genomic DNA. The binding site is specified by the protospacer element of the crRNA (green bar). This element recognizes 19 or 20 nt on the opposite strand of the NGG PAM site. The PAM site must be present immediately downstream of the protospacer element that binding can occur.

DNA, the Cas9 nuclease requires a guide RNA (gRNA) which is composed of crRNA, fluorescently labeled *trans*-activating crRNA (tracrRNA), and a protospacer adjacent motif (PAM). This specific complex is formed by hybridization of the designed crRNA in combination with the fluorescently labeled tracrRNA [Ishii et al., 2019]. The crRNA contains a 20-nucleotide guide sequence called spacer and the PAM, a short G-rich motif which is positioned next to the gRNA-specific part of the target sequence.

To further develop the CRISPR/Cas9-based RNA-guided endonuclease-in situ labeling (RGEN-ISL) method for the detection of high-copy DNA, we employed telomere and maize 180-bp knob [Peacock et al., 1981; Ananiev et al., 1998] repeat-specific gRNAs. We established a combination of RGEN-ISL, immunostaining, and 5-ethynyl-deoxyuridine (EdU) labeling to detect specific repeats, histone marks, and DNA replication sites, respectively. We compared the effect of non-denaturing RGEN-ISL and standard denaturing FISH on the structure of chromatin using super-resolution microscopy.

Materials and Methods

Material

Nuclei and chromosomes of *Zea mays* L. (genotype B73) and *Scadoxus multiflorus* (Martyn) Raf. (also known as *Haemanthus multiflorus*) were used.

Sample Preparation

Maize nuclei and chromosomes were isolated from root meristems and leaves of young seedlings. *S. multiflorus* nuclei were isolated from roots of mature plants. Leaves were fixed for 30 min in Tris buffer with 2% formaldehyde (10 mM Tris, 10 mM Na₂EDTA, 100 mM NaCl, 0.1% Triton X-100, 2% formaldehyde, pH 7.5 [prepared from formaldehyde solution 37%, Carl Roth GmbH, cat. No. 7398.1]) at 4°C. The first 5 min of fixation were done under vacuum condition using a Vacufuge concentrator (Eppendorf, model 5301) according to Doležel et al. [1992]. After fixation, leaves were washed 3 times in Tris buffer, using a rotating shaker (100 rpm) on ice. For the preparation of chromosomes and nuclei from the root meristems, Tris buffer with 3% formaldehyde was used for 35 min (5 min under vacuum and 30 min on ice only) for fixation. The meristems of four 1-cm-long root tips were chopped into thin slices with a razor blade in 500 µL LB01 buffer (15 mM Tris, 2 mM Na₂EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol, 0.1% Triton X-100, pH 7.5) [Doležel et al., 1989]. The suspension was passed through a 35-µm nylon mesh, and nuclei and chromosomes were spun onto standard microscopic slides using a Thermo Shandon Cytospin 3 (700 rpm for 5 min for leaf- and 400 rpm for 5 min for root-derived material). The slides were checked by phase contrast microscopy and kept in ice-cold 1× PBS. Before use, slides were washed in 1× PBS for 5 min on ice while shaking (100 rpm).

RNA-Guided Endonuclease-in situ Labeling

Target-specific crRNAs for the 180-bp maize knob repeat [Peacock et al., 1981] and the vertebrate telomere repeat were designed using the software crCRISPRdirect (<https://crispr.dbcls.jp/>) (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000502600). We employed the 2-part gRNA (crRNA and tracrRNA) system (Alt-R® CRISPR-Cas9, Integrated DNA Technologies, <https://eu.idtdna.com>) for RGEN-ISL according to Ishii et al. [2019]. For the assembly of 10 µM gRNA, 1 µL 100 µM crRNA, 1 µL 100 µM ATTO550-labeled tracrRNA, and 8 µL duplex buffer were used. Afterwards, the gRNA was denatured for 5 min at 95°C to hybridize. In the next step, the ribonucleoprotein (RNP) complex was prepared: 1 µL 10 µM gRNA, 1 µL 6.25 µM dCas9 proteins (D10A and H840A; Novateinbio, PR-137213), 10 µL 10× Cas9 buffer (200 mM Hepes pH 7.5, 1 M KCl, 50 mM MgCl₂, 50% (v/v) glycerol, 10% BSA, and 1% Tween 20), 10 µL 10 mM DTT, and 80 µL double distilled water were mixed, incubated at 26°C for 10 min, and stored at 4°C. Per slide, 100 µL of 1× Cas9 buffer/1 mM DTT was applied for 2 min at room temperature. The slides were tilted to remove the buffer, and 25 µL RNP complex per slide was applied. Slides were covered with parafilm and kept in a humid chamber at 26°C for 2–4 h, or at 4°C overnight. After incubation, the slides were washed in ice-cold 1× PBS for 5 min. To prevent the dissociation of the RNP complex, post-fixation was performed with 4% formaldehyde in 1× PBS for 5 min on ice. Then, the slides were washed with 1× PBS for 5 min on ice and dehydrated in ethanol (70, 90, and 96%; 2 min each) at room temperature. Slides were embedded and counterstained with DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Combination of RGEN-ISL and FISH

After RGEN-ISL, the slides were fixed in 4% formaldehyde in 1× PBS for 5 min and treated with freshly prepared ethanol:glacial

acetic acid (3:1) at room temperature for 7 h in the dark. Then, the slides were dehydrated in an ethanol series (70, 90, and 96%; 2 min each) at room temperature. After brief drying, 15 μ L of a prehybridization solution of DS20 (20% dextran sulfate [Sigma-Aldrich, cat. No. D8906], 50% deionized formamide [Sigma-Aldrich, cat. No. 4767], 300 mM NaCl, 30 mM tri-sodium citrate dehydrate, 50 mM phosphate buffer, pH 7.0) was applied per slide and covered with a 22 $\times$ 22 mm cover slip for overnight incubation at 37°C. The next day, coverslips were removed, and the slides were washed in 2 $\times$ SSC with 0.1% Triton and 2 $\times$ SSC at room temperature for 5 min each, and dehydrated in an ethanol series (70, 90, and 96%; 2 min each) at room temperature. After short drying, DNA was denatured in 0.2 N NaOH/70% ethanol at room temperature for 3 min and then dehydrated sequentially in an ethanol series. The hybridization solution consisting of 15 μ L DS20 and 1 μ L of probe specific for the 180-bp Knob 2 (oligo probe FAM-GAAGGCTAACACCTACGGATTTTGTACCAAGAAATGGTCTCCAC-CAGAAATCCAAAAAT [Zhu et al., 2017]) was denatured at 95°C for 5 min, immediately transferred on ice, and kept for 5 min. The hybridization mix was applied to the slide, covered with a coverslip, and hybridized overnight in a humid chamber at 37°C. The next day, slides were washed in 2 $\times$ SSC with 0.1% Triton X-100 and 2 $\times$ SSC at room temperature for 5 min each. Slides were dehydrated using the ethanol series and finally embedded and counterstained with DAPI in Vectashield mounting medium.

Combination of DNA Replication Analysis and RGEN-ISL

Roots of 3-day-old maize seedlings were incubated with 20 μ M EdU solution (baseclick GmbH, cat. No. BCK-EdU647) in ddH₂O (stock solution contains 10 mM EdU in DMSO) for 30 min at 28°C. Then, the roots were washed thoroughly in H₂O, and the nuclei were prepared as described for RGEN-ISL.

Combination of DNA Replication Analysis, Immunostaining, and RGEN-ISL

Slides carrying nuclei isolated from EdU-treated roots were washed for 5 min in ice-cold 1 $\times$ PBS and incubated with 70 μ L of primary antibodies per slide (1:100 dilution, anti-methylation H3K9me2 and H3K4me2 [Abcam, cat. No. Ab1220 and Ab7766]) at 4°C overnight in a humid chamber. The next day, the slides were washed twice for 5 min in 1 $\times$ PBS, and then 100 μ L of 1 $\times$ Cas9 buffer per slide was used for equilibration for 2 min. After that, 25 μ L of the RNP complex together with the secondary antibody anti-rabbit Alexa 488 (1:200 dilution; Jackson ImmunoResearch Laboratories, cat. No. 711-545-152) was applied per slide. First the RNP complex was prepared, afterwards the secondary antibodies were diluted in the RNP mix. Slides were covered with parafilm and incubated overnight at 4°C in a humid chamber. For the visualization of the EdU-incorporated DNA, the slides were washed in 1 $\times$ PBS on a shaker (5 min; 100 rpm), and the freshly prepared EdU reaction cocktail (250 μ L per slide) for detection was applied, covered with parafilm, and incubated for 30 min at 28°C in a humid chamber protected from light. After incubation, the reaction cocktail was removed, and the slides were washed for 5 min in 1 $\times$ PBS on a shaker (100 rpm). Post-fixation was performed with 4% formaldehyde in 1 $\times$ PBS for 5 min on ice. Then, the slides were washed in 1 $\times$ PBS for 5 min on ice and dehydrated in ethanol (70, 90, 96%; 2 min each) at room temperature. The slides were embedded and counterstained with DAPI in Vectashield.

Fig. 2. Workflow of RGEN-ISL and its combination with FISH (RGEN-ISL + FISH) and with EdU-based DNA replication analysis and immunolabeling (EdU + RGEN-ISL + IM). FA, formaldehyde; IM, immunolabeling.

All protocols for RGEN-ISL and its combination with FISH (RGEN-ISL + FISH) and with EdU-based DNA replication analysis and immunolabeling (EdU + RGEN-ISL + IM) are summarized in Figure 2.

Microscopy

Widefield fluorescence imaging was performed using an Olympus BX61 microscope equipped with an ORCA-ER CCD camera (Hamamatsu). All images were acquired in grayscale and pseudo-colored with Adobe Photoshop 6 (Adobe Systems). To analyze the ultrastructure and spatial arrangement of signals and chromatin at a lateral resolution of ~120 nm (super-resolution, achieved with a 488-nm laser), spatial structured illumination microscopy (3D-SIM) was applied using a Plan-Apochromat 63 $\times$ /1.4 oil objective of an Elyra PS.1 microscope system and the software ZENblack (Carl Zeiss GmbH). Image stacks were captured separately for each fluorochrome using 561-, 488-, and 405-nm laser lines for excitation and appropriate emission filters. Maximum intensity projections were calculated based on 3D-SIM image stacks employing the ZENblack software [Weissart et al., 2016].

Results and Discussion

RGEN-ISL and FISH Signals Differ at the Subchromosomal Level

The 180-bp knob repeat of maize [Peacock et al., 1981; Ananiev et al., 1998] was used to compare the structure

Fig. 3. Application of RGEN-ISL for the detection of high copy repeats. **A, B** Comparison of the chromatin ultrastructure after Knob 2-specific labeling by RGEN-ISL (red) and subsequent FISH (green) of fixed chromosomes (**A**) and interphase nuclei of maize (**B**). **C** Nucleus of *S. multiflorus* exhibiting vertebrate-specific telomere signals. To analyze the ultrastructure and spatial arrangement of signals and chromatin at a lateral resolution of ~140 nm, 3D structured illumination microscopy was applied. A higher resolution is achieved by FAM labeling (~120 nm).

of chromatin and fluorescence signals after applying the newly developed RGEN-ISL method and standard FISH. Specific gRNAs (Knob 1, Knob 2, Knob 3) which differ in GC content, PAM, melting temperature, and target copy number were designed (online suppl. Table 1). A positive correlation between signal intensity and reliability of designed probes and their copy number of target repeats was observed as the Knob 2-specific gRNA with the highest copy number resulted in the strongest signals (online suppl. Fig. 1). A negative correlation between RGEN-ISL signal intensity and the degree of chromatin structure preservation was found as previously noted by Ishii et al. [2019]. Fixation of leaf tissue in 2% formaldehyde resulted in the strongest RGEN-ISL signals, but the chromatin structure was of low quality. The opposite was observed using 4% formaldehyde-fixed nuclei. Application of 3% formaldehyde fixation in combination with a Knob 2-specific gRNA provided the most reliable result in all experiments (online suppl. Fig. 2).

To evaluate the influence of denaturation on the morphology of chromatin, non-denaturing RGEN-ISL was performed first and super-resolution microscopy (3D-

SIM) images were acquired. Afterwards, the same specimen was used for standard FISH, recorded again, and the images were compared. The depicted overall morphology of chromosomes and nuclei was similar for both methods (Fig. 3A, B). However, the application of 3D-SIM revealed subtle differences. The width of chromosomes increased after standard FISH, and the chromosome structure labeled by DAPI was less defined (Fig. 3A). It seems that FISH, a method which is based on denaturation and acetic acid fixation, impaired and flattened the chromatin. In case of non-denaturing RGEN-ISL, the chromatin structure stays more compact. FISH-positive chromosome regions were about one fifth larger in total. Hence, RGEN-ISL is the method of choice for the visualization of repeats if the ultrastructure of chromatin is of interest.

RGEN-ISL Enables the Detection of Vertebrate-Type Telomeres in S. multiflorus

The chromosome termini of the blood lily *S. multiflorus* ($2n = 18$) are sealed by vertebrate-type (TTAGGG)_n and not *Arabidopsis*-type (TTTAGGG)_n telomere repeats [Monkheang et al., 2016]. To check whether a visualiza-

Fig. 4. RGEN-ISL in combination with EdU-based DNA replication analysis (EdU + RGEN-ISL) (**A, B**) and immunolabeling (EdU + RGEN-ISL + IM) (**C, D**) using maize nuclei. **A** The early replicating nucleus shows euchromatin labeling by EdU (white), whereas the Knob 2 repeats marked by RGEN-ISL (red) are not yet replicated. **B** The late replicating nucleus shows Knob 2 repeat-specific EdU labeling. **C** After triple staining of early S phase, the late replicating Knob 2 repeats are free of EdU, but stained by anti-histone H3K9me2. **D** During late S phase the late replicating Knob 2 repeats are EdU marked and free of anti-histone H3K4me2 signals. **A, B** 3D structured illumination microscopy. **C, D** Standard fluorescence microscopy.

tion of this repeat is possible by RGEN-ISL, *Arabidopsis* and vertebrate telomere-specific gRNAs were used. Telomere signals were only detected with the vertebrate telomere-specific gRNA (Fig. 3C; online suppl. Fig. 3). The RGEN-ISL resulted in 29.6 dot-like signals per nucleus ($n = 50$). A slightly higher number of telomere signals per nucleus (32.2) was found after standard FISH ($n = 50$). Hence, RGEN-ISL allows the differentiation between vertebrate-type (TTAGGG)_n and *Arabidopsis*-type (TTTAGGG)_n telomere repeats.

Combination of RGEN-ISL and DNA Replication Analysis

The combination of FISH and EdU-based DNA replication detection has been used to determine the replication timing of defined genomic sequences [Klemme et al., 2013]. To test whether RGEN-ISL could be used instead of FISH, maize roots were pulse-labeled with 20 μ M EdU. After application of RGEN-ISL, the EdU click-reaction was performed without a negative effect on the RGEN-ISL signal intensity. Post-fixation with 4% formaldehyde before EdU labeling, reduced the EdU click-reaction efficiency and resulted in a weaker EdU signal. Therefore,

the final post-fixation step should be done after both methods were employed. SIM shows clearly the overlap between the Knob 2 signals and EdU-labeled chromatin in the late phase of replication (Fig. 4B) and no colocalization during early replication (Fig. 4A). Using this fast, reproducible, and sensitive technique, we were able to deliver in only 1 week the same information as obtained after a laborious repli-Seq project in maize, in which it was shown that the Knob 180-bp repeat is replicated in late S phase [Wear et al., 2017].

Combination of EdU-Based DNA Replication Analysis, RGEN-ISL, and Indirect Immunostaining

Finally, we tested whether the combined EdU-based replication analysis and RGEN-ISL-based DNA detection could be linked with indirect immunolabeling to visualize the replication behavior of knob repeats and the distribution of post-translational histone marks simultaneously. Therefore, maize nuclei after EdU-pulse labeling were isolated, and RGEN-ISL was employed in combination with immunostaining. The triple combination resulted in knob-specific RGEN-ISL signals colocalizing with early and late replicating histone H3K9me2 and

H3K4me2 chromatin, respectively (Fig. 4C, D). It is possible that H3K9me2 contributes to the early replication of heterochromatin in maize. Contrary, H3K4me2 may have an opposite function H3K4me2 may have an opposite function in late replicating euchromatin. To conclude, a triple combination method under non-denaturing conditions for the simultaneous detection of specific DNA repeats, proteins, and DNA replication sites has been developed.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

References

- Ananiev EV, Phillips RL, Rines HW: Complex structure of knob DNA on maize chromosome 9. Retrotransposon invasion into heterochromatin. *Genetics* 149: 2025–2037 (1998).
- Anton T, Bultmann AS, Leonhardt H, Markaki Y: Visualization of specific DNA sequences in living mouse embryonic stem cells with a programmable fluorescent CRISPR/Cas system. *Nucleus* 5:163–172 (2014).
- Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, et al: Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155:1479–1491 (2013).
- Deng WL, Shi XH, Tjian R, Lionnet T, Singer RH: CASFISH: CRISPR/Cas9-mediated in situ labeling of genomic loci in fixed cells. *Proc Natl Acad Sci USA* 112:11870–11875 (2015).
- Doležel J, Binarová P, Lucretti S: Analysis of nuclear DNA content in plant cells by flow-cytometry. *Biol Plant* 31:113–120 (1989).
- Doležel J, Čiháliková J, Lucretti S: A high-yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba* L. *Plant* 188:93–98 (1992).
- Dreissig S, Schiml S, Schindele P, Weiss O, Rutten T, et al: Live-cell CRISPR imaging in plants reveals dynamic telomere movements. *Plant J* 91:565–573 (2017).
- Ishii T, Schubert V, Khosravi S, Dreissig S, Metjesprink J, et al: RNA-guided endonuclease – in situ labelling (RGEN-ISL): a fast CRISPR/Cas9-based method to label genomic sequences in various species. *New Phytol* 222: 1652–1661 (2019).
- Klemme S, Banaei-Moghaddam AM, Macas J, Wicker T, Novak P, Houben A: High-copy sequences reveal distinct evolution of the rye B chromosome. *New Phytol* 199:550–558 (2013).
- Monkheang P, Chaveerach A, Sudmoon R, Tanee T: Karyotypic features including organizations of the 5S, 45S rDNA loci and telomeres of *Scadoxus multiflorus* (Amaryllidaceae). *Comp Cytogenet* 10:637–646 (2016).
- Peacock WJ, Dennis ES, Rhoades MM, Pryor AJ: Highly repeated DNA sequence limited to knob heterochromatin in maize. *Proc Natl Acad Sci USA* 78:4490–4494 (1981).
- Wear EE, Song J, Zynda GJ, LeBlanc C, Lee TJ, et al: Genomic analysis of the DNA replication timing program during mitotic S phase in maize (*Zea mays*) root tips. *Plant Cell* 29: 2126–2149 (2017).
- Weisshart K, Fuchs J, Schubert V: Structured illumination microscopy (SIM) and photoactivated localization microscopy (PALM) to analyze the abundance and distribution of RNA polymerase II molecules on flow-sorted *Arabidopsis* nuclei. *Bio-protocol* 6:e1725 (2016). <http://www.bio-protocol.org/e1725>
- Zhu M, Du P, Zhuang L, Chu C, Zhao H, Qi Z: A simple and efficient non-denaturing FISH method for maize chromosome differentiation using single-strand oligonucleotide probes. *Genome* 60:657–664 (2017).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.H. designed the experiments. A.N., C.W., and V.S. conducted the study and processed the data. A.N., C.W. and A.H. wrote the manuscript. A.N., C.W., V.S., T.I., E.H., and A.H. discussed the results and contributed to manuscript writing. All authors read and approved the final manuscript.