HuluFISH non-denaturing in situ detection of genomic DNA opened by CRISPR-Cas9 Nickase and Exonuclease

Kang Han¹, Sheng Liu¹, Yongsheng Cheng^{1*}

*Corresponding author: yongsheng.cheng@pixelbiotech.com

1. PixelBiotech GmbH, Waldhofer str. 104, 69123, Heidelberg, Germany

Abstract

DNA fluorescence in situ hybridization (FISH) has been widely used in diagnosis and genetic research. Traditional Bacterial artificial chromosome (BAC) or oligonucleotide based probe to detect DNA in situ is only effective when the target is relatively large, usually over 150Kb DNA fragments. And it involves heat denaturation steps to open the DNA for in situ hybridization. The heat process can affect the fine structure of nuclei. Here we reported a novel method based on Cas9 nickase and exonuclease digestion of double strand DNA and permanently mark the DNA in single strand state for FISH. With this novel design, we detected non-repetitive genomic loci as small as 2Kb.

Introduction

DNA FISH is a powerful technique in detecting chromosomal structures and has been widely used in study chromatic structure and clinical diagnosis of chromosomal abnormalities. However, the DNA FISH needs the genomic DNA to be opened by heat denaturation and relatively long target region to generate sufficient hybridization signal for detection. The usual BAC (Bacterial Artificial Chromosome) based DNA FISH probe spans dozens of kilobases (kb) or even hundreds of kb in genomic DNA. For fine chromatic structure study, the heat denaturation and long target region sometimes are prohibitive, especially when the target region is less than 10 kb like an exogenous gene introduced into the genome in cell and gene therapy.

The CRISPR-Cas9 (*Streptococcus pyogenes*) system has been extensively explored in studying genome structure both in vivo and in vitro (Chen et al., 2013; Deng et al., 2015; Ishii et al., 2019; Knight et al., 2015; Wang et al., 2021). The CRISPR-Cas9-sgRNA ribonucleoprotein complex can target a specific loci on genomic and label or open genome for hybridization.

Here we are presenting a novel method (CRISPR-ExoFISH) based on the CRISPR-Cas9 system to open the genome loci and digest the specific loci to be single strand DNA (ssDNA) for permanent accessibility for DNA FISH probes (Figure 1). Once the DNA is opened by CRISPR-Cas9 (H840A mutant) on the target strand (TS), a T7 exonuclease starts to digest the non-targeting strand (NTS) of the CRISPR-Cas9 system and leave the targeting strand intact for hybridization. For the following FISH probe hybridization, heat denaturation is not needed and a

shorter region below 5kb is sufficient for the DNA FISH probe to bind and generate specific signals.

Figure 1. CRISPR-ExoFISH workflow. Genomic DNA loci is binded by Cas9 nickase (H840A)-sgRNA (cyan) ribonucleoprotein complex and cut at NTS strand (grey). Exonuclease find the flapped DNA on NTS strand and digest the NTS stand. Then HuluFISH probe is hybridizing to the TS stand (red).

Method and Materials

sgRNA synthesis by in vitro transcription (IVT)

sgRNA was produced with the ENGen SgRNA synthesis kit (NEB, E3322). sgRNA guide sequences were designed by customized script at PixelBiotech GmbH for Human Telomere, alpha-satellite DNA (ASAT), and the non-repetitive region of MUC4 gene (Intron 1, 2kb long target). Target-specific DNA oligo templates were designed according to the protocol for ENGen SgRNA synthesis kit. The sgRNA IVT was following the protocol from the kit. For multiple sgRNA synthesis in one reaction, the total concentration of oligo DNA template was adjusted to 250nM final concentration. The sgRNA was purified with the Monarch RNA Cleanup kit (NEB, T2040).

CRISPR-ExoFISH

Hela cells were fixed by cold methanol:acetic (3:1, stored at -20°C) for 10 min and stored at -20°C or immediately used. RNase A (NEB, T3018) treatment at 0.1mg/ml in the 1xPBS for 30 min at 37°C is optional to remove background staining from cellular RNA. After RNase A treatment, the cells were washed with 1xPBS at 65°C for 10 min each, 3 times. Cas9-sgRNA ribonucleoprotein complex was reconstituted in binding buffer (20mM HEPES, pH 7.5, 100mM KCl, 5mM MgCl₂, 5% (v/v) Glycerol, 0.1% (v/v) Tween-20, 1% (w/v) Bovine serum albumin (BSA), 1mM Dithiothreitol (DTT), 0.4U/ul RNase inhibitor Murine (NEB, M0314)) at the concentration of 200nM each (1:1 ratio for Cas9:sgRNA concentration) for 10 min at room temperature. Fixed cells were washed with the binding buffer for 10 min at room temperature. Then the cells were incubated with 200nM Cas9-sgRNA ribonucleoprotein complex in the binding buffer with 1U/ul T7 exonuclease (NEB, M0263) for 1 hour at 37°C. Then the cells were washed with 1xPBS for 5 min. After washing with 1xPBS for 3 times in 5 min, cells were hybridized with a 320nM HuluFISH probe in HuluHyb (PixelBiotech GmbH, PB102) for 1 hours at 37°C. After hybridization, the cells were washed with 2xSSC, 20% formamide at 37°C for 10 min for two times, and then washed with 1xPBS for 5 min before mounting in the VectorShield HardSet (Vector Laboratories, H-1400). Stained cells were imaged under Leica TCS SP8 confocal microscope.

Results

We tested CRISPR-ExoFISH on 3 targets in human cells. In Figure 2A-B, telomere sgRNA were designed against the repeat TAAGGG in Human telomeres (sequence details in table 1). Without sgRNA and Cas9, HuluFISH probes for telomeres could not bind it all onto the genome without heat denaturation. In the facilitation of Cas9-sgRNA complex, HuluFISH probes efficiently hybridize to the telomere region. This indicates the ssDNA nature of telomeres after Cas9-sgRNA binding and T7 exonuclease digestion. Another repeat region in the genome, alpha satellite DNA (ASAT) was chosen for further validation of this novel method (Figure 2C-D). All repeated genomic regions show specific staining compared to the negative control without Cas9-sgRNA opening and digesting the genomic DNA.

Despite the repeat region, CRISPR-ExoFISH was also employed to detect non-repetitive genomic loci. MUC4 intron 1 was chosen for the validation (Figure 2E-F). A 2kb region was selected for sgRNA design and 9 sgRNAs were selected for in vitro transcription and assembly with Cas9. Additionally 63 HuluFISH probes were designed for the 2kb region in MUC4. The results showed specific staining of the MUC4 gene intron 1.

Figure 2. CRISPR-ExoFISH of Human genomic targets. (A, C, E) HeLa cell stained with HuluFISH probe for telomere, ASAT, and MUC4 respectively. (B, D, F) HeLa cell were fixed and stained with HuluFISH probe against Human telomere, ASAT, and MUC4 respectively, after genomic DNA of telomere, ASAT and MUC4 is opened and digested as ssDNA.

References

- Chen, B., Gilbert, L.A., Cimini, B.A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E.H., Weissman, J.S., Qi, L.S., et al. (2013). Dynamic Imaging of Genomic Loci in Living Human Cells by an Optimized CRISPR/Cas System. *Cell* 155, 1479–1491.
- Deng, W., Shi, X., Tjian, R., Lionnet, T., and Singer, R.H. (2015). CASFISH: CRISPR/Cas9-mediated in situ labeling of genomic loci in fixed cells. *Proc. Natl. Acad. Sci.* 112, 11870–11875.
- Ishii, T., Schubert, V., Khosravi, S., Dreissig, S., Metje-Sprink, J., Sprink, T., Fuchs, J., Meister, A., and Houben, A. (2019). 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.
- Knight, S.C., Xie, L., Deng, W., Guglielmi, B., Witkowsky, L.B., Bosanac, L., Zhang, E.T., Beheiry, M.E., Masson, J.-B., Dahan, M., et al. (2015). Dynamics of CRISPR-Cas9 genome interrogation in living cells. *Science* 350, 823–826.
- Wang, Y., Cottle, W.T., Wang, H., Feng, X.A., Mallon, J., Gavrilov, M., Bailey, S., and Ha, T. (2021). Genome oligopaint via local denaturation fluorescence in situ hybridization. *Mol. Cell* 81, 1566–1577.e8.

Table 1 Sequence of oligos used in this study.

Sequence	Name	Note
TTCTAATACGACTCACTATAGGGATGAATaagggttaagggttaagggttaaGTTTTAGAGCTAGA	Telomere_gRNA	Oligo DNA template for sgRNA in Telomere
TTCTAATACGACTCACTATAGGGATGAATActttttagtagaatctgcaagGTTTTAGAGCTAGA	ASAT_gRNA	Oligo DNA template for sgRNA in alpha satellite
TTCTAATACGACTCACTATAGGGATGAATAgcgatgtctggaaggatccaGTTTTAGAGCTAGA	MUC4NR_gRNA_7	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAggtgagacgcagacaaactgGTTTTAGAGCTAGA	MUC4NR_gRNA_210	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAttgggtcaccgacctgctgGTTTTAGAGCTAGA	MUC4NR_gRNA_410	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATActggggtccagagttcaagcGTTTTAGAGCTAGA	MUC4NR_gRNA_620	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAcaggactaggctgagggcaaGTTTTAGAGCTAGA	MUC4NR_gRNA_821	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAgaggctgggcttggggcgcGTTTTAGAGCTAGA	MUC4NR_gRNA_1025	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAaagctgagcccaaagtgtgctGTTTTAGAGCTAGA	MUC4NR_gRNA_1225	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATActgagtccttgcgtcgctaGTTTTAGAGCTAGA	MUC4NR_gRNA_1434	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAacctgcctatacttgagtctGTTTTAGAGCTAGA	MUC4NR_gRNA_1662	Oligo DNA template for sgRNA in MUC4 intron 1
TTCTAATACGACTCACTATAGGGATGAATAgatggaaatttggtgtctacGTTTTAGAGCTAGA	MUC4NR_gRNA_1870	Oligo DNA template for sgRNA in MUC4 intron 1
TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG	HuluFISH_Telomere	HuluFISH probe sequence for Telomere
CTCACAGAGTTGAACCTTCCT	ASAT_GSO_AS	HuluFISH probe sequence for alpha satellite DNA
CCCAGGCTTACTCGCAGA	MUC4_NR	HuluFISH probe MUC4 intron

	_52	1
GAGAAAGACAGGCAGAGC	MUC4_NR	HuluFISH probe MUC4 intron
	_70	1
CAGAGCAAGAGGAACAGAGTC	MUC4_NR	HuluFISH probe MUC4 intron
	_91	1
AAGGAGAAAGATGTACACCC	MUC4_NR	HuluFISH probe MUC4 intron
	_111	1
TTGTGTACAGAGCTGGGGG	MUC4_NR	HuluFISH probe MUC4 intron
	_130	1
TAGAGGGGATGCCAGGAAAG	MUC4_NR	HuluFISH probe MUC4 intron
	_150	1
CTGGGTGATGGAGACGGAAG	MUC4_NR	HuluFISH probe MUC4 intron
	_170	1
AACTCATGTAAAGCTGCAGGG	MUC4_NR	HuluFISH probe MUC4 intron
	_193	1
AGGACCCTGGGAATGGAGA	MUC4_NR	HuluFISH probe MUC4 intron
	_247	1
GAGGAGAAGATCGGGAG	MUC4_NR	HuluFISH probe MUC4 intron
	_264	1
CAGCAAGCAAGGGAAGC	MUC4_NR	HuluFISH probe MUC4 intron
	_285	1
ACAAGGAGGAGAGGGGCA	MUC4_NR	HuluFISH probe MUC4 intron
	_304	1
CCGTGGGCTTCTTACCTCTG	MUC4_NR	HuluFISH probe MUC4 intron
	_385	1
AGCTCGGGTTTAAAAGC	MUC4_NR	HuluFISH probe MUC4 intron
	_402	1
CCTTGGGCTCCTCCGTGTACA	MUC4_NR	HuluFISH probe MUC4 intron
	_471	1
CCAGGCCTGTGGGAGATGTT	MUC4_NR	HuluFISH probe MUC4 intron
	_511	1
CCCGTCCTCTTCCCCACACTT	MUC4_NR	HuluFISH probe MUC4 intron
	_544	1
GCTGTCCCTCTGGCTTCA	MUC4_NR	HuluFISH probe MUC4 intron
	_567	1
ATTCTGTGTTTCTGGGTGTC	MUC4_NR	HuluFISH probe MUC4 intron

	_599	1
TGAGTCTTTGGGGGAGAGT	MUC4_NR	HuluFISH probe MUC4 intron
	_618	1
TGGGGTTAGAGTCTCAGCTCC	MUC4_NR	HuluFISH probe MUC4 intron
	_659	1
CCTCTCAGCAGGCTAAGAACA	MUC4_NR	HuluFISH probe MUC4 intron
	_687	1
GTCGCCGAGGGAAAATATTTTC	MUC4_NR	HuluFISH probe MUC4 intron
	_708	1
TTGGGCGCATATTTGAGGAG	MUC4_NR	HuluFISH probe MUC4 intron
	_728	1
CTTCCTGGGAGTGAGTCAGA	MUC4_NR	HuluFISH probe MUC4 intron
	_748	1
CGAGTGCCGTTTAAAGG	MUC4_NR	HuluFISH probe MUC4 intron
	_768	1
CTGCAAGAGAAGCCATGCTG	MUC4_NR	HuluFISH probe MUC4 intron
	_788	1
CCTTCCACCTCGGGATGTT	MUC4_NR	HuluFISH probe MUC4 intron
	_818	1
GCTCCAGTGCTCATCCCA	MUC4_NR	HuluFISH probe MUC4 intron
	_929	1
TGCCCTGCAGATGTGGTTG	MUC4_NR	HuluFISH probe MUC4 intron
	_989	1
GGAGTCTCCACGGGAATCA	MUC4_NR	HuluFISH probe MUC4 intron
	_1010	1
CGGGGAGGCAGCTTGGATTT	MUC4_NR	HuluFISH probe MUC4 intron
	_1063	1
CCCCAGGGCCAAGTCTTT	MUC4_NR	HuluFISH probe MUC4 intron
	_1086	1
GCCGTGAACTGTTCTGG	MUC4_NR	HuluFISH probe MUC4 intron
	_1103	1
GCCCCGGTCCCCTGTGTAAAA	MUC4_NR	HuluFISH probe MUC4 intron
	_1160	1
AGCAGTGGTGAACGGTTGG	MUC4_NR	HuluFISH probe MUC4 intron
	_1179	1
ACCTCCTGACGCCCAAGTT	MUC4_NR	HuluFISH probe MUC4 intron

	_1198	1
CTGGACACTCAGCTCCATGT	MUC4_NR	HuluFISH probe MUC4 intron
	_1272	1
CAGGAAGTGGGGTCTGTGGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1297	1
ACCTGGGGTGGGCGTGGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1338	1
CGTGACCCGGAGAAGGAGTGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1368	1
AGGACTGTTGGTGTGCAAGG	MUC4_NR	HuluFISH probe MUC4 intron
	_1388	1
GTCTCCATGACGACCCGAA	MUC4_NR	HuluFISH probe MUC4 intron
	_1409	1
GAAGCTAGGCATGTCGTGGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1429	1
AAGTGGAGCTGGGCCAGGAGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1481	1
GAGATGGTCGTGGCTGGGAGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1503	1
TGGCACCCACACATCTGAC	MUC4_NR	HuluFISH probe MUC4 intron
	_1522	1
CTTGGCAGGAAAAGCAGTCAC	MUC4_NR	HuluFISH probe MUC4 intron
	_1559	1
CCAGCCAGCCCCGTTCTTTT	MUC4_NR	HuluFISH probe MUC4 intron
	_1635	1
CCTGAAGGGGTTTACAGATG	MUC4_NR	HuluFISH probe MUC4 intron
	_1660	1
AGGGTCTGTTTGCACACTTG	MUC4_NR	HuluFISH probe MUC4 intron
	_1700	1
CCAGGCTGGGTCACACTGAA	MUC4_NR	HuluFISH probe MUC4 intron
	_1736	1
GCCCAGGCCAGAGGAAAAA	MUC4_NR	HuluFISH probe MUC4 intron
	_1755	1
CACAGGGTTTCCACAAAG	MUC4_NR	HuluFISH probe MUC4 intron
	_1773	1
GCTGCCGCAATGAGGGTTT	MUC4_NR	HuluFISH probe MUC4 intron

	_1794	1
CCTTAAGGAACAGCCCTGGC	MUC4_NR	HuluFISH probe MUC4 intron
	_1814	1
CAAGGGTTAAAGGATAAGGC	MUC4_NR	HuluFISH probe MUC4 intron
	_1837	1
CAGCAGACAGAGGTGGGCTA	MUC4_NR	HuluFISH probe MUC4 intron
	_1858	1
CAGGCAGGAATGACTCAGA	MUC4_NR	HuluFISH probe MUC4 intron
	_1916	1
CCGTCCTGGTTCCATGCCACA	MUC4_NR	HuluFISH probe MUC4 intron
	_1948	1
GGCAACTGGGTCGAAATAGG	MUC4_NR	HuluFISH probe MUC4 intron
	_1974	1
CCTTGGTCTCCAGCACTATCA	MUC4_NR	HuluFISH probe MUC4 intron
	_1995	1