Visualizing DNA Methylation (5mC) on a Specific Genomic Locus (*SEPTIN 9*) in Individual Human Cancer Cells using In-situ Hybridization and Proximity Ligation Assays

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Abstract

Although there are many techniques to study epigenetic marks such as DNA- and histone-methylation, on a genomic scale, there exists a need in the field to visualize these epigenetic marks at a single genomic locus in individual cells. Such an application requires a highly sensitive detection method. With this aim, a protocol was developed to perform in-situ hybridization (ISH) followed by proximity ligation assay (a.k.a. Duolink®) and cell imaging to visualize DNA-methylation (5meC) on the SEPTIN 9 promoter. SEPTIN 9 promoter methylation is a known biomarker for colon cancer[1]. After optimizing cross-linking, cell permeabilization and chromatin accessibility, the genomic specificity was ascertained by hybridizing with a pool of biotinylated-oligo probes that target the CpG islands in the human SEPTIN 9 promoter. The Duolink assay was performed using anti-biotin and anti-5meC antibodies, corresponding proximity ligation assay probes, and Far Red detection reagents.

Imaging by fluorescent microscopy revealed two red punctate spots in metastatic prostate cancer DU145 cells (diploid for chromosome 17 – location of SEPTIN 9 gene) and three red spots in colon cancer SW480 cells (triploid for Chr17). No signal was observed in normal cells (BJ) or with non-specific oligo probes (LacZ). A decrease in Duolink signal was observed when the DU145 cells were treated with 5-AzaC, a drug known to block DNA-methylation. This proof of concept study will be extended to frozen and formalin fixed paraffin embedded human cancer tissue samples.

Fluorescent imaging data from a Duolink assay was used to monitor the interaction of EZH2 histone methyltransferase with the H3K27me3 epigenetic mark in prostate cancer (DU145) cells. Reduction in the Duolink signal demonstrated inhibition of EZH2 activity by the small molecule inhibitors SAHA, Panobinostat and GSK343.

ISH-Duolink[2] Assay Workflow

Grow cells on cover slips with silicone gasket

↓

Cross-link cells with 4% para-formaldehyde

↓

Permeabilize cells with Permeabilization buffer*

↓

Denature cells with Denaturing solution*, neutralize with PBS

↓

Protease treatment* for 1 minute, inactivate and quench protease

↓

Block wells with Duolink blocking buffer

↓

Block wells with hybridization buffer*

↓

Co-denature biotinylated-oligo probes and cellular DNA at 80º C, 5 min. on Thermobrite

↓

Hybridize at 37º C overnight (16 hours) on Thermobrite

↓

Wash with 2X SSC, 0.1% NP40

↓

Block with Duolink blocking buffer

↓

Primary Ab's: O/N @ 4º C with anti-Biotin (Rb) and **anti-5MeC** (Ms) Ab's, washes

↓

Secondary Ab's-PLA probes: anti-Rb-(+PLA probe) and anti-Ms-(-PLA probe), washes

↓

Ligation, washes

↓

Amplification, washes

↓

Imaging on fluorescent microscope

* **Novel**

Sigma Duolink® products available at: http://sigma.com/duolink

Results and Discussion

SEPTIN 9 Probes

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GCGTCGCCCGTCCCTGGCTTCTCTGACAGCCGTGTTCCATCCCCGCCCTG
TGCCCCTTCTCCCGGACAGTGCCTTCTCCAGGGCTCACCCAGGAGGGTGC
AGCGGTGGCCCCCGGGGCGGTGGTCGTGGTGGGGGTGTTAGCTGCAGGGG
TGCCCTCGGTGGGTGGGAGTTGGTGGCCTCTCGCTGGTGCCATGGGACTC
GCATGTTCGCCCTGCGCCCCTCGGCTCTTGAGCCCACAGGCCGGGATCCT
GCCTGCCAGCCGCGTGCGCTGCCGTTTAACCCTTGCAGGCGCAGAGCGCG
CGGCGGCGGTGACAGAGAACTTTGTTTGGCTGCCAAATACAGCCTCCTG
CAGAAGGACCCTGCGCCCGGGGAAGGGGAGGAATCTCTTCCCCTCTGGGC
GCCCGCCCTCCTCGCCATGGCCCGGCCTCCACATCCGCCCACATCTGGCC P7
GCAGCGGGGCGCCCGGGGGGAGGGGCTGAGGCCGCGTCTCTCGCCGTCCC
CTGGGCGCGGGCCAGGCGGGGGAGGAGGGGGGCGCTCCGGTCGTGTGCCCA
CpG GGACTGTCCCCCAGCGGCCACTCGGGCCCCAGCCCCCCAGGCCTGGCCTT
Island GACAGGCGGGCGGAGCAGCCAGTGCGAGACAGGAGAGCCGGTGCGGGTGC
GGGAACCTGATCCGCCCGGGAGGCGGGGGCGGGGCGGGGGCGCAGCGCGC
GGGGAGGGGCCGGCGCCCGCCTTCCTCCCCCATTCATTCAGCTGAGCCAG P8
GGGGCCTAGGGGCTCCTCCGGCGGCTAGCTCTGCACTGCAGGAGCGCGGG
CGCGGCGCCCCAGCCAGCGCGCAGGGCCCGGGCCCCGCCGGGGGCGCTTC
CTCGCCGCTGCCCTCCGCGCGACCCGCTGCCCACCAGCCATCATGTCGGA
CCCCGCGGTCAACGCGCAGCTGGATGGGATCATTTCGGACTTCGAAGGTG
GGTGCTGGGCTGGCTGCTGCGGCCGCGGACGTGCTGGAGAGGACCCTGCG
GGTGGGCCTGGCGCGGGACGGGGGTGCGCTGAGGGGAGACGGGAGTGCGC
TGAGGGGAGACGGGACCCCTAATCCAGGCGCCCTCCCGCTGAGAGCGCCG
CGCGCCCCCGGCCCCGTGCCCGCGCGCCGCCTACGTGGGGGACCCTGTTAGG
GGCACCCGCGTAGACCCTGCGCGCCCTCACAGGACCCTGTGCTCGTTCTG
CGCACTGCCGCCTGGGTTTCCTTCCTTTTATTGTTGTTTGTGTTTGCCAA
GCGACAGCGACCTCCTCGAGGGCTCGCGAGGCTGCCTCGGAACTCTCCAG
GACGCACAGTTTCACTCTGGGAAATCCATCGGTCCCCTCCCTTTGGCTCT
CCCCGGCGGCTCTCGGGCCCCGCTTGGACCCGGCAACGGGATAGGGAGGT
CGTTCCTCACCTCCGACTGAGTGGACAGCCGCGTCCTGCTCGGGTGGACA
GCCCTCCCCTCCCCCACGCCAGTTTCGGGGCCGCCAAGTTGTGCAGCCCG
TGGGCCGGGAGCACCGAACGGACACAGCCCAGGTCGTGGCAGGGTCTAGA
GTGGGATGTCCCATGGCCCCCATCCAGGCCTGGGGATATCCTCATCCGCC
TCCCAGAATCGGGCCGTGGGGGACAGAAGGGGCCTGCGTGCGGGCAGGGA
GAGTATTTTGGCTCTCTCCTGTCTTCGGGGTTTACAAAGTGTGTTGGGAC
TTGCGGGGCTGCTCTGTCCAAGCCTGGGTCTGGCGTCCGCGTCTCTGAGC
CTGTGAGTGCGTGCGCTTTCCTGCGTCCTCTTGACTGCCGGTGCTGGGGC
TCTGCGTCCTGCGTCCGCG
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Figure 1. ISH SEPTIN 9 probes. 18 Oligo probes with 3'TEG-Biotin were designed using Stellaris FISH probe designer (Biosearch Technologies) targeting the human Septin 9 promoter (1819 bp). The CpG island is shown in cyan and the probes are in red. Probes # 7 and 8 (P7,P8) flanked the CpG island.

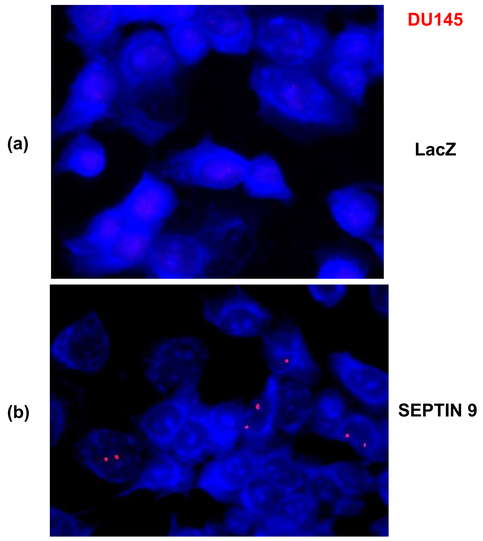

Figure 2. Septin 9 promoter methylation visualized by ISH-Duolink in prostate cancer cells (DU145). Two punctate red dots were seen in prostate cancer cells hybridized with Septin 9 probes (b) but not with Lac Z probes (a). DU145 cells are diploid for chromosome 17, where the Septin 9 gene is located, indicating both copies of the promoter are methylated. No signals were observed with normal cells (BJ-foreskin fibroblast) or pancreatic cancer cells (BxPC3).

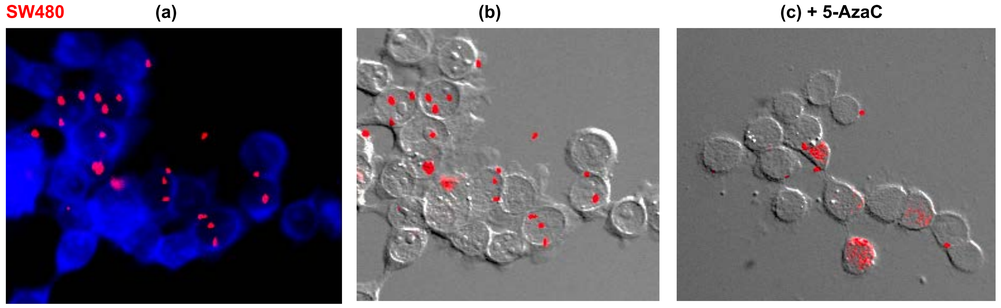

Figure 3. Septin 9 promoter methylation visualized by ISH-Duolink in colon cancer cells (SW480). Three punctate red dots were seen in colon cancer cells hybridized with Septin 9 probes (a, b). SW480 cells are triploid for chromosome 17, where the Septin 9 gene is located, indicating all three copies of the promoter are methylated. These signals were dramatically reduced to non-specific levels in cells following 5-Azacytidine (5-AzaC) treatment (c). 5-AzaC is a known inhibitor of DNA methylation.

Acknowledgments

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EZH2 + H3K27me3 Duolink Assay

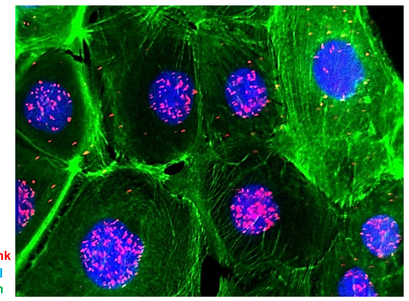


Figure 4. Interaction between EZH2 and H3K27me3 was visualized by Duolink in prostate cancer cells (DU145). Duolink signal in red was overlaid with nuclei stained in blue with DAPI and actin fibers stained in green with Phalloidin-Atto (SIGMA 49409).

Duolink assay to monitor small molecule inhibition of EZH2 in individual cells

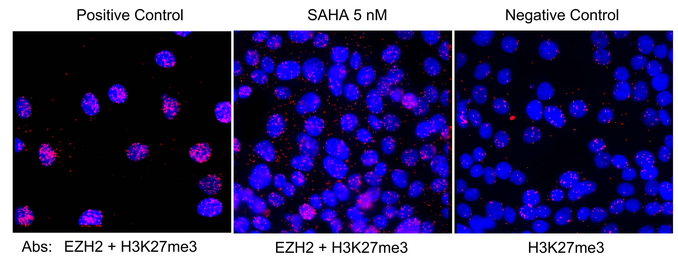
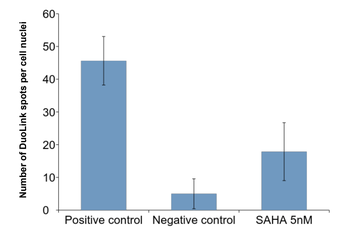


Figure 5. Inhibition of EZH2 and H3K27me3 interaction by small molecules (SAHA and GSK343) was monitored by Duolink in prostate cancer cells (DU145). DU145 cells were treated with SAHA (5 nM, 3 days) and then Duolink assay was performed using EZH2 and H3K27me3 antibodies. High concentration of H3K27me3 antibody alone gave some background Duolink signal (right panel). GSK343 (500 nM) and Panobinostat (50 nM) gave similar results (data not shown).



Key Observations

- DNA methylation on a specific genomic locus can be visualized in individual cancer cells using the ISH-Duolink assay.
- Two punctate red dots were observed in prostate cancer cells DU145 (diploid for chromosome 17 – where Septin 9 is located) and three punctate red dots were observed in colon cancer cells SW480 (triploid for chromosome 17).
- Septin 9 promoter methylation was abolished following 5-AzaC treatment
- Duolink assay was also used to monitor small molecule inhibition of EZH2-H3K27me3 interactions in DU145 cells treated with SAHA, Panobinostat and GSK343.
- Future applications under development include analysis of frozen and FFPE human tissue samples for cancer diagnostics.

References

1. Wasserkort *et. al. BMC Cancer* 2013, **13**:398. Aberrant septin 9 methylation in colorectal cancer is restricted to a single CpG island.
2. Gomez *et. al. Nature Methods* 2013, **10**:171. DOI:10.1038/NMETH.2332 Detection of histone modifications at specific gene loci in single cells in histological sections.

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