NicE-viewSeq: an integrative visualization and genomics method to detect accessible chromatin in fixed cells

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Abstract

A novel genome-wide accessible chromatin visualization, quantitation and sequencing method is described which allows in situ fluorescence visualization and sequencing of the accessible chromatin in the mammalian cell. The cells are fixed by formaldehyde cross-linking, and processed using a modified nick translation method, where a nicking enzyme nicks one strand of DNA, and DNA polymerase incorporates biotin-conjugated dCTP, 5-methyl-dCTP, Fluorescein-12-dATP or Texas Red-5-dATP, dGTP and dTTP. This allows accessible chromatin DNA to be labeled for visualization and on bead NGS library preparation. This technology allows cellular level chromatin accessibility quantification and genomic analysis of the epigenetic information in the chromatin, particularly accessible promoter, enhancers, nucleosome positioning, transcription factor occupancy, and other chromosomal protein binding.

1. Introduction

Eukaryotic chromosomes are built of packaged chromatin fibers, a complex made of DNA, histone and chromatin associated proteins. Each chromosome maintains its territory in the nucleus. During development, spatial separation of active and inactive fractions of the genome in the cell nucleus is crucial for gene expression (1-3). Depending on the transcriptional activity and degree of compaction, chromatin can be functionally and structurally distinguished to two types, euchromatin and heterochromatin, and are spatially separated within the nucleus. The euchromatin is less condensed and enriched in genes encompassing the active fraction of the genome. It is less densely packed, and therefore more accessible to the transcriptional protein machineries. This fraction of the genome is also known as accessible chromatin (4). The other fraction is heterochromatin, highly condensed and generally contain few genes and mostly transcriptionally inactive (5). This general pattern of the nuclear genome organization described above is found in virtually all eukaryotic cell types. For nuclear structure studies, particularly specific protein or DNA staining, including labeling technologies for visualization is routinely performed. Specific DNA sequences in an intact nucleus can be probed and visualized by hybridization with fluorescently labelled DNA probes with a complementary sequence. This technique is called fluorescence in situ hybridization (FISH) (6-8). In this case the hybridization probe is itself made fluorescent, generally by the incorporation of FITC, rhodamine or far-red fluorophore conjugated deoxynucleotides that would fluorescent upon excitation. Alternatively, modified nucleotides (e.g., bromodeoxyuridine), or conjugated deoxynucleotides may be used, that could be further revealed by fluorescence conjugated antibodies to the modified nucleotide using immunocytochemistry/histochemistry methodologies. Apart from sequence specific interrogation, total nuclear DNA can be fluorescently labelled using a number of fluorescent DNA binding dye, such as DAPI or Hoechst, although these dyes exhibit non-specific affinity for RNA, as well as exhibit DNA sequence preferences (9-12).

Labeling accessible chromatin was demonstrated for the first time using a modified assay of transposase-accessible chromatin using a hyperactive Tn5 transposon, that allowed direct imaging of the accessible chromatin in situ, and deep sequencing to reveal the identity of the imaged DNA elements. The basic principle relied on a prokaryotic Tn5 transposon, that is loaded with fluorescently labeled sequencing adapters creating an active dimeric transposome complex. The complex can provide the cut in the accessible chromatin, and simultaneous ligation of the cargo sequences. This technology is known as ATAC-see and it provides information that other chromatin accessible technologies such as DNase-seq, MNase-seq, FAIRE-seq, ATAC-seq alone or NicE-seq could not generate (13-19). Indeed, ATAC-see is a direct modification of ATAC-seq technology (13, 18). ATAC-see has been used in a variety of studies with protocol optimization (20, 21).

We subsequently modified our universal nicking enzyme assisted sequencing (UniNicE-seq) for direct fluorescent labeling and sequencing of accessible chromatin genome-wide (22). The modified UniNicE-seq protocol included fluorescein-dATP or Texas Red–5-dATP in the mix to allow sequential visualization, cell sorting, and sequencing of the accessible regions from the same sample. We named this technology, Nicking enzyme-assisted viewing and sequencing (NicE-viewSeq) (23). It is a versatile method that combines quantitative imaging and sequencing of the accessible chromatin regions across various cell types using NGS. This technology has been used to study HDAC inhibitor effect on chromatin accessibility in both quantitatively and using NGS (Fig. 1-2). Here, we describe a stepwise protocol for NicE-viewSeq on mammalian culture cells that is fixed by formaldehyde.

2. Materials

Prepare all solutions using ultrapure water (example: milliQ water or equivalent by purifying deionized water, to attain a conductivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare all reagents at room temperature and store at 4 ˚C (unless indicated otherwise). Formaldehyde must be handled with gloves and in a laboratory fume hood.

2.1. Crosslinking cells on micro cover glass

1. For HCT116 cells: Culture in McCoy's 5A medium (Thermo Fisher Scientific #16600082) supplemented with 10% Fetal Bovine Serum (GemCell #100-500); for MCF7, HeLa cells use DMEM medium (Cytiva #SH30285.1) supplemented with 10% Fetal Bovine Serum (GemCell #100-500). For HUT 78 cells use RPMI 1640 medium (Thermo Fisher Scientific #61870036) supplemented with 10% Fetal Bovine Serum (GemCell #100-500). Grow cells on micro cover glass (VWR #48366067) in a 6 well plate format. HUT 78 cells were grown in suspension and spun at 1,000 rpm on micro cover glass before fixation with formaldehyde.
2. 16 % formaldehyde (w/v), methanol free (Thermo Fisher Scientific #28908)
3. 1X PBS (Thermo Fisher Scientific #70011-044)
4. 2.5 M Glycine (Sigma-Aldrich #G7126)

2.2. Accessible chromatin labeling

1. Prepare cytosolic buffer: 15 mM Tris-HCl pH 7.5, 5 mM MgCl2, 60 mM KCl, 15 mM NaCl, 0.1% Tween-20 and 300 mM sucrose. Add 0.5 mM fresh DTT (NEB #B7705S).
2. Prepare a 10x dNTP mix: 240 μM dATP, 180 μM 5-methyl-dCTP (NEB #N0356S), 60 μM biotin-14-dCTP (Thermo Fisher Scientific #19518018), 300 μM dGTP, 300 μM dTTP and 60 μM of Fluorescein-12-dATP (PerkinElmer #NEL465001EA) or Texas Red-5-dATP (PerkinElmer #NEL471001EA).
3. Prepare 800 μl of accessible chromatin labelling buffer for one well for a 6 well plate: 80 μl of 10X NEBuffer^{TM} 2 (NEB #B7002S), 2.5 U of Nt.CviPII (NEB #R0626S), 50U of DNA Polymerase I (NEB #M0209S) with 30 μM of each dNTP including 6 μM of biotin-dCTP, 6 μM of Fluorescein-12-dATP (PerkinElmer #NEL465001EA) or 6 μM of Texas Red-5-dATP (PerkinElmer #NEL471001EA). Fluorescent dNTPs will be used for visualization, biotin-dCTP for on bead DNA library construction.
4. 37 °C Incubator
5. 0.5 M EDTA, pH 8.0 (Thermo Fisher Scientific #15575-038)
6. RNase A (Thermo Fisher Scientific #12091021)

2.3. Mounting slides and open chromatin index using Zeiss LSM 880 confocal microscope

1. Microscope slides (VWR Vista Vision #16004-368)
2. ProLong gold antifade reagent with DAPI (Thermo Fisher Scientific #P36935)
3. Visualization of Fluorescein-dATP or TexasRed-dATP and Hoechst using Argon 488, DPSS 561 and diode 405 nm laser respectively.

2.4. NicE-seq view library construction

1. 65 °C heat block
2. Proteinase K (NEB #P8107S)
3. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris,pH 8.0, 1 mM EDTA (Sigma-Aldrich #P3803)
4. Ethanol (Sigma-Aldrich #E7023)
5. Monarch Genomic DNA Purification Kit (NEB #T3010S)
6. Covaris S2 sonicator
7. Covaris microtubes (Covaris #500330)
8. NEB Ultra II DNA Library Prep Kit for Illumina (NEB #E7645)
9. PCR machine
10. Prepare High Salt Buffer: 10 mM Tris-HCl, pH 8, 2 M NaCl and 1 mM EDTA
11. Prepare High Salt Buffer with Triton X-100: 10 mM Tris-HCl, pH 8, 2 M NaCl, 1 mM EDTA and 0.05 % Triton X-100.
12. 1.5 ml DNA LoBind Eppendorf tubes (Eppendorf #022431021)
13. Gelatin from cold water fish skin (Sigma-Aldrich #G7765)
14. Streptavidin Magnetic Beads (NEB #S1420S)
15. Rocking platform/End-over end rotator
16. Nuclease free water (Thermo Fisher Scientific #AM9932)
17. NEBNext® Oligos for Illumina (NEB #E7335S or E7500S)
18. NEBNext® Sample Purification Beads (E7104S) or AMPure XP beads (Beckman Coulter #A63881)
19. 0.1X TE buffer (NEB #E7763AA)
20. Qubit fluorometer 2.0
21. Bioanalyzer

3. Methods

3.1. Crosslinking cells on micro cover glass

1. Grow from 5K to 1 million cells on micro cover glass in 2 ml media in a 6 well plate. Remove media at 50-70% confluency and add 937.5 μl 1X PBS per well. Add 62.5 μl of 16% formaldehyde to crosslink the cells for 10 min at RT on rocking platform.
2. Quench reaction by adding 125 mM glycine (52.5 μl of 2.5M stock) and incubate 5 min at RT on a rocking platform
3. Wash cells twice with 1x PBS

3.2. Accessible chromatin labeling

1. Add 1 ml of cytosolic buffer to the cells and incubate for 10 min at 4 °C.
2. Wash twice with 1X PBS, nuclei can be visualized under the microscope at this point (circular with smooth edges).
3. Add 800 μl of accessible chromatin labeling buffer for at least 30 min (can be extended up to 2 h) at 37 °C away from light.
4. Add 20 μl 0.5M EDTA and 2 μl RNase A to each well to stop the reaction. Incubate for 20 min at 37°C to digest RNA.
5. Remove supernatant and wash once with 1X PBS at 55 °C to remove autofluorescence background.
6. Wash twice with 1X PBS at RT.
7. Remove and dry the cover glass for at least 30 min at RT away from light.
8. Mount the coverslip on microscope slide using ProLong gold antifade reagent with DAPI. At this point, slides are ready to be visualized by confocal microscopy.

3.3. Mounting slides and open chromatin index using Zeiss LSM 880 confocal microscope

1. To visualize Fluorescein-dATP or TexasRed-dATP and DAPI, set up the 488 or 561 nm laser power to 2% and diode (405 laser) at 0.5% (Fig.1a).
2. Set up the frame size at optimal, scan time at 5-7 min, averaging number of image frames at 16 and bit depth at 16.
3. Histograms will give the mean pixel intensity for Fluorescein-dATP or TexasRed-dATP.
4. Open chromatin index (OCI) is defined by mean pixel intensity/number of nuclei (Fig.1b).

3.4. NicE-seq view library construction

1. After labeling chromatin on slides, cells can be removed using 1% SDS, 2mg/ml. proteinase K and 200 mM NaCl at 65 °C overnight to reverse crosslinking.
2. Genomic DNA can be extracted and purified using phenol/chloroform/isoamyl alcohol method or Monarch Genomic DNA purification kit.
3. Take 200-500 ng of genomic DNA and sonicate:
    a. Transfer to Covaris microtube and add up to 50 µL 1X TE buffer
    b. Sonicate using the following settings to obtain 150 bp fragments. To do this, insert the tube into the holder, and simply select "Open" and select the program named "Covaris 200 for 50µL" on the computer. Click "start".
        i. Intensity: 5
        ii. Duty Cycle: 10 %
        iii. Cycles per burst: 200
        iv. Treatment time: 2 min

4. DNA pull-down:
    a. Transfer the biotinylated fragmented DNA to DNA LoBind Eppendorf tubes
    b. For low # of cells add 15 µL of Blocked Streptavidin beads. For high # of cells add 30 µL of beads.
    c. Add 1 mL of 1X High Salt Buffer
    d. Incubate for 2 h at 4 °C on end-over end rotator.
    e. Place the eppendorf tube on the magnetic rack. When solution is clear, remove the liquid carefully using a pipet, and wash beads for 5 min with 1 mL cold High Salt Buffer containing 0.05% Triton X-100.
    f. Repeat the above wash steps 2 times
    g. Wash beads once with 1 mL 1X TE buffer for 5 min.
    h. Resuspend beads in 50 µL 1X TE buffer.

5. Prepare library:

    a. End-repair: Combine the following per sample
        i. 50 µL of fragmented DNA on Streptavidin beads from the above step
        ii. 3 µL of NEB Next Ultra II End Prep Enzyme Mix
        iii. 7 µL of NEB Next Ultra II End Prep Reaction Buffer

        Mix well and incubate at 20 °C for 30 min and at 65 °C for 30 min (Use a PCR machine)

    b. Adaptor Ligation:
        i. 60 µL of End Prep Reaction Mixture
        ii. 30 µL of NEB Next Ultra II ligation Master Mix
        iii. 1 µL of NEB Next ligation Enhancer

        iv. 1 µL of 1:10 diluted (recommended by NEB) NEB Next Adaptor for Illumina
        v. Incubate 2 h-O/N at RT.
        vi. Add 3 µL USER enzyme for 15 min @ 37 °C.
        vii. Repeat step 4e to 4g.
        viii. Resuspend the beads in 19 µl 0.1X TE buffer.

6. PCR amplification.
    a. Mix:
        i. 19 µL of Streptavidin beads
        ii. 3 µL Index primer (10 µM)
        iii. 3 µL Universal primer (10 µM)
        iv. 25 µL NEB Ultra II Q5 Master Mix
    b. Set up PCR:
        i. 30 sec @ 98 °C; initial denaturation
        ii. 10 sec @ 98 °C; denaturation
        iii. 30 sec @ 65 °C; 10 cycle annealing
        iv. 45 sec @ 65 °C; extension
        v. 5 min @ 72 °C; final extension
        vi. Hold @ 4 °C

7. Clean up PCR using NEBNext® Sample Purification Beads.
    a. Place PCR reaction on a magnetic rack to remove the magnetic streptavidin-biotinylated-DNA bead complexes. Transfer the supernatant that contains the PCR products to new DNA LoBind tube and add 0.9X volume (45 µL) of NEBNext® Sample Purification Beads.
    b. Incubate 5 minutes at RT and quick-spin the tube in a microcentrifuge.
    c. Put samples on magnetic rack to separate beads from the supernatant. When the solution looks clear, carefully remove the supernatant. DO NOT DISTURB THE BEADS.
    d. Add 200 µL of freshly prepared 80% EtOH. DO NOT REMOVE THE PCR TUBES OF THE RACK OR RESUSPEND THE BEADS. Wait for 30 sec, remove the 80% EtOH from the beads and repeat once. After removing the supernatant for a second time, quickly spin down the tubes and completely remove the residual EtOH.
    e. Air dry the beads for 5 min while the tube is on the rack with the lid open. DO NOT OVERDRY THE BEADS, THIS MAY RESULT IN LOWER RECOVERY OF DNA.
    f. Remove the tube from the magnet and resuspend the beads in 20 µL 0.1X TE buffer for 5 min at RT to elute the DNA.
    g. Put back the tube on to the magnetic rack until the solution is clear and transfer the supernatant to a clean PCR tube and store it at -20 °C.

h. Measure the amount of DNA using the Qubit HsDNA protocol. A successful library preparation should have at least a DNA concentration of 1 ng/µl.
i. Analyze the DNA on the Bioanalyzer (Agilent DNA 1000 Chip) to assess the library quality (size distribution and concentration). After Illumina DNA sequencing mapping and peak analyses, genomic open chromatin regions can be visualized using IGV browser (Fig.2).

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