Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA

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Eviction or destabilization of nucleosomes from chromatin is a hallmark of functional regulatory elements in eukaryotic genomes. Historically identified by nuclease hypersensitivity, these regulatory elements are typically bound by transcription factors or other regulatory proteins. FAIRE (formaldehyde-assisted isolation of regulatory elements) is an alternative approach to identify these genomic regions and has proven successful in a multitude of eukaryotic cell and tissue types. Cells or dissociated tissues are cross-linked briefly with formaldehyde, lysed and sonicated. Sheared chromatin is subjected to phenol/chloroform extraction and the isolated DNA, typically encompassing 1–3% of the human genome, is purified. We provide guidelines for quantitative analysis by PCR, microarrays or next-generation sequencing. Regulatory elements enriched by FAIRE have high concordance with those identified by nuclease hypersensitivity or chromatin immunoprecipitation (ChIP), and the entire procedure can be completed in 3 d. FAIRE has low technical variability, which allows its usage in large-scale studies of chromatin from normal or diseased tissues.

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INTRODUCTION

Understanding the regulation of transcription by sequence-specific regulatory factors and subsequent remodeling of chromatin is central to studies of health and disease. The activities of regulatory factors at promoters, enhancers, silencers and insulators typically cause nucleosomes to be evicted from chromatin in eukaryotic cells[1]. Therefore, one of the most effective means of discovering transcriptional regulatory elements is through the identification of nucleosome-depleted regions ('open chromatin'). Historically, this was accomplished by exploiting regional hypersensitivity to nucleases such as DNase I[2–9]. More recently, we demonstrated an alternative methodology for the detection of open chromatin, which we termed FAIRE[10–13]. FAIRE was first characterized in yeast and subsequently applied to human cells and tissues[11,13–15]. The technique has proven useful for a wide range of eukaryotes, from *Plasmodium*[16] to maize[17]. Here we present recent methodological enhancements that improve the utility and reliability of FAIRE, especially for use on tissues or lipid-laden cells such as adipocytes.

Overview

FAIRE does not rely on the use of antibodies or enzymes, and it is based on differences in cross-linking efficiencies between DNA and nucleosomes or sequence-specific DNA-binding proteins. DNA in nucleosome-depleted regions of chromatin (for example, through the activity of a sequence-specific regulatory factor) is much less efficiently cross-linked to protein[12]. DNA not cross-linked to protein will segregate to the aqueous phase during phenol/chloroform extraction. In contrast, DNA covalently linked to proteins will demonstrate hydrophilic properties, and it will become trapped between the organic and aqueous phase. To perform FAIRE (**Fig. 1**), cells or dissociated tissues are cross-linked briefly with formaldehyde, lysed and sonicated. Sheared chromatin is then subjected to phenol/chloroform extraction. The DNA in the aqueous phase is then purified and assayed. FAIRE-enriched chromatin is detected using one of several quantitative approaches. Options include quantitative amplification by PCR (FAIRE-qPCR)[13], hybridization to a tiling DNA microarray (FAIRE-chip)[11,13] or sequencing via next-generation sequencing technologies (FAIRE-seq)[13,15]. Owing to declining costs of sequencing and higher quality and resolution of sequencing-based data, FAIRE-seq has now nearly fully supplanted FAIRE-chip and FAIRE-qPCR, especially for larger genomes, but also for smaller genomes through multiplexing. Analysis by next-generation sequencing requires alignment of high-quality reads to a reference genome (e.g., Bowtie[18]) followed by detection of regions of significant enrichment (we recommend ZINBA[19]). Bowtie and ZINBA are both freely available.

Applications

Our lab has used FAIRE extensively to characterize active regulatory elements of several human cell lines as part of the ENCODE consortium[20], as well as to investigate different cell, tissue and tumor samples from humans, mice and other eukaryotes. FAIRE has been used to create catalogs of regulatory elements in normal or diseased cells[11,13,15], narrow the search space for causal sequence variants in human disease[13,21], and understand the interactions between transcription factors and chromatin remodeling[22,23]. When coupled with high-throughput sequencing, FAIRE can also be used to identify both large- and small-scale structural variations such as copy number variants[19].

Comparison with other methods

We have previously shown that regions in the yeast genome enriched by FAIRE were anti-correlated with occupancy of histones H3 and H4 (ref. 10), and that FAIRE regions encompass promoters, enhancers, insulators and other regulatory elements, most of which are also captured by DNase I hypersensitivity assays[10–13,15]. An in-depth comparison between regulatory elements captured by FAIRE, DNase I hypersensitivity and ChIP-seq found that although

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a large set of elements were identified by all methods, each assay also identified a unique set of features[15]. FAIRE was able to detect some distal regulatory elements, such as transcriptional enhancers, that DNase-seq could not, whereas DNase-seq identified some promoters that FAIRE did not.

Advantages of FAIRE

Antibody and enzyme independency. In contrast to ChIP, which is highly subject to antibody reliability and variability issues[24], FAIRE offers the consistency of a chemical-based isolation. Moreover, FAIRE does not require enzymes, such as DNase or MNase, which are commonly used in analogous methods for detecting nucleosome-free regions. Avoiding the optimization and extra steps necessary for enzymatic processing or immunoprecipitations eliminates a major source of variation, and thus makes it a much more reliable and robust method.

Enhancer detection. As described here and in Song *et al.*[15], FAIRE may identify additional transcriptional enhancers and other distal regulatory elements in comparison to other methods such as DNase-seq.

Sequenced input control not required. As discussed in Rashid *et al.*[19], a sequenced input control is generally not required for proper analysis of FAIRE-enriched regions. This reduces next-generation sequencing costs as well as the cost of reagents.

Applicability to tissue samples. As FAIRE does not require a single-cell suspension or nuclear isolation, it is easily adapted for use on tissue samples. The only additional step needed is pulverization of frozen tissue into a coarse powder before fixation.

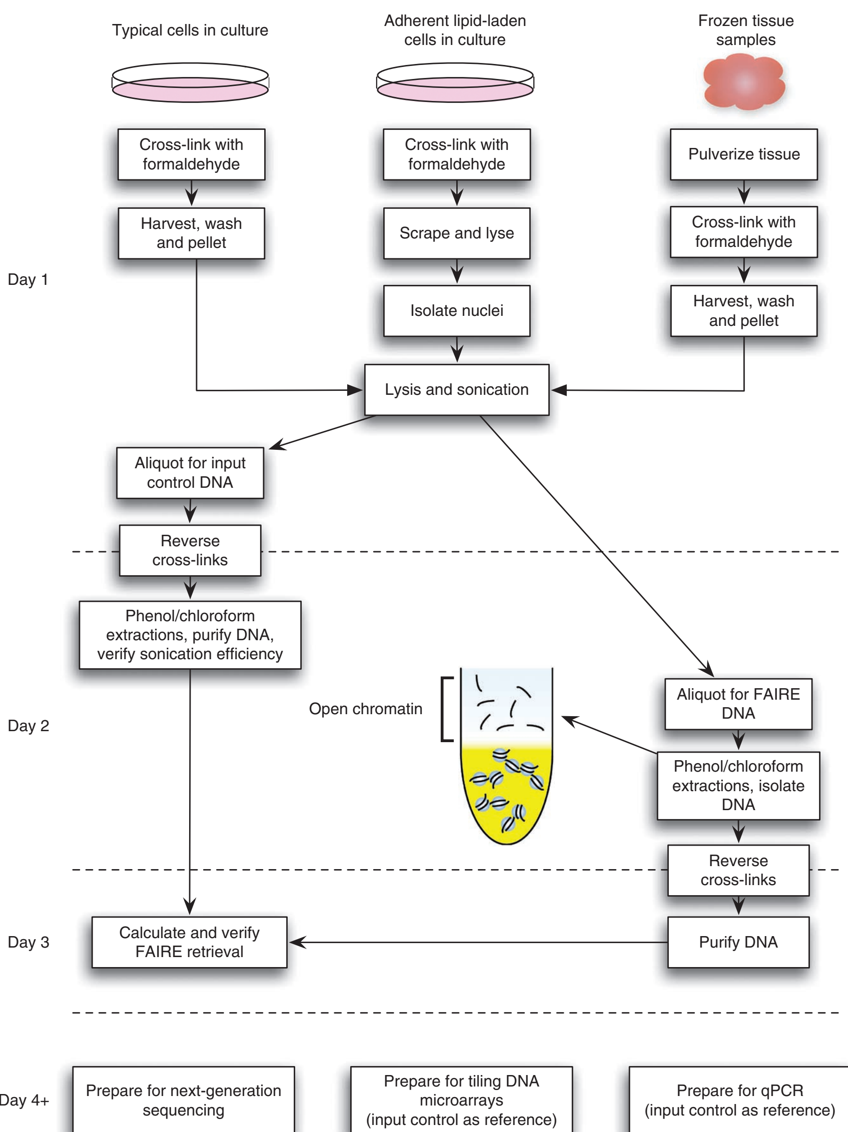

Figure 1 | Example timeline for FAIRE protocol. Steps are grouped by day for the typical timeline, but using the Pause Points will extend the duration.

Limitations

Promoter detection. As described here and in Song *et al.*[15], other methods, such as DNase-seq, may be better at identifying nucleosome-depleted promoters of highly expressed genes.

Analysis. As noted in Experimental design, although FAIRE is relatively straightforward experimentally, an extensive amount of computational processing and analysis is required for comprehensive interpretation of genome-wide results. Groups without access to bioinformatics specialists and to computers with sufficient memory, computing power and storage capacity may experience challenges in interpreting their results. Quantification of FAIRE signal by qPCR or microarrays may be more straightforward.

Absence of transcription factor footprinting. Transcription factor motifs can be identified in regions of open chromatin identified by FAIRE. However, the higher resolution and increased signal-to-noise of DNase-seq permits detection of specific transcription factor footprints in very deeply sequenced data[1].

Low signal-to-noise ratio. Relative to ChIP-seq or DNase-seq experiments, FAIRE has a lower signal-to-noise ratio. Therefore, the sites detected by FAIRE can, at times, be only marginally enriched above the background signal. This leads to a reduced confidence in the sites identified. This effect can be exacerbated when using non-sequencing-based detection methods. Consequently, primer and array design as well as the selection of control regions are crucial. Despite the low signal-to-noise ratio, we note that FAIRE is remarkably reproducible from experiment to experiment.

Fixation variation among tissues. Fixation efficiency can vary considerably for many reasons, including differences in cellularity, permeability, purity, fat content and surface area. Although dissociation by pulverization seems to make fixation slightly more consistent compared with mincing or other methods, this variability can still lead to inconsistent results; optimization is thus recommended.

Experimental design

Replicates. Studies using FAIRE, similarly to those using many other genome-wide assays, should include biological replicates. This entails the use of multiple independently grown batches of cells or tissues treated in the same manner. Several methods have been developed for the assessment of concordance among replicates, such as irreproducible discovery rate (IDR)[25], which is currently used by the ENCODE consortium. Methods such as IDR often require a ranked set of statistically enriched regions, which can be obtained by most peak-calling algorithms, including ZINBA[19].

Control sample. For sequencing-based detection of FAIRE enrichment, we have found that a control sample, such as genomic or input DNA, although always better to have, is not strictly necessary for samples that have been sequenced to sufficient depth and coverage[19]. When you are detecting enrichment by qPCR or tiling DNA microarrays, a genomic or input DNA sample is necessary for use as a reference.

Analysis. Although FAIRE is a relatively straightforward experimental protocol that can be completed in ~3 d, extensive computational processing and analysis are required for interpretation of the results. This includes quality assessment of the sequencing library and the sequencing reactions themselves, reference genome alignment, detection of enrichment and assessment of replicate concordance. We recommend a combination of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and TagDust[26] for quality control of the sequencing reactions and libraries, respectively. Although we typically use Bowtie[18] for reference genome alignment, other similar algorithms such as BWA[27] are equally suitable. To detect regions of significant FAIRE enrichment (peaks), we found that methods such as MACS[28] and Fseq[29], though commonly used successfully for ChIP-seq or DNase-seq data, do not perform well on FAIRE-seq data; this is likely to be a result of its relatively lower inherent signal-to-noise ratio. We thus developed a novel statistical algorithm called ZINBA[19]. The regions identified by ZINBA can then be used to assess concordance among replicates using algorithms such as IDR[25]. If possible, the data should be compared with existing maps of open chromatin, such as DNase-seq and FAIRE-seq data made available by the ENCODE consortium[20], or with gene expression data. FAIRE enrichment at gene promoters is strongly linked to gene expression. Therefore, strong FAIRE enrichment is expected around genes known to be highly expressed. A large fraction (approximately 30–50%) of the regions enriched by FAIRE are in intergenic regions of the genome. Typically, only approximately 5–15% of all FAIRE sites are at proximal promoters[13,15]. To determine whether an experiment was successful, we often examine the pattern from a locus on human chromosome 19 that produces a remarkably consistent level of FAIRE enrichment across cell types (see ANTICIPATED RESULTS).

Detection method. In cases where a reference genome assembly is available, FAIRE coupled with high-throughput sequencing is likely to be the most cost-effective option, especially if multiplexing is applicable. In smaller eukaryotes or for very targeted experiments, detection by microarray or quantitative PCR may be preferable, but array and primer design will have a key role in the overall success of the experiment (see FAIRE-chip microarray design and FAIRE-qPCR primer design below).

Fixation. The most common reason for a failed FAIRE experiment is underfixation of the cells. We have found that for a majority of mammalian cells in culture, fixation for 5 min with formaldehyde is both adequate and ideal. The protocol below includes quantification of both input control and FAIRE DNA, and we describe a diagnostic for determining if the sample has not been fixed sufficiently. For tissues, samples must first be pulverized into a coarse powder and then fixed for 7–9 min. The adequacy of fixation will depend heavily on the tissue size and composition, and thus may need to be optimized. Other techniques or adaptations for fixation may be required for plants or fungi, such as substantially increased fixation time[10] or modified fixation solutions[30]. For lipid-laden cells, it may be beneficial to perform both fixation and cell lysis (to extract nuclei) before attempting to collect the cells, as outlined below in PROCEDURE Step 1B.

Sonication. Sonication parameters must be optimized for each experiment because of variation in cell number, composition, sonicator and probe type, and fixation. In **Figure 2**, we present a representative agarose gel that provides examples of over-, under- and sufficiently sonicated chromatin. Ideally, chromatin is sheared to a range of about 150–750 bp with an average fragment length around 300–400 bp. Sonication yielding average fragment sizes smaller than

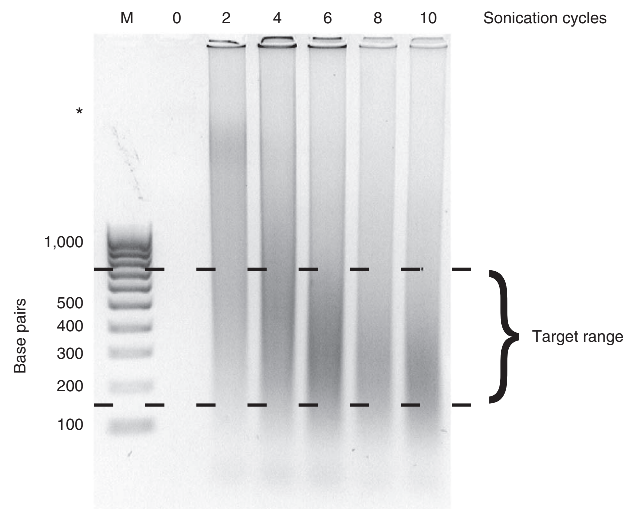

Figure 2 | Representative gel image showing varying degrees of sonication. NIH3T3 cells were fixed and lysed as described here. Chromatin was then sheared by sonication for 0, 2, 4, 6, 8 and 10 cycles using the parameters outlined in Step 2A. After clearing cell debris, cross-links were reversed, and purified DNA was run on a 1% (wt/vol) agarose gel. A 100-bp ladder (lane marked M) is included for reference. The target range for fragment sizes is shown. Six cycles yield an ideal distribution of fragment lengths; fewer than six cycles of sonication is insufficient for solubilization and shearing of chromatin, whereas sonication beyond six cycles leads to oversonication. A high-molecular-weight band is slightly visible and marked with an asterisk.

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this can result in reduced detection of highly nucleosome-depleted regions. High-molecular-weight bands may be visible especially when beginning with frozen tissue, but their presence in lieu of a distribution of smaller fragments is indicative of under-sonication or poor cell lysis.

FAIRE-chip microarray design. The two main considerations for microarray design are the resolution (or spacing) of the probes throughout the genome and the set of genomic loci covered by the probes. Resolution is the genomic distance from one probe to the next and must be sufficiently dense to capture the physiologically relevant size of the DNA fragments recovered by FAIRE (~200 bp). Probe spacing should allow a minimum of three probes per FAIRE DNA fragment or ~65 bp resolution. The set of genomic regions represented on the array is important as it provides a relative interpretation of the results. This is because of all the measurements being expressed as a ratio of the FAIRE signal over a reference sample, which is normalized by centering on the basis of the mean ratio. The majority of probes should span regions that correspond to background (not open) chromatin. There are a number of published protocols that address specific aspects of array design and include recommendations for reliable detection[31–40].

FAIRE-qPCR primer design. When detecting FAIRE enrichment via quantitative PCR, careful consideration of experimental design will maximize the chance of success. In addition to the methodology for quantification of the results, selection of an appropriate set of control regions and locations of primers have an important role in calculating relative enrichment. This is often difficult because of the lack of a priori knowledge of both true FAIRE-positive and -negative sites for most cell or tissue types or growth conditions. The data made available by the ENCODE consortium may be helpful in this regard[20]. We often employ a tiling approach for detection of open chromatin sites using qPCR, such that primer pairs are designed so the amplicons are either directly overlapping or closely spaced across the assayed genomic regions. As a control, we recommend using primer sets that flank the regions isolated by FAIRE. As primers spanning or near the edges of sonication breakpoints of FAIRE fragments are unlikely to properly amplify, primer pairs should be designed such that they amplify 60–100 bp within the center of the region of interest. Primer sets should be validated on a dilution series of input DNA to confirm consistent and proportionate amplification characteristics. For these and other reasons, FAIRE-chip and FAIRE-seq are strongly preferred over FAIRE-qPCR.

MATERIALS

REAGENTS

- Formaldehyde (37% (wt/vol); Fisher Scientific, cat. no. F79-500) **! CAUTION** Formaldehyde is toxic by inhalation or if swallowed; it is irritating to the skin, eyes and respiratory system and may be carcinogenic. Formaldehyde should be used with appropriate safety measures, such as protective gloves, glasses, clothing and sufficient ventilation. All waste should be handled according to hazardous waste regulations.
- Glycine (2.5 M; Fisher Scientific, cat. no. BP381-500)
- Dulbecco's phosphate-buffered saline (PBS, 1×; Cellgro, cat. no. 21-031)
- Tris-HCl (pH 8.0; Fisher Scientific, cat. no. BP152-500)
- Tris-HCl (pH 7.4; Fisher Scientific, cat. no. BP152-500)
- Triton X-100 (Fisher Scientific, cat. no. BP151-500)
- Sodium dodecyl sulfate (SDS, Fisher Scientific, cat. no. BP166-500)
- NaCl (Mallinkrodt, cat. no. 7581)
- EDTA (Fisher Scientific, cat. no. BP120-500)
- KCl (Fisher Scientific, cat. no. BP366-500)
- NP-40 (Sigma, cat. no. I8896-100)
- Sucrose (Gibco, cat. no. 15503-014)
- Protease inhibitors (Roche, cat. no. 11836153001)
- DNase-free RNaseA (10 $\mu g\ \mu l^{-1}$; Roche, cat. no. 11119915001)
- Proteinase K (20 mg ml^{-1}; Roche, cat. no. 03115836001)
- Phenol/chloroform/isoamyl alcohol (25:24:1; Sigma, cat. no. P3803) **! CAUTION** Phenol/chloroform is harmful if swallowed or in contact with skin, causes severe skin burns and eye damage, is fatal if inhaled and potentially carcinogenic. It should be used with appropriate safety measures, such as protective gloves, glasses, clothing and sufficient ventilation. All waste should be handled according to hazardous waste regulations.
- Chloroform/isoamyl alcohol (24:1; Sigma, cat. no. C0549) **! CAUTION** Chloroform/isoamyl alcohol is harmful if swallowed, causes skin and eye irritation and is potentially carcinogenic. It should be used with appropriate safety measures, such as protective gloves, glasses, clothing and sufficient ventilation. All waste should be handled according to hazardous waste regulations.
- Ethanol (95% (vol/vol); Decon, cat. no. 2801)
- Glycogen (20 mg ml^{-1}; Roche, cat. no. 901393)
- Sodium acetate (3 M, pH 5.2; Mallinckrodt, cat. no. 7364)
- Ethanol (70% (vol/vol), ice-cold, diluted from 95% ethanol)
- Double-distilled water (ddH_2O)
- Lysis buffer A (see REAGENT SETUP)
- Lysis buffer B (see REAGENT SETUP)
- Sucrose pad (see REAGENT SETUP)
- SYBR Green master mix (Applied Biosystems, cat. no. 4309155)
- Reagents for agarose gel electrophoresis and ethidium bromide staining

EQUIPMENT

Lab equipment

- Cell scrapers (Corning, cat. no. 3008)
- Liquid nitrogen and appropriate container
- Tissue pulverizer (Biospec, cat. no. 59012N)
- Nutator (such as BD/Clay Adams 421105)
- Temperature-controlled swinging bucket centrifuge (Fisher Scientific, Accuspin 1R)
- Dounce (Kimble-Chase, cat. no. 885300-0000)
- Bead-beater (Mini-BeadBeater-8, Biospec)
- Tubes with metal beads (2.38 mm; MoBio, cat. no. 13117-50) **▲ CRITICAL** We have found that the specified metal beads dissociate tissues or cell clumps better than other materials.
- Sonicator (Branson Sonifier 450D equipped with microtip)
- Zymo-I spin columns (Zymo, cat. no. C1003-250)
- Zymo DNA binding buffer (Zymo, cat. no. D4004-1-L)
- Zymo wash buffer (Zymo, cat. no. D4003-2-4)
- Fluorometer with DNA quantification reagents and standards (Invitrogen, cat. no. Q32866) or NanoDrop ND-1000. Quantification of DNA is necessary, and a fluorescence-based system is recommended as it is much more accurate
- MicroAmp optical 96-well reaction plate (Applied Biosystems, cat. no. 4306737)
- Conical tubes
- Tabletop centrifuge
- Microcentrifuge
- Vacuum concentrator (e.g., SpeedVac (Savant))
- Illumina sequencing platform (Illumina)
- Nimblegen (Roche)
- AMPure XP beads (Agencourt)

Computer and software

- A computer with at least 8 GB RAM and 100 GB hard drive (preferably with a UNIX subsystem, such as Mac OS X or LINUX, and multiple processing cores)

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- Bowtie[18] for reference genome alignment, ZINBA[19] for peak calling and TagDust[26] and FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) for quality control. All are freely available and run easily in a command-line context

REAGENT SETUP

Lysis buffer A Lysis buffer A is prepared by mixing 10 mM Tris-HCl (pH 8.0), 2% (vol/vol) Triton X-100, 1% SDS, 100 mM NaCl and 1 mM EDTA. Store at 4 °C for a maximum of 6 months.

Lysis buffer B Lysis buffer B is prepared by mixing 10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1% (vol/vol) NP-40, 5% (wt/vol) sucrose and 1× protease inhibitors. Prepare the stock solution, excluding protease inhibitors, and store at 4 °C for a maximum of 6 months. Add protease inhibitors immediately before use.

Sucrose pad Prepare by combining 10 mM Tris (pH 7.4), 15 mM NaCl, 60 mM KCl, 10% (wt/vol) sucrose and 1× protease inhibitors. Prepare stock solution excluding protease inhibitors, sterile filter and store at 4 °C for a maximum of 6 months. Add protease inhibitors immediately before use.

EQUIPMENT SETUP

Tissue preparation To pulverize tissues, prechill both the mortar and the pestle of a tissue pulverizer in liquid nitrogen. Tissues should be pulverized until a coarse powder is achieved, with a granularity similar to that of cornmeal or granulated sugar. Samples should only be allowed to begin to thaw after pulverization is completed and before PBS is added, as otherwise the PBS will freeze.

Cell dissociation For dissociation of cells and tissues we use the Mini-BeadBeater-8 set to homogenize. Although settings will depend on cell or tissue type, we begin with five 2-min cycles at 4 °C, allowing the sample to cool on ice for 2 min between each cycle. We have found that the specified metal beads dissociate tissues or cell clumps better than other materials.

Sonication We typically use a Branson Sonifier 450D equipped with a microtip. Although the settings will depend on cell or tissue type, growth conditions and cross-linking, we begin with 30% amplitude and six cycles (where each cycle has a 1.0-s burst followed by a 0.5-s pause and a total length of 30 s) and allow the sample to cool in an ice-water bath during and/or between cycles.

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PROCEDURE

Formaldehyde cross-linking and cell lysis (day 1) ● TIMING 4–6 h

1| For formaldehyde cross-linking of cells, choose one of the following three procedures depending on whether you are using (A) typical adherent or suspension cells, (B) adherent lipid-laden cells such as adipocytes (or if a bead-beater is unavailable) or (C) frozen tissues. The procedure described in (C) has been tested in our lab on human and mouse samples. Alterations such as increased fixation length may be necessary for nonmammalian eukaryotes such as plants or fungi; we recommend reviewing existing publications on performing chromatin immunoprecipitation for such necessary adaptations. Other modifications may also be necessary, such as those made for pancreatic islets[41].

(A) For typical adherent or suspension cells

(i) Culture 1×10^6 – 5×10^7 cells for each experiment. If available, 1×10^7 cells is ideal for most applications.
(ii) Add 37% (wt/vol) formaldehyde directly to the medium to a final concentration of 1%.
(iii) Fix for 5 min while rocking on a Nutator at room temperature (25 °C).
(iv) Add 2.5 M glycine to a final concentration of 125 mM to quench the formaldehyde.
(v) Rock on a Nutator for an additional 5 min at room temperature.
(vi) Scrape cells if needed and pool them in 50-ml conical tubes.
(vii) Spin at 300–500*g* for 5 min at 4 °C to collect fixed cells. Decant or pipette the supernatant into formaldehyde waste.
(viii) Wash the pellet with 10 ml of 1× PBS.
(ix) Repeat Steps 1A(vii–viii) twice for a total of three washes. During the final wash, transfer cells in PBS to 15-ml conical tubes.
(x) Flash-freeze the fixed cell pellets in liquid nitrogen and either store them at −80 °C or proceed directly to Step 1A(xi). Alternatively, pellets can be resuspended without freezing.
■ PAUSE POINT Once cells have been frozen, they can be stored at −80 °C indefinitely.
(xi) Resuspend fixed cells in 1 ml of cold lysis buffer A and transfer them to the tubes containing the metal beads.
(xii) Dissociate and lyse cells by bead beating (see EQUIPMENT SETUP). For most cell and tissue types, five 2-min cycles followed by 2 min of rest (on ice) is sufficient, but additional cycles may be required.
▲ CRITICAL STEP This step must be performed in a 4 °C room.
(xiii) Transfer lysate to a 15-ml conical tube. Wash the beads that are left behind with an additional 1 ml of cold lysis buffer A. Add this wash to the 15-ml tube for a final lysate volume of 2 ml.

(B) For adherent lipid-laden cells (or if a bead-beater is unavailable)

(i) Begin with 1×10^6 – 5×10^7 cells for each experiment. If available, 1×10^7 cells is ideal for most applications.
(ii) Add 37% (wt/vol) formaldehyde directly to the medium to a final concentration of 1%.
(iii) Fix for 5 min while rocking on a Nutator at room temperature.
(iv) Add 2.5 M glycine to a final concentration of 125 mM to quench the formaldehyde.
(v) Rock on a Nutator an additional 5 min at room temperature.
(vi) Pipette and remove the formaldehyde, glycine and medium mixture without disturbing the cells.
(vii) Scrape cells in 7 ml of lysis buffer B added directly to the plate. Multiple plates can be pooled by scraping cells from each plate in the same 7 ml of lysis buffer B such that the final volume does not exceed 7 ml.
(viii) Dounce cells with five to ten smooth strokes using a tight fitting pestle.
(ix) Transfer to a 15-ml conical tube and add a 2-ml sucrose pad slowly to the bottom of the tube using a Pasteur pipette.

(x) Centrifuge at 2,100*g* for 20–25 min at 4 °C to collect nuclei.
(xi) Aspirate to remove supernatant.
(xii) Resuspend the nuclear pellet in 2 ml of lysis buffer A.

(C) For frozen tissues

(i) Begin with 20–200 mg of frozen tissue.
(ii) Pulverize frozen tissue into a coarse powder (see EQUIPMENT SETUP). Allow the sample to begin to thaw.
▲ CRITICAL STEP Pulverization of the tissue to a coarse powder, rather than mincing or Douncing, allows for a more uniform fixation.
(iii) Add 5 ml of PBS to resuspend the powder, avoiding freezing of the PBS. Transfer the suspension to a 15-ml conical tube.
(iv) Wash the pulverizer mortar and pestle with an additional 5 ml of PBS and add it to the conical tube for a total volume of 10 ml.
(v) Add 37% (wt/vol) formaldehyde to a final concentration of 1%.
(vi) Fix for 7–9 min while rocking on a Nutator at room temperature.
(vii) Add 2.5 M glycine to a final concentration of 125 mM to quench the formaldehyde.
(viii) Rock on a Nutator for an additional 5 min at room temperature.
(ix) Spin at 300–500*g* for 5 min at 4 °C to collect fixed tissue. Pipette supernatant into formaldehyde waste.
(x) Wash pellet with 10 ml 1× PBS.
(xi) Repeat Step 1C(vii–viii) twice for a total of three washes. During the final wash, transfer the tissue in PBS to 15-ml conical tubes.
(xii) Flash-freeze fixed tissue in liquid nitrogen and either store them at –80 °C or proceed directly to Step 1C(xiii). Alternatively, pulverized tissue can be resuspended without freezing.
■ PAUSE POINT Once tissues have been frozen, they can be stored at −80 °C indefinitely.
(xiii) Resuspend fixed tissue in 1 ml of cold lysis buffer A and transfer it to the tubes containing the metal beads.
(xiv) Dissociate the tissue and lyse the cells by bead beating (see EQUIPMENT SETUP). For most cell and tissue types, five 2-min cycles followed by 2 min of rest (on ice) is sufficient, but additional cycles may be required.
▲ CRITICAL STEP This step must be performed in a 4 °C room.
(xv) Transfer the lysate to a 15-ml conical tube. Wash the beads that are left behind with an additional 1 ml of cold lysis buffer A. Add this wash to the 15-ml tube for a final lysate volume of 2 ml. Note that some clumps or cloudiness may remain, especially if the tissue was particularly vascular.

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Sonication (day 1) ● TIMING 1–2 h

2| Sonicate the cell lysate to achieve an average DNA fragment size of approximately 300–400 bp. For most cell and tissue types, six 30-s cycles with 1-s bursts followed by 0.5 s of rest at 30% amplitude (using the Branson Sonifier 450D) is sufficient, but optimization may be required for some cell or tissue types or for other sonicators. Samples should be allowed to cool in an ice-water bath during and/or between sonication cycles or for at least 1–2 min.
▲ CRITICAL STEP Foaming should be avoided, as this is likely to decrease sonication efficiency. If foaming occurs, let the sample settle on ice until bubbles have subsided or centrifuge it briefly and gently resuspend all material. Probe positioning heavily influences both sonication efficiency and whether or not samples will foam. In most cases, the probe should be placed in the center of the tube approximately one-quarter to one-half an inch from the bottom.

Preparation of input control DNA (day 1) ● TIMING 1.5 h plus overnight incubation

3| Remove a 100-µl aliquot of cell lysate to check efficiency of sonication. The remaining lysate can be stored temporarily at 4 °C until it is required at Step 30 or 31.

4| Centrifuge the aliquot of lysate at 15,000–20,000*g* for 5 min at 4 °C to pellet the cell debris.

5| Transfer the supernatant to a fresh 1.5-ml tube.

6| Add 1 µl of DNase-free RNaseA and incubate for 30 min at 37 °C.
▲ CRITICAL STEP RNaseA must be DNase free.

7| Add 1 µl of proteinase K, incubate at 55 °C for 1 h, and then incubate overnight at 65 °C to reverse cross-links.

Purification and assessment of input control DNA (day 2) ● TIMING 3–4 h

8| If needed, collect the sample by brief centrifugation in a microcentrifuge or tabletop centrifuge. Add 200 µl of 10 mM Tris-HCl (pH 7.4) to a final volume of 300 µl and ensure that all materials are fully resuspended.

9| Add 300 μl of phenol/chloroform/isoamyl alcohol solution.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

10| Vortex for 10 s and then centrifuge at 12,000*g* for 5 min in a tabletop centrifuge.

11| Transfer the aqueous (top) layer to a fresh 1.5-ml tube.

12| To ensure complete retrieval of aqueous material, add 150 μl of 10 mM Tris-HCl (pH 7.4) to the tube containing the interphase and organic layers.

13| Vortex for 10 s and then centrifuge at 12,000*g* for 5 min in a tabletop centrifuge.

14| Transfer the aqueous (top) layer and combine it with the previously isolated aqueous material.

15| Add 1 volume of phenol/chloroform/isoamyl alcohol.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

16| Vortex for 10 s and then centrifuge at 12,000*g* for 5 min in a tabletop centrifuge.

17| Transfer the aqueous (top) layer to a fresh 1.5-ml tube.

18| Add 200 μl of chloroform/isoamyl alcohol to remove traces of phenol.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

19| Vortex for 10 s and then centrifuge at 12,000*g* for 5 min in a tabletop centrifuge.

20| Transfer the aqueous layer to a fresh 1.5-ml tube.

21| Add a one-tenth volume of 3 M sodium acetate (pH 5.2), two volumes of 95% (vol/vol) ethanol and 1 μl of 20 mg ml^{-1} glycogen to tube containing the aqueous layer.

22| Incubate the sample at −80 °C for 30 min or longer.
■ PAUSE POINT Precipitations can be left at −80 °C overnight or longer.

23| Centrifuge at 12,000*g* for 15 min at 4 °C to precipitate DNA.

24| Carefully aspirate the supernatant without disturbing the DNA pellet.

25| Wash the pellet with 500 μl of ice-cold 70% (vol/vol) ethanol.

26| Centrifuge at 12,000*g* for 5 min at 4 °C.

27| Carefully aspirate the supernatant without disturbing the DNA pellet.

28| Dry the pellet with a SpeedVac or by leaving tubes open for 10–20 min, then resuspend the pellet in 20 μl of 10 mM Tris-HCl (pH 7.4).
▲ CRITICAL STEP Remaining traces of ethanol can affect downstream steps. Make sure pellets are completely dry before resuspending.

29| Quantify 1 μl of input control DNA with a fluorometer or NanoDrop. We recommend fluorometry-based quantification because of its improved accuracy. For experiments beginning with around 1×10^7 cells or 150 mg of tissue, input control yields should be around 50–100 ng μl^{-1} with a total volume of 20 μl.
? TROUBLESHOOTING

30| Run 500 ng or half of input control DNA on a 1% (wt/vol) agarose gel and visualize it with ethidium bromide staining.
▲ CRITICAL STEP Sufficient sonication has been achieved if fragments range in size from 100 to 1,000 bp, with an approximate average fragment length of between 200 and 500 bp. If only a large-molecular-weight band is detected or if the average fragment size is markedly larger than 500 bp, additional rounds of sonication are necessary. Retrieve the lysate from 4 °C storage (Step 3) and repeat Steps 2–30 until this optimal range of fragment sizes has been achieved.
■ PAUSE POINT Input control DNA can be frozen and stored indefinitely at −80 °C. Cell lysates can be stored at 4 °C for up to several days or frozen and stored for several weeks at −80 °C.
? TROUBLESHOOTING

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Preparation of FAIRE DNA (day 2) ● TIMING 3–4 h plus overnight incubation

31| Aliquot the remaining cell lysate (from Step 3) into 1.5-ml tubes each with no more than 500 μl. One aliquot can be stored at −80 °C indefinitely as a backup.

32| Centrifuge aliquots of lysate at 15,000–20,000*g* for 5 min at 4 °C to pellet cell debris.

33| Transfer supernatants to fresh 1.5-ml tubes.

34| Add 1 volume of phenol/chloroform/isoamyl alcohol to each aliquot.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

35| Vortex each tube for 10 s and then centrifuge it at 12,000*g* for 5 min in a tabletop centrifuge.

36| Transfer the aqueous (top) layers to fresh 1.5-ml tubes.
? TROUBLESHOOTING

37| To ensure the complete retrieval of aqueous material, add 150 μl of 10 mM Tris-HCl (pH 7.4) to the tubes containing the interphase and organic layers.

38| Vortex each tube for 10 s and then centrifuge it at 12,000*g* for 5 min in a tabletop centrifuge.

39| Transfer the aqueous (top) layers and combine them with previously isolated aqueous material.

40| Add 1 volume of phenol/chloroform/isoamyl alcohol to each tube.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

41| Vortex each tube for 10 s and then centrifuge it at 12,000*g* for 5 min in a tabletop centrifuge.

42| Transfer the aqueous (top) layers to fresh 1.5-ml tubes.

43| Add 200 μl of chloroform/isoamyl alcohol to each tube to remove traces of phenol.
! CAUTION See REAGENTS for precautions when using phenol and chloroform.

44| Vortex for 10 s and then centrifuge tubes at 12,000*g* for 5 min in a tabletop centrifuge.

45| Transfer the aqueous layer to fresh 1.5-ml tubes.

46| Add a one-tenth volume of 3 M sodium acetate (pH 5.2), two volumes of 95% (vol/vol) ethanol and 1 μl of 20 mg ml^{-1} glycogen to each tube containing the aqueous layer.

47| Incubate the tubes at −80 °C for 30 min or longer.
■ PAUSE POINT Precipitations can be left at −80 °C overnight or longer.

48| Centrifuge at 12,000*g* for 15 min at 4 °C to precipitate the DNA.

49| Carefully aspirate the supernatants without disturbing the DNA pellets.

50| Wash the pellets with 500 μl of ice-cold 70% (vol/vol) ethanol.

51| Centrifuge at 12,000*g* for 5 min at 4 °C.

52| Carefully aspirate the supernatants without disturbing the DNA pellets.

53| Dry the pellets with a SpeedVac or by leaving tubes open for 10–20 min, then resuspend the pellets in 50 μl of 10 mM Tris-HCl (pH 7.4).
▲ CRITICAL STEP Remaining traces of ethanol can affect downstream steps. Make sure pellets are completely dry before resuspending.

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54| Add 1 µl of DNase-free RNaseA and incubate for 30 min at 37 °C.
▲ **CRITICAL STEP** RNaseA must be DNase-free.

55| Add 1 µl of proteinase K, incubate at 55 °C for 1 h, then incubate overnight at 65 °C to reverse any DNA-DNA cross-links.

Purification and assessment of FAIRE DNA (day 3) ● TIMING 1 h
56| If needed, collect samples with brief centrifugation in a microcentrifuge or tabletop centrifuge.

57| Purify with Zymo-I spin columns using two volumes of DNA binding buffer and 200 µl of wash buffer for each washing step. Elute twice with 10 µl of 10 mM Tris-HCl (pH 7.4), allowing the buffer to sit on the column at room temperature for 1–2 min.

58| Quantify 1 µl of FAIRE DNA with a fluorometer or NanoDrop. We recommend fluorometry-based quantification because of its increased accuracy. For experiments beginning with around 1×10^7 cells or 150 mg of tissue, FAIRE yields should be around 6–12 ng μl^{-1} with a total volume of 20 µl.
▲ **CRITICAL STEP** To test whether the FAIRE yield is within an acceptable range, we recommend dividing the total FAIRE yield (in ng) by the volume of cell lysate used for FAIRE (in µl, the number of lysate aliquots multiplied by the aliquot volume). A similar value should be calculated for the input control (total yield in ng over lysate aliquot volume). The volume-normalized ratio of FAIRE DNA isolated with respect to input control DNA isolated should not exceed 5% and will ideally fall in the 1–3% range. A retrieval ratio substantially higher than 5% is often indicative of underfixation and may predict experimental failure due to poor signal enrichment.
■ **PAUSE POINT** FAIRE DNA can be frozen and stored indefinitely at −80 °C.
? TROUBLESHOOTING

Detection of FAIRE enrichment and basic data analysis ● TIMING variable
59| For detection of FAIRE enrichment, one of three procedures can be used depending on whether you are using (A) next-generation sequencing (FAIRE-seq)[13,15], (B) tiling DNA microarrays (FAIRE-chip)[11,13] or (C) quantitative PCR (FAIRE-qPCR)[13]. The procedure described in A has been tested on the Illumina sequencing platform and thus optimization may be required for other platforms. The procedure described in option B has been tested on multiple tiling human DNA microarray platforms including Nimblegen (Roche) and Agilent, but optimization may be required for certain platforms or array types.
(A) Detection and analysis by next-generation sequencing (FAIRE-seq)
(i) Prepare sequencing libraries using the manufacturer's protocols. We recommend beginning with 100–200 ng of FAIRE DNA. We typically incorporate two rounds of purification with Agencourt AMPure XP beads before 18 cycles of amplification by PCR, and size-select the final library to 200–500 bp, avoiding adapter bands, which typically run under 100 bp. Alternatively, libraries can be prepared for sequencing by using the TruSeq kit (Illumina), which may be useful if multiplexing is desired.
▲ **CRITICAL STEP** We have found that sufficient depth and coverage of the human genome is typically achieved with no less than 3×10^7 aligned reads.
(ii) Remove sequencing reads with significant adapter contribution using an algorithm such as TagDust[26]. We typically set the false discovery rate parameter to 0.001.
▲ **CRITICAL STEP** For clean libraries, an average of about 0.1–0.2% of reads are filtered at this step. Substantially higher fractions (>10%) are indicative of poor library quality.
(iii) Assess sequencing quality, including confidence scores and nucleotide distributions, using algorithms such as those in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).
▲ **CRITICAL STEP** It is important that a relatively even nucleotide distribution is observed for all nucleotides across every read sequence. An overrepresentation of 'N', abundance of a specific sequence or wide variability in sequence quality is indicative of poor library complexity or sequencing errors.
(iv) Align high-quality reads to the reference genome using an algorithm such as Bowtie[18]; use default parameters, with the exception that the maximum number of allowable alignments should be restricted to four and Bowtie should be forced to pick the highest-scoring alignment when multiple possibilities exist. For most genomes and experiments, approximately 75–85% of sequencing reads are successfully aligned.
(v) Create files for visualization and detect regions of significant enrichment with respect to local background using ZINBA[19].
? TROUBLESHOOTING
(vi) Assess cross-replicate correlation using an algorithm such as IDR[25].

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(B) Detection by tiling DNA microarrays (FAIRE-chip)

(i) Amplify FAIRE and input control DNA using ligation-mediated PCR[31,42].

(ii) Follow the manufacturer's recommended protocols and refer to Lee *et al.*[31] for sample labeling, hybridization and image acquisition procedures.

(iii) For dual-channel platforms (such as NimbleGen), data for each probe on the microarray are expressed as a $\log_2$ ratio, which is normalized by calculating the *z*-score for each probe.

(iv) Identify regions enriched by FAIRE; this can be accomplished using most peak-finding algorithms used for ChIP-chip. We recommend ChIPOTle[43] or Mixer[44]. If applicable, the window size should be sufficiently large enough to contain approximately ten probes and the step size should be set to equal the resolution of the microarray.
? TROUBLESHOOTING

(C) Detection by quantitative PCR (FAIRE-qPCR)

(i) In each well of a MicroAmp optical 96-well reaction plate, add 12.5 µl of 2× SYBR Green master mix, 5 µl of DNA (2.5–25 ng μl^{-1}, ideal is 5 ng μl^{-1}), 1 µl of primers (20 µM concentration) and 6.5 µl of ddH_2O.

(ii) Seal the plate with optical adhesive film.

(iii) Cycling parameters will vary, but they are typically 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and finally, 60 °C for 1 min.

(iv) Calculate the relative enrichment for each amplicon using the comparative C_t method[45], such that a ratio is calculated for the signal from the FAIRE sample relative to the signal from input control DNA.
? TROUBLESHOOTING

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
29	Low input control yield	Low starting cell number	Start the experiment with more cells or tissue (Step 1)
		Poor cell lysis	Vary dissociation and cell lysis conditions (Step 1)
30	Sheared chromatin has incorrect average fragment length	Solution has foamed	Make sure the sonicator tip is centered and located one-quarter to half an inch from the bottom of the tube and that the sample has been cooled in an ice-water bath (Step 2)
		Undersonication	Increase the number of sonication cycles (Step 2)
		Underfixation	Insufficiently cross-linked chromatin will lead to the production of very small fragments. Increase fixation time or vary fixation conditions (Step 1)
36	Aqueous layer is cloudy	Phenol may be overloaded because of a high cell number	Start the experiment with fewer cells or less tissue (Step 1)
58	High DNA yield	Underfixation	Insufficiently cross-linked chromatin will lead to high DNA yields with respect to input control. Increase fixation time or vary fixation conditions (Step 1)
	Low DNA yield	Low starting cell number	Start the experiment with more cells or tissue (Step 1)
		Overfixation	Excessive cross-linking will reduce the recovery of nucleosome-depleted regions. Reduce fixation time or vary fixation conditions (Step 1)
59	Poor signal-to-noise ratio	Underfixation	Insufficiently cross-linked chromatin will lead to decreased enrichment by FAIRE. Increase fixation time or vary fixation conditions (Step 1)

● TIMING

Steps 1–7 (day 1), formaldehyde cross-linking and cell lysis: approximately 7–8 h plus overnight incubation
Steps 8–30 (day 2), purification and assessment of input control DNA: 3–4 h
Steps 31–55 (day 2), preparation of FAIRE DNA: approximately 3–4 h plus overnight incubation
Steps 56–58 (day 3), purification and assessment of FAIRE DNA: approximately 1 h
Step 59, detection of FAIRE enrichment and basic data analysis: variable

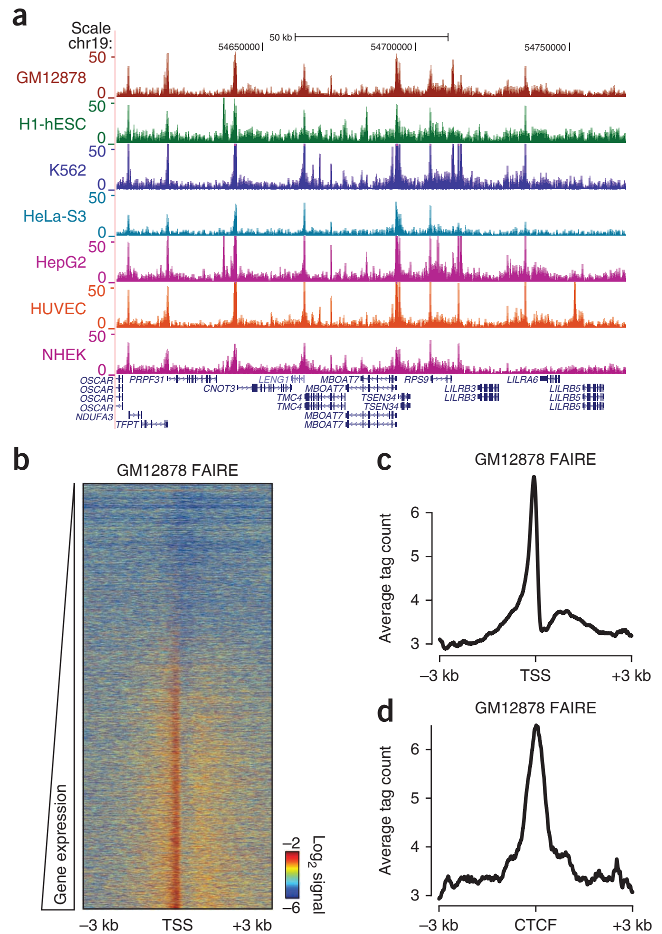

Figure 3 | Expected results from FAIRE-seq experiments. (**a**) Genomic locus residing on chromosome 19 as visualized with the UCSC Genome Browser[46] shows consistent FAIRE enrichment at TSSs across seven ENCODE cell lines (GM12878, H1-hESC, K562, HeLa-S3, HepG2, HUVEC and NHEK)[15]. Data are presented as number of aligned, *in silico* extended reads per base, on a scale of 0–50 reads. The pink coloring atop tall peaks of enrichment represent those areas where the signal exceeded this range. (**b**) Heat map of normalized GM12878 FAIRE signals ±3 kb around TSSs ranked by gene expression in GM12878 cells. Color was assigned on a $\log_2$ scale of −6 (background) to −2 (enriched). (**c**) Average GM12878 FAIRE signal ± 3kb around TSSs across all genes. Enrichment peaks around −125 bp. (**d**) Average GM12878 FAIRE signal ±3 kb around GM12878 CTCF sites, representing a class of distal regulatory elements.

ANTICIPATED RESULTS

Visualize FAIRE-seq or FAIRE-chip data in a browser such as the University of California Santa Cruz (UCSC) Genome Browser[46]. For data from human cells or tissues, we expect to see enrichment similar to that presented in **Figure 3a**. This genomic locus on chromosome 19 contains several genes that each contain a nucleosome-depleted promoter detectable in nearly every cell or tissue type assayed to date, including all Tier-1 and Tier-2 cell types assayed by ENCODE (a total of 19 cell types to date)[20]. In addition, there are some cell type–selective regions of open chromatin, such as the region immediately upstream of *CNOT3*, which is selective for embryonic stem cells and HepG2. The aggregated FAIRE signal around all transcription start sites (TSSs) ranked by gene expression should be similar to that presented in **Figure 3b**, showing a strong nucleosome-free region ~125 bp upstream of the TSSs and depletion (representing a well-positioned nucleosome) immediately downstream of the TSSs. The average signal across all genes is presented in **Figure 3c**. The number of regions of the genome enriched by FAIRE should be ~100,000 in any given cell or tissue type. FAIRE additionally detects distal regulatory regions, such as those marked by the transcription factor CTCF (**Fig. 3d**).

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