Mapping open chromatin with Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE).

Takao Nammo[1,2], Santiago A. Rodríguez-Seguí[1,2], Jorge Ferrer[1,2,3,4]

[1]Genomic Programming of Beta Cells, Institut d'Investigacions Biomediques August Pi i Sunyer, Barcelona, Spain, 08036.

[2]Centro de Investigación Biomédica en Red (CIBER) de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain, 08036.

[3]Endocrinology, Hospital Clinic de Barcelona, Barcelona, Spain, 08036.

[4]Correspondence should be addressed to

Jorge Ferrer

Endocrinology, Hospital Clinic de Barcelona,

Villarroel 170, Barcelona, Spain, 08036

jferrer@clinic.ub.es;

Tel +34 932275400 (2598);

Fax +34 93 4516638.

Abstract

Non-coding regulatory genomic elements are central for cellular function, differentiation, and disease, but remain poorly characterized. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) has emerged as a simple method to identify and analyze active regulatory sequences based on their decreased nucleosomal content. More recently FAIRE was combined with high-throughput sequencing (FAIRE-seq) to locate tissue-specific regulatory elements at a genome scale in purified human pancreatic islets. Here we describe the implementation of the FAIRE method in human pancreatic islet cells.

Key Words: FAIRE, Formaldehyde-Assisted Isolation of regulatory elements; gene transcription; open chromatin; diabetes; epigenetics; high throughput sequencing; pancreatic islets

1. Introduction

The dissection of functional non-coding sequence elements is currently one of the most important challenges in genome biology. Such sequence elements are believed to instruct genomic regulatory programs that underlie cellular phenotypes, and are likely to play an important role in disease mechanisms. The analysis of functional regulatory sequences can thus provide an opportunity to increase our understanding of cellular function, differentiation, and disease.

Genomic regions that are directly involved in transcriptional regulatory functions can be discriminated because they exhibit distinct structural chromatin features. Studies of sequences that contain binding sites for cis-regulatory proteins have thus revealed either local nucleosome eviction, or altered nucleosome-DNA interactions. This correlation could result from an increased ability of DNA binding factors to access their cognate sites at sequences that have decreased intrinsic binding affinities for nucleosomes, or because transcription factors promote local depletion of nucleosomes (1, 2).

Several methods have been developed to identify open chromatin regions. Some exploit the increased accessibility of DNA in open chromatin to nucleases such as DNAse I or restriction enzymes (3, 4). Another method, named FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements), exploits the fact that upon exposure to formaldehyde, crosslinks are

formed between intimately interacting nucleosomal histones and DNA(5-7). In this method, formaldehyde-treated cells are first lysed and sonicated. DNA fragments that are not bound by nucleosomes do not establish nucleosomal-DNA crosslinks and are thus preferentially recovered from the aqueous phase of a phenol-chloroform reaction(8, 9). FAIRE has now been employed by diverse investigators to study open chromatin in a variety of eukaryotic cells, including yeast, protozoan parasites, and mammalian cells(10-14). A recent study (15)combined FAIRE with high-throughput sequencing (FAIRE-Seq) in purified human pancreatic islets –which are clusters of endocrine cells that produce insulin and other polypeptide hormones. That study verified that FAIRE indeed provides a signature of active regulatory elements in a primary tissue; it accordingly revealed prominent FAIRE enrichment in active promoters, as well as in predicted and known long range regulatory elements(16). The same study also uncovered thousands of clusters of tissue-selective open chromatin sites, many of which appear to be unexpectedly broad regulatory domains(17).

FAIRE is easy to perform, and is applicable to very small amounts of primary tissue. It can therefore be readily employed to study chromatin states linked to lineage-specific differentiation processes in living organisms or during *in vitro* differentiation protocols. It can also be employed to identify chromatin changes in human disease samples, or to understand how sequence variation impacts open chromatin states. Here we describe a detailed

implementation of the FAIRE protocol in isolated human pancreatic islets. Adaptations of this method are generally applicable to cultured cell lines and primary tissue fragments.

2. Materials

2.1. Tissue culture

1. Dithizone stock solution. Add 10 mg dithizone (Sigma, St. Louis, MO) to 2 mL dimethyl sulfoxide (DMSO) (Sigma). Store the solution at -20˚C.
2. RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS, Lonza) and penicillin/streptomycin 100 units/mL (Lonza).
3. Hanks' balanced salt solution (Lonza) and Phosphate Buffered Saline (PBS, Lonza).

2.2. FAIRE

1. 37% Formaldehyde (Calbiochem, Darmstadt, Germany).

3. 2 *M* Glycine (Sigma).
4. Protease inhibitor cocktail (Roche, Basel, Switzerland).
5. Dry ice and ethanol.
6. Lysis buffer (2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1m*M* EDTA). Add 20 µL Protease inhibitor cocktail (Roche) to 1000 µl of Lysis buffer freshly

before use.

7. TE buffer (10 m*M* Tris, pH 8.0, 1 m*M* EDTA, pH 8.0).
8. 25 gauge needles.
9. 2mL screw cap tubes (1 per sample).
10. Glass beads 0.5 mm (Biospec, Bartlesville, OK).
11. Conical 75 mm tall polypropylene tube (Sarstedt #57.512, Nümbrecht, Germany).
12. Sodium Acetate 3*M*, pH 5.2.
13. Ethanol 70% (Store at -20˚C), Ethanol 95% (Store at -20˚C), Ethanol 100% (Store at -20˚C).
14. Glycogen (Invitrogen, San Diego, CA).
15. Proteinase K 20 mg/mL (Roche).
16. RNase A 10 mg/mL (Qiagen, Hilden, Germany).
17. Tris-saturated Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (USB, Cleveland, OH), Chloroform (Sigma).
18. 5*M* NaCl.
19. 0.5*M* EDTA pH 8.0.
20. QIAquick PCR Purification Kit (Qiagen).

3. Methods

The analysis of primary human samples for functional genomic studies entails considerable experimental variability due to unavoidable differences in donors' history and tissue procurement procedures. Human pancreatic endocrine islets are a typical . They are obtained from organ donors after a lengthy experimental procedure that involves collagenase digestion and gradient purification that separates islets from the more abundant exocrine tissue(18). To mitigate environmentally induced variation, we culture islets in suspension under uniform conditions for 3 days prior to performing FAIRE.

The FAIRE method described here is based on that developed by Lieb and colleagues (19-21) and has been adapted for purified primary human islets, which are essentially tissue fragments of typically ~500-2000 cells that can be cultured in suspension. Adequate fixation of human islets requires slightly longer times of exposure to formaldehyde than cells grown in monolayer. Fixation conditions for other types of primary tissue samples may need to be optimized empirically. Proper sonication of primary tissues is also challenging. It is not only crucial to ensure that most chromatin fragments are smaller than 1 Kb, but also that fragment length is uniform across samples that need to be compared. Our protocol overcomes difficulties in the sonication of isolated islets, and can assist efforts to optimize sonication of other primary tissues.

3.1. Tissue culture

1. Prior to culture, islets are quantified and assessed for purity using Dithizone, which binds to zinc ions present in ß-cells and thus permits discrimination from pancreatic exocrine contaminants. Dilute 200 µL dithizone solution with 800 µL Hanks' balanced salt solution, centrifuge at maximum speed at room temperature for 5 min. After uniform dispersion of islets, an aliquot of 400 µL is taken and transferred to a 1.6 mL eppendorf tube (a larger volume is needed if there are not at least 50 islets). Let the islets sediment, then add 900 µL diluted dithizone solution to this, and incubate for 15 min at 37°C. Islets are next rinsed with PBS twice, and then examined under a microscope. Islet number in this aliquot is extrapolated to the total islet count based on the total culture volume. These islets can be spun, snap frozen and stored at -80˚C for subsequent extraction of unfixed DNA that can be employed as the input control (see below).
2. To place islets in culture after isolation or transportation, they are first transferred to 50 mL Falcon tubes in a tissue culture hood, and spun at 500 rpm (45 g) for 1 min in a benchtop centrifuge at room temperature. After discarding the supernatant, 10 mL of the culture medium is added for every ~2,000 islets. Islets are very gently resuspended, 10 mL medium is transferred to each 100 mm bacterial culture dish, which are then placed in

culture at 37ºC for 3 days.

3.2. *Fixation*

1. After culture, collect islets in the center of the culture dish by very slow constant anticlockwise swirling of the dish. Use a pipette to remove as much debris and non-islet material as possible.
2. Aspirate islets from two culture dishes (~4000 islets) and transfer to a 50 mL Falcon tube. Spin at 500 rpm (45 g) for 1 min in a benchtop centrifuge at RT.
3. Remove most supernatant, and transfer all islets into a single new 15 mL tube.
4. Rinse islets with 10 mL PBS and spin at 500 rpm (45g) for 1 min. Remove the supernatant. Repeat this twice (see **Note 1**).
5. After removing supernatant add PBS up to 10 ml.
6. Add 278 µL 37% formaldehyde (final concentration 1%) and fix cells shaking gently for 10 min at room temperature (see **Note 2**).
7. Stop fixation by adding 685 µL 2*M* glycine (final concentration 125m*M*) and incubate for 5 minutes shaking gently at room temperature.
8. Centrifuge islets at 500 rpm (45g) for 1 minute at room temperature.
9. Remove the supernatant, resuspend islets in 1 mL ice-cold PBS. Centrifuge at 500 rpm for 1

min at 4˚C. Repeat this step twice.

10. Remove the supernatant, snap-freeze fixed islets in liquid nitrogen or a dry ice/ethanol bath, and store the samples at -80˚C or liquid nitrogen. We have stored chromatin samples at -80˚C for over 6 months without any apparent deterioration of FAIRE enrichment.

3.3. Sonication

1. Allow a tube containing ~4000 fixed islets to briefly thaw on ice and add 1 mL Lysis buffer with protease inhibitor cocktail. Pipette the resuspended islets into a 2 mL screw-top tube. Add 1 mL of 0.5 mm glass beads to each tube.
2. Lyse cells by vortexing vigorously for one minute. Repeat this 10 times, placing tubes on ice 1 min. between each vortexing session.
3. Open the tube cap, insert a 25G needle in the bottom of the tube, allow the extract to drip into a conical 75 mm-tall tube on ice . Add 0.7 mL Lysis buffer to the beads, mix gently, and allow that to drip into the same tube. Aim for ~1.5-1.7 mL total lysate volume.
4. Label Eppendorf tubes for checking DNA sonication (e.g. 0, 5, 10 cycles). Take a 25-50 µL aliquot for cycle 0. Prepare a dry ice/ethanol bath.
5. Clean the sonicator probe by squirting water, ethanol, and then water again. Adjust sonication settings as follows (Branson 450D sonifier with tapered micro-tip

(#101-148-062)): 30”-1”ON/0.5” OFF; Amplitude 15% (set manually).

6. Sonicate for 10 cycles, placing the tube in the dry ice bath 2 seconds between each cycle (see **Note 3**). Take 25-50 µL aliquots at 5 and 10 cycles.
7. To assess the efficiency of sonication, add 150-175 µL TE buffer (to make 200 µL) to the 0, 5, 10 cycle samples. Spin at full speed in the microfuge at 4ºC for 5 min to clear the extract of debris and unlysed cells. Transfer the supernatants to new tubes. Add 1 µL RNase A (10 mg/mL) to each tube, tap the tubes lightly to mix and incubate at 65ºC for 20 min.
8. Add 2 µL Proteinase K 20 mg/mL, tap the tube to mix, and incubate at 65ºC for 3 hr.
9. Add 160 µL TE to make 360 µL total volume. µ
10. Add phenol-cholorofom solution (450 µL), vortex to mix. Spin at room temperature at maximum speed in a microfuge for 5 min. Recover the aqueous phase and transfer to new tubes.
11. Add chloroform (450 µL), vortex to mix. Spin at room temperature at maximum speed for 5 min. Recover the aqueous phase and transfer to new tubes.
12. Add 1/10 volume of Sodium Acetate (3*M*, pH 5.2), X 2.5 volume ice-cold 100 % ethanol and 1 µL glycogen (5 mg/mL). Invert several times and keep at -20ºC for >15 min.
13. Spin at 4ºC at maximum speed for 20 min and remove the supernatants.
14. Rinse with 500 µL ice-cold 70% ethanol. Spin at 4ºC at maximum speed for 5 min and

remove the supernatants.

15. Speed-vac ~10 min. at room temperature. Do not allow the pellet to overdry. Resuspend in 10 µL dH2O. Incubate at 37ºC for 30 minutes.
16. Run 5-8 µL of the resuspended DNA on a 1% agarose gel in 0.5X TBE buffer (110 V, 1 hour).
17. After electrophoresis, check the DNA size. If the chromatin has the intended size at 10 cycles (see **Note 4**), aliquot 300 µL chromatin samples in labeled eppendorf tubes, snap freeze and store at -80ºC, or use fresh. If fragments are too large, sonicate for 2-5 further cycles, check DNA quality again, and then store chromatin aliquots and/or use fresh.
18. Aliquot 75 µL of chromatin extract in one tube and label it as fixed chromatin input.

3.4. Phenol-chloroform extraction

1. Take 300 µL sonicated extract, add TE buffer to make a total volume of 500 µL. Spin for 10 min at 4˚C at full speed to clear debris. Recover the supernatant and transfer to a new tube.
2. Add an equal volume of phenol-chloroform solution to this supernatant. Vortex. Spin at top speed in microfuge for 5 min. Recover the supernatant and transfer to a new tube.
3. Add an equal volume of phenol-chloroform solution, vortex, and spin as in the previous step. Recover the supernatant ensuring that the interphase content is not collected and transfer to

a new tube.

4. Add an equal volume of chloroform, vortex, spin as in the previous step, and recover supernatant.
5. Add 1/10 volume Sodium Acetate (3*M*, pH5.2). Mix. (Optional: Add 1 µL of 20 mg/ml Glycogen). Add 2.5 X volume ice-cold 95% ethanol. Mix. Place at -20˚C for > 1 hour.
6. Centrifuge at full speed for 20 min at 4˚C. Remove supernatant, add ice-cold 70% ethanol, spin for 5 min. at full speed. Remove supernatant and speed-vac at room temperature. Be careful not to overdry the pellet.
7. Resuspend in 50 µL of TE buffer and add 1 µL RNase A (10 mg/mL). Flick the tube and incubate at 37˚C for >30 min.
8. DNA concentration can be quantified using a fluorometry-based method such as Qubit (Invitrogen) (see **Note 5**).

3.5. Preparation of input DNA

1. Place the sonicated extract labeled as input on ice.
2. Add TE to make 300 µL. Add 1.5 µL RNase A (10 mg/mL). Incubate at 65˚C for >30 min.
3. Add 4.5 µL Proteinase K (20 mg/mL). Incubate at 65˚C for 5 hr to O/N (see **Note 6**).
4. Purify DNA with a spin columns such as the QIAquick PCR Purification Kit (QIAGEN)

following the manufacturer's instructions. Elute DNA in 50 μL TE buffer.

3.6. Overview of FAIRE DNA analysis

1. The analysis of FAIRE-enriched DNA is in many ways analogous to that of chromatin immunopreciptation experiments, and can therefore be performed using either locus-specific assays (22), hybridization to miroarrays (23), or high throughput sequencing(24). Several general features of this analysis are nevertheless distinct in FAIRE as outlined below.
2. FAIRE can be analyzed with semi-quantitative PCR assays that target specific genomic sites. As in chromatin immunoprecipitation experiments, this involves designing oligonucleotides for the experimental target site as well as for negative control sites that presumably do not harbor open chromatin. FAIRE DNA is then assayed for enrichment at experimental and control sites relative to input DNA. There are however important caveats to bear in mind when performing this type of analysis. First, without *a priori* knowledge of where the FAIRE enrichment sites are located in the cell of interest, any PCR assay can easily fail to detect an open chromatin site even if it encompasses an open chromatin site. This can occur, for example, if one of the two oligonucleotides targets a sequence that flanks a true open chromatin site but is intimately bound by nucleosomes; DNA fragments

containing this flanking sequence will not be retained in the aqueous phase of the phenol extraction, and therefore the PCR assay will not detect FAIRE enrichment. Another critical issue is the selection of appropriate negative control regions. For unknown reasons FAIRE-seq experiments have shown detectable differences in “background” DNA between different broad genomic *loci*. This difference can occur between two regions that do not necessarily harbor any discrete open chromatin sites, which warrants the use of local negative control regions for PCR assays. These limitations can theoretically be overcome by designing a tile of overlapping short amplimers across a region of interest. In general, however, target–specific assays in FAIRE are ideally suited for interrogating previously characterized open chromatin sites.

3. High-throughput short-read sequencing provides a very powerful means of assessing FAIRE-enriched DNA at a genomic scale. Library construction, sequencing, and alignment can be performed analogously to chromatin immunoprecipation sequencing experiments. Some noteworthy differences are nonetheless also applicable here. First, the fraction of the genome that exhibits open chromatin is higher than that enriched in typical transcription factor chromatin immunoprecipitations, and furthermore the assay inherently leads to the recovery of a low level of DNA from non-enriched regions. These factors determine that a high sequencing depth is required to attain a reliable read coverage at any given site. As a

guideline, >50 million high-quality mapped 36-nucleotide reads from a single sample can provide a highly informative genome-scale open chromatin map in human tissue. A second related issue is that FAIRE does not divide a cellular genome into discrete closed regions and open chromatin peaks. Instead, this method provides a diverse range of enrichment values at different sites, and different sites can exhibit considerable length heterogeneity. Calling enriched sites thus provides a greater challenge than in typical transcription factor binding ChIP-seq experiments. A previous report of FAIRE-seq in human islets employed F-seq(25), a method that calculates a read density probability for each base, and identifies enriched regions of varying lengths that exceed different threshold standard deviations above a background mean. In general, different epigenomic features require different types algorithms for optimal detection, and any additional candidate algorithms need to be tested to assess their performance in FAIRE-seq datasets.

4. Notes

1. Do not expose islets to unnecessary physical stress, such as inverting or vigorous shaking of tubes. Vigorous pipetting of fixed islets will cause significant loss of the sample due to adherence to the plastic surface.
2. These fixation conditions are adequate for FAIRE in human islets, but need to be optimized

for other tissues or cell types. The same conditions are valid for chromatin immunoprecipitation assays for histone modifications.

3. Keep the probe at the highest possible point that does not lead to foaming and in a well-centered position, avoiding high-pitched sounds. The number of cycles varies according to several parameters, including fixation time and number of cells, and needs to be determined empirically by checking the DNA size ranges after 10 cycles. Because primary human samples can vary considerably in ways that affect sonication efficiency, we generally check sonication for all samples to ensure that we obtain consistent sonication lengths in different samples.
4. We aim for fragment sizes ranging from 200 to 1000 bp. When assessing this by simple inspection, it is worth remembering that ethidium bromide stains larger fragments more intensely than smaller DNA fragments. The optimal DNA size is empirical, yet different size ranges can yield different results for both FAIRE and chromatin immunoprecipitation experiments. It is therefore critical to ensure that the size range is similar across samples that are going to be compared to each other at some point.
5. Avoid using spectrophotometric methods as they can significantly overestimate DNA in FAIRE samples. FAIRE-enriched DNA can also be both quantified and assessed for size range using the Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA reagents

(Agilent Technologies, Palo Alto, CA).

6. It is important to ensure that the removal of crosslinks is exhaustive, otherwise "open" chromatin may theoretically be preferentially recovered when decrosslinking is insufficient in the input sample. This may influence any downstream assays that use input as a reference. Using unfixed DNA is a valid control for sequence analysis of FAIRE DNA.

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