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Supplementary information

RASER-FISH: non-denaturing fluorescence in situ hybridization for preservation of three-dimensional interphase chromatin structure

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SUPPLEMENTARY INFORMATION

RASER-FISH: non-denaturing fluorescence *in situ* hybridization for preservation of three-dimensional interphase chromatin structure

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SUPPLEMENTARY METHOD 1 | DENATURING FISH

This protocol describes the denaturing FISH approaches 4 min, 4 min + dry and 30 min + dry, as depicted in **Figure 1**, that we used for comparison to the RASER-FISH technique.

ADDITIONAL MATERIALS

REAGENTS

- Glycerol (Sigma Aldrich cat. no. G5516)
- Formamide (deionized, RNase free Invitrogen cat. no. AM9342) **! CAUTION** Formamide may damage fertility or the unborn child if swallowed, suspected of causing cancer if swallowed, may cause damage to organs through prolonged or repeated exposure. Wear appropriate personal protective equipment and use in a fume hood.
- Liquid nitrogen **! CAUTION** Liquid nitrogen is an extremely cold liquid and gas held under pressure. Wear appropriate personal protective equipment. Liquid nitrogen can cause rapid suffocation and severe frostbite. It should be dispensed slowly to a container suitable to withstand cryogenic temperatures. It boils rapidly therefore do not enclose in a sealed container. In the event of suffocation from lack of oxygen the casualty should be removed to fresh air. If the casualty is not breathing, give artificial respiration. If breathing is difficult, give oxygen. Obtain prompt medical attention. For skin or eye

splashes/contact, flush with copious amounts of tepid water and arrange immediate medical attention.

EQUIPMENT

Dilvac liquid nitrogen portable dewar (Cole Parmer cat.no. OU-03774-14 or similar)

REAGENT SET UP

20× SSC pH 5.5 Add 175.3 g NaCl and 88.2 g tri-sodium citrate to a final volume of 1L in MQW. Adjust pH with HCl. Store at room temperature for several months.

70% Ethanol Mix 70 ml ethanol with 30 ml DEPC MW. Store, tightly capped, for several months at room temperature.

85% Ethanol Mix 85 ml ethanol with 30 ml DEPC MW. Store, tightly capped, for several months at room temperature.

90% Ethanol Mix 90 ml ethanol with 30 ml DEPC MW. Store, tightly capped, for several months at room temperature.

HCl solution To make 0.1 N HCl solution, add 8.3 ml of concentrated HCl to 991.7 ml of MQW. Store at room temperature for several months.

Glycerol solution To make 20 % (vol/vol) glycerol solution add 20 ml glycerol to 80 ml MQW. Mix well on a rotary flatform and store at room temperature for several months.

Equilibration solution To make 50% (vol/vol) formamide in 2× SSC with 0.05% Tween; combine 50 ml formamide, 10 ml 20× SSC pH 5.5, 40 ml MQW and 500 µl 10% Tween solution and mix well. Store at 4 °C for several months

Denaturation solution To make 70% (vol/vol) formamide in 2× SSC; combine 70 ml formamide, 10 ml 20× SSC pH 5.5 and 20 ml MQW and mix well. Store at 4 °C for several months.

Cold 2× SSC. Chill 2× SSC to 4 °C and store for several months**.**

EQUIPMENT SET UP

Liquid nitrogen bath. Fill a liquid nitrogen dewar with liquid nitrogen to a safe level. Transport with care to the place of use.

Equilibration and Denaturation waterbaths Set two waterbaths in a fume hood; one set at 37 °C and one at 78 °C.

PROCEDURE

Seeding and fixation of cells ● TIMING 1 h + 20–24 h seeding and growth

1 | This part is as described previously under the same subheading within the main text, but without BrdU/C incorporation.

Denaturing FISH pre-treatment and hybridization ● TIMING 2.5–3h

Follow the steps as outlined in **Prehybridization treatment to generate single-stranded DNA, followed by hybridization** in the main Procedure, up to and including step 17**,** then proceed as follows.

2 | Equilibrate the coverslip in glycerol solution at room temperature for at least 30 min.
■ **PAUSE POINT** Equilibration in glycerol solution can be carried out for a few hours when convenient.

3 | Using forceps carefully lift the coverslip from glycerol solution, very briefly wick away excess liquid by touching the coverslip edge on a lint free tissue. Dip the coverslip into liquid nitrogen bath and hold until boiling stops (usually around 6 s). Carefully lay the coverslip, cell side up, on a clean paper towel until the frozen glycerol thaws. Transfer the coverslip briefly to glycerol solution before dipping again. Repeat this process a total of three times.
▲ **CRITICAL STEP** Coverslips can easily break during this dipping process owing to the rapid cooling. To minimize the risk of breakage avoid using excessive force on the forceps whilst holding the coverslip. Also, between dipping, keep the forceps free of ice build-up which can impair their good hold on the coverslip. We usually prepare an extra coverslip in case of accidental loss at this point. As the coverslip is not in an excess of liquid, be careful that does not dry out.

4 | Wash the coverslip three times for 5 min in 1× PBS at room temperature.

5 | Briefly rinse the coverslip in HCl solution and then incubate in HCl solution at room temperature for 5 min.
▲ **CRITICAL STEP** The HCl incubation partially removes proteins which allows better access for the FISH probes. Where poor hybridization signals are seen the HCl step can be lengthened however can prove detrimental to the preservation of chromatin morphology.

6 | Wash the coverslip three times each for 5 min in 1× PBS at room temperature.

7 | Where dehydration is required then incubate the coverslip in a series of ethanol washes (70%, 85%, 90% and 100%) each for 5 min at room temperature.

8 | Equilibrate the coverslip in equilibration solution at room temperature for 5 min.

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9 | Change the equilibration buffer to one warmed to 37 °C and incubate at 37 °C for 20 min by floating the 6-well plate in a waterbath, or by placing individual dishes in a floating box.

10 | Transfer the coverslips using forceps to the preheated denaturation solution in a 6-well plate, floated in a waterbath heated to 78 °C and denature for the length of time required.

! CAUTION See Step 8 for caution on formamide use

▲ CRITICAL STEP The denaturation solution is first warmed to temperature by heating in a 50 ml conical tube placed in the waterbath. This solution is then transferred to the wells of a floating, at temperature, 6 well plate in the same waterbath. The plate lid is quickly replaced, and the solution is allowed a few minutes to reach temperature. Working quickly, coverslips are transferred using forceps to the 6-well plate, with timing commencing from the first coverslip being immersed in the denaturation solution. Too short a denaturation time will result in poor hybridization signal whereas too long will impair the chromatin morphology. The denaturation time required may change from cell type to cell type.

11 | As soon as the denaturation time is complete, the coverslip is transferred using forceps to a 6-well plate on ice containing cold 2× SSC.

▲ CRITICAL STEP The cold 2× SSC acts to halt the denaturation. In order to keep the time in denaturation solution as accurate as possible, coverslips should be transferred out of the denaturation solution in the order that they were added to it.

12 | Change the cold 2× SSC washes on the coverslip twice.

13 | Take the prepared coverslip and quickly wick away excess 2× SSC by touching the coverslip edge on a lint-free tissue.

14 | Invert the coverslip, cell side down, on the prepared probe in hybridization buffer on the cleaned Superfrost glass slide, as detailed in subheading **Probe preparation for hybridization.**

▲ **CRITICAL** Avoid trapping any air bubbles in the hybridization mix by slowly and carefully lowering the coverslip. We find that setting one edge of the coverslip on the slide surface and then lowering the coverslip (akin to lowering a trapdoor) helps minimize trapped bubbles. If they are close to the edge, the gentlest pressure using forceps (to the side of the bubble away from the coverslip edge) can encourage them out. If they are more centrally located, the coverslip itself can be very gently eased across the slide surface to allow the bubble to reach the coverslip edge.

15 | Seal coverslip edges with vulcanising rubber cement.

▲ **CRITICAL STEP** It is not necessary to wait for the rubber cement to dry before transferring to the hybridization chamber, and then the waterbath.

16 | Place these slides into the hybridization chamber and allow to hybridize to commence by floating the hybridization chamber in a waterbath at 37–42 °C.

Post-hybridization washing and hapten detection ● TIMING 1–3 h

This is as described under subheading **Post-hybridization washing and probe detection** within the main Procedure. An array of alternative washes exists and can be employed where necessary[1-5].

SUPPLEMENTARY METHOD 2| PROBE LABELLING

Suitable bacterial based clones for probe use can be selected by viewing their genomic positions within the UCSC genome browser (https://genome.ucsc.edu/). Within the 'Mapping and Sequencing' area, the human hg19 build shows positions for FISH-validated clones, BACs and cosmids, and likewise the murine mm9 build shows positions for BACs. Additionally, within the mouse libraries listed at https://bacpacresources.org one can locate murine chromosome .bed files detailing positions of WIBR-1 library fosmid clones for each mouse chromosome. These files can be uploaded and viewed at the UCSC genome browser, under the 'add custom tracks' option. Clones can be ordered via https://bacpacresources.org or http://www.brc.riken.jp/lab/dna/. Plasmid clones, unless gifted, are usually subcloned from larger constructs.

ADDITIONAL MATERIALS

REAGENTS

- $MgCl_2$ (1 M solution Sigma-Aldrich cat. no. M1028)

- β-mercaptoethanol (β-ME, pure Sigma-Aldrich, cat. no. M3148)
- dNTPs (Invitrogen cat.no. 10297-018)
- Agarose (Invitrogen cat.no 16500-500)
- 10× TBE solution (Severn Biotech cat. no. 20-6000-50 or similar)
- DNase I, recombinant, RNase-free (10 U/µl Roche cat. no. 04716728001)
- DNA polymerase I, (10 U/µL NEB cat. no. M0209S)
- Hapten or fluor-labelled dUTP: e.g., digoxygenin-11-dUTP (Roche cat. no. 11093088910), Cy3 dUTP (GE Healthcare cat. no. PA53022, Alexa Fluor 594-5-dUTP (Thermo Fisher cat. no. C11400). Other suppliers of labelled dUTPs are also available.
- Illustra G-50 Microspin columns (GE Healthcare Life Sciences cat. no. 27-5330-02)
- Ethidium bromide solution (10 mg/ml Sigma-Aldrich cat. no. E1510)
  ! CAUTION It is strongly recommended that ethidium bromide is bought as a ready-made solution. Ethidium bromide is toxic if inhaled and is suspected of causing genetic defects. If inhaled, remove victim to fresh air and keep at rest in a position comfortable for breathing and seek emergency medical attention. If exposed or concerned, seek medical advice/attention.
- DNA size ladder (PCR Ranger 100 bp ladder Norgen cat. no. 11300 or similar).
- RNase (20–40 mg/ml solution Sigma-Aldrich cat. no. R4642)

EQUIPMENT

- Lo-bind 1.5 ml tubes (Camlab cat no. EP01466)
- Gel electrophoresis tank and powerpack

REAGENT SET UP

BSA solution for 10× NTB To make 50 mg/ml BSA solution, dissolve 500 mg BSA in a final volume of 10 ml molecular biology grade water. Dissolve well by mixing on a rotary platform or by incubating at 37 °C. BSA solution foams easily, to avoid this do not shake or pipette unnecessarily. Filter sterilise through 0.22 µm filter, aliquot and store and -20 °C.

10× NTB 10× nick translation buffer is composed of 0.5 M Trizma-HCl pH 8.0, 50 mM $MgCl_2$ and 0.5 mg/ml BSA (Sigma, Fraction V). Combine 5 ml 1 M Trizma-HCl pH 8.0, 500 µl 1 M $MgCl_2$ and 100 µl 50 mg/ml BSA and 4.4 ml molecular biology grade water. Store in aliquots at –20 °C.

0.1 M β-ME Mix 0.1 ml β-mercaptoethanol and 14.4 ml molecular biology grade water. Store in aliquots at –20 °C.

0.5 mM dAGC Mix 1 µl of each d A/G/CTP in 200 µl of molecular biology grade water. Store in aliquots at –20 °C.

DNase I solution Each batch of DNase I should be empirically tested for its cutting potency. It may be diluted with molecular biology grade water where required. If requiring dilution before use, make fresh and discard any remainder. DNase I is easily denatured, do not vortex, rather gentle flicking will suffice to mix. It is also a sticky enzyme, so use low-binding tubes.

RNase solution To make a 200 ng/µl solution, dilute RNase 1/170 in molecular biology grade water. Use immediately.

Gel staining solution Add ethidium bromide to a final concentration 0.5 µg/ml in 1× TBE buffer or deionised water. Use on the same day.

2% (wt/vol) agarose gel Dissolve 2 g agarose in 100 ml 1× TBE by heating, pour into casting tray with comb in place and allow to set. Can be stored, wrapped well to prevent drying, at 4 °C for 1–2 weeks.

PROCEDURE

Probe labelling ● TIMING 3 h

1 | Mix 1 µg DNA with 5 µl each of 10× NTB, 0.1 M β-ME, and 0.5 mM dAGC mix in a 1.5 ml low-binding tube. Add 1 nmol of hapten dUTP or fluorescently labelled dUTP, 3 µl DNase I and 10 U DNA polymerase I, then make up to 50 µl with molecular biology grade water. Residual RNA present in the DNA preparation can be removed prior to nick translation by first mixing only the DNA and water, together with 200 ng RNase and incubating for 30 min at 37 °C prior to adding the remaining reaction components.

▲ **CRITICAL STEP** Following DNA extraction we strongly recommend using a fluorometry-based approach for DNA quantitation. We use the Qubit fluorometer which yields consistent DNA quantitation in our hands.

▲ **CRITICAL STEP** For all clone types, we strongly recommend using a self- made alkaline lysis approach[6] to extract the cloned DNA rather than using a commercially available kit. We have found that with some kit-prepared DNA the nick translation labelling can be suboptimal.

▲ **CRITICAL STEP** DNase I is easily denatured therefore vortexing should be avoided. We mix by gentle flicking. Also, DNase I is a sticky enzyme so low-binding tubes are recommended.

2 | Incubate reaction mix at 16 °C for ~2 h. Put on ice to halt further enzymatic reaction during size assessment.

■ **PAUSE POINT** The nick translation mix can be stored at –20 °C for a few days before assessment on a gel and clean-up. If the DNA requires further cutting after freezing, add fresh enzymes as freezing will have destroyed those originally added.

▲ **CRITICAL STEP** The required nick translation incubation time is dependent on the DNase I and DNA polymerase I potencies, the insert size of the construct being labelled, the label being incorporated, and the purity of the DNA. Incubation times therefore vary and should be empirically assessed. With each new batch of DNase I the optimal concentration for controlled cutting needs to be empirically assessed.

3 | Run a 3 µl aliquot of the reaction mix on 2 % TBE agarose gel at 100 V for ~1 h with an appropriate size DNA ladder.

4 | Stain the gel using gel staining solution to allow visualisation of the DNA**.** This should show a smear predominantly 200–500 bp in size. If the DNA requires further cutting, add additional DNase I and re-incubate at 16 °C.

! CAUTION Ethidium bromide is hazardous, please use with care and dispose of staining solution and stained gels according to local rules.

▲ **CRITICAL STEP** To view the nick translation products, we find it necessary to stain the gel using ethidium bromide after electrophoresis. We find this best to visualise the relatively faint smear of nick translation products. We find that dUTPs with different modifications can differentially affect the processivity of DNA polymerase I, sometimes requiring small alterations in the incubation period.

? TROUBLESHOOTING

5 | When the smear size is correct, clean up the reaction mix using an illustra G-50 Microspin column.

■ **PAUSE POINT** Probes can be stored at –20 °C until required. Probes can be stored for varying lengths of time, depending on the label/hapten used.

▲ **CRITICAL STEP** Labelled DNA is variably labile depending on the fluorochrome/hapten used. We note that Alexa Fluor 594-5-dUTP as a probe label is particularly labile when stored and should be prepared when needed, whereas digoxygenin 11-dUTP and Cy3-dUTP as probe labels are reasonably stable and can be stored for at least 1 year. Green-emitting fluorochome dUTPs label less well than red or far-red dyes.

? TROUBLESHOOTING

SUPPLEMENTARY REFERENCES

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