

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

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15 **Editorial summary: RASER-FISH is a DNA fluorescence *in situ* hybridization technique which uses exonuclease digestion, instead of heat denaturation, to generate single-stranded target DNA for efficient probe binding, enabling chromatin preservation for super-resolution microscopy.**

20 **Proposed Tweet: #NewNProt RASER-FISH a non-denaturing FISH labelling technique that preserves 3D chromatin structure for super-resolution microscopy**

Proposed Teaser: RASER-FISH for preservation of chromatin structure

25 **KEY REFERENCES USING THIS PROTOCOL**

Brown JM, Roberts NA, Graham B, Waithe D, Lagerholm C, Telenius JM, De Ornellas S, Oudelaar AM, Scott C, Szczerbal I, Babbs C, Kassouf MT, Hughes JR, Higgs DR, Buckle VJ. (2018) A tissue-specific self-interacting chromatin domain forms independently of enhancer-promoter interactions. *Nat Commun* **9** (1):3849. DOI: 10.1038/s41467-018-06248-4.

Ochs F, Karemore G, Miron E, Brown J, Sedlackova H, Rask M-B, Lampe M, Buckle V, Schermelleh L, Lukas J, Lukas C. (2019) Stabilization of chromatin topology by 53BP1 and RIF1 safeguards genome integrity. *Nature* **574**: 571-574. DOI: 10.1038/s41586-019-1659-4.

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Miron E, Oldenkamp R, Pinto DMS, Brown JM, Faria AR, Shaban HA, Rhodes JDP, Innocent C, de Ornellas S, Buckle V, Schermelleh L. (2020) Chromatin arranges in chains of nanodomains with 3D functional zonation. *Sci Adv* **6**: eaba8811. DOI:

40 10.1126/sciadv.aba8811.

ABSTRACT

DNA fluorescence *in situ* hybridization (FISH) has been a central technique in advancing our understanding of how chromatin is organized within the nucleus. With the increasing
45 resolution offered by super-resolution microscopy, the optimal maintenance of chromatin structure within the nucleus is essential for accuracy in measurements and interpretation of data. However, standard three-dimensional (3D)-FISH requires potentially destructive heat denaturation in the presence of chaotropic agents such as formamide to allow access to the DNA strands for labelled FISH probes. To avoid the need to heat-denature, we developed
50 Resolution After Single-strand Exonuclease Resection (RASER)-FISH, which uses exonuclease digestion to generate single-stranded target DNA for efficient probe binding over a two-day process. Furthermore, RASER-FISH is easily combined with immunostaining of nuclear proteins or the detection of RNAs. Here, we provide detailed procedures for RASER-FISH in mammalian cultured cells to detect single loci, chromatin tracks and
55 topologically associating domains with conventional and super-resolution 3D structured illumination microscopy. Alongside, we provide a validation and characterization of our method, demonstrating excellent preservation of chromatin structure and nuclear integrity, together with improved hybridization efficiency, compared to classical 3D-FISH protocols.

INTRODUCTION

60 The combination of DNA fluorescence *in situ* hybridization (FISH) with super-resolution
microscopy (SRM) has recently gained a lot of traction. FISH is being used alongside super-
resolution imaging to inform on many measurable aspects of chromatin organization,
including spatial relationships between given genomic sites¹⁻⁵, compaction of specific
chromatin regions⁶, and their conformation^{3,4,7}. The understanding of genome organisation
65 and its relation to development, gene regulation and transcription has been transformed by
the powerful combination of FISH with chromosome conformation capture (3C)
technologies⁸⁻¹², where the complementary application of orthogonal approaches have
underlined the importance of genome partitioning into topologically associating domains
(TADs) at the size scale of hundreds of kilobases. The results using these two approaches
70 are mostly concordant, however there are instances where results diverge¹. Whilst careful
and thoughtful FISH approaches have been devised with super-resolution imaging in mind,
'swelling or dispersal of chromatin, in particular as a result of the heat denaturation step' has
been noted and the suggestion made that 'future studies should also focus on approaches
modifying or replacing heat denaturation'¹³. Specialized approaches, such as COMBO-
75 FISH^{14,15}, low temperature FISH¹⁶, and CASFISH¹⁷ have been developed to detect repetitive
sequences, however their broader applicability is limited as there are strictures so far on the
genomic sequences that can be probed. The recently reported GOLD-FISH method¹⁸ offers
promise in minimising denaturation by targeting super-helicase activity to unwind DNA at
specific genomic sites, however at present this technique works only with a denaturing
80 fixation involving methanol and acetic acid, which reduces compatability with some protein
labels, and lacks the stabilization created by covalent crosslinking.

Development of the protocol

To overcome the limitations of classical DNA FISH approaches, we sought an alternative
85 technique compatible with maintaining mesoscale chromatin structure (i.e. physical size
range of several tens to a few hundred nanometer and genomic size range of a few 10s kb
to ~1 Mb), that was not reliant on physical denaturation by heat and exposure to formamide
to generate single-stranded DNA (ssDNA). We developed a non-denaturing alternative
termed RASER-FISH, adapted from chromosome orientation (CO)-FISH^{19,20}, a technique
90 previously applied to detect repetitive sequences on metaphase chromosome preparations.
The rationale is that DNA labelled with bromine-nucleotide analogues can be nicked by UV
light exposure (following a sensitization process using Hoechst or DAPI). These nicks can
then serve as a substrate for subsequent digestion by Exonuclease III (Exo-III) to create
single-stranded DNA regions, available for hybridization with labelled FISH probes. Through

our modifications to the original protocol, the RASER-FISH technique robustly generates ssDNA throughout the interphase genome, allowing hybridization of single copy probes within structurally maintained interphase nuclei.

Applications of the method

3D genome organization and its interplay with genomic function is currently the focus of intense research²¹⁻²³. At an averaged cell population level, the evolving repertoire of 3C based techniques has increased our understanding of 3D genome architecture, especially at the TAD level, where genes and their enhancer elements interact to regulate gene expression²⁴⁻²⁷. Single cell 3C approaches have been developed and suggest broad heterogeneity in genome structure between nuclei^{28,29}, however FISH remains critical to fully understand the organization of the genome within single cells and its integrated application with 3C-based technologies will be essential to reach a balanced understanding of the principles underlying functional genome organization³⁰⁻³³. From a FISH perspective, recent step-change developments in probe and imaging strategies have allowed visualization across genomic regions at an increasingly fine scale^{8-10,34,35}, which is where preservation of chromatin structure in three dimensions becomes vital.

RASER-FISH allows for the detection of FISH probes of varying sizes, including plasmids, fosmids, bacterial artificial chromosomes (BACs) and BAC contigs, as well as oligonucleotide probe pools. Further, existing FISH methodologies cannot always be easily combined with the detection of nuclear features described by protein occupancy or RNA expression. We demonstrate that RASER-FISH can be combined with both immunostaining and RNA detection. We have shown that a range of probe sizes from plasmids to BAC contigs can be detected together with proteins. RNA can also be detected alongside genomic DNA, either by direct detection of the RNA or by detecting engineered stem loops in the RNA (see **Anticipated Results** section).

RASER-FISH has been applied successfully to studies of the α -globin locus as a model for gene regulation, where we were able to show that formation of a decompacted chromatin domain occurs during erythroid differentiation, independently of enhancer-promoter interactions³⁶. More broadly within the nucleus, the technique was critical for studies of mesoscale chromatin organisation³⁷, the functional operation of 53BP1 bodies across several TADs in DNA double-strand break repair,³⁸ and the disruption by cohesin of long-range interactions between sites occupied by the polycomb repressive system³⁹.

It is feasible that RASER-FISH in the absence of bromodeoxyuridine/bromodeoxycytidine (BrdU/C) incorporation could be applied to studies of DNA damage, due to DNA resection from sites of breakage, although this is not an

approach that we have tried. One caveat may be that certain sites of damage, depending on factors including cell cycle and chromatin environment, would be capped too rapidly to allow exonuclease access on a meaningful scale.

Comparison with other 3D-FISH methods

To assess the effects of RASER-FISH [RASER] on chromatin, we compared it alongside two FISH protocols, heat denaturation at 78°C for 4 minutes [4 min] and heat denaturation at 78 °C for 4 minutes after dehydration through an ethanol series [4 min+dry] (**Fig. 1, Supplementary Method 1**), derived from commonly used 3D-FISH approaches for super-resolution imaging^{1,7,13,35}. We found treatments at 78 °C for 4 minutes were the minimum steps necessary to obtain a reasonable FISH signal. One report used 76 °C for 2 minutes¹³ but this involved prior storage in 50% formamide which could have increased accessibility for hybridization. This highlights the delicate situation that experimentalists face when attempting to balance successful FISH outcomes with preservation of nuclear structure. Heat denaturation methods often also include liquid nitrogen dipping (freeze-thawing) and acid incubation steps to improve probe accessibility, which we also include in our comparison (**Fig. 1**). Where appropriate, we compared conventional immunofluorescence (IF) preparations as the standard where chromatin has not been disturbed. In addition, to highlight the damage that heat denaturation causes, we include a prolonged heat denaturation of 30 min with dehydration [30 min+dry]⁴.

Good hybridization efficiency is key to FISH being a reliable and productive approach. When we compare paired allelic hybridization efficiencies of plasmid probes for the four hybridization strategies, RASER-FISH scores very favourably relative to heat-denaturing FISH approaches (88% for RASER vs 52% for 4 min, 39% for 4 min+dry and 58% for 30 min+dry, using 7kb probes) (**Fig. 2a**). The increased hybridization efficiencies reported here and elsewhere⁴⁰ for RASER-FISH are particularly promising where accuracy is required for assessing volume shape and size of the hybridized regions; high efficiency of hybridization would give confidence that a region is fully available as a single-stranded template. Additionally, it is not surprising, for heat-denatured preparations, that not all alleles are accessible as a single-stranded template⁴¹, given the delicate balance at play between sample preservation and appropriate DNA denaturation. RASER-FISH should be free from such issues relating to differential denaturation.

To compare the level of ssDNA achieved by 4 min, 4 min+dry, and RASER, we utilized immunostaining with an antibody against ssDNA (**Fig. 2b**). For RASER-prepared DNA, we see uniform ssDNA staining throughout the nucleus volume and staining that is smoothly concordant with the nuclear periphery as denoted by a DNA counterstain (**Fig. 2b**).

This contrasts with the 4 min and 4 min+dry preparations where ssDNA staining appears more speckled and less uniform throughout the nuclear volume and where we see spillages of DNA outside the nuclear periphery (**Fig. 2b**). These spillages are most often not visible with DAPI staining, which is known to fluoresce less strongly when bound to ssDNA templates⁴² and hence would be under-recognized in DAPI stained, heat-denatured preparations. This informative approach demonstrates robust single-stranded template generation within the nuclei of RASER-FISH preparations whilst maintaining the integrity of the nuclear boundary.

A question that arises is whether the more densely packaged regions of nuclei, for example heterochromatic regions, are less physically accessible to Exo-III for resection. To assess this, we hybridized murine cells with a FISH probe against the gamma-satellite sequences which are located pericentromerically and form the characteristic chromocenters seen in mouse cells (**Fig. 2c**). The heterochromatic regions appear to be labelled equally well by all FISH approaches. However, we again observe occasional spillage beyond the nucleus periphery with heat denaturation, but not with the RASER approach (data not shown).

When analyzing the effect on the mesoscale structure of chromatin by super-resolution 3D structured illumination microscopy (3D-SIM), we observe a qualitatively similar internal nuclear morphology after RASER-FISH as compared to the IF control, that is characterized by a rather inhomogeneous distribution of SYTOX Green stained chromatin with filamentous chains of 200–300 nm wide domains separated by distinctive interchromatin space of similar dimensions³⁷ (**Fig. 3a**, left). In contrast, already after 4 min heat denaturation (independent of dehydration) the chromatin distribution appears far more homogeneous with an apparent reduction in interchromatin space. This is further exacerbated after 30 min heat denaturation (**Fig. 3a**, right). Qualitative assessment of corresponding TAD FISH signals reveals consistently more distinct, defined edges of the RASER signal, compared to 4 min (\pm dry) or 30 min+dry heat denaturation (**Fig. 3b**). We conclude that chromatin organization at the increased resolution level of 3D-SIM is visibly changed already after 4 min of heat denaturation, whereas RASER-FISH results in a less affected mesoscale nuclear topography.

Limitations

The main restriction in application of RASER-FISH is that cells must be cycling for incorporation of nucleotides to occur, prior to fixation. Certain cell lines can show differential metabolism of nucleotides, such as thymidine kinase deficient cell lines, and may require BrdU/C levels to be adjusted to ensure good labelling. Very occasionally certain cell types

may not be suitable for use with RASER-FISH as they may not label sufficiently well with BrdU/C and this would result in insufficient nick sites for creating ssDNA as hybridization target sites therefore affecting the success of an experiment. Theoretically, exonuclease III could utilize RNA-DNA hybrids as a substrate which could impact RNA signal strength, however we are able to detect RNAs in combination with DNA. Finally, it is theoretically possible that incorporation of BrdU/C into the DNA strand, subsequent UV light exposure after a sensitization process and nicks could have an unquantified effect on chromatin organisation. For instance, it is known that BrdU has an effect on the structure of DNA and that may in turn affect interactions with DNA binding proteins/molecules. However we have no evidence that this might be the case. At least on the resolution level of SIM, we do not see any major differences in chromatin topography between RASER samples and IF controls.

Experimental design

The RASER-FISH approach harnesses the ability to introduce breaks within BrdU/BrdC substituted DNA that can then be resected to leave single-stranded regions available for hybridization^{43,44}. A mixture of BrdU and BrdC (to avoid bias and label both AT or GC nucleotide pairs) is incorporated into the DNA during cell culture or *in vivo*. The cells are then taken through the standard 3D FISH prehybridization steps of fixation, permeabilization and quenching. At this point, instead of being pre-treated with liquid nitrogen dipping and acid and eventually heat-denatured, the cells are stained with an ultra-violet (UV) light sensitizer (DAPI or Hoechst 33258) and exposed to UV light to induce breaks into the DNA strands. Incubation with an exonuclease then creates runs of single-stranded DNA available for hybridization with appropriately labelled probes (**Fig. 4**). The RASER-FISH workflow provided below describes the seeding and fixation of cells, probe preparation for hybridization, pre-hybridization treatment to generate single-stranded DNA followed by hybridization, post-hybridization washing, and probe detection and assessment of ssDNA generation. Advantageously, immunostaining for protein can be introduced between the quench and sensitizer steps, and detection of nascent RNA can be undertaken as a process leading into the sensitizer step (**Fig. 5**). We describe these RASER-FISH add-ons in **Box 1** (RNA-DNA RASER-FISH) and **Box 2** (Immuno-RASER-FISH).

Reagent considerations: Whilst it is not strictly necessary, we would recommend the use of Milli-Q® grade (MQW) water (defined as having a minimum resistivity of 18.0 MΩ/ cm with maximum ten parts per billion total organic carbon). This offers a consistent starting basis for all solutions, thereby aiding reproducibility between experiments.

240 **Sample preparation considerations:** Incorporation of BrdU/C into replicating DNA is central to the success of RASER-FISH. The care of cells preceding any experiments is paramount to a successful experimental outcome. Cells should be as viable as possible and in growth phase, with contact inhibition of cell growth avoided. Ensure that seeding densities at the beginning of the experiment take into account the projected growth of the cells. The
245 concentration of BrdU/C used to label cells may need adjustment from cell type to cell type, for example certain cells lines are known to have differential metabolism of nucleotides, such as thymidine kinase deficient cell lines, and may require a higher BrdU concentration to ensure good labelling or indeed may not be suitable for use with RASER-FISH. It is sensible to monitor the cell viability and proliferative capacity of the cells to ensure that BrdU/C
250 incorporation does not unduly affect the cells in use. If any chemical or genetic treatment of the cells is required for your experimental design, an assessment that BrdU/C labelling has not been unduly perturbed is recommended. The timing of BrdU/C labelling does not need to be of a strict length i.e., one cell cycle. Instead, we have found that an estimation of the shortest time taken for the majority of cells to be labelled with BrdU, by using antibody
255 assessment of BrdU incorporation, is sufficient. Although we have not used cells where BrdU/C incorporation was performed *in vivo*⁴⁵, we envisage no reason as to why these would not yield success with RASER-FISH. Again, assessment of the optimal BrdU/C concentration, delivery method and incorporation time is advised. Other available deoxynucleotide analogues (e.g., EdU/EdC) are not suitable for RASER-FISH. In using
260 BrdU/BrdC, it is photodissociation of the bromine atom upon UV excitation that degrades the DNA backbone⁴⁶. RASER-FISH was developed with sub-diffraction microscopy in mind, and we emphasise that where super-resolution imaging is required, consideration must be given to the fine details of optimal sample preparation, using published experimental protocols⁴⁷.

265 **Successful single-stranded DNA generation:** Another feature foundational to RASER-FISH success is the generation of sufficient nick sites in the DNA to act as substrates for subsequent resection. Key to this is using a UV wavelength which delivers sufficiently high energy. The UV light used must be of a short wavelength, with peak emission at 254 nm. Longer wavelengths such as 365 nm will not be successful. Our experiments have been
270 empirically optimised using a given bulb type. It is possible to use bulbs of differing wattage, however the timing needed for successful creation of ssDNA should be empirically assessed by immunofluorescent detection with an antibody to ssDNA. We have not used the more recently available UVC LED light sources. Again, empiric assessment of the optimal conditions for ssDNA generation using such light sources could be undertaken. A sensitizer

275 is also required in order to allow the UV light to optimally nick and break the BrdU/C substituted DNA and RASER-FISH does not work without the inclusion of this step (unpublished results). We have successfully used DAPI and Hoechst 33258, which are fluorescent DNA minor groove binders that are excited by UV wavelength light. Consideration of the final experimental design should be made when deciding which
280 sensitizer to use, for instance if DAPI is your preferred nuclear counterstain, then it is sensible to use this within the RASER-FISH workflow. Irrespectively, signal intensity will be reduced due to the lower affinity to ssDNA of both dyes. Antibody detection of ssDNA provides confidence that the RASER-FISH technique is working well, which is especially helpful when difficulties are encountered in obtaining FISH signals. When performing ssDNA
285 staining, a positive control (achieved by standard formamide denaturation) and negative control (non-denatured, fixed cells) should be included alongside the RASER-FISH prepared samples. Single-stranded DNA can occasionally be seen within untreated nuclei, usually within the nucleoli of particularly transcriptionally active cells.

290 **Probe generation:** We and others have successfully used a wide range of probe types with RASER-FISH³⁶⁻⁴⁰. Labelled probes can be produced from custom-designed oligonucleotides by amplification and label incorporation using rolling circle amplification⁴⁸ or by the Oligopaint approach^{49,50} and from various types of cloned DNA (typically plasmids, fosmids/cosmids and BACs) using nick translation or random prime labelling. We describe in **Supplementary**
295 **Method 2** a process for generating nick translated probes that we have found reliable.

We recommend paying careful attention to probe generation. Where DNA needs to be prepared from bacterial cultures, home-made alkaline lysis reagents produce DNA that labels well by nick translation. We occasionally find DNA prepared using commercially available kits can be resistant to optimal labelling by nick translation. We also find that, when
300 using fluorochrome labelled dUTPs, nick translation labelling works best when no dTTP is present in the nucleotide mix. However, there can be occasional fluorochrome dUTPs that buck this trend, in which case we suggest following the manufacturer's instructions. We also note that different fluorochrome labelled dUTPs offer variability in their longevity of storage e.g., Cy3 and digoxigenin labelled probes store well, whereas Alexa 594 labelled probes are
305 more labile. In planning your experiments, consider the requirements of the imaging system that you will use to analyse the outcome of your experiments and label your probes accordingly. When combining RASER-FISH with immunostaining we find it is sensible to use green fluorochromes for FISH probes and red or far-red fluorochromes for immunostaining. This is owing to the unavoidable photoconversion of DAPI following ssDNA generation by
310 UV exposure which can give some fluorescence in the FITC channel⁵¹. This typically does

not interfere with FISH signals but can make nuanced protein detection more difficult to assess. Also, many far-red fluorochromes can produce probes that result in higher levels of spurious background by virtue of their increased hydrophobicity.

315 **Experiment size considerations:** We find it comfortable to handle up to 12 coverslips in one experiment, in order to keep timings accurate, although 24 is possible. An experiment should include enough coverslips for all comparisons and controls for the experimental question, plus a few extra in case of coverslip loss especially where the experimenter is not used to handling coverslips. We score 200-500 nuclei (easily achievable from one coverslip)
320 for each condition and the experiment should be repeated three times, where possible, for statistical analysis.

MATERIALS

REAGENTS

- 325 • Milli-Q grade water (MQW) (Millipore)
- NaCl (Sigma-Aldrich cat. no. S3014)
- Tri-sodium citrate dihydrate (Sigma-Aldrich cat. no. C7254)
- NH₄Cl (Sigma-Aldrich cat. no. A9434)
- 5-Bromo-2'-deoxyuridine (BrdU Sigma-Aldrich cat. no. B5002) **! CAUTION** BrdU may
330 cause genetic defects. Order an appropriate amount that does not require weighing, wear appropriate personal protective equipment and resuspend in a laminar flow hood to maintain sterility and protect the user.
- 5-Bromo-2'-deoxycytidine (BrdC, Jena Bioscience cat. no. N-DN-6496)
- 10x PBS solution pH 7.0 (Severn Biotech cat. no. 20-7400-10)
- 335 • Poly-L-lysine solution (0.1% wt/vol solution Sigma-Aldrich cat.no. P8920)
- Formaldehyde (16 % methanol free Alfa Aesar cat.no. 43368.9M) **! CAUTION**
Formaldehyde is carcinogenic and harmful to the skin and respiratory tract. Wear appropriate protective equipment and use in a fume hood.
- HEPES (1 M solution Gibco cat. no. 15630080)
- 340 • Tween 20 (Sigma-Aldrich cat. no. P9416)
- Triton X-100 (Sigma-Aldrich cat. no. T9284)
- Hoechst 33258 (10 mg/ ml solution Invitrogen cat. no. H3569)
- 4', 6'-diamidino-2-phenylindole (DAPI) (Roche cat. no.10236276001)
- SYTOX Green (5 mM solution Invitrogen cat. no. S7020)
- 345 • Exonuclease III (100 U/μl NEB cat. no. MO206L)

- Mouse *Cot*-1 DNA (1 mg/ ml Invitrogen cat. no. 18440016)
- Human *Cot*-1 DNA (1 mg/ ml Invitrogen cat. no. 15279011)
- Single-stranded salmon testes DNA (10 mg/ ml Sigma-Aldrich cat. no. D9156)
- Anhydrous sodium acetate (Sigma-Aldrich cat. no. S2889)
- 350 • Ethanol (absolute VWR cat. no. 20821.330) ! **CAUTION** Ethanol is flammable.
- DNA hybridization buffer (Leica Biosystems cat. no. KBI-FHB)
- Vulcanising rubber cement solution (Weldtite Products cat. no. 02002)
- Bovine serum albumin (BSA)-fraction V (Sigma-Aldrich cat. no. 05482)
- VECTASHIELD antifade mountant (Vector Laboratories cat. no. H-1000-10)
- 355 • Slowfade Diamond mountant (ThermoFisher Scientific cat. no. S36963)
- Foetal bovine serum (FBS) (Sigma-Aldrich cat. no. F7524)
- Trizma base (Sigma-Aldrich cat. no. T1503)
- Trizma-HCl solution (pH 8, 1 M Sigma-Aldrich cat.no. T2694)
- Hydrochloric acid (concentrated, 37% Sigma-Aldrich cat. no. 258148) ! **CAUTION**
- 360 Hydrochloric acid may be corrosive to metals, causes severe skin burns and eye damage, and may cause respiratory irritation. Wear appropriate personal protective equipment and use in a fume hood.
- Molecular biology grade water (Sigma Aldrich cat. no. W4502)
- Rabbit anti-single-stranded DNA (IBL International cat. no. JP18731)
- 365 https://scicrunch.org/resolver/RRID:AB_2341405
- Sheep anti-DIG FITC (Roche cat. no. 11207741910 or similar)
- https://scicrunch.org/resolver/RRID:AB_514498
- Rabbit anti-sheep FITC (Vector cat. no. FI-6000 or similar)
- https://scicrunch.org/resolver/RRID:AB_2336218
- 370 • Goat anti-rabbit Alexa 488 (ThermoFisher Scientific cat. No. A-11001)
- https://scicrunch.org/resolver/RRID:AB_2534069

BIOLOGICAL MATERIALS

375 **Cell line of interest.** RASER-FISH requires cycling cells, in order to incorporate BrdU/BrdC into the replicating DNA. Certain cell lines can show differential metabolism of nucleotides, in which case BrdU/C levels may need to be adjusted. In unusual cases, cells may not label sufficiently well with BrdU/C and would therefore not be compatible with RASER-FISH. None of the cell lines used here are known to be frequently misidentified or cross contaminated. Cell types we used to produce the data reported in this protocol include:

- 380
- C127; mouse mammary epithelial cell line ATCC Cat No CRL-1804
https://scicrunch.org/resolver/RRID:CVCL_6580
 - U2OS; human epithelial like osteosarcoma cell line ATCC Cat No HTB-96
https://scicrunch.org/resolver/RRID:CVCL_0042
 - HeLa; human epithelial like cervical carcinoma cell line ATCC Cat No CCL-2,
- 385
- https://scicrunch.org/resolver/RRID:CVCL_0030
 - RPE1; human epithelial retinal cell line ATCC Cat No CRL-4000
https://scicrunch.org/resolver/RRID:CVCL_4388
 - mES ROSA-Tir1 (here referred to as mES Tir); a gift from Prof Robert Klose (Rhodes 2020)
- 390
- ! CAUTION** Cells should be regularly checked to ensure they are not infected with mycoplasma.

EQUIPMENT

- Laminar flow hood
- 395
- Stratalinker 2400 (Agilent Genomics)
 - 15 W 254 nm bulbs (15 W 254 nm GE Healthcare cat. no. G15T8).
 - 6-well tissue culture grade plates (Corning cat. no. 3516)
 - Borosilicate glass coverslips, 22x22 mm, No 1.5H (tol. $\pm 5 \mu\text{m}$; Marienfeld Superior cat. no. 0107052).
- 400
- Plastic 3 ml pastettes (SciLabWare Ltd cat. no. 726128)
 - Vacuum aspirator (VACUUBRAND BVC Professional cat. no. 20727402)
 - Fine-tipped glass pipettes (Fisher cat. no. FP50253)
 - Parafilm (Starlab cat. no. 13080-1075)
 - Whatman paper 3MM CHR grade (GE Healthcare cat. no. 3030-917)
- 405
- Refrigerated centrifuge (Eppendorf 5424R or similar)
 - Superfrost glass slides (VWR cat. no. 631-0911 or similar)
 - 0.2 μm filters (Sartorius cat. no. 16534 or similar)
 - Fine-tipped forceps (Dumont no. 5; Fine Science Tools, cat. no. 11251-20 or similar)
 - Autoclavable 10 l carboy (Nalgene cat. no. C2692 or similar)
- 410
- 15 ml tubes (Corning cat. no. 430791)
 - 50 ml tubes (Corning cat. no. 430829)
 - Gel electrophoresis tank and powerpack
 - FISH hybridization chamber: any tight-lidded box that holds slides flat and in darkness that can be floated in a waterbath is suitable.

- 415 • Antibody incubation chamber: Bioassay dish (Corning cat. no. 431111 or similar).
- Thermomixer C (Eppendorf)
- Lint free tissue e.g., Kimwipe soft tissue (Kimberley-Clark Professional cat. no. 34155 or similar)
- Paper towels (Tork cat. no. 290158 or similar)
- 420 • Coverslip sealant (transparent nail polish)
- Lidded, light-proof microscope slide storage box (Fisher Scientific cat. no. 11354135 or similar)
- Lidded glass staining trough with slide rack (Pyramid Innovation cat. no. R60015-E and R61015-E or similar)
- 425 • Rotary platform (IKA Roller 10 Digital, IKA cat. no. 4013000 or similar)
- Gel imaging system, e.g., GelDoc XR+ with Image Lab software (BioRad) or similar.

REAGENT SETUP

- 10 mM BrdU/C mix** Add 100 mg BrdU to 32.5 ml molecular biology grade water (10 mM) and 100 mg BrdC to 32.5 ml molecular biology grade water (10 mM). Mix the 10 mM solutions at a ratio of BrdU: BrdC 3:1. Store in aliquots at -20°C , protected from light, for several years. Avoid repeated freeze/thaw cycles.
- 10x PBS** 10x PBS is 80 g NaCl, 2 g KCl, 4 g Na_2HPO_4 , 2.4 g KH_2PO_4 , pH 7.4; for working dilutions, dilute with MQW to make 1x PBS. Store at room temperature for up to one year.
- 435 **10x TBE** 10x TBE is 890 mM Tris, 890 mM boric acid, 20 mM EDTA. Dilute to 1x TBE using MQW. Store at room temperature for a few months. 10x TBE can precipitate easily, do not store at low temperatures. Filtering can prolong the storage time. Reheat briefly to dissolve if precipitates do form.
- PBST** PBST is composed of 1x PBS with 0.05% (vol/vol) Tween 20. Add 500 μl Tween 20 to 1 l of 1x PBS. Store at room temperature for up to 6 months.
- 440 **Coverslip cleaning solution** Add 8 ml HCl to 492 ml MQW. Make fresh.
- 0.01% (vol/vol) poly-L-lysine solution** dilute one volume poly-L-lysine solution with nine volumes MQW. Prepare fresh.
- 10% (vol/vol) Triton X-100 solution** Add 10 ml of Triton X-100 to 90 ml of 1x PBS.
- 445 Dissolve the solution overnight by rotation on a rotary platform. Can be stored at room temperature for several weeks or at 4°C for several months.
- 10% (vol/vol) Tween 20 solution** Add 10 ml of Tween 20 to 90 ml of MQW. Dissolve the solution overnight by rotation on a rotary platform. Can be stored at room temperature for several weeks or at 4°C for several months.

450 **Fixation solution** Prepare 4% (wt/vol) formaldehyde by mixing 10 ml 16% formaldehyde, 10 ml 1 M HEPES, 20 ml MQW. Use immediately upon preparation.

Post-detection fixation solution Prepare 4% (wt/vol) formaldehyde in 1× PBS by mixing 10 ml 16% formaldehyde, 4 ml 10× PBS and 26 ml MQW. Use immediately upon preparation.

455 **Quenching solution** Add 0.13 g NH₄Cl to 50 ml 1× PBS. Make fresh.

DAPI stock solution Make a stock DAPI solution of 5 mg/ml by dissolving 10 mg DAPI in 2 ml MQW. Store stock solution protected from light at –20 °C for up to one year.

Sensitizer solution Add 2.5 µl 10 mg/ml Hoechst 33258 to 50 ml PBS to give a working solution of 0.5 µg/ml or 5 µl 5 mg/ml DAPI to 50 ml 1× PBS solution to give a working

460 solution of 0.5 µg/ml. Both solutions can be stored at 4 °C for up to 3 months.

Exonuclease III solution Prepare ~60 µl per coverslip; for 6 coverslips, mix 20 µl 100 U/µl Exonuclease III, 40 µl supplied buffer and 340 µl molecular biology grade water. Make fresh.

3 M sodium acetate solution Make 24.6 g anhydrous sodium acetate up to 100 ml with MQW. Store at room temperature for several years.

465 **70% (vol/vol) ethanol** Mix 70 ml absolute ethanol and 30 ml MQW. Store at room temperature for up to 1-2 months. Ethanol evaporates readily, ensure the bottle is tightly sealed and do not store for prolonged periods.

Wash buffer (PBST; 0.02% (vol/vol) Tween 20 in PBS) To 1 L of 1× PBS add 2 ml 10% Tween 20. Mix well. Can be stored at room temperature for several months.

470 **Permeabilization solution (0.2% Triton-X 100 (vol/vol) in 1× PBS)** To 500 ml 1× PBS add 1 ml Triton-X 100. Mix well by gentle rotation on a rotary platform. Can be stored at room temperature for several months. Permeabilization concentrations can be varied from 0.1 to 0.5 % (vol/vol) to optimize for different cell types.

20× saline-sodium citrate (SSC) Dissolve 175.3 g NaCl and 88.2 g tri-sodium citrate to a

475 final volume of 1 L with MQW. Autoclave and store at room temperature for up to one year. From this stock, make working solutions of 4× SSC (one volume 20× SSC with 4 volumes MQW), 2× SSC (one volume 20× SSC with 9 volumes MQW) and 1× SSC (one volume 20× SSC with 19 volumes MQW). All solutions can be stored, tightly capped, at room temperature for up to one year.

480 **FISH Blocking solution (3% [wt/vol] BSA)** Add 3 g BSA to final volume of 100 ml of 4× SSC. Filter through 0.2 µm filter to remove particulates. This can be stored at 4 °C for up to one month, check for any signs of contaminating growth before use.

FISH Wash solution (SSCT; 4× SSC with 0.05% [vol/vol] Tween 20) To 1 L 4× SSC add 500 µl Tween 20. Mix well by gentle rotation on a rotary platform. Can be stored for several

485 months at room temperature.

Antibodies Dilute antibodies appropriately in FISH blocking solution.

DNA staining solution Make 1 µg/ml DAPI in 1× PBS (10 µl of 5 mg/ml stock in 50 ml 1× PBS). Store in dark at 4 °C for 1-2 months.

SYTOX Green staining solution Make a 250 nM working solution by diluting 2 µl 5 mM SYTOX Green in 2× SSC. Store in dark at 4 °C for 1-2 months.

EQUIPMENT SETUP

Preparation of coverslips for seeding adherent cells Coverslips should be immersed in coverslip cleaning solution with gentle shaking for 1 h at room temperature to remove residual machining contaminants. Rinse copiously with MQW (at least 10 changes) and then store in 70% ethanol until required. Under laminar flow, transfer coverslips with forceps from their storage container and place them vertically into 6-well dishes (against the well's wall), until all the ethanol has evaporated. Flip them into the well when fully dried. Flame sterilising of coverslips is not recommended as they deform.

Preparation of coverslips for settling suspension cells Prepare the coverslips as described for seeding adherent cells in **Preparation of coverslips for seeding adherent cells**. Coat the dried coverslips in 0.01% (vol/vol) poly-L-lysine solution at room temperature for 5 min and remove the poly-L-lysine solution by vacuum aspiration using a fine-tipped glass pipette attachment. Coated coverslips can be rinsed with water following removal of excess poly-L-lysine and then air-dried (optional).

▲ **CRITICAL** Prepare poly-L-lysine coated coverslips no sooner than the preceding day of the experiment. They can become permanently anchored to their holding well if small drops of poly-L-lysine ingress under the coverslip.

Humidified dark chamber Any tight lidded waterproof box which can hold slides flat and be floated in a waterbath will work. We do not find it necessary to add additional wetted paper.

Exonuclease III incubation chamber Prepare a plastic lidded box with a Parafilm strip (clean side facing up) laid on the bottom. Exonuclease III solution is spotted on the Parafilm, onto which the coverslips are inverted to place the cells in contact with the Exonuclease III solution.

Antibody incubation chamber To perform the antibody incubation steps, we use a Bioassay dish (any lidded tray or box would suffice) with a piece of wetted Whatman paper laid on the bottom and a Parafilm strip laid on this, clean side facing upwards. Working antibody solution is dotted on to the clean Parafilm strip and the coverslips are inverted (i.e., cell side down) to place the cells in contact with the antibody solution.

Preparation of slides Superfrost slides are racked in a metal holder which is then submerged in a glass trough containing methanol. Slides are lifted out, and air-dried as needed by resting, vertically, on clean tissue.

525 **PROCEDURE**

▲ **CRITICAL** It is crucial that the cells do not dry out at any point during the entire procedure. To minimise delays when changing solutions, ensure you have sufficient volumes of everything required at hand before beginning.

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

1 | Seed adherent cells ~20–24 h before fixing on pre-cleaned and dried coverslips and grow under appropriate conditions with BrdU/C, at a final concentration of 10 μ M, added to the culture media. For each coverslip in a single on a 6 well plate in 2ml media, we use 2 μ l of 10 mM BrdU/C to give final concentration of 10 μ M BrdU/BrdC. Skip to Step 4.

535 **! CAUTION** BrdU may cause genetic defects, wear appropriate personal protective equipment and use in a laminar flow hood.

▲ **CRITICAL STEP** Seeding densities will vary from cell type to cell type. The desired end point is cells that are not overgrown or confluent; we find that confluency of ~50% is optimal at the fixation point. When growing embryonic stem cells, aim to seed single cells to yield
540 small colonies for optimal imaging.

2 | For cells growing in suspension, set up at an appropriate density to encourage maximal growth, add BrdU/C to media (to 10 ml media we add 10 μ l of BrdU/C to give final concentration of 10 μ M BrdU/BrdC) and allow to grow for 20–24 h.

545

3 | Centrifuge an appropriate number of suspension cells ($1-2 \times 10^5$ cells per coverslip) at 300g for 5 min. Decant off culture media and wash cell pellet in an excess of 1 \times PBS. Centrifuge again at 300g for 5 min. Resuspend at around $1-2 \times 10^5$ cells/ 100 μ l of 1 \times PBS. Pipette 100 μ l of cell suspension onto each poly-L-lysine coated coverslip and allow cells to
550 anchor to the coverslip for 5 min.

4 | For adherent cells, remove the medium by quickly but gently inverting the 6-well plate and wash the cells with 1 \times PBS. Remove 1 \times PBS wash and quickly but gently add fixation solution. Incubate for 15 min at room temperature. For suspension cells settled on coated
555 coverslips, gently add the fixation solution and incubate for 15 min at room temperature.

! CAUTION Formaldehyde is carcinogenic. Wear appropriate protective equipment, and work under a fume hood.

▲ CRITICAL STEP Carefully add the fixation solution into the well containing the coverslip with suspension cells attached. Always add solutions to the side of the coverslip, not directly on top of the coverslip to avoid detaching or disturbing the cells.

▲ CRITICAL STEP Where protein staining is also required within the experiment, varying the fixation conditions may be necessary for optimization. Where 4% (wt/vol) PFA solution gives suboptimal protein staining, we suggest using 2% (wt/vol) PFA fixation (see Table 2).

5 | In a stepwise manner exchange the fixative solution for wash buffer by removing about half of the solution in the well using a 3 ml plastic pastette and replacing with wash buffer. Repeat this 4–5 times. If storing coverslips, exchange the wash buffer for 1× PBS.

▲ CRITICAL STEP Stepwise fixative exchange with wash buffer ensures that the cells do not dry out and that cellular structures are optimally preserved. The gradual exchange of fixative with washing buffer containing Tween 20, a surfactant that decreases surface tension, helps with wetting of the cells.

■ PAUSE POINT Coverslips may be stored in 6-well plates containing 1× PBS for up to one month at 4 °C.

▲ CRITICAL STEP In order to prevent slow evaporation of 1× PBS during storage, wrap the plates well, either in a plastic bag or with Parafilm.

Probe preparation for hybridization ● TIMING 1.5–2 h

▲ CRITICAL Many types of FISH probes are available, including commercial ready to use, custom synthesised oligonucleotides and DNA clones which require labelling. Prior to hybridization, DNA clone labelled probes require precipitation and resuspension in hybridization buffer. We typically label our probes by nick translation (see Supplementary Method 2). The amounts of probe required for each hybridization are variable depending on probe type, genomic location and label type. In **Table 1** we suggest starting amounts, however we do recommend empiric tailoring.

6 | To precipitate nick translated probes add all required labelled probes (using the amounts suggested in **Table 1**), 3 µl *Cot*-1 DNA per hybridization area if needed, 20 µg salmon sperm DNA, 0.1 volume 3 M sodium acetate and 2.25 volumes absolute ethanol.

▲ CRITICAL STEP We do not find it necessary to use cold ethanol to precipitate DNA.

? TROUBLESHOOTING

7 | Mix by flicking and pulse centrifuge to collect tube contents, then precipitate at -80°C for 1 h. Follow by centrifugation in a refrigerated microcentrifuge at $15,300g$ at 4°C for 30 min.

595 ▲ **CRITICAL STEP** The precipitated DNA pellet is not always easily visible. Occasionally the precipitated DNA can smear along the side of the tube. In this case, gently wash the precipitate off using 70% ethanol in order to allow a pellet to form at the bottom of the tube on the upcoming spin step.

600 8 | Wash the pellet with 70% ethanol.

9 | Spin for 10 min at $15,300g$ at 4°C in a refrigerated microcentrifuge and then air-dry the pellet.

605 ▲ **CRITICAL STEP** Do not allow the precipitated probe pellet to over-dry as this will make its subsequent resuspension difficult.

10 | For each coverslip being hybridized, resuspend precipitated probe in $12\ \mu\text{l}$ DNA hybridization buffer. Shake at 37°C for at least 10–15 min on a Thermomixer.

610 ▲ **CRITICAL STEP** It is convenient but not necessary to use a Thermomixer for resuspension of precipitated probes. Incubation on a heat block, with regular flicking, also works. Probes can be mixed for longer times when convenient.

■ **PAUSE POINT** Once resuspended in hybridization buffer, probes can be stored at -20°C until required.

615 11 | Once well resuspended, probes should be denatured. For probes precipitated without $C_{ot}-1$, denature at 95°C for 10 min, then put on ice immediately. For fosmids/cosmids or BACs where $C_{ot}-1$ is present, the denaturation is followed with a pre-annealing step for 10 or 15 min respectively at 37°C , then put on ice.

620 ▲ **CRITICAL STEP** $C_{ot}-1$ is a repeat rich DNA fraction. It is required to suppress nonspecific hybridization of repeat DNA present within the labelled probes which would otherwise generate spurious signals at genomic locations aside from the region of interest. The pre-annealing step allows the unlabelled $C_{ot}-1$ to hybridize to the labelled repeat probe fragments, therefore removing these from the pool of labelled DNA available for hybridization to the target DNA.

625 ? **TROUBLESHOOTING**

12 | Avoiding air bubbles, carefully pipette the prepared probe in DNA hybridization buffer onto the surface of a prepared Superfrost glass slide.

630 13 | Continue to Step 26, after completing the **Prehybridization treatment** described in Steps 14-25). As described in Step 26, the prehybridized coverslip is placed cell-side down on this prepared probe.

Prehybridization treatment to generate single-stranded DNA, followed by

635 **hybridization** ● **TIMING 1.5 h + overnight**

▲ **CRITICAL** From this point, the washing steps can be done by quickly but gently inverting the 6-well plate. The coverslip will remain in the well owing to capillary tension between the coverslip and the dish surface. Add the buffers/wash solutions by gently pouring from a 50 ml conical tube. Do not pour directly onto the coverslip to avoid disturbing the cells unduly.

640 Alternatively, coverslips may be washed individually using a fine-tipped forceps and dipping them into one or two 50-ml beakers of washing solution before transferring into a second clean 6-well plate filled with fresh washing solution. In between each transfer, coverslip edges should be blotted on a tissue to remove excess liquid.

645 14 | Exchange wash buffer (or 1× PBS, if coverslips have been stored) with permeabilization solution and incubate for 10 min at room temperature.

▲ **CRITICAL STEP** Permeabilization solution can be varied in detergent concentration (and also detergent type) depending on the requirements of the cell type.

? TROUBLESHOOTING

650

15 | Wash the coverslip 3 times for 5 minutes with 1× PBS at room temperature.

16 | Quench free aldehydes by incubating the coverslip in quenching solution for 10 min at room temperature.

655 **? TROUBLESHOOTING**

17 | Wash the coverslip 3 times with 1× PBS at room temperature.

18 | Incubate the coverslip in 3 ml sensitizer solution for 15 min at room temperature

660 ▲ **CRITICAL STEP** Both Hoechst 33258 and DAPI sensitize the BrdU/C-labelled DNA to UV light treatment. We typically use DAPI.

? TROUBLESHOOTING

19 | Rinse the coverslip in 1× PBS, then reduce 1× PBS volume to just cover the coverslip (~3 ml in an individual well on 6-well plate).

20 | Leaving the lid off the plate or dish, expose the coverslip to 254 nm UV light for 15 min.

▲ **CRITICAL STEP** This step is central to the success of RASER-FISH. Ensure that the bulbs you are using are 254 nm in their main emission. Bulbs emitting light at a longer UV wavelength do not work for RASER-FISH. To be sure the UV bulbs are functioning we suggest running a short time cycle and checking the bulbs are slightly warm to the touch, before exposing coverslips. It is normal for the plate/dishes to warm slightly (usually to around 30 °C) during this process owing to the heat emitted by the bulbs. In order to minimise heating effects of the UV bulbs on the samples and to reduce variability, we recommend using these at a fixed distance from the samples. We use a Stratalinker 2400, which positions the bulbs at a distance of 15 cm from the sample.

? TROUBLESHOOTING

21 | Rinse the coverslip in 1× PBS at room temperature.

22 | Invert the coverslip, cell-side down, on to 60 µl Exonuclease III solution in an Exonuclease III incubation chamber. Float this chamber in a waterbath at 37 °C for 15 min.

? TROUBLESHOOTING

23 | Transfer the coverslip, cell-side facing up, back into a 6-well plate/dish.

▲ **CRITICAL STEP** In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.

▲ **CRITICAL STEP** to assess success of the generation of single-stranded DNA by detection with the ssDNA antibody, proceed to step 50.

24 | Wash three times for 3 min each in 1× PBS at room temperature.

25 | Lift the prepared coverslip using forceps and quickly wick away any excess 1× PBS by touching the coverslip edge on a lint-free tissue.

26 | Invert the prepared coverslip, cell-side down, on the probe in hybridization buffer placed on a cleaned glass slide, as described in Step 12.

▲ **CRITICAL STEP** 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.

? TROUBLESHOOTING

27 | 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 into the waterbath.

28 | Place slides into the hybridization chamber and allow hybridization to commence by floating the chamber in a waterbath at 37–42 °C overnight and up to 2 days.

▲ **CRITICAL STEP** The optimal hybridization temperature is assessed empirically. Depending on the probe DNA sequence, increased hybridization temperatures may be required in order to increase the hybridization stringency, thereby reducing non-specific annealing which gives rise to spurious background FISH signals. For DNA FISH, hybridization can be performed over one or two nights. When in combination with protein detection, in order to preserve proteins optimally, hybridize FISH probes over one night only and preferably at 37 °C, although more robustly bound proteins can withstand a slightly increased temperature.

? TROUBLESHOOTING

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

▲ **CRITICAL** As with all previous steps, it is critical to prevent any drying out of the coverslip in order to preserve cellular architecture and to prevent artefactual background arising during the detection steps.

29 | After carefully peeling off rubber cement, place the slide with coverslip in a reservoir holding 4× SSC and allow the coverslip to lift away from the slide surface.

▲ **CRITICAL STEP** To prevent the coverslip accidentally lifting off with the rubber cement, we find that carefully holding one corner whilst peeling the cement helps.

735

30 | Lift the coverslip and place it, cell-side upwards, into 2× SSC, either in 6-well plate or 35 mm dish depending on how many coverslips require processing.

740

31 | Wash twice in ~ 3 ml 2× SSC at 37 °C for 30 min then once in ~ 3 ml 1× SSC at room temperature for 30 min.

▲ CRITICAL STEP The stringency of the washes can be changed by altering the salt concentration or temperature e.g., 0.5× SSC at 42 °C is more stringent than the suggested washes, whereas 2× SSC at room temperature is less stringent. To perform the wash steps, we float the dishes/plates in a tray in a waterbath as this offers the best heat transfer.

745

? TROUBLESHOOTING

32 | Where the probe is hapten-labelled then its detection will be required. In this case, follow Steps 33-38. If no hapten detection is needed, then skip to Step 39.

750

33 | Incubate coverslip in ~ 3 ml FISH blocking solution for at least 30 min at room temperature.

■ PAUSE POINT Coverslips can be incubated in FISH blocking solution for up to a few hours when convenient.

? TROUBLESHOOTING

755

34 | Prepare sheep anti-DIG FITC (1/50 dilution) and rabbit anti-sheep FITC (1/100 dilution) in FISH blocking solution, allowing 100 µl per coverslip. Leave at 4 °C for at least 10 min, then centrifuge at 15,300g for 15 min at 4 °C before use.

760

▲ CRITICAL STEP Centrifuging the antibody solution pellets any aggregates that may form. By preparing slightly more antibody than required one can avoid any aggregates that are pelleted at the bottom of the tube.

? TROUBLESHOOTING

765

35 | Dot 100 µl prepared sheep anti-DIG FITC solution in the antibody incubation chamber. Remove the coverslip from the FISH blocking solution and briefly blot any excess liquid away by touching the coverslip edge on a clean tissue and then invert the coverslip, cell-side down, on to the antibody solution for 30 min at room temperature, avoiding trapped air-bubbles.

? TROUBLESHOOTING

770 **36 |** Transfer the coverslip, cell-side facing up, back into a plate/dish containing FISH wash solution.

▲ CRITICAL STEP In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and
775 allows the coverslip to be easily lifted off.

37 | Wash three times for 3 min each with FISH wash solution at room temperature.

38 | Repeat steps 35–37 using the prepared secondary rabbit anti sheep FITC solution
780 instead.

39 | Wash once in 1× PBS for 3 min each at room temperature.

40 | Add post-detection fixation solution to the coverslip in order to fix the FISH signals.
785 Leave for 15 min at room temperature.

! CAUTION Formaldehyde is carcinogenic. Wear appropriate protective equipment, and work under a fume hood.

▲ CRITICAL STEP This post-fixation step extends the length of storage of the samples considerably.
790

41 | Wash twice in PBST for 3 min each at room temperature.

42 | Stain in DAPI DNA staining solution for 10 min at room temperature.

795 **43 |** Wash four times in PBST for 3 min each at room temperature.

44 | Rinse in 1× PBS, then rinse briefly in MQW at room temperature.

▲ CRITICAL STEP This MQW rinse step removes residual salts from the coverslip and prevents formation of unwanted precipitates which can interfere with subsequent imaging.
800

45 | Equilibrate coverslips in mountant of choice by inverting the coverslip, cell-side down, on an excess amount (~0.5–1 ml) of mountant dotted onto the clean side of a Parafilm strip.

▲ CRITICAL STEP This equilibration step avoids unwanted dilution of the mounting media and alteration of its properties. There are many mountant types available, however we
805 routinely use VECTASHIELD or Slowfade Diamond mountant, which are both non-

hardening. Unlike curing mountants which can flatten cells upon hardening, neither of these mountants distort the 3D morphology of the cells. It is sensible to check that your chosen mountant will protect your fluorochromes of choice against photobleaching as certain mountants are not uniformly protective across the fluorescence spectrum.

810

46 | Lift the coverslip carefully and wick away the excess mountant by touching the corner of the coverslip against a clean, lint-free tissue.

815

47 | Mount the coverslip. Invert cell-side downwards on a small spot (~10 µl) of the same mountant on a cleaned, labelled Superfrost glass slide.

▲ CRITICAL STEP Avoid trapping any air-bubbles in the mountant 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 air-bubbles 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.

820

48 | Blot away any excess mountant carefully using clean tissue and without applying any pressure. Seal with coverslip sealant.

825

▲ CRITICAL STEP Allow sealant to air-dry before storage. Repeated exposure to coverslip cleaning agents may degrade the sealant, in which case it can be reapplied.

49 | Image slides under an appropriate microscope.

830

■ PAUSE POINT Slides may be stored for a several weeks at 4 °C in a lidded light-proof microscope slide storage box or long-term at -20 °C.

Assessment of single-stranded DNA generation ● TIMING 3.5h

▲ CRITICAL From Step 23 carry on as follows:

835

50 | Block the coverslip in antibody blocking solution for 30 min at room temperature.

■ PAUSE POINT Coverslips can be incubated in antibody blocking solution for up to a few hours when convenient.

840

51 | Prepare rabbit anti ssDNA antibody at 1/50 dilution in antibody blocking solution.

▲ CRITICAL STEP Antibodies should be handled with care. Where possible store at the lowest recommended temperature and where long-term freezer storage is recommended, we aliquot into small volumes to minimise repeated freeze-thaw cycles. Always keep antibodies on ice whilst in use.

845

52 | Dot 100 µl of prepared antibody solution in the prepared antibody incubation chamber. Lift the coverslip from the blocking solution and wick away excess liquid by touching the coverslip edge on a clean tissue. Gently place the coverslip, cell-side down, on the primary antibody solution and incubate for 1 h at room temperature or overnight at 4 °C.

850

53 | Lift the coverslip back into a 6-well plate/ dish containing 1× PBS, being careful to place cell side upwards.

▲ CRITICAL STEP In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.

855

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

860

55 | Prepare a secondary antibody, for example goat anti-rabbit Alexa 488 at 1/500 dilution in antibody blocking solution, allowing 100 µl per coverslip.

56 | Lift the coverslip from 1× PBS and wick away excess liquid by touching the coverslip edge on a clean tissue. Gently place the coverslip, cell-side down, on the secondary antibody solution and incubate for 1 h at room temperature.

865

57 | Lift the coverslip back into a 6-well plate/ dish containing 1× PBS, being careful to place the cell-side upwards.

▲ CRITICAL STEP In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.

870

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

875

59 | Follow steps 40-49, under subheading **Post-hybridization washing and probe detection** for post fixation, DNA staining and mounting steps.

Box 1 | RNA-DNA RASER-FISH

ADDITIONAL MATERIALS

REAGENTS

▲ **CRITICAL** Chemicals used in RNA-FISH experiments should be purchased RNase free. For all dry chemicals, these should be reconstituted in DEPC MQW (or RNase free water).

- Diethyl pyrocarbonate (DEPC Sigma-Aldrich cat. no. D5758) **! CAUTION** DEPC is harmful if swallowed, wear appropriate personal protective equipment and use in a fume hood.
- RNaseZap (Invitrogen cat. no. AM9780)
- 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.
- Triton X-100 (RNase free Sigma-Aldrich cat. no. 93443)
- Tween 20 (RNase free Promega cat. no. H5152)
- Dextran sulfate (Sigma cat. no. D8906)
- ELISA blocking reagent (Roche cat. no. 11112589001)
- PBS tablets (Sigma-Aldrich cat. no. P4417)
- Sodium acetate (3M RNase free Invitrogen cat. no. AM9740)

REAGENT SETUP

DEPC MQW Add 10 ml DEPC to 10 L MQW in a 10 L carboy in a chemical fume hood. Leave at room temperature for 24 h, then autoclave to inactivate any residual DEPC. This should be used as the base diluent for all RNase free solutions. Store at room temperature for several months.

20× SSC Add 175.3 g NaCl and 88.2 g tri-sodium citrate to a final volume of 1 l in DEPC MQW. For 2× SSC dilute 1 in 10 using DEPC MQW. Store at room temperature for several months.

RNase free 70% ethanol Mix 70 ml ethanol with 30 ml DEPC MQW. Store in a tightly capped bottle at room temperature for several months.

1× PBS solution Add 5 tablets to 1 l DEPC MQW. Autoclave and store at room temperature for several months.

RNA-FISH permeabilization solution 0.5 % (vol/vol) Triton-X 100 in 1× PBS: add 0.5 ml of Triton-X 100 to 100 ml 1× PBS. Store at 4 °C for several months.

50% (wt/vol) dextran sulfate Dissolve 25 g dextran sulfate in a final volume of 50 ml DEPC treated MQW. This is extremely viscous and benefits from heating at 37 °C. Avoid unnecessary agitation as this introduces air bubbles. Aliquot and store at –20 °C for several years. Warm to 37 °C when ready to use to reduce viscosity.

RNA-FISH hybridization buffer Mix 25 ml (final 50% [vol/vol]) formamide, 10 ml 50 % (final 10% [wt/vol]) dextran sulfate, 500 µl (final 1% [vol/vol]) Tween-20 with 500 µl 20× SSC (final 2× SSC) and DEPC MQW to a final volume of 50 ml at pH 7.0. Store in aliquots at –20 °C. These can be kept for several years.

10× Saline Solution For 9% (wt/vol) NaCl solution dissolve 90g NaCl in 1 l DEPC MQW. Autoclave, then can be stored at room temperature for several months. Mix 10 ml 10× saline solution and 90 ml DEPC MQW to give a 1× saline solution.

2 M Tris-HCl solution pH 7.5 To ~300 ml of DEPC MQW add 242.2 g of Trizma base. This gives a very alkaline solution. Using conc. HCl, adjust pH to 7.5. Make up to 500 ml using DEPC MQW. Store at room temperature for several months.

TST (Tris/Saline/Tween 20 solution; 0.15 M NaCl, 0.1 M Tris-HCl and 0.05% (vol/vol) Tween 20) For 1 L, mix 100 ml 10× saline solution, 50 ml 2 M Tris-HCl pH 7.5, 850 ml MQW and 500 µl Tween 20. Mix well by rotation on a rotary platform. Store at room temperature for several months.

20× RNA-FISH blocking solution Make 20× RNA-FISH blocking stock solution (27% [wt/vol]) by dissolving 27 g ELISA blocking reagent in a final volume of 100 ml with MQW. Aliquot and store 20× RNA-FISH blocking solution at –20 °C. Freshly prepare 1× working solution by diluting 1 volume stock with 19 volumes TS solution.

RNA FISH Probes For RNA-FISH probes use 25-50 ng of each oligo probe set or ~150 ng nick translated DNA. For oligo probes for RNA-FISH add a maximum volume of 1.2 µl oligos to 10.8 µl RNA-FISH hybridization buffer. For nick translated probes, resuspend precipitated probes in 12 ml RNA-FISH hybridization buffer. For precipitation of probes see sub-heading **Probe preparation for hybridization (Steps 6-13)** for details.

Supplementary Method 1 gives further details on probe labelling.

▲ **CRITICAL** Probe labelling for subsequent use in RNA-FISH should be carried out without an RNase treatment step.

EQUIPMENT SET UP

▲ **CRITICAL** All equipment used should be RNase free wherever possible (this cannot be the case for all equipment used e.g., centrifuges. Be sure to swab items that have been in a potentially contaminating environment with RNaseZap before continuing). Where possible, use disposable RNase free plasticware.

RNase treated glassware, pipettes and forceps Glassware and forceps can be rendered free of RNases by spraying with RNaseZap, leaving for a few minutes and then rinsing twice with DEPC MQW. Allow to dry and then store in a clean dust free environment for a maximum of 1 week. Given the cost of RNaseZap, a less expensive way to prepare RNase free glassware and forceps is to bake items that have been washed in detergent and rinsed well, wrapped in aluminium foil, at 180 °C for several hours. These can be stored, but this should be kept to as short a time period as possible.

RNase free glass slides In an RNase free lidded glass trough, submerge Superfrost slides in RNaseZap buffer for 5 min. Wash three times with DEPC MQW and store in RNase free 70% ethanol for one or two months in a dust-free environment (e.g., closed cupboard). When needed, carefully lift out the requisite number of slides using RNase free forceps and prop vertically to air-dry on clean, lint free tissue.

▲ **CRITICAL** We find that the RNaseZAP from this slide preparation step can be reused once without experimental detriment. Transfer to an RNase free storage bottle until required again.

PROCEDURE

RNA-FISH seeding and fixation of cells ● **TIMING** 1 h + 20–24 h seeding and growth

Follow **Steps 1-5** for Seeding and fixation of cells as described in subheading **Seeding and fixation of cells**, however, once the cells are removed from culturing media, ensure all solutions/reagents/equipment used are RNase-free.

▲ **CRITICAL** Coverslips for use in RNA-FISH experiments should not be stored as the RNA can degrade easily. Instead, use immediately.

RNA-FISH pre-treatment and hybridization ● **TIMING** 30 min + overnight

▲ **CRITICAL** The workflow described here, Box1 Steps 1-9, that is to the point of hybridization, should be RNase-free. For extended RNA-FISH details, see Brown and Buckle⁵².

1. Exchange 1× PBS with cold RNA-FISH permeabilization solution. Place the 6-well plate or 35 mm dish on ice for 6 min.

2. Wash the coverslip three times for 3 min in 1× PBS at room temperature.
3. Rinse in 2× SSC at room temperature.
4. Add RNA FISH probes in RNA-FISH hybridization buffer (as detailed in **Box 1 | RNA-DNA RASER-FISH; Reagent Setup**) to a RNase-free glass slide.

? TROUBLESHOOTING

5. Lower the prepared coverslip, cell-side down onto this, avoiding air bubbles.
▲ CRITICAL STEP 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.
6. Seal with vulcanising rubber cement.
7. Place the slides in a hybridization chamber and allow to hybridize overnight by floating the chamber in a waterbath at 37 °C.

RNA-FISH post-hybridization washing and detection ● TIMING 1–3.5 h

▲ CRITICAL For the subsequent Steps, 8-20, RNase free working is not a requirement.

8. After carefully peeling off rubber cement, place the slide with coverslip in a reservoir holding 4× SSC and allow the coverslip to lift away from the slide surface.
▲ CRITICAL STEP To prevent the coverslip accidentally lifting off with the rubber cement, we find that carefully holding one corner whilst peeling the cement helps.
9. Lift the coverslip and place it cell-side upwards into 2× SSC, either in a 6-well plate or 35 mm dish depending on how many coverslips require processing.
10. Wash the coverslip three times for 10 min in 2× SSC at 37 °C.
▲ CRITICAL STEP We float dishes/plates in a tray in a waterbath as this offers the best heat transfer.

11. Wash in TST for 5 min. For probes directly labelled with fluorophores, skip to Step 20. For hapten-labelled probes, proceed with Step 12.
12. Where hapten-labelled probes are to be detected, incubate the coverslip in RNA-FISH blocking solution for at least 30 min.
 - **PAUSE POINT** Coverslips can be incubated in antibody blocking solution for up to a few hours when convenient.
13. Dilute sheep anti-DIG FITC antibody (1/50 dilution) and rabbit anti-sheep FITC antibody (1/100 dilution) in RNA-FISH blocking solution. Leave at 4 °C for at least 10 min, then centrifuge at 15,300 g for 15 min at 4 °C before use.
 - ▲ **CRITICAL STEP** Centrifuging the antibody solution pellets any aggregates that may form. By preparing slightly more antibody than required one can avoid any aggregates that are pelleted at the bottom of the tube.
14. Dot 100 µl of prepared sheep anti-DIG FITC antibody solution in the prepared antibody incubation chamber. Lift coverslip from the RNA-FISH blocking solution and wick away excess liquid by touching the coverslip edge on a clean tissue. Gently place the coverslip, cell-side down, on the primary antibody solution and incubate for 30 min at room temperature.
15. Using forceps, lift the coverslip back into 6-well plate/ dish containing 1× PBS, being careful to place cell-side upwards.
 - ▲ **CRITICAL STEP** In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.
16. Wash three times for 5 min in TST at room temperature between each antibody layer.
17. Repeat Steps 14–16, using the prepared secondary rabbit anti sheep antibody instead.
18. Following the final TST wash after final antibody layer, rinse for 3 min in 1× PBS at room temperature.

19. Add post-detection fixation solution to the coverslip in order to fix the FISH signals.

Leave for 15 min at room temperature.

! CAUTION Formaldehyde is carcinogenic. Wear appropriate protective equipment, and work under a fume hood.

20. Rinse in 1× PBS three times for 5 min at room temperature.

21. To proceed with RASER-FISH to completion, follow the main Procedure Steps 18-49 .

Box 2 | Immuno RASER-FISH

▲ **CRITICAL** Always carry out a standard immunofluorescence alongside to determine protein staining alone as a comparison to the Immuno-RASER-FISH. We have found it necessary to perform the immunostaining before the RASER-FISH preparation and hybridization.

ADDITIONAL MATERIALS

REAGENTS

- Appropriate primary antibodies against proteins of interest.
- Appropriate secondary antibodies against primary antibodies.
- (optional) MAXblock Blocking medium (Active Motif cat. no. 15252)

REAGENT SET UP

Immuno blocking solution Add 10% (vol/vol) fetal bovine serum (FBS) in 1× PBS, filter through 0.2 µm filter and store at 4 °C for 1–2 months. Alternatively, MAXblock blocking medium can be used.

PROCEDURE

Immunostaining ● **TIMING 2 h 30 min (plus optional overnight)**

1. Follow Steps 1-5, then 14-17 from the main Procedure.

? **TROUBLESHOOTING**

2. Block the coverslip for at least 30 min at room temperature in immuno-blocking solution.

■ **PAUSE POINT** Coverslips can be incubated in antibody blocking solution for up to a few hours when convenient.

3. Dilute appropriate primary and secondary antibodies of choice in immuno-blocking solution.

4. Dot 100 µl of prepared antibody solution in the prepared antibody incubation chamber. Lift the coverslip from the immuno-blocking solution and wick away excess liquid by touching the coverslip edge on a clean tissue. Gently place the coverslip, cell-side down, on the primary antibody solution and incubate for 30 min at room temperature, or overnight at 4 °C.

5. Lift the coverslip back into 6-well plate/ dish containing 1× PBS, being careful to place cell-side upwards.

▲ **CRITICAL STEP** In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.

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

7. Dot 100 µl of prepared antibody solution in the prepared antibody incubation chamber. Lift the coverslip from the immuno-blocking solution and wick away excess liquid by touching the coverslip edge on a clean tissue. Gently place the coverslip, cell-side down, on the primary antibody solution and incubate for 30 min -1 h at room temperature.

8. Lift the coverslips back into a 6-well plate/ dish to perform washing steps, being careful to place the cell side upwards.

▲ **CRITICAL STEP** In order to reduce cohesive tension between the coverslip and the Parafilm surface, we use a 3 ml plastic pastette to gently introduce an excess volume of 1× PBS around each coverslip. This fluid-bed raises the coverslip away from the Parafilm and allows the coverslip to be easily lifted off.

9. Wash the coverslips three times for 5 min each in 1× PBS at room temperature.

10. Add post-detection fixation solution to the coverslips in order to fix the FISH signals. Leave for 15 min at room temperature.

! CAUTION Formaldehyde is carcinogenic. Wear appropriate protective equipment, and work under a fume hood.

11. Rinse once for 5 min in 1× PBS at room temperature.

12. To proceed with RASER-FISH to completion, follow the main procedure Steps 18-49.

▲ **CRITICAL STEP** In order to preserve the protein detection, the hybridization temperature should not exceed 37 °C and should be carried out overnight only. More robust proteins can withstand slightly higher hybridization temperatures which can be determined empirically.

TIMING

The time taken for each step is included throughout the protocol and given here:

885

MAIN PROTOCOL:

Steps 1-5, seeding and fixation of cells: 1 h + 20–24 h seeding and growth

Steps 6-13, prehybridization treatment to generate single-stranded DNA: 1.5 h

Steps 14-28, probe preparation for hybridization: 1.5–2 h

890 Steps 29-49, post-hybridization washing and probe detection: 1–3 h

Steps 50-59, assessment of single-stranded DNA generation: 3.5 h

RNA-DNA RASER-FISH additional steps:

Box 1 Main Protocol Steps 1-5, RNA-FISH seeding and fixation of cells: 1 h + 20–24 h

895 seeding and growth

Box 1 Steps 1-7, RNA-FISH pre-treatment and hybridization: 30 min + overnight

Box 1 Steps 8-21, RNA-FISH post-hybridization washing and detection: 1–3.5 h

Immuno RASER-FISH additional steps:

900 Box 2 Steps 1-12, Immunostaining: 2 h 30 min (plus optional overnight)

Figure 5 provides an overview of the technique and timeframes, over the two days. This also gives visual explanations of how protein detection can be incorporated into the procedure and how nascent RNA can be detected as a first process, thereby extending the protocol to three days.

905

TROUBLESHOOTING

Troubleshooting advice is provided in **Table 2**. Any problems encountered during the protocol are only detectable when the nuclei are viewed under a microscope at Step 49. The Step numbers provided in Table 2 indicate points in the protocol where problems could occur.

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ANTICIPATED RESULTS

In general, when assessed with conventional widefield microscopy, specimens look broadly comparable to those achieved by standard FISH procedures and FISH probe signals should be similar. We note that it is easier to attain a high hybridization frequency for smaller sized probes with RASER-FISH compared to standard FISH approaches, where for example 7.5 kb probes had a reported hybridization efficiency of <20%⁵³. We assume this is owing to an

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increased availability of single-stranded DNA template in RASER-FISH preparations.

920 Additionally, when using 3D-SIM imaging, we have anecdotally noted that RASER-FISH signals can look more defined and distinct at their edges, however we emphasise this is observational and has not been quantifiably compared to standard FISH preparations. With RASER-FISH there can be an increased level of background within the green wavelength emission range, owing to the photoconversion of DAPI by UV light, however, this is usually
925 not problematic. We see that DAPI counterstaining of DNA in RASER-FISH preparations is more weakly fluorescent compared to that seen in standard FISH preparations, which we assume is owing to the predominantly single-stranded nature of DNA in these preparations. In some RASER-FISH preparations there are occasional cells that have not incorporated BrdU/BrdC and these can be easily ascertained by their brighter, conventional DAPI
930 staining. As expected, these cells do not show FISH signals. We note that SYTOX Green, when used as a stain for single stranded DNA, works extremely well, showing more intense fluorescence than when bound to undenatured double-stranded DNA (unpublished results, see **Fig. 6c**). FISH can fail for many reasons; we list common problems encountered, such as weak, patchy or non-specific signals, or general background, in the troubleshooting guide,
935 for the benefit of users.

Figure. 6 provides examples of the quality of results expected using variously sized probes, by standard (**Fig. 6a, b**) and super-resolution microscopy (**Fig. 6c–e**). We also provide examples of RASER-FISH combined with detection of nascent RNA (**Fig. 6f**) and with immuno-detection of proteins (**Fig. 6g–i**). Comparisons with denaturing FISH
940 approaches are provided in **Figures 2** and **3**.

The plasmids depicted in **Figure 6a** encompass the α -globin domain at 146 kb apart. They are equidistant to the α -globin genes in the mouse, with pEx located at the enhancer elements and pCx acting as a control sited outside the α -globin domain. They have been informative in examining the timing of domain formation during erythropoiesis³⁶. Fosmids
945 NKX2 and Pax2 (**Fig. 6b**) are 1,143 kb apart and were used to examine polycomb-dependent chromatin interactions in the absence of cohesin³⁹. **Figure 6c** demonstrates the discrete signals of a BAC probe obtained by 3D-SIM. Six pools of directly labelled oligonucleotide probes encompassing a contiguous region of over 1 Mb at the α -globin locus and imaged by 3D-SIM (**Fig. 6d**) demonstrate the curvilinear arrangement of
950 chromatin domains³⁷. The oligonucleotide probe pools detected by 3D-STED imaging in **Figure 6e** cover 78.5 kb across an active α -globin domain where the internal blue region covering the domain is distinct from the two flanking red and green sites, which are now adjacent³⁶. **Figure 6f** provides an example of RNA-DNA RASER-FISH where both active α -globin loci (red) can be visualized against the nascent products of transcription (green).

Figures 6g-h demonstrate discrete plasmid FISH signals against the detection of proteins HP1 α and fibrillarin using the immuno-RASER-FISH protocol. This protocol was also used to demonstrate the accumulation of 53BP1 protein at the KIF23 gene (**Fig. 6i**) where the 53BP1 body is stabilising chromatin topology at sites of DNA breakage³⁸.

Supplementary Information

Supplementary Method 1: Denaturing FISH

Supplementary Method 2: Probe Labelling

Supplementary References

Author contributions

JMB and VJB developed the protocol, hybridized, imaged and analysed RASER-FISH preparations, SdO synthesized, hybridized and analyzed the oligonucleotide probe preparations, LS and EP hybridized, imaged and analysed preparations using structured illumination microscopy, and JMB, LS and VJB wrote the paper.

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Competing interests

The authors declare no competing interests.

Data availability statement

Figures 2, 3 and 6 have associated raw image data plus one dataset. All raw data files are archived in Figshare: Figure 2 doi.org/10.6084/m9.figshare.16778899, Figure 3

995 doi.org/10.6084/m9.figshare.16778902, Figure 6 doi.org/10.6084/m9.figshare.16755394.

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1150

Figure Legends

1155 **Figure 1 | Comparison of pre-hybridization workflow for RASER-FISH with heat**
denaturation FISH techniques. The RASER-FISH prehybridization steps involve fixation
(Fix), permeabilization (P), quenching (Qu), DAPI or Hoechst staining (DAPI), exposure to
ultra-violet light (UV) and strand resection with exonuclease III (Exo III). The 4 min, 4
min+dry and 30 min+dry prehybridization steps involve fixation (Fix), permeabilization (P)
1160 and quenching (Qu) as for RASER-FISH but then a glycerol step followed by liquid nitrogen
(LN2) and acid (HCl). 4 and 30 min+dry require dehydration through an ethanol series, then
all three approaches are equilibrated in a formamide wash (Form wash), and heat-denatured
in a formamide solution. Dashed lines represent washing steps. All steps are to scale.

1165 **Figure 2 | Comparison of hybridization strategies. a,** Hybridization efficiencies plotted as
the percentage of signal scored (mean with SD) in C127 cells for probes pEx and pCx³⁶ (red
and green respectively). As a lack of hybridization signals was occasionally noted in
RASER-FISH samples, owing to incomplete BrdU labelling, we assessed hybridization
efficiency as follows: on an allelic basis, the presence of a single signal (either green or red)
1170 deemed that allele as scorable. At that same allele, the presence (or absence) of the
neighbouring signal was recorded. Hybridization frequencies were expressed as number of
alleles with both red and green signals / number of all scorable alleles $\times 100$. The
hybridization frequencies shown are calculated from 501 (4 min), 715 (4 min+dry), 755
(RASER) and 218 (30 min+dry) alleles. **b,** Comparison of single-strandedness and nuclear
1175 integrity. Single-stranded DNA detected by antibody in example HeLa and C127 cells after
simple immunofluorescence (IF control) or the three FISH methods, 4 min, 4 min+dry,
RASER. Upper panels show the ssDNA antibody labelling signal (green/white). Scale bar,
5 μm . Lower panels show an expanded view of the nuclear periphery of the same cells with
the ssDNA signal (green) against a DAPI counterstain (purple). The disruption to nuclear
1180 integrity in the heat-denatured samples is evident. Scale bar, 1 μm . **c,** Comparison of access
to blocks of DNA repeats. Example C127 nuclei hybridized by the three FISH methods with a
probe to gamma satellite DNA repeats (green) against a DAPI counterstain, all imaged with
the same settings. RASER-FISH provides the most comprehensive labelling. Scale bar, 5
 μm .

1185

Figure 3 | Comparison of chromatin structure and TAD shapes. Super-resolution 3D-
SIM imaging of human RPE-1 cells after a control IF treatment or indicated FISH protocol

using a KIF23 TAD-specific probe. **a**, Central mid-sections of SYTOX Green stained nuclei show a rather inhomogeneous distribution of chromatin with strings of domain-like features separated by distinctive interchromatin space in both the IF control and after RASER-FISH. In contrast, 4 min and 30 min+dry nuclei show both a more homogeneous chromatin distribution with much reduced interchromatin regions. Insets are pseudocoloured for intensity and demonstrate the retention of interchromatin space in the RASER-FISH sample. Scale bar, 5 μm and 1 μm (insets). **b**, Exemplary TAD FISH signals selected from several cells highlight more defined and distinct edges of the TAD signal after RASER detection compared to 4 min, 4 min+dry (bottom row of middle section) and 30 min+dry heat denaturation. Images show false colour representations of maximum intensity projections. Scale bar, 1 μm .

Figure 4 | RASER-FISH compared to heat-denaturation FISH. Experimental design and effects on the substrate are schematically depicted at the level of DNA (black), ~ 10 nm diameter nucleosomes (dark grey) and >100 nm chromatin domains (light gray). RASER-FISH leaves the structure and integrity of 'TAD-like' chromatin domains largely intact (bottom left). In contrast, heat denaturation causes the denaturation of nucleosomes (together with other proteinaceous chromatin components) and renders chromatin domains less defined (bottom right).

Figure 5 | Workflow for RASER-FISH. The RASER-FISH workflow is depicted over two days (D1 and D2). D1 involves the prehybridization steps as described in **Figure 1**.

Hybridization occurs overnight followed on D2 by washing and immunodetection of hybridized probes. Dashed lines represent washing steps. Immunodetection of protein can be slotted in after the quench step. RNA-FISH detection can be undertaken prior to joining the RASER-FISH workflow at the sensitizer staining stage.

Figure 6 | RASER-FISH hybridization examples with inset magnifications. a, Deconvolved widefield image of plasmids pCx (green) and pEx (red) from the α -globin gene region³⁶ hybridized to C127 nucleus detected with DAPI. **b**, Fosmids recognising NKX2 (green) and Pax2 (red) hybridized to mouse ES cells³⁹ with nuclei detected with DAPI. **c**, BAC RP24-217110⁵ (red) hybridized to C127 nucleus with chromatin stained with SYTOX Green (grey), imaged by 3D-SIM. Orthogonal (top) and lateral (bottom) cross sections are shown. **d**, 3D-SIM image of a C127 nucleus hybridized with 6 pools of oligonucleotide probes directly labelled with Atto 565, Abberior Star Red and Oregon Green and covering 1030 kb of the α -globin gene region³⁷. Maximum projection with the nuclear boundary

defined by DAPI outlined (DAPI not shown). **e**, 3D-STED image (maximum intensity
1225 projection) of a mouse erythroblast nucleus hybridized with 3 pools of oligonucleotide probes
directly labelled with Atto 565, Abberior Star Red and Oregon Green and covering 78.5 kb of
the α -globin gene region³⁶. **(f)** RNA-DNA RASER-FISH image of a mouse erythroblast
showing the α -globin genes detected with plasmid pA³⁶ (red) against nascent α -globin
transcripts⁵⁴ (green) imaged by widefield deconvolution (maximum intensity projection). **g-i**,
1230 Immuno-RASER-FISH examples imaged by widefield deconvolution. **g**, Antibody detection
of HP1 α (red) combined with a plasmid probe pCx (green)³⁶ detected in a DAPI-stained
C127 cell nucleus (maximum intensity projection covering the central region). **h**, Immuno-
RASER-FISH image of an antibody to fibrillarin (detecting nucleoli) (red) combined with a
plasmid probe pCx³⁶ (green) detected in a DAPI-stained C127 cell nucleus. **i**, Immuno-
1235 RASER-FISH image of an antibody to 53BP1 (red) combined with a BAC RP11-347N18
probe partly covering the KIF23 TAD³⁸ (green) detected in a DAPI-stained U2OS cell
nucleus (maximum intensity projection covering the central region). Scale bars are 5 μ m and
1 μ m (insets).

Table 1 | List of recommended DNA amounts per hybridization area

Probe Type	Recommended DNA Amount	Cot-1 DNA?
Plasmids	100 ng	✗
Fosmids/cosmids	100 ng	✓
BACs	60–100 ng	✓
Oligonucleotide pools	100 ng	✗

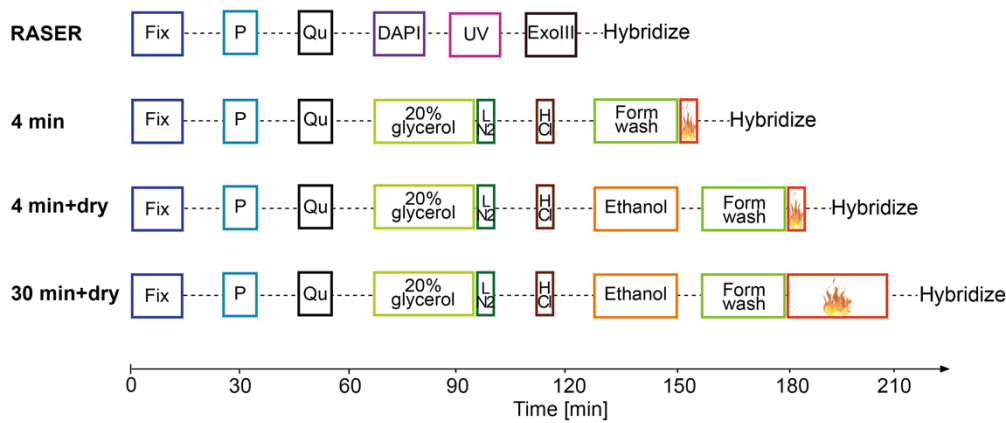
1245 **Table 2** | Troubleshooting advice

Step	Problem	Possible reason	Solution
6, 11, 14, 16, 18, 20, 22, 28, 31, S2.4, S2.5	Very weak or absent FISH signals	Limited nuclear access of probes	Increase detergent in the permeabilization step, or change the detergent used.
		Poor probe preparation	Ensure the probe gives a smear predominantly of 200-500 bp in size. Ensure the dUTPs or dAGC mix have not degraded. Try labelling with an alternatively modified dUTP, or use the dUTP according to the manufacturer's instructions. Check the probes are suitably labelled by performing a standard denaturing FISH.
		RASER-FISH prehybridization steps have failed	Ensure cells have taken up BrdU/C sufficiently by checking levels using an immunostaining approach. Ensure the UV bulbs are functional. Ensure the lid is off the holding plate/dish when coverslip(s) are exposed to UV light. Ensure sensitization and Exonuclease III treatment have been carried out appropriately. Check the RASER-FISH protocol has been successful by using ssDNA immunostaining.
		Hybridization is too stringent	Lower the stringency by decreasing the hybridization temperature.

		Post-hybridization wash conditions are too stringent	Decrease the wash temperature and/or increase the salt concentration.
		Antibodies used to detect probe are at incorrect concentration	Try higher concentration of antibodies. Labelling the probe directly can help to check that the probe indeed gives signal.
6, 11, 28, 31, 33, 34, S2.4	Spurious non-specific FISH signals	Probes are too short	Relabel and cut to the correct length. Increase the probe pre-annealing time.
		Repeats sequences within the probe have not been suppressed	Include COT-1 DNA or increase the preannealing time.
		Hybridization is not stringent enough	Increase the stringency by increasing the hybridization temperature.
		Post-hybridization wash conditions are not stringent enough	Increase the wash temperature and/or decrease the salt concentration.
		Insufficient blocking	Block for longer or choose a different blocking reagent.
		Antibodies used have degraded/aggregated	Do not freeze-thaw antibodies where possible. Centrifuge the antibody solution at 15,300g for 15 min at 4 °C before use, to pellet any precipitates that may have formed.
26, 35	Patchy hybridization signals	Trapped air bubbles in hybridization mix	When dispensing the viscous hybridization mix onto slide, do not introduce bubbles. Lower the coverslip very carefully to avoid trapping air bubbles.
		Trapped air bubbles in antibody detection steps	Take care to avoid air bubbles in the antibody solution.
16	General background signal	Autofluorescence from free aldehydes	Include a quenching step in the workflow.
		Autofluorescence from other sources	Investigate if your chosen cell type has abundant auto-fluorescent molecules. Try an alternative quenching agent such as sodium borohydride.

Box1 (4)		Sample has temporarily dried	Exchange the fixative in a stepwise manner to avoid drying out at this crucial step. Have all solutions ready to hand so no delays are encountered in changing solutions.
	No RNA-FISH signals	Incorrectly prepared probe	Probe has been prepared by nick translation with RNase step included. Oligo probes have been designed against the incorrect DNA strand.
		Potential incompatibility with RASER-FISH	Conduct an RNA-FISH assessment without the RASER-FISH steps to ensure signals are seen.
		RNA-FISH workflow requires optimization	RNA-FISH can be difficult to optimize. Begin by using a cell type and a probe where robust signals are expected, in order to check the workflow.
Box2 (1)	Poor protein staining	Inappropriate fixation	Test alternative fixation such as 2% wt/vol formaldehyde (see Main Procedure Step 4).
		Epitopes are masked and inaccessible to the antibody in use.	Source an antibody that is suitable to use with your chosen fixation method.
		Potential incompatibility with RASER-FISH	For proteins detected using green fluorophores, photoconversion of DAPI by UV light can mask low level/nuanced protein staining. Use a secondary antibody that is red or far red in detection. Conduct an immunostaining without RASER-FISH to ensure the staining is suitable and as expected.

Figure 1



1250

Figure 2

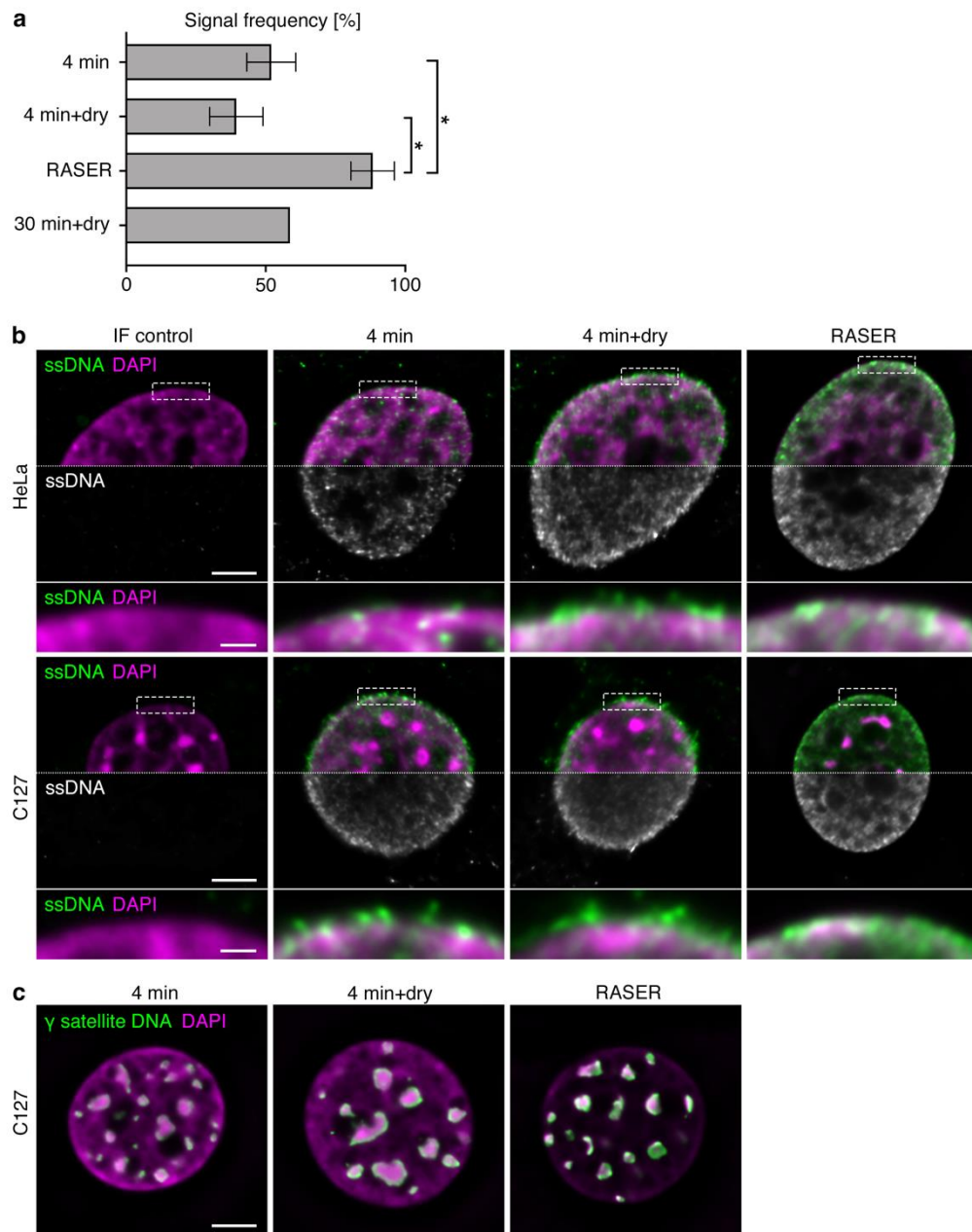


Figure 3

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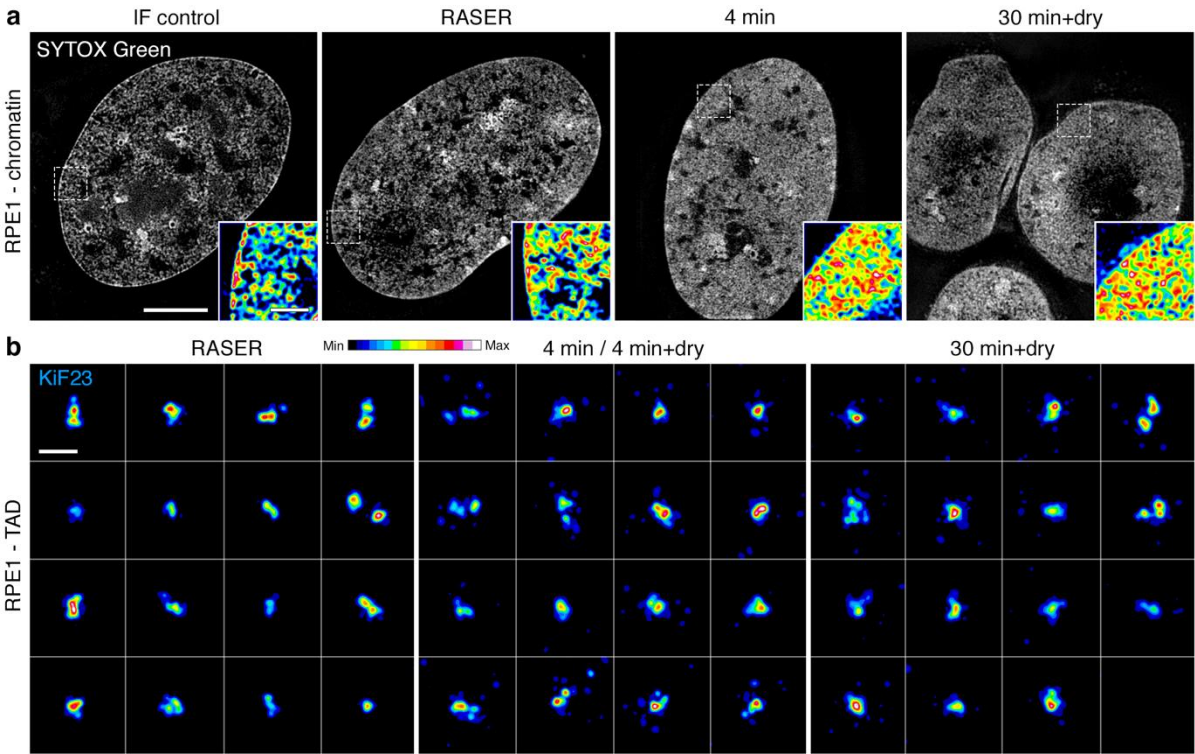
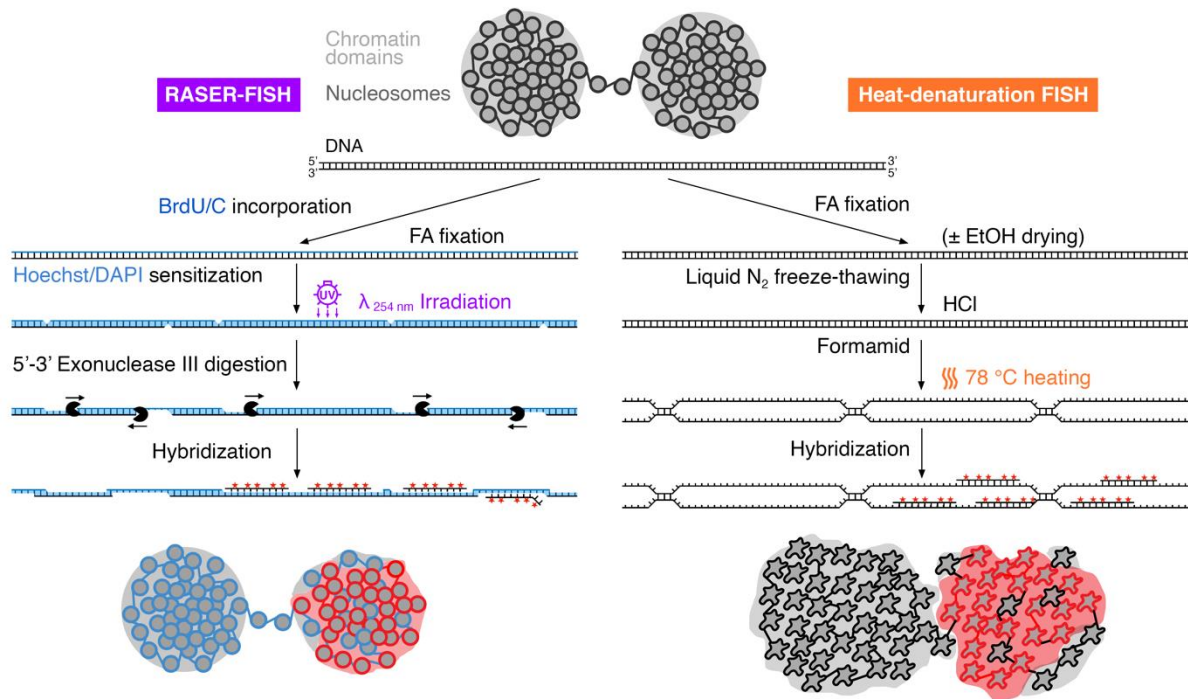


Figure 4



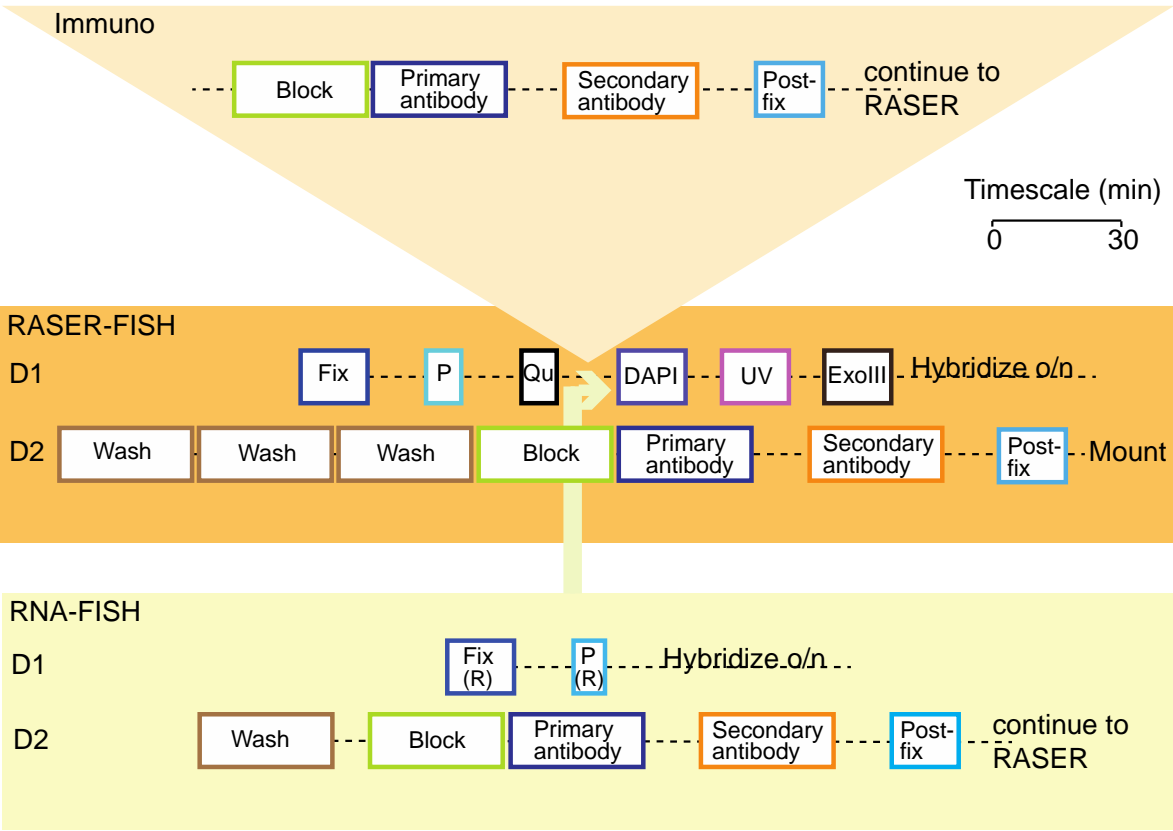
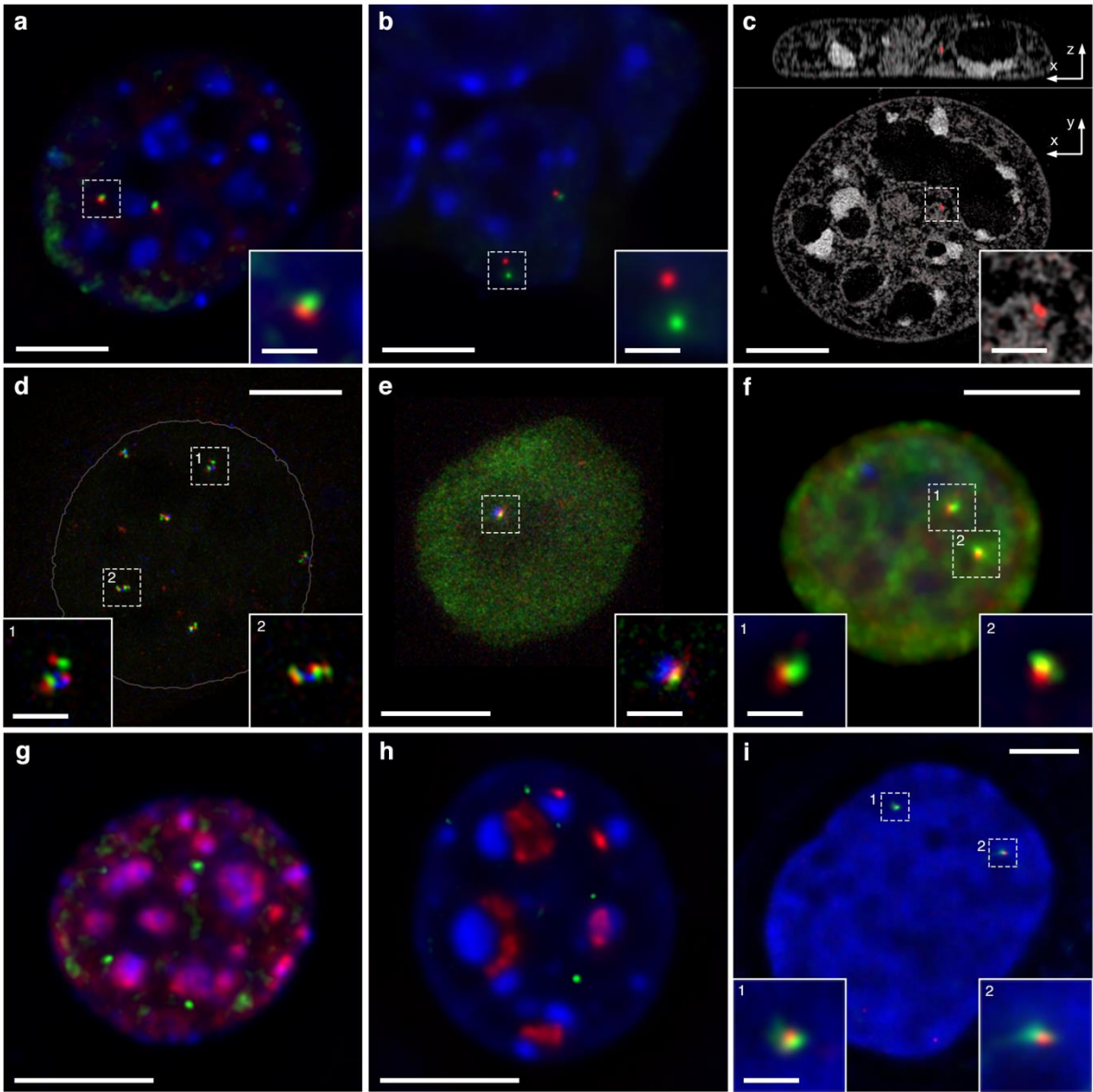


Figure 6



1265

SUPPLEMENTARY METHODS

SUPPLEMENTARY METHOD 1 | DENATURING FISH

1270 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)
- 1275 • 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.
- 1280 • 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
- 1285 splashes/contact, flush with copious amounts of tepid water and arrange immediate medical attention.

EQUIPMENT

- 1290 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.
- 1295 **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
- 1300 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 platform and store at room temperature for several months.

1305 **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
1310 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.

1315 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

1320 **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**

1325 Follow the steps as outlined in **Prehybridization treatment to generate single-stranded DNA** until Step 9 and then proceed as follows.

2. Equilibrate the coverslip in glycerol solution at room temperature for at least 30 min.

1330 ■ **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
1335 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.

! 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.

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** See Step 18 for critical comment

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 earlier under subheading **Post-hybridization washing and probe detection** within the main text. An array of alternative washes exists and can be employed where necessary^{1,4,7,35,55}.

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

- MgCl₂ (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., digoxigenin-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

1445 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 at -20 °C.

1450 **10× NTB** 10× nick translation buffer is composed of 0.5 M Trizma-HCl pH 8.0, 50 mM MgCl₂ and 0.5 mg/ml BSA (Sigma, Fraction V). Combine 5 ml 1 M Trizma-HCl pH 8.0, 500 µl 1 M MgCl₂ 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.

1455 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.

1460

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

1465 **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.

1470

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.

1475

1480 **▲ 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 approach⁶ 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.

1485 **▲ 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.

1495 **▲ 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.

1500 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.

1510 **▲ 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

1515 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.

1520 ▲ **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 digoxigenin 11-dUTP and Cy3-dUTP as probe labels are reasonably stable and can be stored for at least 1 year. Green-emitting fluorochrome dUTPs label less well than red or far-red dyes.

? TROUBLESHOOTING

1525

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