

Video Article

Combined Immunofluorescence and DNA FISH on 3D-preserved Interphase Nuclei to Study Changes in 3D Nuclear Organization

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Abstract

Fluorescent *in situ* hybridization using DNA probes on 3-dimensionally preserved nuclei followed by 3D confocal microscopy (3D DNA FISH) represents the most direct way to visualize the location of gene loci, chromosomal sub-regions or entire territories in individual cells. This type of analysis provides insight into the global architecture of the nucleus as well as the behavior of specific genomic loci and regions within the nuclear space. Immunofluorescence, on the other hand, permits the detection of nuclear proteins (modified histones, histone variants and modifiers, transcription machinery and factors, nuclear sub-compartments, etc). The major challenge in combining immunofluorescence and 3D DNA FISH is, on the one hand to preserve the epitope detected by the antibody as well as the 3D architecture of the nucleus, and on the other hand, to allow the penetration of the DNA probe to detect gene loci or chromosome territories¹⁻⁵. Here we provide a protocol that combines visualization of chromatin modifications with genomic loci in 3D preserved nuclei.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50087/>

Introduction

Epigenetic mechanisms trigger establishment and inheritance of developmental and cell-type specific transcriptional profiles. At one level this involves modulation of chromatin packaging that defines active or silent genomic regions. On a larger scale, global 3D organization of the genome and nuclear architecture also play a role in the control of transcriptional patterns. Thus, dissection of these epigenomic features is essential for a full understanding of how genes are regulated⁶⁻¹¹.

Combined immunofluorescence and 3D DNA FISH provide a unique opportunity to complement molecular and biochemical analyses by assessing specific interactions/associations of DNA sequences and/or proteins within the nucleus. Furthermore, while genome-wide high throughput techniques such as chromatin immunoprecipitation (ChIP-seq) or chromosome capture conformation coupled with deep sequencing (4C-seq, 5C, Hi-C) provide global data on cell populations¹², immunofluorescence/DNA FISH techniques enable analyses at the single cell level.

Here we describe a protocol for simultaneous detection of histone modifications by immunofluorescence and DNA sequences by DNA FISH followed by 3D microscopy and analyses (3D immuno-FISH). The advantage of this protocol is the combined visualization of DNA and preservation of protein structures. Our experience in this field has enabled us to improve and simplify existing protocols. Although we have used this protocol to detect DNA double-stranded breaks in lymphocytes undergoing recombination, this method can be applied to other proteins and other cell-types.

Protocol

1. DNA Probe Labeling with Fluorophores: Nick Translation (~ 6 hr)

1. Clean BAC DNA (prepared by maxi-prep) or plasmids or PCR products, all resuspended in H₂O, can be used for labeling. **Note** that for a robust FISH signal, probes should span at least 10 kb.
2. Incubate DNA in RNase A for 30 min at 37 °C (All reagents are listed in **Table 1**).

3. Incubate the nick translation reaction for 2 hr at 16 °C (see **Tables 2 and 3**). Alternative methods of direct labeling may be used (Example: FISH Tag, Invitrogen).
4. Inactivate the reaction for one hr at -80 °C or overnight at -20 °C.
5. Determine probe size on a 2% agarose gel. The smear should be between 100 and 1,000 bp, with the majority at approximately 300-500 bp (**Note** that the smear of Cy5-probes is not visible). If the DNA is not digested enough, more DNA Pol I and DNase I can be added to the reaction and incubated for two more hr at 16 °C.
6. Purify probes on 0.025 mm filters in two liter beakers filled with H₂O for two hr in the dark.
7. Add blocking factors to the probes as follows: 10 µg of Cot-1 DNA, 10 µg of Hybloc DNA and 100 µg of salmon sperm for 10 µg of DNA probes. Store DNA probes at -20 °C.

2. Probe Precipitation/Denaturation/Pre-annealing (3-4 hr)

1. Between 0.3 µg and 1 µg of DNA probe is used per coverslip. Mix probes with 0.1 volume of 3M NaAc (pH 5.2) and 2.5 volumes of 100% ethanol, incubate for one hr at -80 °C or overnight at -20 °C and spin down at 13,000 rpm at 4 °C for 30 min. **Note that the amount of DNA probe may be adjusted depending on the quality of the DNA and the nuclear region of hybridization. Alternative methods of direct labeling may allow the use of reduced amounts of probe. For detection of multiple DNA FISH signals on one slide, the different probes can be precipitated together (except probes that should not be pre-annealed, like repeat sequences). Probes for several experiments performed at the same time can also be precipitated together. Pellets should be lightly colored according to the fluorophores used (if small amounts of probes are precipitated, the pellet may not be visible).**
2. Dry pellets and resuspend them in 15 µl hybridization buffer (see **Table 4**) per coverslip with shaking (using an Eppendorf thermomixer) in the dark at 37-45 °C (for approximately 20 min).
3. Denature probes for 5 min at 75-95 °C.
4. Pre-anneal probes at 37 °C for 30-60 min. Length of pre-annealing may be adjusted depending on the complexity and quality of the probes.
5. Apply probes to the coverslips for overnight hybridization (see Section 3).

3. 3-dimensional DNA FISH - First Day (~ 3 hr)

1. Non-adherent cells are concentrated in a small volume of 1x PBS (~ 2x10⁵ cells in 5-10 µl 1x PBS per coverslip) and dropped on the center of a poly-L-lysine coated coverslip. Adherent cells should be cultured on coverslips for at least 24 hr (~ 70% confluence) (coverslips may be coated with 0.1% gelatin). Square 18x18 to 24-24 mm coverslips are placed in 6-well plates, and throughout the protocol, two ml of each solution is used per well (alternatively round 1.5 mm coverslips can be placed in 12/24-well plates).
2. Fix cells in 2% paraformaldehyde / 1x PBS, pH 7-7.4, for 10 min at RT (4% PFA stocks can be aliquoted, stored at -20 °C). Paraformaldehyde can be purchased in solution (16%), or 4% stock solutions can be prepared from powder/prills. Paraformaldehyde powder is dissolved in 1x PBS at > 60 °C (pH may be increased to 8-9 to facilitate dissolution), cooled-down on ice, adjusted to pH 7-7.4, filtered, aliquoted in falcons and stored for weeks at -20 °C.
3. After three rinses in 1x PBS, permeabilize cells in iced-cold 0.4% Triton-X-100 / 1x PBS for 5 min on ice. Length of permeabilization may be adjusted depending on the cell-type. **N.B.:** If DNA FISH is combined with immunofluorescence, the immunostaining should be performed at this stage (see **Section 4**).
4. After three rinses in 1x PBS, incubate cells with 0.1 µg/µl RNase A in 1x PBS for one hr at 37 °C. Coverslips are placed cell-side down onto a drop of 100 µl of solution on parafilm or a slide in a humid chamber. Coverslips are then carefully removed with forceps. If any resistance is encountered, coverslips should be flooded with 1x PBS in order to avoid damaging the cells.
5. After three rinses in 1x PBS, permeabilize cells in iced-cold 0.7% Triton-X-100 / 0.1 M HCl for 10 min on ice.
6. After three rinses in 1x PBS, denature cells in 1.9 M HCl for 30 min at RT. Alternately, cells can be denatured in 50% formamide / 2x SSC at 75-80 °C for at least 30 min. **Note** that hybridization length (and temperature) may be optimized depending on the cell-type.
7. After three rinses in ice-cold 1x PBS, hybridize cells with probes overnight at 37 °C in a dark and humid chamber. Coverslips are placed cell-side down onto a drop of 15 µl of probe on a slide, and sealed with rubber cement.

4. 3-dimensional Immuno-FISH - First Day (~ 6-7 hr)

1. For combined immunofluorescence /DNA FISH, immuno-staining should be performed after the permeabilization in 0.4% Triton-X-100 (**Step 3.3**).
2. Incubate cells in blocking solution for 30 min at RT (2.5% bovine serum albumin (BSA), 10% Normal Goat Serum, 0.1% Tween-20). The blocking solution is filtered, aliquoted in 1.5 ml Eppendorfs and stored for months at -20 °C.
3. Incubate cells with the primary antibody raised against the protein or the histone modification of interest diluted in blocking solution, for one hr at RT in a humid chamber. Here we show the example of an antibody against phosphorylated serine-139 of H2AX (γ-H2AX, Millipore; See **Table 5** for details). Coverslips are placed cell-side down onto a drop of 100 µl of solution on parafilm or a slide in a humid chamber. Coverslips are then carefully removed with forceps (flooded with 1x PBS if necessary). Note that dilution and incubation length may be adjusted depending on the quality of the antibody and cell-types.
4. Wash cells in 0.2% BSA / 0.1% Tween-20 / 1x PBS, three times 5 min at RT with shaking.
5. Incubate cells with the appropriate fluorophore-conjugated secondary antibody diluted in blocking solution for one hr at RT in a dark and humid chamber. Here we show an example of detection with a secondary goat anti-mouse antibody (Alexa Fluor 488 or 555, Invitrogen; See **Table 5** for details). Hapten-conjugated secondary antibodies (biotin, digoxigenin, etc) could also be used. In this case, an extra step for appropriate detection (streptavidin, anti-dig, etc) can be performed either before or after over-night DNA-FISH hybridization.
6. Rinse cells in 0.1% Tween-20 / 1x PBS, three times 5 min at RT with shaking in the dark.
7. Post-fix cells in 2% paraformaldehyde / 1x PBS for 10 min at RT in the dark.
8. Continue DNA FISH as above from incubation in RNase A (**Step 3.4**).

5. 3-dimensional DNA FISH / Immuno-FISH - Second Day (~ 2 hr)

1. Carefully remove rubber cement, flood coverslips with 2x SSC, carefully remove with forceps and place them in wells containing washing solution.
2. Wash cells in 2x SSC for 30 min at 37 °C in the dark with shaking.
3. Wash cells in 2x SSC for 30 min at RT in the dark with shaking.
4. Wash cells in 1x SSC for 30 min at RT in the dark with shaking. *Note that in case of denaturation with formamide, washes should be replaced by the following: 3 washes in 50% formamide / 2x SSC and 3 washes in 2x SSC, 5 min each, at 37 °C.*
5. Mount cells in ProLong Gold mounting medium containing 1.5 µg/ml DAPI (4,6-diamidino-2-phenylindole) for counterstaining of total DNA. Coverslips are placed cell-side down onto a drop of 10-15 µl of mounting medium on a slide and sealed with nail polish. Slides are kept for months at -20 °C.

6. 3D Microscopy and Analysis

1. 3D images can be acquired with different microscope systems. In our experiments, optical sections separated by 0.3 µm are collected on a Leica SP5 AOBS confocal system (Acousto-Optical Beam Splitter). *Note that the separation between optical planes may be adjusted depending on the type of analysis.*
2. 3D image stacks can be analyzed using different software and tools. We use the Image J software to assess distance measurements between loci of interest and to analyze different types of association of the loci of interest with sub-nuclear compartments or proteins.
3. All statistical analyses are performed to compare two (or more) different biological conditions with pairwise assessment(s) of significance: comparison between different cell types or stages, comparison between different loci of interest. In all statistical tests, P -values $\leq 5.00E-2$ ($\alpha = 0.05$) are taken to be significant ($1.00E-2 \leq P \leq 5.00E-2$ * significant; $1.00E-3 \leq P \leq 1.00E-2$ ** very significant; $P < 1.00E-3$ *** highly significant).

Statistical analysis of the entire distributions of distances between two alleles. We first construct cumulative distribution frequency curves over the entire range of measured distances between the two alleles. To assess the significance of differences in the distributions of empirical inter-allelic distances we use the nonparametric two-sample Kolmogorov-Smirnov (KS) test^{13,14}.

Statistical analysis of close association (i.e. pairing) of two alleles using an optimal distance cut-off. To identify the range of most robust differences in distances between two alleles or loci, we use a series of two-tail Fisher exact test¹⁵ s. Namely, at each measured distance we test whether one condition is significantly over-represented at shorter distances between the two samples. The minimum in the distribution of P -Values indicates the range of distances that should be considered as a cut-off for close association (i.e. pairing) of the two alleles. Subsequently the two-tail Fisher exact test is used to assess the significance of pairing of two alleles at the identified cut-off value¹⁵. Multiple testing corrections are applied to account for the total number of Fisher tests performed¹⁶.

Statistical analysis of association of the locus of interest with sub-nuclear compartments or proteins. The two-tail Fisher-exact test is used to analyze the significance of association of the locus of interest with different compartments or proteins¹⁵.

Representative Results

DNA and immuno-FISH are used in the Skok lab to study the changes in nuclear organization associated with the process of V(D)J recombination of antigen receptor loci during B and T lymphocyte development. The techniques detailed above enable us to i) measure distances between the two ends of a locus (contraction) ii) measure distances between alleles or loci (pairing), iii) analyze the DNA damage occurring within loci, iv) assess the location of alleles and loci relative to nuclear sub-compartments (repressive pericentromeric heterochromatin in our study), and v) assess the association of nuclear proteins on alleles and loci (association of the phosphorylated histone γ -H2AX in our study). In particular, we analyzed the role of locus dynamics in the regulation of V(D)J recombination of the *Immunoglobulin* loci in developing B cells¹⁷⁻³⁰, and in the regulation of lineage commitment between CD4⁺ and CD8⁺ T cells¹⁴. We also assessed DNA damage at one of the T-cell receptor locus, *Tcra/d*, in developing T cells carrying a mutant form of the recombinase protein RAG2³¹.

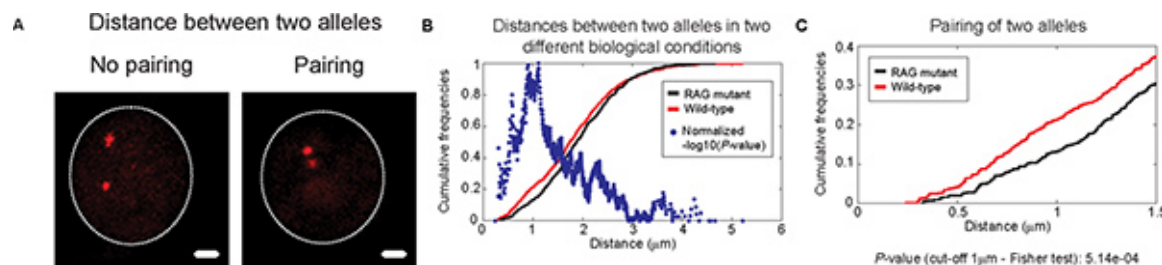


Figure 1. Analysis of distances between two alleles or loci. (A) Confocal sections showing representative unpaired (right) or paired (left) alleles in individual nuclei. The DNA FISH probe was generated from a BAC (~200 kb) spanning the locus of interest. Distances between alleles were measured between the center of mass of each BAC signal in 3D image stacks. Dashed circles outline nucleus shapes. Scale bars = 1 µm. (B) Cumulative frequency distribution curves showing the entire range of distances between the two alleles measured in two different biological conditions (wild-type vs. mutant). The nonparametric two-sample KS test was used to assess the significance of the differences between the two distributions. The range of most robust differences in inter-allelic distances was assessed using a series of two-tail Fisher exact tests (blue dots). The most robust *P*-values (higher dots) were found for the distances around 1 µm. Thus the cut-off for close association (*i.e.* pairing) of the two alleles was set up at 1 µm. (C) Cumulative frequency curves showing the distances between the two alleles from 0 to 1.5 µm in the two different biological conditions. The significance of pairing of the two alleles was assessed using the two-tail Fisher exact test for a cut-off at 1 µm. [Click here to view larger figure.](#)

Location of a locus relative to PCH

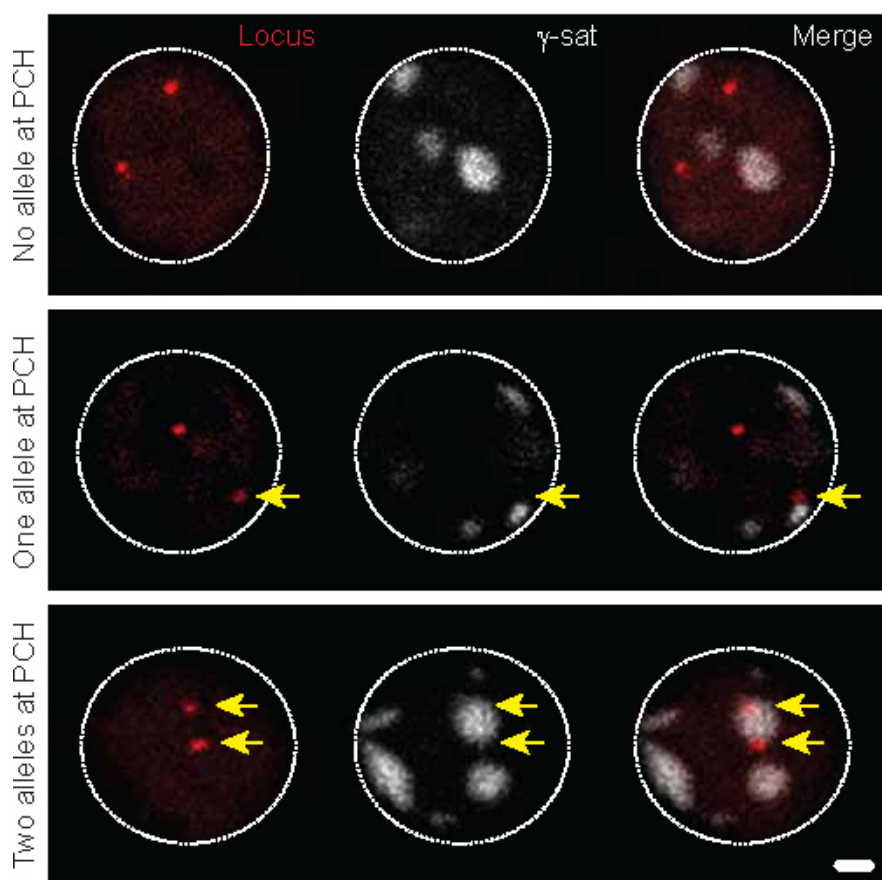


Figure 2. Analysis of the location of a locus relative to sub-nuclear compartments. In our studies we analyze the relationship between our loci of interest and the repressive sub-nuclear compartment formed by the pericentromeric heterochromatin (PCH)³². Alleles are considered as located at PCH when the BAC signal for the locus of interest is adjacent or overlapping with the γ -satellite signal that hybridizes to PCH (no pixel in between the edges of the BAC and γ -satellite DNA FISH signals). Confocal sections representative of the location of *Tcra* alleles relative to PCH: (Top) no allele located at PCH, (Middle) one allele at PCH, (Bottom) two alleles at PCH. Locus in red; and γ -satellite in white. Yellow arrows show the alleles located at PCH. Dashed circles outline nucleus shapes. Scale bar = 1 µm.

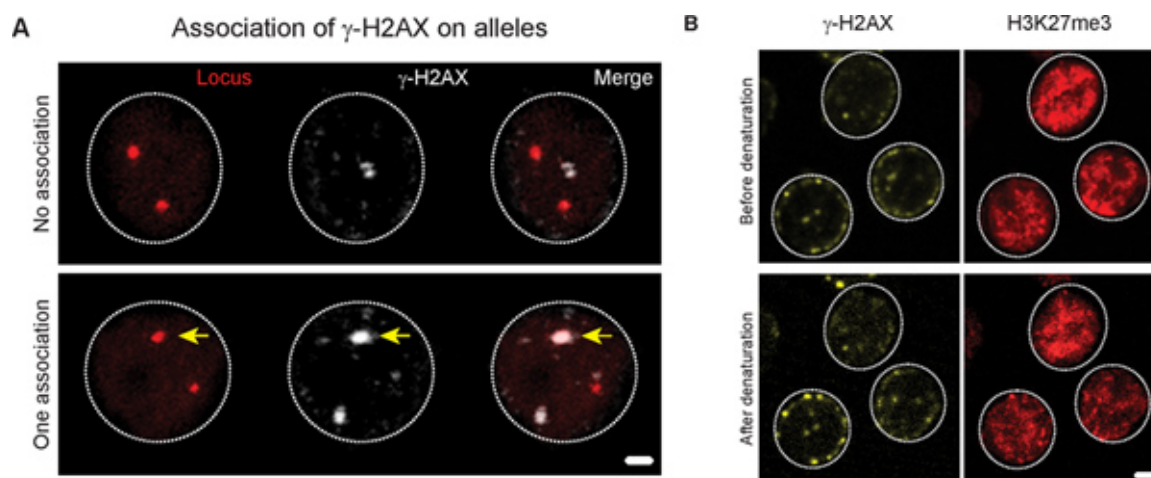


Figure 3. Analysis of the association of nuclear proteins and histone modifications on a locus. (A) In our studies we analyze the relationship between our loci of interest and the phosphorylated form of the histone H2AX (γ -H2AX), as read-out for DNA double-stranded breaks³³. Confocal sections show examples of no γ -H2AX association (Top) or association on one *Tcra* allele (Bottom) in individual nuclei. Alleles were defined as associated with γ -H2AX if the BAC signals and immunofluorescence foci were at least partially overlapped (at least one pixel of colocalization). Locus is shown in red and γ -H2AX in white. Dashed circles outline nucleus shapes. Scale bar = 1 μ m. *It is of note that colocalization analyses of the BAC signal with PCH or γ -H2AX foci are performed manually using Image J software without automated threshold.* (B) Loss of part of the γ -H2AX signal after denaturation is one of the main technical questions raised by this immuno-DNA FISH protocol. Here we show an example of immunofluorescence before (Top) and after (Bottom) HCl-based denaturation treatment (Formamide-treatment gave similar results (not shown)). Although the signal was weaker, the main features of the staining, in particular the number of foci, remain after the treatment. Using an SP5 Leica confocal, the settings for the 561 laser before and after denaturation were the following: 35% power / smart gain at 730V, and 45% power / smart gain at 750V, respectively. Furthermore, we show here an example of an immunofluorescence staining for another histone modification, H3K27me3 (Right panels) showing that this protocol can be applied to other histone marks, as has previously been published^{1,3}. γ -H2AX is shown in yellow and H3K27me3 in red. Dashed circles outline nucleus shapes. Scale bar = 2 μ m. [Click here to view larger figure.](#)

Name of Reagent/Material	Company	Catalogm Number	Comments
H ₂ O	Fisher	# BP2470	
RNase A	Sigma	# R4642	
dNTP	Sigma	# DNT100	
Alexa dUTP	Invitrogen	# C11397 to C-11401	
Cy3 or Cy5 dUTP	Fisher	# 45-001-xxx	
DNase I	Roche	# 04536282001	
DNA Pol I	Biolabs	# M0209	
0.025 μ m filters	Millipore	# VSWP02500	
Cot-1 DNA 1 mg/ml	Invitrogen	# 18440	
Hybloc DNA 1 mg/ml	Applied Genetics	# MHB	
Salmon sperm	Sigma	# D1626	powder to be resuspended at 10 mg/ml in H ₂ O
NaAc (Sodium Acetate, pH 5.2, buffer solution)	Sigma	# S7899	
Ficoll 400 (Mol Biol grade)	Fisher	# 525	
Polyvinylpyrrolidone (Mol Biol grade)	Fisher	# BP431	
Dextran sulfate powder	Sigma	# D8906	
SSPE (Saline-Sodium Phosphate-EDTA) 20x solution	Fisher	# BP1328	
Formamide	Fisher	# BP227	
Coverslips	Fisher	# 12-548-B	
Slides	Fisher	# 12-550	
6-well plates	Fisher	# 0720080	

PBS, 10x	Fisher	# MT-46-013-CM	
Poly-L-lysine solution	Sigma	# P8920	
Paraformaldehyde, prills, 95%	Sigma	# 441244	
Triton-X-100, Mol Biol grade	Sigma	# T8787	
BSA (Bovine Serum Albumin) Fraction V	Fisher	# BP 1600	
Normal goat serum	Vector Labs	# S-1000	
Tween-20, Mol Biol grade	Sigma	# P9416	
SSC (Saline Sodium Citrate) 20x solution	Fisher	# BP1325	
ProLong Gold antifade reagent	Invitrogen	# P36930	
DAPI (4',6-diamidino-2-phenylindole)	Sigma	# D9542	
Best test one coat rubber cement	Art or office supply stores		

Table 1. Specific reagents and small equipment.

NT with dUTP coupled with Alexa-fluorophores	Amount	Final Concentration
10 µg DNA	X µl	
RNase A - 0.17 µg/µl	10 µl	
H ₂ O	Fill to 260 µl	
TOTAL - 30 min at 37 °C	260 µl	
10x NT buffer	50 µl	1x
100 mM β-mercaptoethanol	50 µl	10 µM
dNTP mix	40 µl	40 µM each dNTP
0.1 mM dTTP	50 µl	10 µM
1 mM Alexa dUTP	12 µl	30 µM
DNase I (working solution, see Table below)	30 µl	
DNA Pol I	7.5 µl	15 U/ml
TOTAL - 2 hr at 16 °C	500 µl	
NT with dUTP coupled with Cy3 or Cy5	Amount	Final Concentration
10 µg DNA	X µl	
RNase A - 0.17 µg/µl	10 µl	
H ₂ O	Fill to 300 µl	
TOTAL - 30 min at 37 °C	300 µl	
10x NT buffer	50 µl	1x
100 mM β-mercaptoethanol	50 µl	10 µM
dNTP mix	50 µl	50 µM each dNTP
1 mM Cy3- or Cy5-dUTP	12 µl	30 µM
DNase I	~30 µl	~1.2 U/µl
DNA Pol I	7.5 µl	15 U/ml
TOTAL - 2 hr at 16 °C	500 µl	

Table 2. RNase A treatment and Nick Translation for 10 µg DNA.

Solutions		Stocks
RNase A	Working solution: 0.17 µg/µl	Made up fresh
10x NT buffer:	0.5M Tris-HCl, pH 8; 50 mM MgCl ₂ ; 0.5 mg/ml BSA	1.5 ml tubes at -20 °C.

dNTP mix:	dATP, dCTP, and dGTP: 0.5mM each in H ₂ O	1.5 ml tubes at -20 °C
DNase I:	<i>Stock solution:</i> reconstituted at 20,000 U/ml in 20 mM Tris pH 7.5/50% glycerol/50 mM NaCl	0.5 ml tubes at -20 °C
	<i>Working solution:</i> Each new batch of DNase I stock solution should be tested at different dilutions (10 U/ml, 20 U/ml and 40 U/ml in H ₂ O). The dilution giving a DNA smear between 100 and 1,000 bp (see step 5) should be used as the working solution.	Made up fresh

Table 3. Solutions for RNase A treatment and Nick Translation.

Hybridization buffer		Solutions	Stocks
10%	50x Denhardt's solution:	1 g Ficoll 400, 1 g Polyvinylpyrrolidone and 100 ml H ₂ O - Filtered	50 ml falcon at -20 °C
40%	25% Dextran Sulfate:	Dextran sulfate powder is dissolved in 12.5x SSPE at 37-65 °C	50 ml falcon at -20 °C
50%	Formamide		Bottles at +4 °C
Aliquots in 1.5 ml Eppendorfs are stored at -20 °C			

Table 4. Hybridization buffer.

Antibodies	Supplier	Catalog number	Dilution
γ-H2AX - phospho H2A.X, clone JBW301, mouse monoclonal	Millipore	# 05636	1/200
H3K27me3, clone , rabbit polyclonal	Millipore	# 07449	1/200
Alexa Fluor 555 Donkey Anti-mouse	Invitrogen	# A-31570	1/500
Alexa Fluor 488 Goat Anti-mouse	Invitrogen	# A-11001	1/500
Alexa Fluor 488 Goat Anti-rabbit	Invitrogen	# A-11008	1/500

Table 5. Primary and secondary antibodies.

Discussion

The techniques detailed above were used in our lab to analyze the regulation of V(D)J recombination of the *Immunoglobulin* and *Tcra/d* loci in developing lymphocytes^{30,31}. We are confident that this technique can be adapted for detection of various nuclear proteins, nuclear compartments and loci, in different cell-types. Modifications of the protocol may be necessary, and in this case the major steps to focus on are the following. First, the length of permeabilization can be adjusted depending on the cell-type. Second the length of the primary antibody incubation can also be adjusted according to the quality of the antibody and the abundance of the protein of interest (for example in some cases, incubation may be performed overnight at 4 °C). Importantly, the denaturation step can be adapted, and HCl treatment may be replaced by denaturation in Formamide^{3,4,34} and NaOH for DNA FISH²⁰. Also repeated freeze-thaw cycles of liquid nitrogen may be applied to the cells after fixation / permeabilization for DNA FISH alone^{4,34}. Finally, while several histone modifications, RNA Polymerase II and co-factors have been successfully detected using this method^{30,35}, immuno-staining for other nuclear proteins may be performed after the DNA FISH hybridization⁴.

3D image analysis can involve the use of different types of microscopes, including wide-field epifluorescence (coupled with deconvolution) and confocal laser microscopy³⁶. The choice of the imaging method will depend on the resolution required, the number of fluorophores, the depth of the samples and the quality of the DNA and protein signals. The development of new high-resolution microscopes opens an entirely new avenue in the accurate visualization of nuclear and cellular processes³⁷⁻³⁹. Furthermore, new techniques for the generation of custom-made probes that contain no repetitive sequence enable the clean detection of genomic regions, from a 15-kb specific locus, to complex pools of exons⁴⁰. This increases the accuracy and sensitivity of high-resolution microscopy in tracking the dynamics of nuclear processes.

Disclosures

The authors declare that they have no competing financial interests.

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