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TITLE:

3D Multicolor DNA FISH Tool to Study Nuclear Architecture in Human Primary Cells

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KEYWORDS:

fluorescence in situ hybridization, 3D multicolor DNA FISH, preserved interphase nuclei, nuclear architecture, 3D genome folding, DNA contacts, nuclear positioning, human primary cells

SUMMARY:

3D multicolor DNA FISH represents a tool to visualize multiple genomic loci within 3D preserved nuclei, unambiguously defining their reciprocal interactions and localization within the nuclear space at a single cell level. Here, a step by step protocol is described for a wide spectrum of human primary cells.

ABSTRACT:

A major question in cell biology is genomic organization within the nuclear space and how chromatin architecture can influence processes such as gene expression, cell identity and differentiation. Many approaches developed to study the 3D architecture of the genome can be divided into two complementary categories: chromosome conformation capture based technologies (C-technologies) and imaging. While the former is based on capturing the chromosome conformation and proximal DNA interactions in a population of fixed cells, the latter, based on DNA fluorescence in situ hybridization (FISH) on 3D-preserved nuclei, allows contemporary visualization of multiple loci at a single cell level (multicolor), examining their interactions and distribution within the nucleus (3D multicolor DNA FISH). The technique of 3D multicolor DNA FISH has a limitation of visualizing only a few predetermined loci, not permitting a comprehensive analysis of the nuclear architecture. However, given the robustness of its results, 3D multicolor DNA FISH in combination with 3D-microscopy and image reconstruction is a possible method to validate C-technology based results and to unambiguously study the position and organization of specific loci at a single cell level. Here, we propose a step by step method of 3D multicolor DNA FISH suitable for a wide range of human primary cells and discuss

all the practical actions, crucial steps, notions of 3D imaging and data analysis needed to obtained a successful and informative 3D multicolor DNA FISH within different biological contexts.

INTRODUCTION:

Higher eukaryotes need to systematically condense and compact a huge amount of genetic information in the minute 3D space of the nucleus[1-4]. Today, we know that the genome is spatially ordered in compartments and topologically associated domains[5] and that the multiple levels of DNA folding generate contacts between different genomic regions that may involve chromatin loop formation[6,7]. The 3D dynamic looping of chromatin can influence many different biological processes such as transcription[8,9], differentiation and development[10,11], DNA repair[12,13], while its perturbations are involved in various diseases[14-16] and developmental defects[15,17,18].

Many approaches have been developed to study the 3D genome organization. Chromosome conformation capture-based technologies (C-technologies, 3C, 4C, 5C, Hi-C and derivatives) have been developed to study genome organization in fixed cells[3,4,19,20]. Such approaches are based on the ability to capture the contact frequencies between genomic loci in physical proximity. C-technologies, depending on their complexity, catch the global 3D genome organization and nuclear topology of a cell population[3,4,19,20]. Nevertheless, 3D interactions are dynamic in time and space, highly variable between individual cells consisting of multiplex interactions, and are extensively heterogenous[21,22].

3D multicolor DNA fluorescence in situ hybridization (FISH) is a technique that allows the visualization of specific genomic loci at a single cell level, enabling direct investigation of the 3D nuclear architecture in a complementary manner to C-technologies. It represents a technology currently used to unambiguously validate C-results. 3D multicolor DNA FISH uses fluorescently labeled probes complementary to the genomic loci of interests. The use of different fluorophores and suitable microscopy equipment allow contemporary visualization of multiple targets within the nuclear space[23,24]. In recent years, FISH has been combined with technological advances in microscopy to obtain the visualization of fine-scale structures at high resolution[25,26] or with CRISPR-Cas approaches for the visualization of the nucleic acids in live imaging[27,28]. Despite wide adoption, the 3D multicolor DNA FISH approach is still considered difficult in many laboratories because the biological material used must be adapted.

Here, we provide a comprehensive protocol for 3D multicolor DNA FISH (from cell/probe preparation to data analysis) applicable to a wide range of human primary cells, enabling the visualization of multiple genomic loci and preserving the 3D structure of nuclei. In order to study nuclear architecture, the 3D structure of nuclei must be preserved. For this reason, contrasting from other existing protocols[29-31], we avoid the use of an alcohol gradient and the storage of the coverslips in alcohol that can affect chromatin structure[32]. The method is adapted from preserved 3D DNA FISH protocols[24,33] to be applied to a wide range of human primary cells, both isolated ex vivo or cultured in vitro. There are permeabilization and deproteinization parameters for different nuclear morphology and cytological characteristics (e.g., different degrees of nuclear compaction, cytoskeleton abundance)[34]. These parameters are often generally described in other protocols[24,33], without providing a clear discrimination of the procedure within different cell

types. Furthermore, we developed a specific tool named NuCLεD (nuclear contacts locator in 3D)[16], providing principles for data analysis that will improve the 3D proximity between different loci and their nuclear topological distribution within the nuclear space in an automated way.

PROTOCOL:

1. DNA probe preparation and labelling procedures with nick translation

1.1. Purify and clean bacterial artificial chromosomes (BACs), plasmids or PCR products with specific kits (**Table of Materials**), resuspend in ddH_2O, check by electrophoresis on an agarose gel and quantify.

1.2. Perform nick translation on 1.5−2 µg of DNA from step 1.1 in a final volume of 50 µL by mixing all the reagents in a 0.5 mL low binding DNA tube according to **Table 1**.

NOTE: Directly labelled probes can improve the signal to noise ratio. Kits to produce indirectly and directly labelled probes with various Alexa fluorochromes by nick translation are commercially available.

1.3. Incubate the nick translation mix in a thermal mixer at 16 °C for a time depending on the length of the starting DNA material: 45 min for PCR products (a pool of PCR products of 2,000 bp each) and up to 4 h for BAC DNA and plasmids.

1.4. Check the size of the probes produced in step 1.3 by electrophoresis on a 2.2% agarose gel.

NOTE: The optimal probe size is <200 bp (**Figure 1A**).

1.4.1. If the DNA is not digested enough, add 5 U of DNA polymerase I and 0.05 U of DNase I to the reaction and incubate for 1−2 h at 16 °C and subsequently re-check. Stop the reaction with 0.5 mM EDTA (final concentration). Store probes at -20 °C.

1.5. For each DNA FISH experiment, precipitate the following quantities of probes depending on the starting DNA material from which the probes are produced: 200 ng from nick translated PCR products, 100 ng from nick translated BACs, or 300 ng from nick translated plasmid. Add ddH_2O up to 150 µL, 20 µg of unlabelled salmon sperm DNA, 3.5 µg of species-specific Cot-1 DNA, 3 volumes of 100% EtOH, and 1/10 volume of 3 M sodium acetate pH 5.2. Precipitate at -80 °C for 1 h.

1.6. Centrifuge at maximum speed for 1 h at 4 °C and discard the supernatant. Wash the pellet twice with 70% EtOH.

1.7. Resuspend the pellet in 2 µL of 100% formamide pH 7.0, shake at 40 °C for 30 min (can take up to a few hours) and then add an equal volume of 4x saline-sodium citrate (SSC)/20% dextran sulfate.

1.7.1. Prepare 20x SSC by mixing 175.3 g of NaCl and 88.2 g of sodium citrate in a final volume of 1 L of H_2O. Autoclave, filter and prepare aliquots.

NOTE: For multicolor DNA FISH, the different probes can be precipitated together except for probes that can anneal with one another. In these specific cases, treat them separately, precipitate and resuspend the probes dividing the reagents mentioned in steps 1.5−1.7 with respect to the number of probes. Pool the probes only after resuspension in formamide. If glass coverslips greater than 10 mm or less than 10 mm are used, scale up/down the volume of the reagents used in steps 1.5−1.7 proportionally to the slide size. Hybridization probes can be stored at -20 °C for a long period of time (up to two months).

2. Cell fixation, pre-treatment and permeabilization

NOTE: Permeabilization and deproteinization passages are crucial steps. The time of reaction and concentration of the reagents strongly depend on the cell type, the cytoplasm abundance, and the nuclear morphology.

2.1. Cell fixation, pre-treatment and permeabilization for human primary T lymphocytes

NOTE: This protocol is suitable for small cells, with small nuclei and a low amount of cytoplasm.

2.1.1. Use glass coverslips (10 mm, thickness No. 1.5H).

2.1.2. Wash the glass with ddH_2O, then with 70% EtOH and let them dry. Use one glass for each well of a 24-well plate.

2.1.3. Add 200 µL of 0.1% poly-L-lysine (w/v) (**Table of Materials**) directly on the glass to form a drop. Pay attention as the drop must remain on the glass surface without touching the well for 2−5 min. Leave out the drop carefully, and let the glass dry for 30 min.

2.1.4. Perform step 2.1.3 twice more.

2.1.5. Put 200 µL of suspension cells (2 x 10^6/mL, PBS suspension of ex vivo primary T lymphocytes) directly on the glass, allow the cells to seed at room temperature (RT) for 30 min.

2.1.6. Quickly remove the drop. Add freshly made 4% PFA (prepared in PBS/0.1% TWEEN 20, pH 7.0, filtered) for 10 min and fix the seeded cells at RT.

2.1.7. Wash three times for 5 min each with 0.05% Triton X-100/PBS (TPBS) at RT. Permeabilize with 0.5% TPBS for 10 min at RT.

2.1.8. Perform RNase treatment by adding 2.5 µL of RNase cocktail (**Table of Materials**) in 250 µL of PBS/well in a 24-well plate for 1 h at 37 °C.

2.1.9. Rinse in PBS, add 20% glycerol/PBS, and incubate overnight (ON) at 4 °C.

NOTE: This step can range from 1 h to ON. Slides can be kept in 20% glycerol/PBS at 4 °C up to 7 days.

2.1.10. Freeze on dry ice (15−30 s), thaw gradually at RT, and soak in 20% glycerol/PBS. Repeat step 2.1.9 another three times.

2.1.11. Wash in 0.5% TPBS for 5 min at RT. Wash in 0.05% TPBS, twice for 5 min at RT. Incubate in 0.1 N HCl for 12 min at RT. Rinse in 2x SSC.

2.1.12. Incubate in 50% formamide pH 7.0/2x SSC ON at RT.

NOTE: The incubation time can be optimized and eventually reduced. Slides can be kept in 50% formamide/2x SSC for several days.

2.2. Cell fixation, pre-treatment and permeabilization for human primary myoblasts

NOTE: This protocol is suitable for large cells, with a high amount of cytoplasm.

2.2.1. Grow human primary myoblasts directly on the coverslip glasses in 24-well plates in growth medium (Dulbecco's modified Eagle medium (DMEM), 20% fetal bovine serum (FBS), 25 ng/mL fibroblast growth factor (FGF), 10 ng/mL epidermal growth factor (EGF), 10 µg/mL human insulin, 1x glutamine, 1x penicillin/streptomycin) for at least 24 h (reaching 50−70% of confluence).

NOTE: To facilitate the adhesion, gelatin or collagen or poly-L-lysine can be used to coat the coverslip prior the seeding.

2.2.2. Rinse cells in 2−3 changes of PBS. Add freshly made 4% PFA and fix the cells for 10 min at RT.

2.2.3. Wash three times for 3 min each in 0.01% TPBS at RT. Permeabilize in 0.5% TPBS for 10 min at RT.

2.2.4. Perform RNase treatment by adding 2.5 µL of RNase cocktail (**Table of Materials**) in 250 µL of PBS/well in a 24-well plate for 1 h at 37 °C.

2.2.5. Rinse in PBS, add 20% glycerol/PBS, and incubate ON at RT.

NOTE: This step can range from 1 h to ON. Slides can be kept in 20% glycerol/PBS at 4 °C up to 7 days.

2.2.6. Freeze on dry ice (15−30 s), thaw gradually at RT, and soak in 20% glycerol/PBS. Repeat this step three times.

2.2.7. Wash three times for 10 min each in PBS. Incubate in 0.1 N HCl for 5 min at RT. Rinse in 2x SSC.

2.2.8. Incubate in 50% formamide pH 7.0/2x SSC ON at RT.

NOTE: The time of incubation can be optimized and eventually reduced. Slides can be kept in 50% formamide/2x SSC for several days.

2.2.9. Equilibrate slides (kept in 50% formamide/2x SSC) in 2x SSC for 2 min. Then, equilibrate in PBS for 3 min.

2.2.10. Treat with 0.01 N HCl/0.0025% pepsin from a few seconds up to 5 min, depending on the cell type. During this step, observe the cells under an optical microscope and stop the reaction (step 2.2.11) as soon as the nuclei are free from the cytoplasm, while maintaining their structure intact (e.g., nucleoli are still visible and intact).

2.2.11. Inactivate the pepsin by washing twice for 5 min each in 50 mM $MgCl_2$/PBS.

2.2.12. Post-fix in 1% PFA/PBS for 1 min. Wash for 5 min in PBS. Wash twice for 5 min each in 2x SSC, and then add 50% formamide/2x SSC for at least 30 min.

3. 3D multicolor DNA FISH hybridization

3.1. Denature the probes at 80 °C for 5 min, and then put quickly on ice.

3.2. Load the hybridization probes on a clean microscope slide. Turn the coverslip with cells upside down on the drop of hybridization probes.

3.3. Seal the coverslip with rubber cement. Let the rubber cement dry completely. Then place slides on a heating block and denature at 75 °C for 4 min.

NOTE: The timing of denaturation and temperature of denaturation can be augmented, up to 80 °C.

3.4. Hybridize at 37 °C ON in a metallic box floating in a water bath.

NOTE: To improve the signal to noise ratio, the hybridization temperature can go up to 42 °C.

3.5. Peel off rubber cement, immerse the slides in 2x SCC, strip off the glass coverslip and transfer it to 2x SSC in 6-well plate.

3.6. Wash in 2x SSC three times for 5 min each at 37 °C, shaking at 90 rpm in an incubator shaker. Wash in 0.1x SSC three times for 5 min each at 60 °C, shaking at 90 rpm in an incubator shaker. Rinse briefly in 4x SSC/0.2% TWEEN 20.

4. 3D multicolor DNA FISH detection

NOTE: For directly labelled probes, skip steps 4.1 and 4.2.

4.1. Block in 4x SSC/0.2% TWEEN 20/4% bovine serum albumin (BSA) for 20 min at 37 °C in a 24-well plate, shaking at 20 rpm in an incubator shaker.

4.2. Incubate with the appropriate concentration of anti-digoxygenin (1:150), and/or streptavidin (1:1,000) (**Table of Materials**) diluted in 4x SSC/0.2% TWEEN 20/4% BSA for 35 min in a dark and wet chamber at 37 °C.

4.3. Wash in 4x SSC/0.2% TWEEN 20 three times for 5 min each at 37 °C, shaking at 90 rpm in a 6-well plate in an incubator shaker.

4.4. Equilibrate in PBS and post-fix in 2% formaldehyde/PBS for 2 min at RT in a 24-well plate.

NOTE: Directly labelled probes do not need post-fixation.

4.5. Wash 5x briefly in PBS. Stain with 1 ng/µL 4,6-diamidino-2-phenylindole (DAPI)/PBS for 5 min at RT.

4.6. Wash 5x briefly in PBS. Mount with antifade solution (**Table of Materials**).

5. 3D multicolor DNA FISH microscopy and analysis

5.1. Acquire 3D images with a microscope system.

NOTE: Here, a widefield microscope with an axial distance of 0.2–0.25 µm between consecutive sections (**Figure 1B**) is used.

5.2. Analyze 3D image stacks using different software and tools (here, NuCLεD or Nuclear Contacts Locator in 3D).

NOTE: The tool NuCLεD has been developed in order to automatically analyze 3D multicolor DNA FISH in fluorescence cell image z-stacks. NuCLεD reconstructs the nuclei from cell image stacks in 3D as well as detects and localizes fluorescent 3D spots. It measures the relative positioning of spots in the nucleus (e.g., distance from the centroid of the nuclei and/or periphery of the nuclei) and the maximum radius for each nucleus and inter-spots distances. The tool and the algorithm used are fully described in Cortesi et al.[16].

5.3. Analyze the data (e.g., 3D distances between specified genomic loci and the nuclear centroid and contact frequencies) retrieved by NuCLεD.

NOTE: For 3D distances between specified genomic loci and the nuclear centroid, normalize distances on the maximum radius for each nucleus and represent these data as frequency distributions of normalized distances from the nuclear centroid. For long range interactions studies, contact frequencies are supposed to be in the range of 10−20%[21] with a threshold inter-distance that can vary and can be put around 2 µm[35].

5.3.1. To perform statistical analysis, analyze approximately 100 nuclei per biological replicate. Represent 3D distances between specified genomic loci as cumulative frequency distributions of distances that are below the threshold inter-distance selected and use a *t*-test to assess the significance of differences in the distributions. Also, calculate the percentage of nuclei positive for the interactions that are below the threshold inter-distance selected, and use Fisher's exact test to assess the significance of differences in the percentages.

REPRESENTATIVE RESULTS:

The method of 3D multicolor DNA FISH described in this article allows contemporary visualization of different genomic loci within preserved 3D nuclei (**Figure 1B**). This protocol permits the measurement of distances between alleles, and different genomic loci in order to evaluate their spatial proximity, and to assess their location within the nuclear space (e.g., loci distance from the centroid or the periphery of the nuclei)[16]. However, there are many crucial steps that must be accurately and specifically set up for each cell type used; it is highly recommended to pay particular attention to the following steps for success of 3D multicolor DNA FISH.

For DNA probe preparation, check that probe size is <200 bp (**Figure 1A**). This size ensures a successful procedure of 3D multicolor DNA FISH (**Figure 1B**). Suboptimal DNA FISH probes produced by nick translation can be partially digested (**Figure 2A**) or over digested (**Figure 2B**). With partially digested probes, the procedure will have no signal in the cells, due to the inability of the probe to enter the nuclei and properly hybridize to the complementary genomic loci. Over digested probes will result in a nonspecific signal, due to a loss of specificity in the hybridization and a consequent increase of the background. A representative example of over digested probes is shown in **Figure 3A** in comparison to an optimal digested probe in **Figure 3B**.

For deproteinization and pepsinization, follow these steps according to the cell type. In particular, take into consideration nuclear size and cytoplasm abundance. For human primary ex vivo isolated T lymphocytes and cells with small, highly compacted nuclei and low abundant cytoplasm, HCl deproteinization is crucial. Treatment with 0.1 N HCl for 5 min is not sufficient for DNA FISH visualization. 0.1 N HCl treatment for 12 min is recommended to promote nuclei accessibility to DNA probes and preserve nuclear integrity (**Figure 4A**). Pepsin digestion of the cytoplasm is not needed to obtain a good signal of DNA FISH (**Figure 4B**).

For human primary myoblasts and cells that have large nuclei and abundant cytoplasm, the pepsinization step is fundamental. A short and suboptimal pepsinization of the cytoskeleton will

hamper the entry of the probe in the nuclei (**Figure 4C**), ending in the absence of a DNA FISH signal. However, if the cells are over pepsinized, nuclei will not remain intact (**Figure 4D**), losing their 3D structure. An example of successful 3D multicolor DNA FISH is provided in **Figure 4E**.

During hybridization, seal the coverslip accurately; otherwise, the probe will disperse and dry. Denaturation and hybridization steps must be performed rapidly such that the probe and genomic DNA will not reanneal. The duration of the denaturation can be increased.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative DNA probes and 3D multicolor DNA FISH. (**A**) Nick translated DNA probes of optimal size run on an 2.2% agarose gel (lane 1, 2), 50 bp marker (M). (**B**) Representative 3D multicolor DNA FISH nucleus using probes mapping to 3q11.2 region (green), 10q26.3 region (red) and 8q24.13 region (magenta) in human primary myoblasts. Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 5 µm.

Figure 2: Examples of not optimally digested DNA FISH probes. (**A**) Not digested (lane 1, 2) or partially digested (lane 3) nick translated DNA probes run on an 2.2% agarose gel, 2log marker (M). (**B**) Over digested nick translated DNA probes run on an 2.2% agarose gel, 2log marker (M).

Figure 3: Comparison of 3D multicolor DNA FISH using suboptimal or optimal DNA FISH probes. (**A**) Representative 3D multicolor DNA FISH nuclei using over digested probe mapping to 8q24.13 region (magenta) in human primary myoblasts. Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 10 µm. (**B**) Representative 3D multicolor DNA FISH nuclei using optimally digested probe mapping to 8q24.13 region (magenta) in human primary myoblasts. Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 10 µm.

Figure 4: Possible outcomes of suboptimal deproteinization and pepsinization steps on 3D multicolor DNA FISH results. (**A**) Representative 3D multicolor DNA FISH nuclei of human primary T lymphocytes treated for 5 min (left) or 12 min (right) with 0.1 N HCl, using probe mapping to 8q24.13 region (green). Nuclei are counterstained with DAPI (blue). 100x magnification. Scale bar = 10 µm. (**B**) Representative 3D multicolor DNA FISH nuclei of human primary T lymphocytes treated for 12 min with 0.1 N HCl (left) or coupled with 0.01 N HCl/0.0025% pepsin for 2 min (right), using probe mapping to 8q24.13 region (green). Nuclei are counterstained with DAPI (blue). 100x magnification. Scale bar = 10 µm. (**C**) Representative 3D multicolor DNA FISH nuclei of human primary myoblasts treated with short and suboptimal pepsinization, using probe mapping to 8q24.13 region (magenta). Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 10 µm. (**D**) Representative 3D multicolor DNA FISH nuclei of human primary myoblasts treated with prolonged pepsinization step, using probe mapping to 8q24.13 region (magenta). Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 5 µm. (**E**) Representative 3D multicolor DNA FISH nuclei of human primary myoblasts treated with optimal HCl/pepsin conditions using probe mapping to 8q24.13 region (magenta). Nuclei are counterstained with DAPI (blue). 63x magnification. Scale bar = 25 µm.

Table 1: Nick Translation. Table describing all the reagents, their concentration and suggested timing for nick translation reaction.

DISCUSSION:

The current method describes a step by step protocol to perform 3D multicolor DNA FISH on a wide range of human primary cells. Although DNA FISH is a technology in wide use, 3D multicolor DNA FISH on preserved 3D interphase nuclei is still difficult to perform in many laboratories, mainly due to the characteristics of the samples used[23,24].

Probe nick translation is a fundamental step for successful 3D multicolor DNA FISH; many different substrates (BAC, fosmid, plasmid, PCR products) can be used for this reaction, and the timing of the reaction and enzyme concentration can be accordingly adjusted with respect to the length of the substrate. A proper probe digestion is fundamental (**Figure 1**), as nonoptimal probes (**Figure 2**) will result in no signal or a nonspecific signal (**Figure 3A**). Permeabilization, deproteinization, and pepsinization steps are crucial passages that strongly depend on the cell type used. Cells with small nuclei and low cytoskeleton abundance, such as ex vivo isolated T lymphocytes, require deproteinization with a prolonged 0.1 N HCl treatment. Also, washes in PBS with higher percentages of Triton X-100 can help the probe entry in the nuclei of these cells. On the contrary, in vitro cultured human primary myoblasts that present larger nuclei, with a high content of cytoskeleton, need digestion of the cytosolic structures with pepsin. These general roles can be applied to a wide range of cells, eventually combining the different steps depending on the specific cellular characteristics.

The use of freshly prepared biological material, fresh solutions (in particular solutions with detergent), and fluorescent reagents are strongly suggested: filtered PFA at pH 7.0; autoclaved and filtered 20x SSC at pH 7.0; filtered formamide at pH 7.0; nuclease free water; and disposable aliquots of modified UTP. Prolonged incubation with 20% glycerol/PBS, or 50% formamide/2x SSC can facilitate the hybridization. HCl and/or pepsin treatment can be further increased. The timing of hybridization, the quantity of probes, the concentration and the timing of incubation of anti-digoxigenin and streptavidin can all be further adjusted to improve the signal to noise ratio.

3D multicolor DNA FISH represents a complementary tool to C-technologies, the standard method to validate C-based results. If combined with 3D microscopy and analysis, 3D multicolor DNA FISH can monitor the proximity between genomic loci and their topological distribution within the nuclear space at single cell level. 3D multicolor DNA FISH can be further integrated with other methodologies such as RNA FISH and immunofluorescence for a comprehensive overview of the dynamics and interactions between genomic loci, RNAs (messenger RNA or regulatory non coding RNA) and a wide range of proteins, providing a unique opportunity to visualize the nuclear structure and investigate the epigenetic mechanisms that subtend cellular identity.

Despite the huge improvement of FISH technologies with super resolution[25,26], live cell imaging[27,28,36], single molecule detection[37], and contemporary visualization of multiple targets with oligonucleotide arrays such as Oligopaint[37,38] with 3D high-throughput approaches[39], a

limitation of the technology remains the discrete number of predetermined genomic loci that can be visualized. This prevents a wide-ranging analysis of nuclear architecture. Several studies have recently described sequential methods of hybridization to address genome organization in single cells such as barcode DNA FISH[40-43]. Further efforts will be needed to couple the single cell nature of 3D multicolor DNA FISH to genome wide features to broadly visualize nuclear architecture heterogeneity with imaging technologies, as the number of loci that can be tested at a time will increase.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

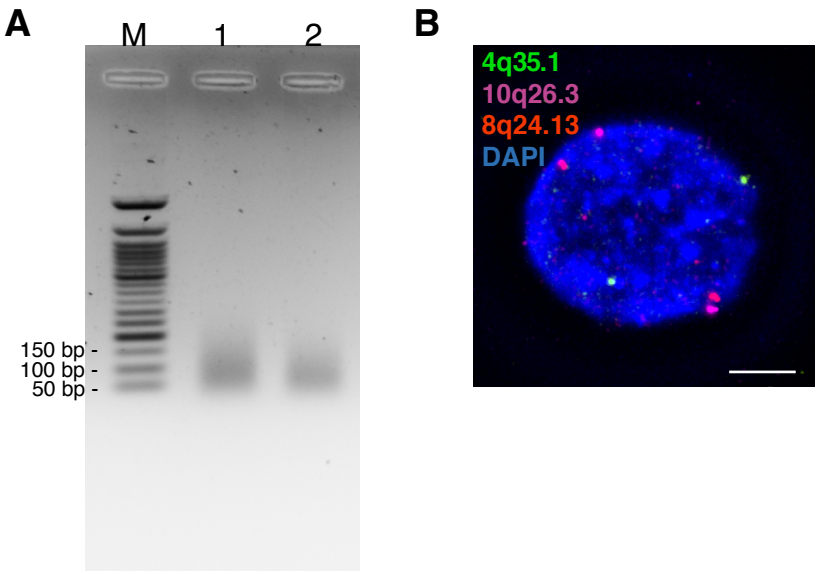

Figure 2

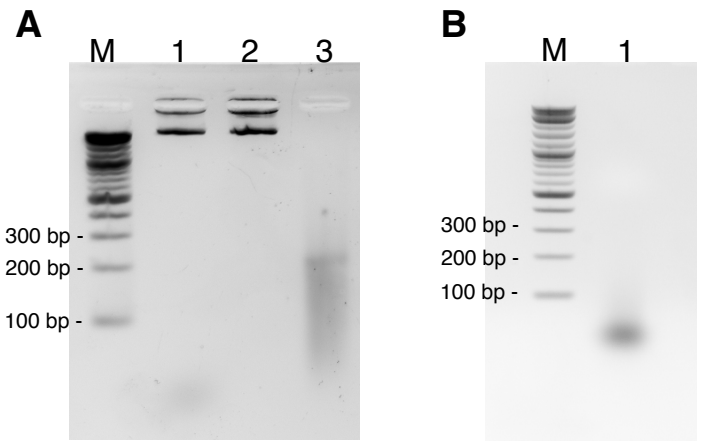

Figure 3

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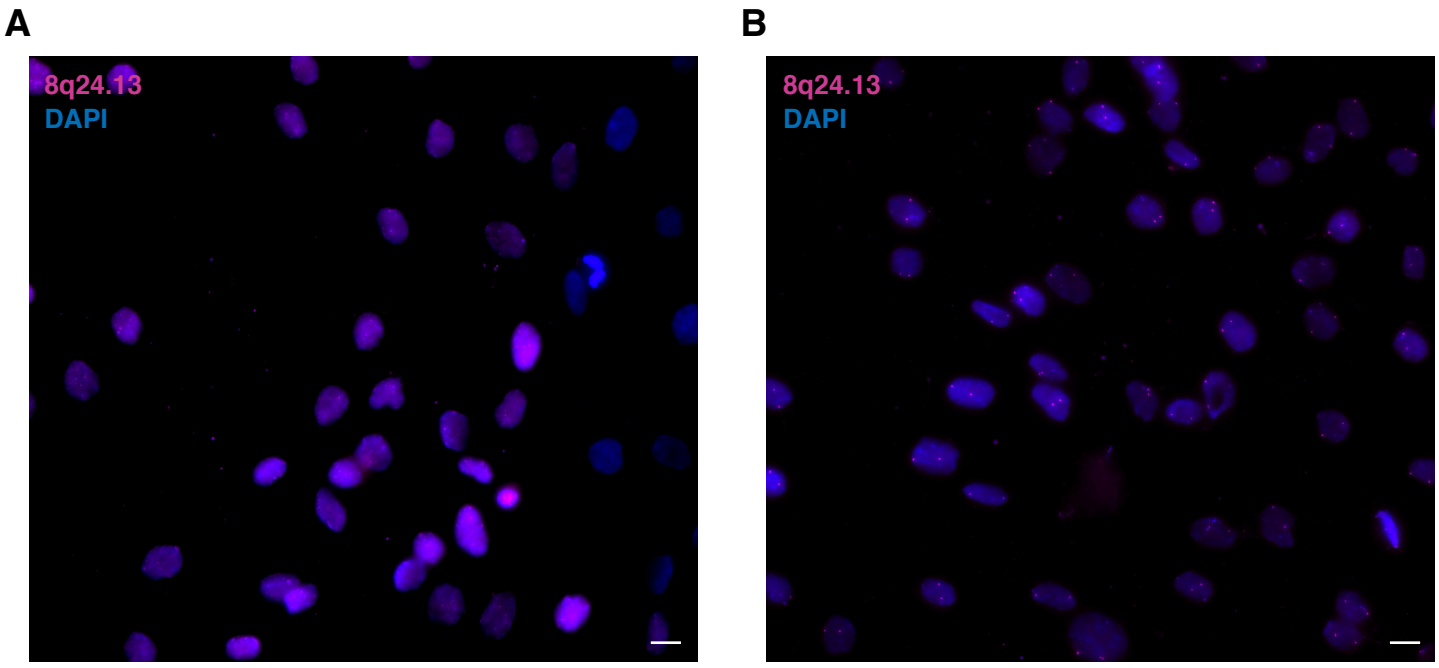

Figure 4

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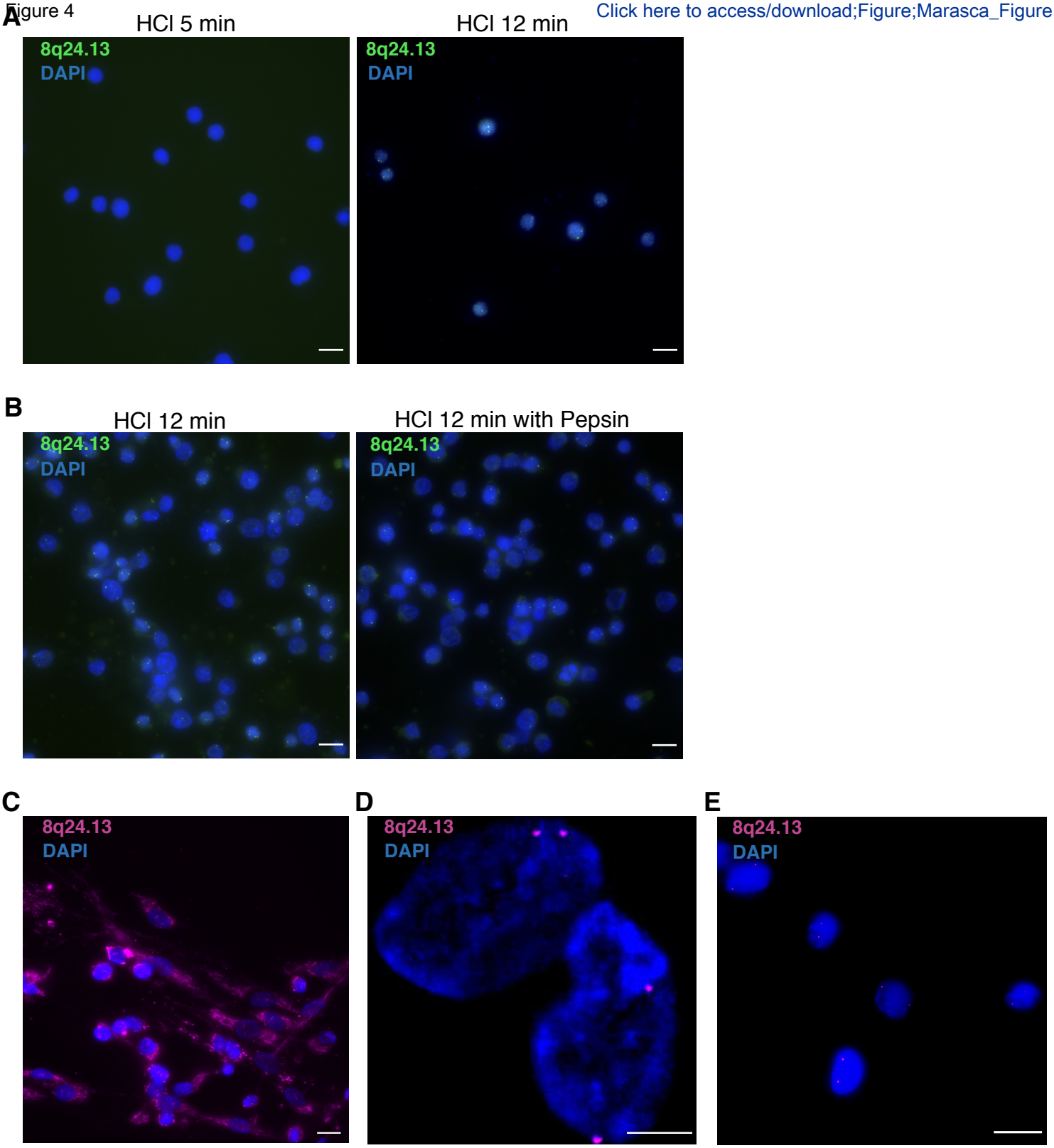


Nick translation reagents	**Initial concentration**	**Final concentration**
dNTPs (C-G-A)	0.5 mM	0.05 mM
dTTP	0.1 mM	0.01 mM
Biotin/Dig/Cy3 dUTP	1 mM	0.02 mM
Tris HCl pH 7.8	1 M	50 mM
$MgCl_2$	100 mM	5 mM
β-mercaptoethanol	100 mM	10 mM
BSA	100 ng/µL	10 ng/µL
DNA Pol I	10 U/µL	0.1 U/µL
DNase I	1 U/µL	0.002 U/µL
DNA 2 µg	x	x
ddH_2O		Up to 50 µL

Name of Material/Equipment	Company	Catalog Number
24-well plates	Thermo Fisher Scientific	142475
6-well plates	Thermo Fisher Scientific	140675
Anti-Digoxigenin 488	DBA	DI7488
b-Mercaptoethanol	Sigma	M3148
bFGF	PeproTech	100-18B
Biotin 11 d-UTP	Thermo Fisher Scientific	R0081
BSA (bovine serum albumine)	Sigma	A7030
Coverlsips	Marienfeld	117500
CY3 d-UTP	GE Healthcare	PA53022
DAPI (4,6-diamidino-2-phenylindole)	Thermo Fisher Scientific	D21490
Deoxyribonucleic acids single strand from salmon testes	Sigma	D7656
Dextran sulfate (powder)	Santa Cruz	sc-203917A
Digoxigenin 11 d-UTP	Roche	11093088910
DMEM	Thermo Fisher Scientific	21969-035 500mL
DNA polymerase I	Thermo Fisher Scientific	18010-017
DNase I	Sigma	AMPD1
dNTPs (C-G-A-T)	Euroclone	BL0423A/C/G
EGF	Sigma	E9644.2MG
Ethanol	Sigma	02860-1L
FBS Hyclone	Thermo Fisher Scientific	SH30109
Formaldehyde solution	Sigma	F8775-25mL
Formamide	Sigma	F9037
Glutammine	Thermo Fisher Scientific	25030-024 100mL
Glycerol	Sigma	G5516-100mL
Glycogen	Thermo Fisher Scientific	AM9510
HCl	Sigma	30721
Human *Cot-1* DNA	Thermo Fisher Scientific	15279-001
Insulin Human	Sigma	I9278-5 mL
$MgCl_2$	Sigma	63069
NaAc (Sodium Acetate, pH 5.2, 3 M)	Sigma	S2889
NaCl	Sigma	S9888

Paraformaldehyde	Sigma	158127-25G
PBS (phosphate-buffered saline)	Sigma	P4417
Pennycillin/Streptavidin	Thermo Fisher Scientific	15070-063 100mL
Pepsin	Biorad	P6887
PhasePrep BAC DNA Kit	Sigma	NA0100-1KT
Poly-L-lysine solution	Sigma	P8920
ProLong Diamond Antifade Mountant	Thermo Fisher Scientific	P36970
PureLink Quick Gel Extraction & PCR Purification Combo Kit	Thermo Fisher Scientific	K220001
PureLink Quick Plasmid Miniprep Kit	Thermo Fisher Scientific	K210010
RNAse cocktail	Thermo Fisher Scientific	AM2288
Rubbercement	Bostik	
Slides	VWR	631-0114
Streptavidina Alexa fluor 647	Thermo Fisher Scientific	S21374
Tri-Sodium Citrate	Sigma	1110379026
Tris-HCl	Sigma	T3253-500g
Triton X-100	Sigma	T8787-250mL
TWEEN 20	Sigma	P9416-100mL

INGM
ISTITUTO NAZIONALE GENETICA MOLECOLARE
"Romeo ed Enrica Invernizzi"

Milan, 14/10/2019

Beatrice Bodega, PhD, INGM

Rebuttal letter to the reviewers of the manuscripts JoVE60712

Below we provide detailed answers to the editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We have carefully checked the entire manuscript for any spelling or grammar issues.

2. Title: Please revise to avoid the use of punctuation (colon, dash, etc.) and abbreviations.
We changed the title removing the colon.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.
We have carefully checked through the manuscript for the proper use of abbreviations and symbols.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Marienfeld, Nikon Instruments, etc.
We have removed any company names before instruments and reagents.

5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
We have revised the protocol following editorial suggestions.

6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."
We have revised the protocol following editorial suggestions.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please specify all volumes and concentrations used throughout. We need these details to film. See examples below.
Following editorial suggestions, we have added more details to the protocol throughout the text.

8. 1.1: Please specify nick translation procedure. Table 2 only shows reagents and concentrations.
Reagents and concentrations are now specified in the section 1.2.

9. 1.2: What is incubated?

The subject is now specified.

10. 1.3: What probes? From which step? It is unclear. What volume of DNA Pol I, DNase I, and EDTA are added? Please specify.
Please note that all the details required are now present in the mentioned section.

11. 1.4: What happens after centrifugation, discard the supernatant?
We have added the required details in the 1.3 section.

12. 2.1.1: What does 1.5 H mean?
It means "thickness No. 1.5H". We have added the proper nomenclature in the text.

13. 2.1.3: What does "repeat other two times" mean exactly? Make two drops?
We have specified that the step 2.1.3 should be performed three times.

14. 2.2.1: Please specify the cell type and growth conditions.
As requested, we have specified the cell type and growing conditions.

15. Section 5: Please describe in the imperative tense how images are collected and analyzed.
We have specified the editor requirement within the text.

16. Figure 4: Please ensure that the panels are of the same dimensions if possible.
The panels shown are checked to be of the same dimensions.

17. Table of Materials: Please sort the materials alphabetically by material name.
Materials in the table are alphabetically sorted.

18. References: Please do not abbreviate journal titles; use full journal name.
We have used journal full name in the References.

Below we provide detailed answers to the referees' comments:

Reviewer #1:
Major Concerns:

- Paragraph 3/hybridization should include the post-hybridization washes in SSC, i.e. until step 4.4., before the blocking step which is dedicated to the FISH detection per se (paragraph 4).
We have re-organized the paragraph following reviewer suggestion.

- I understand that the Freeze-thaw steps in 20% Glycerol/PBS are for permeabilization, but is it really necessary since there is already a permeabilization step with high Triton concentration and a deproteination step with HCl or HCl/pepsin. This step is time consuming, need dry ice or liquid nitrogen, but is probably facultative (as we experienced with different mammalian cells), especially with small probes (<200 bp as described here).
We thank the reviewer for this comment. Indeed, as he noted, freeze-thaw steps are facultative and dependent on the cell type and probes sizes; therefore, we have now indicated a range from 1h to over night (O.N.) for the step of 20 % Glycerol/PBS, in a way that the reader can vary the protocol adjusting to the cell type used. However, in our experience, the freeze and thaw step in general ameliorates the result; in agreement with this, while this step is not included in protocols that propose the alcoholic scale to fix and to permeabilize cells (Chaumeil, J. 2008; Byron, M. 2013; Takizawa, T. 2008), it is always suggested when the protocol avoid alcoholic scale, like our one (Solovei, I. 2010; Cremer, M. 2008).

- If using only directly labeled probes (which often give better signal to noise ratio), the blocking/post-fix steps are unnecessary, again lightening the protocol. Kits to produce directly labeled probes by nick-translation with various Alexa fluorochromes are commercially available.
Following reviewer suggestion, we have removed the blocking and post-fixing steps for directly labelled probes, and we have better specified the different possibilities with an appropriate note in the section 1.2.

- In the representative results/discussion: may be worth to mention that "Timing of denaturation" as well as temperature of denaturation can be augmented, up to 80°C. Otherwise, to improve signal to noise ratio, temperature of hybridization can go up to 42°C.
We thank the reviewer for the suggestion, we have added a note in the section 3.5 and 3.6 to provide this information.

- In Figure 4C-D, would need to have a FISH example with optimal HCl/pepsin conditions for primary myoblasts to appreciate the importance of the pepsinization step. Unfortunately, only suboptimal conditions are shown here. Also, the same probe should be used for comparison.
As requested, we have added in Fig. 4E a DNA FISH example with optimal HCl/pepsin conditions; we have replaced the panel 4C with a new one in order to have the same probe used for panels C-D-E.

Minor Concerns:

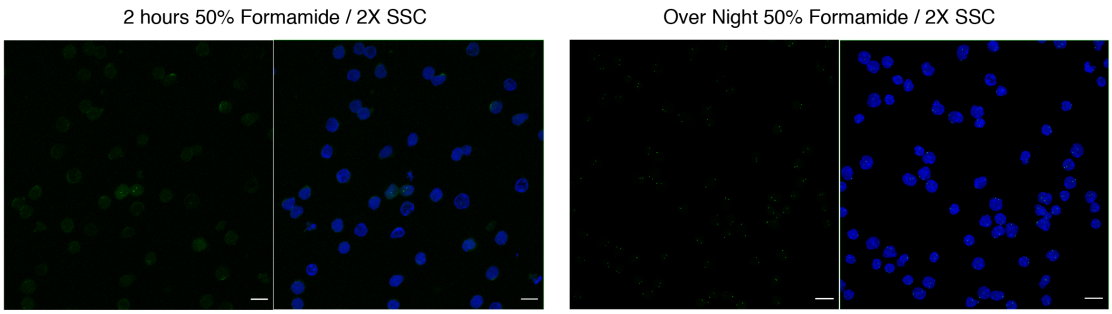
- step 2.1.3: precise for how long the Poly-Lysine drop need to remain on the glass surface. Not clear here. Alternatively, you can put the glass coverslips in a solution of 0.01% Poly-Lysine for at least 5 min.
Following the reviewer suggestion, we have specified the Poly-lysine step.

- 2.1.5: why adding few drops at the end of the fixation step. Don't think that this step is necessary.
The reviewer is right, we have removed this step from the protocol.

- 2.1.8: ON incubation in 20% Glycerol/PBS. I think 30-60 min could be sufficient.
Following reviewer recommendation, we have now indicated a range from 1h to over night (ON) for the step of 20 % Glycerol/PBS, in a way that the reader can vary the protocol adjusting to the cell type used. See also answer to Major concern point 2.

- 2.1.10: Why using DEPC water here?
This was a mistake, we have removed it.

- 2.1.11: same as above, an ON incubation seems a lot. I think 30-60 min could be sufficient. All these ON incubations render this protocol quite very long! (4 days in total, including the hybridization step).
In this particular case, although we recognized that this step could extend the time of the protocol, we have noticed that prolonged incubation in 50 % formamide/ 2× SSC seems crucial for nuclei denaturation for the subsequent hybridization step and to obtain a successful 3D DNA FISH. As the reviewer can appreciate below, 2 hours incubation is much less efficient than ON incubation with 50 % formamide/ 2× SSC. However, we have added a note at this step and at step 2.2.8, specifying that the time can be optimized and eventually reduced.



- 2.2.12: replace FA by formamide.
FA has been replaced with formamide.

- 3.1 (note): Why the denaturation of probes seems only to apply to human primary T lymphocytes. This seems an important step.
The reviewer is right; therefore, we have removed the note and extended the step as general.

- 4.6: don't understand why it is needed to block additionally when using single fluorophore, directly labeled probes.
This passage has been removed and specified, with an appropriate note in section 4.1, that for directly labelled probes the blocking is not needed.

- In the discussion: I don't think that ref . 27 (Ricci et al.) are using FISH technologies with super resolution.
As noticed by the reviewer, the citation has been corrected.

- In the discussion: "The limitation of the technology remains the small number…", Several papers have recently described sequential methods of hybridization, i.e. barcode DNA FISH (Bintu Zhuang 2018; Nir Wu 2018; Cardozo Nollmann 2019; (Nir et al. 2018; Cardozo Gizzi et al. 2019; Mateo et al. 2019)) to address genome organization in single cells. The number of loci that can be tested at a time will therefore be less and less limited.
Following reviewer suggestion, we have toned down this concept in the discussion, introducing and commenting the suggested papers.

Reviewer #2:
Major Concerns:

1. The introduction focuses on the comparison between DNA FISH and Hi-C approaches. However, this final statement is not valid: "For these reasons, although "C-technologies" are high evaluable tools to study general roles of genome folding, they need to be complemented with single cell approaches in order to dissect cellular heterogeneity". This is because single-cell HiC has already been established in other studies (Nagano et al, Nature 2013 and others) and because Hi-C approaches have already been used to complement FISH methods in previous studies (Bintu et al, Science 2018 and others). The author may extensively cut down this comparison between Hic and FISH in the introduction section.
As requested by the reviewer, we have cut and toned down the comparison between Hi-C and FISH in the introduction.

2. On the other hand, the introduction did not describe any specific feature of this particular DNA FISH protocol. The protocol appears to be a standard DNA FISH method. I do not understand how the authors have expanded on or improved the original DNA FISH protocol. This information will enable readers to understand the specific

advantage of this protocol compared to many other similar protocols. This information needs to be clarified in the introduction section in comparison with other protocols with proper citations.
We thank the reviewer for this useful suggestion. In the introduction we have now highlighted the improvements of our protocol in respect to other similar protocols.

i) In respect to other protocols (Chaumeil, J. 2008; Byron, M. 2013; Takizawa, T. 2008), we avoid the use of alcohol to maximize the preservation of the nuclear structure.
ii) In respect to protocols of 3D DNA FISH on preserved nuclei (Solovei, I. 2010; Cremer, M. 2008), we highlight the punctual adjustments in terms of permeabilization and deproteinization that we use on the basis of the cytological characteristics of the cells (e.g. different degree of nuclear compaction, cytoskeleton abundance), that in our experience can be crucial in 3D DNA FISH setting on different cell types.
iii) For data analysis, we have developed and designed a novel algorithm named NuCLεD (Nuclear Contacts Locator in 3D), described in Cortesi et al 2019 and in Gregoretti et al (accepted in Methods in Molecular Biology); in this ms we have further implemented the description of the method, providing principles for data analysis.

Minor Concerns:

1. In the abstract, ''C-technologies" is not clear. This term should be spelled out.
We have spelled out “C-technologies” in the abstract.

2. Preparation of pure DNA fragments is important to reduce the background of DNA FISH. How is DNA purified for BAC clones, plasmids, and PCR products? This information can be described.
We thank the reviewer for this comment. We have added this step in the protocol and the information regarding the kits used for DNA purification in Table 1 of reagents.

3. Why are the experimental conditions for nick translation different between BAC clones/plasmids and PCR products?
The time of incubation required for the reaction of nick translation is different between BACs, plasmids and PCR products since these templates have different length (ranging from 150 kb of a BAC to 2 kb of PCR products); thus, in our experience, to produce probes < 200 bp, DNAse I and DNA Polymerase I require different incubation times. This is now specified in the ms (see 1.3), as requested.

4. Regarding step 1.4, why are different units used for DNA measurement ("5 □L" of nick translated PCR pool vs. "100 ng" of nick translated BAC)?
The reviewer is right, we have modified the text specifying the same units of measurements.

5. Although the authors used their own protocol for nick-translation, the use of nick-translation kits can make the preparation of DNA probes much easier. Did the author try the commercial kits?
Yes, we have tried commercial kits and they worked as well as our in house nick-translation protocol. However, we would like to suggest our method as *i)* it reduces the costs of the nick translation procedure *ii)* it can be easily modified and adapted to different targets (i.e. small DNA fragments or repetitive elements), *iii)* it is very versatile for getting probes efficiently labelled directly or indirectly. We have added a note in 1.2 section to make the reader aware of the possibility to use also commercial nick-translation kits.

6. It would be informative to describe how 3D FISH data can be analyzed.
Following reviewer suggestion, we implemented this paragraph with the description of NuCLεD algorithm for data analysis. Moreover, we highlight in text that the tool is already reported in Cortesi et al 2019 and in Gregoretti et al (accepted in Methods in Molecular Biology).