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Running Head: Higher-order chromatin organization using 3D DNA FISH

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Fluorescent in Situ Hybridization

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Abstract

The comprehensive analysis of the tri-dimensional (3D) organization of the genome is crucial to understand gene regulation. 3D DNA fluorescent in situ Hybridization (3D-FISH) is a method of choice to study nuclear organization at the single-cell level. The labeling of DNA loci of interest provides information on their spatial arrangement, such as their location within the nucleus or their relative positioning. The single-cell information of spatial positioning of genomic loci can thus be integrated with functional genomic and epigenomic features, such as gene activity, epigenetic states, or cell-population averaged chromatin interaction profiles obtained using Chromosome Conformation Capture methods. Moreover, the development of a diversity of super-resolution (SR) microscopy techniques now allows the study of structural chromatin properties at subdiffraction-resolution, making a finer characterization of shapes and volumes possible, as well as allowing the analysis of quantitative intermingling of genomic regions of interest. Here, we present and describe a 3D-FISH protocol adapted for both conventional and SR microscopy such as 3D Structured Illumination Microscopy (3D-SIM), which can be used for the measurement of 3D distances between loci and the analysis of higher-order chromatin structures in cultured *Drosophila* and mammalian cells.

1. Introduction

Our understanding of the mechanisms underlying genome regulation has been recently expanded by a deeper characterization of the relationships between structural and functional properties of chromatin folding. DNA Fluorescent in situ Hybridization (FISH) has been a pioneer technique in the study of nuclear organization, revealing for example the presence of chromosome territories or the differential location of active and inactive genes [1,2]. In the last decade, molecular biology experiments have allowed the genome-wide characterization of several chromosomal features, such as chromatin epigenetic status, chromatin interaction profiles, or attachment to the nuclear periphery. These methods have provided crucial information, but they cannot address the cell-to-cell variability within populations and therefore they do not provide information as to what extent these features reflect the single cell states. For example, the high-throughput version of the “Chromosome Conformation Capture” method, Hi-C [3], revealed the presence in many species of sub-megabase domains of preferential chromatin interactions, commonly named Topologically Associating Domains (TADs) [4-7]. TADs are often characterized by the enrichment of

chromatin interactions within a given genomic region compared to its surrounding chromatin. However, Hi-C experiments represent averaged chromatin interaction profiles typically obtained using millions of cells, hampering the understanding of the structural organization of the chromatin in single-cell. Therefore, FISH appears highly complementary to “Chromosome Conformation Capture” methods in order to decipher how TADs, or any other layer of chromosome organization, fold in individual cells. Recently, different studies have been using FISH to explore the correspondence between single-cell chromatin organizations and genomic-based assays [8-14], showing for instance differential degree of condensation for different chromatin states [9], the large-scale organization of chromosomes into active or repressive compartments [15,13], the absolute contact frequencies between TAD borders [10], or the TAD-based physical organization of chromosomes in *Drosophila* and human cells [8,12]. In this chapter, we will describe a detailed 3D-FISH protocol that can be applied in both *Drosophila* and mammalian cells, which allows investigating the 3D organization of specific genomic regions. Two complementary approaches will be presented: 3-color FISH used to systematically measure 3D distances using confocal laser scanning microscopy (CLSM), and 2-color FISH to characterize chromatin structure using 3D-SIM SR microscopy. This FISH protocol can be combined with immunostaining. In addition, we will briefly describe probe design, and present some guidelines for quantitative image analysis.

2. Materials

2.1 Materials for nick-translation based probe production and labeling

1. Genomic DNA
2. Primers
3. GoTaq G2 Hot Start Green Master Mix (Promega, M7422).
4. PCR purification kit.
5. DNA Marker and ladder
6. LE Agarose
7. FISH Tag DNA kits (Invitrogen Life Technologies, F32951 for the multicolor kit).

2.2 Materials for FISH

2.2.1 General equipment

1. Coverslips 0.170 ± 0.005 mm (also labeled as #1.5H) (Zeiss) (*see Note 1*).
2. Inox Tweezers (such as Dumont N°5).
3. Kimwipes Kimtech.
4. 6-well plates for cell culture.
5. Dark humid chamber for slides.
6. Rubber cement.
7. Transparent nail polish.
8. Aluminum block for heating.
9. Water bath with cover (such as VWR digital water bath 5L).
10. Vacuum.

2.2.2 Solutions

1. 0.01% Poly-L-Lysine: 1/10 (vol/vol) of 0.1% Poly-L-Lysine in distilled and deionized water (ddH₂O).
2. PBS.
3. 4% Paraformaldehyde (make it fresh): 1/10 (vol/vol) of 10× PBS and 1/4 (vol/vol) of 16% formaldehyde in ddH₂O.
4. 0.5% Triton X-100 (make it fresh): 1/200 (vol/vol) of Triton X-100 in PBS (*see Note 2*).
5. 0.1M HCl: 415 μL of 37% HCl in 50 mL ddH₂O (make it fresh).
6. 2× SSCT: 1/10 (vol/vol) of 20× SSC and 1/1000 (vol/vol) of Tween 20 in ddH₂O.
7. 50% Formamide, 2× SSCT (make it fresh): 1/2 (vol/vol) of deionized Formamide (Sigma, F-9037) and 1/2 (vol/vol) of 4× SSCT (4× SSCT made from 1/5 (vol/vol) of 20× SSC and 1/500 (vol/vol) of Tween 20 in ddH₂O).
8. 2× SSC: 1/10 (vol/vol) of 20× SSC in ddH₂O (*see Note 3*).
9. 0.1× SSC: 1/200 (vol/vol) of 20× SSC in ddH₂O (*see Note 3*).
10. 0.2× SSC: 1/100 (vol/vol) of 20× SSC in ddH₂O (*see Note 3*).
11. DAPI (4,6-diamidino-2-phenylindole): 1/100 to 1/1000 of DAPI stock solution (stock solution at 0.1 mg/mL in 180mM Tris-HCl, pH 7.5, stored at -20°C) diluted in PBS (*see Note 4*).

2.2.3 Denaturation, hybridization and mounting

1. FISH Hybridization Buffer (FHB): 1/2 (vol/vol) of deionized Formamide, 1/10 (vol/vol) of 20× SSC, 1/5 (vol/vol) of 50% Dextran sulfate solution (made from Dextran sulfate Mw 500,000), sheared Salmon Sperm DNA at final concentration 0.5 mg/mL, volume adjusted in ddH₂O. Store FHB in aliquots at -20°C (*see Note 5*).
2. RNase A stock in 10 mg/mL solutions at -20°C.
3. Probes.
4. Vectashield.

2.3 Materials for Immunostaining (if combined with FISH, see Note 17)

1. PBT: 1/1000 (vol/vol) Tween 20 diluted in PBS.
2. Blocking solution: 2% BSA diluted in PBT.

2.4 Software

1. Primer design: Primer 3 (for the nick-translation based probes, see section 3.1.1)
2. Image analysis: A wide range of software can be used for FISH analysis (see section 3.4)

3. Methods.

3.1 Probe design and production

Choose the genomic region of interest. After selecting the region, different approaches can be used to obtain DNA fluorescent probes. Here, we will use two different methods. The first one consists in amplifying the genomic region of interest using PCR, followed by direct labeling of DNA fragments by nick-translation. The second one uses the Oligopaint technology, developed in the laboratory of C.-T. Wu (Harvard Medical School Boston) and described in [16]. An example of probe design based on Hi-C map and using these two methods is shown in **Fig. 1**.

3.1.1 Probes labeled by nick-translation

Extract the genomic sequence of interest covering 10-12 kb (for *Drosophila* probes) or 20-24 kb (for mammalian probes) and subdivide the sequence into contiguous 2 kb segments, i.e. representing a total of 5-6 fragments for *Drosophila* and 10-12 fragments for mammals (*see Note 6*). It is crucial to

avoid including repeat sequences such as transposable elements. For each segment, design PCR primers using Primer3 software (<http://primer3.ut.ee/>) in order to obtain amplicons of 1.2-1.7 kb, again avoiding repeat sequences by using the appropriate reference genome. After purification, the products can be re-amplified using a second round of PCR in order to increase the concentration, and purified again. PCR fragments are then directly labeled using the FISH Tag DNA kit, starting one given probe with 1µg of total DNA from every PCR fragments pooled in equimolar amount. Follow the procedure describe in the FISH Tag DNA kit protocol (*see Note 7*).

3.1.2 Oligopaint probes

To produce Oligopaint probes, we recommend to follow the procedures described by Beliveau et al. [16,17]. Additional information can be found on the Oligopaint website: <https://oligopaints.hms.harvard.edu/>. Briefly, this technique consists in producing series of DNA oligos composed of a homology genomic sequence targeting the region of interest and a non-genomic “MainStreet” tail labeled with a fluorophore. The MainStreet tail can be targeted with secondary oligos carrying additional fluorophores to increase the fluorescence signal.

3.1.3 Control probes

The very nature of light or, sometimes, the design of the microscope, introduces biases and systematic errors during image acquisition. Depending on the type of the analysis, it can thus be necessary to assess errors such as chromatic aberrations by producing a set of probes labeled simultaneously with different fluorophores and/or produce color-swapped probes (two sets of probes where fluorophores are swapped) (see section 3.4.5). This is more accurate than simply imaging fluorescent labeled beads in mounting medium because the optical path between the control and the actual experiment environments is the same. In the case of nick-translation based probes, the series of the 1.2-1.7 kb fragments of one probe can be alternatively labeled with the different fluorophores, i.e. in a 6-fragments scenario and a 3-color FISH experiments, one color for fragments 1 and 4, another color for fragments 2 and 5, and another color again for fragments 3 and 6. For Oligopaints, secondary oligos labeled with a different fluorophore than the primary one can be used.

[Fig 1 near here]

3.2 FISH procedure

3.2.1 FISH

This protocol is designed for FISH on cells attached on coverslips (*see Note 8*). Individual coverslips are placed within a well of a 6-well plate. For each incubation step, 3 mL (or 4 mL for washes) of the appropriate solution are deposited within the well, and aspirated after the incubation time using vacuum before the next step. Unless specific indications, incubations are performed at room temperature.

1. Prepare coverslips: deposit each coverslip in a well of a 6-well plate, rinse with 100% ethanol, allow coverslips to dry 5-15 min, incubate for 5 min in 0.01% Poly-L-Lysine, and allow coverslips to dry again (*see Note 9*).
2. Deposit coverslips within a humid chamber, i.e. with water deposited in the bottom of the chamber without touching the coverslips (*see Note 10*).

3. Prepare a cell suspension of $\sim 1.5\text{-}2 \cdot 10^6$ cells/ml in growth media and apply 100 μl of the cell suspension in the center of each coverslip. Allow cells to adhere to coverslips for approximately 1 h (*see Note 10*).
4. Take coverslips with tweezers, plunge them briefly in a recipient containing PBS, and deposit them (cells facing up) within wells of a 6-well plate (*see Note 10*).
5. Fix for 10 min in 4% Paraformaldehyde.
6. Wash three times for at least 2 min each in PBS.
7. Incubate for 10 min in 0.5% Triton X-100.
8. Wash three times for at least 2 min each in PBS.
9. Incubate for 10 min in 0.1 M HCl.
10. Wash twice for 2 min each in $2\times$ SSCT.
11. Incubate for at least 30 min in 50% Formamide, $2\times$ SSCT (*see Note 11*).
12. Prepare probe mixture: for nick-translation probes, mix 15-30 ng of each probe with 0.8 μL of RNase A in FHB for a total volume of 15-25 μL (*see Note 12 and 13*); for Oligopaint probes, mix probes (each at $\sim 1\text{-}3 \mu\text{M}$ final concentration) with the same amount of their secondary oligo and 0.8 μL of RNase A in FHB for a total volume of 15-25 μL . For both nick-translation and Oligopaint probes, keep the highest possible ratio of FHB/total volume (*see Note 14*).
13. Use tweezers to take out coverslips from wells, lay the coverslips with cells facing up and drain off the excess of liquid with Kimwipes without touching the cells.
14. Add probe mixtures directly on coverslips.
15. Invert coverslips onto slides, i.e. cells and probe mixture between the coverslip and the slide, and seal coverslips on slides with rubber cement. Allow rubber cement to air-dry about 10-15 min.
16. Denature DNA by putting slides, coverslips facing up, for 3 min at 78°C or 80°C on a heating block immersed in a water bath.
17. Remove slides from the heating block, and deposit them in a dark humid chamber for O/N hybridization (14 to 20 h), at $37\text{-}52^\circ\text{C}$ (*see Note 15*).
18. Use tweezers to peel off rubber cement and deposit coverslips (cells facing up) into wells containing $2\times$ SSC (*see Note 16*).
19. Wash three times for 5 min each at 37°C in $2\times$ SSC by putting the 6-well plate floating within a water bath (*see Note 16*).
20. Wash three times for 5 min each at 45°C in $0.1\times$ SSC by putting the 6-well plate floating within a water bath (*see Note 16*).
21. Wash twice in PBS (*see Note 17*).
22. Incubate for 10 min with DAPI (*see Note 4* for concentrations), with gentle agitation.
23. Wash three times with PBS (*see Note 18*).

24. Use tweezers to take out coverslips from wells, lay the coverslips with cells facing up and drain off the excess of liquid with Kimwipes without touching the cells.
25. Deposit one drop of Vectashield (~12 μL) on the cells.
26. Invert coverslips onto slides, i.e. cells and Vectashield between the coverslip and the slide, and seal coverslips on slides with nail polish. Allow nail polish to air-dry for ~1h (*see Note 19*).
27. Slides can be stored up to 2 weeks in the dark at 4°C before imaging.

3.2.2 FISH combined with Immunostaining

To combine FISH with immunostaining, proceed as following after step n° 21 of section 3.2.1 of the FISH procedure.

1. Wash three times for at least 1 min each with PBT.
2. Incubate in blocking solution for 30-60 min.
3. Use tweezers to take out coverslips from wells, lay the coverslips with cells facing up and drain off the excess of liquid with Kimwipes without touching the cells.
4. Add primary antibody diluted in blocking solution (total volume of ~20 μL) directly on coverslips.
5. Invert coverslips onto slides, i.e. cells and antibody solution between the coverslip and the slide, and incubate for 120 min at room temperature or overnight at 4°C in a dark humid chamber.
6. Wash for 1 min, 3 min and twice 5 min with PBT.
7. Use tweezers to take out coverslips from wells, lay the coverslips with cells facing up and drain off the excess of liquid with Kimwipes without touching the cells.
8. Add secondary antibody diluted in blocking solution (total volume of ~20 μL) directly on coverslips.
9. Invert coverslips onto a slide, i.e. cells and antibody solution between the coverslip and the slide, and incubate for 60 min at room temperature in a dark humid chamber.
10. Wash for 1 min, 3 min and twice 5 min with PBT.
11. Proceed as described in section 3.2.1 from step n° 21 (included).

3.3 Image acquisition

Conventional wide-field microscopy, CLSM, and SR methods such as 3D-SIM can be used to image 3D-FISH experiments. A good image quality with a good resolution and signal-to-noise ratio (SNR) is essential for analysis. Use objectives with the highest numerical aperture (NA), they will optimize both signal and resolution. To maximize the signal, use bright and photo-stable fluorophores. Increase the SNR by increasing exposure time and laser power rather than using detector gain. While doing so, keep under control the eventual photobleaching. The images should use as much as possible of the dynamic range of the camera, avoiding saturated pixels because the correspondence emitted photons and electrons (and therefore between the sample and the intensity) will be lost. To take advantage of the resolution offered by the microscope, the sampling distances in lateral (xy) and axial (z) directions should ideally be less than half the theoretical resolution accordingly to the Nyquist criterion. Most modern microscopes do provide tools to calculate those sampling densities.

1. **CLSM:** Here, CLSM images were acquired with a Leica SP8 microscope equipped with a $\times 63/1.4\text{NA}$ Plan Achromat oil immersion objective and photomultiplier tube and hybrid detectors, for a pixel size of 59 nm and z-step size of 300 nm.
2. **3D-SIM:** To successfully perform 3D-SIM imaging, we strongly recommend to refer to the protocol published by Demmerle et al. [18], which provides comprehensive and detailed guidelines to obtain super-resolution images of quality. Here, 3D-SIM images were acquired with an OMX V4 microscope (GE Healthcare) equipped with a $\times 100/1.4\text{NA}$ PSF Plan Super Achromat oil immersion objective (Olympus), electron-multiplying charge-coupled device (EMCCD) (Photometrics) cameras, for a pixel size of 80 nm and z-step size of 125 nm. Z-stacks were acquired with five phases and three angles per image plane. Raw images were reconstructed using SoftWoRx v6.5 or 7.0 (GE Healthcare), using channel-specific optical transfer functions (pixel size of reconstructed images: 40 nm). Quality of super-resolution reconstructed images were assessed using the SIMcheck plugin [19] on Fiji software [20]. Alignments of colors used for FISH probes was done with Chromagnon [21], using as reference image Oligopaint FISH probe simultaneously labeled with Alexa-488 and ATTO-565 fluorophores (*see section 3.1.3*).

3.4 Image analysis

The following part is a basic guideline for FISH analysis. Quantitative image analysis can be addressed using a variety of software packages. For example, Image J is a widely used, free and open-source software enriched by many plug-ins. Other commercial software packages, such as Imaris or Volocity, allow image analysis with a polished and user-friendly graphic user interface. Programming using for example Matlab, Python or ImageJ's own scripting interfaces offer other advantages such as a high versatility and adaptability to specific aims. All the following steps of this part can be addressed using a wide range of software or programs and do not require particular knowledge in programming for image analysis.

- 3.4.1 **Image pre-processing:** Before starting the analysis, data can first be processed in order to reduce the noise of microscopic images. Image analysis software often proposes different types of filters, such as Gaussian, median or average filters. For example, a 3×3 average filter will replace each pixel value with the neighboring mean in a 3×3 pixel region. This will have a blurring effect on the image and thus reduce the noise, which can facilitate further segmentation processes. It will, however, come at the cost of losing resolution. Other more sophisticated techniques, such as image deconvolution, will increase SNR and resolution at the same time, but a certain experience is necessary to avoid the introduction of artifacts.
- 3.4.2 **Segmentation:** To separate and identify the labeled objects an intensity threshold value is defined in order to obtain a binary image in which pixels will be divided into two classes, one under the threshold corresponding to the background, and one above the threshold corresponding to the labeled objects. To define the threshold, it can be helpful to visualize the grayscale intensity value distribution of the image using histograms. Image analysis software often provides automatic thresholding algorithms (such as Otsu's method) that can be tested in order to obtain satisfying segmentation. However, it may be difficult to obtain acceptable thresholding using an automatic method, and a threshold value may be manually defined. It is important to have a visual feedback to verify the quality of the segmentation. Examples of segmentations using thresholding are shown in **Fig. 2b** and **Fig. 3b**. It can be very useful for FISH analysis to segment DAPI staining in order to define nuclei in which FISH signals will be analyzed (DAPI mask), i.e. FISH signals outside DAPI segmented objects will be automatically discarded for the analysis. This can be particularly helpful if some non-specific residual fluorescent signal remains

outside nuclei. Moreover, it can be useful to identify individual nuclei for further single-cell analysis. Volume range can be defined to discard too small or too large objects, the latest often corresponding to clumped nuclei segmented as one object. However, if nuclei are too crowded, it may be difficult to automatically segment individual nuclei.

- 3.4.3 FISH object identification:** FISH objects can be segmented using intensity-based routines as presented above. Keep in mind that structures of loci of tens to hundreds of kb cannot generally be resolved using conventional wide-field or CLSM microscopy (~200 nm lateral and ~500 nm axial resolution). In this case, segmented objects are larger than the real genomic loci. Loci can thus be defined by the centroids of segmented FISH objects. Local intensity maxima approaches can also be used, which identify high intensity pixels that stand out from the surrounding pixels by more than a defined intensity value. However, if the labeled genomic regions are large enough to be resolved, FISH segmentation can also provide information such as the volume of the regions and their intermingling (number of segmented voxels multiplied by the voxel volume, **Fig. 3c, d**), although, segmented volume may not accurately corresponds to the absolute volume of the real genomic loci (the error increasing with the volume decreasing). Depending on the quality of the FISH probes, the SNR and the efficiency of the intensity-based FISH identification, some nuclei may display false positive signals and, on the other hand, not all nuclei may display FISH objects. If individual nuclei have been segmented, an efficient way to minimize errors is to define a minimum and maximum number of FISH objects per nucleus such that nuclei that do not respect these criteria will be discarded for the analysis. Further data processing can also be used to ensure proper analysis (see the following step).
- 3.4.4 3D distance measurements:** After FISH objects identification, their x , y and z coordinates are extracted. If objects were identified as segmented objects instead of single voxels, these coordinates can be defined by their centroids. The Euclidean distance is then calculated between coordinates of the FISH signals of different colors, allowing for example identification of nearest neighbors using the shortest 3D distance for a given FISH object (**Fig. 2c**). Alternatively, computing the Euclidean distance transform of binary images can provide nearest distances between segmented objects. Keeping only the mutual nearest neighbors between the different channels can also remove outliers. For example, the nearest distance of a FISH object within a nucleus missing the FISH object from the other color may actually represent a distance between FISH objects from different nuclei or unpaired loci. The distance distribution can also inform on the presence of outliers, a distance cutoff can thus be used to remove them. Moreover, in 3-color FISH experiments, having triplets of mutually nearest neighbors allows paired analysis of distances, i.e. distance between one pair of colors compared to the other pairs from the same nucleus (**Fig. 2d**).
- 3.4.5 Control measurements:** Errors such as chromatic aberrations or other biases induced by the different wavelengths of the probes, which can lead to systematic shifts between the different probes, have to be assessed. A way to do so is to perform the same experiment and analysis from control experiments with a set of probes labeled simultaneously with the different fluorophores. This control can be used to correct the positioning of target loci and/or to estimate the error of the measurement and calculate the distance distribution corresponding to a truly co-localized genomic region (**Fig. 2c**).

[Fig 2 near here]

[Fig 3 near here]

4. Notes

1. The thickness of the support of the cells is crucial for optimal imaging, especially with SR microscopy. We recommend to use coverslips of $170 \pm 5 \mu\text{m}$.
2. Triton X-100 is highly viscous. It can be convenient to do an initial 1/10 (vol/vol) solution in ddH₂O before further dilutions.
3. Prepare the SSC buffers according to the chosen washing steps of the FISH procedure (see section **3.2.1** and **Note 16**).
4. For conventional microscopy, we recommend a final DAPI concentration of $0.1 \mu\text{g/mL}$, for 3D-SIM, we recommend to increase up to $1 \mu\text{g/mL}$. When using DAPI, we recommend a relatively fresh batch. DAPI staining is very efficient and may last for many years, however, the quality of a DAPI staining for 3D-SIM is remarkably reduced using aged DAPI.
5. First prepare a 50% Dextran sulfate solution dissolving 5 g of Dextran sulfate with 7 mL of pre-warmed ddH₂O at 60°C (total volume of 10 mL) in a 50 mL falcon tube. Solution can be stored at 4°C .
6. The higher is the number of fragments, the better will be the fluorescent signal. 5-6 PCR fragments in *Drosophila* and 10-12 PCR fragments in mouse generally ensure good signals, although this number may eventually be reduced.
7. The labeling of probes is described in the FISH Tag DNA kit, however, we adapted the nick-translation reaction to optimize using a pool of 1.2-1.7 kb DNA fragments, representing $1 \mu\text{g}$ of total DNA. For this, we use 1.4-1.7 μL of DNase I working solution for a $50 \mu\text{L}$ nick-translation reaction during 90-120 min. We also recommend to first run a time-course test of nick-translation, for which the reaction is performed in the same manner, but $5 \mu\text{L}$ are taken out from the reaction mix every 30 min (from 0 to 120 min). As soon as $5 \mu\text{L}$ aliquots are taken out, vortex well to inhibit DNase I (you can also add EDTA), and freeze at -20°C before running every aliquots in gel electrophoresis. Gels should display smears decreasing gradually in size with time. Smears ranging from approximately 100 to 300 bp represent the optimal condition for the nick-translation reaction. You can also further increase or decrease the amount of DNase I in order to reach this optimal condition for a 90-120 min reaction. At the end of the labeling procedure, the efficiency of dye incorporation will depend on the fluorophore, and good probes should have approximately 5-10 pmol/ μL of A488 dye, 1-3 pmol/ μL of A555 dye, and 4-8 pmol/ μL of A647 dye.
8. Because of the smaller thickness, we recommend to use coverslips rather than slides for optimal imaging (especially for SR microscopy). However, this protocol can also be used with cells attached

to slides. In this case, incubation steps are performed with slides deposited in Coplin jars instead of coverslips deposited in 6-well plates.

9. Alternatively, grow cells on autoclaved and coated coverslips, each coverslip within a well of a 6-well plate.
10. If cells were directly grown in 6-well plates, aspirate the media with a vacuum and rinse with PBS instead of steps 2, 3 and 4.
11. Alternatively, incubate for at least 20 min in 50% Formamide, 2× SSCT, then incubate for 20 min in 50% Formamide, 2× SSCT at 60°C by putting the 6-well plate floating in a water bath. We obtain better results with mammalian cells using this step instead.
12. If you use nick-translation-based probes, denature probes for 10 min at 80°C and put them on ice until usage.
13. If you use mammalian cells and nick-translation-based probes, include within the probe mixture Cot-1 DNA. For example, for mouse cells, use mouse Cot-1 DNA (Invitrogen life technologies, 18440-016) at a final concentration of 0.1-0.2 µg/µL to suppress potential hybridization with repetitive sequences.
14. The higher the FHB/total volume ratio is, the better is the hybridization. A typical experiment uses approximately 2 µL of probes plus 0.8 µL of RNase, which gives a ~8-9/10 ratio for 20 µL of total volume. Do not go lower than a 3/4 ratio of FHB/total volume.
15. The hybridization temperature may vary depending on the probes, with stringency increasing with temperature. We obtain efficient hybridization at 37°C using both nick-translation based probes and Oligopaints with 42-mer genomic sequences in *Drosophila* cells; For Oligopaints, we recommend to refer to [17] to choose the optimal hybridization temperature.
16. This note is an alternative of the 18, 19 and 20 washing steps. Use tweezers to peel off rubber cement and deposit coverslips (cells facing up) into wells containing 2× SSCT. Wash for 15 min at 60°C in 2× SSCT, then wash for 10 min at room temperature in 2× SSCT, then wash for 10 min at room temperature in 0.2× SSC. We obtain better results with mammalian cells using this washing procedure instead.
17. FISH can be followed by immunostaining against proteins of interests (*see* Section 3.2.2). However, the FISH procedure can alter protein integrity and/or epitopes, and immunostaining may not be suitable with some proteins or antibodies.
18. If samples are going to be imaged using 3D-SIM, extensive washing of DAPI is crucial. Wash three times for at least 5 min each instead.
19. Manage the amount of Vectashield so that there is not too much. Clean the excess of mounting medium before sealing.

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Figure Captions

Fig. 1 Example of FISH experimental design to study TAD folding. Hi-C map (visualized with Juicebox [22]) from *Drosophila* S2R+ cells representing a specific chromosomal region along with the location of FISH probes. TADs are highlighted with dashed lines. *P1*, *P2*, and *P3* probes were produced using the nick-translation method starting from 6 PCR fragments each, and were labeled with Alexa-488, Alexa-555 and Alexa-647 fluorophores, respectively. *P1*-*P2* and *P2*-*P3* are at equivalent genomic distances, i.e. 125 kb. Control probes (2 PCR fragments each) were produced by alternatively labeling the PCR fragments of the *P2* region with Alexa-488, Alexa-555 and Alexa-647 fluorophores, respectively. *O1* and *O2* Oligopaint probes were labeled with Alexa-488 and ATTO-565, respectively. On one side, this design with the nick-translation based probes allows studying 3D distances between loci inside TADs versus between loci located in two adjacent TADs (see **Fig. 2**). On the other side, this design with the Oligopaint probes allows studying some physical properties of TADs in single cells, such as their size or the degree of overlap between two adjacent TADs (see **Fig. 3**).

Fig. 2 Example of 3D-FISH analysis using CLSM. **(a)** *P1* (in green), *P2* (in red) and *P3* (in blue) probes were designed to measure 3D distances within a TAD ($d(P1-P2)$) and between two adjacent TADs ($d(P1-P3)$ and $d(P2-P3)$). **(b)** Left, example of CLSM imaging of DAPI (in gray) and FISH probes in S2R+ cells; Right, zoom in the square showing processed image. Segmented object contours are shown (dashed line for DAPI, segmentation using Otsu's method automatic thresholding; colored lines for FISH, segmentation using a defined threshold value from intensity distribution and visual inspection), and circles indicate centroids of segmented FISH objects (mutual nearest neighbors linked to each other with white lines). Maximum intensity projections are shown. Scale bar: 2 μm . **(c)** Histograms and distribution fits of mutual nearest neighbor 3D distances (from 130 nuclei). On top right, mutual nearest neighbor 3D distance distributions between the different control probes (*P2a*, *P2b*, and *P2c* for Alexa-488, Alexa-555, and Alexa-647, respectively, from 49 nuclei). The differences in distance distribution observed in the actual experiments (*P1*, *P2* and *P3* probes) are not observed with the control probes, indicating that they are not due to technical bias. *** $P < 0.0001$ with Kruskal-Wallis and Dunn's multiple comparisons tests. **(d)** Single-cell paired distance analysis, with the *P1-P2* distance compared to the *P2-P3* distance from each triplets of mutual nearest FISH neighbors. The *P1-P2* distance is shorter than the *P2-P3* distance in 85% of the analyzed nuclei. This analysis indicates that the probes located in the same TADs are closer compare to probes located into adjacent TADs in a large majority of single-cells, although they are at the same genomic distance.

Fig. 3 Example of 3D-FISH analysis using 3D-SIM. **(a)** *O1* (in green) and *O2* (in magenta) probes were used to label two adjacent TADs. **(b)** Examples of 3D-SIM imaging of the probes with their segmentation (Otsu's method automatic thresholding) contour in S2R+ cells. Maximum intensity projections are shown, scale bar: 500 nm. **(c)** Super-resolution 3D-SIM allows the volume quantification of the segmented probes (from 38 nuclei). **(d)** Volume overlap fraction (using the Jaccard index) between *O1* and *O2* probes. This analysis illustrates the physical partitioning of the genome into TADs in *Drosophila* cells.





