Chapter 1

The Relationship Between Human Nucleolar Organizer Regions and Nucleoli, Probed by 3D-ImmunoFISH

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Abstract

3D-immunoFISH is a valuable technique to compare the localization of DNA sequences and proteins in cells where three-dimensional structure has been preserved. As nucleoli contain a multitude of protein factors dedicated to ribosome biogenesis and form around specific chromosomal loci, 3D-immunoFISH is a particularly relevant technique for their study. In human cells, nucleoli form around transcriptionally active ribosomal gene (rDNA) arrays termed nucleolar organizer regions (NORs) positioned on the p-arms of each of the acrocentric chromosomes. Here, we provide a protocol for fixing and permeabilizing human cells grown on microscope slides such that nucleolar proteins can be visualized using antibodies and NORs visualized by DNA FISH. Antibodies against UBF recognize transcriptionally active rDNA/NORs and NOP52 antibodies provide a convenient way of visualizing the nucleolar volume. We describe a probe designed to visualize rDNA and introduce a probe comprised of NOR distal sequences, which can be used to identify or count individual NORs.

Key words 3D-immunoFISH, Nucleolus, Nucleolar organizer region (NOR), Ribosomal genes, Human acrocentric chromosomes

1 Introduction

Nucleoli form around arrays of ribosomal gene (rDNA) repeats that are transcribed by RNA polymerase I, to produce the major RNA components of ribosomes, 18S, 28S, and 5.8S ribosomal RNAs. In humans, these arrays, termed nucleolar organizer regions (NORs), range in size from 50 kb to >6 Mb and are positioned on the short or p-arms of each of the five acrocentric chromosomes HSA13-15, HSA21, and HSA22 [1]. During cell division, the nucleolus disappears and NORs can be observed as achromatic gaps on DAPI-stained metaphase chromosomes. These chromosomal features are sometimes referred to as 2° constrictions. Unlike the highly condensed heterochromatic 1° constrictions, centromeres, the rDNA in 2° constrictions is under-condensed compared to the surrounding heterochromatin. Upstream binding factor (UBF), a nucleolar-specific HMG box protein that binds extensively

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across the rDNA array is directly responsible for this morphology [2, 3]. In many or possibly most human cell types, a proportion of NORs fail to show this morphology. In these silent NORs, the rDNA is condensed and was not transcriptionally active in the previous interphase and will not be active in the subsequent interphase [4]. These silent NORs lack UBF and will not contribute to nucleolar formation. Upon reactivation of transcription in anaphase, nucleoli begin to form around individual active NORs [5]. Then in most cell types, these small nucleoli will fuse producing the characteristic large nucleoli observed in most human cells.

Nucleoli are subdivided into three distinct components reflecting the stages of ribosome biogenesis [6]. Fibrillar centers (FCs) contain unengaged pools of transcription factors and nontranscribed rDNA sequences. rDNA transcription occurs at the interface between FCs and the associated dense fibrillar component (DFC) where early processing of resulting pre-rRNA takes place. Late processing and ribosome assembly occurs in the surrounding granular component (GC).

Combining fluorescent in situ hybridization (FISH) with antibody staining on cells in which three-dimensional structure is preserved, 3D-immunoFISH, is a powerful tool for examining the intimate relationship between NORs and nucleoli [3, 7, 8]. In principle, it allows one to distinguish active from silent NORs. It is possible to count the individual NORs associated with each nucleolus. Finally, using 3D-immunoFISH, alterations in NOR activity and organization can be visualized in cells as they respond to changes in growth status, drug treatments, or genetic manipulation. In this chapter, we describe DNA probes, antibodies, and protocols for performing 3D-immunoFISH to study the relationship between NORs and nucleoli in human cells.

The 3D-immunoFISH procedure we use is similar to a previously described protocol (*see* ref. [9]). It consists of four essential steps: (1) preparation of slides, (2) preparation and validation of probes, (3) hybridization of slides, and (4) antibody staining.

To visualize nucleoli, we commonly use antibodies against UBF or NOP52. UBF is an FC/DFC protein that associates with active rDNA repeats and NORs [10, 11]. In contrast, NOP52, also known as RRP1, is a GC protein involved in pre-rRNA maturation [12]. It provides a convenient marker for delineating the extent of the nucleolar volume (Fig. 1a).

The human rDNA repeat is approximately 43 kb in length, 13 kb of which are transcribed to produce pre-rRNA and a 30 kb intergenic spacer (IGS) [13]. To visualize rDNA by FISH, we use a 12 kb EcoR I restriction fragment that is positioned in the IGS immediately upstream of the gene promoter (Fig. 1b). As these sequences are nontranscribed, we can be certain that we are visualizing rDNA as opposed to rRNA. This rDNA FISH probe can readily identify individual NORs on metaphase spreads and reveal the variation in their rDNA content. However, it cannot distinguish individual NORs within

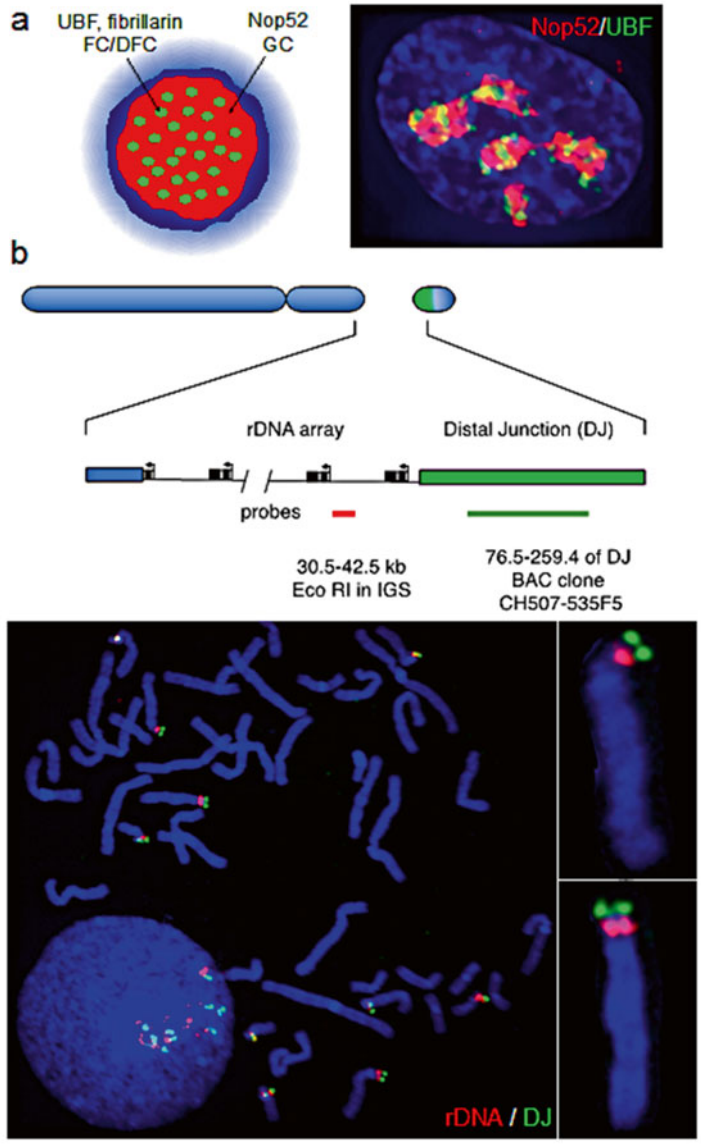

Fig. 1 Antibodies and DNA probes used in probing the relationship between NORs and nucleoli by 3D-immunoFISH. (**a**) Nucleolar fibrillar centers (FC) and dense fibrillar components (DFC) are typically not resolved in the light microscope and can be stained with antibodies against UBF or fibrillarin, resulting in a punctate staining pattern within the nucleolar interior. Antibodies against NOP52, located in the granular component (GC), provide a convenient way of visualizing the nucleolar volume. Staining of an individual human nucleolus is shown in *cartoon* form on the *left*, note the perinuclear heterochromatin (*dark blue*). An individual DAPI-stained human nucleus containing five nucleoli visualized with UBF and NOP52 antibodies is shown on the *right*. (**b**) rDNA is visualized using a 12 kb EcoRI DNA fragment that lies immediately upstream of the gene promoter in the nontranscribed intergenic spacer. This fragment represents sequences between 30.5 and 42.5 kb of the rDNA repeat described in GenBank Accession U13369. Sequences immediately distal to rDNA arrays are referred to as the distal junction (DJ). These sequences are virtually identical on all five human acrocentrics. BAC clone CH507-535F5 contains the sequences that lie between 76.5 and 259.4 kb distal to the rDNA array. FISH using rDNA and DJ probes performed on a normal human male metaphase chromosomes spread is shown below a cartoon representation of the probes. Enlarged individual acrocentric chromosomes are shown on the *right*

interphase nucleoli. The identification of sequences immediately distal to rDNA arrays and the demonstration that these are virtually identical on each acrocentric p-arm, offers a unique opportunity for visualizing and counting NORs [7]. Thus far nearly 400 kb of rDNA distal sequences, distal junction (DJ), has been identified. BAC clone CH507-535F5 (GenBank: CT476834) covers the interval between 76.5 and 259.4 kb distal to rDNA (Fig. 1b). Probing of metaphase spreads with this DJ BAC results in signal that is consistent between NORs (Fig. 1b). Application of this DJ probe to interphase cells can therefore easily enumerate NORs associated with individual nucleoli. DJ sequences associated with active NORs are usually associated with perinucleolar heterochromatin, visualized by DAPI staining (Fig. 2a). Silent NORs are typically observed as isolated DJ signals dissociated from nucleoli. By employing fluorescently labeled secondary antibodies, nucleoli, rDNA, and DJ can be visualized within the same cell (Fig. 2b, c). BACs derived from the long or q-arms can be used to identify each individual acrocentric chromosomes (*see* ref. 7).

2 Materials

The list of materials that we present here is cumulative. Within each section, we list only those materials that are required for the first time.

2.1 Preparation of Slides

1. Superfrost® Plus Microscope slides (Menzel-Glaser) (*see* **Note 1**).
2. QuadriPerm (Sarstedt) or 150 mm cell culture dishes (*see* **Note 2**).
3. The 3D-immunoFISH experiments described here were performed on hTERT-RPE1 cells [14] (ATCC) maintained in DMEM/Nutrient Mixture F-12 Ham containing 2 mM L-Glutamine, 10% v/v FBS, and 0.25% v/v sodium bicarbonate.
4. 10× and 1× Phosphate buffered saline (PBS).
5. Fixative: 4% w/v paraformaldehyde (PFA) in PBS; 4 g in 100 mL PBS, heat at 60 °C until dissolved, then cool to room temperature. Prepare freshly on the day.
6. Rocking platform.
7. Permeabilization buffer: 0.5% w/v saponin, 0.5% v/v Triton X-100 in 1× PBS; 0.5 g saponin and 0.5 mL Triton X-100 in 100 mL PBS. Filter sterilize with a 0.45 μm syringe filter.
8. 20% v/v Glycerol in 1× PBS.
9. Liquid nitrogen.
10. Plastic slide boxes.

2.2 Preparation and Validation of Probes

1. BAC clone CH507-535F5 (GenBank: CT476834) obtained from the BACPAC Resource Center, Children's Hospital Oakland Research Institute in Oakland, California, USA (*see* **Note 3**).

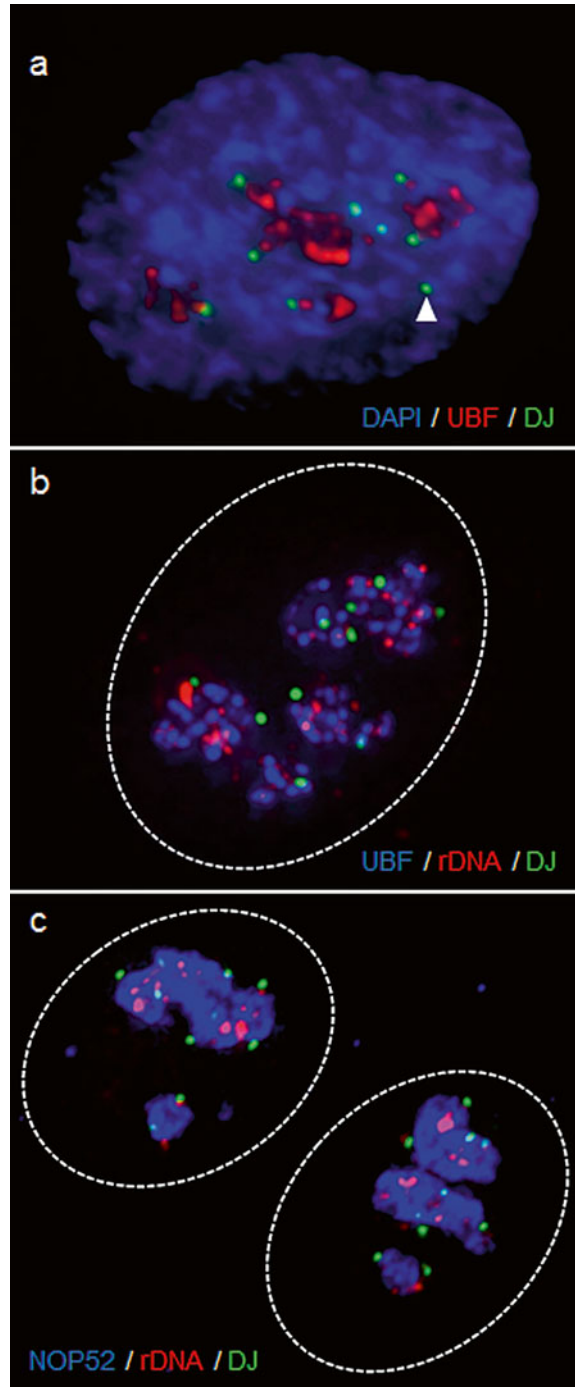

Fig. 2 3D-immunoFISH performed on hTERT-RPE1 cells. (**a**) An individual DAPI-stained cell probed with UBF antibodies and DJ FISH probe. Note that DJ signals are associated with DAPI dense perinucleolar heterochromatin. A silent NOR, lacking UBF signal, is indicated by an *arrowhead*. (**b**) An individual cell probed with UBF antibodies and both rDNA and DJ FISH probes. The nucleus is outlined. (**c**) Two hTERT-RPE1 cells probed with NOP52 antibodies and both rDNA and DJ FISH probes. Note how NOP52 antibodies more clearly delineate the nucleolar volume than UBF antibodies

2. Human rDNA plasmid pUC-hrDNA-12.0: A plasmid containing a 12.0 kb EcoRI fragment that corresponds to sequences between 30.5 and 42.5 kb of the human rDNA repeat (GenBank: U13369) (*see* **Note 4**).
3. Nick translation kit, Green dUTP, and Red dUTP (Abbott Molecular 07J00-001, 02N32-050, and 02N34-050, respectively).
4. Aluminum foil.
5. Dryblocks at 15 and 37 °C.
6. Waterbaths (ranging from 42 to 75 °C).
7. 1 mg/mL Human Cot-1 DNA (Applied Genetics, Hybloc™ Competitor DNA, HHB) (*see* **Note 5**).
8. 10 mg/mL Herring or Salmon sperm DNA.
9. 3 M sodium acetate, pH 5.5.
10. 100, 85, and 70% Ethanol.
11. Hybridization buffer, Hybrisol® VII (MP Biomedicals, 11RIST139010).
12. Human Male Metaphase Slides (Applied Genetics, HMM).
13. Coplin Jars (*see* **Note 6**).
14. Formamide, ACS grade deionized with Amberlite® mixed bed ion exchange resin (Merck) (*see* **Note 7**).
15. 20× SSC: 88.2 g sodium citrate tribase dihydrate, 175.3 g sodium chloride, adjust the pH to 7 with HCl and adjust the volume to 1 L.
16. M-FISH Denaturation buffer: 70% v/v deionized formamide, 2× SSC.
17. Coverslips, 24 mm × 40 mm.
18. Rubber cement (Marabu-Fixogum).
19. Humidity chamber at 37 °C (*see* **Note 8**).
20. Single edge razor.
21. M-FISH Wash buffer I: 0.4× SSC, 0.3% NP-40.
22. M-FISH Wash buffer II: 2× SSC, 0.1% NP-40.
23. VectorShield mounting medium +/− DAPI (4,6-diamidino-2-phenylindole, Vector Laboratories).
24. Nail varnish.
25. Fluorescent microscope, camera, and image capture/analysis software package (*see* **Note 9**).

2.3 Hybridization to Fixed Cells on Slides

1. Formamide, molecular biology grade.
2. 0.1 N HCl.
3. 2× SSC.

4. 3D-FISH Equilibration buffer: 50% v/v molecular biology grade formamide, 2× SSC.
5. Dry slide incubator capable of heating slides to 73 °C for denaturation (*see* **Note 10**).
6. 3D-FISH Wash I: 50% v/v deionized formamide, 2× SSC.
7. 3D-FISH Wash II: 0.1× SCC.

2.4 Antibody Staining

1. Primary antibodies recognizing UBF and/or Nop52 (*see* **Note 11**).
2. 1% w/v BSA in 1× PBS: 0.1 g BSA (IgG free) in 10 mL PBS.
3. Fluorophore (Fluorescein FITC, Rhodamine, or Coumarin AMCA) conjugated whole IgG secondary antibodies (Jackson Immuno Research).

3 Methods

3.1 Preparation of Slides

1. Open a box of Superfrost Plus slides in a tissue culture hood and place slides, frosted side up, in either quadriPERM (1 slide per well) or 150 mm (up to five slides) cell culture dishes, add media, and seed with logarithmically growing cells (*see* **Note 12**).
2. When cells reach 50–70% confluence, replace culture media with the same volume of fixative and incubate for 10 min at room temperature on a rocking platform.
3. Pour off fixative, add the same volume of PBS and incubate for 10 min at room temperature on a rocking platform. This wash step should be repeated a further two times.
4. Pour off the final PBS wash solution, replace with the same volume of permeabilization buffer and incubate for 10 min at room temperature on a rocking platform.
5. Wash the slides three times in PBS as in **step 3**.
6. Pour off the final PBS wash solution and incubate the slides in 20% glycerol in 1× PBS for 2 h at room temperature on a rocking platform. Alternatively, the slides can be incubated overnight at 4 °C.
7. Snapfreeze the slides in liquid nitrogen. Slides can be stored in plastic slide boxes at −80 °C indefinitely.

3.2 Preparation and Validation of Probes

1. Label 1 μg of BAC or plasmid DNA with Green dUTP or Red dUTP by nick translation in a 50 μL reaction according to the manufacturers (Abbott Molecular) protocol. Labeling reactions are protected from light by aluminum foil and incubated at 15 °C for 8 h and terminated by heat inactivation at 70 °C for 10 min. Labeled DNA can be stored at −20 °C in the dark indefinitely.

2. For hybridization to normal human male metaphase chromosome slides, combine 100 ng BAC DNA (5 μL of labeling reaction) and/or 50 ng rDNA (2.5 μL of labeling reaction) with 2.5 μL Human Cot-1 DNA and 5 μL Herring Sperm DNA. Add one-tenth volume of 3 M sodium acetate, pH 5.5 and precipitate the DNA by addition of 2.5 volumes of 100% ethanol. Following washing with 70% ethanol and brief drying, resuspend the DNA pellet in 25 μL Hybrisol® VII by incubation at 37 °C with intermittent mixing.
3. Denature normal human male metaphase chromosome slides by incubating for 5 min in 40–50 mL of M-FISH Denaturation buffer in a Coplin jar sitting in a 75 °C water bath.
4. After denaturation, remove slides and dehydrate by incubating for 1 min in 70% ethanol, then 1 min in 85% ethanol and finally 1 min in 100% ethanol. Air-dry slides.
5. Denature the DNA probe mix in Hybrisol® VII by incubating at 75 °C for 5 min. Apply the denatured probe immediately to a coverslip placed on a 37 °C heating block. Invert the denatured chromosome slide and lower onto the coverslip/probe, holding it in position until the meniscus of the hybridization mix reaches the edge of the coverslip. Turn the slide the right way up and seal the edges of the coverslip with rubber cement.
6. Incubate slides at 37 °C in a humidity chamber for between 16 and 48 h (*see* **Note 13**).
7. After hybridization, remove coverslips and rubber cement using a single edge razor blade. Immediately immerse slides in M-FISH Wash buffer I in a Coplin jar equilibrated to 73 °C in a water bath. Agitate each slide after immersion for 2–3 s and let stand for 2 min.
8. Remove the slide to M-FISH Wash buffer II at room temperature. Agitate each slide after immersion for 2–3 s and let stand for 1 min. Remove slides and air-dry in darkness.
9. Place a drop of Vectashield plus DAPI onto a coverslip. Lower the inverted slide onto it, as before. Remove excess mounting media and seal coverslips using nail varnish (preferably clear).
10. Image slides using an appropriate fluorescent microscope, camera, and image capture/analysis software package (Fig. 1b) (*see* **Note 9**).

3.3 Hybridization to Fixed Cells on Slides

1. Prepare hybridization mix containing labeled probe, human Cot-1 DNA, and Herring sperm DNA exactly as described above (*see* Subheading 3.2, **step 2**) (*see* **Note 14**).
2. Remove slides of fixed and permeabilized cells (*see* Subheading 3.1, **step 7**) from the −80 °C freezer and rinse three times in PBS.

3. Depurinate the slide by placing in a Coplin jar containing 0.1 N HCl for 5 min. Then wash in 2× SSC.
4. To pre-equilibrate the slide, prior to applying the probe and denaturing, remove the slide from the 2× SSC, shake off excess liquid and dry the back and sides of the slide with absorbent tissue. Pipet 250 μL of 3D-FISH Equilibration buffer onto the center of the slide, loosely apply a coverslip and incubate for 15 min in humidity chamber at 37 °C. Care must be taken here that the fixed cells on the slide surface do not dry out.
5. Just prior to the end of the pre-equilibration period, apply the hybridization mix to a coverslip that has been placed on a 37 °C heating block.
6. Remove the coverslip from the pre-equilibrating slide, shake off excess liquid and lower the inverted slide onto the hybridization mix. Again care must be taken here that the cells do not dry out.
7. Seal the coverslip using rubber cement, allowing it to harden by placing the slide (right way up) on the 37 °C heating block for a few minutes.
8. Denature the slide, now containing the hybridization mix, by placing it in a dry slide incubator set at 73 °C for 12 min.
9. After denaturation remove the slide to a humidity box and incubate at 37 °C for between 16 and 48 h (*see* **Note 13**).
10. After hybridization, remove the coverslip and rubber cement and wash the slides in a Coplin jar, three times for 5 min each, with 3D-FISH Wash I at 42 °C.
11. Place the slides contained within a Coplin jar on a rocking platform at room temperature, and wash three times for 5 min each with 3D-FISH Wash II preheated to 60 °C.
12. After the final hybridization wash, prepare the slides for antibody staining by washing three times (5 min each) in PBS.

3.4 Antibody Staining

1. Dilute the primary antibody in 200 μL 1% BSA/PBS at an appropriate dilution and pipet onto the center of the slide. Loosely apply a coverslip. Incubate the slide for 1 h in humidity chamber at 37 °C. Care must be taken here that the fixed cells on the slide surface do not dry out.
2. Remove the coverslip and wash the slide in PBS at room temperature three times for 10 min each.
3. Dilute the fluorophore-conjugated secondary antibodies in 200 μL 1% BSA/PBS (1/200 for FITC and Rhodamine-labeled secondary antibodies and 1/100 for AMCA) and pipet onto the center of the slide. Loosely apply a coverslip. Incubate the slide for 40 min in humidity chamber at 37 °C.
4. Remove the coverslip and wash the slide in PBS at room temperature three times for 10 min each.

5. Place a drop of Vectashield with or without DAPI onto a coverslip. Lower the inverted slide onto it, as before. Remove excess mounting media and seal coverslips using nail varnish (preferably clear).
6. Image slides using an appropriate fluorescent microscope, camera, and image capture/analysis software package (Fig. 2) (*see* **Notes 9** and **15**).

4 Notes

1. SuperFrost® Plus glass slides are made by a process that places a permanent positive charge on standard microscope slides. This forms a bridge so that covalent bonds develop between formaldehyde fixed cells and the glass.
2. In QuadriPerm (Sarstedt) dishes, parallel cell culture on up to four slides can be performed. This is useful for comparing various treatments on cells. 150 mm cell culture dishes can be used to grow cells on up to five slides at a time in the same conditions.
3. BAC DNA is prepared from 800 mL cultures using a Nucleobond Xtra Maxi kit (Macherey Nagel) and following the manufacturer's instructions for low-copy number plasmids.
4. Although direct labeling of the rDNA containing plasmid works in 3D-immunoFISH, we prefer to label gel purified insert.
5. Both the human rDNA IGS and the DJ BAC probes contain repetitive elements, such as *alu* repeats, found elsewhere in the genome. It is therefore essential that hybridization signals from these be suppressed by inclusion of Cot-1 DNA [7, 13].
6. We prefer polypropylene Coplin jars with lids (Fisher 10082844) to glass.
7. Add 5 g of mixed bed resin to 1 L of ACS grade formamide in a glass beaker and mix on a magnetic stirrer for 30 min. Then filter through a 0.2 μM bottle top filter into a brown glass bottle.
8. Slides are placed within sealed plastic food storage boxes with clip-on lids and containing water soaked absorbent tissue to maintain humidity. Sealed boxes are placed in a standard 37 °C incubator.
9. In our opinion, the major determining factors for producing high quality 3D-immunoFISH images are the slide processing, quality of the probes and antibodies used. Images can be captured using a basic fluorescent microscope and imaging software. Currently, we use a Photometric® Coolsnap HQ camera and Volocity 6 imaging software (PerkinElmer) with a 63× Plan Apochromat Zeiss objective mounted on a Zeiss Axioplan2 imaging microscope. Typically we capture Z-stacks of fluorescent images, 30–40 slices for interphase cells and ~5 for meta-

phase spreads. Images are deconvolved by iterative restoration in Volocity 6 with a confidence limit of 95% and a maximum of 50 iterations. In most cases, extended focus projections of deconvolved Z-stacks are presented (*see* Fig. 2).

10. We use a TruTemp DNA MicroHeating System with heating block capable of holding up to 16 slides, originally supplied by Robbins Scientific. While this is discontinued, other systems that could substitute include CytoBrite and CytoBrite Duo Slide incubation systems (SciGene) and ThermoBrite Programmable Temperature Controlled Slide Processing System (Abbott Molecular).
11. While we typically use our own antibodies raised in sheep against recombinant UBF and Nop52, there are many commercial UBF antibodies available and we have good experience with Nop52 antibodies supplied by Novus (NBP1-85338). Examples of other antinucleolar antibodies that work in 3D-immunoFISH can be found in other publications from this laboratory [2, 3, 8, 15].
12. The protocol we describe here works in every established adherent human cell line that we have tested thus far, ranging from primary, to hTERT immortalized and cancer cell lines. Care should be taken using cancer cell lines as they often have complex poorly defined karyotypes. While cells in metaphase are rounded up and make minimal contact with the surface of the slide, they can still be observed if care is taken in slide handling.
13. For repetitive probes, such as rDNA, 16 h hybridization is sufficient. For low-copy number probes, such as the DJ BAC, signals are improved by longer hybridization times, up to 48 h.
14. In the protocol described here, probe and cellular DNA are denatured together in the sealed slide. Accordingly, separate denaturation of the probe mix is not required.
15. Antibody staining is usually performed after the hybridization. However in some cases, determined empirically, it is preferable to perform antibody staining prior to depurination and hybridization. In this case cells are stained with both primary and secondary antibodies. The antibodies are then fixed with 2%PFA/PBS prior to depurination and hybridization as outlined above (*see* Subheading 3.3).

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