Chapter 2

Analysis of the *C. elegans* Nucleolus by Immuno-DNA FISH

Christian Lanctôt

Abstract

Caenorhabditis elegans is a well-established model organism which allows, among others, to investigate the link between nucleolar structure/function on the one hand and cell fate choices and cellular differentiation on the other. In addition, *C. elegans* can be used to study the role of the nucleolus in processes that can be difficult to faithfully reproduce in vitro, such as gametogenesis, disease development, and aging. Here I present two complementary techniques, immunofluorescent staining and DNA fluorescence in situ hybridization, that have been adapted to label nucleolar components at various stages of the life cycle of the worm.

Key words *Caenorhabditis elegans*, Nucleolus, Immunofluorescence, DNA FISH, Embryogenesis

1 Introduction

Caenorhabditis elegans is a small nematode that is well established as a premier model organism for biomedical research. However, it is fair to say that so far *C. elegans* has not been widely used to study the biology of the nucleolus. Yet, the worm offers a well-characterized system to investigate the various aspects of nucleolar functions, especially the link between nucleolar activity and cell fate choices/differentiation [1]. In addition, as a whole organism, *C. elegans* allows one to study nucleolar structure and function in processes that can be difficult to faithfully reproduce in culture, such as gametogenesis, disease development, and aging [2, 3]. Furthermore, the model is easily amenable to genetics and cell biology approaches. For instance, it was recently found that mutations in the *C. elegans* ortholog of the Nopp140 nucleolar protein lead to abnormal gonadal development and oogenesis [4]. Similarly, depletion of RBD-1, an homolog of the yeast RNA-binding protein Mrd1p, was found to be associated with abnormal processing of pre-rRNA, growth retardation, and larval arrest in the worm [5]. *C. elegans* was also used in an elegant study to show that the embryonic nucleoli form through condensation of a fixed number of nucleolar components when these reach a certain threshold concentration, an assembly which the authors modeled based on the

Attila Németh (ed.), *The Nucleolus: Methods and Protocols*, Methods in Molecular Biology, vol. 1455,
DOI 10.1007/978-1-4939-3792-9_2,

physics of phase transition [6]. In the present paper, I describe how to use immunostaining combined with DNA fluorescence in situ hybridization (FISH) to investigate the *C. elegans* nucleolus.

2 Materials

2.1 Preparation of Samples

1. Microscope slides, 76 mm × 26 mm × 1 mm, precleaned, twin frosted end.
2. Coverslips, 18 mm × 18 mm, standard no. 1, and high quality no. 1.5.
3. Filter paper: e.g., no. 1 circles (cat. 1001 150), cut in quadrants.
4. Diamond pen.
5. Razor blade.
6. Metal block in dry ice: a block from a dry bath (e.g., 10 cm × 8 cm) is turned upside down, half-buried in pellets of dry ice and stored at −80 °C.
7. Gelatin, from porcine skin.
8. Chrome alum.
9. 1 M sodium azide (NaN_3), *caution*, this is a toxic substance.
10. Poly-L-lysine (hydrobromide, M_w > 300,000 g/mol, Sigma-Aldrich).
11. PLL solution (*see* **Note 1**): heat 40 mL of ddH_2O to 60 °C. Dissolve 0.08 g of gelatin. Cool the solution to 37 °C. Add 0.008 g of Chrome Alum. Mix well. Add 40 µL of 1 M NaN_3 (final concentration is 1 mM). Dissolve 40 mg of poly-L-lysine (final concentration is 1 mg/mL). Transfer to a 50-mL tube. Store at 4 °C.
12. 10 N NaOH.
13. Sodium hypochlorite solution ("bleach," available chlorine contents 4.00–4.99 %, Sigma-Aldrich).
14. M9: dissolve the following in 200 mL of ddH_2O: 0.6 g of KH_2PO_4 (anhydrous, final concentration 22 mM), 1.495 g of $Na_2HPO_4 \cdot 2H_2O$ (42 mM), 1 g of NaCl (86 mM). Add 0.2 mL of 1 M $MgSO_4$ (1 mM). Sterilize on 0.22 µm filter (optional). Store at room temperature.
15. Egg Buffer: dissolve the following in 450 mL of ddH_2O: 3.448 g NaCl (final concentration 118 mM), 1.789 g KCl (48 mM), 0:147 g $CaCl_2 \cdot 2H_2O$ (2 mM), 0.203 g $MgCl_2 \cdot 6H_2O$ (2 mM), 2.979 g HEPES (M_w 238.3 g/mol, 25 mM). Adjust pH to 7.3 with 1 N NaOH (no more than 10 mL). Complete to 500 mL with ddH_2O. Sterilize on 0.22 µm filter (optional). Store at room temperature.
16. 100 mM levamisole-HCl (in water, Sigma-Aldrich), *caution*, this is a toxic substance.

2.2 Immuno-fluorescent Staining

1. 1× PBS.
2. 10× PBS.
3. Tween-20™, polyoxyethylene-sorbitan monolaurate.
4. Coplin jars.
5. Silicon isolator rings, Press-to-seal Gasket, 13 mm diameter, 2.5 mm depth (Electron Microscopy Sciences Inc.).
6. Methanol, in a Coplin jar, at –20 °C.
7. Acetone, in a Coplin jar, at –20 °C.
8. 70, 50 and 30% acetone, (v/v) in ddH_2O, precooled at 4 °C in Coplin jars.
9. 4% formaldehyde: mix 26 mL of ddH_2O, 4 mL of 10× PBS and one vial (10 mL) of 16% paraformaldehyde solution, EM Grade (Electron Microscopy Sciences Inc.).
10. PBST (PBS-0.02% Tween): 10 µL of Tween-20™ in 50 mL of 1× PBS.
11. PBST-BM: dissolve 0.05 g bovine serum albumin fraction V and 0.05 g dry milk (low fat) in 10 mL of PBST.
12. Primary antibodies.
13. Secondary antibodies coupled to fluorophores, e.g., goat anti-mouse IgG, Alexa Fluor 488 conjugate (Life Technologies) or goat anti-rabbit IgG, Cy3 conjugate (Jackson Immunoresearch).
14. 0.5 mg/mL DAPI, in 1× PBS, Sigma-Aldrich.
15. Vectashield mounting medium, Vector Laboratories.
16. Nail polish.
17. Microbeads stained with multiple fluorophores (e.g., TetraSpeck™ from Life Technologies).
18. Wide field or confocal fluorescence microscope.

2.3 DNA FISH

1. 5 U/µL Taq DNA polymerase, or equivalent.
2. 10× Taq enzyme buffer.
3. 1.5 mM dTTP, in water.
4. 2 mM dAGC: dATP, dGTP, dCTP, 2 mM each (in water).
5. 1 mM Cy3-dUTP, GE Healthcare Amersham.
6. Forward primer, 25 µM, 5′-TTGTGCAAGCGGCCGAGGTC-3′.
7. Reverse primer, 25 µM, 5′-AGACTCAAGCGCCTCGACGC-3′.
8. PCR cycler.
9. Ion-exchange columns for the purification of DNA, e.g., Zymoclean™, QiaQuick™, or equivalent.
10. Nano-spectrophotometer.
11. 0.1 N HCl: add 0.44 mL of concentrated (11.3 N) HCl to 49.5 mL of ddH_2O.

12. 0.01 N HCl: add 5 mL of 0.1 N HCl to 45 mL of ddH_2O.
13. 20× SSC: add 17.5 g of NaCl (3 M) and 8.8 g of sodium citrate tribasic dehydrate (0.3 M) to 80 mL of ddH_2O. Adjust the pH to 7 with 1 N HCl. Complete to 100 mL with ddH_2O.
14. 2× SSC.
15. 0.2× SSC.
16. 10 mg/mL RNAse A, in water, store at −20 °C.
17. Formamide, molecular biology grade (deionized).
18. 2× SSC/50% formamide: add 5 mL of 20× SSC to 20 mL of ddH_2O. Add 25 mL of molecular biology-grade (deionized) formamide.
19. Hybridization chambers: 13 mm diameter, 20 μL volume (Electron Microscopy Sciences Inc.).
20. 2× Hyb Buffer: weigh 1 g of dextran sulfate (Sigma-Aldrich) in a 15 mL tube. Add 4× SSC to 5 mL. Vortex and rotate to dissolve completely. Make 0.5 mL aliquots and store at −20 °C.

3 Methods

3.1 Preparation of Samples

Worms are easily and cheaply maintained according to standard procedures [7]. At 20 °C, *C. elegans* goes from embryo to adult in 3–4 days, passing through four larval stages [8]. The animals are transparent and their typical lifespan is on the order of 15–30 days, depending on the culture conditions. The vast majority of adults are self-fertilizing hermaphrodites. A number of well-maintained websites cover various aspects of the worm's biology: anatomy (http://www.wormatlas.org/), strains (http://cbs.umn.edu/cgc/home), genomics (www.wormbase.org), general (www.wormbook.org), to name just a few.

3.1.1 Treatment of Microscope Slides

Although the procedures described here can be performed in solution, I prefer to stain samples that are first affixed to a microscope slide. This ensures a better penetration of reagents and, to some extent, a better preservation of the sample. However, it is crucial to treat the slides in order to promote adhesion of the worms. The importance of this step cannot be overstated, especially if working with adult worms. Indeed, partial or even complete loss of the sample is a frequent problem when using the protocol described here. To minimize this problem, slides are usually coated with high molecular weight poly-L-lysine, which provides a positive surface to which the negatively charged cuticle of the worm can attach (*see* **Note 2**).

1. Bring the poly-L-lysine solution (PLL) to room temperature.
2. Place two precleaned microscope slides back to back, dip in the PLL solution, and incubate 2 min.

3. Remove the slides from the PLL solution and place them upright against a support. Leave to dry at least 20 min, protected from dust.
4. Use on the same day (*see* **Note 3**).

3.1.2 Isolation of Early Embryos

C. elegans embryos develop in the uterus until shortly after gastrulation (~25–30-cell stage). The easiest way to obtain a large number of embryos is therefore to dissolve the hermaphrodite body in conditions that minimally affect the embryos. This is done by a brief incubation in a NaOH/bleach solution, followed by several washes in a neutral buffer.

1. Collect healthy young adult worms in ddH_2O and transfer to a 15 mL tube. Let the worms settle by gravity. Wash once with 10 mL of ddH_2O.
2. Resuspend the worm pellet in a total of 3.75 mL of ddH_2O.
3. Mix 0.25 mL 10 N NaOH and 1 mL of sodium hypochlorite solution. (This mix must be made fresh). Add to the worm solution. Vortex briefly.
4. Incubate at room temperature until the worms are no longer visible with the naked eye (usually 5–7 min). Vortex briefly every other minute.
5. Centrifuge 30 s at 1200 × *g*. Quickly remove the supernatant and add 5 mL of Egg Buffer to the pellet. Vortex briefly.
6. Repeat **step 5** three times.
7. After the last wash, leave ~50 µL/60 mm petri of worms above the egg pellet. If needed, let embryos grow at room temperature until they reach the desired stage (e.g., ~60 min to get to the ~200-cell stage).

3.1.3 Freeze-Crack

The *C. elegans* embryonic egg shell and larval/adult cuticle are largely impermeable. To be able to stain internal structures, it is necessary to break open and tear away part of the egg shell or cuticle. This is done by freezing the sample under a glass coverslip and then popping off the coverslip in order to rip up, so to speak, the egg shell/cuticle [9]. Please note that this step takes some practicing before the optimal "tearing" conditions can be reproducibly generated.

1. If starting with isolated embryos, please go to **step 4**.
2. Collect worms in M9 and transfer to a 15 mL tube. Spin 30 s at 200 × *g* at room temperature. Wash two times with 5 mL of M9 and once in ddH_2O (*see* **Note 4**).
3. After the last wash, leave ~50 µL of supernatant/60 mm petri of worm. Add 0.5 µL of 100 mM levamisole. Mix gently.
4. Label a PLL-treated slide and, with a diamond pen, gently draw a circle of approximately 1 cm in diameter *on the reverse side*, in the center. This slight etching helps to locate the sample in subsequent steps.

5. Using a tip rinsed in M9 + 0.002 % (v/v) Tween-20™ (*see* **Note 5**), pipette 25 µL of the embryo/worm solution inside the circle onto a PLL-treated slide.
6. Using forceps, cover gently with an 18 mm × 18 mm coverslip. Working at low magnification under the stereomicroscope, adsorb the liquid using a filter paper until the worms/embryos are slightly compressed between the slide and the coverslip (*see* **Note 6**).
7. Place the slide on a metal block in dry ice. Incubate at −80 °C for at least 30–60 min.
8. Holding the slide almost vertically, wedge a razor blade under the corner of the coverslip and pop it off. Take care not to thaw the slide before popping.
9. Proceed immediately to fixation.

3.2 Immunofluorescence Staining

Although a large collection of transgenic strains that express a variety of GFP fusion proteins is now available to the *C. elegans* research community [10], immunostaining remains a powerful technique due to its ability to reveal multiple antigens simultaneously and its high sensitivity when compared to GFP-based labeling. It is estimated that up to 80 % of human genes have orthologs in *C. elegans* [11]. Unfortunately, due to the relatively limited homology of proteins between these two species, typically around 40 %, many of the antibodies that were raised against mammalian antigens fail to detect their counterpart in the worm. It is therefore advisable to use antibodies raised against *C. elegans* proteins, many of which can be searched for in Wormbase (www.wormbase.org) and obtained directly from *C. elegans* researchers or from the Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/). If a *C. elegans*-specific reagent cannot be located, then it is worth testing several antibodies against the target protein to find one that reacts against the worm protein. Figure 1 shows examples of an embryo and a young larva immunostained for both a nucleolar protein (DAO-5) and a germ line marker (PGL-1). These results indicate that the formation of the nucleolus is delayed in the embryonic germ line, thus highlighting how *C. elegans* can be used to reveal unique features of nucleolar biology in vivo.

3.2.1 Fixation

The fixation conditions have to be optimized depending on the target protein(s) and the extent of structural preservation that is wished for. I have found that fixation with acetone followed by progressive rehydration usually provides the most intense immunofluorescent signal and satisfactorily preserves intracellular structures, especially in L4 larvae and adult worms. However, it should be kept in mind that formaldehyde fixation is preferred if one is to perform DNA FISH after immunostaining, since acetone-fixed material does not withstand well the FISH procedure.

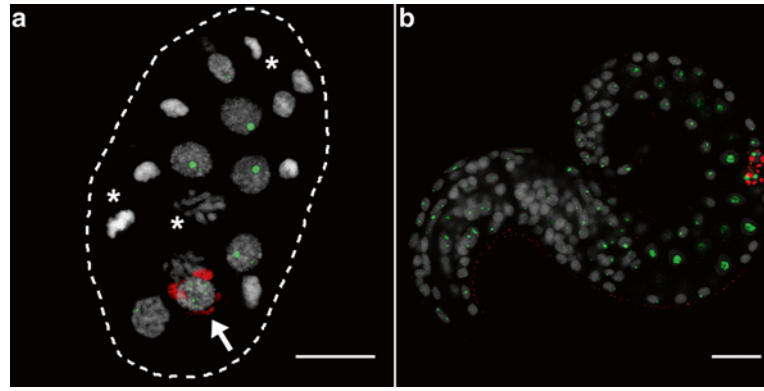

Fig. 1 Nucleologenesis is delayed in the *C. elegans* embryonic germ line. Samples were immunostained with an antibody against DAO-5 (*green*), a Nopp140 homolog, and PGL-1, a marker of germ line cells (*red*). DNA was counterstained with DAPI (*gray*). Scale bar, 10 μm. (**a**) In the 24-cell embryo nucleoli have formed in somatic cells, but not in the single germ cell precursor (*arrow*), in which the DAO-5 signal is seen as ~10–15 *small dots* found throughout the nucleoplasm. Note that this embryo contains numerous mitotic cells (some labeled with *asterisks*), none of which display nucleoli. The contour of the embryo is indicated by a *dotted line*. Shown here is only a cross-section of the embryo (4 μm in thickness). (**b**) In the L1 larva, the two germ cell precursors (*red*) show prominent DAO-5-labeled nucleoli

1. After freeze-crack and removal of the coverslip, immediately fix embryos in cold 100% methanol, 2 min at −20 °C.
2. Transfer the slide(s) to acetone at −20 °C and fix a further 5 (for embryos) or 10 min (for larvae and adults).
3. Incubate the slides successively in precooled 70% acetone, 50% acetone, and 30% acetone, 1 min each at 4 °C (*see* **Note 7**) (*see* **Note 8** if DNA FISH is to be performed after immunostaining).
4. Rinse slides in PBS. Wash 3 min in fresh PBS at room temperature.

3.2.2 Staining

From this point on, all incubations and washes are performed at room temperature unless otherwise stated. These steps are performed in small volumes (~200–500 μL) pipetted in a well that is created around the sample by gluing a silicon ring adaptor on the slide. Solutions should not be squirted directly onto the samples but rather dispensed on the side of the well.

1. Remove the slides one at a time from PBS. Carefully wipe out the liquid around the sample, i.e., outside the etched circle. Glue a 13 mm silicon ring adaptor around the sample. Press firmly using a second microscope slide. Immediately pipette 500 μL of PBS in the well thus created. Repeat for every slide.
2. Permeabilize the sample by incubating for 5 min in PBST (*see* **Note 9**).

3. Wash 3 min in PBS.
4. Dilute the primary antibody(ies) in 200 µL PBST-BM/sample (*see* **Note 10**).
5. Pipette the primary antibody solution onto the samples. Place the slides in a humidified chamber and incubate 2 h at room temperature or overnight at 4 °C.
6. Wash two times 3 min with 500 µL of PBST.
7. Dilute the secondary antibody(ies) in 200 µL PBST-BM, typically 1/400 (*see* **Note 11**).
8. Pipette the secondary antibody solution onto the samples. Place the slides in a humidified chamber and incubate 2 h at room temperature or overnight at 4 °C.
9. Wash two times 3 min with 500 µL PBST.
10. Wash once 3 min with 500 µL PBS.
11. If DNA FISH is to be performed, fix the immunocomplexes using 2 % formaldehyde in PBS (10 min), wash twice with PBS, and proceed to Subheading 3.3.2.
12. Counterstain with a 1/500 dilution of DAPI (final concentration of 1 µg/mL) for 2 min.
13. Rinse with 500 µL PBS. Remove the silicon ring adaptor. Mount in Vectashield. Use high quality 18 mm × 18 mm no. 1.5 coverslips. Seal with nail polish. Store slides at 4 °C until and between observations. Proceed to Subheading 3.4.

3.3 DNA FISH

While in some cases immunostaining is sufficient to reveal nucleolar structure, in others it is necessary to combine this technique with DNA FISH in order to highlight the position of the rDNA repeats in the nucleus. For instance, using immuno-DNA FISH, we have found that nucleolar proteins such as DAO-5 and FIB-1 label extranucleolar structures in the early *C. elegans* embryo (Fig. 2), the identity of which remains to be determined [12]. There is a single array of about 50 rDNA repeats (the *rrn* genes) in the *C. elegans* genome [13]. It is located at the end of chromosome I and encodes the 18S, 5.8S, and 26S rRNA molecules [14].

3.3.1 Probe Synthesis

Since the rDNA sequence is repeated many times in the genome, a short fragment that is synthesized and labeled by PCR can serve as an efficient FISH probe against rDNA. In the following protocol, a 390-bp fragment encompassing nt 172–561 upstream of the 18S rRNA sequence is amplified from genomic DNA and labeled with Cy3-dUTP.

1. Prepare the following reaction.

C. elegans genomic DNA	200 ng
Forward primer	1 µL (final 500 nM)
Reverse primer	1 µL (final 500 nM)
2 mM dAGC	5 µL (final 200 µM)
1.5 mM dTTP	4.3 µL (final 130 µM)
1 mM Cy3-dUTP	3.25 µL (final 65 µM) (*see* **Note 12**)
10× Taq Buffer	5 µL
H_2O	complete to 49.5 µL

2. Program the following PCR.

   94 °C, 3 min (initial denaturation).

   25 cycles [94 °C 30 s, 58 °C 30 s, 72 °C 30 s].

   72 °C 10 min (final elongation).

3. Hot start the PCR by adding 2.5 U (0.5 µL) of Taq DNA polymerase.
4. Analyze a 2.5 µL aliquot of the PCR reaction (1/20) on an agarose gel.
5. If a single band is visible (*see* **Note 13**), purify the rest of the probe on an ion-exchange column (Zymoclean™, QiaQuick™ or equivalent). Elute in 50 µL of elution buffer.

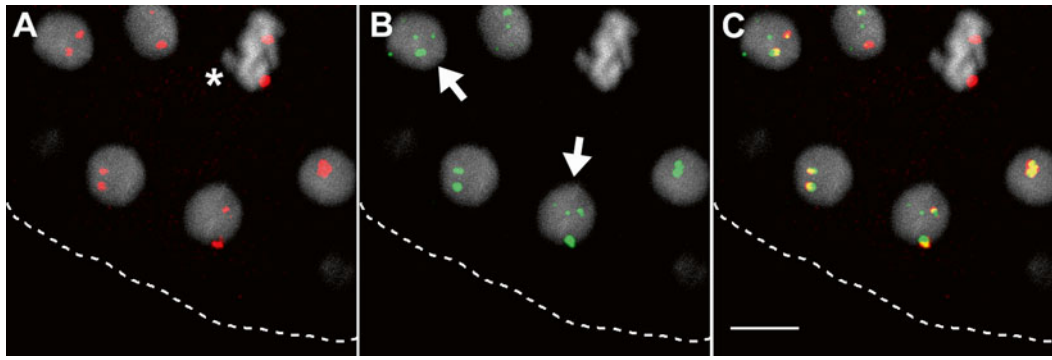

Fig. 2 Immuno-DNA FISH can be used to identify nucleoli. Samples were first immunostained with an antibody against DAO-5 and then hybridized with a labeled DNA probe against the first intervening sequence (ITS1) of *C. elegans* rDNA. Shown here is a cross-section (4 µm in thickness) of part of a ~40-cell embryo. The *dotted line* indicates the egg shell. Scale bar, 10 µm. (**a**) The DNA FISH signal (*red*) appears as two distinct dots in each nucleus, including in mitotic cells (*asterisk*). (**b**) The DAO-5 signal (*green*) localizes to rDNA spots, but is also found in ~4–5 *smaller dot*-like structures throughout the nucleoplasm. The *arrows* point to nuclei in which these structures are clearly seen. (**c**) Merged image showing only partial colocalization of the immunofluorescence (DAO-5, *green*) and DNA FISH (rDNA, *red*) signals

6. Assay the probe concentration by nano-spectrophotometry. Store the probe at –20 °C.

3.3.2 DNA FISH

1. Wash 2 min with 500 µL PBS.
2. Rinse once in 0.01 N HCl.
3. Incubate *precisely 2 min* in 0.1 N HCl at room temperature.
4. Wash once with PBS, then with 2× SSC, each time for 2 min.
5. Treat with 50 µg/mL RNAse A in 2× SSC, 45 min at 37 °C.
6. Remove the silicon ring adaptor.
7. Transfer the slides to a Coplin jar filled with 2× SSC.
8. Incubate in 2× SSC/50% formamide for at least 2 h.
9. Dilute the probe to a concentration of 2 ng/µL in molecular biology-grade (deionized) formamide (12 µL per slide). Add an equal volume of 2× Hyb. The final probe concentration is 1 ng/µL.
10. Mix well by pipetting and vortexing. Spin 20 s in a tabletop centrifuge to remove air bubbles. Keep the probe at room temperature.
11. Pipette 20 µL of the probe solution into a hybridization chamber.
12. Take a slide out of the 2× SSC/50% formamide solution. Gently wipe off excess liquid around the sample using a soft paper.
13. Invert the sample onto the hybridization chamber prepared in **step 11**. Press firmly to seal the chamber.
14. Repeat **steps 11–13** for each slide.
15. Prehybridize the slides at 37 °C overnight (*see* **Note 14**).
16. Denature the probe and the genomic DNA simultaneously by leaving the slides for 3 min upright on a heating block set at 76 °C.
17. Incubate at 37 °C for 2 days.
18. Fill four Coplin jars with 2× SSC. Preheat three of those in a water bath at 37 °C. Fill two other Coplin jars with 0.2× SSC and preheat at 55 °C.
19. Using the tip of a scalpel or a razor blade, gently lift and remove the hybridization chamber.
20. Place the slide in a Coplin jar filled with 2× SSC at room temperature.
21. Wash three times 5 min in 2× SSC at 37 °C. During these and subsequent washes, the slides should occasionally be gently pulled out of the solution and plunged back in.
22. Wash two times 5 min in 0.2× SSC at 55 °C.
23. Counterstain with a 1/500 dilution of DAPI (final concentration of 1 µg/mL) for 2 min. Pipette 500 µL of the DAPI dilution onto the sample.

24. Rinse with 500 μL of 2× SSC. Mount in Vectashield. Use high quality 18 mm × 18 mm no. 1.5 coverslips. Seal with nail polish. Store slides at 4 °C until and between observations.

3.4 Imaging

The labeled samples can be imaged on a wide field or confocal fluorescence microscope. We routinely image *C. elegans* samples on a Leica TCS SP5 scanning confocal microscope equipped with a 63× oil immersion objective (numerical aperture 1.4). The distance between individual optical sections (*z* interval) is set at 400 nm and the *xy* pixel size, at ~80 nm. In the case of multicolor imaging experiments designed to assess the extent of colocalization of two different nucleolar or cellular components, care should be taken to correct for the chromatic aberration that is introduced by the optical system (if any). This can be done by imaging microbeads stained with multiple fluorophores (e.g., TetraSpeck™ from Life Technologies), measuring the distance between the focal points for the different fluorophores and using this measure to align image stacks.

4 Notes

1. The poly-L-lysine solution should be stored at 4 °C. It is stable for at least 2–3 months and can be reused a number of times. However, it should be made fresh once a decrease in the adhesion of samples to the slides is noticed.
2. Microscope slides can also be treated with a silane derivative to increase adhesiveness (e.g., aminoethylamine-propyltrimethoxysilane APTS). However, in my experience, this treatment is less efficient than the one with the poly-L-lysine solution.
3. To provide even greater adhesiveness, a small drop of the PLL solution (~20 μL) can be pipetted in the center of the slide, which is then placed on a hot plate (~75 °C) and left there until the PLL solution has evaporated. The spot of evaporated PLL should be very sticky.
4. It is important to thoroughly wash out the bacteria and to resuspend the worms in water, otherwise they will not stick as well to the PLL-treated slide.
5. Worms and embryos tend to stick to plastic tips. To prevent loss of material, the tips are first rinsed in a solution that contains traces of detergent (0.002% v/v) before being used to pipette worms or embryos.
6. This is a critical step. The coverslip should touch the worms and embryos, but not compress them to the point that these are grossly deformed.

7. The rehydration solutions should be cooled to 4 °C in ice or in the refrigerator before use. I have found this precooling to be very important in order to avoid detachment of the samples from the slides.
8. Alternatively, the slides can be rinsed in cold (4 °C) PBS after the incubation in methanol, transferred to cold (4 °C) 4% formaldehyde, and fixed for 10 min *at room temperature*. As earlier, the use of precooled solution is essential to prevent loss of material.
9. Permeabilization with low concentrations of Tween-20™ is sufficient in most cases. If needed, a more thorough permeabilization can be achieved using PBS supplemented with 0.2% (v/v) Triton X-100.
10. The optimal dilution must be determined empirically. The typical range is 1/20 to 1/400.
11. The immunofluorescent signal afforded by strong fluorophores such as Alexa Fluor 488 or Cy3 generally withstands well a subsequent FISH procedure. However, if a higher sensitivity is required, the secondary antibody can be coupled to a biotin moiety instead of a fluorophore. In this case, the immunocomplexes should be labeled after DNA FISH using streptavidin or avidin fluorescent conjugates.
12. The ratio of Cy3-dUTP to dTTP is 1:2. A higher ratio is not recommended.
13. Cy3-labeled double stranded DNA does not bind SyBr Green efficiently, which means that only the quality, but not the quantity of the PCR product can be assessed by agarose gel electrophoresis.
14. Hybridization can be carried out on a hybridization plate. Alternatively, slides can be placed in a small, flat-bottom metal container, which is left to float for the duration of the hybridization in a water bath set at 37 °C.

Acknowledgements

I gratefully acknowledge the financial support of the Czech Science Foundation (grant P305/12/1246). This work was also supported by Charles University in Prague (PRVOUK P27/LF1/1). The Imaging Center at the institute is supported by the European Regional Development Fund (OPPK CZ.2.16/3.1.00/24010).

References

1. Lee LW, Lee CC, Huang CR et al (2012) The nucleolus of *Caenorhabditis elegans*. J Biomed Biotechnol 2012:601274
2. Baumeister R, Ge L (2002) The worm in us - *Caenorhabditis elegans* as a model of human disease. Trends Biotechnol 20:147–148

3. Gonzalez-Aguilera C, Palladino F, Askjaer P (2014) *C. elegans* epigenetic regulation in development and aging. Brief Funct Genomics 13:223–234
4. Lee CC, Tsai YT, Kao CW et al (2014) Mutation of a Nopp140 gene *dao-5* alters rDNA transcription and increases germ cell apoptosis in *C. elegans*. Cell Death Dis 5:e1158
5. Saijou E, Fujiwara T, Suzaki T et al (2004) RBD-1, a nucleolar RNA-binding protein, is essential for *Caenorhabditis elegans* early development through 18S ribosomal RNA processing. Nucleic Acids Res 32:1028–1036
6. Weber SC, Brangwynne CP (2015) Inverse size scaling of the nucleolus by a concentration-dependent phase transition. Curr Biol 25:641–646
7. Stiernagle T (2006) Maintenance of *C. elegans*. In: Community TCeR (ed) WormBook, doi: 10.1895/wormbook.1.101.1, pp 1–11
8. Corsi AK, Wightman B, Chalfie M (2015) A transparent window into biology: a primer on *Caenorhabditis elegans*. Genetics 200:387–407
9. Strome S, Wood WB (1982) Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 79:1558–1562
10. Sarov M, Murray JI, Schanze K et al (2012) A genome-scale resource for in vivo tag-based protein function exploration in *C. elegans*. Cell 150:855–866
11. Lai CH, Chou CY, Ch'ang LY et al (2000) Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. Genome Res 10:703–713
12. Korčeková D, Gombitová A, Raška I et al (2012) Nucleologenesis in the *Caenorhabditis elegans* embryo. PLoS One 7:e40290
13. Albertson DG (1984) Localization of the ribosomal genes in *Caenorhabditis elegans* chromosomes by in situ hybridization using biotin-labeled probes. EMBO J 3:1227–1234
14. Ellis RE, Sulston JE, Coulson AR (1986) The rDNA of *C. elegans*: sequence and structure. Nucleic Acids Res 14:2345–2364

http://www.springer.com/978-1-4939-3790-5

The Nucleolus
Methods and Protocols
Németh, A. (Ed.)
2016, XI, 279 p. 55 illus., 43 illus. in color., Hardcover
ISBN: 978-1-4939-3790-5
A product of Humana Press