Chapter 32

Automated 3D Gene Position Analysis Using a Customized Imaris Plugin: XTFISHInsideNucleus

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Abstract

Fluorescence in situ hybridization (FISH) is commonly used to visualize chromosomal regions or genomic loci within the nucleus, and can largely contribute to unraveling the link between structure and function in the nucleus. Three-dimensional (3D) analyses are required to best capture the nucleus' organizing principles, but the experimental setup and computational analyses are far from trivial. Here, we present a robust workflow for 3D FISH against repeats and single copy loci in embedded intact nuclei from Arabidopsis leaves. We then describe in detail the image acquisition, subsequent image deconvolution before 3D image processing, and the image reconstruction. We developed an automated batch image processing pipeline using a customized, open source plugin implemented in the Imaris environment.

Key words Fluorescence in situ hybridization, 3D FISH, Confocal imaging, Deconvolution, Automated image processing, 3D gene position, Imaris

1 Introduction

The nuclei of plant and animal cells at interphase share several organizing principles. This includes the presence of microscopically visible heterochromatic domains composed of genomic regions enriched in transcriptionally repressive chromatin modifications, and the occurrence of discrete chromosome territories in animal [1, 2] and plant [3–5] nuclei. Studies on yeast and animal model systems have shown that the nucleus has a functional organization. Notably, the position of certain genes relative to different nuclear compartments correlate with their expression status [6]. This suggested an influence of the 3D arrangement of chromatin on genome expression [7]. For instance, in mammalian cells, gene-rich chromatin tends to cluster away from the nuclear periphery and from the nucleolus. Active genes are also found on chromatin loops that protrude away from chromosome territories [6]. In yeast, the relocation of loci toward the nuclear periphery leads to gene silencing [8]. However, much less is known in plants. Yet a recent study

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on Arabidopsis reported the relocation of specific loci in response to light stimuli, which correlated with the induction of their expression [9].

In order to uncover whether plant nuclei also follow specific organizing rules with respect to gene position in the nucleus and expression, it is necessary to develop robust methods for probing and analyzing gene position in intact, three-dimensional (3D) nuclei. Here, we present a step-by-step protocol to generate and interpret data pertaining to the gene's position with regard to the nucleus periphery, the nucleolus and the chromocenters.

Fluorescence in situ hybridization (FISH) protocols have been developed in the past decades to allow the detection of repeat elements or chromosomal regions (using ~100 kb BAC probes) on isolated Arabidopsis nuclei. However, 3D FISH has proven difficult and only a few studies report successful applications in plants using whole-mount tissues [10]. In addition, single copy gene detection is also known as a challenging process likely due to the low density of fluorophores along the probes generated with classical labeling techniques. Oligo-painting [11] or padlock-type probes allow amplification of the target sites of immunolabeling [9], thereby increasing the signal. Here, however, a protocol is shown that uses a traditional nick translation-mediated incorporation of modified nucleotides and indirect immunolabeling that permits single copy gene detection. The probe template consists of a mix of PCR amplicons spanning a region of approximately 30 kb, thereby being large enough for high-confidence signal detection and allowing a specific location of targeted gene. The success of this method also relies on the embedding of isolated nuclei in a polyacrylamide matrix to preserve their 3D structure, while providing a sufficient optical clarity for high-resolution imaging. Image processing includes signal deconvolution, 3D reconstruction and threshold-based detection of signal maxima. For these steps we used the Huygens image deconvolution software (Huygens, Netherlands) and the 3D visualization and image processing software Imaris (Bitplane, CH), but alternative software packages are available. In addition, in order to analyze the position of FISH signals relative to the nuclear periphery, chromocenters and nucleolus, we developed a customized plugin implemented in Imaris. The methodology described in this chapter is summarized in the flowchart in Fig. 1.

2 Materials

General material for all DNA-FISH sections: 1.5 mL microcentrifuge tubes, microcentrifuge with temperature control, shaker, thermomixer, 15 mL tubes, and flat-end forceps.

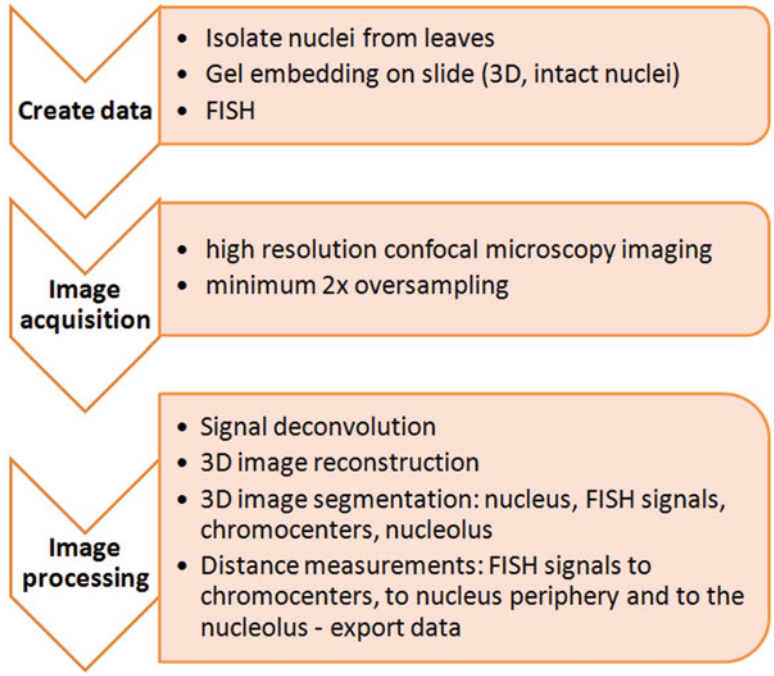

Fig. 1 3D gene position analysis flowchart

2.1 Nuclei Isolation

1. Arabidopsis plants (*see* **Note 1**).
2. Small petri dishes of about 3 cm diameter.
3. Large glass petri dish of about 80 mm × 15 mm.
4. 10× PBS:

   Solution A: Dissolve in 900 mL: 16.02 g Na_2HPO_4, H_2O and 73.84 g NaCl.

   Solution B: Dissolve in 200 mL: 2.76 g Na_2HPO_4, H_2O and 16.56 g NaCl.

   Adjust pH of Solution A to 7.0 with Solution B.
5. Buffer A: 4% Formaldehyde, 0.01% Triton in PBS.
6. Buffer B: 45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.3% Triton X-100. Adjust to pH 7 using 0.1 N HCl (*see* **Note 2**).
7. 30 μm cell strainers.
8. Eppendorf tubes.
9. 2 mL Dounce tissue grinder set with large and small clearance pestles.
10. Single-edge carbon steel razor blade.

11. DAPI (4′,6-diamidino-2-phenylindole) dissolved in antifade mounting medium (Vectashield or similar), 1.5 μg/mL.
12. Fluorescence microscope with epifluorescence illumination.

2.2 Gel Embedding

1. Buffer C: 30% acrylamide, 3.3% bisacrylamide in PBS (*see* **Note 3**).
2. 20% sodium sulfite (NaS) in sterile ddH_2O.
3. 20% ammonium persulfite (APS) in sterile ddH_2O.
4. Superfrost microscope slides.

2.3 Probe and Slide Preparation

1. Phusion DNA polymerase with 5× Phusion buffer or equivalent.
2. 10 mM dNTPs.
3. Specific primers for the region of interest.
4. Thermocycler.
5. 70% EtOH.
6. 100% EtOH.
7. 3 M NaOAc.
8. 0.5 M EDTA pH 8.0.
9. 10 mL 0.5 M sodium phosphate buffer pH 7.0: 5.77 mL Na_2HPO_4 0.5 M and 4.23 mL NaH_2PO_4 0.5 M; check pH and adjust by adding some extra Na_2HPO_4 or NaH_2PO_4 if necessary.
10. Fluorimetric-based nucleic acid quantification device (*see* **Note 4**).
11. 20× SSC: 3 M NaCl, 0.3 M Na-citrate, adjust to pH 7 with 5 M HCl.
12. Digoxigenin (DIG) and/or Biotin (BIO) nucleic acid labeling kit.
13. HB50: 50% deionized formamide, 0.05 M sodium phosphate buffer pH 7, in 2× SSC (*see* **Note 5**).
14. DS: 20% dextran sulfate in HB50 (*see* **Note 5**).
15. FB: 1% formaldehyde in PBS (*see* **Note 6**).
16. 2.5 mg/mL Pepsin stock.
17. 0.1 M HCl stock (*see* **Note 7**).
18. Coplin jars.

2.4 Hybridization and Immunodetection

1. Heating blocks for microscope slides.
2. Digoxigenin (DIG) and/or Biotin (BIO) nucleic acid labeling kit.
3. HB50: 50% deionized formamide, 0.05 M sodium phosphate buffer pH 7, in 2×SSC (*see* **Note 6**).

4. Blocking buffer: 5% BSA, in 4× SSC (*see* **Note 8**).
5. 10× TN: 1 M Tris–HCl pH 7, 1.5 M NaCl.
6. TNB: 0.5% blocking reagent in 1× TN (*see* **Note 8**).
7. Primary antibodies: Mouse anti-DIG and Biotinylated Anti-Avidin (*see* **Note 9**).
8. Secondary antibodies: Alexa Fluor 488-coupled anti-mouse antibody and Avidin coupled with Texas-Red (*see* **Note 9**).
9. SF50: 50% deionized formamide in 2×SSC.
10. 4T: 0.05% Tween 20 in 4× SSC, pH 7.
11. TNT: 0.05% Tween 20 in 1× TN, pH 7.
12. FB: 1% formaldehyde in PBS (*see* **Note 6**).
13. Programmable hybridization incubator for microscope slides (*see* **Note 10**).
14. DAPI (4′,6-diamidino-2-phenylindole) dissolved in antifade mounting medium (Vectashield or similar), 1.5 μg/mL.
15. Transparent nail polish.

2.5 Image Acquisition and Processing

1. Confocal laser scanning microscope ideally equipped with a resonance scanner, new generation hybrid detectors and a 63× glycerol immersion, confocal-grade lens with chromatic and planar aberration corrections.
2. System Requirements: OS: Windows 7 or higher 64 bit or Mac 10.9 or higher (*see* **Note 11**).
3. Image deconvolution software (e.g., Huygens Professional, SVI, Netherlands).
4. Imaris (Bitplane AG, Switzerland).
5. Python 2.7 or higher (https://www.python.org/downloads/).
6. Imaris plugin: XTFISHInsideNucleus.py available on the open source Imaris repository (http://open.bitplane.com/tabid/235/Default.aspx?id=131).

3 Methods

3.1 Nuclei Isolation

Here, intact nuclei are isolated, while preserving their three-dimensional (3D) structure. The nuclei isolation protocol described in this section follows the procedure originally described in [12], with slight variations.

1. Prepare a fresh solution of Buffer A and keep it on ice.
2. Collect 5–6 healthy rosette leaves from Arabidopsis plants.
3. Place them in a small petri dish containing 10 mL Buffer A, make sure that all leaves are submerged.

4. Incubate with gentle shaking for 20 min at 4 °C
5. Rinse the leaves twice with PBS, remove the solution after the last wash.
6. Place the leaves in a large glass petri dish on ice (*see* **Note 12**).
7. Add immediately 200 µL Buffer B and chop finely with a razor blade, rapidly for about 30 s.
8. Repeat **step 7** three times. Make sure you get a fine and homogenous suspension.
9. Transfer the suspension to a clean Dounce tissue grinder (*see* **Note 13**).
10. Grind with the large clearance pestle, by moving it gently up and down (five times maximum) (*see* **Note 13**).
11. Repeat **step 10** with the small clearance pestle (five times maximum) (*see* **Note 13**).
12. Filter the homogenate through two superimposed 30 µm cell strainers into a sterile 1.5 mL Eppendorf tube.
13. Centrifuge at 500 × *g* for 5 min at room temperature.
14. Discard the supernatant.
15. Add 100 µL of Buffer B, and gently resuspend the pellet by pipetting up and down with a cut-end tip.
16. Keep on ice.
17. Verify the quality of the preparation: add a 5 µL drop on a clean microscope slide. Add 5 µL of DAPI Vectashield. Add a coverslip and inspect with epifluorescence microscopy. The nuclei should show regular contours and chromocenters should be well visible.
18. Keep the rest of the suspension on ice. If the preparation quality is good, proceed immediately to gel embedding.

3.2 Gel Embedding

In this step, the nuclei are immobilized while their 3D structures are preserved. The protocol is scaled to capture ca 100 nuclei on a single slide, thus allowing to multiplex downstream FISH or immunostaining experiments. The nuclei extraction allows the preparation of 15 slides. In our hands, embedding in acrylamide pads on microscope slides gives better results than dried nuclei on slides. Acrylamide preparation and embedding is based on a protocol originally established in the Bass lab [13], and also shown in a video protocol [14]. Minor variations were introduced for FISH.

1. Place 15 clean microscope slides horizontally on a clean surface.
2. Prepare fresh solutions of 20% APS in ddH_2O and 20% NaS in ddH_2O.

3. In a 1.5 mL microcentrifuge tube add the following reagents on ice:

   166 μL PBS.

   34 μL Buffer C.

   70 μL nuclei suspension (use a 200 μL cut-end-tip).

   12 μL 20% APS.

   12 μL 20% NaS.

4. Mix gently by pipetting up and down with a 200 μL cut-end tip.
5. Add 15 μL of the sample solution to the center of each microscope slide with a cut-end tip.
6. Wait for 30 s before placing 18 × 18 mm cover slips.
7. Slides can be stored at 4 °C for 1 month, in dry, vertical racks.

3.3 Probe Preparation

We experienced robust signals with probes spanning 30 kb of a single-copy region. Here, the DNA probes are detected by indirect immunostaining of the modified nucleotides using commercially available antibodies. Alternative protocols use direct DNA labeling avoiding immunodetection. In our hands, however, the direct labeling did not yield reproducible results, while indirect labeling allowed for robust signals.

The FISH procedure essentially follows original protocols [15] with minor modifications. PCR amplification of the 30 kb DNA template was performed using the high-fidelity Phusion enzyme. Alternative probe labeling and detection may be used instead of this one (*see* Chapter 25 [16]).

The following proportions are set for probe preparation for one slide/one hybridization.

1. Set up the PCR reaction as described by the manufacterer (https://www.neb.com/protocols/1/01/01/pcr-protocol-m0530):

Component	20 μL reaction	Final concentration
Nuclease-free water	To 20 μL	
5× Phusion HF or GC buffer	4 μL	1×
10 mM dNTPs	0.4 μL	200 μM
10 μM forward primer	1 μL	0.5 μM
10 μM reverse primer	1 μL	0.5 μM
Template DNA	Variable	<250 ng
DMSO (optional)	(0.6 μL)	3%
Phusion DNA polymerase	0.2 μL	1.0 units/50 μL PCR

2. Run the PCR using the following program:

Initial denaturation	→	98 °C for 30 s
Number of cycles	→	25–35
Denaturation	→	98 °C for 5–10 s
Annealing	→	45–72 °C for 10–30 s
Extension	→	72 °C for 15–30 s per kb
Final extension	→	72 °C for 5–10 min
Hold	→	4–10 °C

3. Use 2 μL to verify that a specific amplicon is produced with 1% agarose gel electrophoresis.
4. Measure the DNA concentration of the PCR product using a fluorimetric-based nuclei acid quantification device, then calculate the volume needed for 900 ng (*see* **Note 4**).
5. If the PCR reaction does not yield ~1 μg, scale up to 50 μL reactions.
6. Transfer 900 ng into a fresh tube, add sterile standard ddH_2O to a final volume of 30 μL.
7. Proceed to the DNA precipitation: add 90 μL chilled 100% EtOH, 3 μL 3 M NaOAc, mix well, leave at −20 °C for at least 20 min and centrifuge at maximum speed in a benchtop microcentrifuge at 4 °C for 15 min. Remove the supernatant, briefly rinse the pellet with 70% EtOH and spin briefly at full speed, 4 °C for 1 min.
8. Remove the supernatant and resuspend in 16 μL sterile ddH_2O.
9. Add 4 μL DIG or BIO nucleic acid labeling mix, and gently pipette to mix.
10. Incubate for 90 min at 15 °C (*see* **Note 14**).
11. Stop the labeling reaction. Add 1 μL 0.5 M EDTA and incubate at 65 °C for 10 min. Spin briefly. At this point the probes can be stored at −20 °C until use.
12. Add 5 μL of each DNA probe (differently labeled probes either with DIG or BIO) and complete the volume with sterile ddH_2O up to 50 μL.
13. Precipitate with 150 μL 100% EtOH and 5 μL 3 M NaOAc following the same process as in **step 7**. Carefully dry the pellet on bench.
14. Add 10 μL HB50, and incubate at 42 °C for 10 min—do not pipette at this stage.

15. Add 10 μL DS, mix well by pipetting gently.
16. Denature probe for 15 min at 75 °C.
17. Immediately put on ice.

3.4 Slide Preparation

All steps are performed at room temperature (*see* **Note 15**) unless otherwise specified. For incubation at 38, 80, and 37 °C the heating blocks for microscope slides are used.

1. Label the slides with the FISH experiment number.
2. Immerse them in FB for 10 min for a mild fixation before the FISH treatment (*see* **Note 16**).
3. Rinse two times in PBS by exchanging the solution.
4. Drain the excess of liquid by holding the slide for a few seconds vertically.
5. Add 1 mL of 2.5 mg/mL pepsin in 100 mL 0.01 M HCl.
6. Add 100 μL of pepsin/0.01 M HCl on the gel pad and incubate for 1 min 25 s at 38 °C in a preheated slide heating block for microscope slides (*see* **Note** 17).
7. Transfer the slides to a Coplin jar filled with a PBS solution containing FB for 10 min.
8. Rinse two times in PBS.
9. Remove the last wash, remove as much liquid as possible, and keep the slides in the Coplin jar to air dry.

3.5 Hybridization

1. Cut small pieces of Parafilm that would cover the gel pads (around 18 × 18 mm) for all the slides.
2. Precondition the embedded nuclei with hybridization buffer: add 20 μL of HB50:DS (1:1), and cover the gel pads with Parafilm.
3. Incubate for 10 min at room temperature.
4. Remove the Parafilm, add 20 μL of probe and cover again with the same Parafilm.
5. Transfer the slides quickly to 80 °C in a preheated slide heating block for microscope slides and incubate for 2 min.
6. Hybridize for at least 16 h at 37 °C (*see* **Note 18**).

3.6 Immunodetection

All steps are performed at room temperature unless otherwise specified (*see* **Note 15**). For incubation at 37 and 42 °C, the heating blocks for microscopy slides are used.

1. Add 100 μL of SF50 and incubate for 5 min at 42 °C.
2. Remove the liquid by holding the slide vertically on a paper towel.

3. Add 100 μL of 2× SSC and incubate for 5 min at 42 °C, repeat **step 2**.
4. Add 100 μL of 4T and incubate for 5 min at 42 °C, repeat **step 2**.
5. Add 100 μL of blocking buffer for 30 min at 37 °C, repeat **step 2**.
6. Add 100 μL of 4T and incubate for 5 min, repeat **step 2**.
7. Add 100 μL of TNT, incubate for 5 min and repeat **step 2**. Repeat this step twice.
8. Add 100 μL of mouse anti-DIG in TNB (1:250) (for DIG nick translation) or Biotinylated Anti-Avidin in TNB (1:250) (for BIO nick translation), or 100 μL of TNB containing both antibodies (*see* **Note 19**).
9. Incubate for 90 min at 37 °C, repeat **step 2**.
10. Repeat **step 7**.
11. Add 100 μL of Alexa Fluor 488-coupled anti-mouse antibody (2.5:1000) in TNB (for DIG nick translation) or Avidin coupled with Texas-Red in TNB (1:1000) (for BIO nick translation), or 100 μL of TNB containing both antibodies (*see* **Note 19**).
12. Incubate for 90 min at 37 °C, repeat **step 2**.
13. Repeat **step 7**.
14. Counterstain with 10 μL of DAPI, add precision cover glass and seal with nail polish.
15. Leave for 10 min in the dark.
16. Slides can be stored vertically at 4 °C in the dark.

3.7 Image Acquisition

Objective: Obtain 3D images of individual nuclei with gene-FISH signals and counterstained with DAPI. We used a Leica TCS SP5 resonance CSLM, a 63 Glycerol immersion objective, NA 1.3, CS2 PL APO grade with corrective lenses for chromatic and planar aberrations. Other instruments with similar setup can be used. The important aspects are the following:

1. Image acquisition resolution affects the margin of error of all the distances and positions calculated by the plugin described under Subheading 3.10. The resolution of the images illustrated here is 20 nm × 20 nm × 80 nm, which is largely oversampled (*see* **Note 20**). This resolution is achieved using a pinhole opening of 1 airy unit, 20-fold zoom factor and 256 × 256 image format, and a z-step of 80 nm. An example of an acquired image is shown in Fig. 2.
2. Laser power and gain (if photomultiplier tubes, PMTs, are used) should be balanced to use the optimal dynamic range of

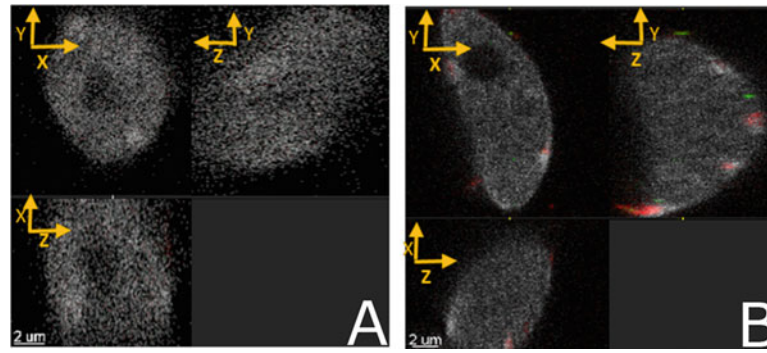

Fig. 2 Example of an acquired FISH image. (**a**) Image of nuclei after FISH without labeled probes. (**b**) Image of nuclei after FISH with labeled probes: CEN-repeat in the red channel and AT1G15690 in the green channel

pixel intensities, and avoid local saturation (using the Look Up Table (LUT) inspection tool) and bleaching.

3. Simultaneous three-channel acquisition should be controlled for the absence of cross-talk. Otherwise sequential scanning is recommended.
4. To speed up the acquisition of multiple nuclei from one preparation, automated z-series scans can be setup using a multiposition recorder grid following the provider's recommendation.
5. The objective lens should ideally allow for glycerol immersion (matching the refractive index (RI) of Vectashield). If available, use the lens correction for coverslip RI. Make sure to use a lens with confocal, fluorescence microscopy grade with best possible chromatic and spherical aberration corrections (particularly important for resolving the FISH signals in the *z* dimension).

3.8 Image Processing

The objective of the image processing part is to get numerical data out of digital image data. Processing consists of two steps: (1) image deconvolution and (2) image segmentation and distance computing. (1) Image deconvolution can in principle be achieved with any software, but we describe a workflow here using the Huygens platform. For alternative software, the users should follow the providers' instructions. (2) Image segmentation and the computing of FISH signal distances relative to the nuclear periphery, chromocenters, and nucleolus are achieved in the Imaris environment. While Imaris is a commercially available software often provided by academic microscopy facilities, the plugin used for this analysis is freely available in the Imaris Xtension open repository. We propose an automated batch processing in order to facilitate large data analysis, and to insure all images are processed in the

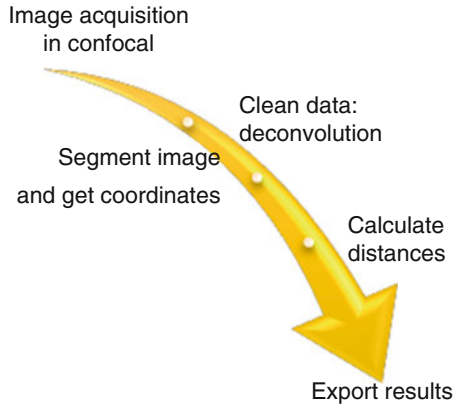

Fig. 3 Image processing flowchart

same manner but single image processing is possible and recommended the first time to become familiar with the process (described in the scheme in Fig. 3).

3.9 Deconvolution

1. Open one image in Huygens.
2. Set up the parameters for deconvolution using the Parameter editor (Edit menu or Right click on the image). The software should read the Meta data of the image and automatically detect the values of all the parameters, except for Lens immersion and Embedding medium. The user needs to set the values for these two parameters to glycerine 80% (this is the medium we used for lens immersion). Verify the values for all the other parameters. Validate the parameters ("Set all verified" and "Save") and save the template file corresponding to these parameters as *Temp1*. Validate ("Accept").
3. Set up the batch analysis mode (Batch tab), add a task. A step-by-step wizard opens, guiding you through the different steps. Validate each step via the arrow to (1) select the files to be processed using a browsing menu, (2) specify the deconvolution parameters to be used by selecting your formerly saved *Temp1* file, (3) adjust the parameters per channel by selecting the option "New Template." Table 1 shows the parameters recommended for each channel. Repeat this process for all channels.
4. Save the workflow and parameters you just set up as a template for future batch operations (Template tab: set the name of the template, for instance *Temp2* and save) and close the task ("Done").
5. Setup the output format and output location: in the Option tab, set Output format to "Imaris Classic." In the "Save

Table 1
Deconvolution parameters for huygens

Parameters	Values for all channels
Algorithm	Classic MLE
PSF mode	Theoretical
Max. iteration	40
Iteration mode	Optimized
Quality change threshold	0.05
Signal to noise ratio	15
Background mode	Lowest value
Background estimation radius	0.7
Relative background	0.0
Bleaching correction	If possible
Brick mode	Auto
PSFs per brick mode	Off
PSFs per brick, manual mode	1

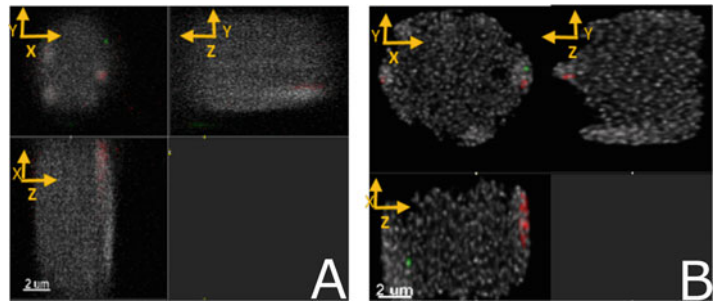

Fig. 4 Example of deconvolution output. (**a**) Raw image before deconvolution. (**b**) Image after deconvolution with Huygens. FISH signal: AT1G15690 in the red channel and AT5G25860 in the green channel

location" field, browse for the folder where all the resulting image files will be saved: *folder2*.

6. Start the deconvolution. An example of a deconvolution result is shown in Fig. 4.

3.10 Imaris Empowered Image Processing

The objective of our customized plugin is to (1) segment the image and (2) compute distances. Segmentation works on the principle of intensity thresholds and voxel size and uses processing modules implemented in Imaris. Segmentation produces four types of 3D

objects: a nuclear surface, spots corresponding to FISH signals, surfaces corresponding to chromocenters and a nucleolus surface. For each FISH signal (spot), its distance to the nuclear surface (PF), to the nucleolus (NF) and to the closest chromocenter (CF) is calculated. All distances are normalized to the nuclear volume. The scheme of the analysis is described in Fig. 5.

The images produced by the plugin contain the features illustrated in Fig. 6. The figure shows that several mask channels are created and used to do the following segmentations: The DAPI

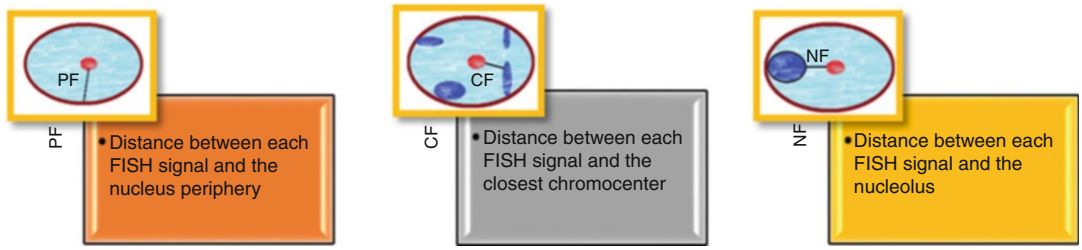

Fig. 5 Scheme of distances calculated with the plugin

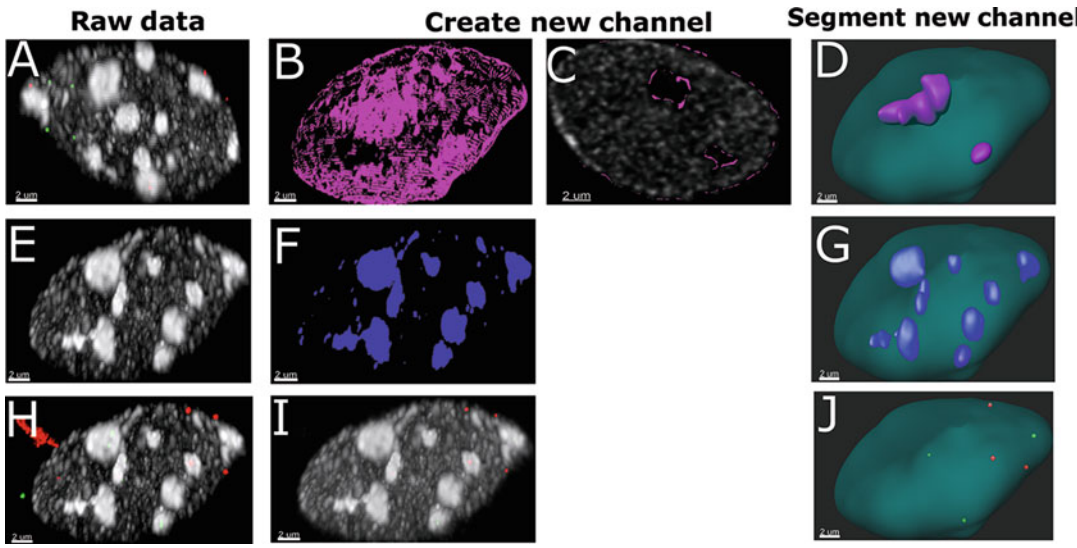

Fig. 6 Segmentation result. (**a**) Raw data: DAPI channel in gray. (**b**) Low intensity DAPI channel (LDI) in magenta, is created by the plugin to select voxels with intensity lower than average. 3D view. (**c**) DAPI and LDI channels, Z-slice view of the nucleoli. (**d**) Nucleus surface and the nucleoli are segmented using the DAPI and LDI channels. (**e**) Raw data: DAPI channel in gray. (**f**) Chromocenters channel, in purple, is created, by selecting voxels within the nucleus with intensity higher than average in the DAPI channel. (**g**) Using the latter, chromocenters are segmented. (**h**) Raw data: DAPI channel in gray, FISH signals: FISH_Ch0: AT1G15690 in the red channel and FISH_Ch1: AT5G25860 in the green channel. (**i**) High intensity FISH channels are created selecting voxels within the nucleus with intensity higher than average in each FISH channel; High intensity FISH_Ch0 in the red channel and High intensity FISH_Ch1 in the green channel. (**j**) High intensity FISH channels are segmented; AT1G15690 spots in red, AT5G25860 spots in green

channel is used to produce the nucleus surface (Fig. 6a and c), the Low intensity DAPI channel is used for the nucleolus surface (Fig. 6b and c), the Chromocenters channel is used for the Chromocenters surfaces (Fig. 6d and e), High intensity FISH channels are created for each FISH channel and are used to segment *FISH* spots (Fig. 6g and h). Options to fine tune segmentation are described in the Troubleshooting section.

The plugin produces four tables for each FISH channel:

FISHPeripheryDistanceTable_ChX.csv.

FISHNucleolusDistanceTable_ChX.csv.

FISHChromocenterDistanceTable_ChX.csv.

FISHPositionTable_ChX.csv.

Each table contains as many columns as there are images and as many rows as there are FISH spots. These tables are contained in the "XTFISHInsideNucleus_Result" folder. The latter is created by the plugin in the directory containing the images to be processed. For each image processed, an .ims file is created in this new folder.

Step by step

5. Download the two files: "XTFISHInsideNucleus.py" and "XTFISHInsideNucleus_Parameters.csv", from http://open.bitplane.com/tabid/235/Default.aspx?id=131. Both files are required to run the plugin. Save both files in the same folder: *folder1*.
6. Download and install python 2.7 or higher. Set installation directory to *pythonFolder* (in windows this is usually C:\Program Files).
7. Open Imaris, and under File/Preferences/Calculation/, set memory limit to 4 GB.
8. Go to the File tab and click on "Preferences" in the drop down menu.
9. In the "Preferences" window, click on "Custom Tools."
10. In the "Custom Tools" window, select "Browse" in the "Python application" field to locate the python.exe file: *pythonFolder*.
11. On the "XTension Folders" field, select "Add" and browse for *folder1*.
12. Select "Ok" on the bottom of the window.
13. In the top tool bar, select Image Processing/FISHInsideNucleus (on the bottom of the menu).
14. Set parameters:
    - Select "Batch of images" in Window1 (Fig. 7a) for batch processing (*see* **Note 21**).

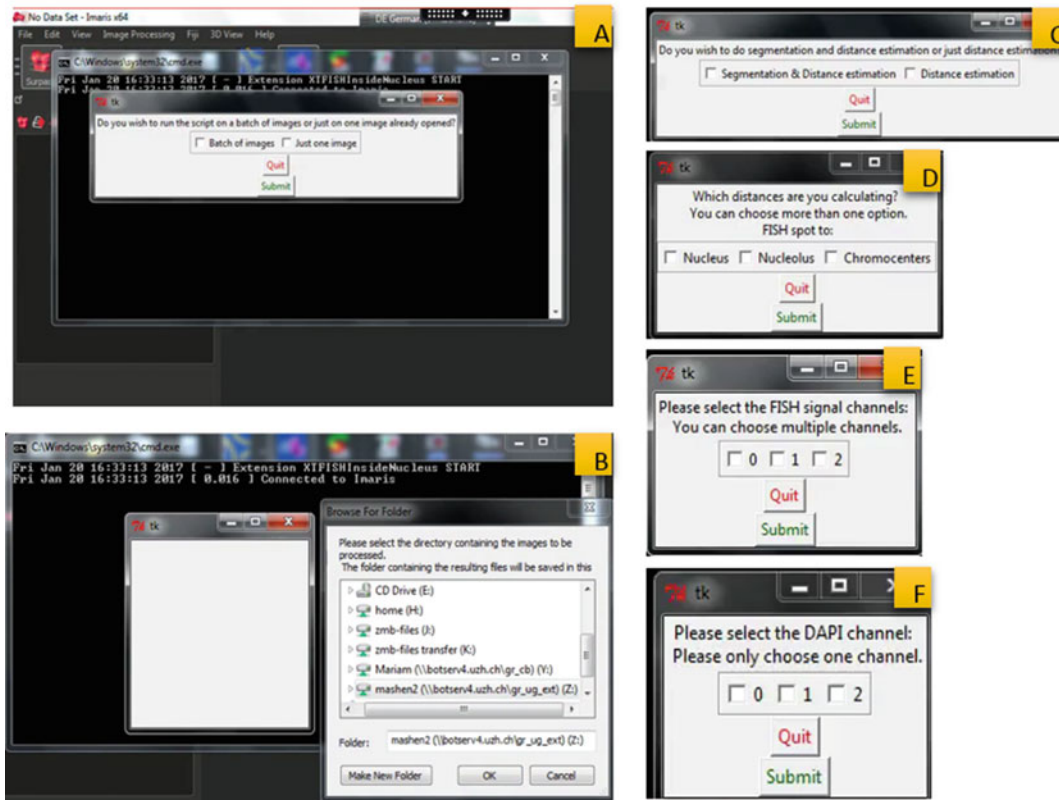

Fig. 7 Running the plugin. (**a**) Window1—Select if the plugin should process: "Batch of images" or "just one image." (**b**) Window2—If Batch of images is selected in Window1, browse for the folder (folder2) containing all the images to be processed. (**c**) Window3—if the plugin should perform segmentation, select "Segmentation & Distance estimation," if the segmentation has already been performed manually, select "Distance estimation." (**d**) Window4—To calculate the distance between FISH spots and nucleus periphery: select "nucleus," to the nucleolus: select "nucleolus," to the closest chromocenter: select "Chromocenters." It is possible to choose multiple options. (**e**) Window5—select FISH channels that should be segmented FISH spots. (This window pops up only if "Segmentation & Distance estimation" is selected in Window3). (**f**) Window6—select DAPI channel that should be segmented into nucleus, nucleolus and chromocenters. (This window pops up only if "Segmentation & Distance estimation" is selected in Window3)

- Browse for *folder2* in Window2 (Fig. 7b), the plugin will batch process the images contained in that folder.
- Select "Segmentation & Distance estimation" in Window3 (Fig. 7c), to run a two-step program: segmentation and distance calculation (*see* **Note 21**).
- Select options according to the distances desired (PF, NF or CF) in Window4 (Fig. 7d). For PF choose: "Nucleus," for NF: "Nucleolus," for CF: "Chromocenters." More than one option can be selected.

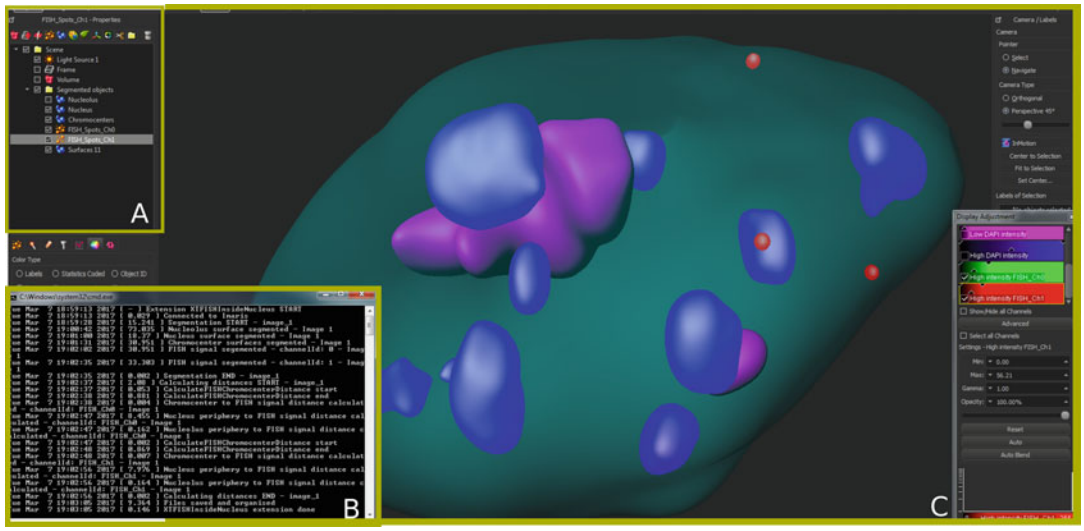

Fig. 8 Extension progress and result. (**a**) All instances (objects) created by the plugin are stored in the container "Segmented Objects." (**b**) While the plugin is running, a python terminal displays the progress of the plugin by logging in tasks that have been completed. (**c**) All objects created are displayed in the 3D viewer: magenta surface = nucleolus, red surfaces = chromocenters, red and green spots = FISH signals

- Select which channels are FISH channels in Window5 (Fig. 7e). More than one option can be selected.
- Select which channel is the DAPI channel in Window6 (Fig. 7f) (*see* **Note 21**).

While the plugin is running, the user can track the progress of the program by tracking the python terminal (Fig. 8b) that logs completed tasks, and by tracking the success of the segmentation process by viewing the objects in the 3D viewer (Fig. 8c).

Once the plugin has finished running, a window pops up, indicating that the program is completed, displaying the number of processed images.

All the produced files are saved in the folder XTFISHInsideNucleus_Result that is created by the plugin in *folder2*.

3.11 Handling Segmentation Errors

It is recommended to go over all the .ims files produced to review the segmentation success, before analyzing the data produced. This can be done by a visual verification: check that the nucleus, chromocenters, and nucleolus surfaces and FISH spots correlate with the signals in the 3D image.

Troubleshoot option 1—for systematic segmentation error:

The segmentations are calibrated to our experiment. As a consequence, some parameters are set to a specific value. So in case the segmentations are erroneous for several images, the user can

calibrate these values to fit their images, by using gradient values and doing several runs of the plugin. These values can be set in the "XTFISHInsideNucleus_Parmaeters.csv" file (*see* **Note 22**).

Troubleshoot option 2—for individual/random segmentation error:

An individual/random segmentation error corresponds to an inaccurate segmentation occurring on only one or a few images where the signal distribution and level does not allow an unambiguous thresholding by the plugin. One example: when the nucleolus is close to the nucleus surface, the plugin cannot distinguish between the voxels with low DAPI intensity inside the nucleolus and those outside the nucleus surface, as shown in Fig. 9a. An erroneous nucleolus segmentation is illustrated in Fig. 9b. To correct this, the segmentation must be done manually using the low DAPI intensity channel.

Manual segmentation steps:

1. Figure 9c Select the "create a surface" icon (1). Then select next (2).
2. Figure 9d Set the channel to Low DAPI Intensity channel (3), set smooth surface detail to 0.4 (4) and diameter of the largest sphere to 1.6 (5). Both values can be adjusted according to the quality of nucleolus segmentation.
3. Figure 9e Set the lowest intensity threshold (7) so that the entire nucleolus surface is segmented.
4. Figure 9f Select Volume and set a threshold of minimum value so that only the nucleolus surface is visible.

Same method shown here can be used for nucleus and chromocenter manual segmentation.

In order to perform the automated distance calculation on the corrected segmented image(s) follow the steps below:

1. If more than one image has been corrected, put all the modified images in one folder, *folder3*.
2. Run FISHInsideNucleus.
3. Set parameters:
   - Select "Batch of images" in Window1 (Fig. 6a) if there is more than one image otherwise select "Just one image."
   - Browse for *folder3* in Window2 (Fig. 6b).
   - Select "Distance estimation" in Window3 (Fig. 6c), to run a one-step program: distance calculation only.

The rest of the steps are the same as those described in the step-by-step section.

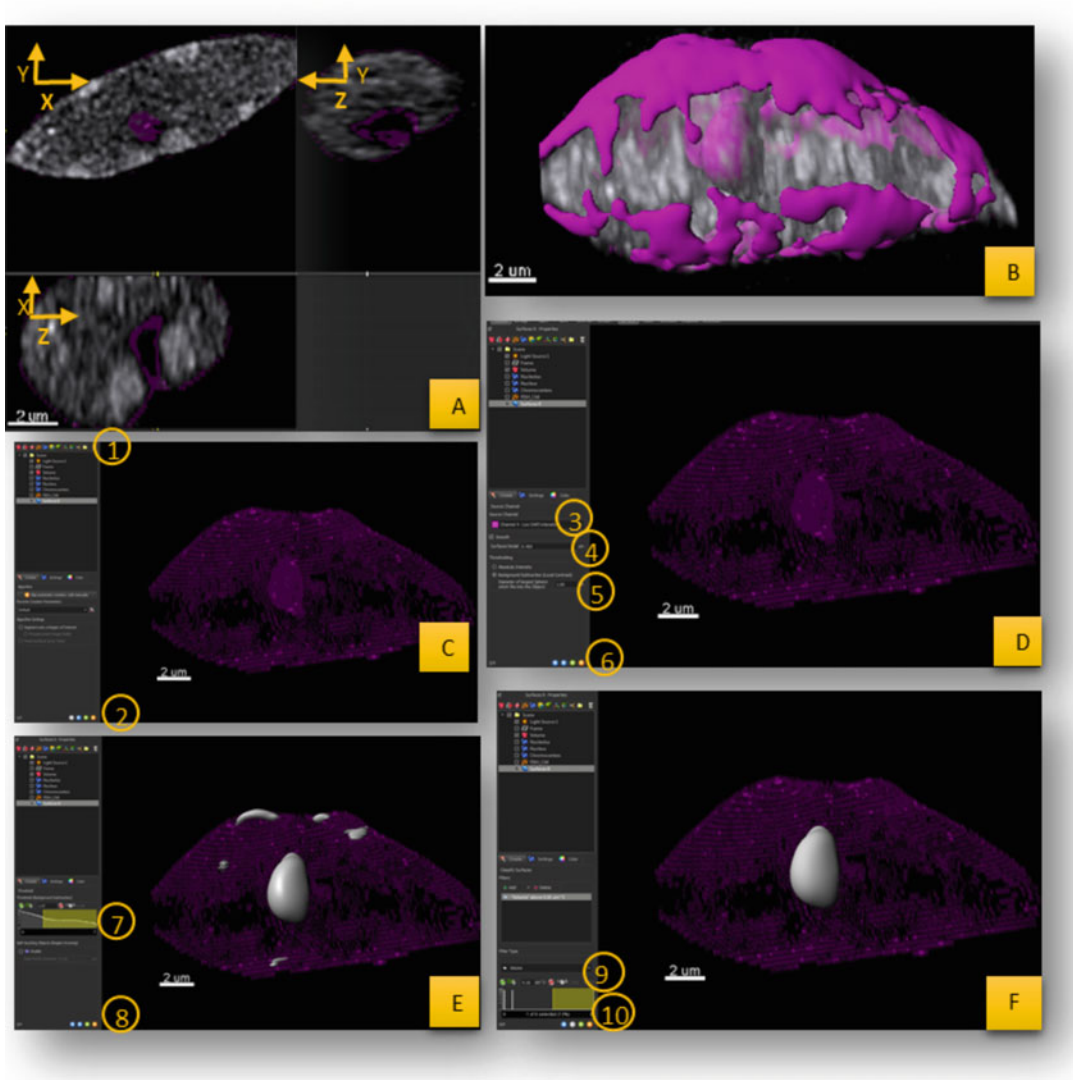

Fig. 9 Manual segmentation. (**a**) DAPI channel is in gray and LDI in magenta. (**b**) Erroneous nucleolus segmentation. (**c**) Create a new surface (*1*) and click on the next arrow (*2*). (**d**) Set the channel index (*3*), the smooth surface detail (*4*) and the diameter of the largest sphere, which fits into object (*5*) and click on the next arrow (*6*). (**e**) Set intensity threshold (*7*) and click on the next arrow (*8*). (**f**) Select volume and set a threshold of minimum value (*9*) and click on the next arrow (*10*)

4 Notes

1. After sterilization, the seeds are sown directly on soil. It is important to extract nuclei from healthy tissue (i.e., stressed, pigmented, or necrotic leaves should be avoided), from well-watered plants. To have a homogeneous population of nuclei, tissue should ideally be collected from the same leaf positions, at the same developmental stage, and around the same time of

the day, as these factors can influence chromatin organization. In this study, we used 35-days old Arabidopsis plants cultivated in a short-day growth chamber. The developmental stage of the plant should be adapted to the study conducted.

2. Prepare 10 mL aliquots of Buffer B and store at −20 °C up to 6 months.
3. After preparing 10 mL fresh Buffer C in a 50 mL bottle in the fume hood, sterilize by filtration and store in the bottle at 4 °C [13]. At this high concentration, this solution should be handled under the fume hood. When the concentration is lowered to 5%, the fume hood is no longer necessary.
4. Accurate quantification of double stranded DNA is critical. It is highly recommended to use a fluorimetric-based quantification discriminating single and double stranded DNA, and RNA. A Qubit system from Invitrogen or similar technology can be used. Alternatively, one can add a purification step after PCR, using a kit, and use the NanoDrop instead.
5. Prepare 10–20 mL of HB50/DS. Sterilize by filtration and prepare 50–100 μL aliquots, store at −20 °C up to 6 months. Use a fresh aliquot for each FISH.
6. FB can be stored at 4 °C for up to 6 months.
7. Prepare 10 mL 2.5 mg/mL pepsin stock, prepare 1 mL aliquots and store at −20 °C. Prepare 10 mL 0.1 M HCl stock and store at room temperature. Both preparations can be stored up to 6 months. Use a fresh aliquot of Pepsin for each FISH.
8. Prepare 10 mL Blocking buffer and TNB solution, sterilize by filtration, prepare 1 mL aliquots and store at −20 °C for up to 6 months. Use a fresh aliquot for each FISH.
9. Antibodies should be stored according to the manufacturer's instructions. We recommend preparing 5 μL aliquots, which should be stored as described by the manufacturer. In our experience, once an aliquot stored at −20 °C is used, it can be stored at 4 °C for 2 weeks only.
10. Instead of the heating block for microscope slides, one can use humid chambers with moist paper towels. However in our experience, the heating block produces a more controlled environment for our experiment and thereby allows replicable results.
11. Recommended computer system requirements: "RAM: 4 GB; 3 GHz CPU (Intel or AMD) dual core; Graphics: AMD Radeon R7 250 2 GB for Windows, nVidia GeForce GT 650M 1024 MB (slow triangle rendering) or Intel HD 4000 graphics or later (OS X only) for Mac; Monitor: 1280 × 1024

pixels or better; Mouse: 3 button wheel" http://www.bitplane.com/systemrequirements.aspx#sthash.yw2tN98V.dpuf.

12. In order to keep the nuclei intact, it is important not to leave the leaves or the suspension dry during **steps 6–8** of Subheading 3.1. Also, putting a layer of aluminum between the ice and the glass petri dish keeps ice from getting into the petri dish during **steps 7** and **8**.
13. After each use of the Dounce tissue grinder, rinse with ddH_2O.
14. If the probe preparation is done for the first time for a particular DNA sequence, the nick translation reaction duration should be adjusted. To determine the proper conditions, load 2 μL of a test reaction on a 1% agarose gel after 10, 30, 60, and 90 min nick translation and more if necessary. It is important that most of the smear is found between 200 and 500 nt. Deviations from this will result in failed or suboptimal FISH signal.
15. The room temperature in our laboratory varies from 20 to 24 °C. Change in room temperature can introduce variability in FISH signal strength.
16. Please use the formaldehyde under the fume hood. The FB can be reused several times and can be stored at 4 °C up to 3 months.
17. Precise incubation duration is critical, underexposure of the nuclei to Pepsin solution would result in suboptimal FISH signal and an overexposure would result in damage of nuclei.
18. If FISH signal strength is too weak, one can try to increase the duration of the hybridization.
19. Antibody should be freshly prepared in TNB, be kept on ice in the dark and used only once. So during and after incubation with antibodies, the slides should be kept in the dark. We recommend using aluminum foil to cover the slides during **step 14** of Subheading 3.6.
20. The margin of errors of the distances calculated is indirectly proportional to the values of image acquisition resolution (the latter is the size of one voxel). It is therefore better to aim for a small voxel size. However the smaller the voxel size is, the longer one image acquisition will take. A good method for estimating the image acquisition resolution is to use the Nyquist calculator: https://svi.nl/nyquist/. The resolution can be adjusted using the zoom factor, the image format and z-step.
21. For these parameters, only one option should be selected. If the user selects more than one option, by default the first option will be the value of that parameter.

22. To have a better understanding of the meaning of these parameters, please look into XTFISHInsideNucleus_Manual.pdf that can be downloaded from http://open.bitplane.com/tabid/235/Default.aspx?id=131 /.

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