G-Quadruplex Visualization in Cells via Antibody and Fluorescence Probe

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Chapter 24

G-Quadruplex Visualization in Cells via Antibody and Fluorescence Probe

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Abstract

G-quadruplexes (G4s) are noncanonical nucleic acids structures involved in key regulatory and pathological roles in eukaryotes, prokaryotes, and viruses: the development of specific antibodies and fluorescent probes represent an invaluable tool to understand their biological relevance. We here present three protocols for the visualization of G4s in cells, both uninfected and HSV-1 infected, using a specific antibody and a fluorescent G4 ligand, and the effect of the fluorescent ligand on a G4 binding protein, nucleolin, upon binding of the molecule to the nucleic acids structure.

Key words G-quadruplex-specific antibodies, G-quadruplex ligands, Fluorescence probe, Immunofluorescence staining, Confocal microscopy, Nucleoli, HSV-1

1 Introduction

G-quadruplexes (G4s) are unique, noncanonical nucleic acids structures adopted by guanine-rich sequences. The building block of these structures is the so-called guanine quartet (G-quartet): two or more G-quartets, stacking on each other, form the G-quadruplex. From a structural point of view, G4s are characterized by a high polymorphism: their topology can be classified as parallel, antiparallel, or hybrid basing on strands orientation and the multiple orientations adopted by the nucleotide linkers between guanine tracts (loops) contribute to increase G4 diversity.

G4s are involved in key regulatory and pathological roles in eukaryotes [1–5], prokaryotes, and viruses [6–10]: given their biological significance, many efforts have been devoted to the development of specific and selective G4 stabilizing molecules [11–14], as well as of probes able to modify their fluorescence behavior upon G4 binding [15–17]. Both antibodies and fluorescence probes that specifically recognize G4 structures represent invaluable tools to visualize G4s in cells and to understand their biological relevance. Recently, two antibodies recognizing G4s

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have been developed: BG4 [18] and 1H6 [19]. BG4 is a single-chain fragment variable antibody generated by phage display employing a library of different single-chain antibody clones and selecting the best G4 binder, while 1H6 is a monoclonal antibody produced immunizing mice with stable G4 DNA structures. Both antibodies were used to detect G4s in cells [20–22], in our studies the monoclonal antibody 1H6 was used.

Many G4-specific fluorescent probes have been developed in the last years [23–25], but only a few of them can be used in both fixed and live cells, because of their cellular and subcellular permeability. The core-extended NDI (*c-exNDI*) is a potent G4 binder with an antiviral and anticancer activity [9]. Given its light-up properties upon G4 binding and its very fast cellular and nuclear entry, *c-exNDI* was used to visualize G4, in combination with the 1H6 antiG4 antibody [23], both in uninfected and in HSV-1-infected cells.

2 Materials

All solutions and materials used for cell culturing must be sterile.

2.1 Cell Culture and Virus

1. Cell line of interest (must be chosen, depending on the experiment purpose: in our case HEK293T and Vero).
2. Cell culture medium, DMEM–Dulbecco's Modified Eagle Medium: NaCl 110.34 mM, $NaHCO_3$ 44.05 mM, D-Glucose 25.00 mM, KCl 5.33 mM, L-Glutamine 3.97 mM, $Fe(NO_3)_3$ 2.47 mM, $CaCl_2$ 1.80 mM, NaH_2PO_4 0.92 mM, $MgSO_4$ 0.81 mM, L-Valine 0.80 mM, L-Isoleucine 0.80 mM, L-Leucine 0.80 mM, L-Lysine 0.80 mM, L-Threonine 0.80 mM, L-Phenylalanine 0.40 mM, L-Serine 0.40 mM, Glycine 0.40 mM, L-Tyrosine 0.40 mM, L-Arginine 0.40 mM, L-Cystine 0.20 mM, L-Methionine 0.20 mM, L-Histidine 0.20 mM, L-Tryptophan 0.08 mM, i-Inositol 0.04 mM, Phenol Red 0.04 mM, Niacinamide 0.03 mM, Choline 0.03 mM, Pyridoxine 0.02 mM, Thiamine 0.01 mM, (Thermo Fisher Scientific).
3. Fetal Bovine Serum (FBS) (Thermo Fisher Scientific).
4. Trypsin-EDTA 0.05% (Thermo Fisher Scientific).
5. Dulbecco's phosphate-buffered saline (DPBS) pH 7.4137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (Thermo Fisher Scientific), (optional: poly-D-lysine) (*see* **Note 1**).
6. HSV-1 wt, strain F (*see* **Note 2**).
7. Six-well plates for cell culture, microscope slides, and coverslips (alternatively: chamber slides for cell culture).
8. *c-exNDI* (or any other fluorescent compound reported to bind G4) (*see* **Note 3**).

9. Fixative: 2% (w/v) paraformaldehyde (PFA) in 1× DPBS.
10. Humidified 37 °C, 5% CO_2 incubator.

2.2 Immunofluorescence and Confocal Microscopy

1. Permeabilizing solution: 0.5% (v/v) Tween-20 in DPBS (*see* **Note 4**).
2. Washing solution after permeabilization (PBST): 0.1% (v/v) Tween-20 in DPBS.
3. 40 μg/mL RNaseA (Invitrogen) or 200 units DNase I (Invitrogen).
4. Blocking agent: BlockAid (Invitrogen) or any other suitable reagent.
5. Anti-G4 antibody: 1H6 [19] (*see* **Note 5**).
6. Anti-nucleolin C23 antibody (H-250) (SantaCruz Biotechnology).
7. Anti-fibrillarin antibody (38F3) (Abcam).
8. Appropriate fluorescent secondary antibody: Alexa 488 anti-mouse IgG antibody, Alexa 488 anti-rabbit IgG antibody and Alexa 546 anti-mouse IgG antibody (*see* **Note 6**).
9. Fluorescent DNA dye: DRAQ5® (Cell Signaling Technology).
10. Antifade mounting medium: Glycergel Mounting Medium (Dako-Agilent) or ProLong™ Gold Antifade Mountant (ThermoFisher Scientific).
11. Nail polish.
12. Confocal microscopes: Leica TCS SP2 and Nikon A1Rsi + Laser Scanning.

3 Methods

3.1 Cell Culture

1. Grow cells in appropriate medium supplemented with Fetal Bovine Serum (FBS) at 37 °C in a 5% CO_2 humidified atmosphere. HEK293T cells were used for fluorescent probe experiments and Vero cells for HSV-1 experiments.
2. Determine compound cytotoxicity (MTT assay or any other cell proliferation assay, according to manufacturer's instructions).
3. Harvest cells using Trypsin-EDTA and seed them onto glass coverslips in a six-well plate (*see* **Note 7**).
4. Allow cells an overnight period for attaching and grow.

3.2 G4 Visualization via 1H6 Antibody and c-exNDI Fluorescent Probe

All the following steps have to be carried out under dim light (*see* **Note 8**).

1. Dilute compound in cell culture medium and treat cells. Compound concentrations have to be nontoxic, exposure times

have to be chosen depending on cell permeability to the compound. For *c-exNDI* in HEK293T cells, we used 1 μM compound for 2.5–30 min at 37 °C in incubator.

2. Remove cell culture medium and wash cells with 1× DPBS at least three times to remove cell medium and compound residuals (*see* **Note 9**).
3. Fix cells with 2% PFA for 20 min at RT in the dark.
4. Remove PFA and wash cells with 1× DPBS at least five times to remove PFA residuals (*see* **Note 10**).
5. Permeabilize cells with 500 μL permeabilizing solution for 15 min on a rocker.
6. Remove permeabilizing solution and wash slides three times with PBST.
7. Treat slides with 40 μg/mL RNaseA for 30 min at 37 °C on a rocker (*see* **Note 11**).
8. Incubate with blocking agent (BlockAid) for 1 h at 37 °C, placing slides face-down in a humidified chamber (*see* **Note 12**). Use tweezer and a needle to pick the slides from the plate and place them in the humidified chamber.
9. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1 μg/mL anti-G4 antibody 1H6 for 2 h at RT in a humidified chamber.
10. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1:250 Alexa 488 anti-mouse IgG antibody for 1 h at 37 °C in a humidified chamber.
11. Put slides back in the six-well plate and wash them three times with PBST.
12. Dip the slides twice in distilled water to remove salts.
13. Place a drop of mounting medium on the microscope slide, and put the coverslip face-down on the mounting medium. Carefully press the coverslip over the slide and remove the excess of liquid with absorbent paper.
14. Use nail polish to seal the edge of the coverslip, and let it dry (*see* **Note 13**).
15. Proceed with confocal microscopy. We used 488 nm excitation wavelength and 500–530 nm emission range for G4 visualization, and 543 nm excitation wavelength and 609–617 nm emission range for *c-exNDI* visualization (*see* **Note 14**).

The fluorescent probe *c-exNDI* enters the cell and localizes in the cell nucleus, with peaks in subnuclear compartments corresponding to nucleoli. Moreover, it shows a good colocalization with the anti-G4 antibody 1H6 (Fig. 1).

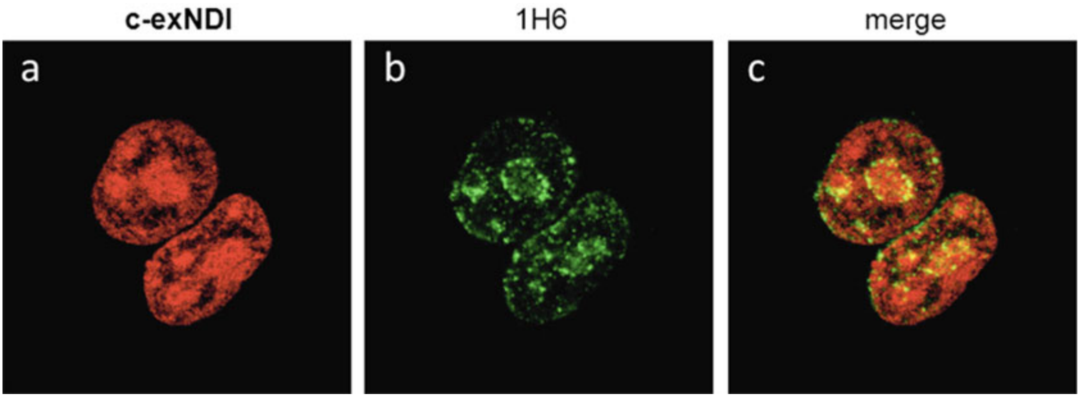

Fig. 1 Colocalization of *c-exNDI* and G4s by confocal microscopy. Cells were incubated with *c-exNDI* (red signal, left panel) and with the anti-G4 antibody 1H6 (green signal, middle panel). The image on the right (merge) shows *c-exNDI* (red) and G4 (green) overlapping

3.3 Effects of Fluorescent Probe upon G4 Binding

Since the fluorescent probe *c-exNDI* is able to bind G4, we investigated its effect on nucleolin, a G4-binding protein mainly localized in the nucleolus [26]. It was already reported that treatment with Quarfloxin (QFX), a potent G4 ligand, induces a displacement of NCL from nucleoli and a relocalization to the nucleoplasm, without affecting the distribution of fibrillarin, a component of nucleolar snRNPs [12]. The following protocol can be used to compare the effect of *c-exNDI* and QFX on nucleolin and fibrillarin distribution.

Proceed from **step 4** of Subheading 3.1.

All the following steps have to be carried out under dim light (*see* **Note 8**).

1. Dilute QFX in cell culture medium to reach a final concentration range 1–5 μM, treat cells, and place them for 2 h at 37 °C in incubator.
2. Dilute *c-exNDI* in cell culture medium to reach a final concentration range 1–5 μM, treat cells, and place them for 30 min at 37 °C in incubator.
3. Remove cell culture medium and wash cells with 1× DPBS at least three times to remove cell medium and compound residuals (*see* **Note 9**).
4. Fix cells with 2% PFA for 20 min at RT in the dark.
5. Remove PFA and wash cells with 1× DPBS at least five times to remove PFA residuals (*see* **Note 10**).

6. Permeabilize cells with 500 μL permeabilizing solution for 15 min on a rocker.
7. Remove permeabilizing solution and wash slides three times with PBST.
8. Treat slides with 40 μg/mL RNaseA for 30 min at 37 °C on a rocker (*see* **Note 11**).
9. Incubate with blocking agent (BlockAid) for 1 h at 37 °C, placing slides face-down in a humidified chamber (*see* **Note 12**). Use tweezer and a needle to pick the slides from the plate and place them in the humidified chamber.
10. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1:500 anti-nucleolin C23 antibody or with 1:500 anti-fibrillarin antibody for 1 h at 37 °C in a humidified chamber.
11. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1:250 Alexa 488 anti-mouse IgG antibody or with 1:250 Alexa 488 anti-rabbit IgG antibody for 1 h at 37 °C in a humidified chamber.
12. Put slides back in the six-well plate and wash them three times with PBST.
13. Dip the slides twice in distilled water to remove salts.
14. Place a drop of mounting medium on the microscope slide, and put the coverslip face-down on the mounting medium. Carefully press the coverslip over the slide and remove the excess of liquid with absorbent paper.
15. Use nail polish to seal the edge of the coverslip, and let it dry (*see* **Note 13**).
16. Proceed with confocal microscopy, using a 488 nm excitation wavelength and 500–530 nm emission range for nucleolin or fibrillarin visualization, and a 543 nm excitation wavelength and 609–617 nm emission range for *c-exNDI* visualization.

The comparative nucleolin displacement induced by *c-exNDI* and QFX confirms not only its specific localization at nucleoli, but also binding to nucleolar G4s (Fig. 2).

3.4 G4 Visualization in HSV-1 Infected Cells via 1H6 Antibody

The herpes simplex virus-1 (HSV-1) genome has a very high GC content (68%) which peaks at 84.7% GC in simple sequence repeats (SSRs): recently, our research group provided evidence for the presence of very stable G4-forming regions located in the HSV-1 inverted repeats [8]. Given the extraordinary extension of G4 forming regions in the HSV-1 genome, it is possible to visualize G4s in eukaryotic cells infected with HSV-1 [27]. HSV-1 infected cells are highly enriched in G4s: in particular, the amount of G4s

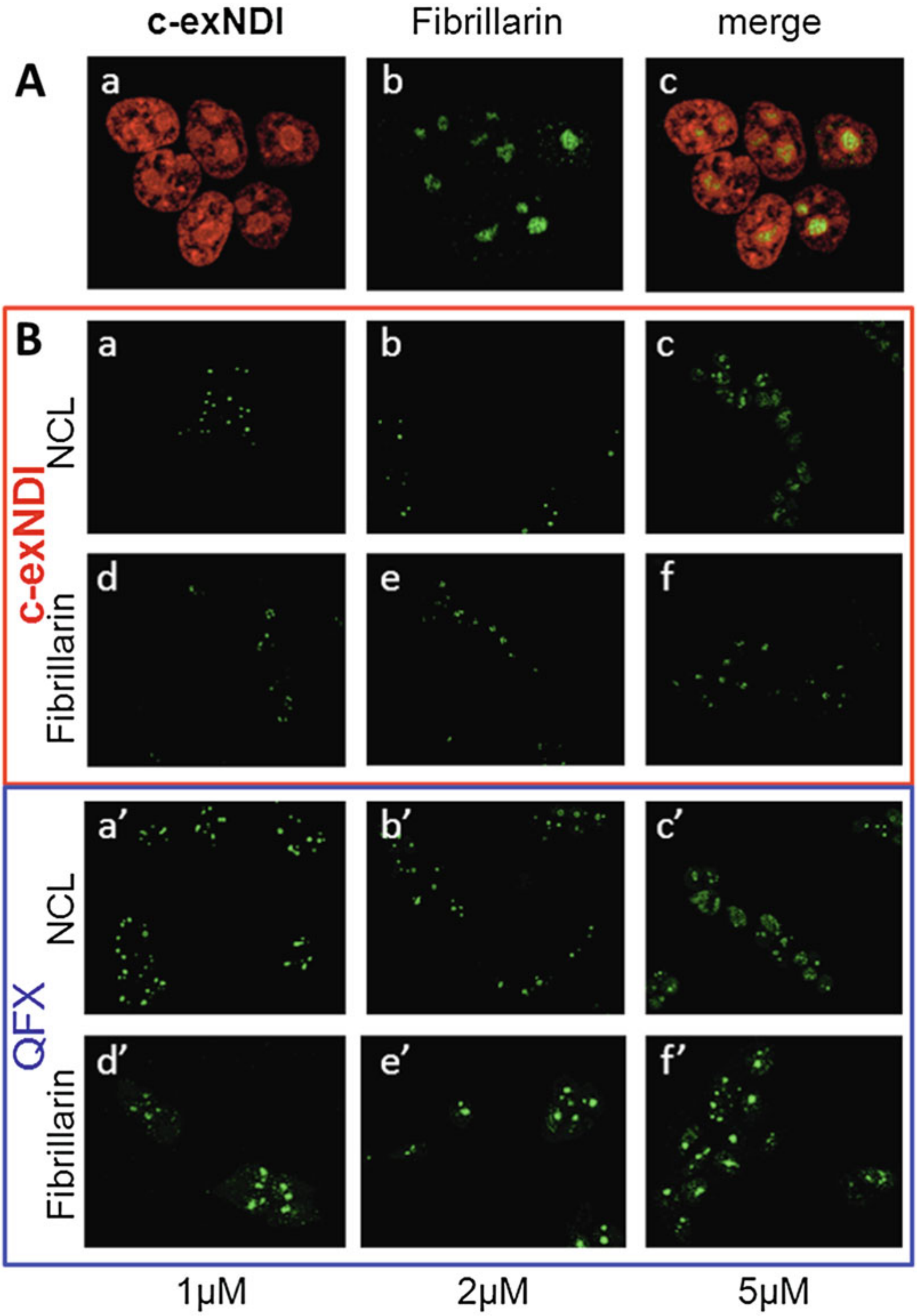

Fig. 2 Cellular localization and targeting of *c-exNDI*. (**a**) Nucleolar localization of *c-exNDI*. Cells treated with *c-exNDI* (red signal, panel a) were incubated with an anti-fibrillarin antibody (green signal, panel b).

depends on the virus amount (MOI) and on the viral step, being more intense around the time of viral DNA replication.

Proceed from **step 4** of Subheading 3.1.

1. Infect Vero cells at MOI 2.5 and 5 in serum-free medium for 1 h at 37 °C in incubator (*see* **Note 15**).
2. Remove serum-free medium and replace it with complete medium.
3. After 6–8 h, remove medium and wash with 1× DPBS.
4. Fix cells with 2% PFA for 20 min at RT in the dark.
5. Remove PFA and wash cells with 1× DPBS at least five times to remove PFA residuals (*see* **Note 10**).
6. Permeabilize cells with 500 μL permeabilizing solution for 15 min on a rocker.
7. Remove permeabilizing solution and wash slides three times with PBST.
8. Incubate with blocking agent (BlockAid) for 1 h at 37 °C, placing slides face-down in a humidified chamber (*see* **Note 12**). Use tweezer and a needle to pick the slides from the plate and place them in the humidified chamber.
9. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1 μg/mL anti-G4 antibody 1H6 for 2 h at RT in a humidified chamber.
10. Put slides back in the six-well plate and wash them three times with PBST and incubate with 1:500 Alexa 546 anti-mouse IgG antibody for 1 h at 37 °C in a humidified chamber.
11. Put slides back in the six-well plate and wash them three times with PBST.
12. Incubate with 1:200 FITC-conjugated anti-HSV-1 ICP8 at room temperature for 1 h.
13. Put slides back in the six-well plate and wash them three times with PBST.
14. Stain nuclei with far-red fluorescent DNA dye (DRAQ5®, 1:1000) for 5 min at room temperature.
15. Dip the slides twice in distilled water to remove salts.
16. Place a drop of mounting medium on the microscope slide, and put the coverslip face-down on the mounting medium.

◄

Fig. 2 (continued) Colocalization is shown in panel **c**. (**b**) **c-***exNDI*-mediated displacement of the G4 binding protein nucleolin from the nucleoli. Cells were treated with increasing concentrations of *c-exNDI* (panels **a–f**) or quarfloxin (QFX) (panels **a'**–**f'**). Nucleolin (NCL) and fibrillarin behavior upon treatment with **c-***exNDI* or QFX was visualized by staining the cells with anti-nucleolin (panels **a–c** and **a'**–**c'**) and anti-fibrillarin (panels **d–f** and **d'–f'**) antibodies

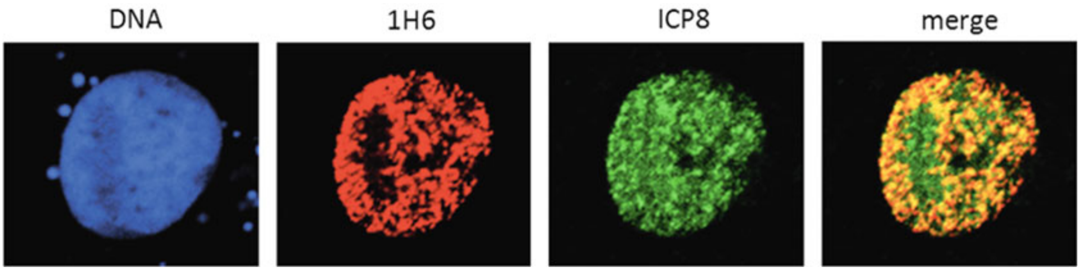

Fig. 3 Colocalization of G4s and the viral protein ICP8 by 3D confocal microscopy. ICP8 is a marker for HSV-1 replication compartments (RCs). Cells were infected with wt HSV-1 (strain F), MOI 5. At 8 h p.i. cells were stained with the anti-G4 (1H6) and anti-ICP8-FITC antibodies. Blue, red, and green indicate DNA, G4s, and ICP8-dependent viral RCs, respectively. The images on the right (merge) show G4 (red) and ICP8 (green) overlapping as a yellow/orange signal

Carefully press the coverslip over the slide and remove the excess of liquid with absorbent paper.

17. Use nail polish to seal the edge of the coverslip, and let it dry (*see* **Note 13**).
18. Proceed with confocal microscopy. We used 488 nm excitation wavelength and 496–519 nm emission range for ICP8 visualization, 546 nm excitation wavelength and 556–573 nm emission range for G4 visualization, and 646 nm excitation wavelength and 681–697 nm emission range for nuclei visualization.

Confocal microscopy colocalization analysis (Fig. 3) shows an almost complete overlapping between G4s induced during the viral infection and replication compartments (RCs) where ICP8, an essential component of the HSV-1 DNA replication machinery implicated in the assembly of viral pre-replication and RCs, localized. This evidence supports formation of viral G4s during viral replication.

3.5 Analysis of Microscopy Images

Different open-source software can be used for the analysis of the acquired images, for example, ImageJ (https://imagej.nih.gov/ij/).

1. Save images for the different channels as TIFF files.
2. Load images for different channels separately on ImageJ and merge them.
3. Using the ImageJ Plot profile tool, draw an ideal line across the cell and obtain the 2D-intensity profile (Fig. 4), or the JACoP colocalization plugin [28] to obtain the overlapping coefficient.

For more information, see the ImageJ tutorial (https://imagej.nih.gov/ij/docs/examples/index.html).

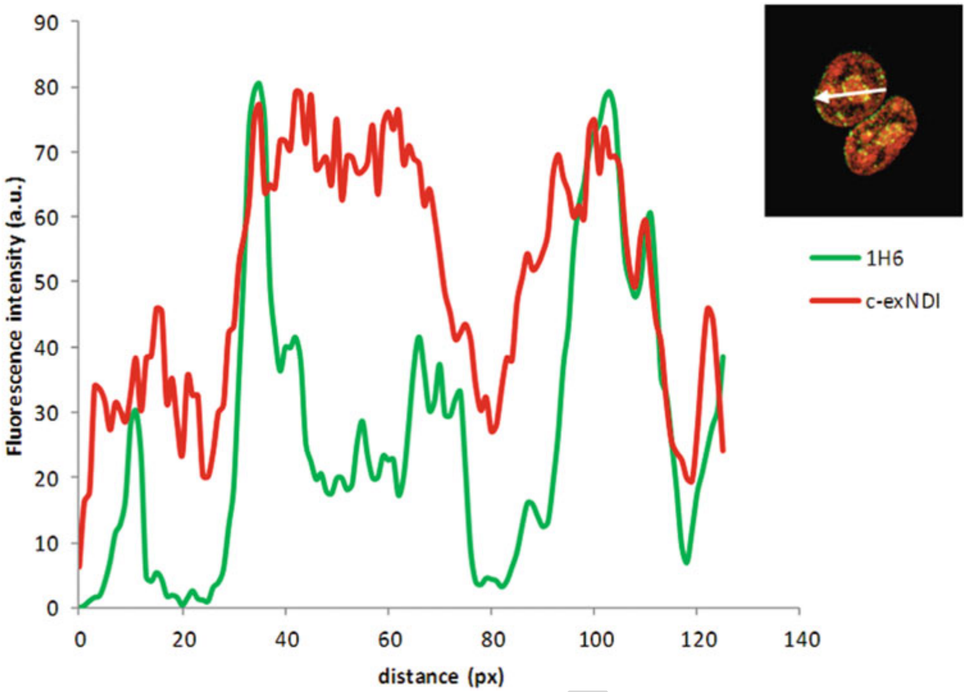

Fig. 4 Colocalization of *c-exNDI* and G4s by confocal microscopy. Intensity profiles of *c-exNDI* (red) and G4s (green) obtained using ImageJ software, along an ideal straight line (white) crossing the nucleus of a representative cell (right inset). Intensity profiles refer to Fig. 1

4 Notes 293

1. The amount of fetal bovine serum (FBS) to supplement cell culture medium depends on the cell line. Typically, it spans from 5% to 10%, but check cell line specifications. Poly-D-lysine promotes the adhesion of cells to the culture vessel, it should be used only if cells tend to detach easily from the culture vessel.
2. HSV-1 strain F was a kind gift from Bernard Roizman, University of Chicago, IL, USA. Other HSV-1 strains can be chosen, depending on the purpose of the experiment. Particular care must be taken when choosing the virus strain and host cell line. Produce virus stock and titrate it according to virological protocols.
3. *c-exNDI* was synthetized by Prof. Freccero's group [23, 29]. Any other fluorescent compound reported to bind G4 and to enter cell nucleus can be used. Attention should be given to the fluorescence emission spectrum of the compound.
4. The choice of the permeabilizing agent is particularly critical. If permeabilization is too strong, anti-G4 antibody recognition

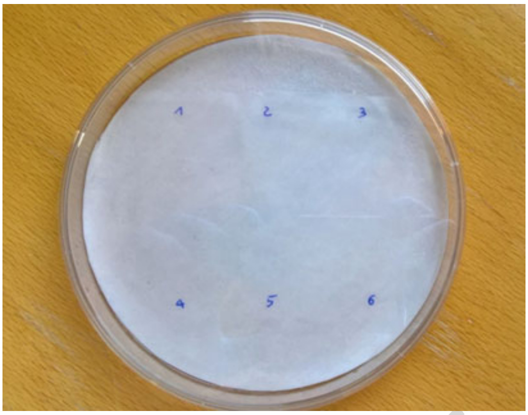

Fig. 5 Humidified chamber made using a petri dish

could be lost. We obtained our best results using 0.5% Tween-20.

5. 1H6 antibody, specific for G4 DNA, was kindly provided by P. Lansdorp, European Research Institute for the Biology of Ageing, University of Groeningen, the Netherlands.
6. The choice of the fluorescent secondary antibody has to be done taking in consideration the source of primary anti-G4 antibody and fluorescence emission properties of G4-binding compound. Any cross talk between antibody and compound has to be avoided.
7. Coverslips can be sterilized by dipping them in ethanol. Subsequently let them dry and wash with DPBS. The number of cells to be seeded depends on cell morphology and doubling time. Optimal confluency is around 70% on the day of cells fixation.
8. Dim light is required to avoid *c-exNDI* and fluorescent secondary antibody bleaching.
9. To visualize *c-exNDI* staining in live cells, proceed to fluorescence or confocal microscopy: place a drop of DPBS onto microscope slide, put the coverslip face-down on the drop and image cells.
10. After fixation, cells can be kept at 4 °C in the dark.
11. Treatment with RNaseA is used to digest RNA and visualize DNA G4. If you wish to visualize RNA G4, treat slides with 200 units DNase I for 30 min at 37 °C on a rocker.
12. An easy way to have a humidified chamber is using a 15 cm petri dish with water-soaked filter paper and a parafilm layer (Fig. 5). Place a drop of reagent (about 30 μL) on the parafilm layer, and coverslips face-down on the drop.
13. Fixed, mounted, and sealed slides can be stored at 4 °C in the dark.

14. According to lab/facility procedure for confocal microscopy acquisition. In particular, be careful not to saturate the fluorescence signal. We preferred to perform single laser scanning instead of sequential scanning, to avoid any undesired and unspecific fluorescence signal due to laser-fluorophores cross talks.
15. Use serum-free medium to dilute viral stock and to infect cells, and complete medium to grow and maintain cells.

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