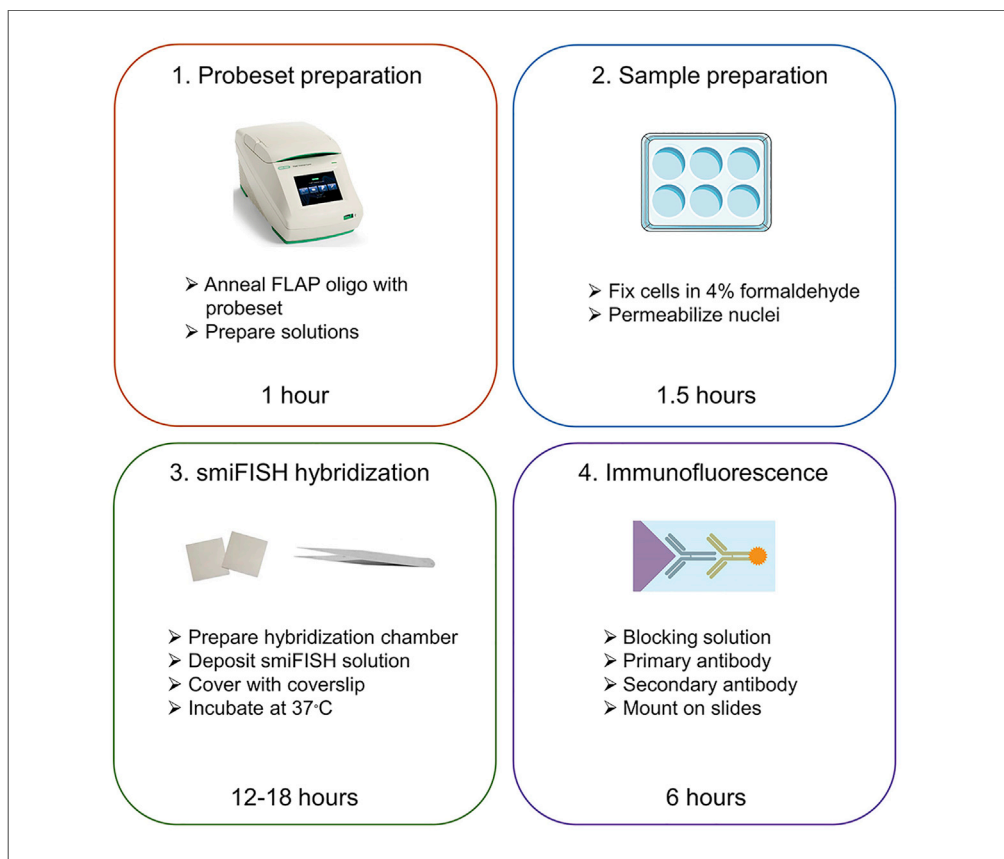


## Protocol

# Imaging of Telomerase RNA by Single-Molecule Inexpensive FISH Combined with Immunofluorescence



Fluorescent *in situ* hybridization (FISH) on the RNA moiety of human telomerase (hTR) with 50-mer probes detects hTR RNA accumulated in Cajal bodies. Using both live-cell imaging and single-molecule inexpensive FISH, our published work revealed that only a fraction of hTR localizes to Cajal bodies, with the majority of hTR molecules distributed throughout the nucleoplasm. This protocol is an application guide to the smiFISH method for the dual detection of hTR RNA and telomeres or Cajal bodies by immunofluorescence.

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### HIGHLIGHTS

RNA smiFISH with multiple small probes reveals single molecules of hTR RNA in nucleus

The smiFISH technique is compatible with immunofluorescence for colocalization assay

Colocalized regions can be mapped in 3D images with the open source 3D ImageJ Suite

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## Protocol

## Imaging of Telomerase RNA by Single-Molecule Inexpensive FISH Combined with Immunofluorescence

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<https://doi.org/10.1016/j.xpro.2020.100104>

## SUMMARY

Fluorescent *in situ* hybridization (FISH) on the RNA moiety of human telomerase (hTR) with 50-mer probes detects hTR RNA accumulated in Cajal bodies. Using both live-cell imaging and single-molecule inexpensive FISH, our published work revealed that only a fraction of hTR localizes to Cajal bodies, with the majority of hTR molecules distributed throughout the nucleoplasm. This protocol is an application guide to the smiFISH method for the dual detection of hTR RNA and telomeres or Cajal bodies by immunofluorescence.

For complete details on the use and execution of this protocol, please refer to Laprade et al. (2020).

## BEFORE YOU BEGIN

## Design and Order smiFISH Probes

⌚ Timing: 2–3 weeks to receive probes

**Note:** smiFISH is a single-molecule RNA FISH method developed by the labs of Florian Mueller and Edouard Bertrand, and is extensively described in their publication “smiFISH and FISH-quant – a flexible single RNA detection approach with super-resolution capability” by Tsanov et al. (2016). The user should refer to that paper for the complete guide to the smiFISH method and probeset design considerations.

## Generate Probeset with Oligostan R Script

1. Oligostan software was developed by Thierry Gostan of the SERANAD platform of IGMM France. Please refer to the Oligostan documentation for full details (link provided in the [Key Resources Table](#)).
  - a. Save the hTR 451 bp sequence as a FASTA file.
  - b. Run Oligostan script with the default settings (gene-specific probe length 26 to 32 nucleotides, probe GC content between 40%–60%, probe obeys PNAS rules 1, 2, and 4 as described in the Oligostan documentation). Only two probes are obtained for hTR.
  - c. Change the Oligostan settings so that the PNAS filters are not applied (set to FALSE) and raise the maximum GC content to 70%. With these changes 8 probes are obtained for hTR, identified as TERC1 to TERC8 in [Table 1](#).
  - d. To obtain more probes, reduce minimum probe size to 23 and increase maximum GC percentage to 85%. With these permissive settings, 15 probes are obtained for



hTR with delta G 37°C between –36 and –28 kcal/mol as recommended in [Tsanov et al. 2016](#) (sequences TRProbe1 to TRProbe15 in [Table 1](#)). The 15 smiFISH probes are illustrated in [Figure 1](#).

- e. BLAST all the probes with blastn to ensure they meet criteria as described in the Oligostan documentation.

**Note:** We chose the FLAP-Y sequence for all the probes and ordered both the 8 probes and 15 probes sets from Integrated DNA Technologies Inc., along with the FLAP-Y-Cy3 and FLAP-Y-Cy5 oligos (listed in [Table 1](#)). This allowed us to test both sets to check if they generated specific signal for hTR RNA, as shown in [Figure 2](#). The hTR smiFISH signal was nuclear in HeLa cells with both sets, while positive control GAPDH mRNA was mainly cytoplasmic. The WI38-VA13 clone cells were negative for hTR smiFISH signal with both probesets. With the smiFISH probes, hTR was detected all over the nucleus, unlike what is obtained with 50-mer probes for hTR FISH ([Zhu et al., 2004](#)). It is likely that the combination of multiple smiFISH probes for hTR and the lower formamide hybridization conditions favor probe binding to hTR RNA and thus enable single-molecule detection.

△ **CRITICAL:** To validate a new set of smiFISH RNA probes, perform a negative control FISH on a cell line that does not express the target RNA. If such cells are not available for the RNA of interest, you can deplete the target by RNA interference as shown in [Querido et al. \(2017\)](#). RNase-treated cells are not a sufficient control since they will be negative even if many probes in the new set cross-react with other RNAs.

### Set Up the Immunofluorescence Detection

⌚ **Timing:** 2 days or more

2. Before combining smiFISH and immunofluorescence (IF), the antibody detection step should be validated. This step should be as short as possible to minimize smiFISH signal loss.
  - a. Test several concentrations of your primary antibody with a 2-h incubation time at 20°C–22°C (using the permeabilization, blocking, and IF antibody buffer conditions listed in this protocol).
  - b. Test higher dilution of the secondary antibody to minimize background. This is especially important for super-resolution microscopy.
  - c. Once antibody incubation is optimized, test smiFISH and IF separately, and combined. Compare the signals obtained with each condition.

△ **CRITICAL:** Always include controls with no antibody and secondary antibody alone.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal Anti-TRF2 antibody produced in rabbit	Novus Biologicals	Cat# NB110-57130; RRID: <a href="#">AB_844199</a>
Monoclonal Anti-Coilin antibody produced in mouse, Clone pdelta	Sigma-Aldrich	Cat# C1862; RRID: <a href="#">AB_476827</a>

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor®488 AffiniPure Donkey Anti-Mouse IgG(H+L)	Jackson ImmunoResearch	Cat# 715-545-150; RRID: <a href="#">AB_2340846</a>
Alexa Fluor® 594 AffiniPure Donkey Anti-Rabbit IgG(H+L)	Jackson ImmunoResearch	Cat# 711-585-152; RRID: <a href="#">AB_2340621</a>
Alexa Fluor® 647 Anti-Coilin mouse monoclonal antibody, Clone pdelta	Novus Biologicals (Discontinued)	Cat# NB600-1275AF647; RRID: <a href="#">AB_608469</a>
Alternative: Alexa Fluor® 647 Anti-Coilin mouse monoclonal antibody, Clone pdelta	Santa Cruz	Cat# sc-56298 AF647
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
32% Paraformaldehyde (formaldehyde) aqueous solution	Electron Microscopy Sciences	15714(EM)
Formamide (certified ACS)	Thermo Fisher Scientific	F84-1
Dextran Sulfate	Thermo Fisher Scientific	BP1585
Ribonucleoside-Vanadyl Complex (VRC)	New England Biolabs	S14025
NEBuffer 3	New England Biolabs	B7003S
Triton™ X-100 BioXtra	Sigma-Aldrich	T9284
Bovine Serum Albumin 98%	Sigma-Aldrich	A3059
tRNA from <i>E. coli</i> MRE 600 (Roche)	Sigma-Aldrich	10109541001
Gelatin from porcine skin, Type A Cell culture tested	Sigma-Aldrich	G1890
Hoechst 33342 – FluoroPure™ Grade	Thermo Fisher Scientific	P10144
ProLong™ Glass Antifade Mountant	Thermo Fisher Scientific	P36982
VECTASHIELD® Antifade Mounting Medium with DAPI	Thermo Fisher Scientific	H1200
<b>Experimental Models: Cell Lines</b>		
HeLa 1.3 (HeLa 1.2.11 clone 1.3)	De Lange Laboratory, Rockefeller University	N/A
HeLa 1.3 hTR-5'-3xMS2	<a href="#">Laprade et al. 2020</a>	N/A
WI-38 VA13	ATCC	CCL-75.1
<b>Oligonucleotides</b>		
Probes for smiFISH	See <a href="#">Table 1</a>	N/A
<b>Software and Algorithms</b>		
Fiji ImageJ software	<a href="#">Schneider et al., 2012</a>	<a href="https://imagej.net/Fiji">https://imagej.net/Fiji</a>
3D ImageJ Suite with RoiManager 3D	<a href="#">Ollion et al., 2013</a>	<a href="https://imagejdocu.tudor.lu/plugin/stacks/3d_ij_suite/start">https://imagejdocu.tudor.lu/plugin/stacks/3d_ij_suite/start</a>
FigureJ ImageJ plugin	N/A	<a href="https://imagejdocu.tudor.lu/plugin/utilities/Figurej/start">https://imagejdocu.tudor.lu/plugin/utilities/Figurej/start</a>
BLASTN suite	National Center for Biotechnology Information	<a href="https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&amp;PAGE_TYPE=BlastSearch&amp;PROGRAM=blastn">https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&amp;PAGE_TYPE=BlastSearch&amp;PROGRAM=blastn</a>
Oligostan R script	<a href="#">Tsanov et al., 2016</a>	<a href="https://bitbucket.org/muellerflorian/fish_quant/src/master/Oligostan/Oligostan.r">https://bitbucket.org/muellerflorian/fish_quant/src/master/Oligostan/Oligostan.r</a>
Oligostan documentation	<a href="#">Tsanov et al., 2016</a>	<a href="https://bitbucket.org/muellerflorian/fish_quant/src/master/Oligostan/Documentation/">https://bitbucket.org/muellerflorian/fish_quant/src/master/Oligostan/Documentation/</a>
R software	The R Project for Statistical Computing	<a href="https://www.r-project.org/">https://www.r-project.org/</a>

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
R Studio Desktop software	R Studio	<a href="https://rstudio.com/">https://rstudio.com/</a>
Zen Microscopy software	Carl Zeiss Microscopy GmbH	<a href="https://www.zeiss.com/microscopy/int/products/microscope-software.html">https://www.zeiss.com/microscopy/int/products/microscope-software.html</a>
Other		
TetraSpeck™ Microspheres, 0.1 μm, fluorescent blue/green/orange/dark red	ThermoFisher Scientific	T7279
SSC buffer, 20×, Sterile, Mol. grade	Wisent	880-040-LL
PBS 10×, pH 7.4 without Ca <sup>2+</sup> and Mg <sup>2+</sup>	Wisent	311-012-CL
Fisherbrand Cover glasses 22 × 22 mm no1.5	ThermoFisher Scientific	12-541B
Pkg of 2, 16 × 20 cm, glass plates	Bio-Rad	1651821
PARAFILM® M 4 in. wide roll	Bemis	PM999
RNaseZAP™ cleaning agent	Sigma-Aldrich	R2020

**Table 1. Sequences of the smiFISH Probes for hTR and hGAPDH RNA, and the FLAP-Y Oligos**

Name	Sequence
FLAP-Y-Cy3	/5Cy3/AATGCATGTCGACGAGGTCCGAGTGTA/3Cy3Sp/
FLAP-Y-Cy5	/5Cy5/AATGCATGTCGACGAGGTCCGAGTGTA/3Cy5Sp/
TRProbe1	GCATGTGTGAGCCGAGTCTGGGTGCTTACTCGGACCTCGTCGACATGCATT
TRProbe2	CGCGCGGGGACTCGCTCCGTTCTTACTCGGACCTCGTCGACATGCATT
TRProbe3	TTCTGCGCCTGAAAGGCTGAATTACTCGGACCTCGTCGACATGCATT
TRProbe4	GGGCCAGCAGCTGACATTTTTGTTTACTCGGACCTCGTCGACATGCATT
TRProbe5	GGCTTTCCGCCCGCTGAAAGTCAGTTACTCGGACCTCGTCGACATGCATT
TRProbe6	GTCCACAGCTCAGGAATCGCGTTACTCGGACCTCGTCGACATGCATT
TRProbe7	GCCCAACTCTTCGCGGTGGCAGTGTACTCGGACCTCGTCGACATGCATT
TRProbe8	GCGGCCTCCAGGCGGGTTCGGTTACTCGGACCTCGTCGACATGCATT
TRProbe9	CCGCAGTCCCCGGGAGGGCGATTACTCGGACCTCGTCGACATGCATT
TRProbe10	AGAATGAACGGTGAAGGCGGCAGGCTTACTCGGACCTCGTCGACATGCATT
TRProbe11	CGCCTACGCCCTTCTCAGTTAGGTTTTACTCGGACCTCGTCGACATGCATT
TRProbe12	CCCCGAGAGACCCGCGGCTGACATTACTCGGACCTCGTCGACATGCATT
TRProbe13	CCTCCGGAGAAGCCCCGGGCCGATTACTCGGACCTCGTCGACATGCATT
TRProbe14	GAAAAACAGCGCGGGGAGCAAAAGCACTTACTCGGACCTCGTCGACATGCATT
TRProbe15	ACAAAAATGGCCACCACCCTCCCTTACTCGGACCTCGTCGACATGCATT
TERC1	GGCGAACGGGCCAGCAGCTGACATTTTACTCGGACCTCGTCGACATGCATT
TERC2	TGTTTGTCTAGAAATGAACGGTGAAGGCGGTTACTCGGACCTCGTCGACATGCATT
TERC3	CATGTGTGAGCCGAGTCTGGGTGCTTACTCGGACCTCGTCGACATGCATT
TERC4	CGTTCCTTCTGCGCCTGAAAGTTACTCGGACCTCGTCGACATGCATT
TERC5	CTGACAGAGCCCACTTTCGCGGTGTTACTCGGACCTCGTCGACATGCATT
TERC6	CCGAGGCTTTCCGCCCGCTGAAAGTTACTCGGACCTCGTCGACATGCATT

(Continued on next page)

**Table 1. Continued**

Name	Sequence
TERC7	CGCCTACGCCCTTCTCAGTTAGGGTTTTACTCTCGGACCTCGTCGACATGCATT
TERC8	GCGAGAAAAACAGCGCGCGGGGAGCAAAGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_1	GCTGGCGACGCAAAGAAGATGCGGCTTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_2	TGACCAGGCGCCCAATACGACCAAATTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_3	CAATATCCACTTTACCAGAGTAAAAGCAGCCTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_4	AACCATGTAGTTGAGGTCAATGAAGGGGTCAATTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_5	CCATGGAATTTGCCATGGGTGGAATCATATTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_6	TGACAAGCTTCCCCTTCTCAGCCTTGTACTCTCGGACCTCGTCGACATGCATT
GAPDH_7	TCGCTCCTGGAAGATGGTGTGGGATTTCTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_8	CCCAGCCTTCTCCATGGTGGTGAAGATTACTCTCGGACCTCGTCGACATGCATT
GAPDH_9	GAGGGGGCAGAGATGATGACCCTTTTGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_10	ACACCCATGACGAACATGGGGGCATTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_11	GATCTTGAGGCTGTTGTCATACTTCTCATGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_12	TAAGCAGTTGGTGGTGCAGGAGGCATTGCTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_13	AGTTGTATGGATGACCTTGGCCAGGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_14	GACTGTGGTCATGAGTCCTTCCACGATATTACTCTCGGACCTCGTCGACATGCATT
GAPDH_15	CCACAGTCTTCTGGGTGGCAGTGTGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_16	TTGGCAGGTTTTTCTAGACGGCAGGTCAGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_17	CCTGCTTACCACCTTCTTGTGTCATTACTCTCGGACCTCGTCGACATGCATT
GAPDH_18	CTCAGTGTAGCCAGGATGCCCTTGATTACTCTCGGACCTCGTCGACATGCATT
GAPDH_19	TGGGTGTCGCTGTTGAAGTCAGAGGAGACCTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_20	TGTCATACCAGGAAATGAGCTTGACAAAGTGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_21	ATGAGGTCCACCACCTGTTGCTGTATTACTCTCGGACCTCGTCGACATGCATT
GAPDH_22	CCAGCAGTGAGGGTCTCTCTTCTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_23	CAGTAGAGGCAGGGATGATGTTCTGGTTACTCTCGGACCTCGTCGACATGCATT
GAPDH_24	GTTCAGCTCAGGGATGACCTTGCTTACTCTCGGACCTCGTCGACATGCATT

## MATERIALS AND EQUIPMENT

### FLAP hybridization reaction mix for PCR cycler

Reagent	Final Concentration	Volume ( $\mu$ L)
MilliQ water	n/a	6.5
10 $\times$ NEBuffer 3	1 $\times$	1
20 $\mu$ M hTR 15 probeset	4 $\mu$ M	2
100 $\mu$ M FLAP-Y-Cy5 oligo	5 $\mu$ M	0.5
Total	n/a	10

**△ CRITICAL:** The FLAP oligo must be in excess of the smiFISH probeset molecules. Throughout this protocol molecular grade nuclease-free ddH<sub>2</sub>O is used to prepare solutions and reaction mixes. Freshly filtered MilliQ or commercial nuclease-free water are good options.

#### Fixation buffer

Reagent	Final Concentration	Volume (mL)
MilliQ water	n/a	31
10× PBS nuclease-free	1×	4
32% Paraformaldehyde (formaldehyde)	4%	5
Total	n/a	40

△ **CRITICAL:** Prepare fresh. Formaldehyde is a carcinogen. Use in a chemical fume hood. We recommend the Electron Microscopy Sciences 10 mL ampoules sealed under inert gas to minimize artifacts.

#### smiFISH wash buffer

Reagent	Final Concentration	Volume (mL)
MilliQ water	n/a	80
20× SSC nuclease-free	1×	5
Formamide	15%	15
Total	n/a	100

Prepare fresh. Formamide is toxic and should be used in a chemical fume hood. The 6-well plate can be wrapped with plastic film to prevent exposure when smiFISH washes are performed.

#### smiFISH hybridization mix

Reagent	Final Concentration	Volume (μL)
MilliQ water	n/a	26.3
20× SSC nuclease-free	1×	5
Formamide	15%	15
2% BSA nuclease-free*	4.5 mg/mL	22.5
40% dextran sulfate**	10.6%	26.5
VRC (200 mM)	2 mM	1
tRNA (25 mg/mL)***	0.425 mg/mL	1.7
FLAP-annealed probeset	0.08 μM	2
Total	n/a	100

Prepare on ice and mix well by vortexing, spin down, and keep on ice. Try to minimize bubble formation.

**Note:** A rocking platform adjusted to a low angle and speed should be used to provide gentle agitation of the 6-well plates with the coverslips during incubation and washing steps.

\*Prepare BSA 20 mg/mL (2%) solution in nuclease-free water, filter with 0.2 μm, aliquot and store at −20°C. Note that a higher BSA concentration than Tsanov et al. 2016 is used in the hybridization mix with the goal of reducing non-specific probe sticking.

\*\*Heat 40% Dextran in nuclease-free water to 65°C to dissolve fully, 0.2-μm filter, and keep at 4°C. Dextran sulfate concentration in the hybridization solution is critical to successful RNA *in situ* hybridization.

\*\*\*Prepare tRNA 25 mg/mL solution in sterile nuclease-free water, aliquot and keep at −20°C.

#### IF antibody buffer

Reagent	Final Concentration	Volume (mL)
MilliQ water	n/a	to 100
10× PBS nuc-free	1×	10
Triton X-100	0.1%	0.1
BSA nuclease-free	2%	n/a
Total	n/a	100

Prepare fresh. Dissolve well and pass through a 0.2-μm filter to sterilize and remove undissolved BSA. Keep on ice.

**Alternatives:** Triton™ X-100 is included in European REACH Annex XIV. TERGITOL™ 15-S-9 is a suggested alternative, but we have not tested it.

### STEP-BY-STEP METHOD DETAILS

#### Anneal smiFISH Probeset with FLAP Oligo

⌚ Timing: ~1 h

The principle of smiFISH is that the oligo marked with the two dye molecules (the FLAP) is complementary to the 3' end of each probeset oligo. They must be annealed together before using the probeset.

1. Anneal FLAP oligo to the probeset in a PCR machine
  - a. The smiFISH probeset is prepared as a 20 μM equimolar mixture of the 15 hTR probes in Tris-EDTA pH8.0 (TE).
  - b. Anneal in PCR cycler then spin down and store on ice protected from light.

PCR conditions		
Steps	Temperature	Time
Denaturation	85°C	3 min
Annealing	65°C	3 min
Annealing	25°C	5 min
Lid: 99°C		

**Note:** For dual-color FISH as shown in [Figure 2](#), the same FLAP sequence can be used since each probeset is annealed with its FLAP-dye oligo in a separate tube. Once annealed, we have not seen any exchanges between FLAP-dye oligos in dual-color FISH.

#### Prepare Samples

⌚ Timing: ~1.5 h

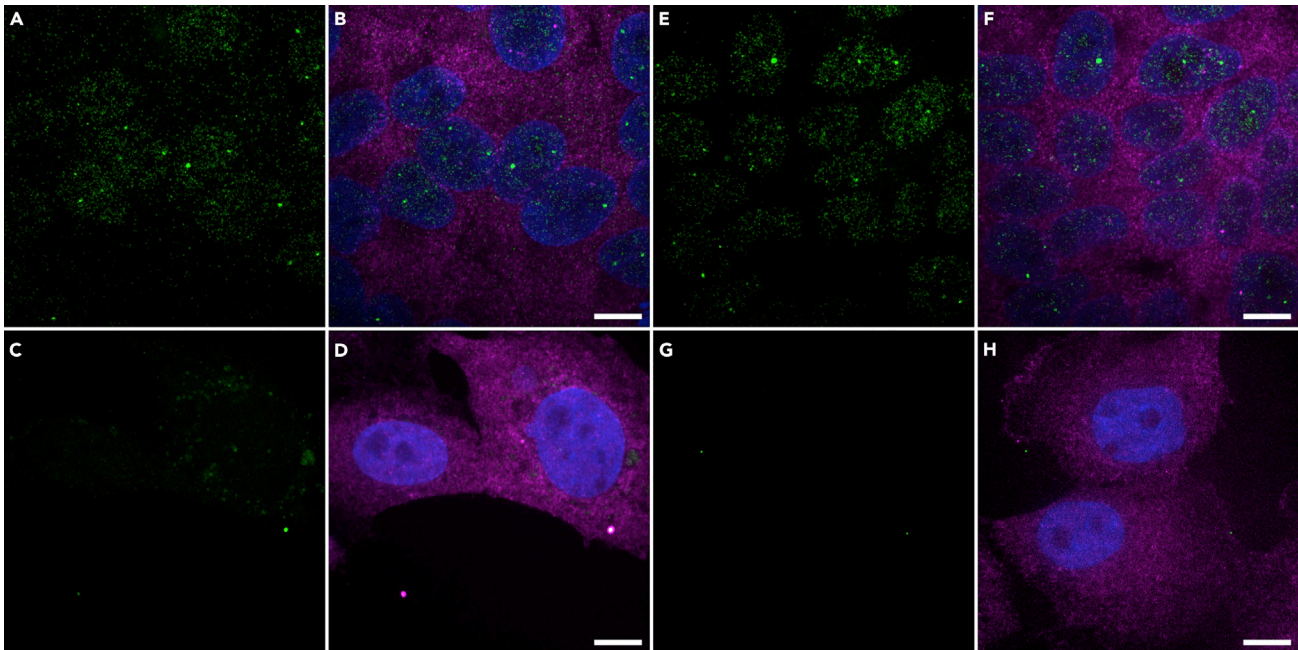
To detect RNA and proteins by microscopy, the cells must be grown on glass coverslips. Once they reach an appropriate density, the cells are fixed by formaldehyde cross-linking, then permeabilized to create openings for the fluorescent probes and antibodies to enter.

**Note:** Prepare gelatin-coated coverslips fresh for every experiment. Autoclave acid-washed coverslips in a beaker of water with 0.5% gelatin. Allow the gelatin beaker to cool then place coverslips in a 6-well plate, rinse with 1× PBS, replace with media and cells. Do not let the gelatin-coated coverslips dry. Alternative coverslip preparation methods appropriate to the cell type are also compatible with smiFISH.

2. Cells grown in a 6-well plate on 22 × 22 mm no. 1.5 coverslips are fixed in 4% formaldehyde (fixation buffer).
  - a. Bring 6-well plate to the chemical fume hood. Remove media and replace with 2 mL fixation buffer per well. Wrap plate in plastic film and incubate for 20 min at 20°C–22°C with gentle agitation.
  - b. Rinse twice with 2 mL per well of 1× PBS.







**Figure 2. Validation of the 8 Probes and 15 Probes Sets for hTR smiFISH**

Dual-color smiFISH with 8 hTR smiFISH probes (A–D) or 15 hTR smiFISH probes (E–H), and 24 GAPDH smiFISH probes. smiFISH was performed on HeLa cells (A, B, E, and F) and hTR-negative WI38-VA13 cells (C, D, G, and H). hTR smiFISH probes labeled with FLAP-Y-Cy3 are shown in green. (B, D, F, and H) Merge with GAPDH smiFISH probes labeled with FLAP-Y-Cy5 (magenta) and DNA in blue. Maximum intensity projection of Z stacks acquired on a Zeiss Axio-Observer Z1 Yokogawa CSU-X1 spinning disk confocal microscope with a Photometrics Evolve EMCCD camera (resolution  $x, y = 0.133 \mu\text{m}$ ). Scale bars,  $10 \mu\text{m}$ .

**Note:** Clean needle-tip tweezers with RNaseZAP or similar solution, rinse in 70% ethanol.

**Pause Point:** If you are only performing smiFISH, the coverslips are now ready for DNA stain and mounting.

### Immunofluorescence

⌚ **Timing:** ~6 h

Before detecting the protein of interest with antibody, the cells are incubated with high protein solution to reduce non-specific sticking. After the primary antibody incubation, secondary antibody cross-linked to dye molecules is used to detect the primary antibody. The nuclear boundary is marked by staining the DNA with a fluorescent dye.

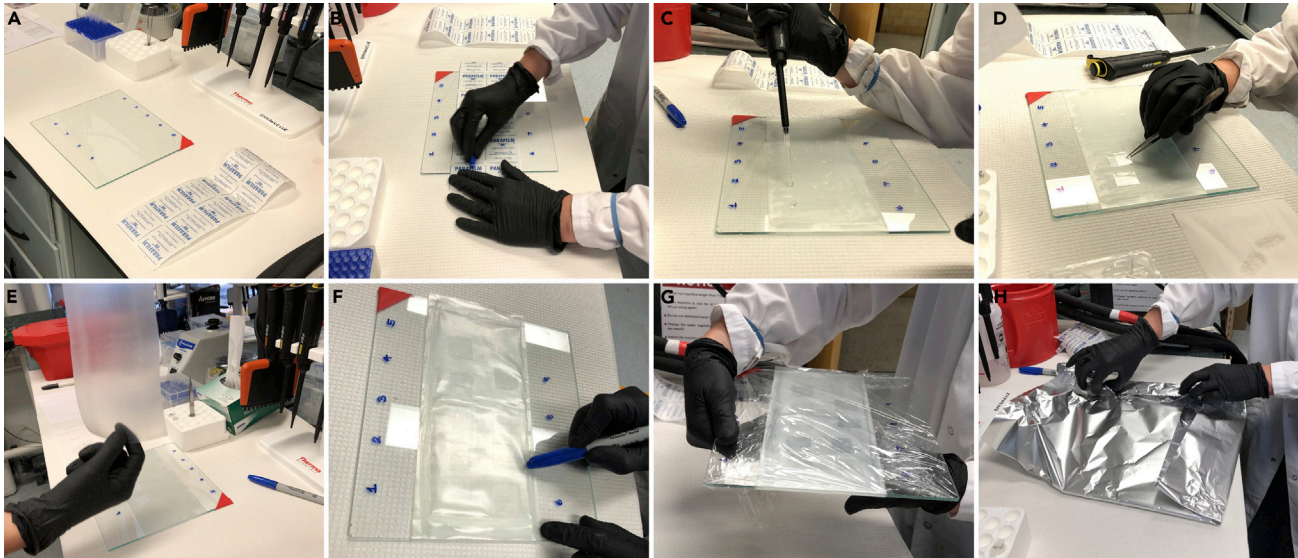
8. Incubate with 2 mL per well of IF antibody buffer for 1 to 1.5 h at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  with gentle agitation to block non-specific binding.

⚠ **CRITICAL:** Protect from light during all steps.

9. Dilute primary antibodies in IF antibody buffer. For [Figure 4](#), we combined anti-coilin mouse mAb 1: 500 and anti-TRF2 rabbit pAb 1:200.

10. Prepare antibody incubation chamber

- a. Use a clean 15 cm plastic dish with lid. Cut out a Parafilm circle and fix to the bottom of the dish with the paper side up.

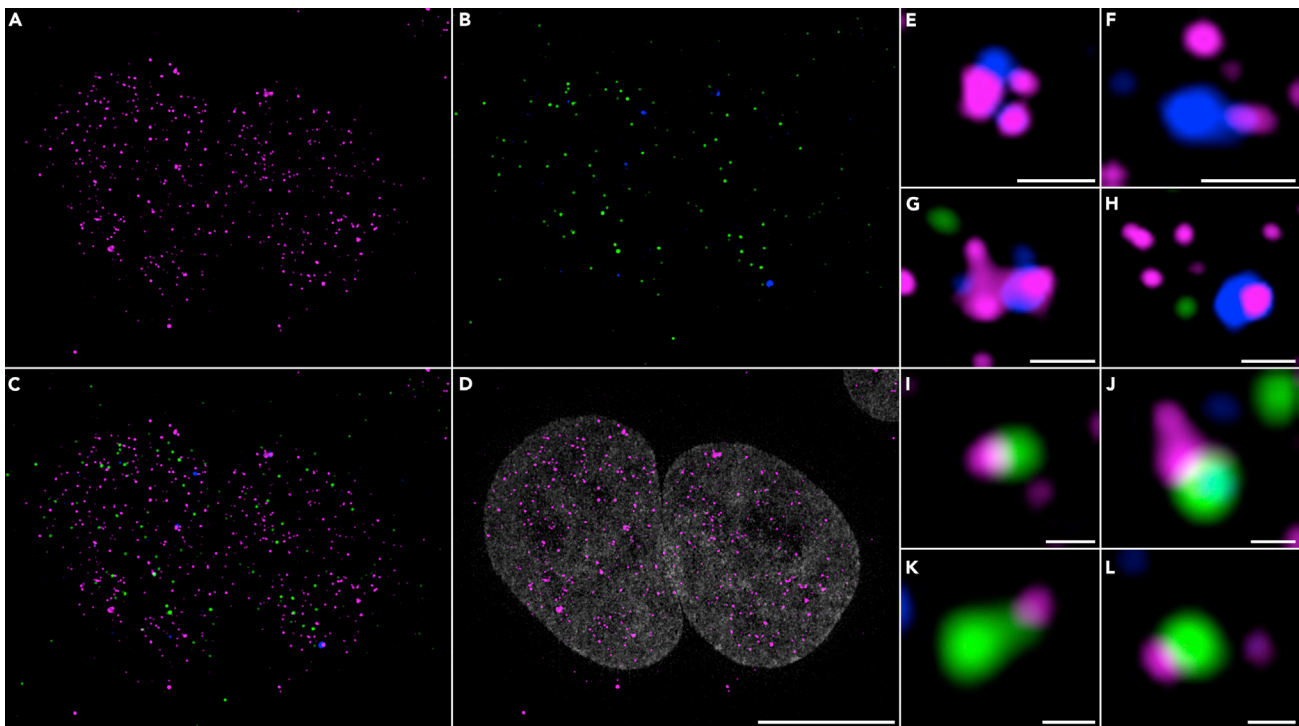


**Figure 3. Setup of an Airtight Hybridization Chamber on a Glass Plate**

- (A) Start with a clean 16 × 20 cm glass plate.  
 (B) Fix Parafilm to glass by pressing lightly with a rounded pen or PCR film paddle tool.  
 (C) Pipet 50 µL of smiFISH hybridization mix on Parafilm.  
 (D) Invert coverslip cell-side down on hybridization mix.  
 (E and F) (E) Cover with second sheet of Parafilm and (F) seal four sides.  
 (G and H) (G) Wrap in plastic film and (H) aluminum foil.

- b. Remove paper. Pick up coverslip and gently blot the edge on a Kimwipe to drain excess liquid. Deposit on Parafilm cell-side up.
  - c. Pipet 150 µL of 1st antibody solution on top of the coverslip. Replace dishcover without moving the dish.
11. Incubate primary antibody for 2 h at 20°C–22°C.
  12. Pick up coverslip and place in 6-well plate containing 1 × PBS 0.1% Triton X-100 wash buffer
  13. Wash 4 times (5 min each with gentle agitation) in 2 mL per well 1 × PBS 0.1% Triton X-100 wash buffer at 20°C–22°C.
  14. Wash twice in 2 mL per well of IF antibody buffer.
  15. Dilute secondary antibodies in IF antibody buffer. For [Figure 4](#), we combined anti-mouse AF488 1: 6500 and anti-rabbit AF594 1:6500.
  16. Prepare antibody incubation chamber as in step 10. Incubate 2nd antibody for 50 to 60 min at 20°C–22°C.
  17. Wash twice in 2 mL per well of IF antibody buffer.
  18. Wash 4 times in 2 mL per well of 1 × PBS 0.1% Triton X-100 wash buffer.
  19. Incubate with H33342 staining solution (0.5 µg/mL in 1 × PBS) for 5–10 min at 20°C–22°C.
  20. Wash twice with 1 mL/well 1 × PBS.
  21. Pick up coverslip and gently blot the edge on a Kimwipe to drain excess liquid. Mount one coverslip per slide with Prolong mounting media. Let it harden at 20°C–22°C protected from light for the recommended 24–72 h. Seal the four corners of the coverslip with a drop of nail polish and let dry completely before imaging.

**Alternatives:** Use other antifade mounting medium. We have also had good results with Vectashield (skip step 19 if mountant contains DAPI).



**Figure 4. Super-Resolution Imaging of hTR smiFISH Combined with IF against Coilin and TRF2**

(A) HeLa 1.3 cells hybridized with the 15 probes set for hTR smiFISH labeled with FLAP-Y-Cy5 shown in magenta.

(B) Coilin (in blue) and TRF2 (in green) were detected by immunofluorescence.

(C) Overlay of coilin, TRF2, and hTR smiFISH.

(D) Overlay of hTR with DNA stain in gray, scale bar, 10  $\mu\text{m}$ .

(E–L) Zoom on regions of image C showing Cajal bodies (E–H) or telomeres (I–L) with partially overlapped hTR signal. Panels (E–H) scale bars, 0.5  $\mu\text{m}$ .

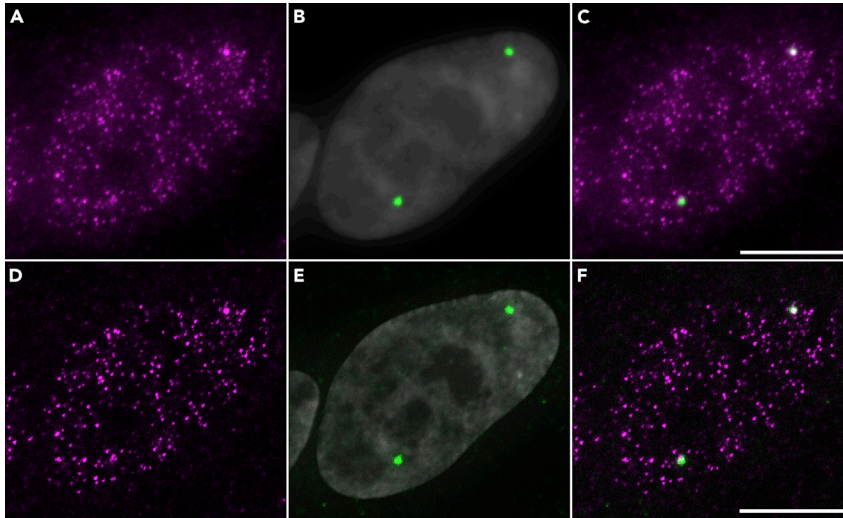
Panels (I–L) scale bars, 0.2  $\mu\text{m}$ . Maximum intensity projection of Z stacks acquired on a Zeiss Elyra PS.1 super-resolution microscope by structured illumination using an Andor iXon3 EMCCD camera (resolution after SR-SIM processing  $x, y = 0.040 \mu\text{m}$ ).

## EXPECTED OUTCOMES

Single-molecule RNA FISH typically has a low signal-to-noise ratio. Negative and positive controls should be included, as they are essential to determine appropriate settings for image acquisition and image analysis steps. The control cells should be negative or depleted for the RNA target. Highly expressed mRNAs are good positive controls for smiFISH, although they may not be resolved as single molecules. The sequences for a 24 probes set for human GAPDH are provided in [Table 1](#). [Figure 2](#) shows GAPDH smiFISH results in HeLa and VA13 cells photographed with a spinning disk confocal microscope.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Several software packages are available for image analysis of single-molecule RNA FISH but many work only on 2D images, usually by applying a maximum intensity projection (MIP) on the 3D Z stack. For the quantification of hTR single-molecule FISH, analysis of the image stacks in 3D was necessary as the nuclear volume contained many spots that could appear to falsely overlap in a MIP image. The 3D ImageJ Suite created by Thomas Boudier has a variety of segmentation options with the needed flexibility for this application ([Ollion et al., 2013](#)). A brief outline of the steps to segment the nuclei, hTR spots, and Cajal body regions with the 3D Suite, is given below. Values for background subtraction and seeds threshold will need to be set experimentally dependent on the signal-to-noise ratio of the smiFISH and IF spots.



**Figure 5. IF against Coilin Followed by hTR smiFISH Imaged by Epifluorescence Microscopy**

For this experiment, HeLa 1.3 hTR-5'-3xMS2 cells were incubated with coilin antibody labeled with AF647 for 1 h, followed by overnight smiFISH hybridization with the 15 hTR probeset plus 4 probes for the MS2 region (as described in [Laprade et al. 2020](#)) labeled with FLAP-Y-Cy3.

(A and D) hTR smiFISH shown in magenta.

(B and E) Coilin IF in green merged with DAPI in gray.

(C and F) Overlay of coilin and hTR.

(D–F) Images were deconvolved with the ZEN Fast Iterative algorithm default settings (Zeiss software). Maximum intensity projection of Z stacks acquired on a Zeiss Axio Imager Z2 epifluorescence microscope with a Photometrics sCMOS Prime camera (resolution  $x, y = 0.065 \mu\text{m}$ ). Scale bars,  $10 \mu\text{m}$ .

Steps for nuclei segmentation:

- 1 Save a copy of the DAPI or H333342 single-channel 16-bit Tiff Z stack
- 2 Process>Filters>GaussianBlur3D
- 3 Adjust Threshold - Li method
- 4 Plugin>3D>3D Simple Segmentation  
Save as segmented nuclei image
- 5 If necessary, separate nuclei that touch by drawing on the image, save it as a seed image, then apply seeded watershed (Plugin>3D>3D Watershed)
- 6 Import segmented nuclei into 3D RoiManager

Steps for hTR spot segmentation:

- 1 Save a copy of the hTR smiFISH single-channel 16-bit Tiff Z stack
- 2 Process>Math>Subtract background
- 3 Plugin>3D>3DFastFilters - MaximumLocal  
Save as seed image
- 4 Plugin>3D>3D Watershed  
Save as segmented spots image
- 5 Import segmented spots from one nucleus by selecting it into 3D RoiManager
- 6 Select hTR spots in 3D RoiManager and press 3D Quantify to measure on unmodified hTR 16-bit Tiff

Steps for Cajal body (CB) segmentation:

- 1 Save a copy of the coilin single-channel 16-bit Tiff Z stack
- 2 Process>Math>Subtract background

- 3 Plugin>3D>3DFastFilters - MaximumLocal  
Save as seed image
- 4 Plugin>3D>3D Spot segmentation-Gaussian fit classical  
Save as segmented CB image
- 5 Import segmented CB from one nucleus by selecting it into 3D RoiManager
- 6 Select CB and hTR spots in 3D RoiManager and press Colocalization to list the hTR spots and CB that overlap

**Note:** Prior to colocalization image analysis, the hTR, TRF2 and coilin image stacks should be aligned using reference multicolor bead images (Tetraspeck 0.1  $\mu\text{m}$  beads listed in the [Key Resources Table](#)). Prepare beads slide in the same mounting media used for the cells, and photograph in the same imaging session.

### LIMITATIONS

The length and sequence of the RNA could make probeset design challenging for some targets. The main limitation of single-molecule RNA FISH however is the microscope system itself. Epifluorescence microscopes have been shown to work well for single-molecule RNA FISH, as shown in [Raj et al. 2008](#) and [Tsanov et al., 2016](#), but the images might require deconvolution, as illustrated in [Figure 5](#). Scanning confocal microscopes may not be sufficiently sensitive for the weak single-molecule RNA FISH signal, but the spinning disk microscope has worked in our hands ([Figure 2](#)). We recommend lasers as the lightsource and an ultra-sensitive camera to capture the emission with a high magnification high NA objective. Structured illumination super-resolution microscopy (SR-SIM) has been particularly successful in resolving single RNA molecules as unique spots ([Figure 4](#)), as shown in [Adivarahan et al. 2018](#) and [Laprade et al. 2020](#).

### TROUBLESHOOTING

#### Problem 1

RNA smiFISH signal indistinguishable from background.

#### Potential Solutions

Change reagent(s) that might be contaminated with RNase. Bovine serum albumin (BSA) is a likely suspect. Collaborators have reported that they omitted the BSA from the smiFISH hybridization buffer and this solved the low signal problem. Use filter tips when pipetting.

If there is high endogenous fluorescence in your cell type in one channel, changing fluorophores for the FLAP oligo may help. In addition, if the lightsource is a Metal Halide lamp, there may not be enough power to excite Cy5 sufficiently for single-molecule imaging, Cy3 or AF488 could work much better.

If your RNA length permits, try adding more probes.

#### Problem 2

Immunofluorescence detection no longer works when combined with smiFISH.

#### Potential Solutions

A solution of 2 $\times$  SSC 10% formamide can be used for smiFISH incubation and washing, this lower formamide concentration might help to maintain the epitope for some antibodies.

The order of steps can be reversed, i.e., permeabilization, blocking, and antibody incubation can be performed on the first day, followed by smiFISH incubation overnight. However, the risk of RNA degradation is greater if antibody incubation is done first. [Figure 5](#) shows the results of an IF first-smiFISH second experiment.

The use of a primary antibody directly tagged with the dye of choice can sometimes resolve a high background problem and be ideal for smiFISH combined with IF.

If the protein of interest is GFP or mCherry-tagged, the fluorescence of these tags can be conserved during fixation and smiFISH procedure. Make sure that the pH of the fixation solution is close to pH 7.4 in order to preserve fluorescence.

## RESOURCE AVAILABILITY

### Lead Contact

Pascal Chartrand ([p.chartrand@umontreal.ca](mailto:p.chartrand@umontreal.ca)).

### Materials Availability

For cell lines, please refer to [Laprade et al., 2020](#). All other materials are available commercially.

### Data and Code Availability

The 16 bit TIFF files for the microscopy images in [Figures 2, 4, and 5](#) are available on a Mendeley archive created for this paper. The ImageJ 3D Suite analysis files are included for [Figure 4](#). Software is listed in the [Key Resources Table](#).

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## AUTHOR CONTRIBUTIONS

E.Q. developed the protocol. A.S. and P.C. supervised and provided comments on the text.

## DECLARATION OF INTERESTS

A.S. is a co-founder of Repare Therapeutics and a member of its scientific advisory board.

## REFERENCES

- Adivarahan, S., Livingston, N., Nicholson, B., Rahman, S., Wu, B., Rissland, O.S., and Zenklusen, D. (2018). Spatial organization of single mRNPs at different stages of the gene expression pathway. *Mol. Cell* 72, 727–738.
- Laprade, H., Querido, E., Smith, M.J., Guérit, D., Crimmins, H., Conomos, D., Pourret, E., Chartrand, P., and Sfeir, A. (2020). Single-molecule imaging of telomerase RNA reveals a Recruitment – Retention model for telomere elongation. *Mol. Cell* 79, 115–126.
- Ollion, J., Cochenec, J., Loll, F., Escudé, C., and Boudier, T. (2013). TANGO: A Generic Tool for High-throughput 3D Image Analysis for Studying Nuclear Organization. *Bioinformatics* 29, 1840–1841.
- Querido, E., Dekakra-Bellili, L., and Chartrand, P. (2017). RNA fluorescence in situ hybridization for high-content screening. *Methods* 126, 149–155.
- Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* 5, 877–879.
- Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675.
- Tsanov, N., Samacoits, A., Chouaib, R., Traboulsi, A.M., Gostan, T., Weber, C., Zimmer, C., Zibara, K., Walter, T., Peter, M., et al. (2016). smiFISH and FISH-quant - a flexible single RNA detection approach with super-resolution capability. *Nucleic Acids Res.* 44, e165.
- Zhu, Y., Tomlinson, R.L., Lukowiak, A.A., Terns, R.M., and Terns, M.P. (2004). Telomerase RNA accumulates in Cajal bodies in human cancer cells. *Mol. Biol. Cell* 15, 81–90.