# Single-cell Visualization of Chromosome Transcriptional Territories by RNA-paint

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**[Abstract]** We developed a FISH-based method to directly assess chromosome-wide transcriptional activity, thereby enabling the visualization of the actively transcribed fraction of a chromosome at the single-cell level. We applied this method to probe the activity of X-chromosomes and its instability in the context of human embryonic stem cells and cancer cells.

## Materials and Reagents

1. 24-well plates (Sigma-Aldrich, catalog number: Z707791-126EA)
2. 13 mm round coverslips (Thermo Fisher Scientific, catalog number: 174950)
3. Glass slides
4. Filter (0.2 µm)
5. Filtration unit (Merck Millipore, catalog number: SCGPS05RE)
6. Human embryonic stem cells (H9, WIBR2 and HUES1) and cancer cells (TCCSUP and RT112)
7. Matrigel
8. PBS solution (Life Technologies)
9. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
10. Sucrose (Sigma-Aldrich, catalog number: S0389)
11. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
12. PIPES (Sigma-Aldrich, catalog number: P6757)
13. NaOH
14. Triton X-100 (Sigma-Aldrich, catalog number: X100)
15. Ribonucleoside vanadyl complex (New England Biolabs, catalog number: S1402)
16. Paraformaldehyde (PFA) (16%, EM grade) (VWR International, catalog number: 100503-916)
17. EtOH
18. Human Cot-1 DNA (Life Technologies)
19. Sodium acetate anhydrous (NaOAc) (Sigma-Aldrich, catalog number: W302406)
20. Deionized formamide (Sigma-Aldrich, catalog number: F9037)
21. Rubber cement
22. 20x SSC (Sigma-Aldrich, catalog number: 93017)
23. 20 mg/ml BSA (New England Biolabs, catalog number: B9000S)
24. Dextran sulfate (Sigma-Aldrich, catalog number: 67578)

25. Formamide (Sigma-Aldrich, catalog number: 47670)
26. Mounting medium (Vector Laboratories, catalog number: H-1200)
27. Cy3-labelled human X-Chromosome paint (Metasystem, catalog number: D-0323-050-OR)
28. FITC-labelled human X-Chromosome paint (Metasystem, catalog number: D-0323-050-FI)
29. CSK buffer (see Recipes)
30. 3% PFA/PBS solution (see Recipes)
31. Hybridisation buffer (2x) (see Recipes)
32. Washing solution (50% formamide/2x SSC) (see Recipes)
33. Denaturating solution (70% formamide/2x SSC) (see Recipes)

## Equipment

1. Hybridisation table (Boekel Scientific, model: 240000)
2. Shake 'N Bake™ hybridisation oven (Boekel Scientific, model: 136400)
3. Centrifuge (VWR, Ependorf®, model: 5417R)
4. Millivac-mini vacuum pump (Merck Millipore, catalog number: XF5423050)
5. Ependorf® Thermomixer® R (Sigma-Aldrich, catalog number: T3317)
   *Note: This product has been discontinued.*
6. Fluorescent microscope (motorized stage) (Leica, model: DMI-6000)

## Procedure

1. Prepare 24-well plates with 13 mm round coverslips at the bottom of the wells. Coat coverslips with matrigel for human embryonic stem cells. Split cells in colonies with a ratio of 1:5 on coverslips. Grow cells for 24 to 48 h.
2. Wash cells with 1 ml PBS per well.
3. Incubate cells for 5 min on ice with 0.5 ml per well of ice-cold CSK buffer freshly supplemented with 0.5% Triton X-100 and 2 mM vanadyl ribonucleoside complex. The core CSK buffer can be kept at 4 °C several months.
4. Fix cells for 10 min at room temperature with 0.5 ml per well of PBS/3% PFA supplemented with 2 mM vanadyl ribonucleoside complex.
5. Rinse cells with 1 ml per well 3 times with cold EtOH (70%) for 4 min.
6. Either proceed to step 8 or the protocol can be stopped here by tightly wrapping the 24-well plate with parafilm, stretching it gently to ensure a good fit and avoid evaporation. Cells can be stored at -20 °C for one month.
7. On the day of the experiment, bring coverslips to 4 °C in 70% EtOH.
8. For probe preparation, for each coverslip, add 290 µl of water to 5 µl of concentrated chromosome paint and 5 µl of human Cot-1 DNA (1 µl/µg). Precipitate DNA by adding 1/10 3 M

NaOAc and 2.5 volumes of 100% EtOH and storing tubes overnight at -20 °C. Probes are prepared individually for each coverslip: 1 mix for 1 coverslip.

9. Spin probes at 15,000 *x g* at 4 °C for 20 min. The pellet should be visible and is washed 2 times with 70% EtOH and spun for 10 min at 15,000 *x g* at 4 °C.
10. Resuspend the pellet in 5 µl of 50% deionized formamide/50% hybridisation buffer (2x), and incubate for 10 min at 37 °C.
11. Denature the chromosome paint 7 min at 75 °C, incubate for 30 min at 37 °C and store it on ice.
12. In the meantime, dehydrate coverslips in 90% and 100% EtOH for 4 min each. Coverslips are then air dried on Kimwipes, cells face up. Heat clean glass slides on the hybridisation table at 37 °C, where the moats have been prefilled with water.
13. Dispense 5 µl of probe per coverslip on glass slides, and place coverslips on the probe, with cells facing down (Figure 1). Incubate the coverslips at 37 °C overnight, and evaporation around the coverslips is prevented using rubber cement to seal the coverslips to the glass slides.

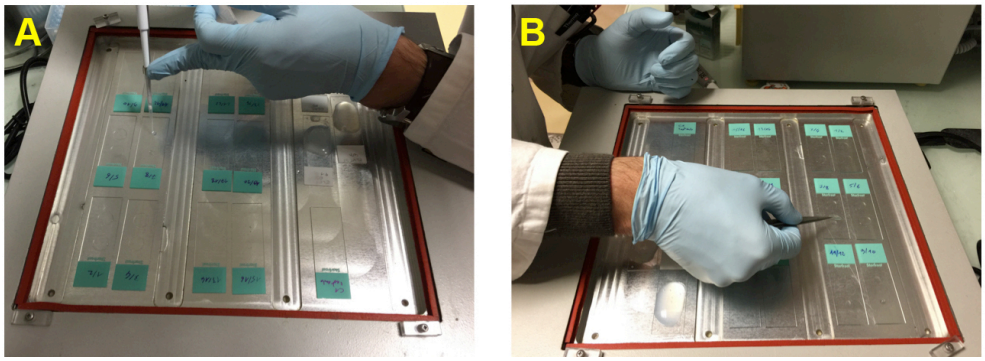


**Figure 1. Manipulating coverslips.** A. Putting 5 µl of probe on slides. B. Putting coverslips with cells face down on probe preparation.

14. The next day, remove rubber cement by gently drawing it from the coverslips. Spill 2 ml of 2x SSC over the coverslips and glass slides, and gently start pushing around the coverslips by making them float in 2x SSC. Fully detached coverslips are transferred to 24-well plates for further washes. Wash coverslips 3 times with 1 ml per well of 50% formamide/2x SSC, and 3 times in 2x SSC, all washes at 42 °C. Incubate 24-well plates with coverslips in hybridization oven with low agitation (shaker speed set at medium speed).
15. Mount coverslips on glass slides using 5 µl of mounting medium containing DAPI. Use transparent nail polish to seal the coverslips to the slides.
16. Observe fluorescent signals with a fluorescent microscope (magnification 100x).
    a. For dual RNA-FISH involving chromosome paint (Vallot *et al*., 2015)
        i. RNA-FISH is performed in two-steps. First, RNA-FISH with chromosome paint is performed overnight as described above (steps 1-14).

ii. Coverslips are washed 3-times in 50% formamide/2x SSC at 37 °C and re-incubated overnight with the second probe.

iii. Coverslips are washed 3-times in 50% formamide/2x SSC and 3-times in 2x SSC at 37 °C. Observe both signals simultaneously.

b. For successive RNA/DNA-FISH with chromosome paint (Vallot *et al.*, 2015)

i. RNA-FISH is performed on slides as described above using a Cy3-labelled paint to visualize RNA, take pictures and note cell coordinates using a stage-motorized Leica microscope.

ii. Wash slides, cells are permeabilized for 10 min in 0.1 N HCl/0.7% Triton X-100 on ice, treated with 100 µg/µl RNase in 2x SSC for 1 h at 37 °C.

iii. Denature slides for 10 min at 80 °C in 70% formamide/2x SSC.

iv. DNA is visualized using a FITC-labelled X chromosome paint; it is denatured for 2 min at 75 °C and incubated with slides overnight.

v. Wash slides 3 times with 2x SSC at 45 °C and 0.1x SSC at 60 °C.

## Representative data

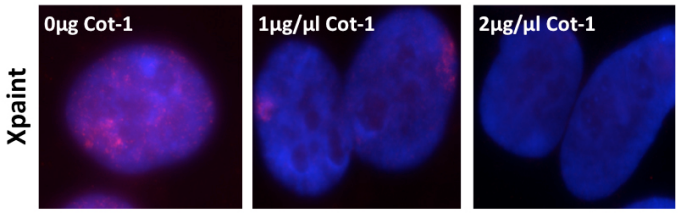


**Figure 2. Optimizing the X-chromosome paint RNA FISH.** 5 µl of cy3-X-chromosome paint are co-precipitated with various amounts of human Cot-1 DNA prior to over-night hybridisation. Optimization of the quantity of Cot-1 should be performed when changing supplier for chromosome paint.

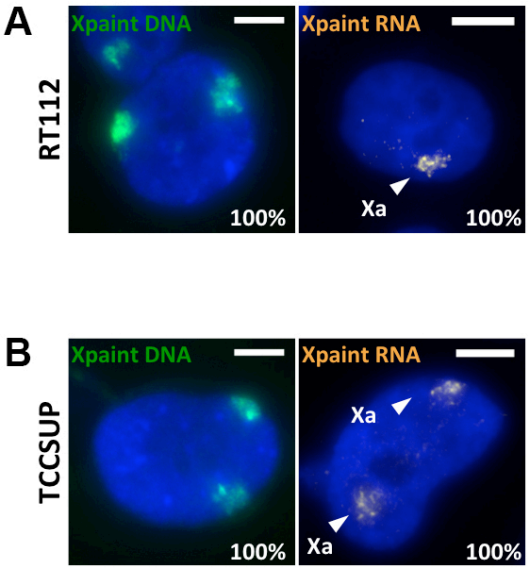


**Figure 3. Single-cell detection of X-chromosome activity in cancer cells.** X-chromosome content of two bladder cancer cell lines RT112 (A) and TCCSUP (B) was assessed by DNA-FISH using FITC-labeled X-chromosome paint (left panel) and X-chromosome activity by RNA-paint using cy3-labeled X-chromosome paint (right panel).The percentage in the right lower corner indicates the percentage of nuclei with displayed pattern.

## Recipes

1. CSK buffer
   100 mM NaCl
   300 mM sucrose
   3 mM $MgCl_2$
   10 mM PIPES
   Adjust pH to 6.8 with NaOH to facilitate dissolution of chemicals in water
   Filter (0.2 µm)
2. 3% PFA/PBS solution
   9.4 ml of 16% PFA solution
   40.6 ml of PBS
3. Hybridisation buffer (2x)
   4x SSC
   4 mg/ml BSA
   20% dextran sulfate
   40 mM vanadyl ribonuclside complex
4. Washing solution (50% formamide/2x SSC) (50 ml), pH = 7.2
   25 ml of formamide
   5 ml of 20x SSC

20 ml of $H_2O$
60 µl of 2.5 N HCl

5. Denaturating solution (70% formamide/2x SSC) (50 ml), pH = 7.2
   35 ml of formamide
   5 ml of 20x SSC
   10 ml of $H_2O$
   145 µl of 2.5 N HCl

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**References**

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