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IF-FISH of cells on 22mm glass coverslips

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1 Works for me dx.doi.org/10.17504/protocols.io.bqtdmwi6

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Fixing cells on coverslips

1 Adherent cells were cultured under the same conditions on glass 22mm x 22mm square coverslips one to two days before they were to be permeabilized and fixed.

2 At the start of the permeabilization and fixing procedure, coverslips were transferred into Coplin jars filled with cold CSK buffer (100mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM PIPES) and making sure to note which direction the cell coated side of the coverslip was facing. 5m

3 The coverslips were then transferred into cold CSK buffer containing 0.5ml of Triton-X in 10ml of CSK buffer for 8-10 minutes. 10m

4 The permeabilized coverslips were then transferred into 10ml of 4% paraformaldehyde in PBS for 8-10 minutes at room temperature to fix them. 10m

5 The coverslips were washed for 3-4 minutes in 70% ethanol then stored in jars of 70% ethanol at 4°C. 5m

IF-FISH 1d 0h 56m

6 Permeabilized and fixed cells on coverslips were incubated face down on 100ul droplets of PBT consisting of 1% v/v Bovine Serum Albumin and 0.1% v/v Tween-20 in PBS supplemented with 0.4U/µl RiboLock RNAse Inhibitor (RI) from Thermo Fisher for twenty minutes. 20m

7 The coverslips were incubated at room in 100ul PBT supplemented with 0.4U/µl RI and 1ul of primary antibody for 4-6 hours at room temperature or overnight at 4°C. All primary antibodies were diluted 1:100 (1ul in 100ul of PBT). To prevent the coverslip and antibody solution from drying, each coverslip was individually sealed between two layers of Parafilm, creating a semi-air tight Parafilm pocket. 5h

8 Just prior to retrieving the coverslips from their Parafilm pockets, hybridization mixtures were created from 5µl of the nick translated fluorescent RNA probe mixed with 12µl of human Cot-1 and 2µl of Salmon Sperm DNA (both from ThermoFisher). The hybridization mixtures were then completely dried in a SpeedVac system from Savant Integrated with the vacuum itself produced by a pump from A Vac Industries. 15m

9 While the probes were being dried, the coverslips were retrieved from their Parafilm pockets and washed three times in room temperature PBST (PBS and 0.1% Tween-20) 15m

10 The coverslips were then incubated for 1 hour at room temperature with the secondary fluorescently-labelled antibody (1:100 PBT supplemented with 0.4U/µl RNase Inhibitor, 1ul secondary antibody resulting in an antibody 1:100 dilution). From this point onwards the coverslips were kept in dark containers to avoid photobleaching 1h

11 The coverslips were then washed for 5 minutes in PBST three times, to remove the unbound secondary antibodies. 15m

12 Coverslips were then fixed in 4% paraformaldehyde in PBS for 10 minutes. 10m

13 The coverslips were washed once in PBS to remove most of the paraformaldehyde then dehydrated by submerging them for two minutes each in 70%, 80% and finally 100% ethanol. 11m

14 The coverslips were then air dried in a dark container at room temperature for 10-20 minutes. 15m

15 The dried FISH probes were resuspended in 10ul of deionized formamide from Sigma and heated to 80°C for 10 minutes.

16 10ul of hybridization buffer consisting of 20% v/v 20mg/ml Bovine serum albumin (Sigma Aldrich), 20% v/v Dextran

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Sulfate and 20% 4x SSC in DEPC treated ddH_2O) was added to the freshly heated FISH probes

17 The fluorescent probe mixture was gently stirred using the pipette tip and pipetted as a droplet on a piece of parafilm onto which a coverslip was placed, cell side down onto the droplet. A second piece of parafilm was placed on top and the edges were sealed creating a parafilm pocket where the probes hybridized overnight at 37°C. 16h

18 The next day the coverslips were retrieved and incubated in a mixture consisting of equal volumes of deionized formamide (Sigma) and 4x SSC (Invitrogen) at 37°C for 20 minutes. 20m

19 The coverslips were then incubated in 2x SSC at 37°C for 15 minutes. 15m

20 The coverslips were then incubated in 1x SSC at room temperature for 15 minutes. 15m

21 DAPI staining was performed by placing the coverslips in a 0.1µg/ml solution of DAPI in pure methanol at 37°C from 15 minutes. 15m

22 The excess DAPI was rinsed off in methanol and the coverslips were mounted on glass slides using 25µl of hard set antifade mounting media from Vectashield. 10m

23 After letting the media harden, cells were imaged using a confocal fluorescence microscope, either a Leica DMI 6000B or Olympus FluoView FV1000, to capture and compile the fluorescent signals from the secondary antibody, RNA probe and DAPI at 100x magnification.

Quantitation of IF-FISH images for XIST RNA enrichment of heterochromatin

24 The identity of all conditions to be tested should be blinded prior to assessment to reduce bias. Prior to analysis the identities of all the images were blinded by assigning them random alpha numeric names (e.g. A1, A2 etc.).

25 quantification of IF-FISH images was carried out using the program ImageJ produced and maintained by Fiji (Fiji is Just ImageJ) contributors. To analyze the photos taken required the plugin Broadly Applicable Routines (BAR).

26 To analyze a single cell, an image within one of these folders was opened and converted to a composite image from its source format (Image > Color > Make Composite). The image was then despeckled (Process > Noise > Despeckle), a median filter process that decreases the random variability inherent with high magnification fluorescent microscopy by normalizing central pixel intensity to its neighboring 8 pixels

27 The straight-line tool was used to draw a line through the nucleus and *XIST* RNA signal. To try to prevent operator biasing of where to draw the line, the following rules were used to determine where the line was drawn. First, the line had to pass through the point or points of maximal *XIST* signal intensity. Second, the line was drawn to be of maximum length without intersecting the nucleoli or leaving the nucleus.

28 Once the line was drawn, a multi-channel plot profile (BAR > Analysis > Multichannel Plot Profile) of the intensities for each of the colours (red, green and blue) was produced.

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29 The data of position and three colour channel intensities were then saved as a .csv file (data > save data) and 60 cells were analyzed for each condition.

30 Analysis of the data was performed using R studio. The three colour channel intensities at each pixel (the base unit of measurement) for each cell were divided into two categories, *XIST*+ve or *XIST*-ve. This was done by determining the range of *XIST* RNA (green) fluorescent intensity in every cell. The pixels that had a level of green intensity greater that 50% of the maximum along the range of *XIST* fluorescence were defined as *XIST*+ve. Those that had a level of green intensity below 25% of the maximum along the range of *XIST* fluorescence were defined to be *XIST*-ve. Those pixels between 50% and 25% of the maximum green intensity value were discarded to better delineate the two groups.

31 To quantify whether a factor's concentration in the *XIST*+ve region of the nucleus was different from the control *XIST*-ve region a two-sample standard score (z-score) calculation was performed for every cell to determine the relative level of a chromatin marks intensity at the XIST +ve region relative to the nuclear average (XIST -ve region).

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