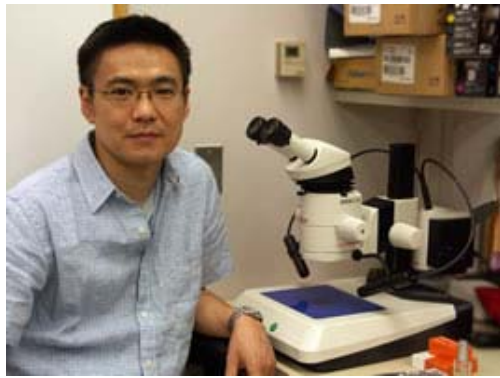


## Sequential RNA and DNA fluorescence in situ hybridization (PROT39)



**Takumi Takizawa and Tom Misteli**

Cell Biology of Genomes, National Cancer Institute, NIH  
41 Library Drive, Bldg. 41  
Bethesda, MD 20892, USA

Email feedback to:

[takizawt@mail.nih.gov](mailto:takizawt@mail.nih.gov)

[mistelit@mail.nih.gov](mailto:mistelit@mail.nih.gov)

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Babraham Institute, Cambridge, UK.

### Introduction

An increasing body of evidence indicates that the spatial positioning of genes in the interphase nucleus is highly relevant for their function (Lanctot et al, 2007; Meaburn & Misteli, 2007; Misteli, 2007). Fluorescence in situ hybridization (FISH) is a powerful technique to map gene loci in the interphase nucleus. Depending on protocol FISH can either detect DNA or RNA. Both methods have limitations. DNA FISH only detects the physical location of a gene, but can not detect gene activity. RNA FISH, on the other hand, detects transcripts, but might miss a significant number of alleles, since not all alleles of a gene are necessarily transcribed simultaneously. The most efficient way to map gene loci and their activity is sequential RNA and DNA FISH. This is an important technique to uncover how gene positioning is linked to activity.

Simultaneous detection of RNA and DNA for a gene loci is non-trivial. Procedures during DNA FISH particularly denaturation of cellular DNA, can cause significant loss of RNA FISH signals. To overcome this problem, we have employed tyramide signal amplification (TSA) to detect RNA FISH signals in a combined DNA/RNA FISH protocol in which RNA is first detected followed by DNA detection. Since tyramide reacts with adjacent tyrosine residues in the presence of peroxidase activity and covalently binds to the residues, the RNA FISH signal is protected in the subsequent DNA FISH procedure. This protocol uses biotin-labeled single stranded DNA probes for RNA detection designed against nascent transcripts or mRNA of the gene of interest, and hybridized probes are visualized using TSA. After RNase treatment, DNA FISH is carried out with a conventional method. The signal enhancement by TSA may give rise to some background both in the nucleoplasm and remaining cytoplasm, but DNA FISH differentiates them from active loci.

With this method, we have successfully mapped active and inactive alleles of IL-4 in lymphocytes. This enabled us to compare the radial distribution of active IL-4 alleles to the inactive ones, where we found a more internal positioning of the active alleles in the interphase nucleus (Takizawa et al, 2008).

### Procedure

The RNA FISH procedures are essentially adapted from Chakalova et al, 2004.

Cells are grown on glass cover slips.

### Fixation

1. Fix cells with 4% PFA in PBS containing 10% acetic acid for 15 minutes at RT after removal of media;
2. Wash with PBS 3 times for 3 minutes each at RT;
3. Replace PBS with 70% ethanol and keep cells at -20°C until use ([note 1](#));

### RNA FISH

1. Wash cells with [TN buffer](#) 2 times at RT;
2. Then incubate cells in [TN buffer](#) for 10 minutes at RT;
3. Digest cytoplasm with 0.01% pepsin/0.01N HCl for 3 minutes at 37 °C ([note 2](#));
4. Quickly rinse cells with H<sub>2</sub>O ([note 3](#));

5. Fix cells with 3.7% PFA in PBS for 5 minutes;
6. Wash with PBS at RT 3 times;
7. Immerse cells into an ethanol series (70%-90%-100% ethanol, for 5 minutes each step) and air dry;
8. Denature a biotin-labeled RNA FISH probe for 10 minutes at 85 °C and keep at 37 °C for 30 minutes ([note 4](#));
9. Add the RNA FISH probe as a drop onto a microscope slide. Place the coverslip cell-side down on top of the RNA FISH probe, wipe the excess solution from the cover slip and seal using rubber solution. Leave the nuclei and probe to hybridize overnight (O/N) at 37 °C in a humidified chamber;
10. Wash the cells with 2XSSC 3 times for 5 minutes each at 37°C;
11. Preincubate with [TNT buffer](#) for 5 minutes at RT;
12. Block with [TNT buffer](#) containing 3% BSA for 20 minutes at RT;
13. Inactivate endogenous peroxidase activity by incubation with 1% H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes at RT ([note 5](#));
14. Incubate with HRP-conjugated streptavidin diluted 1:100 in [TNT buffer](#) containing 3% BSA for 1 hour at RT ([note 6](#));
15. Wash with PBS 3 times;
16. Incubate with fluorophore-conjugated tyramide diluted in 0.0015% H<sub>2</sub>O<sub>2</sub> at 1:100 for 10 minutes at RT ([note 7](#));
17. Wash with PBS 3 times;
18. Incubate with RNase A (100 µg/ml in PBS) for 30 minutes at 37°C;
19. Wash with PBS 3 times;
20. Repeat step 7;

## DNA FISH

1. Denature cells in 70% formamide/2XSSC for 10 minutes at 75°C;
2. Repeat step 7 but the 70% ethanol should be ice-cold here;
3. Denature a Digoxigenin-labeled DNA FISH probe for 10 minutes at 85 °C ([note 8](#));
4. Hybridize with the probe overnight at 37 °C;
5. Wash the cells with 50% formamide in 2XSSC 3 times for 5 minutes each at 45 °C;
6. Wash with 1XSSC 3 times for 5 minutes each at 60 °C;
7. Incubate with 1% H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes at RT ([comment 1](#));
8. Block with 3% BSA/ 2XSSC/ 0.1% Tween 20 for 15 minutes at RT;
9. Incubate with a fluorophore-conjugated anti-DIG antibody diluted in blocking buffer at 1:200 for 1 hour at RT;
10. Wash with PBS three times for 5 minutes each;
11. Mount cells with mounting media containing anti-fade containing DAPI such as Prolong Gold.

## Materials & Reagents

<b>Hybridization buffer</b>	10% dextran sulfate 50% formamide 2XSSC 1mM EDTA 50mM sodium phosphate buffer (pH 7.0) 10 ng/µl Cot-1 DNA 0.5 ug/µl tRNA
<b>TN buffer</b>	0.1 M Tris-HCl (pH 7.5) 0.15M NaCl
<b>TNT buffer</b>	<b>TN buffer</b> + 0.05% Tween 20
<b>Paraformaldehyde 16% solution</b>	(Electron Microscopy Sciences; # 15170)
<b>TSA kit</b>	(Invitrogen; e.g. #T-20935)
<b>Anti-Digoxigenin-Fluorescein, Fab fragments</b>	(Roche; #11207741910)
<b>ProLong Gold antifade reagent with DAPI</b>	(Invitrogen; #P-36931)
<b>Mouse cot-1 DNA</b>	(Invitrogen; #18440-016)
<b>Yeast tRNA</b>	(Invitrogen; #15401-011)

## Author Notes

1. It is better to keep cells at -20°C at least O/N rather than directly going to the next step. In our hands, cells can be stored for a few weeks without significant RNA degeneration.
2. Concentration of pepsin, temperature and incubation time for appropriate digestion can vary between cell types

and need to be optimized. Ideally, the cytoplasm is digested but the nucleus is left intact. If cells are in pepsin too long nuclear morphology becomes affected; too short an incubation in pepsin can result in poor probe penetration, weak nuclear signals and strong background in the undigested cytoplasm. Make sure the solution is at the right temperature before incubation. Since pepsin is activated and digests itself, we warm up 0.01N HCl first and add pepsin just before use. For optimization experiments, it is easiest to carry out the digestion on a phase contrast microscope and follow digestion kinetics in real time.

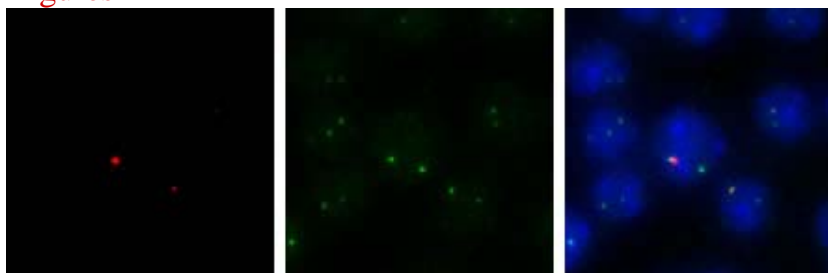
3. Cells can easily detach from coverslips after pepsin digestion. Solutions should be replaced gently.
4. We use 10  $\mu$ l of 2 ng/ $\mu$ l single strand cDNA (ssDNA) probe dissolved in [Hybridization buffer](#) for a 12 mm round coverslip. ssDNA probe is made by reversed transcription from *in vitro* transcribed RNA templates. Details for preparation of the probe are in (Chakalova et al, 2004). Either biotin or digoxigenin can be used as a hapten but we obtained better results using biotin to label RNA FISH probes. Probes can be designed either against mRNA or intron sequences. For the mRNA probe, we use plasmids containing bacterial promoters such as T7, SP6 in the 5' flanking region of the cDNA sequence as template for *in vitro* transcription. For the intron probe, we use PCR for the targeting sequence (~1000 bp) with primers containing a bacterial promoter to make a template.
5. This step can be skipped depending on cell types.
6. We are using a [TSA kit](#) from Invitrogen (e.g. #T20935).
7. Green or Red fluorescence can be used.
8. We use 100 ng of a nick-translated BAC probe for a 12 mm round cover slip. We commonly purchase our BACs from BACPAC Resources Center, C.H.O.R.I.. Details for nick-translation is in our website, <http://rex.nci.nih.gov/RESEARCH/basic/lrbge/protocols.html>. The length of the probes should be 200-400 bp. Longer probes give increased background signals and shorter probes result in homogenous, non-specific staining.

## Reviewer Comments

Reviewed by: Lyubomira Chakalova and [Peter Fraser](#). Babraham Institute, Cambridge, UK.

1. We had just one question about step 7 of the DNA FISH. We couldn't figure out what this step was for since the TSA is all over by this point. **[Author Response:** We have included the step because we got a high background for the DNA FISH without it. We have not tried the protocol without this step since then. Perhaps it could be skipped. Users might try both, with or without this step, and empirically chose the method giving the best results.]

## Figures



An example of sequential RNA and DNA FISH. RNA FISH was performed against IL-4 mRNA in mouse T helper type 2 cells and visualized with Alexa594-conjugated tyramide using the TSA technique (left). After RNase treatment, DNA FISH was performed against IL-4 using a DIG-conjugated nick-translated BAC probe (middle). Nuclei were counter-stained with DAPI (blue).

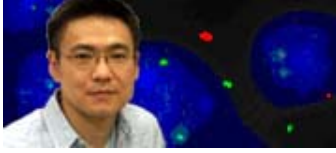
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