Optimizing the detection of nascent transcripts by RNA fluorescence *in situ* hybridization

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ABSTRACT

An unusual feature of the mammalian genome is the number of genes exhibiting monoallelic expression. Recently random monoallelic expression of autosomal genes has been reported for olfactory and *Ly-49* NK receptor genes, as well as for *Il-2*, *Il-4* and *Pax5*. RNA fluorescence *in situ* hybridization (FISH) has been exploited to monitor allelic expression by visualizing the number of sites of transcription in individual nuclei. However, the sensitivity of this technique is difficult to determine for a given gene. We show that by combining DNA and RNA FISH it is possible to control for the hybridization efficiency and the accessibility and visibility of fluorescent probes within the nucleus.

INTRODUCTION

A surprising feature of gene expression in mammalian cells is the number of genes that are expressed exclusively from one allele. The best studied examples of this epigenetic phenomenon are X chromosome inactivation, where one of the two X chromosomes in females is inactivated at random, and genomic imprinting, in which the use of alleles is determined by the parent of origin. More recently Chess *et al.* (1) showed that individual olfactory neurons express a single olfactory receptor from one allele only. Since then there have been a number of reports describing the random monoallelic expression of *Pax5*, *Ly-49* NK receptor genes, *Il-2* and *Il-4* (2–5).

When monoallelic expression is achieved through a random choice mechanism the pattern of expression of the gene within a tissue is mosaic and, therefore, gene expression must be examined at the single cell level. The number of tools available to study gene expression at this level is limited. Single cell RT–PCR, while powerful, is not always reliable for genes that are expressed at very low levels (6,7; L.L.Sandell and S.M.Tilghman, unpublished results). One method that has been adopted for allelic analysis is RNA fluorescent *in situ* hybridization (FISH) (5). This technique allows visualization of RNA at the site of transcription in individual nuclei and, at the same time, reveals the position of the gene within the nucleus. RNA FISH can be remarkably sensitive (8) and has been used to visualize tracks of fibronectin RNA as it is processed and to study temporal regulation and splicing of the globin genes (9,10).

Nutt *et al.* (5) used RNA FISH to show that 49% of interphase B cell nuclei exhibited a single site of hybridization to *Pax5*, rather than the two 'dots' expected for a biallelically expressed gene. Based on these and other results they argued that the *Pax5* gene was expressed monoallelically at least part of the time. This finding has since been challenged by Rhoades *et al.* (7), who argued that the percentage of B cells with one dot depended upon the stringency of the hybridization conditions and demonstrated that under more optimal conditions the gene was shown to be biallelic. This difference reflects the fact that there is no accepted control for RNA FISH experiments. While examining the expression of *Pax6* in embryonic cells (11) we investigated the sensitivity and consistency of the RNA FISH technique. We suggest a simple control to ensure that RNA FISH is accurately detecting gene expression.

MATERIALS AND METHODS

RNA–DNA FISH

Neural retinas from E12.5 mouse embryos were dissected, pooled and dissociated into single cell layers by pipetting in 1× trypsin–EDTA for 3 min at 25°C. Cells were spun onto slides using Cytofunnels in a Shandon Cytospin 3 centrifuge operated at 400 r.p.m. for 4 min. Slides were rinsed in phosphate-buffered saline (PBS) for 15 s and cells were permeabilized in cytoskeletal buffer (CB) (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH 6.8), CB plus 0.5% Triton X-100 and CB for 30 s each step. Slides were then fixed in 4% paraformaldehyde for 10 min and stored in 70% ethanol at 4°C for up to 2 weeks. For RNA FISH, slides were dehydrated through 80, 95 and 100% ethanol, dried and hybridized to probe overnight at 37°C. The probe was prepared as follows. Aliquots of 200 ng biotin-labeled genomic DNA encompassing exons 2–13 of *Pax6* (~20 kb), 50 µg tRNA, 4 µg mouse Cot-1 DNA and 50 µg salmon sperm DNA were precipitated, resuspended in 10 µl of Hybrisol VII (Oncor), denatured and pre-annealed for 1 h at 37°C. After hybridization, slides were washed in 50% formamide, 2× SSC at 39°C for 3 × 5 min and 2× SSC for 3 × 5 min, and at room temperature using 1× SSC for 10 min and 4× SSC for 5 min with agitation. Slides were blocked with 4 mg/ml BSA (New England Biolabs) for 20 min at 37°C. Biotin was detected by incubating slides with FITC–avidin (1:200), biotinylated anti-avidin D (1:100) and FITC–avidin (Vector) at 37°C for 40 min each step, with three washes of 5 min each in 4× SSC after each incubation. The buffer for the blocking and detection steps was 4× SSC. Avidin was post-fixed for 10 min in 4% paraformaldehyde. Immediately

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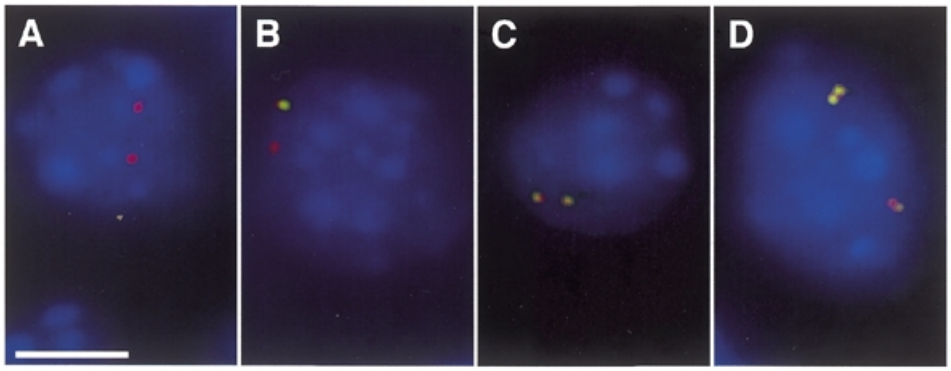

Figure 1. RNA–DNA FISH examples. *Pax6* RNA–DNA FISH performed on E12.5 neural retina cells reveals DAPI-stained nuclei (blue) with no (**A**), one (**B**), two (**C**) or four (**D**) spots of *Pax6* RNA (green) co-localizing to either two (A–C) (G_1 cells) or four (D) (G_2 cells) *Pax6* DNA alleles (red). The scale bar in (A) represents 5 μm.

following fixation, DNA FISH was performed. Slides were treated with 0.1 mg/ml RNase for 1 h at 37°C, denatured with 70% formamide, 2× SSC at 70°C for 2 min and quenched in 70% ethanol at 4°C. Slides were dehydrated and hybridized as above to rhodamine-labeled BAC 255020 from the 129/SvJ II library (Genome Systems), which contains ~150 kb of genomic DNA including the entire *Pax6* gene. Slides were washed as above. Total DNA was visualized with DAPI. Digital images of fluorescent signal were acquired with a Nikon Eclipse E800 microscope and CCD camera. Images were merged and analyzed using Metamorph imaging software.

Table 1. Summary of RNA FISH results

RNA signal	Number of cells (%)	
	With amplification/ with DNA FISH	No amplification/ no DNA FISH
None	1 (2%)	14 (11%)
1	6 (12%)	53 (43%)
2 or 4	43 (86%)	56 (46%)

RNA FISH was performed on E12.5 neural retina cells either with or without a FITC signal amplification step and DNA FISH.

RESULTS AND DISCUSSION

Monoallelic expression is detected by RNA FISH as a single dot of hybridization in a diploid non-dividing interphase nucleus. Because the absence of two dots is a negative result, hybridization to a known biallelic gene has been used as a positive control (5). This control assumes that the two probes will have equal access to the target genes in the nuclear compartment and will hybridize with equal efficiency. When a field of cells displays <100% efficient hybridization to either the control or target gene it is impossible to know whether this reflects a technical problem or the fact that some cells do not express the gene.

We used RNA FISH to assess allelic expression of *Pax6*, a member of the *Pax* transcription factor family that is required for development of the eye, in E12.5 mouse neural retina cells (11,12). We first used a standard protocol and observed that the percentage of cells with one spot of RNA signal was 43%, but the number of cells with no signal was also high (11%), suggesting the possibility that the protocol was not efficient (Table 1). When an anti-avidin and subsequent FITC–avidin signal amplification step was included in the protocol there was a reduction in the number of one dot cells to 12%, while the number of cells with no hybridization signal was only 2% (Table 1). When RNase treatment was included prior to hybridization no FITC signal was detected, indicating that the probe was detecting RNA (11). This result illustrates the sensitivity of RNA FISH to the experimental conditions, highlighting the need for an independent assessment of efficiency of hybridization.

A second technical limitation we encountered was that some cells had one bright spot and one or more faint spots, leaving the interpretation ambiguous as to whether this was monoallelic or biallelic expression. One way to ensure that one is measuring hybridization at the site of transcription is to combine RNA FISH with DNA FISH. Furthermore, by restricting the RNA analysis to those nuclei in which both alleles are visible by DNA FISH (those that display two or four dots of DNA, depending on the phase of the cell cycle) one can be sure that the RNA probe has access to the gene. Using two-color FISH we detected two foci of *Pax6* DNA in G_1 cells (Fig. 1A–C) or two pairs of alleles in G_2 cells (Fig. 1D) in the majority of nuclei (>95%). Importantly, the majority of cells (86%) had either two or four spots of RNA (Table 1). Thus when the site of transcription and target accessibility was controlled for with DNA FISH, the RNA FISH analysis detected biallelic *Pax6* expression in a majority of cells.

We noticed that among cells with RNA detected at both *Pax6* alleles the intensity of the RNA signal appeared more variable between the two alleles than the DNA signal. To confirm this observation we measured the pixel brightness of the signals in digitally acquired images of RNA–DNA FISH nuclei using Metamorph imaging software, normalized for exposure time. Analyzing 53 randomly selected nuclei with two spots of *Pax6* RNA we determined the maximum brightness of each spot of DNA and RNA and then found the ratio of the two DNA or RNA intensities for each cell. The average

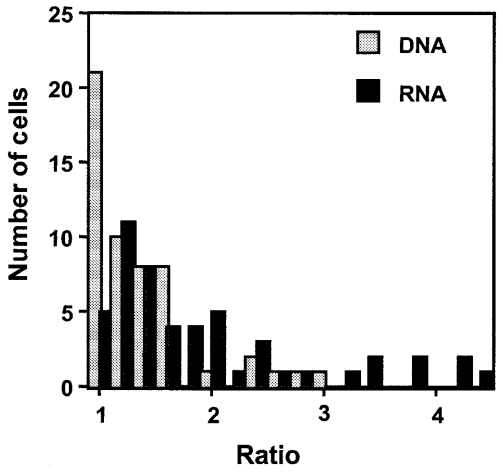

Figure 2. Ratio of signal intensities within cells. Histogram showing the distribution of the ratios of the two DNA (gray) or two RNA (black) signal intensities at individual loci in 53 cells.

ratio for DNA was 1.4 ± 0.07 (standard deviation 0.48), while the ratio for the RNA signals was 2.1 ± 0.14 (standard deviation 1.03) (Fig. 2). Furthermore, 42% of cells had at least a 2-fold difference in RNA intensities, while only 11% of cells had this great a difference in DNA intensities (Fig. 2), confirming that the *Pax6* RNA signals are more variable between the two alleles than the DNA signals. This may be the result of fluctuations in the rate of *Pax6* transcription or RNA stability or some inherent difference in the RNA FISH technique that makes it less consistent than DNA FISH. We conclude that it should not be assumed that RNA FISH uniformly detects RNA within single nuclei.

We further noticed that often the brighter RNA and DNA signals were coincident. To quantify this we examined 30 randomly selected cells with two RNA spots and found that in 73% of the cells the same allele was brighter for both RNA and DNA. There was a statistically significant correlation between the brightness of the co-localized RNA and DNA spots ($P < 0.025$, χ^2 analysis), suggesting that certain positions and/or conditions in the nucleus can influence the ability of both probes to access their targets. Therefore, DNA FISH provides an independent method to identify accessible alleles for RNA FISH.

In conclusion, the reliability of RNA FISH to study allelic expression of genes will be enhanced by routinely using a DNA FISH control and monitoring only those alleles that are detected by DNA hybridization.

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