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Cell-to-cell transcription variability as measured by single molecule RNA FISH to detect epigenetic state switching

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Running Title: Single Molecule RNA FISH

Abstract

Single molecule RNA fluorescent *in situ* hybridisation (smRNA FISH) allows for the visualisation, localisation and quantification of RNA transcripts within individual cells and tissues using custom designed fluorescently labelled oligonucleotide probes. Here we describe a protocol for the preparation, imaging and analysis of a smRNA FISH experiment that can be applied to any RNA of choice. We also provide insights as to how this powerful tool can be used to study epigenetic regulation, for example following the epigenetic editing of genes.

Key words RNA FISH, Fixation, Permeabilisation, Hybridisation, Fluorescence microscopy, Image analysis, Epigenetics

1. Introduction

Since its conception in 1982 [1], single molecule RNA fluorescent *in situ* hybridisation (smRNA FISH) has grown to become an indispensable tool for cellular biologists worldwide, allowing visualization and quantification of individual RNA molecules within individual cells and tissues. Following fixation and permeabilisation of the cells, a set of tiled fluorescently labelled oligonucleotide probes is introduced, each of which has specificity for a given section of a target transcript. In this way, many probes are capable of binding each transcript, giving a strong fluorescence signal per RNA molecule that is significantly higher than the background generated by unbound residual probes. These bound transcripts can then be imaged using conventional wide-field or confocal microscopes [2] (*see* **Figure 1**). For analysis of tissue slices that are only a few μm thick, wide-field microscopy might provide suitable signal detection, but for quantitative analysis of single RNAs in single cells (which requires nuclear and cytoplasmic volume and transcript measurements), it is essential to use confocal microscopy imaging [3].

Besides removing the need for reference genes, which themselves could be influenced by treatment conditions, the primary benefit of using smRNA FISH is the ability to assess the heterogeneity in gene expression at the RNA level across cell populations. The mild procedure and imaging based approach allows researchers to measure fluorescent protein expression or antibody labelling simultaneously with smRNA FISH. These features make smRNA FISH in single cells an attractive technique for researchers interested in studying cell-to-cell variability of transcription and cellular decision making based on cell heterogeneity. Additionally, it is now possible to measure the expression of other RNA species, such as long non-coding RNAs [4], and even enhancer RNAs [5], making it attractive for those wishing to explore the expression of these species.

Epigenetic gene regulation plays a major role in establishing and maintaining cell lineage-specific transcriptional programs, while allowing changes in gene expression to environmental cues. It is also well accepted that many diseases are associated with an altered epigenetic landscape and since epigenetic regulation is in principle flexible and reversible it opens unique opportunities for (therapeutic) intervention. With the emergence of epigenetic editing techniques capable of making site specific epigenetic alterations, smRNA FISH becomes and incredibly powerful tool with which to study the subsequent transcriptional changes.

There is a clear need to unravel the impact of epigenetic plasticity in health and diseased conditions to advance epigenetic intervention in clinical settings [6]. Understanding how dynamic epigenetic interactions emerge and contribute to biological functionality in single living cells is still largely unknown which is in part due to the complex nature of the epigenome. SmRNA FISH allows one to determine the precise relationship between transcript numbers and the epigenetic chromatin composition [7]. Quantitative sampling of single transcripts at single cell level in combination with stochastic modelling in an altered epigenetic context (either experimentally induced or endogenously altered) opens-up an unexplored field of research with great potential towards individualized behaviour and precision medicine[8, 9].

2. Materials

2.1 Probe storage

1. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2. 0.5 mL amber microcentrifuge tubes.

2.2 Fixation and permeabilisation

1. 2-well culture chamber glass slides (*see* **Note 1**).

2. Fixation buffer: 3.7 % (vol./vol.) formaldehyde in 1 X PBS, RNase-free.

3. 70 % (vol./vol.) ethanol.

4. Parafilm.

5. RNaseZap cleaning agent (Sigma-Aldrich).

2.3 Hybridisation

1. Wash buffer A: 70 % (vol./vol.) Stellaris® Wash Buffer A, 10 % (vol./vol.) deionised formamide, nuclease-free water (*see* **Note 2**).

2. Hybridisation buffer: 90 % (vol./vol.) Stellaris® RNA FISH Hybridisation Buffer, 10 % (vol./vol.) deionised formamide (*see* **Note 2**).

3. Hybridisation chamber: petri dish wrapped in foil, or any opaque container big enough for the slide and water tray.

4. Small water tray (such as the lid of a second chambered slide).

5. #1.5 (0.13 to 0.17 mm) coverslips, using a glass cutter, carefully cut a coverslip to the appropriate size for the wells.

6. Glass cutter.

7. Tweezers.

8. DAPI staining buffer: Wash buffer A, 5 ng/mL DAPI.

9. Stellaris® wash buffer B (*see* **Note 2**).

10. 2x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0

3. Methods

Stellaris® (Biosearch Technologies) provides a general method for performing smRNA FISH on many types of biological material. Here we have adapted the method for optimal signal-to-noise detection. In all steps, RNase free solutions should be used, and the workbench and all tools should be cleaned with RNaseZap cleaning agent:

3.1 Probe design and handling

1. Design the oligonucleotide probes for a specific target RNA. This can be done using the Stellaris® Probe Designer tool online (*see* **Note 3**).

2. For probes purchased from Stellaris® (5 nmol), add 400 µL of TE buffer and then vortex and centrifuge briefly. For probes purchased elsewhere, add the appropriate amount of TE buffer such that a probe concentration of 12.5 µM is obtained.

3. Aliquot this probe stock solution into 0.5 mL amber microcentrifuge tubes and store at -20 °C till required (*see* **Note 4**).

3.1 Fixation and permeabilisation

1. Transfer cells to a 2-well culture chamber glass slide and allow to adhere (*see* **Note 5**).

2. Gently remove the media and wash with 1 X PBS.

3. Add 1 mL of fixation buffer to each well and incubate at room temperature for 10 minutes.

4. Wash twice with 1 mL of 1 X PBS.

5. Permeabilise cells in 1 mL of 70 % ethanol for at least 1 hour at room temperature, or overnight at 4 °C. Cells in ethanol can be stored at 4 °C for a week, or at -20 °C for several months.

3.2 Hybridisation and imaging

1. Aspirate the 70 % ethanol from the slide.

2. Add 1 mL of wash buffer A and incubate at room temperature for 5 minutes.

3. Add 0.5 µl of stock probe solution to 100 µl of hybridisation buffer and mix thoroughly (see **Note 6**).

4. Aspirate wash buffer A.

5. Evenly dispense the hybridisation buffer with probe onto the slide. For a 2-well slide, add 50 μl to each well (*see* **Note 7**).

6. Slowly lower the coverslip on the cells such that the hybridisation buffer is evenly distributed within the well. If any bubbles appear, gently lift the coverslip with tweezers and reapply.

7. Place the slide in the hybridisation chamber, alongside a small water tray to ensure that the slide does not dry.

8. Incubate in the dark at 37 °C for at least 4 hours (*see* **Note 8**).

9. Gently remove the coverslip from the slide, and add 1 mL of wash buffer A.

10. Incubate in the dark at 37 °C for 30 minutes (*see* **Note 9**).

11. Aspirate wash buffer A and add 1 mL of DAPI staining buffer.

12. Incubate in the dark at 37 °C for 30 minutes.

13. Aspirate the DAPI staining buffer and add 1 mL of wash buffer B. Incubate for 5 minutes at room temperature.

14. Aspirate wash buffer B. To prevent evaporation of the solution during scanning 1 mL of 2 x SSC is added to the well.

15. Proceed to image the cells using a confocal microscope. We use a Nikon Ti-E scanning-laser confocal inverted microscope with 60x oil objective in conjunction with Nikon NIS-Elements software. Optical sections are captured at 0.3 μm intervals at a resolution of 256 x 256 pixels (*see* **Note 10**).

16. To ensure that the spots detected in the images are true signals, we recommend performing a control experiment where 0.1 mg/mL RNase A is added to the cells for at least an hour at 37 °C prior to hybridisation (*see* **Figure 2)**.

3.3 Image analysis

We perform image analysis using custom MATLAB scripts (unless stated differently) adapted from Raj et al (2008)[10] and Kempe et al (2015)[3]. Other image analysis software is also possible. Here we will briefly describe the steps to take:

1. Convert and split the resulting ND2 file into TIFF files using ImageJ. Every stack imaged should have one corresponding TIFF file.

2. Filter images with a semi-three-dimensional Laplacian of Gaussian filter to remove noise and enhance the signal-to-noise ratio. The size of the filter can be adjusted depending on the size of the observed spots (*see* **Figure 3**).

3. Apply a threshold to the filtered images and count the number of spots above this threshold. Iterate this for all threshold values and plot against the number of spots counted for each iteration. The threshold should be set where the total number of mRNAs counted is least sensitive to any changes in the threshold i.e. at the plateau (*see* **Figure 4).**

4. If investigating mRNA expression per cell, transcript concentration has been shown to be a more reliable readout. This can be calculated by defining any region with a DAPI signal or probe signal as being intracellular. Convert this region in voxels to a volume by multiplying by the voxel size (*see* **Note 11**).

4. Notes

1. While any glass slide can be used, we generally use 2-well culture chamber slides and so all methods have been given for one chamber of a 2-well slide.

2. If you do not want to purchase the Stellaris® Hybridisation buffer, you can make your own, consisting of 100 mg/mL dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.2 mg/mL RNAse-free BSA, 1 mg/mL *E. coli* tRNA, 2 x SSC and 10 % formamide.

   If you do not want to purchase the Stellaris® buffers A and B, you can make your own, consisting of 10 % formamide and 2 x SSC. Best results regarding signal-to-noise have been obtained using the complete Stellaris® buffer set.

3. Oligonucleotide probes can be designed using many tools, however we find the Stellaris® Probe Designer tool is the simplest, using the standard settings of masking level 5, oligo length 20, maximum of 48 probes and 2 nt spacing length. It is possible to design probes of varying number, length, specificity and spacing, and to select the fluorophore of your choice. It is important to avoid targeting repetitive elements and areas with a high GC content. Initial publications suggested that 48 probes were necessary for a satisfactory signal, however we and others have got a good signal from less than 30 probes [4]. The ability to use fewer probes is especially true for agglomerations of RNA, such as those found at active enhancers [4, 5]. With optimal imaging techniques and fluorophores the necessary probe number may decrease further.

4. It is very important to use amber tubes here, and to ensure that the probes are stored in the dark. Repeated freeze-thaw cycles and exposure to light will degrade the fluorophore and result in poor signals.

5. Cells should be left to adhere for a duration that is dependent on your cells. Some cells require less than an hour to adhere but others need much longer. If you are imaging single cells, it is important to leave cells to adhere for no longer than necessary since cell growth will reduce the chance of finding single cells on the slide.

6. The volume of probe that you should add depends on the specificity and efficiency of the designed oligonucleotides. With every new probe, a test smRNA FISH run should be done to find the optimal concentration. We typically find that 62.5 nM is a suitable concentration.

7. The 100 μL of hybridisation buffer containing the probe should be evenly dispensed for the whole slide, i.e. for a 2-well slide, 50 μL will be used for each well, or 25 μL for each well of a 4-well slide etc.

8. Again, the duration of incubation is probe dependent and will need optimising, but it should be at least 4 hours. If necessary, the hybridisation time can be extended up to 24 hours.

9. If you have a high background signal, this step can be repeated with another 1 mL of wash buffer A for 30 minutes.

10. Any other confocal or wide-field microscope can be used to image the cells. We opt to use a confocal for single cell imaging since it allows us to discriminate single mRNAs in 3D. In addition, confocal microscopy can be used to calculate the cellular and nuclear volumes, giving us transcript concentrations per cell, which has been shown to give a more reliable measurement of total transcription cell-cell variability since it reduces the effect of biological noise due to cell growth.

11. To enable cell volume measurements the following image analysis steps are taken: median filtering (20 × 20 pixels); image thresholding (graythresh, Otsu's method [11]); fill the image regions and holes; morphological closing with a disk (radius = 4 pixels). We used a custom written MATLAB script.

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Figures and legends of Beckman et al. *Cell-to-cell transcriptional variability as measured by single molecule RNA FISH to detect epigenetic state switching* to be published in Springer Methods in Moleculear Biology 'Epigenome Editing'
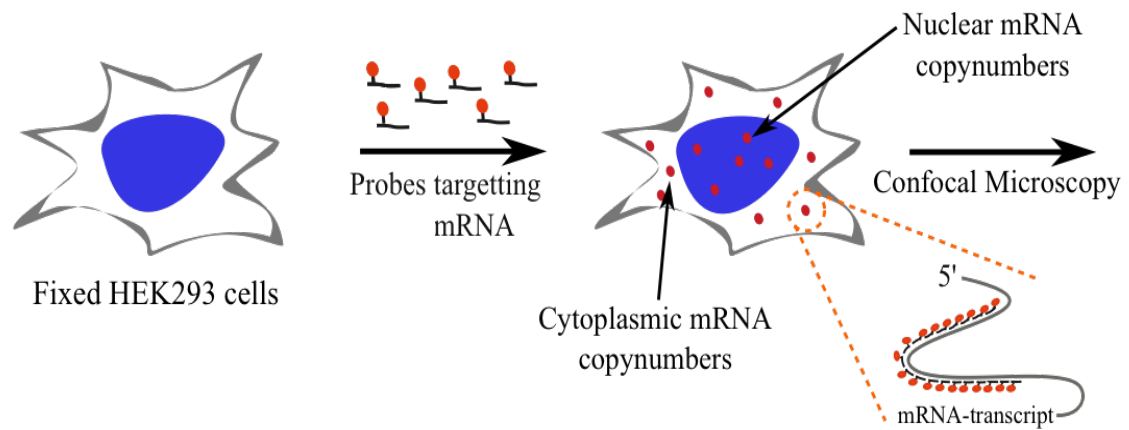
Fig. 1 Overview of smRNA FISH technique. Cells are fixed and permeabilised before the introduction of a set of fluorophore-bound probes. These probes will bind to the target transcript producing spots of high signal intensity, which can then be imaged using a confocal microscope [3].
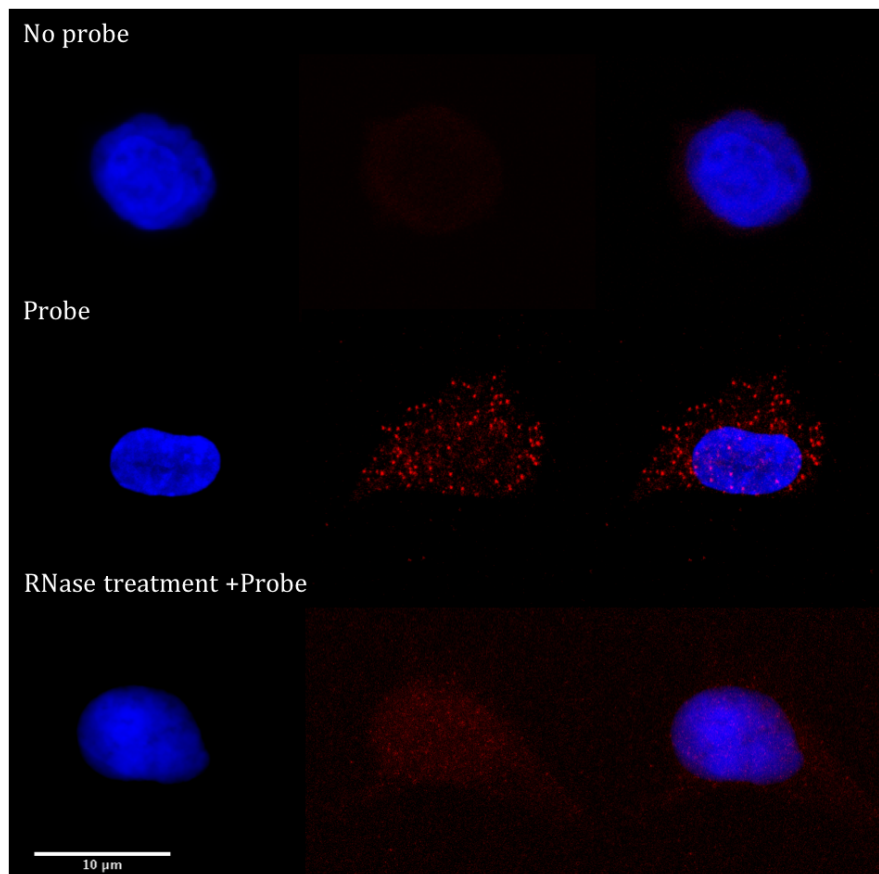

Fig 2. Example images of a smRNA FISH control experiment in MCF-7 cells. Left column shows nuclei stained with DAPI; middle column shows the signal from a probe targeting PLOD2 mRNA tagged with CAL Fluor red 590 fluorophore (Biosearch Technologies); right column shows overlay. Imaging was done with a Nikon A1 confocal as described, and the resulting stack was compressed to a 2D image (Sum slices projection, ImageJ)
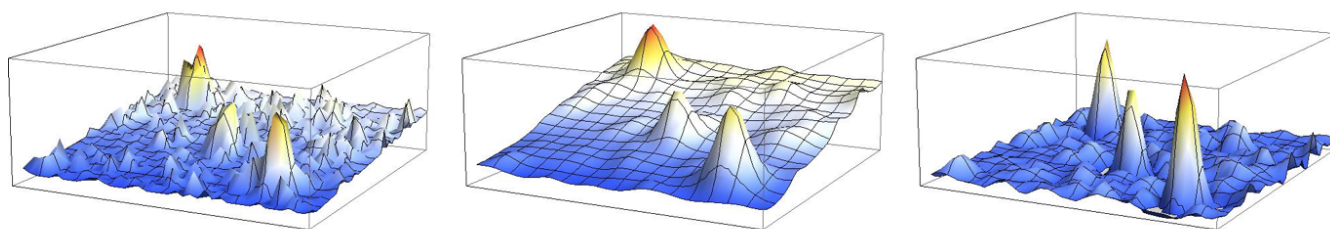
Fig. 3 Image analysis of smRNA FISH signal. (**a**) Raw image intensities. (**b**) Effect of Gaussian filtering. (**c**) Effect of Laplacian of Gaussian filtering (sigma = 1.5, filter width = 15 pixels)
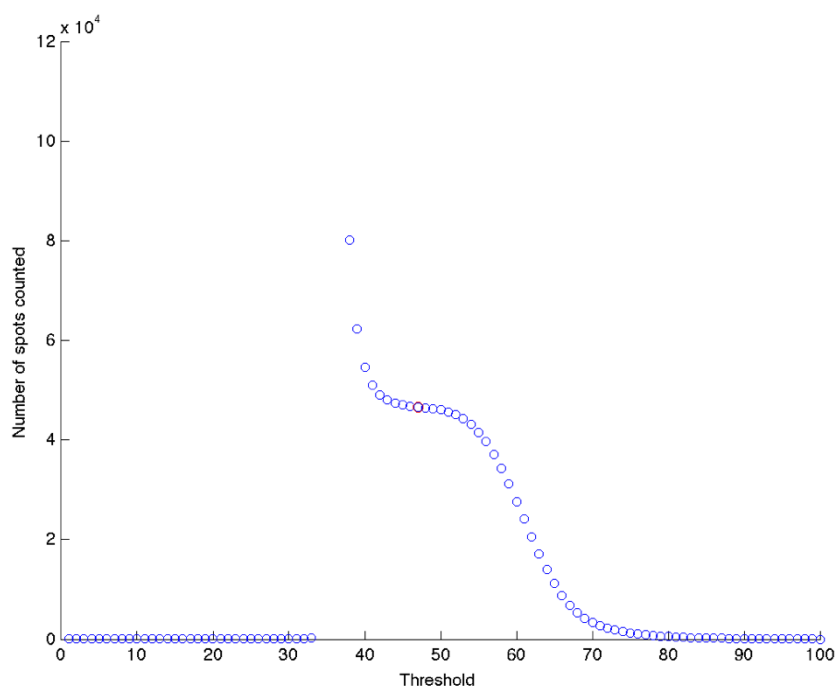

Fig. 4 Determination of the correct threshold for image analysis. By iterating the spot count script using different threshold values, it is possible to find the threshold value that corresponds to the "true" spot count (*red circle*)