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Single cell analysis of transcriptionally active alleles by single molecule FISH

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Abstract

Gene transcription is an essential process in cell biology, and allows cells interpret and respond to internal and external cues. Traditional bulk population methods (Northern blot, PCR, and RNAseq) that measure mRNA levels lack the ability to provide information on cell-to-cell variation in responses. Precise single cell and allelic visualization and quantification is possible via single molecule RNA fluorescence *in situ* hybridization (smFISH). RNA-FISH is performed by hybridizing target RNAs with labeled oligonucleotide probes. These can be imaged in medium/high throughput modalities, and, through image analysis pipelines, provide quantitative data on both mature and nascent RNAs, all at the single cell level. The fixation, permeabilization, hybridization and imaging steps have been optimized in our lab over many years using the model system described herein, which results in successful and robust single cell analysis of smFISH labeling. The main goal with sample preparation and processing is to produce high quality images characterized by a high signal-to-noise ratio to reduce false positives and provide data that are more accurate. Here we offer our protocol describing the pipeline from sample preparation to data analysis in conjunction with suggestions and optimization steps to tailor to specific samples.

SUMMARY:

Single molecule RNA fluorescence *in situ* hybridization (smFISH) is a method to accurately quantify levels and localization of specific RNAs at the single cell level. This technique provides information regarding cell-to-cell and allele-by-allele variation in RNA content and responses to external stimuli, and allows for precise single cell analysis with spatial information. Here, we report our validated lab protocols for wet-bench processing, imaging and image analysis for single cell quantification of specific RNAs.

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DISCLOSURES:
The authors have nothing to disclose.

Keywords

Single molecule FISH; imaging; image analysis; gene transcription

INTRODUCTION:

Gene transcription and translation are the two major processes involved in the central dogma of biology, going from DNA sequence to RNA to protein[1]. In this study, we focus on the production of messenger RNA (mRNA) during transcription. The nascent RNA molecule includes both non-coding introns and coding exons. Introns are co-transcriptionally spliced out before the mRNA moves into the cytoplasm for translation on the ribosomes[2,3].

Traditionally, the measurement of mRNA is performed in bulk populations of cells (hundreds to millions) with classic assays such as RT-qPCR or Northern blotting. While very powerful, these methods are limited as they do not provide insights into cell-to-cell variation (phenotypic heterogeneity), identification of the number of transcriptionally active alleles, and, perhaps more importantly, lack spatial information. More recently, genome wide technologies allowing for single cell RNA sequencing (RNAseq) began to bridge the gap between imaging methods and sequencing[4]. However, RNAseq suffers from relatively low detection efficiencies and the processing steps result in complete loss of spatial information[5]. Although single cell RNAseq can provide insights into phenotypic heterogeneity amongst a population of isogenic cells, RNA fluorescence *in situ* hybridization (RNA-FISH) can facilitate a more complete exploration of target gene expression at the spatial level, and on an allele-by-allele basis[6,7].

RNA FISH is a technique that allows for the detection and localization of target RNA molecules in fixed cells. Unlike earlier, laborious methods, current state-of-the-art RNA-FISH utilizes commercially available nucleic acid probes that are complementary to the target RNA sequences, and these probes hybridize to their targets by Watson-Crick base pairing[8]. The early *in situ* hybridization techniques involved a similar protocol established by Gall and Pardue in 1969, as a sample was processed with nucleic acid probes that specifically hybridized to a target RNA[9]. Originally the assay was performed using radioactive or colorimetric detection; however, in the 1980s, the development of fluorescence *in situ* hybridization (FISH) protocols to DNA FISH and later RNA FISH opened the route towards more sensitive detection, and the potential for multiplexing[10,11].

Further developments in RNA FISH have led to the ability to detect and quantify single RNA molecules (smFISH)[12,13]. Direct detection is a method in which the probes themselves are labeled with fluorophores. A challenge with smFISH is that there is a need for enough hybridizing probes/target to facilitate the detection of fluorescent signals as distinct, diffraction-limited spots. To address this issue, one can use a probe set of short single-stranded DNA oligonucleotides complementary to various regions of the target RNA[8,12,14]. The binding of multiple probes increases local fluorophore density making the RNA visible as a distinct, high intensity spot by fluorescence microscopy. This method is advantageous because off-target binding of oligonucleotides in the probe set pool can be distinguishable from the true RNA spot signal by quantitatively analyzing the spot size and intensity to

differentiate between true signal and spurious oligonucleotide binding, thus facilitating more accurate analysis by reducing false positives[12].

Alternative methods to detect mRNA are the classic BAC clone-based nick translation method and the more recently developed branched DNA system. In the BAC-cloned, nick-translation method, the DNA to be labeled is enzymatically-nicked and a new nucleotide is added to the exposed 3' end. The activity of DNA polymerase I adds a labeled nucleotide resulting in the desired probe[15,16]. The branched DNA technique involves oligonucleotide probes that hybridize in pairs to RNA targets and these probes are amplified utilizing multiple signal amplification molecules. This method can significantly improve the signal-to-noise ratio but the amplification can skew accurate quantitation since fluorescent spots can be wildly different in size and intensity[17].

As a model system for this protocol, which is fully-employed as part of our NIEHS Superfund Research Program participation to quantify the effects of endocrine disrupting chemicals in Galveston Bay/Houston Ship Channel, we used the estrogen receptor (ER) positive breast cancer cell line MCF-7 treated overnight with an ER agonist, 17β-estradiol (E2)[7,18,19]. E2 regulates many ER-target genes, including the prototypical ER-target gene, GREB1[7,20–22]. The smFISH protocol described here utilizes a set of two spectrally-separated probe sets targeting GREB1; one that hybridizes to introns and the other to exons, allowing for the measurement of both mature and nascent RNA[7]. We then use epifluorescence microscopy coupled with deconvolution to image smFISH labeled samples, and then apply image analysis routines to quantify the number of active alleles and mature RNAs per cell.

PROTOCOL:

To ensure optimal results, the wet lab portion of this protocol requires standard RNAse-free precautions at all steps. For example, the use of filtered pipette tips, sterile vessels and RNase-free buffers is highly-encouraged. Using RNase inhibitors such as vanadyl ribonucleoside complexes is also suggested, especially for low abundance target RNAs.

1. Cell culture and experimental set up

1.1. —Maintain adherent MCF-7 breast cancer cells (ATCC #HTB-22) in a T75 tissue culture flask with phenol-red free (only required for hormone stimulation experiments) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 nM sodium pyruvate, 50 I.U./mL penicillin and 50 μg/mL streptomycin.

1.2. —Place acid-etched, poly-D-lysine coated round coverslips (0.16 to 0.19 mm thick, 12 mm diameter) into a 24 multiwell tissue culture plate.

NOTE: Use 1M HCl to acid-etch the coverslips and coat with a 1 mg/ml solution of poly-D-lysine reconstituted in sterile PBS (phosphate-buffered saline without Ca^{++} and Mg^{++}) according to standard protocols.

1.3. —Remove the growth media from the cells and wash once with 5 mL of sterile PBS (phosphate-buffered saline without Ca^{++} and Mg^{++}). Detach MCF-7 cells using 2–3 mL of Trypsin-EDTA 0.25% solution. Once cells are detached, neutralize the Trypsin-EDTA 0.25% solution with an equal volume of growth media.

1.4. —Transfer the cells in the media suspension to a 15 mL conical tube, spin down at 391 *g* for 1 minute to pellet out the cells. Carefully remove the media from the conical tube without disturbing the cell pellet and resuspend the cells in an appropriate amount of treatment media. Treatment media is phenol-red free DMEM supplemented with 5% charcoal-dextran stripped and dialyzed FBS, 2 mM L-glutamine, 1 nM sodium pyruvate, 50 I.U./mL penicillin and 50 μg/mL streptomycin. This media is essential for steroid hormone stimulation experiments as the serum component is largely depleted of steroids by charcoal stripping the media, and has reduced growth factors via dialysis.

1.5. —Seed the cells onto coverslips in the 24 multiwell plate at 60,000 to 70,000 cells per well in 500 μL of treatment media. For this experiment, cell confluency should be around 70–80% at treatment initiation. Optimize cell seeding density depending on cell type, growth rate and length of the experiment to avoid confluency, which complicates image analysis.

3. NOTE: For best imaging and image analysis, avoid cell clumping. For this purpose, mix the aliquot of cells thoroughly by pipetting (or briefly vortex) the cells a few times before dispensing into the wells. Before placing the plated cells back into the incubator, letting the cells settle in the tissue culture hood for about 20 minutes helps reduce cell clumping.

1.6. —Incubate cells for at least two days prior to treatments to ensure cell cycle synchronization and background reduction due to any signaling molecules that remain in the stripped/dialyzed serum of growth media.

1.7. —After a 48-hour incubation in treatment media, treat MCF-7 cells with 10 nM 17β-estradiol (E2) and vehicle control (DMSO), diluted in treatment media for 24 hours. This treatment uses a saturating dose of E2 to induce a maximal response. Perfrom time course and dose-response experiments to empirically determine conditions for different target genes, cell models and type of treatments. As an example, see our recent publication[7].

2. Preparation of buffers

2.1. —To prepare the fixation buffer, dilute purified, monomeric formaldehyde (sold by electron-microscopy suppliers as 16% paraformaldehyde) to 4% in sterile PBS Ca^{++}/Mg^{++} (phosphate-buffered saline plus Ca^{++} and Mg^{++}). Add vanadyl ribonucleoside complexes (VRC) to the fixation buffer for a final concentration of 2 mM to delay RNA degradation. Calculate the total amount of fixation buffer based upon requiring 300–500 μL of fixative per well of a 24 multiwell plate. Store the 4% formaldehyde on ice until it until the fixation step.

NOTE: Make the fixation buffer fresh for each experiment.

CAUTION: Formaldehyde is a teratogen that is absorbed through the skin. Use this chemical in a fume hood along with appropriate PPE according to your institutional regulations.

2.2. —For the permeabilization step, prepare 70% ethanol in nuclease free water. An alternative option is 0.5% Triton-X100 in sterile PBS Ca^{++}/Mg^{++} plus 2 mM of VRC. Calculate the total amount of permeabilization buffer needed by utilizing 500 μL of buffer per well.

2.3. —To prepare 9 mL of the hybridization buffer, add 2 mL of stock 50% dextran sulfate, and 1 mL of 20X SSC (saline sodium citrate) buffer to 6 mL of nuclease free water. Vortex to thoroughly mix the hybridization buffer. This buffer can be stored at 4°C for up to one week.

2.4. —There are five separate wash steps that require 2X saline sodium citrate (SSC) buffer. Calculate the final volume of 2X SSC buffer needed by anticipating 500 μL of buffer per coverslip per wash step. Dilute the required amount of stock 20X SSC buffer in nuclease-free water. 2 mM VRC can be added to wash steps as an extra precaution. This buffer can be stored at room temperature for the duration of the processing steps.

3. Fixation for RNA FISH

3.1. —Following treatment, remove the media from the wells and wash once with sterile, cold PBS Ca^{++}/Mg^{++}. If a low adherence cell model is utilized, do not use a vacuum to aspirate liquid; use manual pipetting to carefully remove media from the side of the well and omit the initial wash step. Add 500 μL fixation buffer for 30 minutes on ice.

NOTE: If the sample is prone to RNA degradation, use sterile PBS with 2 mM VRC for the wash step at this point.

3.2. —Remove fixative and dispose it in chemical waste according to institutional regulations. Wash the cells twice with cold sterile PBS Ca^{++}/Mg^{++} for 3 minutes.

3.3. —Remove PBS and add 500 μL of 70% ethanol to each well. Seal the 24 well plate with a paraffin plastic film. Place on a rotator at 4°C for a minimum of four hours. It is best to leave the samples in 70% ethanol overnight. The protocol can be paused at this and the samples can be stored for up to a week in 70% ethanol.

NOTE: A 0.5% Triton-X100 in PBS with 2 mM VRC solution may be used as alternative. Incubate the samples in 500 μL of this permeabilization buffer for 20 minutes at room temperature on a rotator. If utilizing this permeabilization buffer, the protocol cannot be paused at this point and must continue through the hybridization step.

4. Hybridization for RNA FISH

4.1. —Remove the permeabilization buffer and wash one time in 500 μL of 2X SSC buffer plus 10% formamide for 5 minutes at room temperature on a rotator.

4.2. —Prepare the complete hybridization buffer. Calculate approximately 30 μL of complete hybridization buffer per coverslip. Add formamide to the hybridization buffer to reach the percent needed. For example, if 500 μL of complete hybridization buffer is needed, then it would consist of 450 μL of the previously made hybridization buffer plus 50 μL of molecular grade formamide to reach 10% vol/vol. Vortex briefly to mix.

CAUTION: Formamide is a teratogen that is absorbed through the skin. Use this chemical in a fume hood along with appropriate PPE according to institutional regulations.

4.3. —Dilute the GREB1 intron (labeled with Atto 647N) and GREB1 exon (labeled with Quasar 570) probes 1:300 in hybridization buffer. Mix via pipetting. Protect this buffer from light and use immediately.

NOTE: The probes were designed and manufactured as detailed in Stossi et al[7]. The probes are usually provided as a dried oligonucleotide probe pool that must be reconstituted in RNAse-free TE buffer according to the manufacturer protocol[14]. The stock solution for these probes was 12.5 μM, therefore; the working concentration of the probes is 42 nM.

4.4. —Prepare a humidifying chamber for the overnight hybridization step. Lay down a piece of paraffin plastic film, unexposed side up, into a glass Petri dish cleaned with a surface decontaminant to remove RNases. Saturate two paper towels with sterile water and place them along the edges of the Petri dish to provide humidity, as drying can destroy specific labeling.

4.5. —Aliquot the probes by dotting 30 μL of probes in hybridization buffer onto the clean side of the paraffin plastic film. Distribute the aliquots of probes so that the coverslips do not come into contact with each other.

4.6. —Using sterilized forceps, gently flip the coverslips cell side down onto the probes in the glass Petri dish. Avoid air bubbles and do not apply pressure to the coverslips.

NOTE: Do not discard the tissue culture plate with the 2X SSC buffer, as it will be needed for subsequent steps.

4.7. —Seal the glass Petri dish with paraffin plastic film and cover the plate with foil to prevent light exposure to the fluorophores. Incubate overnight, up to 16 hours, at 37°C on a flat non-rotating surface. Time of incubation can be varied empirically, however a minimum of 4 hours is recommended.

NOTE: The coverslips are susceptible to sliding around when incubating in the hybridization buffer, which can lead to dry spots on the coverslip and damage the sample.

5. Preparing samples for imaging

5.1.

The next day, remove the coverslips from the humidifying chamber using forceps and return them to the 24 well plate with the cell-side up. Add 500 µL of fresh 2X SSC buffer plus 10% formamide to wash out excess probe and non-specific hybridization.

NOTE: Protect the samples from light from this point onwards.

5.2.

Wash the cells twice with 500 µL of 2X SSC buffer plus 10% formamide for 15 minutes each at 37°C on a heated rotator.

5.3.

Counterstain DNA with DAPI at 1 µg/mL in 2X SSC buffer for 10 minutes at room temperature on a rotator.

5.4.

Wash one time with 2X SSC buffer for 5 minutes at room temperature.

5.5.

Mount the coverslips onto glass slides using non-hardening mounting media after removing any excess 2X SSC buffer with a paper towel and a quick wash in nuclease free water to eliminate excess salts. Finally, seal the coverslips with clear nail polish.

6. High resolution microscopy and image processing

6.1.

Image the samples on a wide-field epifluorescence microscope using a 60x or 100x oil objective and a sCMOS camera. See the table of materials for the specific microscope and objective used for this experiment.

NOTE: Complete imaging as soon as the samples are fully processed to avoid time-dependent degradation of the signal.

NOTE: Immersion oil with a refractive index (RI) of 1.516 is used in this experiment. Empirical testing should be performed to match the oil RI to specific samples, as RI mismatches will result in aberrations of the point spread function that will affect image deconvolution.

6.2.

Images are captured with a lateral pixel size of 0.10827 µm.

6.3.

Optimize the amount of illumination from the light source (*i.e.*, percent transmittance) and exposure times for each channel (DAPI, TRITC, CY5 filters in this particular example) using the sample that is expected to have the highest intensity (*i.e.*, positive controls). In this experiment, the E2-treated sample is expected to have higher intensity for both GREB1 probe sets. Generally, the percent transmittance for GREB1 intron and exon probes is 50 – 100%, and the exposure times range from 0.25 – 0.60 seconds.

6.4.

Additionally, acquire images under conditions that avoid photobleaching and/or any saturation of camera pixels. In our experience, there should be a minimum ~ten-fold difference between the background and the signal intensity. Saturation of few pixels/field can occur due to non-specific signals in the sample (*i.e.*, dirt on the coverslip, probe aggregates outside the cell), which can be acceptable, but only if saturated regions are not included in quantitation.

6.5.

To set acquisition of a z-stack, focus on the sample in the DAPI channel. Select the top and the bottom of the z-stack at the distances where the DAPI stained nuclei become out of focus. The z-stack step size we used is 0.25 μm per slice and the total z-stack should span the whole cell (about 10 μm in MCF-7 cells). However, the z-stack step size will change according to the objective used and should follow the Nyquist sampling criterion.

NOTE: Introns are usually present only in the nucleus; however, exons are both in the nucleus and the cytoplasm. Therefore, setting the z-stack as described above will capture most of the intron and exon labeling as the z-stack encompasses the whole cell.

6.6.

We usually image a minimum of 200 cells for quantitative analysis in preliminary experiments to capture a snapshot of the magnitude of response and variation between cells.

6.7.

Some imaged cells will be excluded from the final analysis (*i.e.* drop-out rate) because they will be filtered out by the analysis pipeline (*i.e.*, cells touching the border of the image, apoptotic/mitotic cells).

NOTE: When selecting image areas, there are a few factors to keep in mind. Choose areas with evenly spread, non-overlapping cells as defined by DAPI-labeled, nuclear fluorescence. Additionally, try to select areas that have the least number of nuclei along the edges of the image area to avoid counting partial cells. Automated, unbiased imaging is always preferred for experiments requiring statistical analysis.

6.8.

After acquisition, images are deconvolved using an aggressive restorative algorithm using 10 cycles (*i.e.*, number of iterations). Generate max-intensity projections for image analysis

(omit this step if specific 3D analysis is required). Save all projection images according to the bit depth of the camera used; in our case 16-bit TIFF grayscale images were saved. RGB images have a reduced bit depth resulting in a loss of information; therefore, RGB images are not suitable for image analysis.

7. Image analysis

7.1.

Read the instructions and download the jupyter notebook from github (https://github.com/pankajmath/RNA_FISH_analysis).

7.2.

Install Python anaconda distribution (https://www.anaconda.com/distribution/) version 3.6 or higher.

7.3.

Open anaconda prompt and type in the installation command to Install Simple ITK for anaconda (https://anaconda.org/SimpleITK/simpleitk).

7.4.

Open anaconda navigator and launch jupyter notebook. It will open a web browser showing directories and files. Browse through the directory structures to reach the directory containing the downloaded jupyter notebook from github.

7.5.

Open the notebook and run each cell by pressing “Shift” and “Enter” simultaneously.

7.6.

The details of each function and step are provided in the notebook. For a different set of images, one may have to change some parameter values.

In brief, nuclei are segmented from the DAPI channel using local thresholding with a block size of 251 pixels, holes were filled and objects smaller than 100 pixels removed. Touching nuclei were split using a watershed algorithm. The nuclear mask was then dilated by 100 pixels and watershed was used to separate touching objects using nuclei as basins. To identify steady state RNA (exons), Otsu thresholding was used; for transcriptional active alleles (intron), first a Gaussian blur (sigma=1) was applied and then max entropy thresholding was used.

REPRESENTATIVE RESULTS:

To analyze hormonal responses via smFISH, as an example, we chose our estrogen receptor (ER) model used in high throughput assays to determine the presence of endocrine disrupting chemicals in environmental disasters in the context of participation in our NIEHS Superfund Research Program[7]. In this experiment, adherent MCF-7 breast cancer cells were

treated with the ER agonist 17β-estradiol (E2, 10nM) or vehicle (DMSO) for 24 hours. Spectrally-separated probe sets against GREB1 intronic (Atto 647N, red in Figure 1) and exonic (Quasar 570, green in Figure 1) sequences allow for simultaneous visualization and quantification of nascent and mature mRNA; importantly, marking the number of transcriptionally-active alleles in each cell that has been shown to be part of the estrogen response time course[7].

The completed protocol generates 16-bit maximum intensity projections (TIFF) for each region of interest. Nuclear segmentation is performed using DAPI stained nuclei, and cell boundaries are estimated by expanding the nuclear mask. GREB1 intron and exon signals are then segmented and assigned to each individual cell. Figure 1 displays deconvolved max-projected images and their segmentation for a sample treated with DMSO and E2.

Following the image analysis pipeline, we obtained the number of cells that were not touching the image boundaries based on imaging ten random fields per treatment, and it was determined that there were 150 DMSO treated cells and 149 E2 treated cells. Since the aneuploid MCF-7 cells have four copies of the GREB1 gene, and with intron and exon probes, overlapping signals will determine the number of alleles and cells that are engaged in active transcription[24]. We determined the fraction of the cell population for each treatment that had zero-to-four active GREB1 alleles by counting overlapping intron and exons spots. As in with our previous study, we define transcriptionally active cells as those that displayed two or more active GREB1 alleles[7]. As seen in Figure 2A–B, E2-treated cells have a 4-fold increased fraction of cells that show two or more active GREB1 alleles compared to vehicle treated cells[7].

Introns are spliced out of nascent mRNA to produce mature mRNA that consists of only exon sequences. Therefore, it is also possible to count the number of mature mRNA per cell by quantifying the number of green spots. Figure 2C–D show the shift in distribution of mature GREB1 mRNA/cell and the aggregate fold change (20x) in the cell population after E2 treatment.

In keeping with our original observations over 24 hours of E2-treatment, not all cells respond the same way (*i.e.*, responses are heterogeneous in the MCF-7 population)[7]. For example, the number of mature GREB1 mRNAs per cell shows a vast range of expression (~15 fold) even in the 24-hour E2-treated cells; bulk RNA quantitation methods fail to discern the wide-ranging levels of transcription in cells by statistical averaging. This emphasizes the power of smFISH to explore heterogeneous cell-to-cell and allele-by-allele responses in a population of isogenic cells.

DISCUSSION:

The smFISH methodology described is based upon reviously-published protocols[7,12,14]. In this protocol, we explain the critical steps that were optimized from the wet-lab, (including seeding density, fixation time, permeabilization, and probe concentration), to imaging and image analysis, providing a full experimental pipeline for laboratories interested in performing single cell analysis of gene transcription.

To facilitate single cell analysis, it is important that the cells are subconfluent and do not overlap so that cell borders are estimated without the need for a cell boundary. We suggest completing a cell proliferation assay that spans a wide range of seeding densities for the duration of the experiment to optimize cell density.

Determining the optimal fixation time is critical for a successful smFISH experiment. We have found that fixing the samples with 4% purified formaldehyde, diluted in sterile PBS containing VRC for 30 minutes on ice, significantly improves the consistency of high quality smFISH images, especially if structural analysis is required via combination of antibodies against factors of interest. Formaldehyde fixes samples by cross-linking macromolecules and can better maintain cellular structures than other fixation methods such as those employing organic solvents[25]. However, adjusting fixation time and temperature is necessary for your specific sample to avoid over- or under-fixing the sample[26]. VRC is useful because it reduces RNA degradation and is helpful especially for low abundant RNA[27]. There are two options that we have had success with for the permeabilization step. The first is a 70% ethanol incubation at 4°C and the second is a 0.5% Triton-X100 incubation at room temperature[7,14]. It is possible to perform the same protocol in high throughput using imaging-compatible glass bottom multiwell plates (96 and 384 wells). Consistent success in performing smFISH has depended upon use of glass multiwell plates using fixative containing VRC, and 0.5% Triton-X 100 with VRC for the permeabilization step. Although optical plastic bottom plates are an option, image quality and success has been markedly lower.

A high signal-to-noise ratio is required for single cell analysis to filter out background signal and false positives and correctly identify diffraction-limited spots. Background signal adds to the intensity values of the signal of interest. High background often arises from sub-optimal experimental design, and although it is subtracted from fluorescent intensity measurements, it is best to initially image the regions of interest with as little background signal as possible[28]. A high dynamic range, with a minimum ~10-fold difference, is required to successfully determine if signal is considered background or signal of interest[29]. False positive spots refer to signal residing outside of the cell boundaries, often due to poorly cleaned coverslips, insufficient washing following hybridization, or probe aggregation that occurs when probes self-associate[30]. If non-specific signals due to spurious binding of the oligo set to RNAs that are different from the target happens (*i.e.*, due to pseudogenes or sequence similarities), these can be evaluated by testing the probe sets in a model that does not express the gene of interest (*i.e.*, knock-out MEFs, CRISPR/Cas9 knock-out)[31]. Additionally, as with any fluorescence microscopy experiment, the intron and exon probes must be spectrally-separated. In our example experiment, we chose Atto 647N and Quasar 570 dyes for the GREB1 probe set because they are compatible with the specifications of the filter sets on the microscope utilized, resistant to photobleaching and have a relatively high quantum yield[8,14,32]. Depending on the sample, we recommend making a dilution series of the stock probe (usually a 1:200 to 1:1000 dilution, or 12.5 nM to 62.5 nM) to identify the best signal-to-noise ratio for each specific probe set. Some vendors supply custom-labeled probes with user-selected fluorophores to increase brightness, reduce photobleaching and, if needed, add additional 1–2 channels generally available on most modern fluorescent microscopes [7,8,14]. Although smFISH does have the ability to measure low-abundance RNA,

a key issue is increasing its sensitivity and capability to detect partially degraded RNA, especially for tissue samples. Achieving a higher signal-to-noise ratio is possible by using branched DNA probes designed to amplify the signal, though we have not found this approach is helpful in our studies[33]. Confocal microscopes use a focused light source to illuminate the sample while blocking out-of-focus light from reaching the detectors with one or more pinholes. Point-scanning confocal microscopy is generally discouraged for RNA-FISH imaging because probes will photobleach faster from laser illumination. However, depending on the sample and signal intensity, confocal microscopes can generate images with little to no signal loss[8]. Here, we utilized a wide-field epifluorescence microscope with a high magnification and high numerical aperture objective to collect the maximum number of photons emitted by the FISH probes[34]. Although images can be blurred by out-of-focus light from adjacent planes of the z-stack, restorative deconvolution algorithms are applied to computationally "reassign" diffracted light among the acquired z-stacks to its point of origin[34]. This produces final high-resolution images for image analysis. If possible, it would be best to empirically compare different imaging modalities to determine the system that is best for your experiment[28].

There are many possible extensions and applications of smFISH. In our model experiment, we completed one round of hybridization with one set of probes for the GREB1 gene. However, it is possible to complete several rounds of smFISH in a sequential manner (seqFISH)[13,35]. In this method, the mRNAs in the cell are labeled by sequential rounds of hybridization, imaging, probe-stripping and rehybridization. In order to massively increase multiplexing up to whole transcriptome level, barcoding techniques have been developed (e.g., MER-FISH)[36–38]. This uses a fluorescence barcode strategy to uniquely identify each mRNA[37,39]. These techniques have been adapted to tissues, and when coupled with expansion microscopy, a method that physically expands a sample with a polymer network, can provide increased access of probes to endogenous RNAs[36,40,41]. MER-FISH also allows for increasing the signal brightness of individual molecules by signal amplification techniques[38]. The protocol we described is a two-day process, however, it is possible to shorten the smFISH protocol so that the hybridization of the probes to the target RNA occurs can occur in as little as ~5 minutes (Turbo-FISH)[26]. Immunofluorescence can be combined with smFISH (IF-FISH) to simultaneously detect proteins and mRNA in a single sample[42]. The protocol must be optimized for the FISH probes and antibodies utilized for each experiment to reduce degradation of protein and/or RNA degradation in the sample, as some materials (*i.e.* buffers and fixatives) are not compatible for processing both protein and mRNA[43]. Refer to several publications from our lab as examples of successful IF-FISH experiments in addition to optimized IF-FISH protocols[7,18].

In conclusion, we present a single molecule fluorescence *in situ* hybridization (smFISH) method that provides insight into single cell heterogeneity and allele-by-allele variation in response to stimulus. While this protocol is optimized for the model system previously described, we provide a series of possible adjustments that can enhance other target gene models[7].

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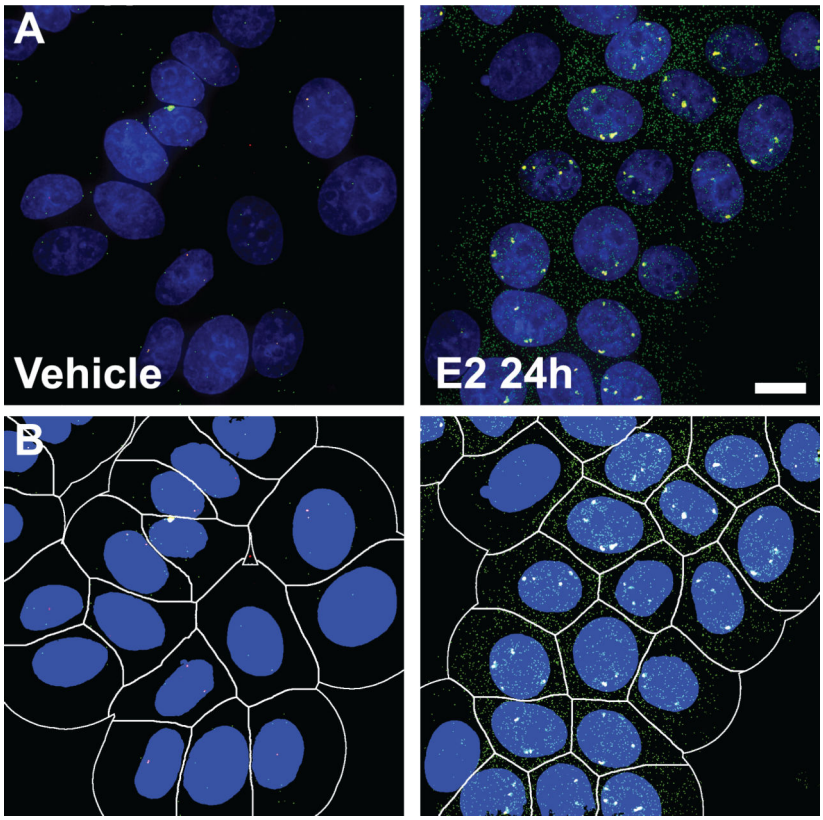

Figure 1:

smFISH sample images and output of image segmentation. (A) MCF-7 cells, treated with DMSO (left panel) and 17β-estradiol (E2, right panel) for 24h, were hybridized to target GREB1 introns (Atto 647N, nascent mRNA, red), and GREB1 exons (Quasar 570, mature mRNA, green). Images are acquired at 60x/1.42NA, deconvolved and maximum intensity projected. (B) Images from panel A are processed through the described image analysis pipeline that defines the nuclear mask, estimates the cellular mask, identifies individual mature mRNA and transcriptionally active alleles. GREB1 intron and exon spots are then assigned to individual cells. Scale bar: 10 μm.

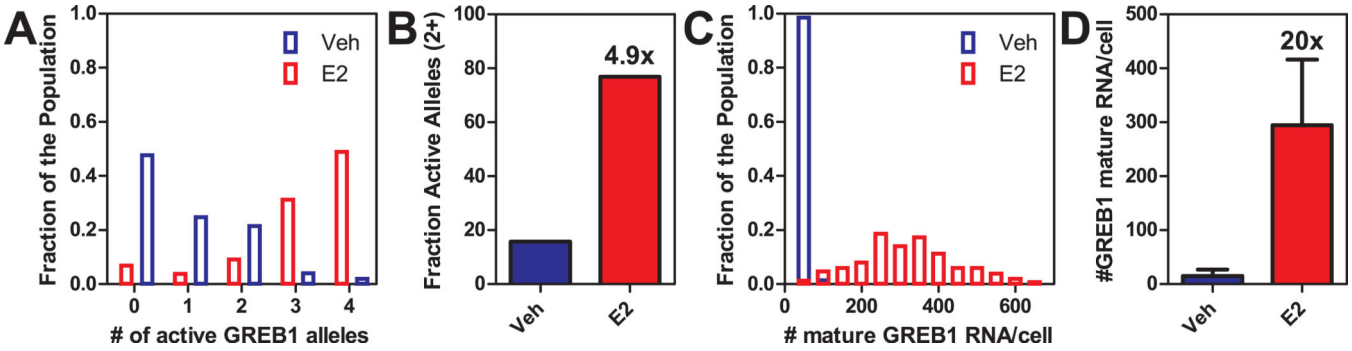

Figure 2:

Quantitation of E2-induced GREB1 at the cell-by-cell and allele-by-allele level. (A) The distribution of the number of active GREB1 alleles per cell comparing vehicle (blue bars) and E2 (red bars) treated cells. (B) Fraction of cells that are considered transcriptionally active, defined here as cells with two or more active GREB1 alleles[7]. (C) Distribution of the number of GREB1 mature mRNA per cell for each treatment. (D) Average number of GREB1 mature RNA/cell represented as mean value for the population. Error bars indicate standard deviation.

Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
17β Estradiol	Sigma-Aldrich	E2257	CAS Number 50-28-2
Ambion SSC (20X) Buffer, Rnase-free	ThermoFisher Scientific	AM9770	
Corning DMEM with L-Glutamine and 4.5 g/L Glucose	Fisher Scientific	MT10017CV	Manufactured by Invitrogen
DAPI	Sigma-Aldrich	D8417	Without sodium pyruvate
Dextran Sulfate, 50% Solution Sterile	Sigma-Aldrich	3730-100ML	
Dulbecco's Phosphate Buffered Saline Ca++/Mg++	Corning	21030CV	Manufactured by Calbiochem
Ethanol, 200 Proof (100%)	Fisher Scientific	07-678-005	
Falcon 24-Well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate	Corning	353047	Manufactured by Decon Laboratories
Fetal Bovine Serum (FBS), 500 mL	Fisher Scientific	50-753-2978	Manufactured by Gemini Bio Products
GE Healthcare DeltaVision LIVE High Resolution Deconvolution Microscope	GE Healthcare		
Hyclone Water, Molecular Biology Grade	Fisher Scientific	SH3053802	
Immersion Oil 1.516	GE Healthcare Life Sciences	29162940	Manufactured by GE Healthcare Bio-Science
L-glutamine Solution	Fisher Scientific	MT25005Cl	Manufactured by Corning
MCF-7 cells	ATCC	HTB-22	
Molecular Grade Formamide	Promega	PAH5051	
Olympus 60x/1.42 NA Objective	Olympus		
Parafilm	Bemis Company, Inc.	52858-076	Distributed by VWR
Paraformaldehyde, 16% Solution, EM Grade	Electron Microscopy Sciences	15710	
Penicilin-Streptomycin Solution	Fisher Scientific	MT3001CI	Manufactured by Corning
Ribonucleoside Vanadyl Complex	VWR	101229-716	Manufactured by New England Biosciences
RNase Away	Ambion	9780	
Sodium pyruvate, 100 mM	Fisher Scientific	11-360-070	Manufactured by Gibco
Thermo Scientific Glass Coverslips, #1.5	Fisher Scientific	12-545-81P	
Triton X-100, 100 mL	Fisher Scientific	BP151-100	Manufactured by Fisher BioReagents
Trypsin-EDTA Solution 0.25%	Sigma-Aldrich	T4049-500ML	
Vectashield Antifade Mounting Medium 10 mL	Fisher Scientific	NC9265087	Manufactured by Vector Laboratories