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# Single molecule analysis of transcription dynamics to understand the relationship between epigenetic alterations and transcriptional variability

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**Running head**:

Analysis of epigenetically altered transcription dynamics

**Key words**:

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## Abstract

Heterogeneity in gene expression largely stems from the discontinuous nature of transcription, with transcripts being produced in bursts with defined frequencies. This cell-to-cell variability in transcription within isogenic cell populations is a known phenomenon across numerous genes. Multiple gene regulatory and epigenetic factors have been identified as key contributors to this pulsatile gene activity. Understanding the effects of epigenetic modulation on transcriptional cell-to-cell variability and kinetics of transcriptional activity is crucial for interpreting changes in treatment responsiveness.

We present a detailed protocol that guides the assessment of fluctuations in gene expression induced by epigenetic modulation using single molecule RNA in situ hybridisation (smRNA FISH) combined with confocal microscopy imaging, data analysis and quantification in breast cancer cells. Through smRNA FISH labeling, both mature and nascent transcripts are identified. Subsequently, the number of mature transcripts and the intensity and frequency of nascent transcripts are quantified, and these measurements are used to calculate the burst size and frequency for the labeled gene. By following this step-by-step methodology, insights are obtained into the intricate relationship between epigenetic alterations and the dynamic nature of gene expression in breast cancer cells.

## 1. Introduction

Genetically identical cells, even being in similar developmental states, often exhibit remarkable differences in their mRNA expression levels [1]. This cell-to-cell variability in gene activity has been observed for many genes. Heterogeneous transcription largely originates from the discontinuous nature of transcription, which occurs in bursts or pulses (the ON state) followed by periods of inactivity (the OFF state) [2]. This pulsatile gene activity is related to a combination of gene regulatory features, i.e. the probabilistic association of transcription factors with gene regulatory regions, the non-continuous elongation of RNA Polymerase II, stochastic enhancer regulation, and changes in epigenetic chromatin structure [3].

Transcriptional variability has been associated with various biological phenomena. It allows genes to adjust their expression in response to environmental fluctuations [4]. At the cellular level, transcriptional cell-to-cell variability provides a mechanism to generate diversity between cells or organisms to adapt differently to a changed environment or exposure condition, for instance during cell differentiation contributing to the establishment of cell types [5, 6]. Importantly, transcriptional variability can lead to the development of therapeutic resistance by adaptation of gene expression profiles and outgrowth of cells that do not respond to the treatment with a more favourable phenotype to a therapeutic response. Such conditions can lead to loss of cellular homeostasis and development of severe diseases [7].

Knowing how to epigenetically regulate variability versus consistency in gene expression for instance with epigenetic editing within tumour cells has the potential to interfere with the development of drug resistance and extend patient survival [8]. Assessing the impact of epigenetic treatments, not only on the transcriptional magnitude but also on the transcriptional variability and kinetics, is essential to evaluate to monitor changes in cell behaviour [5, 9–11].

Single-molecule RNA fluorescence in situ hybridization (smRNA FISH) is a technique utilized for detecting and quantifying individual RNA molecules of a specific gene of interest in individual cells. In smRNA FISH, short oligoribonucleotide probes complementary to different regions of the target RNA (such as exon or intron), are employed [12, 13]. These probes are each tagged with a fluorescent dye, allowing for the single molecule identification and quantification of the transcripts produced by the target gene.

Here, we present a step-by-step protocol for evaluating epigenetic modulation-induced fluctuations in gene expression using smRNA FISH (**Figure 1**). Specifically, we illustrate the procedure for investigating the effect of epigenetic drug treatment in combination with endocrine treatment on the transcription dynamics of hormone-responsive breast cancer MCF7 cells. We describe the methodological analysis of the experimental procedure which includes cell culture and treatment with the epigenetic drug KDM5-C70, a specific inhibitor of histone demethylase KDM5 that induces trimethylation of histone H3 at lysine 4 (H3K4me3), and treatment with fulvestrant, a selective estrogen receptor (ER) degrader (SERD), commonly used in endocrine therapy for premenopausal women with ER+ breast cancer. Our protocol encompasses various stages, such as (epigenetic) drug treatment, smRNA FISH labelling, microscopy analysis as well as data analysis and quantitative interpretation. By following this comprehensive methodology, insights into the effects of epigenetic modulation on the dynamic nature of gene expression are obtained (**Figure1**).

## 2. Materials

### 2.1 Cell treatment with epigenetic and endocrine drugs

1. Cell culture medium: Glutamax DMEM media with 10% foetal bovine serum and Penicillin-Streptomycin-Glutamine (Penicillin, 100 units/mL - Streptomycin, 100 µg/mL) and pre-warm to 37°C (*see* **Note 1**).
2. For cell treatment: culture the cells on µ-Slide 4 Well Ibidi chamber Glass coverslips.
3. For epigenetic drug treatment: Dissolve 25 mg of lyophilized chemical KDM5-C70 (Cat. No.: HY-120400, Xcesbio Biosciences) in DMSO, resulting in a final concentration of 10 mM. For endocrine drug treatment: Dissolve 25 mg of chemical compound Fulvestrant (Cat. No.: 14409, Sigma) in DMSO, resulting in a concentration of 0.1 mM (*see* **Note 2** and **Note 3**).

### 2.2 smRNA Fluorescence In Situ Hybridisation (FISH)

1. TE buffer: 10 mM Tris-HCl, 1 mM EDTA. Create TE buffer and adjust to pH8.
2. Use RNAse-free 10X Phosphate buffered saline (PBS) and RNAse-free water.
3. Use RNase-free pipette tips with filters (10–100 µl, 100–1250 µl).
4. Use Eppendorf tubes that protect or completely block light transmission enabling storage of light sensitive material and dissolve the FISH probes in the hybridization mixture.
5. Formaldehyde fixation solution 37% (vol/vol). To prepare the fixation mixture, create a volume of 10 ml by dissolving 1 ml 37% formaldehyde in 1 ml RNAse-free water and 8 ml RNAse-free 10xPBS. This fixation buffer can be adjusted to accommodate multiple reactions and can be stored at room temperature (RT) for future use (*see* **Note 4**).
6. Use 70% (v/v) ethanol for cell permeabilization (molecular biology grade).
7. Use deionized formamide for creating the probe solution.
8. Use Stellaris Wash Buffer A, Wash Buffer B, and Hybridization Buffer (Biosearch Technologies). For creating wash buffer A, prepare 6 ml buffer in a 15 ml tube. For this solution, first mix 4.2 ml nuclease free water with 1.2 ml Stellaris RNA FISH buffer to which 0.6 ml deionized formamide is added. The solution is mixed well by pipetting the solution up and down or inverting the tube. For creating wash buffer B, mix 88 ml nuclear free water with 20 ml wash buffer B in the tube. The diluted wash buffer B can be stored at 4°C for future use. For creating the hybridization mixture, calculate the necessary amount of hybridization mixture. For a single well of a µ-Slide 4 Well Ibidi chamber, 100 µl mixtures is needed for 4 wells (25 µl/well). Prepare this mixture by mixing 90 µl Stellaris RNA hybridization buffer with 10 µl deionized formamide to which 1 µl FISH probe is added as prepared in step 1 (*see* **Note 5**).
9. Use microscope cover glasses (*see* **Note 6**), parafilm, and razor blades for the FISH hybridization procedure.

10. For DNA probe design: use the Stellaris Web pagehttps://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer) (*see* **Note 7** and **Note 8**).
11. Create 4',6-Diamidino-2-phenylindole, hydrochloride (DAPI 5 ng/ml stock) for cell nuclear staining.
12. 2xSSC (Sodium Chloride-Sodium Citrate) buffer, RNase-free: 3 M NaCl, 0.3 M sodium citrate. Dissolve and adjust pH to 7.0.

### 2.3 Microscopy equipment and imaging analysis

1. Nikon Ti inverted confocal microscope (A1) containing: Optical table; High-numerical-aperture (NA), oil-immersion objective (60x Plan Apo 1.4 NA); Band-pass emission filters to separate fluorescence signals (450/50, 595/50, 640LP); Mercury and transmission lamp; Fluorescent cubes for BFP, CFP, GFP, YFP and RFP imaging; High-sensitivity electron-multiplying charge-coupled device (EM-CCD); 4 PMT Detectors configured for different dichroic; Motorized optical shutter; Motorized XYZ stage; Universal specimen holder.
2. Computer: With all the software listed below. ~~Enough HDD storage~~.
3. Computer software: MATLAB; Python; FIJI Image J; Microscope management software (NIS-Elements).

## 3. Methods

### 3.1 Cell culture, epigenetic and endocrine drug treatment

1. For cell culture, grow the MCF-7 cells in a T25 flask with a vented cap ~~a breathing hole~~ until reaching 70% confluency. Cells with early passage (passage number 10 - 25) need to be employed for the experiment.
2. Seed the cells on Ibidi 8-well chambered culture slides and allow the cells to adhere for at least two hours. The medium needs to be subsequently removed and replaced with pre-warmed medium supplemented with the epigenetic drug (KDM5-B-C70) and/or endocrine drug (Fulvestrant), for 24 hours. Treat cells in one well with DMSO only as vehicle control. To reach the proper treatment concentration, for epigenetic and endocrine drug treatment, dissolve 1 µl KDM5-C70 (10 mM) and 1 µl of Fulvestrant (0.1 mM) each in 1 ml culture media.

### 3.2 Probe design and handling

1. For the probe design process, a number of fluorescently labelled oligonucleotide probes should be designed to bind to the exon sequence (*see* **Note 7**) of the target RNA. The probe length should range between 17 and 22 nucleotides long. To facilitate the design and ordering of custom probes, utilize an online tool via the following link: https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer
2. To suspend the custom probes (5 nmol), add 400 µl of TE buffer to the tube containing the lyophilised probes resulting in the final concentration of 12.5 µM. The solution needs to be thoroughly mixed by vortexing and then centrifuged to pellet any remaining debris or aggregates. Aliquots of 20 µl (*see* **Note 8**) are made. The probes can be stored at -20°C.

### 3.3 Fixation and permeabilisation

1. Gently remove the media after 24 hours treatment with epigenetic and/or endocrine drugs with a 1 ml pipette. The cell culture plate should be tilted to remove the media by touching one of the corners of the well. Wash the cells gently with 1 X PBS at room temperature.

2. Add 1 mL of fixation buffer to each well and incubate at room temperature for 10 minutes. This step needs to be performed carefully to prevent that the cells detach and that the cells dry out during the fixation process (*see* **Note 9**).
3. Wash twice with 1 mL of 1 X PBS.
4. Permeabilize the cells in 1 mL of 70 % ethanol at 4°C for 1 hour. The incubation can be prolonged overnight. Cells in ethanol can be stored at 4 °C for a week.
5. For cell permeabilization, wash the cells twice with 1X PBS. Cells are permeabilized by adding 500 µl of 70% (v/v) ethanol to each well and the cells need to be incubated.

### 3.4 Hybridization with FISH probes

1. For preparing the cells for hybridization, aspirate the ethanol using a 1 ml pipette by touching the corner of the well while 0.5 ml (1 well of a 4 chambered plate) wash buffer A is added per well and incubated for 5 minutes at room temperature.
2. For hybridization of the probes, aspirate wash buffer A and add 50 µl hybridization mixture per well. The sample needs to be covered with a glass coverslip that is cut to fit the size of the well (*see* **Note 6**). Samples are stored in a black box at 37°C for at least 4 hours. The hybridization can be prolonged overnight. When the incubation is finished, remove the glass coverslip and add 0.5 ml wash buffer A per well and incubate for 30 minutes at 37°C in a black box (*see* **Note 10**).
3. For DAPI staining, after removing wash buffer A, add 0.5 ml DAPI nuclear stain to create a final concentration of 5 ng/ml in wash buffer A. Stain the cells with DAPI staining for 30 minutes at 37°C in a black box. After the incubation, replace wash buffer A with wash buffer B and incubate for 5 minutes at room temperature. When wash buffer B is aspirated add 0.25 ml of 2X SSC buffer.

### 3.5 Microscopy imaging and image analysis

1. For imaging the cells, use a Nikon Ti-E inverted confocal microscope (A1) with an automated stage using a 60x oil objective and Nikon NIS-Elements imaging software.
2. For each condition, 100 cells need to be imaged. For excitation, 405nm and 561nm lasers lines need to be used and for detection 450-50 nm and 595-50 nm band-pass filters. Optical 0.35 µm sections in the Z-direction and a resolution of 512x512 pixels (16 bits), resulting in a voxel volume of 0.002 $\mu m^3$. To reduce photon and camera noise, use four times averaging. To obtain microscopy images with appropriate quality, the following microscopy settings are used 512x512 pixels (16 bits), Z-stack of 15 z-steps, 2 channels (450 and 595 nm emission for DAPI and CAL Fluor Red 590), with a pixel size of 0.083 µm in XY and 0.35 µm in Z.
3. For microscopy image analysis two phases are followed: spot counting and the selection and intensity measurement of the most intense spots representing the transcription start sites where nascent transcripts are produced.
4. Store the microscopy images using microscope management software (e.g. NIS-Elements) and convert the images to TIF format with FIJI ImageJ software for further processing. In case the imaged cells occupy the full field of view, cell segmentation is not necessary.
5. For detection and counting of single transcripts in 3D (*see* **Note 11**) utilize a dedicated MATLAB script [12, 13]. Briefly, the spot detection consists of filtering the image with a semi-three-dimensional Laplacian of Gaussian filter (with a filter width of 1.5) removing noise and enhancing the signal-to-noise ratio, after which spurious background noise spots are removed (**Figure 2**).
6. Transcription bursts can be recognized by the presence of large more intense fluorescent spots at the transcription site (TS) where nascent RNA accumulates temporarily in the nucleus [14]. Interactively select these more intense transcription spots in the nucleus with a customized Python script integrated with Napari [15, 16] (**Figure 3**). Calculate the intensity of the transcription burst sizes by manually collecting the intense fluorescent nascent transcription

spots based on maximal image projections. To extract the position of each intense spot in full Z-dimension, apply a region of interest with dimensions of 4x4 pixels and a density of 0.336 $\mu m^2$ across all optical sections. The region of interest enables precise intensity analysis of the intense transcription spots. By dividing the sum of the intensity of the intense nascent transcripts by the average sum of the overall (mature RNA) nuclear spots labelled with the exon FISH probe. This way a relative number of transcripts (bursts) present at the transcription start sites is obtained. Determine the transcription frequency as the number of intense nascent transcripts obtained per total amount of cells analyzed (in this case 100 cells) (*see* **Note 12**).

## 4 Notes

1. MCF7 cells are cultured with medium plus $10^{-8}$ M 17-β-estradiol (Sigma E8875) to keep their luminal estrogen receptor positive phenotype.
2. To determine the most suitable drug treatment concentration, based on literature data, the half maximal effective concentration ($EC_{50}$) needs to be determined experimentally, e.g. using a cell viability analysis with 10-fold dilutions.
3. In accordance with the manufacturer protocol, all drugs need to be stored in DMSO 1000 times more concentrated than used in cell culture. The working concentrations are obtained by dissolving 1 µl in 1 ml media.
4. CAUTION! Formaldehyde is very toxic and a known carcinogen. Handle it in a fume hood while wearing a lab coat and protective gloves.
5. The hybridization mixture has to be prepared right before use. Signal detection is improved by utilizing a freshly prepared hybridization mixture. To ensure optimal quality of the hybridization mixture during longer storage periods, it is advised to prepare the mixture in an Eppendorf tube that protects or completely blocks light transmission to store light sensitive material and keep it at 4°C.
6. Glass cover slips are used to cover the hybridization mixture on the cells. The glass covers can be cut from a glass cover slip with a razor and a liner. The glass pieces should be cut a bit smaller than the exact size of the slide chamber well to enable to easily remove the glass after hybridization.
7. To ensure optimal spot quality, the design of the smRNA FISH probes must adhere to specific requirements. The first requirement is the length of the probe based on the targeted exon and the need for a detectable signal. Typically, a range of 24 to 48 probes is utilized to cover the mRNA sequence of interest. The second requirement involves designing probes to bind to the mRNA prior to its maturation.
8. The probes are expensive and therefore proper storage conditions and handling are important. To prevent the negative effects of sequential thawing-freezing cycles and contamination, it is advisable to aliquot the dissolved probes. Both repeated thawing and contamination can significantly impact the detection signal.
9. Fierce pipetting of the solutions should be avoided, since this can lead to detachment of the cells from the slides.
10. After adding the FISH probes to the samples, the samples should be kept in a black box to avoid long exposure to light (natural or artificial). The sample should be covered with a glass coverslip to prevent that the cells dry out. To remove the glass coverslip, a needle is used to open the glass cover from one of the corners. Sometimes the cover breaks but this will not affect the sample quality. Be careful not to scratch the cells with the needle. A black incubation box can be made from an empty box that contained pipette tips. The lid should be covered with aluminium foil and a piece of tissue should be placed on the bottom of the box to help removing the sample from the box.

11. For finding the optimal threshold of spot counts ($N$), spots are calculated for 100 different spot intensity threshold values ($T$); the flattest point in this spot count-threshold curve, $N(T)$ is used as the optimal threshold value ($T_{opt}$). The flattest point is determined by applying the following sliding window function to this curve: $W_i = \mu_i(k)/\left(\alpha + \sigma_i(k)\right)$ and winding the threshold ($T_i$) that belongs to the largest value of $W_i$. The parameters $\mu_i(k)$ and $\sigma_i(k)$ denote the moving average and moving standard deviation with window size $2k + 1$ respectively and $\alpha$ denotes an empirical constant. This methodology is designed to select a threshold that is less susceptible to changes, a moving window size of 7 ($k$ = 3) and $\alpha$ = 10 gave a reliable estimation of the number of mRNA spots [12, 13].
12. An alternative approach to assess transcription burst size and frequency from confocal microscopy measurements is by determining the fraction of time a promoter is active (the ON state, known as the transcription frequency), and the number of transcripts produced during the ON state, (known as the transcription burst size), using 2-state modelling. A useful procedure is available through Quant FISH and Bay FISH [16]. https://fish-quant.github.io/

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## Figures and figure legends

**Figure 1**: **Cartoon of the involved methodologies to determine the effect of epigenetic modulation on changes in transcription dynamics.**

*Upper part*: Breast cancer cells (MCF-7) are seeded in chambered culture slides and treated with different conditions (treatment A-D) e.g. only culture media as control (A), culture media supplemented with epigenetic drugs, e.g. KDM5-C70, a specific inhibitor of histone demethylase KDM5, and with endocrine treatment, e.g. fulvestrant, a selective estrogen receptor degrader (B-D).

*Middle part*: After removal of the media MCF-7 cells are undergoing various steps to detect single transcripts of the gene of interest. Cells are fixed (step 1), permeabilized (step 2), smRNA FISH labeled (step3), and imaged with confocal microscopy (step 4).

*Lower part*: To determine the effect of epigenetic modulation on the transcription dynamics of the gene of interest, data analysis of the microscopy images is performed. The number and intensity of smRNA FISH transcripts are counted. The intensity of nascent transcripts at the transcription site (TS) and the number of nascent transcripts per total amount of cells are quantified as a measure of the transcription burst size and frequency, respectively.

An alternative approach to assess transcription bursting parameters from smRNA FISH microscopy measurements is by using 2-state modeling through Quant FISH and Bay FISH (https://fish-quant.github.io/). The fraction of time a promoter is in the ON state (transcription frequency), and the number of transcripts produced during the ON state (transcription burst size) can be determined.

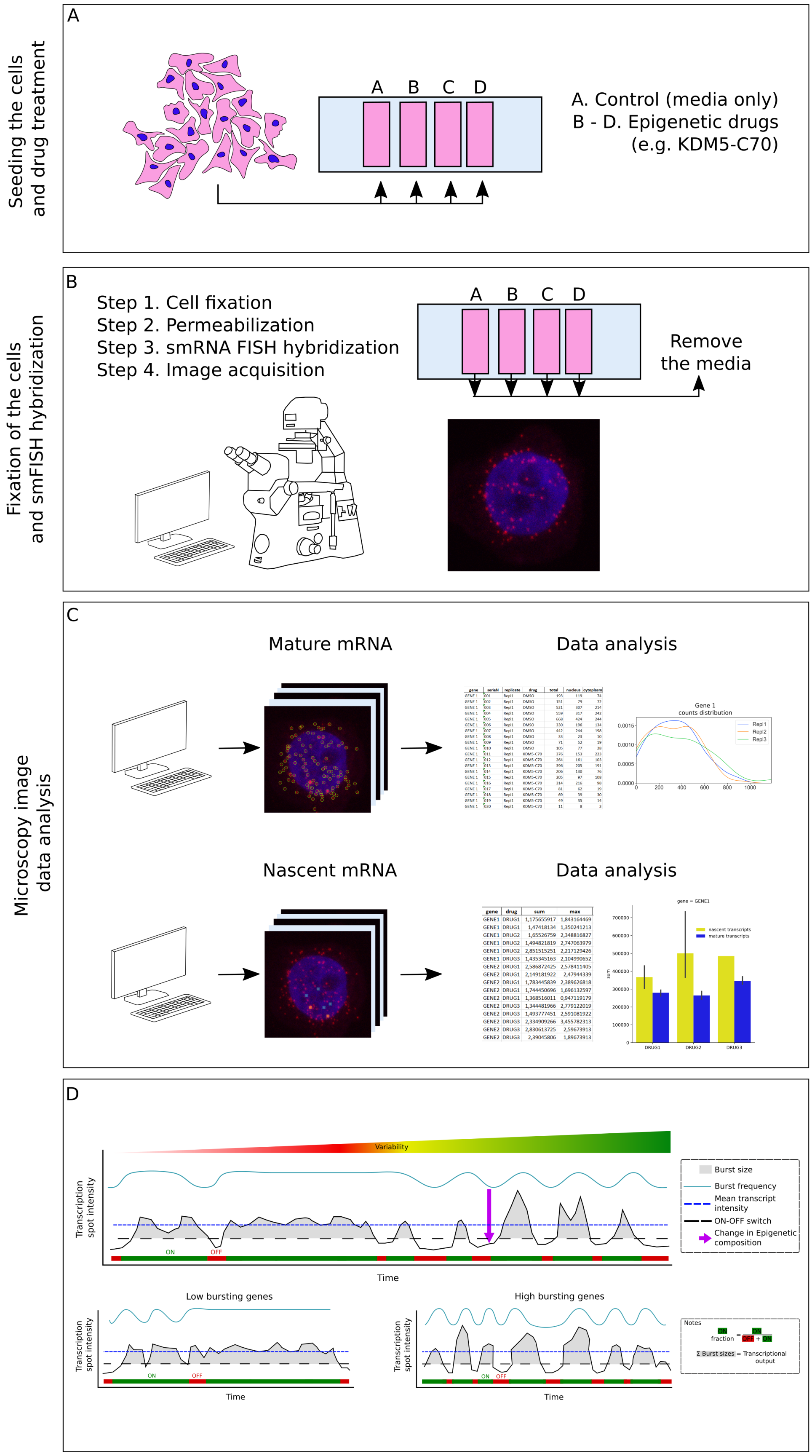
Seeding the cells and drug treatment
A
A B C D
A. Control (media only)
B - D. Epigenetic drugs
(e.g. KDM5-C70)
Fixation of the cells and smFISH hybridization
B
Step 1. Cell fixation
Step 2. Permeabilization
Step 3. smRNA FISH hybridization
Step 4. Image acquisition
A B C D
Remove the media
Microscopy image data analysis
C
Mature mRNA
Data analysis
Nascent mRNA
Data analysis
D
Variability
Transcription spot intensity
ON
OFF
Time
Burst size
Burst frequency
Mean transcript intensity
ON-OFF switch
Change in Epigenetic composition
Low bursting genes
High bursting genes
Notes

**Figure 2. Example of a confocal microscopy imaging and quantification of single molecule RNA FISH labeled transcripts.**

A) Image projection from confocal microscopy images of smRNA FISH labeled amphiregulin (AREG) transcripts in a breast cancer MCF-7 cell. Orange/yellow spots depict single AREG molecules in the cytoplasm and nucleus. The blue signal indicates the DAPI stained nucleus. The right insert is a magnification of single molecule transcription spots in the cytoplasm, and the left insert is a magnification of a newly synthesized transcription site in the nucleus. The 10 μm sized bar enables to depict the size of the MCF-7 cell and smRNA FISH labeled transcripts.

B) Depiction of the smRNA FISH labeled cell showing 3D confocal sections in Z-direction. C) Illustration of counting the number of single molecule transcription spots in the cytoplasm and nucleus. The white encircled spots represent the counted transcripts. D) Determination of the threshold for counting transcription spot numbers. The iterative spot counting script using different threshold values helps to find the optimal threshold corresponding to the actual transcript spot count.

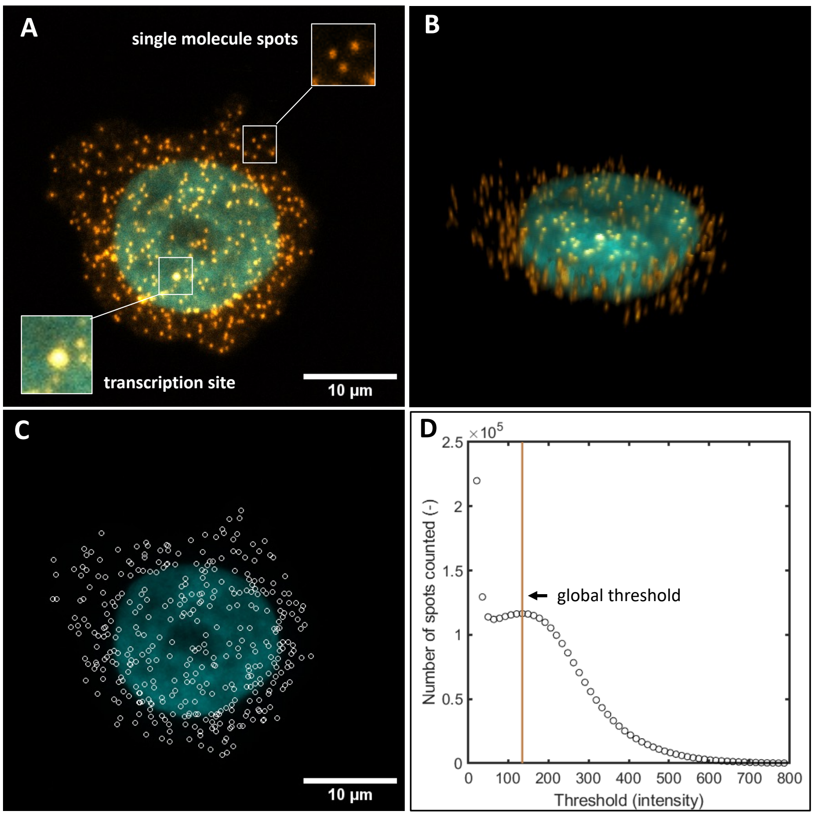

**Figure 3**. **The protocol of microscopy image data analysis is shown.**

The analysis consists of smRNA FISH (with an exon probe) spot selection, spot counting and measuring the intensity of the mature and nascent transcripts. With a customized Python script integrated with Napari, the most intense spots, representing the nascent transcripts at the transcription site (TS) are detected and their intensity is measured. A region of interest (ROIs) is selected (A), and a maximal projection of the nascent transcripts at the TS and the exon FISH labeled mature transcripts are shown. The inserts show the maximal projection of nascent transcripts and mature transcripts in XY axis (B) and in XZ axis (C).

The transcription burst size is calculated by the sum of the intensity of the intense nascent transcripts divided by the average sum of the intensity of all mature transcript nuclear spots detected (D). This calculation provides the relative number of transcription bursts at the transcription start site. The number of nascent transcripts per total amount of cells analyzed represents a measure for the transcription frequency.