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Quantifying the transcriptional output of single alleles in single living mammalian cells

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Abstract

Transcription kinetics of actively transcribing genes *in vivo* have generally been measured using tandem gene arrays. However, tandem arrays do not reflect the endogenous state of genome organization where genes appear as single alleles. We present here a robust technique for the quantification of mRNA synthesis from a single allele in real-time, in single living mammalian cells. The protocol describes how to generate cell clones harboring a tagged allele and how to detect *in vivo* transcription from this tagged allele at high spatial and temporal resolution throughout the cell cycle. Quantification of nascent mRNAs produced from the single tagged allele is performed using RNA fluorescence in situ hybridization (FISH) and live-cell imaging. Subsequent analyses and data modeling detailed in the protocol include measurements of: transcription rates of RNA polymerase II; determining the number of polymerases recruited to the tagged allele; and measuring the spacing between polymerases. Generating the cells containing the single tagged alleles should take up to a month; RNA FISH or live-cell imaging will require an additional week.

INTRODUCTION

The transcription process sits at the heart of the gene expression pathway. The thousands of genes found in the mammalian genome can fluctuate between “on” and “off” states and produce the required amounts of mRNA transcripts that ultimately lead, in conjunction with other processes, to: the correct development of the organism; the precise action of enzymes, and; the control of additional gene expression patterns. Conversely, de-regulation of gene expression is known to result in a wide variety of pathologies. It is therefore of high interest to examine the mechanics of gene expression in both cell populations and in single cells.

The transcriptional output of a gene can be measured by many techniques. Traditional methods, such as northern blotting of radioactively labeled mRNA species¹ and RT-PCR² are restricted to analysis of only a limited number of mRNA transcripts at once. Newer methods, such as real-time PCR (qPCR)³, microarrays⁴ and next-generation sequencing (RNA-Seq)⁵, allow high-resolution genome-wide information on gene expression profiles to be obtained; however these approaches focus mainly on measuring mRNA expression in cell

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Competing Financial Interests

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populations and are less typically applied to analysis of single fixed cells, let alone measurements in single living cells. Over the past decade or so, studies have revealed that most cells in a population, e.g. organ tissue or tissue culture, are not alike with regard to gene expression profiles⁶. This raises many questions as to how a group of individual cells can function as a complete organ. To answer such queries it is imperative to design experimental strategies that will provide information on the output of gene expression pathways from single cells, preferably in living cell systems.

Initially, radioactive in situ hybridization was devised to visually detect nucleic acid molecules in cells and tissues. Modification of the technique to accommodate fluorescent labeling of the probes (FISH) allowed for the high resolution visualization of labeled DNA or RNA within the context of a fixed cell. RNA FISH can be applied to a variety of organisms, and performed with combinations of different fluorescently colored probes⁷. It is possible to detect the transcribing alleles within the nuclear volume of a cell, and the RNA FISH procedure can be used in a quantitative manner to count single mRNA molecules associated with the active gene or dispersed throughout the cell⁸.

With the advent of live-cell imaging^{9,10}, different approaches were designed to directly examine the real-time dynamics of nucleic acids (DNA or RNA) within their natural cellular environment, and not in fixed cells only. One avenue of nucleic acid labeling in eukaryotes has used a repetitive prokaryotic DNA or RNA sequence added to the gene (DNA) or RNA of interest. Then a DNA-binding or RNA-binding GFP-fusion protein is allowed to bind these many repeated sequences, thus labeling the DNA or RNA respectively with numerous DNA/RNA-binding GFP-fusion proteins¹¹⁻¹³. For mRNA labeling, a bacteriophage sequence repeat (MS2) can be cloned downstream of a gene of interest; the resulting transcribed mRNA contains within its 3' UTR a series of these MS2 stem-loop structures, which can be specifically bound by the MS2 coat protein (MS2-CP) fused to GFP, co-expressed in the cells. This approach allows fluorescently bright mRNA particles to be followed at single molecule resolution^{12,14,15}.

Such techniques have enabled several aspects of transcription to be analyzed in real-time and in single living cells, including: visualization of the transcriptional machinery; following of the dynamics of the genome and auxiliary proteins; and measurement of the synthesis rates of RNA molecules transcribed from a variety of genes^{11,16}, even at the single molecule level¹⁷. The kinetics of the transcriptional machinery have now been quantified in living mammalian cells^{11,18-29}, yeast²³, bacteria^{30,31}, *Drosophila*³² and *Dictyostelium*^{33,34}, as well as in fixed cells^{8,35-39}.

Following and measuring transcription in living cells

Tandem arrays and photobleaching—In mammalian cells, when an exogenous gene construct of interest is stably integrated into the genome, it typically results in a multi-copy tandem array of the construct in a single random genomic locus. By incorporating the MS2 tag into the gene construct of interest, an array containing up to several hundred copies of the gene bound by the fluorescent MS2-GFP label, can be generated⁴⁰, thereby providing significant amplification of the signal. The signal can be easily detected using live-cell microscopy assays and kinetic data obtained and used to measure enzymatic RNA transcription rates. The experimental approach to measure transcription rates utilizes the buildup of MS2-GFP fluorescence on the active gene as the tagged mRNA is being transcribed. When the MS2-GFP signal is photobleached (or essentially blackened) using a high-powered laser pulse, the increase in MS2-GFP signal on the tandem array can be followed as new transcripts are being generated and new MS2-GFP molecules are binding to the MS2 stem-loops. This method is termed fluorescence recovery after photobleaching

(FRAP)⁴¹, and the rate of recovery allows the extraction of transcription rates of the studied genes^{20,22,42}

Such approaches have provided a wealth of information regarding the transcription process^{15,22,42-46}. In light of these findings, gene arrays have indeed provided crucial insights on gene expression dynamics. However, since multiple copies of protein-encoding genes are usually not encountered in the mammalian genome, tandem arrays cannot provide a true picture of the endogenous state of single-copy genes

Analysis of transcription from a single gene—The direct visualization of transcriptionally active single-copy genes in living mammalian cells has recently been demonstrated by two approaches^{24,25}. One approach uses knock-in technology to examine the activity of a gene within its endogenous genomic context; the other method (developed in our lab and described in the Procedure) uses the Flp/FRT system to introduce an exogenous gene construct (see Overview of the Procedure). In the former method, MS2 sequence repeats are inserted into the 3'UTR of the gene of interest and site-specific homologous recombination is used to replace the endogenous gene with the tagged gene and generate a GOI-MS2-knock-in mouse. In proof-of-principle experiments, β -actin transcription and mRNA localization could be observed in living primary mouse cells using RNA FISH and live-cell imaging.²⁵ Although the knock-in approach results in a tagged gene at its true genomic location, the user is typically confined to the study of this one particular gene only in mouse cells due to the complexity of mouse knock-in procedures. Our Flp/FRT-based approach enables high resolution kinetic analysis of transcription at the single-allele level in a variety of mammalian cells, particularly in human cells²⁴. It provides a cost-effective and relatively robust comparative experimental set-up for the study of several similar genes within the same genomic context.

Overview of the Procedure

This protocol delineates in a step-by step manner the procedure for performing live-cell microscopy and quantitative FISH for the quantification of transcription kinetics at a single-gene level in single mammalian cells, particularly in human cells. The gene can be visually detected in either fixed or live cells using techniques which label the mRNAs transcribed from the gene and provide a means for transcriptional activity quantification. A prerequisite of this protocol is that the gene of interest contain a series of MS2 sequence repeats, which serve as the region for labeling the mRNA both in the fixed and live-cell experiments. The gene (containing the MS2 sequence repeats) is integrated into the genome as a single copy, using the FRT homologous recombination system, and correct integration is verified. In this protocol, the GOI contains 24 MS2 sequence repeats in the 3'UTR for mRNA detection, and the gene is therefore termed GOI-MS2. Once a stable cell line is established the protocol can be taken in either or both of two directions i.e. analysis in fixed or live cells. In fixed cells, RNA FISH is performed using a fluorescent probe that hybridizes with the repeated MS2 sequence repeats. This procedure allows: the detection of single mRNA molecules transcribed from the single gene; high-resolution quantification of the spatial distribution of the transcripts; and counting of the number of mRNAs being transcribed. For live-cell experiments in which the transcribed mRNA can be fluorescently detected *in vivo*, a MS2-GFP protein is co-expressed in the cells and serves as the specific label that binds the MS2 sequence repeats in the mRNA. The labeled mRNAs can be followed in live cells by time-lapse imaging, or can be further used for the extraction of mRNA transcription rates by photobleaching the active transcription site and following the recovery of fluorescence over time (FRAP). Modeling of the data will enable further analysis with actual numbers, such as transcription rates (kb/min), the spacing between the polymerases associated with the gene, and measurements of promoter firing. Altogether, this protocol uniquely enables the user to

examine the transcriptional activity of a gene of interest, or even better - a series of comparable genes, integrated as single alleles, and to obtain first-hand information of transcription kinetics in living cells.

Generating stable clones—The FLP/FRT homologous recombination system⁴⁷⁻⁴⁹ enables integration of a single copy of a gene of interest into a unique genomic FRT site in mammalian cells; cells containing FRT sites can be purchased from Invitrogen (Flp-In System) or independently generated. Other methods for gene integration are available, such as knock-in procedures in embryonic stem cells (ES cells)⁵⁰ or Zinc-finger nucleases⁵¹. These methods are expensive, laborious and require unique expertise, while the FRT system is readily available and affordable.

The pCDNA5/FRT/GOI-MS2 vector containing the GOI and MS2 sequence repeats is co-transfected with the pOG44 vector that encodes the Flp recombinase enzyme⁵², into the Flp-In host cell line (Zeocin resistant) (Fig. 1a **top**). Due to the recombination event, the cells acquire hygromycin resistance and become Zeocin sensitive (Fig. 1a **bottom**). Thereby, stable Flp-In expression cell lines can now be selected. Furthermore, the recombination event which disrupts the *lacZ*-Zeocin fusion gene, leads to the lack of β -galactosidase activity, so in an X-Gal staining experiment, positive clones will not stain compared to non-recombined clones that will stain blue (Fig. 1b).

Detection of GOI-MS2 expression by RNA FISH in fixed cells—To detect the nascent transcripts on the active GOI-MS2 gene as well as the mature mRNAs distributed throughout the cell, a fluorescent probe designed to specifically hybridize with the MS2 region of the transcript is used. Since the analysed mRNAs contain 24 MS2 sequence repeats, several probes will bind to each mRNA molecule and will generate a bright fluorescent signal at the active transcription site and will label the cellular mRNA molecules. Figure 1c shows a cell with a labeled active cyclin D1 gene. The labeled mRNAs produced from the cyclin D1-MS2 gene are counted (Fig. 2). The fluorescent intensity of a typical single mRNA molecule is measured and used to calculate the number of nascent transcripts associated with the active gene. This analysis can be performed together with markers of cell cycle stages to quantify the transcripts levels during cell cycle phases.

Detection of GOI-MS2 expression in living cells—Expression of the MS2-GFP protein in cells will lead to the fluorescent labeling of the active transcription site, appearing as a bright green dot in the nucleus (Fig. 1d **and** 3a). Within the cell population, some cells will exhibit green dots (active GOI-MS2) while in some the dot will be un-detectable (inactive) (Fig. 3a-d). These fluctuations (Supplementary Video 1) will depend on the promoter driving GOI-MS2 expression, the mode of transcription kinetics (constitutive/bursting), the cell cycle stage, and any signal transduction pathways that reach the promoter.

FRAP is performed to prove that the active transcription sites are indeed transcribing. Photobleaching of the green transcription spot will render the MS2-GFP associated with the nascent RNA transcripts, dark (Fig. 3e). If GOI-MS2 transcription is ongoing, then the bleached/dark mRNAs will be released, whereas newly transcribed mRNAs will be coated with unbleached green fluorescent MS2-GFP proteins (once the RNA polymerase has reached the MS2 sequence repeats). Under the microscope this is observed and measured as a gradual recovery of the green dot (Fig. 3f and Supplementary Video 2). The recovery kinetics can provide information about the elongation rate of RNA polymerase II, which are proportional to the rate of MS2-GFP fluorescence recovery at the transcription site. This part of the protocol can provide information about transcription rates during the cell cycle if the cell is marked with cell cycle markers (Fig. 3g-h).

Deconvolution—Deconvolution is a mathematical process that improves the observed spatial resolution, contrast, and signal-to-noise ratio of images acquired by a fluorescence microscope. Deconvolution is typically required in this protocol and can be used in the two major analysis steps described. It enables accurate measurement and quantification from each voxel (volumetric pixel) in the imaged volume by restoring out-of-focus light back to its original point, so that the restored image more accurately describes the fluorescence intensity originating from each voxel. Deconvolution is performed using the known parameters of the microscope and the diffraction theory of light that affects the quality of every image, known as the point spread function (PSF). Deconvolution on time-lapse movies provides deblurring and noise reduction to the image sequence. We use the Huygens commercial software from Scientific Volume Imaging (SVI) for deconvolution but any other deconvolution software can be used.

Image processing and quantitative RNA FISH data analysis—3D RNA FISH stacks require processing and analysis using software that can perform 3D rendering. Several commercial options exist and we use the Imaris software (Bitplane). The program allows visualization and processing of multi-dimensional datasets, and enables statistical calculations of features in the acquired images. The fluorescence signal of individual mRNAs within the full 3D volume of the cell, can be presented, counted, measured and analyzed without converting the 3D stack into a 2D image. The software detects the objects, and the user manually adjusts the threshold to remove the background objects. The mRNAs are volumetric particles, and after 3D rendering the fluorescence signal from each object volume is measured to provide the sum intensity of each fluorescent particle.

SpotTracker plug-in for computing the transcription site trajectory—The SpotTracker plug-in (ImageJ) is a freely available tool for tracking a single fluorescent spot in a time-lapse movie. Other commercial tracking programs are also available. This algorithm is optimal for analysis of the GOI-MS2 single allele transcription site, which while active appears as a fluorescent spot with relative low signal within a noisy image sequence. The SpotTracker algorithm automatically recognizes only the highest intensity fluorescent spot in every frame. The user can manually correct the track when the spot disappears (i.e. gene inactive). The spot intensity is normalized to the variations in the background fluorescence. Plotting the transcription site intensity over time depicts the transcription profile of the GOI-MS2 single allele (constitutive/bursting), and enables further calculation of the different parameters such as the duration of gene active or inactive periods.

Limitations

The quantitative approach outlined in this protocol provides the intensity only for mRNA molecules that contain the MS2 region, since this is the region detected by the RNA FISH probe or by the MS2-GFP protein. Also, although the RNA FISH conditions open mRNA secondary structures and provide accessibility to the fluorescent probes, it should be kept in mind that there might be variability in the number of probes that bind each mRNA. This is accounted for by measuring a large population of cells and many mRNAs. The histogram of all these measurements results in a Gaussian distribution, with a mean that represents the most likely fluorescent signal of a single molecule. The lower tail of this distribution represents noise (e.g. aggregates of the FISH probe) and the high values tail represents mRNA aggregations. The quantification is based on the mean value of the distributions designating a single mRNA molecule. These are approximations that require controls of cells without any staining and careful analysis by the user. Further in depth analysis of single mRNA molecules with probes that hybridize once to each mRNA can be performed as previously described⁵³.

In the RNA FISH experiment, the number of nascent mRNAs associated with the active transcription site is known only from the MS2 region and downstream, because nascent mRNAs that have yet to reach the MS2 region cannot be detected fluorescently. Still, the total number of mRNAs on the whole gene (including upstream to the MS2 region) can be calculated on the basis of the length of the gene, and the assumption that the nascent RNA polymerases are equally distributed along the gene. Furthermore, due to diffraction limitations, the spatial arrangement of the transcripts along the gene cannot be resolved. Therefore, the number of polymerases at the transcription site might actually be larger than the number of quantified mRNAs, because the sum of intensities of partially transcribed transcripts will be considered in the calculation as the intensity of a full transcript. For simplification of the model, and in order to assess the number of polymerase along the gene, it is assumed that the polymerases are equally distributed. Although it is known that the recruitment of polymerases is a stochastic process, our assumption is justified since the distribution of these events is Gaussian and the mean value can be taken as the spacing between the polymerases along the gene.

Experimental design

Construct design for single allele integration—The GOI is cloned into the pCDNA5/FRT vector (5.1 kb) using the multiple cloning site (MCS) downstream to the human cytomegalovirus (CMV) immediate-early enhancer/promoter. This promoter is considered a strong promoter and results in significant overexpression of the GOI even though it is expressed as a single allele. To analyze the expression of the GOI under different promoters, the CMV promoter sequence can be replaced with a promoter of interest in a separate cloning step. Preferably, the GOI should be expressed under its endogenous promoter to achieve endogenous control of gene expression and to better simulate a functional gene and mRNA. If the endogenous promoter is unavailable and moderate GOI expression levels are required, then replacement with a moderate promoter should be considered (e.g. β -actin promoter).

Additionally, the GOI can also be tagged with a fluorescent fusion protein (XFP) or any epitope tag (HA, Flag, Myc etc.). Tagging of the GOI is preferable since it allows for a means of screening for the positive cell clones, and also proves that a final protein product is generated from the integrated gene. The cloned GOI should contain at least a short 3'UTR sequence downstream of the coding region for insertion of the MS2 sequence repeats. It is also possible to add the endogenous 3'UTR region of the GOI to better simulate a functional gene and mRNA.

If different versions of the GOI are to be examined (e.g. promoters, mutations, deletions, alternatively spliced versions etc.) it is possible to make a series of isogenic cell clones in which the GOI-MS2 is always integrated into the same genomic FRT site. This eliminates position effects and allows expression from the gene variants to be directly compared.

The fragment containing the MS2 sequence repeats (approx. 1300 bp) is cloned from the pSL-MS2 $\times$ 24 vector into the 3'UTR of the GOI. Suitable restriction sites for this purpose will be *Bam*HI and *Bgl*II sites flanking the 24 MS2 sequence repeats in pSL-MS2 $\times$ 24. This step may require prior insertion of the required restriction sites into the 3'UTR of the GOI using an adaptor. It is important to verify that indeed 24 sequence repeats have been cloned into the GOI since bacteria tend to discard repeated sequences. This requires transforming the pCDNA5/FRT/GOI-MS2 using competent bacteria that are specifically designed for cloning unstable inserts (e.g. Stbl2 competent cells, Invitrogen). After bacterial colonies grow, several colonies are taken; plasmid DNA is extracted from them and subjected to restriction with enzymes that cut on both sides of the MS2 repeats. By checking the size of

the restricted 24 MS2 region it is possible to identify which bacterial colonies have retained the full 24 repeats.

The pCDNA5/FRT/GOI-MS2 expression vector and the Flp-recombinase expression vector (pOG44) are amplified using a maxiprep kit for purification of high-quality plasmids. Generation of high-purity plasmids is a critical step for efficient transfection resulting in single gene integration. We typically purify pCDNA5/FRT using the QIAGEN Plasmid Maxi Kit, and pOG44 with the Roche Plasmid Maxi kit, since we found these to give the best purification results respectively. After amplification, it is important to verify that pCDNA5/FRT/GOI-MS2 contains the full 24 MS2 sequence repeats.

Verifying single allele integration—Once stable clones have been generated, it is important to verify that: a single copy of the construct has integrated at the correct site; that the GOI-MS2 is in the correct open reading frame (ORF); that all 24 MS2 repeats are present; and that the GOI-MS2 and hygromycin genes are expressed. PCR-amplified genomic DNA can be sequenced to verify the integration site and ORF. We use two sets of primers (Fig. 1a bottom): The first F1/R1 primer set verifies that the hygromycin gene now has an upstream ATG and so can be expressed. The second F2/R2 primer set uses a forward primer for the end of the GOI and a reverse primer in the LacZ region to confirm that the GOI was correctly inserted in the FRT site. Southern blotting will show that only a single copy of the construct is present⁴³. RT-PCR analysis will confirm expression of GOI-MS2 and hygromycin and will also verify the presence of all the MS2 repeats. If the GOI contains a tag (e.g. Myc, HA, or Flag tag), the presence of a protein product can be confirmed using standard biochemical procedures, e.g. western blotting. Fluorescence microscopy can also be used to detect the protein product, but it should be noted that since the gene is integrated as a single allele, the levels of protein expression are expected to be low. Immunofluorescence can be performed with an antibody to the GOI (which will also identify the endogenous protein), or with an antibody directed at the tag for identification of the exogenous product only. If the GOI contains a fluorescent fusion protein (XFP) tag, then expression of XFP can be monitored in fixed and living cells.

Controls—Several controls are required to confirm that GOI-MS2 homologous recombination has occurred. Each step used to examine the clones after the homologous recombination event should be performed also with the original Flp-In cell line without the GOI-MS2 insertion. The level of expression of the GOI-MS2 expressed from the integrated allele should be compared to the endogenous alleles expressed from their original genomic locus, in order to examine whether they behave similarly. This can be performed by RT-PCR with different sets of primers designed to discriminate between the endogenous and exogenous alleles.

For the quantitative RNA FISH experiments it is important to estimate the threshold that distinguishes between true mRNA signal and background fluorescence. Therefore, a necessary control in this section is to perform the whole RNA FISH procedure, including the deconvolution step, also on cells in which the GOI-MS2 is not expressed (using the same cell line). After performing this control, weak spots observed in these cells will be considered as background signal, which will not be taken into account during the analysis.

MATERIALS

REAGENTS

- pCDNA5/FRT/GOI-MS2 – FRT expression plasmid containing the GOI under the control of the CMV promoter with the MS2 repeats in the 3' UTR. The Procedure

assumes that this construct has been prepared in advance using components from the following plasmids:

- DNA clone containing the gene of interest (GOI)
 - pCDNA5/FRT expression vector (Invitrogen cat. no. V6010-20) <http://products.invitrogen.com/ivgn/product/V601020>
 - pSL24XMS2 vector that contains the 24 MS2 sequence repeats (see Supplementary Sequence)
 - MS2-GFP plasmid (Addgene ID 27121) that expresses the protein that tags the mRNA in living cells. <http://www.addgene.org/27121/>
CRITICAL different fluorescent protein tags can be used if desired (e.g. MS2-YFP-CP).
- pOG44, Flp-recombinase expression vector (Invitrogen, cat. no. V6005-20) <http://products.invitrogen.com/ivgn/product/V600520>
 - Restriction enzymes (Fermentas)
 - T4 DNA Ligase (New England Biolabs, cat. no. M0202)
 - Competent *E. coli* or *Stbl2* competent cells (Invitrogen, cat. no. 10268019)
 - Qiagen Plasmid Maxiprep kit (QIAGEN cat. no. 12162)
 - Genopure Plasmid Maxi Kit (Roche, cat. no. 03143422001)
 - Genomic DNA isolation: PerfectPure DNA cultured cell kit (5PRIME, cat. no. 2302000)
 - NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, cat. no. 740609)
 - Agarose (Sigma, cat. no. A9539)
 - TRIreagent (Sigma, cat. no. T9424)
 - cDNA synthesis kit (Fermentas, cat. no. K1622)
 - PCR mix (ThermoStar, cat. no. JMR-TS5/RTL)
 - Flp-In HEK293 human cell line (Invitrogen, cat. no. R-750-07). **CRITICAL** Different cell lines are available.
 - Dulbecco's Modified Eagle's Medium (DMEM, Gibco, cat. no. 41965)
 - FBS (Biological industries, cat. no. 04-12b-1A)
 - Penicillin-streptomycin (Biological industries, cat. no. 03-031-1B)
 - Trypsin (Biological industries, cat. no. 03-052-1A)
 - PBS (Sigma, cat. no. D8537)
 - DMSO (Sigma, cat. No. D2650)
 - Hygromycin (Sigma, cat. no. H0654)
 - Potassium ferricyanide, $K_3Fe(CN)_6$ (Sigma, cat. no. 244023). **CRITICAL** Light sensitive. Store in light-resistant containers after preparation. **CAUTION** May cause irritation to skin, eyes and respiratory tract. Use a fume hood and wear protective clothing.

- Potassium ferrocyanide, $K_4Fe(CN)_6$ (Sigma, cat. no. P3289) **CRITICAL** Light sensitive. Store in light-resistant containers after preparation. **CAUTION** May cause irritation to skin, eyes and respiratory tract. Use a fume hood and wear protective clothing.
- $MgCl_2$ (USB Corp., cat. no. 18641)
- X-gal (Stock of 40 mg/ml, store at $-20^\circ C$, protect from light, Inalco, cat. no. 1758-0300)
- Paraformaldehyde (4% (wt/vol) diluted in PBS, Electron Microscopy Science, cat. no. 19208). **CAUTION** PFA is a hazardous solution and cross-linking agent. Wear protective gloves and handle it under a fume hood.
- Ethanol (70% (vol/vol)), made from 100% (vol/vol) ethanol and distilled H_2O , Frutarom, cat. no. 2355516400024)
- Saline-sodium citrate (20×SSC, Amresco, cat. no. 0804)
- Formamide (Sigma, cat. no. 101120877). **CAUTION** Formamide is a toxic solution. Avoid contact with eyes or skin and also avoid inhalation and ingestion. Wear protective gloves and handle under the fume hood.
- Triton X-100 (0.1% (vol/vol), Sigma, cat. no. X100)
- BSA (New England Biolabs, cat. no. B90015)
- Sheared salmon sperm DNA (Sigma, cat. no. D7656)
- tRNA (Roche, cat. no. 109541)
- Labeled DNA FISH probe. The MS2 probe sequence: 5'-TTT CTA GGC AAT TAG GTA CCT TAG GAT CTA ATG AAC CCG GGA ATA CTG CAG-3'. The probe is labeled with 5 Cy3 fluorophores: three internal Cy3 and one on the 5' and 3' ends.
- Hoechst 33342 (Sigma, cat. no. B2261)
- Mounting medium active substance (p-Phenylenediamine, Sigma, cat. no. P6001)
- Primers for verifying the correction integration of the GOI-MS2 at the FRT site.
 - SV40-promoter sense (primer F1): 5'-CCAGTTCCGCCCATTCTCC-3'
 - Hygromycin antisense (primer R1): 5'-CTGTTATGCGGCCATTGTCC-3'
 - LacZ-Zeocin antisense (primer R2): 5'-GTAACCGTGCATCTGCCAGTTTG-3'
 - Gene-specific primer (primer F2): user-defined sequence

EQUIPMENT

- 12-well tissue culture plates (Jet Biofil, cat. no. TCP-011-012)
- Glass-bottomed tissue-culture plates with collagen coating (MatTek, Ashland, MA cat. no. P35G-1.5-14-C)
- Incubator (Thermo, cat. no. BB15)
- Centrifuge (Eppendorf, cat. no. 5702)
- Electroporator (Bio-Rad Gene Pulser Xcell, Hercules, CA, cat. no. 165-2660)
- Gene Pulser Cuvette 0.4 cm (Bio-Rad, cat. no. 165-2088)

- Petri dish (Greiner, cat. no. 664160)
- Fume hood (Phoenix Controls)
- Parafilm (Parafilm, cat. no. PM996)
- Cloning cylinders (Sigma, cat. no. CLS-31666-125EA)
- Cryotubes (Greiner, cat. no. 122263)
- PCR tubes (Thermo, cat. no. AB-0620)
- Mastercycler gradient (Eppendorf, cat. no. 5331)
- 18 mm coverslips (Bar-Naor, cat. no. BN/1001/18-1C)
- Microscope slides (Bar-Naor, cat. no. BN1045001CG)
- Wide-field Cell^R Olympus IX81 fluorescence microscope (see EQUIPMENT SETUP)
- Microscope incubation chamber (CUBE & BOX Integral Temperature Control System, Life Imaging Services, cat. no. LIS-500)
- Laser scanning confocal microscope (see EQUIPMENT SETUP)
- ImageJ (Java software for image-processing analysis; freely available at: <http://rsbweb.nih.gov/ij/>)
- SpotTracker ImageJ Plug-in for tracking single particle over images sequence freely available at: <http://bigwww.epfl.ch/sage/soft/spottracker/>
- Huygens software (Scientific Volume Imaging)
- Image analysis software (e.g. Imaris, Bitplane, Switzerland)

REAGENT SETUP

X-gal solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-gal). For 2ml staining solution, add 300 μ l 33mM $K_3Fe(CN)_6$, 300 μ l 33mM $K_4Fe(CN)_6$ and 4 μ l 1M $MgCl_2$ to 1.4 ml 1 $\times$ PBS. Store solution at 4°C protected from light up to 6 months.

CRITICAL Add 50 μ l 40 mg/ml X-gal (1:40 final dilution) to the staining solution immediately before use. **4 $\times$ SSC** (vol/vol) made from 10 ml 20 $\times$ SSC and 40 ml distilled H_2O . Freshly prepare the solution before use.

40% formamide Combine 30 ml 4 $\times$ SSC with 20 ml 100% formamide. **CAUTION** Formamide is toxic and should be handled in a fume hood and discarded according to relevant environmental health and safety instructions. Store formamide at 4°C. **CRITICAL** Freshly prepare the solution before use.

ssDNA/tRNA competitor DNA : Mix equal vol. of 10 mg/ml ssDNA and 10 mg/ml tRNA. Aliquot into 50 μ l aliquots. Store at -20 °C for several years.

Hybridization solution 1 Mix 2.5 μ l probe (40 ng/ μ l stock, for 10 coverslips), 3.6 μ l of 20 $\times$ SSC, 2 μ l of 5 mg/ml of ssDNA/tRNA, in 160 μ l of 100% formamide. Adjust to 200 μ l with DDW.

Hybridization solution 2 Mix 198 μ l of DDW, 2 μ l of BSA, with 50 μ l of 20 $\times$ SSC. **CRITICAL** Freshly prepare the solutions before use.

RNA FISH probe design For this protocol we use a probe sequence (51 nts DNA probe) that binds the MS2 repeated region in the mRNA. The repetitive nature of the MS2 sequence repeats offers identical and well spaced multiple binding sites for the probe. This in turn provides good amplification of the signal and the ability to detect each mRNA molecule as a discrete object. Each probe is conjugated with 5 fluorophores (3 internal and one on each end). We use Cy3 conjugated probes.

We use 10 ng probe per coverslip. The probe aliquots are usually prepared at a concentration of 40 ng/ μ l (from the stock) and so therefore we take 2.5 μ l probe for solution 1, which is sufficient for labeling 10 coverslips.

Hoechst solution Prepare 1 mg/ml Hoechst 33342 solution in 1 $\times$ PBS. Wrap in aluminum foil. Store solution at 4°C protected from light up to 1 year. Before use dilute 1:1000 in PBS.

EQUIPMENT SETUP

Wide-field microscope For acquiring the 3D RNA FISH data use a standard wide-field epifluorescence microscope. We use the Cell[^]R system based on an Olympus IX81 fully motorized inverted microscope (60 $\times$ PlanApo objective, 1.42 NA, 100 $\times$ UPLSAPO objective, 1.4 NA) fitted with an Orca-AG CCD camera (Hamamatsu), rapid wavelength switching, a motorized XY stage (Scan IM, Märzhäuser, Wetzlar-Steindorf, Germany), with a NanoScanZ piezo driven Z stage (Prior), and an integrated Z-drift compensation (ZDC) system. Images are acquired with the Cell[^]R software. Fluorescent filters for the Cy3 and DAPI channels are used. We use Chroma filter sets 49004 for Cy3 and 31000 for DAPI.

Confocal microscope For the FRAP experiments use a confocal microscope. We use the FV-1000 confocal microscope setup (Olympus, Japan). The microscope has 6 laser lines that cover the excitation bands of almost all fluorochromes. It has three photomultiplier detectors; two of them have a flexible selectable spectral range (based on a prism). The same microscope is also equipped with a CCD camera model DU-885 (Andor, Ireland), a motorized stage and a miniature incubator (Tokai, Japan).

PROCEDURE

Generating mammalian cell clones expressing single integrated alleles in the same genomic locus •TIMING 3 weeks

CRITICAL Steps 1-13 are performed for all different versions of the GOI-MS2 to be investigated (e.g. constructs with the same GOI but containing different promoters).

1. Grow the Flp-In cell line in 10 cm tissue culture plates containing 10 ml of the appropriate medium. We typically use DMEM medium supplemented with 10% FBS and Pen/Strep antibiotics. Incubate the cells in a tissue culture incubator at 37 °C and 5% CO₂. Split the cells on the day before transfection to reach 50% confluency on the day of the experiment.
2. On the day of transfection prepare the plasmid DNA to be transfected (pCDNA5/FRT/GOI-MS2 and pOG44) and transfection reagents according to the transfection protocol of choice. Take the cells and the plasmid DNA to a laminar flow hood for sterile working conditions.
3. Co-transfect the pCDNA5/FRT/GOI-MS2 vector containing the GOI and MS2 sequence repeats, together with the pOG44 vector that encodes the recombinase, into the Flp-In host cell line to generate a stable Flp-In expression cell line.

CRITICAL STEP The transfected plasmid ratio is critical and should range between 1:9 - 1:15 in favor of pOG44, to increase the probability of homologous recombination into the genomic FRT site, rather than random integration in the genome. We typically use 1 µg of pCDNA5/FRT/GOI-MS2 and 9-15 µg of the pOG44 using electroporation or calcium phosphate precipitation with the Flp-In HEK293 cell line.

4. After transfection, return the cells to the 37 °C incubator for 24 hours, prior to selection.
5. On the next day, begin antibiotic selection for the establishment of stable positive clones containing the GOI. Aspirate the medium and add fresh medium supplemented with hygromycin antibiotics. Hygromycin resistance is conferred to the cells only if the GOI entered by homologous recombination at the FRT genomic site (Fig. 1a). Replace the medium every 2-3 days for ~3 weeks, until single separated colonies appear.

CRITICAL STEP Prior to transfection perform a kill curve experiment to determine the minimum hygromycin concentration required to kill non-recombinant cells in the Flp-In host cell line. For the Flp-In HEK293 cell line we use 100 µg/ml hygromycin.

6. Using cloning cylinders transfer each clone to a separate well in a 24-well tissue culture plate. After the cells have recovered and begun proliferating and fill up the well, split each well into two duplicate 12-well plates. One plate will serve as the cell stock for further cell growth and colony maintenance. The duplicate plate will serve for X-gal screening.
7. Screen for stable clones by X-Gal staining (Fig. 1b). This step eliminates clones with incorrect or no recombination. Correct integration of the GOI-MS2 into the FRT site will result in the disruption of the *lacZ* gene leading to lack of β-galactosidase activity. Add 0.5 ml X-Gal staining solution to the cells in the duplicate 12-well plate for 6 hrs at 37 °C and check if blue color develops. Blue-stained cells are negative for the integration. Select unstained clones that should contain single integrations into the FRT genomic site.

TROUBLESHOOTING.

8. Expand the positive cell clones in a 10 cm tissue culture plate for further molecular biology analysis to ensure the correct integration of the GOI.
9. Extract genomic DNA from the expanded positive clones using a suitable kit.
10. Perform two separate PCR reactions using genomic DNA as a template and using primers that span the genomic integration area to verify the integration of GOI-MS2 at the FRT site in the genome. In the first PCR reaction use primers that amplify the region between the SV40 promoter (F1 primer) and the hygromycin resistance gene (R1 primer). The second reaction is performed with primers designed to amplify the genomic region between the GOI-MS2 (user designed per GOI, F2 primer) and the *lacZ*-Zeocin fusion gene (R2 primer). (Fig. 1a). Typical PCR reaction components and cycling conditions are tabulated below. Note that annealing temperature is primer dependent and extension time is dependent on the length of the expected product. The table below indicates a typical annealing temperature for F1/R1 primer pair and an extension time typically required to amplify a 1 kb product.

Component	Amount/reaction (μl)	Final concentration
Template DNA	1	
Taq buffer (10×)	2.5	1×
10 mM dNTPs	0.75	300 μM
Taq polymerase 5 unit/μl	0.15	0.03 unit/μl
10 μM Forward Primer	1	0.4 μM
10 μM Reverse Primer	1	0.4 μM
Nuclease free water	Up to 25 μl	

Cycle number	Denature	Anneal	Extend
1	94 °C, 2 min		
2 to 39	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min
40			72 °C, 10 min

11. Run the entire PCR product (25 μl) on an agarose gel and then extract the DNA using a DNA extraction kit.
12. Sequence the genomic area of the FRT site using the PCR products from step 10 using the same primers designed to amplify the PCR products, to show that the GOI-MS2 has been integrated correctly. Verify within the sequence that the SV40 promoter and the ATG codon are in proximity to the hygromycin resistance gene (Fig. 1a).
13. Verify the expression of the gene at the mRNA and protein levels by conventional methods. For instance, RNA expression can be confirmed by RT-PCR using primers specific to the GOI-MS2 (see ref ²⁴) or by RNA FISH with a probe that hybridizes to the 24 MS2 sequence repeat (see step 15A). Detect protein expression using western blotting with antibodies that recognize either the protein product of the GOI (will also recognize the endogenous protein) or preferably the epitope tags added to the gene. Alternatively, if an XFP was fused to the GOI-MS2, detect the fluorescent protein product using fluorescence microscopy. Expect low fluorescence levels since the gene is expressed as a single allele.
14. Freeze down stable positive clones for further use. Freeze by using cyroprotectant medium (90% FBS and 10% DMSO) and store in cryotubes. Finally, store the vials in liquid nitrogen for long term preservation.
15. At this point the user can choose from the following three options to analyze mRNA expression from the integrated GOI-MS2 in fixed or living cells. Option A describes the identification and quantification of the mRNA transcripts in fixed cells using RNA FISH. Option B delineates how to tag the mRNAs in living cells and to follow the expression pattern of the GOI-MS2 in real-time. Option C explains the photobleaching procedure of FRAP performed on the active GOI-MS2 in living cells, and ends with the modeling of the data such that several transcription values and parameters can be extracted from the acquired data.

Option A Quantification of gene expression on active genes using quantitative RNA-FISH

•TIMING 6 days

- i. Grow the cell clone expressing a single allele of GOI-MS2 on 18-mm round coverslips in a 12-well TC dish in a 37 °C incubator, to 50% confluence.
CRITICAL STEP Cells should be separated from each other for later detection of cell borders during single cell image analysis.
- ii. Wash cells in 0.5 ml 1× PBS at room temperature (RT, ~25 °C) for 5 min and then fix in 0.5 ml 4% paraformaldehyde (PFA) in PBS for 20 min at RT.
CAUTION Paraformaldehyde is toxic and should be handled in a fume hood and discarded according to environmental and health safety precautions.
- iii. Wash again in 0.5 ml 1× PBS for 1 min at RT and then add 1 ml 70% ethanol to each well. Seal the plate with Parafilm and leave overnight at 4 °C.
PAUSE POINT Coverslips in 70% ethanol can be kept at 4 °C up to a week.
- iv. On the day of hybridization, aspirate the ethanol and wash twice for 10 min with gentle shaking in 0.5 ml 1× PBS at RT. In parallel to the washes, prepare the probe and the hybridization solutions (see Reagent Setup).
- v. Permeabilize the cells with 0.5 ml 0.1% Triton X-100 in PBS for 3 min at RT. Then wash for 10 min in 1× PBS at RT.
CRITICAL STEP Permeabilization is required for probe accessibility. However, the 70% ethanol treatment also permeabilizes the membrane and therefore the Triton X-100 is not always necessary. It is suggested to test per probe type and also to adjust the permeabilization time according to the cell type.
- vi. Pre-hybridization: Wash coverslips twice for 5 min with 0.5 ml 40% formamide at RT. In parallel to these washes, add the probe directly to solution 1 and immediately boil for 5 min. Then cool on ice for 5 min.
- vii. Prepare the hybridization solution by adding solution 2 (200 µl) to solution 1 (200 µl containing the probe) and mix by pipetting.
- viii. Hybridization: Gently place a 40 µl drop of the hybridization solution onto a petri dish. With forceps, take the coverslip and invert it so that it is placed face down onto the drop. This can be repeated for additional coverslips. Finally, place a small reservoir containing 40% formamide in the dish to prevent drying out of the coverslips during hybridization.
- ix. Close the petri dish and seal with Parafilm. Place the hybridization dish in a 37 °C incubator and allow the probe to hybridize overnight.
- x. Next day, 30 min before washing, warm up the remaining 40% formamide solution to 37 °C.
- xi. With forceps, transfer the coverslips face-up into a 12-well dish containing the pre-warmed 40% formamide and rinse twice in 0.5 ml by placing the plate at 37 °C for 15 min.
- xii. Rinse twice for 1 hr with 0.5 ml 1× PBS at RT, with gentle shaking.
- xiii. Stain the nucleus with 0.5 ml of nuclear staining dye (e.g. Hoechst) for 5 minutes at RT. This step is recommended in order to easily distinguish between the nucleus and the cytoplasm, for further detection of the active gene and mRNAs in the cells.
- xiv. Briefly wash in 0.5 ml 1× PBS at RT. Mount slides in mounting solution.

PAUSE POINT: Slides should be kept in a -20°C freezer until analysis. Although slides can be stored for weeks, for quantifications it is preferable to perform the analysis within 3 days.

- xv. Examine the cells using a fluorescence microscope and detect the active genes within the population of cells by detecting a single dot in the nucleus of the cells (Fig. 1c). Single mRNAs should also be observed, typically more abundant in the cytoplasm and less pronounced than the active transcription site.

TROUBLESHOOTING.

- xvi. Acquire three-dimensional (3D) stacks of total cell volumes using a wide-field fluorescent microscope. High magnification (100 $\times$) is recommended to easily detect individual mRNAs in the cells. Using HEK293 cells we acquire 3D stacks of 76 slices with a step-width of $0.2\text{ }\mu\text{m}$ and exposure times of 2000 ms in the Cy3 channel.

CRITICAL STEP The mode of image acquisition is critical for the next steps of analysis. Acquire the stack with a small Z step between each focal plane (e.g. $0.25\text{ }\mu\text{m}$), so that all fluorescence information is collected from the cell volume. Acquire all 3D datasets using exactly the same microscope parameters such as intensity levels, exposure times, camera gain, binning, and magnification. If there are several samples (different cells or treatments) acquire them on the same day using the same microscope settings, to avoid misinterpretation of the quantitative data. Add negative control cells in which the gene is not expressed. This step will help to distinguish between true mRNA signal and the background.

- xvii. Deconvolve the 3D stacks using deconvolution software in order to drive the out-of-focus light acquired throughout the cell volume during wide-field imaging, back to the original point in the sample (Fig. 2a). Deconvolution software requires knowledge of all the microscope parameters used during imaging: microscope type, lens and medium refractive index, numerical aperture (NA), voxel size, excitation and emission wavelengths.

In addition, deconvolution parameters will be set in the software: the number of iterations, signal to noise ratio (SNR), quality threshold, and the point spread function (PSF) value, which can be obtained theoretically by the software or measured. We typically deconvolve our 3D-stacks using the Huygens software with a theoretical PSF, 500 iterations, SNR of 40 and 0.01 value for quality threshold. These parameters result in significant enhancement of the signal-to-noise ratio and enable clear detection of mRNA molecules. **CRITICAL STEP** Calibrate and adjust the specific abovementioned parameters to your own data in order to obtain optimal image restoration.

TROUBLESHOOTING.

- xviii. Upload the deconvolved 3D-stack into the Imaris software (or any other 3D volume rendering software). By visualizing volume images, all the objects in the 3D volume of the cells can be detected without image projection. Identify mRNAs molecules in the cell volume using the Imaris “Spots” function, which is based on object size and fluorescence intensity (Fig. 2c-d). After estimating the approximate diameter of the 3D objects (mRNAs), set fluorescent-based thresholding to determine true signal spots compared to non-specific spots (Fig. 2e). Use the manual threshold rather than the automatic option for the appropriate selection of spots. This threshold excludes weak spots in the data which present the background. To avoid detection of false signals in this step it is recommended to

compare the data to negative control cells in which no mRNAs are detected using the probe to the MS2 region of the mRNAs. The “Spot” function sorts the data using a filter that is based on a selected criterion of choice (e.g. quality, voxel size, etc.). This minimizes taking into account of background spots.

- xix.** Next, create surfaces for all spherical objects using the “Surface Objects” option (Fig. 2h-j). This creates an artificial solid object superimposed on the fluorescent object (mRNA), in order to visualize a volume object with defined boundaries, which enables statistical calculations. Then the sum of intensities is measured for all objects such that each mRNA is assigned an intensity value (Fig. 2k). The measured mRNA intensities are plotted as a frequency histogram to find the most frequent and thus the most probable single mRNA intensity (Fig. 2l). High quality image data result in a Gaussian-like distribution.
- xx.** Calculate the number of nascent transcripts associated with the single allele by finding the ratio of intensities between the mRNA signal measured at the site of transcription (at which the highest intensity is measured) to the intensity of the single mRNAs.
- xxi.** Repeat steps 15A xvi-xx to perform quantitative FISH on tens of cells. The number of nascent transcripts that are counted for each cell then undergo further statistical analysis. We use MATLAB for statistical analysis and fittings: build a histogram by applying the “hist” function in MATLAB on all the quantified values. We then use the “fit” function to fit a Gaussian distribution to the histogram results, which yields the mean values and the variance for the quantified number of nascent transcripts on the active genes. Altogether, the number of transcripts generated by the GOI-MS2 gene can be counted. It is also possible to measure which of these mRNAs are cytoplasmic and which are still in the nucleus.

It should be noted that this quantitative approach provides the intensity only for mRNA molecules that contain the MS2 region. In other words, the number of nascent mRNAs associated with the active transcription site is probably larger due to the fact that upstream mRNAs (that have yet to reach the MS2 region) cannot be measured.

TROUBLESHOOTING.

Option B Time-lapse microscopy •TIMING 3 days

- i.** Plate the stable cells on 35 mm glass-bottom tissue-culture plates and grow at 37 °C and 5% CO₂ in an incubator to reach 30% confluency (optional: use collagen coating to enhance the adherence of the cells to the plate).

TROUBLESHOOTING.

- ii.** Next day, transiently transfect the cells with MS2-GFP plasmid (1 µg) using calcium-phosphate precipitation (or any other method of transfection). When expressed, the MS2-GFP fusion protein binds as a dimer to each MS2 stem-loop structure in the mRNAs transcribed from the GOI-MS2 single allele. The repeated binding sites (×24) in the 3′UTR result in a detectable concentration of MS2-GFP labeled transcripts at the active transcription site, thereby creating a green fluorescent dot clearly visible above the background of diffuse MS2-GFP (Fig. 1d).

CRITICAL STEP MS2-GFP expression levels are critical for detecting the small active transcription site (see recent report for improving signal to noise issues⁵⁴). Moderate to high MS2-GFP levels may mask the transcription site. Therefore, a

weak background is necessary for obtaining an optimal signal compared to the background. This should be calibrated per transfection procedure.

TROUBLESHOOTING.

- iii. Next day, acquire time-lapse movies in which the single allele is followed over time (Supplementary Video 1). Important issues to consider in these experiments are the relatively low fluorescence signal observed at the site of transcription, the normal motion of living cells, and the dynamic nature of the transcription site within the nuclear volume (Fig. 3b). In order to overcome these technical issues we typically use glass-bottom tissue-culture plates with collagen coating to enhance the adherence of the cells to the plate. Wide-field fluorescence images are obtained while the plate is located in a cell incubator at 37 °C with 5% CO₂ to keep the cells under physiological conditions with minimum fluctuations in temperature.

Record the time-lapse movies in 4D (three dimensions over time): at each timepoint collect a Z-series of several microns (5-10 μm) to prevent loss of the imaged transcription site. Since the active site is rather small it is important that the Z-steps cover a significant portion of the nuclear volume. The use of hardware Z-drift correction (e.g. ZDC on Olympus microscopes) improves performance in long time-lapse experiments. The interval between frames is determined according to the experiment goal (e.g. can range from 300 msec and up to 30 minutes).

CRITICAL STEP Since it is necessary to image many slices at each timepoint, the cells are exposed to relatively high illumination levels, which might lead to photobleaching and phototoxicity. Therefore, it is preferable to minimize the total light exposure by: Setting the exposure time and intensity levels of the excitation light as low as possible (to the lowest level at which the site is still visible); Imaging the cells at 60× magnification instead of 100× (this helps reduce exposure times); Use 2×2 binning while capturing images.

TROUBLESHOOTING.

- iv. Analyze the movies using tracking software. We use the ImageJ SpotTracker plug-in which provides robust tracking of the transcription sites and successfully handles the tracking of low intensity objects. Several pre-processing steps are recommended before analysis: Deconvolve the stack to enhance the SNR; If there are large jumps in the XY plane, correct the XY drift using the Imaris or ImageJ plug-in (use: Align slices), otherwise the track might be lost due to movement constraints of the spot tracker; Correct bleaching over time.
- v. After these steps, upload the 4D stack to ImageJ and convert to 3D (XYT) by choosing the best focus plane with the transcription site.

First, approximate the spot diameter by the number of pixels it covers. The size of the region of the spot that is measured throughout the movie sequence is determined according to this evaluated diameter. Several parameters are adjustable with the SpotTracker plug-in to optimize the tracking (such as: max. displacement, intensity variation, or movement variation). Optional: use the Spot-Enhancing Filter that enhances the spot and reduces the background noise.

After setting all the parameters, the SpotTracker will automatically track the transcription site (will choose the spot with the highest intensity). The software marks the selected bright spot that was chosen in each frame with a red cross that is placed at the center of the fluorescent spot. It is important to then manually validate that the spot was properly detected throughout the time sequence (Fig. 3c).

The output is the average intensity of the spot in each frame. Normalize these values to the background intensity (see details on the normalization process in step 15C vii) and plot in a graph to describe the intensity profile of the site (Fig. 3d). This reflects the kinetics of gene activity over time.

CRITICAL STEP Fluctuations in the transcription profile, namely the turning “on” or “off” of the transcription site will most probably result with the loss of the spot during tracking and automatic placing of the cross elsewhere in the nucleus. Therefore, it is vital to manually place the cross in the right place (i.e. where the transcription site was in the previous frame) in order to obtain accurate measurements.

TROUBLESHOOTING.

Option C FRAP of the transcription site •TIMING ~1 day

- i. Grow the cells as detailed in step 15B i-ii. Perform photobleaching experiments to examine the kinetics of the transcribing gene. Perform FRAP measurements on a confocal microscope, or a fluorescent microscope equipped with a laser capable of photobleaching. Determine the parameters so that a highly detectable fluorescent signal is achieved from the single transcribing gene throughout the whole experiment. For the axial scan we use Z steps of 0.25 μm with a total depth of 10 μm . We set the pinhole to approximately double the default value that is normally selected for the optimal resolution with the specific objective lens (with our Olympus microscope, we use a pinhole diameter of 255 μm instead of the optimal 120 μm). We do this to gain a longer working distance, so that the measurement is much less sensitive to axial shifts of the measured spot.
- ii. Start by focusing on the focal plane at which the site is clearly visible. Then, select the total scanned range symmetrically above and below this plane. Two sets of images are acquired: before and after bleaching (Fig. 3e). Before photobleaching, take three image stacks with no time delay between them. These are used as a reference for the recovery phase. In the second part of the acquisition process, bleach the transcription site and monitor signal recovery over time. Effective bleaching occurs at the focal plane, and thus requires setting the focus correctly to this plane.

TROUBLESHOOTING.

- iii. Define the bleach area in such a way that it contains the whole spot that represents the transcription site. Use 100% of laser intensity and set the activation time for effective bleaching (time depends on the laser power e.g. we use 200 msec bleach time). Set the microscope Z-sectioning and time-lapse measurement parameters, and set a time delay between each consecutive 3D image-stack that is short enough for measuring changes in the recovery process (we typically use 30 sec). Continue imaging until reaching full recovery (Supplementary Video 2). We found that full recovery occurs within approximately 40 minutes and we typically measure 40 focal planes for each time point.
- iv. For FRAP analysis, fit an exponential equation to the intensity data to find the recovery rate (Fig. 3f). Import Z-stacks, and for each pixel, calculate the summation from the whole stack (with the Olympus system this is called ‘sum project’) to create a 2D image for each time-point measurement. Save the files as tiff images (one 2D image for each time-point). **CRITICAL STEP** Summation of Z stacks will sum the fluorescent signal of the whole stack. Because the intensity of

the transcribing site is from a 3D diffraction limited spot, summing all planes will give a true indication to the total signal recovery.

- v. Import the time series images into ImageJ. Combine the pre-bleach and post-bleach images to create a single experiment. Use the SpotTracker plug-in to track the fluorescent intensity of the site over time.

CRITICAL STEP In the first few images after the bleach, the recovery of a single site can be obscured by noise. Set the marker of the SpotTracker plug-in at the approximate location of the site known from the pre-bleach images.

TROUBLESHOOTING.

- vi. Use ImageJ “multi measure” script to extract the average intensity from a selected region in the nucleus at every time point. Choose: Analyze → ROI manager → more → multi measure.
- vii. Import the results into MATLAB and use fitting algorithms to extract the recovery rate.

CRITICAL STEP This analysis focuses on the recovery of a single transcribing allele. It is crucial to measure the intensity of the site itself, without the influence of the nucleus intensity. Subtract for every time point the intensity of the nucleus I_n at that time point, from the measured intensity at the site I_s , and normalize to the same Intensity at time zero (the average intensity for all images before the bleach)

$$I = I_s(t) - I_n(t) / I_s(t_0) - I_n(t_0).$$

- viii. Use an analytical model to extract the elongation rates. One of the parameters that we use is the number of transcripts per site, as determined by the quantitative RNA FISH experiment (see step 15A xx). This number is used as a constraint in order to determine the adequate arrangement of polymerases from the MS2 region and onwards along the GOI-MS2 gene. For simplification, it is assumed that the polymerases are equally distributed along the gene. In this analysis set the quantified total intensity at the transcription site (from RNA-FISH) to be equal to the total number of polymerases, as a first step of an iteration process that is implemented to find the number of polymerases at the site.
- ix. Position the polymerases along the gene, from the MS2 region onwards to the end of the gene, with equal spacing. Calculate the intensity for all the transcripts associated with the polymerases and positioned along the gene depending on the location of the polymerase. For instance, a polymerase that is positioned in the 3'UTR region will contribute full transcript fluorescence intensity, while a polymerase positioned in the MS2 region will contribute less fluorescence depending on how many MS2 stem-loop repeats it has already transcribed. This step should be repeated with the addition of polymerases until the summed calculated intensity is equal to that obtained from the quantified data. It is then possible to estimate the values of the number of polymerases transcribing, the total number of transcribed nucleotides that contribute to the fluorescent signal at the site, the spacing between adjacent polymerases, and the rate of polymerases loading on the gene (“polymerase firing”).
- x. Take the FRAP recovery rates (see step 15C vii) together with the above parameters to calculate the elongation rate of a single polymerase from each

experiment: $k_e = \frac{k_{frap} \cdot \sum_i n_i}{N}$. Where k_e is the elongation rate, k_{frap} is the FRAP recovery rate, n_i is the total number of nucleotides that contribute to the intensity from all the polymerases and N is the number of polymerases that are positioned at

the MS2 region. This analysis was done using MATLAB software but can be performed with any data analysis software.

ANTICIPATED RESULTS

The cell system allows the integration and detection of a single gene of interest in mammalian cells. We generated two comparable cell clones harboring either of two versions of the gene of interest (GOI-MS2), in this case the human *cyclin D1* gene, differing only in the promoter region (Fig. 1) and containing either the endogenous cyclin D1 promoter^{55,56} or the CMV promoter. The experiments presented in this protocol were performed with the human Flp-InTM293-FRT cell line, but any other adherent Flp-In mammalian cell line can be used. Figure 2 shows an example of quantitative RNA FISH experiments performed with a fluorescent MS2 probe that hybridized to the repeats in the 3'UTR of the cyclin D1-MS2 mRNAs. The MS2 probe will provide high resolution images of fixed cells in which transcription sites and cellular mRNAs can be visualized (Fig. 1c; detection of the active cyclin D1-MS2 transcription site). The active gene can also be detected in living cells using MS2-GFP tagging as shown in Figure 1d.

Even though we have described the examination of individual cells, there is advantage in screening the whole cell population, since this can demonstrate upfront whether there are differences in the expression kinetics between the different versions of the GOI that were chosen. For instance in our study, when the CMV promoter was used, the whole cell population showed detectable active transcription sites. In contrast, when using the endogenous cyclin D1 promoter, only 40% of the cells in the population portrayed active transcription sites, demonstrating the different regulation executed by these two promoters.

Figure 2 shows how 3D deconvolution results in the enhanced detection of individual mRNA molecules appearing in the nucleus and cytoplasm as multiple bright spots. The transcribing allele appears as a brighter spot, implying the simultaneous synthesis of several transcripts (Fig 2a-d,m). After determining the correct threshold between true mRNA signal and the background, 3D rendering of the spots is performed and surfaces are created (Fig. 2e-j). Further analysis is performed to quantify the number of nascent mRNAs present on the transcription site during periods of gene activity, as well as count the number of cellular mRNAs in the whole cell volume.

MS2-GFP binding to the nascent transcripts allows the visualization of single allele transcription in individual living cells. The gene should appear as a bright spot above the background of the diffuse MS2-GFP. Tracking the transcription site over time allows measuring the transcriptional kinetics of the single allele (Fig. 3; live-cell imaging and tracking of the cyclin D1 mRNA while transcribing). The spot can appear or disappear over the time sequence in correlation to the activity state of the gene, and plotting the intensities of the transcription site signal over time will indicate the transitioning between ON/OFF states. A constitutively expressed gene would probably show less or no fluctuations, as demonstrated for the CMV promoter.

The fluorescence recovery in the FRAP experiments indicates that new mRNAs are being made and also provides a kinetic ruler of transcriptional kinetics since the rate of transcription can be modeled from the recovery curves. In Figure 3f, showing the recovery curves of the cyclin D1-MS2 gene, note that the plot of the single FRAP experiment shows relatively noisy data, due to the fairly low fluorescence signal from the transcription site and due to photobleaching. Hence, it is necessary to average multiple experiments and fit the data to correctly calculate the elongation rates.

The abovementioned experiments can be performed with a specific focus on the cell cycle stage and enable quantification of the GOI-MS2 allele during the cell cycle. Cell cycle stage can be identified with various markers that stain individual cells (Fig. 3g,h) or cells can be synchronized using different treatments (such as thymidine, etoposide, etc.). For instance, doxorubicin treatment arrests the cells in at G2⁵⁷ (Fig. 3g); PCNA staining shows different structures of replication foci which allow differentiation of the various steps in S phase⁵⁸, and antibody staining to endogenous CENP-F shows cells in G2 since the protein rapidly degrades after mitosis and peaks at G2⁵⁹ (Fig. 3h). We were able to distinguish between cell cycle stages by the number of alleles present in the nucleus. The observed duplication of the single GOI-MS2 allele (appearing as two adjacent sites) due to replication of the GOI-MS2 gene during S phase, allowed the measurements of the transcriptional output from the replicated alleles (i.e. sister chromatids) compared to the pre-replicated allele.

TROUBLESHOOTING.

Troubleshooting advice can be found in Table 1.

•TIMING

Steps 1-14, generating the plasmids and cell clones: user dependent, approx. 3 weeks

Step 15 A, RNA FISH: 2 days; image acquisition of RNA FISH: 2 days; image analysis of RNA

FISH: 2 days

Step 15 B, live cell imaging: 3 days

Step 15 C, FRAP of transcription site: 1 day; data analysis: user dependent

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Generation of a cell system expressing a single GOI-MS2 allele. **(a)** Top: The pcDNA/FRT/GOI-MS2 plasmid harbors the GOI, which is generated to contain all of the following elements: the promoter driving the GOI, the GOI, the MS2 sequence repeats for RNA tagging, the 3'UTR. The MS2 sequences are inserted in the 3'UTR. In addition, the plasmid has one FRT site and a non-functional hygromycin gene (without an ATG). Co-transfection with the pOG44 plasmid that encodes the recombinase leads to the integration of a single allele of the GOI-MS2 by homologous recombination between the FRT site in the plasmid and the FRT site in the Flp-In cells. Bottom: The scheme represents the genomic region of the FRT integration site in the Flp-In cells after the recombination event with the pcDNA/FRT/GOI-MS2 plasmid. The cells are now hygromycin resistant, Zeocin sensitive, and lack β -galactosidase activity. The GOI-MS2 mRNAs expressed from this gene are bound by the MS2-GFP coat protein that enables detection of the transcription site and the mRNAs. **(b)** Flp-In cells stain blue with X-Gal staining when the *lacZ* gene is intact (no integration at the FRT site). When the cells are positive for the recombined GOI-MS2 allele, they do not stain. **(c)** RNA FISH with the MS2 probe labeled with Cy3 fluorophores to a cyclin D1-MS2 gene driven by the CMV promoter. The probe hybridizes to the MS2 sequence repeats in the cyclin D1-MS2 transcripts and enables detection of the transcribing alleles (arrows) and of cellular mRNAs transcribed from the gene (red dots). The image is a single 2D raw image of a cell and is representative of all cells in the population. **(d)** MS2-GFP for tagging the cyclin D1-MS2 transcripts in living cells shows a single transcribing allele (arrow). Nuclear mRNAs are also observed (green dots). The DIC image shows the nucleus, nucleoli and cytoplasm. When using the CMV promoter all cells in the population that express MS2-GFP should show an active transcription site. Scale bars, 5 μ m.

Figure 2.

Detection and quantification of GOI-MS2 mRNAs by RNA FISH. **(a)** An RNA FISH experiment using the Cy3-labeled probe against the MS2 repeats of cyclin D1-MS2 mRNAs expressed from the CMV promoter. Top panel shows the original image, while the bottom panel shows the enhancement of the signal-to-noise ratio after 3D deconvolution. All cells in the population should show active transcription sites and mRNAs when using the CMV promoter. **(b)** The transcription site in the nucleus (green arrow, strong intensity), and single mRNA molecules in the whole cell (low intensity). **(c)** RNA FISH combined with Hoechst staining distinguishes between the mRNAs located in the nucleus and the cytoplasm. Scale bar, 5 μ m. **(d)** Cropped and enlarged area from **(c)** showing single mRNAs. Note similar intensities and sizes. Scale bar, 1 μ m. **(e)** Setting the threshold for mRNA quantification requires correct estimation of the threshold to distinguish between true mRNA signal and background. This will eliminate weak spots from the analysis. Left – threshold value is too low (130) some spots from the background are recognized as true signal (yellow circles). Right - setting a higher threshold value (250) based on control cells, leaves these weak spots unrecognized for further analysis. **(f)** The identified mRNAs are labeled with white spots (inset - enlargement of the boxed area) and then a surface **(g)** is created for each particle that was identified by the Spot function, generating a volume for each object (mRNA). **(h-j)** Representation of the mRNAs as in **(f)** and **(g)** within the entire 3D cell volume. **(k)** The sum of intensities for all the rendered objects (transcription site and single mRNAs) is measured. A box plot illustrates the intensities of all the mRNAs. The transcription site

intensity is several fold higher (indicated as a red star above the box). **(l)** Histogram plot showing the frequencies of all the mRNA intensities. **(m)** Schematic representation of the nascent mRNAs transcribed from the GOI-MS2 allele. When the polymerase reaches the MS2 region, the mRNA MS2 stem-loops are coated by the MS2-GFP proteins.

Figure 3.

Following transcription kinetics of the GOI-MS2 allele in real-time. **(a)** Gene activity of the cyclin D1-MS2 gene driven by the endogenous cyclin D1 promoter is followed in living cells by MS2-GFP tagging. During the tracking, when in the “ON” state a red cross is placed in the center of the bright spot (transcription site), whereas in the “OFF” state the transcription site disappears. **(b)** Left - trajectory (red) of transcription site movement during 3 hours. Right - kymograph representation showing the fluorescence signal (y axis) of the transcribing allele over time (x axis). Periods of gene activity and inactivity can be seen. **(c)** The transcribing allele is imaged for 3 hours and fluctuations in gene activity can be observed. **(d)** The normalized intensity plot of the gene activity from **(c)**. **(e)** Frames of a FRAP experiment in which the transcription site (pre-bleach) is bleached (boxed area) and recovery is followed for 25 min. **(f)** Transcription site intensity is plotted and then fitted. **(g)** Cell arrested with Doxorubicin (red) exhibits two adjacent and transcribing alleles (MS2-GFP, green). DNA is co-stained with Hoechst (blue) and observed with DIC. **(h)** Cells that stain positive for CENP-F (green) are in the G2/M cell cycle phase. RNA FISH (red) shows a cell with one active allele (top) that is unstained for CENP-F, and a cell with 2 active alleles (bottom) that is positive for CENP-F. Scale bars, 5 μ m.

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
7	All selected cell clones stain blue after X-Gal screening	No recombination due to poor quality of plasmids Sub-optimal ratio between the pCDNA5/FRT/GOI-MS2 and pOG44 Transfection method is not efficient for the selected cell line	Purify high quality plasmids using fresh purification reagents. Adjust the ratio between the transfected plasmids. Adjust transfection protocol to obtain high efficiency.
15A	Lack of or low signal of the RNA FISH probe such that individual mRNAs are not detected High background of probe labeling	Poor reagents used for hybridization Sub-optimal amount of probe High amount of probe; hybridization time is too long; insufficient washes	Use fresh formamide and hybridization reagents. Dilute fresh probe from the stock and optimize the amount of the probe. Lower the amount of probe. Shorter hybridization time. Increase the wash times.
15A	Low signal-to-noise ratio after deconvolution	Sub-optimal criteria were chosen for deconvolution; poor quality of original images	Calibrate all the parameters for deconvolution (number of iterations, PSF value, SNR value etc.). Acquire new high quality 3D stacks
15A	Sub-optimal distribution of mRNA intensities in histogram Unreasonable ratio between the mRNA intensities to the transcription site signal	Signal from object in the background was identified as true mRNA signal The created surfaces were not correctly generated to fully superimpose on the particles Poor quality of original images (usually with high background)	Find the correct threshold that distinguishes between specific signal to the background. If necessary use control cells for correct determination. Recreate the mRNA surfaces with precise covering of the particles Acquire new high quality 3D stacks
15B	Transcription sites are not detected using MS2-GFP tagging in living cells	High background of the diffusive MS2-GFP masks the low fluorescence emitted from the single allele; the GOI-MS2 contains a truncated version of the MS2 sequence repeats	If the existence of the GOI-MS2 in fixed cells is verified (fixed cell detection is easier), then: Adjust the expression level of the MS2-GFP until reaching sufficient SNR. Verify the length of the MS2 sequence repeats.
15B	The cells are moving out of focus	Normal mobility Phototoxicity	Attach the cells to the plates using coating (e.g. collagen, poly-lysine). Acquire Z-stacks spanning several microns to cover the possible movements of the cell through the Z axis. Use microscope hardware for autofocus (e.g. ZDC) Lower the exposure times. Use 2×2 binning. Use lower magnification (60× instead 100×) to reduce exposure

Step	Problem	Possible reason	Solution
15B & C	Disappearance of the transcription site during tracking	True disappearance (turning off of the site); the Z-step is too large leading to skipping of the small transcription site between steps; the plane with the transcription site is found above or beneath the acquired stack (the acquired Z-stack is too shallow)	No problem Reduce Z-step size (e.g. to 0.25 μm) Increase the total Z-stack height (by adding more Z-slices)
	Incorrect tracking of the site using SpotTracker plug-in	Large jumps in XY during the time sequence Very low signal from the transcription site causes missing of the spot Shutting down of the transcription site can lead to identification of another spot in the nucleus (with higher intensity)	Correct XY drift before tracking. Adjust to suitable parameters for the tracking procedure (e.g. maximum displacement). Deconvolve the signal to enhance SNR and facilitate tracking. Manually place the cross where the measurement should be performed (where the transcription site appeared in the last frames).
	The obtained time-line plot of transcription site activity is sub-optimal	Incorrect analysis due to high intensity variations of the background during the movie (bleaching/phototoxicity)	Correct bleach and carefully normalize fluorescence intensity at the transcription site according to the variations of the MS2-GFP background (due the relatively low fluorescence signal emitted from single allele, the measurement at the spot is very sensitive to changes in the background).