Visualization of Single RNA Transcripts in Situ
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Fluorescence in situ hybridization (FISH) and digital imaging microscopy were modified to allow detection of single RNA molecules. Oligodeoxynucleotide probes were synthesized with five fluorochromes per molecule, and the light emitted by a single probe was calibrated. Points of light in exhaustively deconvolved images of hybridized cells gave fluorescent intensities and distances between probes consistent with single messenger RNA molecules. Analysis of β-actin transcription sites after serum induction revealed synchronous and cyclical transcription from single genes. The rates of transcription initiation and termination and messenger RNA processing could be determined by positioning probes along the transcription unit. This approach extends the power of FISH to yield quantitative molecular information on a single cell.

The identification of specific nucleic acid sequences by FISH has revealed sites of RNA processing, transport, and cytoplasmic localization (1). Recognition of these sites of hybridization is possible only when sufficient concentrations of the target sequence provide contrast with regions of lesser or no signal. Here we describe a quantitative approach to identify single molecules in these regions of low concentration. The methodology also facilitates accurate quantitation of the regions containing multiple copies of RNA, such as is found at transcription sites. Analysis of individual transcription sites with single molecule accuracy generated precise information on nascent chain initiation, elongation, and termination. FISH images are composed of points of light with variable intensities resulting either from hybridization or from background fluorescent noise. We used multiple probes targeted specifically to β-actin mRNA to generate high-intensity point sources that result from hybridization to individual RNAs. We then quantitated the light intensity from each point source to distinguish hybridization events from spurious fluorescence.

The strategy involves (i) synthesizing several oligonucleotide probes to adjacent sequences on an RNA target such that their collective fluorescence will be emitted as a point source after hybridization; (ii) conjugating fluorochromes to specific sites on each oligonucleotide probe so that the fluorescent output per molecule of probe can be calibrated (Fig. 1, A to C); (iii) acquiring digital images from a series of focal planes through a hybridized cell; and (iv) processing these images with a constrained deconvolution algorithm such that out-of-focus light is quantitatively restored to its original points of origin.

To identify single β-actin mRNA molecules, we hybridized multiple probes to the isomorph-specific 3′-untranslated region (UTR) of the mRNA in normal rat kidney (NRK) cells. The acquired fluorescence image was made up of numerous bright points

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of fluorescence interspersed within a diffuse, lower intensity signal (Fig. 1D). After deconvolution, the image consisted of numerous distinct points of light throughout the cytoplasm (Fig. 1E). The brightest points of light were constrained to a few contiguous volume elements (voxels). The image also contained scattered single voxels and clusters of voxels with low amounts of fluorescent signal attributed to low-level autofluorescence and sources of background noise. Establishing an image threshold removed ~95% of these voxels (Fig. 1, F and G). The discrete, brightest clusters that remained after a threshold was established are referred to as objects. The total fluorescence intensity (TFI) of each object was then calculated. These bright objects had TFI values expected for single molecules and fell within the range of one to five probes hybridized (Fig. 1H). This analysis supports the conclusion that single molecules of β-actin mRNA were being detected and that they corresponded to the points of light. The β-actin mRNA molecules were often far enough apart to be resolved by light microscopy as individual point sources.

Additional evidence that these points of light are single molecules comes from an analysis of their prevalence. A population of cells grown in serum-free medium contained 500 ± 200 β-actin mRNAs per cell. In an exponentially growing population of cells the number increased to ~1500 copies, consistent with activation of actin mRNA transcription and possible stabilization of cytoplasmic actin mRNA by serum (2, 3). This copy number of β-actin mRNA agrees with estimates of the average abundance of β-actin mRNA per cell derived from molecular techniques (4).

Finally, spectrally distinct probes hybridized to different target sequences on the same mRNA molecule were used to verify single molecule detection. When the probes were targeted to the same molecule, the signals were close together (Fig. 2C). The TFI confirmed that they were hybridized to only one molecule. Statistical analysis of 478 signals in the cell determined that 59% of the red signals were ≤3 voxels (279 nm) from a green signal. As a control, two isoforms of actin mRNA, β and γ, were detected simultaneously in the same cell with probes that were specific for each isoform (Fig. 2, A and B). The gene sites for each isoform were distinguishable in the nucleus.

Fig. 1. Methodology for detection of single RNA molecules by quantitative FISH and digital imaging microscopy (13). Probes used are described in (14). (A) The amount of light emitted per probe was calibrated by measuring the TFI from a known number of probes in an imaged volume (15). The TFI was plotted against the number of fluorochrome molecules (five per probe) to generate the regression curve. The slope is equal to the TFI per fluorochrome (curve a = CY3, curve b = CY5, curve c = FITC). A normalized TFI per probe was used to calculate the number of probes hybridized at discrete point sources of light in a restored image (15). (B) The accuracy of the technique was tested by optically sectioning and restoring immobilized CY3-labeled probes adsorbed to glass cover slips. (C) The most frequent occurrences of TFI values per CY3 probe plotted as a histogram are comparable to the normalized value (solid bar) from the solution experiments (16). (D) One optical plane of an NRK cell after in situ hybridization (17) with calibrated CY3 probes to β-actin mRNA shows a number of bright foci superimposed on a diffuse background arising from out-of-focus light. Red arrow, bead = 0.099 μm; yellow arrow, two transcription sites. (E) The fluorescent probe distribution after image restoration with an iterative constrained algorithm, EPR (18). The restored image reveals discrete points of light, which allow accurate measurement of the TFI emanating from each point source (bead is restored to a point source). The image is a two-dimensional (2D) projection of the 3D restored image. (F) The thresholded image (19) is rendered so that the cell is viewed from the bottom looking upward into the nucleus (20). The red surface portrays the boundary of the nuclear envelope. The opening in the center of the nuclear surface is a result of truncating the upper optical sections. (G) Enlargement of (F) showing the dimensions of a restored transcription site in the nucleus (cross hairs), in contrast to a 100-nm bead (yellow arrow), the most intense object in the image, restored to a point source (21). (H) The integrated TFI value of each point was mapped to a single voxel and the number of probes hybridized assigned a color and frequency (1 = violet, 25.2%; 2 = blue, 33.6%; 3 = green, 17.5%; 4 = yellow, 11.6%; 5 = orange, 8.6%; >5 = red, >10 = white, <5%; n = 681 molecules per cell, 95% efficiency of hybridization was estimated from a binomial distribution (22) (23). In (D) to (F), scale bar = 5 μm; in (B) and (G), scale bar = 1 μm.
and the mRNAs as separate point sources in the cytoplasm. In contrast to the cis probes, the signals did not colocalize significantly; they were ≤ 3 voxels apart 13% of the time.

Visualization and measurement of intramolecular distances were possible when the cis probes were sufficiently separated (Fig. 2, C and D). To ascertain that the displacement of the hybridization signal was not an artifact due to misalignment of the respective red and green images, we hybridized three probes, each labeled with distinct fluorochromes, to β-actin mRNA at distances of 1648 nucleotides (nt) (maximum) and 631 nt (minimum) apart. We found that the registered images from the maximally distant probes were not coincident (as much as 487 nm apart), whereas near probes were within the same voxel. This result indicated that the detected mRNA was almost completely extended (1648 kb × 0.3 nm per nucleotide = 494 nm).

To verify that a single copy sequence could be detected by this method, we hybridized 10 probes, each with six CY3 fluorochromes, to the template strand of the β-actin gene (Fig. 3A). Eleven probes, each with five fluorescein isothiocyanate (FITC) fluorochromes, targeted to the β-actin nascent transcripts were used simultaneously (Fig. 3B) and verified the gene site by colocalization (Fig. 3C). The TFI values obtained for hybridization to the single DNA target (60 fluorochromes) were consistent with the TFI predicted from single mRNAs. The nascent transcripts gave a much brighter signal than the gene because they represented multiple RNA molecules concentrated at that site. Measurement of 130 genes revealed the distribution of fluorescence expected from the independent hybridization of 10 probes (Fig. 3D). The hybridization efficiency was close to 100%, because all 10 probes hybridized most frequently. Hybridization to nascent transcripts indicated the presence of multiple copies corresponding to a maximum of 68 probes per transcription site.

This technology allowed us to quantitate the kinetics of β-actin mRNA transcription (Fig. 4). Cells cultured overnight in serum-free medium showed essentially no detectable transcriptional activity (Fig. 4A). The addition of serum (2%) resulted in synchronous activation of transcription detected on one of the β-actin alleles in virtually every cell within a few minutes (Fig. 4B). The number of nascent transcripts per β-actin allele was determined with a single probe to the 5’-UTR. The simultaneous use of 3’-UTR probes provided information on the number of polymerase molecules that had progressed to the terminal portion of the gene. At 3 min after induction, the average gene contained 12 nascent mRNAs, but few 3’-UTR signals (Fig. 4, B and C). By 4 min, both β-actin alleles were transcriptionally active in almost all cells, and four transcripts had entered into the 3’-UTR (Fig. 4B).
Because the released transcripts did not accumulate significantly in the immediate vicinity of the gene after the expected time of termination, we concluded that the initiation of transport took no more than a few minutes. The dispersal of transcripts away from the transcription site took on a variety of spatial patterns (Fig. 4, D and E). Often tracklike distributions were apparent, and the regular spacing between points suggested a constant rate of movement away from the transcription site. Occasionally, the transcripts appeared to spiral away from the site of transcription; however, in at least half of the cases examined, the terminated transcripts simply appeared to diffuse around the site, without any discernible pattern. Nascent transcripts diminished at 60 min after serum induction (Fig. 4F). The decline of transcriptional activity beginning at 30 min fit an exponential decay profile for the next 1.5 hours, at which time it became indistinguishable from the state before induction (Fig. 4H). Over the induction period beginning at 10 min, there was a constant increase in the number of single β-actin mRNA molecules in the cytoplasm (6).

The capacity of these procedures to generate accurate information about single cells was illustrated by a high-resolution, dynamic view of each β-actin mRNA transcription site obtained with three probes positioned along the RNA, each labeled with a different fluorochrome (Fig. 4I). A "snapshot" of a representative gene revealed 23 nascent RNAs in the process of elongation, 14 of which had progressed through the proximal 3′-UTR and 8 of those through the distal 3′-UTR. At least five of the RNAs had passed the cleavage and polyadenylation site. These RNAs may be nascent, cleaved but undergoing polyadenylation, or awaiting transport. These possibilities should be distinguished by using probes downstream from the cleavage and polyadenylation site. The rat β-actin gene has a consensus "termination" sequence at the predicted distance downstream of the polyadenylation sequence that might account for this observation (7). The average spacing between polymerases in the 3′-UTR was ~60 nt, whereas in the 5′-region of the gene, it was ~170 nt. This spacing is consistent with previous observations on polymerase II (Pol II) transcription loading (8). The closer packing of polymerases in the 3′-UTR of the gene suggest-

**Fig. 4.** The kinetics of serum induction and subsequent transcription of the β-actin gene. (A to F) Images captured the progression of transcription into the 3′-UTR from quiescence at 0 min (A), one site active at 3 min (B), both sites at 5 min (C), peak of transcriptional activity and RNA export at 10 min (D) and 15 min (E), to a decline at 60 min (F). (G) Quantitative analysis of the transcription sites. The histogram indicates the mean number of nascent RNA on the gene (green) in the cell population at various times after induction and the mean number of nascent RNA that entered the 3′-UTR (red). The error bars indicate variation in transcriptional activity between the β-actin alleles in the same cell as well as within the cell population. The signal from the 5′- and 3′-end of each nascent mRNA was represented as a scale (c). (H) Separate regression curves were fitted to the following intervals: (section a) 0 to 5 min, before termination; (section b) 5 to 15 min, transcripts were terminating while the total number of transcripts on the gene continued to increase, and the number of RNAs in the 3′-UTR reached a plateau; (section c) 15 to 30 min, a slow decline in the total number of nascent RNA with little change in the number located in the 3′-UTR; and (section d) 30 to 120 min, an exponential decline in the total number of nascent RNA and in RNA reaching the 3′-UTR. (I) A dynamic profile of transcription at one β-actin allele resolved with three spectrally distinct probes (14). The 5′-UTR probe detects all the nascent RNA (green). A second probe detects RNA that has progressed beyond the first 50 bases of the 3′-UTR (red), and the third probe detects RNA that has progressed to the distal region of the 3′-UTR (blue). The triple A’s indicate transcripts beyond the polyadenylation site. Bar = 5 μm.
ed that termination and release were rate limiting. Recent work indicates that Pol II recruits cleavage and polyadenylation factors, which may result in the polymerase slowing at this point (9).

The rate of increase (four transcripts per minute) in the number of nascent transcripts from 0 to 5 min reflects solely the rate of initiation, because fewer polymerases should have reached the termination site, on the basis of the rate of elongation. An in vitro system indicated that Pol II can initiate at a rate of two transcripts per minute (10). Between 5 and 15 min, when termination was occurring, there was a sustained increase of one nascent RNA per minute. Assuming that the initiation rate remained constant, then the rate of termination and release would be three RNA per minute. Surprisingly, there was a precipitous decrease of activity at the transcription site after 30 min. The cycle of the transcriptional pulse in response to serum is similar to that shown for c-fos (11). By interrogating the site with probes along the length of the RNA, we determined that this shut-down resulted from an inhibition of initiation rather than "pausing" of the polymerases. There was no apparent accumulation of transcripts at the nuclear envelope, so it is likely that export is not rate limiting.

The advance herein evaluates FISH images so that single molecule signals are not eliminated through background subtraction or the establishment of a threshold. As a consequence of this approach, single mRNA molecules were detectable with as little as one oligonucleotide probe, containing only five fluorochromes, an increase in sensitivity more than two orders of magnitude greater than previously obtained (12). In addition, the template DNA strand was accessible to the small probes during transcription without denaturation, contrary to other protocols, and may indicate that it contained single-stranded regions. This technique may therefore allow probing of the active regions of chromatin and facilitate detection of specific genes or groups of genes where only short, nonrepetitive sequences can be used. This should bring many physiologically important genes for receptors, signaling molecules, cell cycle regulators, and transcription factors, as well as their nuclear and cytoplasmic transcripts, into range of FISH detection.

REFERENCES AND NOTES

13. For details of the digital imaging microscopy procedures, see F. S. Fay, K. Fogarty, J. M. Coogins, Soc. Gen. Physiol. Ser. 40, 51 (1986); F. S. Fay, W. A. Carrington, K. Fogarty, J. Microsc. 153, 133 (1989); Images were obtained with an inverted Nikon Diaphot 300 epifluorescence microscope equipped with a 100-W mercury lamp and modified to capture images under computer control. Between 20 to 35 optical sections were acquired at 250-nm z-intervals and an effective focus of 187 nm by 187 nm with a thermostatically cooled (~45°C) charge-coupled device (CCD) (model 220; back-thinned RCA CCD chip; 50 kHz; Photometrics, Tucson, AZ). A high quantum efficiency (0.8 at 500 nm) and a low noise (50 photons/s) were required for imaging at the low light levels.
14. Oligonucleotide probes were synthesized, purified, and labeled as described [E. H. Kislauskis, Z. Li, R. H. Singer, K. L. Taneja, J. Cell Biol. 123, 165 (1995)] (23). Below are listed the probes used in this work: (nucleotide number start, length in nucleotides). The sequence is for β-actin unless specified (accession J00691). For Fig. 1, B and D through H, the antisense probes to the 3′-UTR were 3133, 51; 3012, 52; 3434, 53; 3486, 52; and 3542, 52. For Fig. 2, same as Fig. 1 and the antisense probes to the 3′-UTR were 255, 50; to the coding region, 3542, 52. For Fig. 3A, same as Fig. 1 and the antisense probes to the 3′-UTR were 255, 50; to the coding region, 3542, 52 and 3256, 52. For Fig. 4, same as Fig. 1 and the antisense probes to the 3′-UTR were 255, 50; to the coding region, 3542, 52 and 3256, 52. For Fig. 5A, same as Fig. 1 and the antisense probes to the 3′-UTR were 255, 50; to the coding region, 3542, 52 and 3256, 52. For Fig. 6, the antisense probe to the 5′-UTR was 255, 50 and the antisense probe to the 3′-UTR was 3133, 51 and 3542, 52.
15. The TFI per probe was measured by using a known concentration of fluorochrome in a determined volume between a cover slip and slide as follows: (i) Probes were dispersed in a solution equivalent in composition to mounting medium with final concentration ranging from 0.25 to 4.0 ng/μl for cy3- and Cy5-labeled probes and 0.1 to 16 ng/μl for FITC-labeled probes. (ii) Fluorescent beads (200 nm in diameter) adhered to the glass surfaces and delineated the distance between the inner surfaces of the cover glass slip and the surface focus at each surface. The stage position was monitored with an Eddy-current sensor and was used to measure the vertical distance between the two beads to determine the height of the volume element containing the probe either in solution or immobilized on glass coverslips and were compared with a theoretically estimated number of photons emitted by one dye molecule by using the molecule extinction coefficient, the quantum yield, the measured light flux of the microscope, and the quantum efficiency of the CCD camera. This estimate came within 80% of the actual measurement obtained.
16. Fluorescent probes diluted in mounting media (2 pg/μl) were allowed to adsorb to the surface of a cover slip and then optically sectioned. The restored image revealed the distribution of intensities attributed to individual probes. The empirical measurements of the TFI for one probe either in solution or immobilized on glass coverslips were compared with a theoretical estimate of the number of photons emitted by one dye molecule by using the molecule extinction coefficient, the quantum yield, the measured light flux of the microscope, and the quantum efficiency of the CCD camera. This estimate came within 80% of the actual measurement obtained.
17. For cell culture and in situ hybridization, NFRK cells (dfj clone from parent cell line, NPK-52E, American Type Culture Collection CRL 1571) were grown to confluence on 22 mm by 22 mm coverslips and cover slips in F12K media supplemented with 10% fetal bovine serum (FBS). The dfj clone showed serum-responsive β- and γ-actin genes. For serum induction experiments, cells were incubated with F12K media (0.5% FBS) for 24 hours and then stimulated with 10% FBS. Cells were fixed for 10 min at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS), 137 mM NaCl, 8 mM Na2HPO4) after serum stimulation, washed, and stored in PBS. The FSH protocol was modified from K. L. Taneja and R. H. Singer [J. Cell Biol. 109, 929 (1989)]. Cells were hybridized for 3.5 hours at 37°C. Coverslips were washed and mounted on slides with polyvaleninediamine in 90% glycero and PBS. Multicolor (FITC, tetra- methylrhodamine, and Cy3) 9-μm-diameter latex beads (Molecular Probes) were included in the mounting media and used as fiducary markers to align images.
18. To elucidate the true fluorescence distribution of the acquired image, we restored the series of optical sections by using an iterative algorithm (Exhaustive Photon Reassignment, EPR) that used non-negativity constraints. Image intensity of the reconstruction plus 400 iterations and a smoothing factor alpha value = 5 × 10−6 and a convergence value of 0.001. The procedure has been detailed previously [W. A. Carrington et al., Science 288, 1483 (1995); K. L. Taneja, L. M. Lit- shitz, F. S. Fay, R. H. Singer, J. Cell Biol. 119, 1245 (1992)].
19. Background in a restored image comprises point sources of low-intensity light, scattered light, and random noise contributed by the imaging electronics. A threshold value was established and applied to the restored images to eliminate these low-signal point sources. After analyzing the background TFI frequency distribution of nonzero pixels in a cell subjected to a mock hybridization. The mean TFI per nonzero voxel +3 SD was chosen as the threshold value. The 3 SD was chosen as the threshold value. The threshold value was set to zero, whereas those greater were...
In Situ Visualization of DNA Double-Strand Break Repair in Human Fibroblasts

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A method was developed to examine DNA repair within the intact cell. Ultrasoft x-rays were used to induce DNA double-strand breaks (DSBs) in defined subnuclear volumes of human fibroblasts and DNA repair was visualized at those sites. The DSBs remained in a fixed position during the initial stages of DNA repair, and the DSB repair protein hMre11 migrated to the sites of damage within 30 minutes. In contrast, hRad51, a human RecA homolog, did not localize at sites of DNA damage, a finding consistent with the distinct roles of these proteins in DNA repair.

Proteins that mediate certain aspects of DNA metabolism, such as DNA replication, appear to be compartmentalized within the nucleus. DNA replication therefore requires the movement of DNA to and from established sites within the nuclear matrix (1). Cytologic analyses have revealed that the DSB repair proteins hRad51 and the hMre11-hRad50 complex assemble in discrete nuclear foci as part of the normal cellular response to DNA damage (2–5). These findings may indicate that DNA repair does not entail the movement of DNA DSBs to preexisting intranuclear sites. Rather, they suggest that DNA repair proteins move to sites of DNA damage. The inability to detect DSBs in situ has made it difficult to address this issue experimentally. A method to induce and subsequently detect DSBs within a defined subnuclear volume would, in principle, provide a means to determine whether DSB repair requires the movement of DNA repair proteins to the sites of DNA damage.

To that end, we developed a method to examine the temporal and spatial nature of DSB repair within the context of the intact cell. This method relies on synchrotron-generated ultrasonic x-rays (<5000 electron volts (5 keV)), a multilayer monochromator for tunable ultrasonic x-ray energies with sufficient intensity for irradiation of live human fibroblasts (6), and microfabricated irradiation masks to induce DNA damage in discrete subnuclear regions of irradiated cells (Fig. 1) (7). The irradiation masks were fabricated with x-ray lithography and consist of gold stripes (1.85 μm wide with 1.35-μm separation) deposited on thin Si3N4 membranes (7). Dosimetric analyses with the irradiation mask showed that gold-shielded regions receive about 0.5% of the dose absorbed by the nonshielded regions (7). Irradiated cells thus absorb ultrasonic x-rays in 1.35-μm-wide stripes separated by 1.85-μm gaps that remain essentially unirradiated.

The 1.34-keV ultrasonic x-rays used in these experiments act almost exclusively through photoelectric interactions in biological material (8), resulting in low-energy electrons that have very short track lengths (<50 nm), comparable to the dimensions of biologically relevant structures such as chromatin (9). These properties suggested that photoelectrons and Auger electrons as well as free radicals resulting from absorption of ultrasonic x-rays would induce DNA damage almost exclusively within the 1.35-μm stripes imposed by the grids. Human fibroblasts (37Lu) were irradiated and DSBs were labeled with bromodeoxyuridine triphosphate (BrdU) and terminal deoxynucleotidyltransferase (TdT) for visualization with fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody to BrdU (FITC–anti-BrdU) 30, 90, or 300 min later (10–12). Under the conditions used, TdT does not label single-strand DNA nicks (12). Nuclei observed 30 min after irradiation displayed a strong FITC signal in parallel stripes corresponding to BrdU incorporation at DNA ends (Fig. 2A). Each pair of unirradiated-irradiated stripes is 3.2 μm wide (1.85 μm unirradiated plus 1.35 μm irradiated). Hence, most nuclei (average diameter 15 to 20 μm) contained six or seven FITC-staining stripes (Fig. 2, A and B). Confocal microscopy demonstrated that parallel stripes of BrdU incorporation were uniform through-

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Fig. 1. (A) Diagram of the partial volume irradiation scheme (7). Thickness of the Mylar surface (8 μm) is not drawn to scale. (B) Scanning electron micrograph of irradiation mask. Bar, 1 μm.