

Single mRNA Molecules Demonstrate Probabilistic Movement in Living Mammalian Cells

Dahlene Fusco, Nathalie Accornero,
Brigitte Lavoie, Shailesh M. Shenoy,
Jean-Marie Blanchard, Robert H. Singer,
and Edouard Bertrand

Supplementary Experimental Procedures

Plasmids

A first series of MS2 reporter plasmids was generated from pRSV-Z-SV [S1]. This plasmid contains the RSV promoter, followed by the LacZ cDNA, and the SV40 intron and polyadenylation site. Fragments containing n MS2 binding sites ($n = 6, 12, \text{ or } 24$) were cloned between the LacZ cDNA and the SV40 polyadenylation site, to generate pRSV-Z-MS2 n . The plasmid containing 24 MS2 sites was named pLacZ-24-SV. The 3' UTR of chicken β -actin was amplified by PCR and was inserted between the MS2 repeats and the SV40 poly-A region to generate pLacZ-24- β act. The pLacZ-24-hGH MS2 reporter was generated from a modified version of pcDNA3 (Invitrogen). The promoter contained tandemly repeated glucocorticoid-responsive elements (GRE) upstream of a TATA box (note that GRE are not functional in Cos cells), followed by the LacZ cDNA and the human growth hormone polyadenylation site (hGH). Fragments containing the 24 tandemly repeated MS2 sites were cloned between LacZ and hGH sequences. The MS2-GFP fusion protein was generated by cloning the eGFP cDNA (Clontech) into pMS2tam (a gift of T. Grange). This plasmid expresses the MS2dIFG protein from the promoter of the large subunit of RNA polymerase II. The eGFP was PCR amplified and was cloned in frame at the C terminus of the MS2 protein, to generate pMS2-GFP. Oligos were designed to insert the SV40 NLS in frame, at the C terminus of the eGFP. pMS2-YFP was constructed by replacing the eGFP coding sequence of pMS2-GFP by YFP. CFP tubulin was a gift of J. White.

Image Analysis and Quantitation

EPR is a quantitative, constrained-iterative program based on the constrained-iterative algorithm of Dr. Frederic Fay, of the University of Massachusetts Medical School, and it was used as previously described [S2] for single-molecule mRNA identification. Huygens2 is also quantitative, and it uses an iterative algorithm-based maximal likelihood estimation. For EPR deconvolutions, an experimental point spread function was used, and approximately 1000 iterations were performed; while, for Huygens2, a theoretical point spread function was used and only 50 iterations were done. Results obtained by using both imaging/deconvolution systems (in New York or Montpellier) were similar.

The total fluorescent intensity (TFI) per eGFP molecule was obtained with purified eGFP (Clontech). Three-fold serial dilutions (from 0.1 mg/ml to 0.0012 mg/ml) were made in mounting media, and 5 μ l was mounted with 22 \times 22 mm coverslips. For each dilution, four images were taken in different areas of the coverslip and were averaged to compensate for differences in solution thickness. The average fluorescence intensities per voxel were then plotted against the number of molecules imaged, assuming that the volume imaged was the real area of the pixel (67 nm with a 100 \times objective) multiplied by the thickness of the solution (5 μ l/22 \times 22 mm).

The number of probes per particle was determined by calculating the TFI of individual particles and by dividing that value by the TFI per probe [S2]. Particles were selected for analysis based on the colocalization of the fluorescent signals, after thresholding. Thresholds were determined by comparison of experiment and control cells, where experiment cells were cotransfected with the pLacZ-24- β act and MS2-GFP fusion and control cells were transfected with pMS2-GFP. A typical threshold was set to about 60% of the intensity expected for a single RNA particle. The pixels below the threshold were set to zero, while all other pixels retained their original value. TFI per three-dimensional particle was calculated from de-

convolved images with a script written for Iplab, or with Voxelshopro (Bitplane). To compensate for the deconvolution, this value was then divided by either the number of planes in the original image stack when using Huygens2, or by the number of planes in the point spread function when using EPR. TFI per probe was calculated separately for each probe by collecting images from serial probe dilutions. A total of 5 μ l of each probe dilution (ranging from 7.3 ng/ μ l to 7.3 \times 10⁻⁴ ng/ μ l for Cy3 LacZ and from 7.4 ng/ μ l to 7.4 \times 10⁻⁴ ng/ μ l for Cy5 ms2) were placed between a coverslip and a slide, onto which 170 nm blue fluorescent beads had previously been dried. Using the beads as markers, the distance between the coverslip and slide was measured, and the center plane of the dilution was located. A range of interest that excluded beads was identified, and a single image was captured at the center plane, by using an exposure time identical to that used to capture cell images. This procedure was repeated three times, each at a different location on the coverslip, for each dilution. The TFI per probe was then obtained by plotting the integrated fluorescence in the total imaged volume against the number of molecules in that volume, and the slope of the resulting curve was representative of the TFI per fluorescent molecule.

Cell Culture and Transfections

Cos-7 cells were cultured at 37°C in 5% CO₂ and in DMEM containing 10% FCS. Cells were transfected by the calcium-phosphate coprecipitation procedure or by Fugene (Roche). In calcium-phosphate transfections, the precipitate was left overnight on the cells, and it was removed by resuspending in an isotonic solution without calcium and phosphate. Cells were then trypsinized and plated on gelatin-coated glass coverslips, and they were further grown in HEPES-buffered DMEM, containing 10% FCS, but without phenol red and riboflavin, to eliminate fluorescence of the media [S3]. Control experiments showed that cells grew in this media as well as in normal DMEM.

Scoring Analysis

Because direct visual examination of the movies was time consuming, we developed a method based on analysis of maximal image projections to generate a value for the "directedness" of mRNA movement in individual cells. Directedness represents the total number of particles that move >1.5 μ m in a single direction in a given cell. When viewed on maximal image projection looking into the time axis, these appeared as continuous paths, which were visualized as linear traces. In contrast, particles that did not move significantly, but were static or diffusive, appeared as a dot or a patch. When the projection was viewed looking into the Cartesian y axis, these mRNA had a constant x position over time and appeared as a vertical line. In this projection, mRNAs that move along the x axis will appear as diagonal lines, with a different x position for each point in time. This approach is similar to the display method of "kymographs" [S4]. Diagonal lines were counted in addition to linear traces, because some directed mRNA movements were masked by an overlapping trajectory when viewed looking into the time axis and were visible only when looking into the Cartesian y axis. For directedness scores, the numbers of linear traces and diagonal lines, which both represent persistent particles, were added, and the number of vertical lines was subtracted. This procedure was validated by playing movies and classifying RNA particles: the proportion of diffusive particles was slightly higher for localizing mRNA, and the fraction

Table S1. Particle Movement Categories

	Directed	Diffusive	Corralled	Static
LacZ-24-hGH	4 (2)	15 (6)	41 (3)	40 (8)
LacZ-24-SV	2 (1)	25 (5)	40 (5)	33 (6)
GH/Colc	0 (0)	55 (6)	28 (6)	17 (4)
GH/Swh	3 (1)	42 (5)	42 (5)	13 (3)
LacZ-24- β act	22 (3)	39 (4)	21 (2)	18 (2)

The reporter mRNAs are shown in the left-hand column. GH/Colc and GH/Swh are the LacZ-24-hGH reporter treated with colcemid (20 μ M, 1 hr) and swinholide (50 nM, 2 hr), respectively. LacZ-24- β act is the LacZ-24-SV reporter with the β -actin “zipcode” inserted. Data from three experiments are shown, $n > 94$ particles; standard deviations are shown in parentheses.

of directed movements also increased (Table S1). The average displacement of directed particles was 4.8 μ m for LacZ-24- β act, compared to 2.6 for LacZ-24-hGH.

Theoretical Diffusion Coefficients of mRNPs in the Cytoplasm

The expected size of the diffusion constant can be calculated by using the Stokes Einstein equation: $D = KT/6\pi Nr$, where $K =$ Boltzmann’s constant, 1.38×10^{-23} J/K or 1.38×10^{-23} Nm/K; $T =$ absolute temperature, 273.15 (0°C) + $37^\circ\text{C} = 310$ K; $N =$ cytoplasmic viscosity, 8.1 cP [S5]; 1 cP (centiPoise) = $1/1000$ Poiseuille = $1/1000$ Ns/m², $N = 8.1/1000$ Ns/m²; $r =$ molecular radius of the mRNA reporter, where the mRNA reporter contains ~ 1600 nt, and 1 nt = ~ 0.3 nm, so the total length of the reporter = $1600 \times 0.3 = 480$ nm.

If mRNA travels as a circular unit, with a circumference of 480 nm = $2\pi r$, then $r = 480 \text{ nm}/2\pi = 76$ nm, or 7.6×10^{-8} m. Therefore, $D = (1.38 \text{ E} - 23 \text{ Nm/K})(310 \text{ K})/(6 \times 3.14) \times (8.1/1000 \text{ Ns/m}^2) \times (7.6 \times 10^{-8} \text{ m})$. $D = 3.7 \times 10^{-9}$ cm²/s. However, it is most likely that mRNA travels in a complex or locosome, with several proteins and possibly other mRNAs. If we estimate that the radius of this complex is approximately four times the radius of a bare mRNA (similar to the estimate that Politz et al [S6] used in their evaluation of the predicted diffusion coefficients of poly(A) RNA in the nucleus), then the adjusted diffusion coefficient is $D = 0.9 \times 10^{-9}$ cm²/s.

Supplementary References

- S1. Kislauskis, E.H., Li, Z., Singer, R.H., and Taneja, K.L. (1993). Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J. Cell Biol.* 123, 165–172.
- S2. Femino, A.M., Fay, F.S., Fogarty, K., and Singer, R.H. (1998). Visualization of single RNA transcripts in situ. *Science* 280, 585–590.
- S3. Zylka, M.J., and Schnapp, B.J. (1996). Optimized filter set and viewing conditions for the S65T mutant of GFP in living cells. *Biotechniques* 21, 220–221, 224–226.
- S4. Bulinski, J.C., Odde, D.J., Howell, B.J., Salmon, T.D., and Waterman-Storer, C.M. (2001). Rapid dynamics of the microtubule binding of ensconsin in vivo. *J. Cell Sci.* 114, 3885–3897.
- S5. Lang, I., Scholz, M., and Peters, R. (1986). Molecular mobility

and nucleocytoplasmic flux in hepatoma cells. *J. Cell Biol.* 102, 1183–1190.

- S6. Politz, J.C., Browne, E.S., Wolf, D.E., and Pederson, T. (1998). Intranuclear diffusion and hybridization state of oligonucleotides measured by fluorescence correlation spectroscopy in living cells. *Proc. Natl. Acad. Sci. USA* 95, 6043–6048.

Movie 1. Directed, Corralled, Static, and Diffusive mRNA Particles in a Cell Transfected with LacZ-hGH Reporter mRNA and ms2-gfp

Movie 2. Directed, Corralled, and Static mRNA Particles in a Cell Transfected with LacZ-SV Reporter mRNA and ms2-gfp

Movie 3. Directed mRNA Particles in a Cell Transfected with LacZ- β -actin Reporter mRNA and ms2-gfp

Movie 4. A LacZ- β -actin mRNA Particle Undergoing Directed Motion on a CFP-Labeled Microtubule

Table S2. Short- and Long-Range Diffusion Coefficients

	Short-Range (10^{-9} cm ² /s)	Long-Range (10^{-9} cm ² /s)
Diffusional	0.8 (0.3)	0.45 (0.2)
Corralled Diffusional	0.7 (0.2)	0.13 (0.07)
Corralled Diffusional, 25°C	0.8 (0.3)	0.07 (0.06)

The category of movement is shown in the left-hand column. Mean displacement (X) of mRNA molecules measured at given time intervals (t); diffusion coefficients are from the Einstein equation, $D = X^2/(4t)$. For short time periods (0.111 s), $n = 1000$. For long time periods (10 s), $n = 50$. The standard deviations are shown in parentheses.