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Ahmet Yavuz Oral
Zehra Banu Bahsi Oral *Editors*

3rd International Multidisciplinary Microscopy and Microanalysis Congress (InterM)

Proceedings, Oludeniz, Turkey, 19–23 October 2015

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Preface

The 3rd International Multidisciplinary Microscopy Congress (InterM2015) provided all scientists the opportunity to meet, present their work, discuss and mutually interact in order to enhance and promote their research work.

This volume, published by Springer, includes selected papers presented at this congress, held in Oludeniz, Turkey, October 19–23, 2015.

On behalf of the organizing committee we would like to thank all the participants, plenary and invited speakers for their valuable contribution.

We would also like to thank AIGTUR for their support in the organization of the congress as well as the publishers for the quality of this edition.

Gebze, Turkey

Ahmet Yavuz Oral
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Part I
Applications of Microscopy in the Biological Sciences

Structural Analysis of Long Single-Stranded RNA Molecules with Atomic Force Microscopy Imaging

Jamie L. Gilmore, Aiko Yoshida, Katashi Deguchi, Suguru Asai, Hideki Aizaki, Masahiro Kumeta, Kiwamu Hyodo, Tetsuro Okuno, Takaji Wakita and Kunio Takeyasu

Abstract Characterization of the structure of long RNA molecules (>1 kb) is usually a time-consuming and tedious process. In this study, we have developed an imaging procedure for obtaining images of the extended secondary structures of long RNA molecules combined with automated MATLAB-based data processing algorithms for identification of the domain architecture of the molecules in these images. These algorithms include a molecule autoselection procedure based on height and area thresholding, a morphological thinning procedure to generate skeletons of the molecule in order to analyze the branched structure of the molecules, and a procedure to generate local volume profiles along the main chain of the molecule for identification of domains and prediction of the number of nucleotides comprising each domain. The single-molecule nature of this technique also allows for the identification of varying conformations of the molecule and assessment of the conformational flexibility of the identified domain organization.

1 Introduction

Structural characterization of long single-stranded RNA molecules (>1 kb) is a process that often takes many years with each domain identified and studied in independent sets of experiments. In the case the of the Hepatitis C virus (HCV) RNA genome, structural characterization has proceeded gradually, focused mainly

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on the 5′ and 3′ untranslated regions (UTRs), with the internal structures remaining largely uncharacterized [1, 2]. Given the tedious nature of these processes, the development of methods to perform high-throughput characterization of RNA structure could greatly advance our ability to recognize the structural features on long RNA molecules such as viral RNA genomes, messenger RNA (mRNA) transcripts, or ribosomal RNA (rRNA).

Towards this goal, we are attempting to develop Atomic Force Microscopy (AFM) imaging as a method to characterize the secondary structure of these molecules [3]. The first step of this process is the development of procedures to reproducibly image extended secondary structures of various RNA molecules. The next step is the development of automated procedures to extract structural information from the molecules in these images. To do this, we have developed MATLAB-based algorithms to autoselect the molecules from the images by height and area thresholding, generate skeletons of the molecule to analyze the branches of the RNA molecules, and generate local volume profiles along the morphological backbone of the molecule to predict the location of domains along the chain and the number of nucleotides in each.

2 Development of AFM Procedures to Image RNA Secondary Structure

Taking advantage of the fact that Mg^{2+} is necessary for RNA tertiary structure but not for secondary structure [4, 5], we have developed a method to reproducibly observe secondary structures of RNA molecules on a spermidine-modified mica surface using AFM imaging by omitting Mg^{2+} from our reactions and briefly heating the RNA to 65 °C (Fig. 1). This technique has proved effective for a variety of RNA molecules, including the 9.7 kb full-length genome of HCV (JFH-1) [6], a 3.9 kb importin β gene transcript, a polyadenylated importin β transcript, a 5.0 kb 28S rRNA, a 1.9 kb 18S rRNA, and the bipartite genome of the Red clover necrotic mosaic virus (RCNMV) [7] comprised of RNA1 and RNA2 (Fig. 1). Due to the hierarchical nature of RNA folding [5], much of this structure is likely to be conserved in the final folded molecule.

3 Analysis and Domain Recognition of RNA

After development of procedures to obtain reproducible images of RNA molecules, the next stage is to develop data analysis procedures that can extract structural information from these images. To do this, we have developed a series of automated MATLAB-based algorithms to analyze RNA molecules. For now, these algorithms have been applied to a 1.1 kb deletion mutant of the JFH-1 HCV genome with

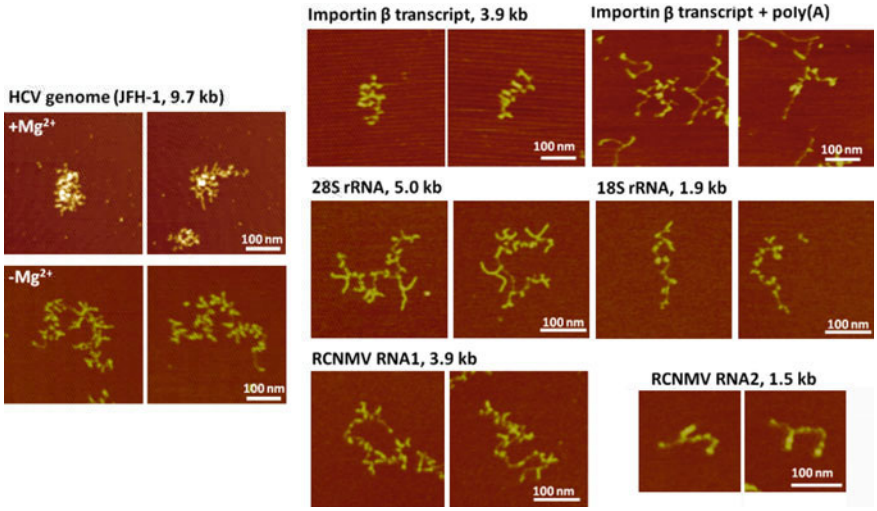

Fig. 1 AFM images of various RNA molecules. Images of the full-length 9.7 kb JFH-1 Hepatitis C Virus (HCV) genome are displayed on the *left* showing the tertiary structure in the presence of 1 mM Mg^{2+} and without the addition of Mg^{2+} (1 mM EDTA). The remaining images all show the secondary structure in Mg^{2+}-free conditions

8.6 kb of the coding region removed (JFH-1dC_5B, Fig. 2a), leaving mainly the 5′ and 3′ UTRs which are important for guiding the viral translation and viral replication processes, respectively. These regions have been reported to be some of the most structurally conserved regions of the genome [8]. Structures in these regions have been well characterized [1, 2], making it ideal to test the validity of our method.

Our algorithms were developed using the MATLAB image processing toolbox [9]. A detailed description of the algorithms will be reported elsewhere. These include autoselection of the molecules through height and area thresholding (Fig. 2b, left), 2D morphological thinning to generate a skeleton of the molecule to analyze its branched structure (Fig. 2b, middle and right), and the generation of 'local' volume profiles along the length of the molecule (Fig. 2c). By plotting the 'local' volume versus the length along the molecule, we can get an idea of the domain structure (Fig. 2c, left). Furthermore, the nucleotide number of each domain structure could be predicted based on the cumulative volume along the chain (Fig. 2c). For the molecule displayed in Fig. 2c, three of the four domains identified corresponded reasonably well to previously reported domains in the HCV genome, including the internal ribosome entry site (IRES) (domain#1), stem loops V-VI (domain #2), and the 3′X RNA (domain #4) located at the end of the genome which is located at the end of a notable single-stranded polyU/UC region (Fig. 2c, right). However, the 5BSL and VSL regions appeared to be contained within a domain structure that encompasses more of the coding region of the genome (domain #4, Fig. 2c, right).

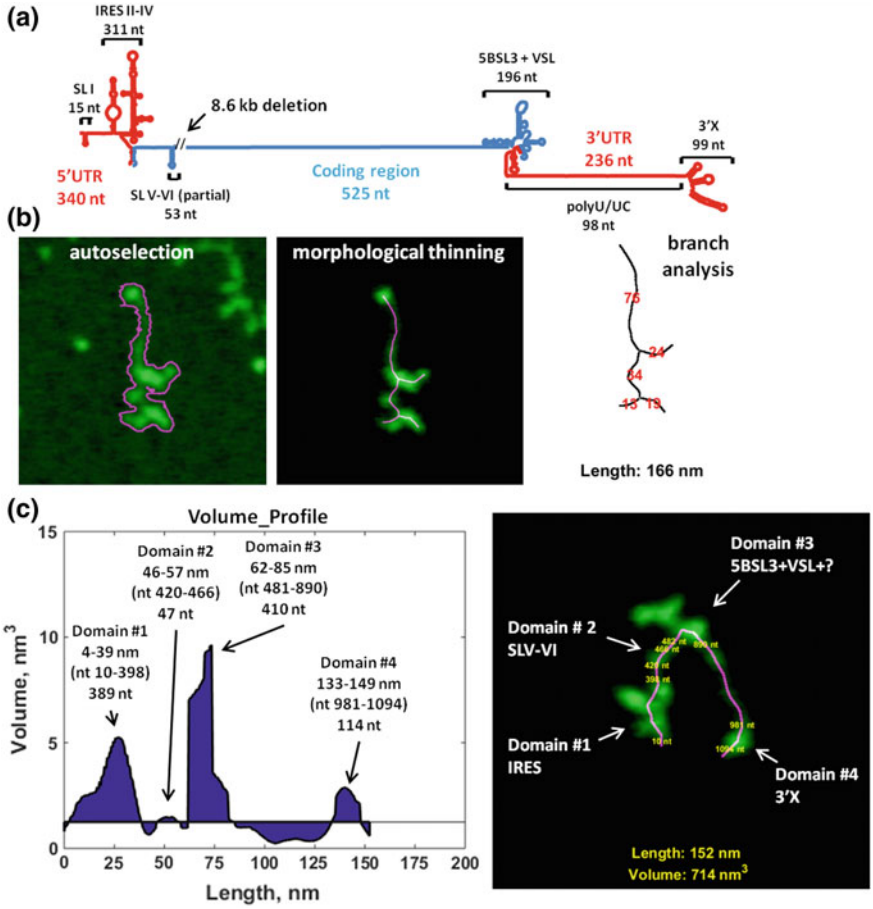

Fig. 2 Automated MATLAB-based algorithms to analyze a 1. 1 kb JFH-1 HCV deletion mutant with 8.6 kb of the coding region deleted. **a** Model of the HCV deletion mutant with well characterized structures labeled. **b** Automated data analysis processes for the deletion mutant include autoselection of the molecule, morphological thinning to produce a skeleton, and branch analysis. The length of each branch in nanometers in displayed in red and the total length is recorded at the bottom of the image. **c** On the *left*, a profile of the 'local' volume along the main chain detects four domains when a threshold of 1.25 nm^3 is used. The profile was generated by identifying the longest end-to-end chain in the skeleton previously generated by morphological thinning. All pixels in the original autoselected region were then assigned to a pixel in the main chain through an image transformation process which iteratively assigns nearby pixels a value corresponding to the value of the line pixel. The volume was then summed for all pixels which were 'local' to the line pixel and the length of the line was determined using a geodesic quasi-Euclidean distance transform and then converted to nanometers according to the image dimensions in order to generate the graph of 'local' volume versus length. The corresponding molecule is shown on the *right* with the predicted domains based on the predicted nucleotide number determined according to the cumulative volume along the main chain. Images are displayed using the MATLAB default falsecolor visualization (*green-magenta*) to overlay the *lines* onto the original image. Image dimensions are 200 × 200 nm^2

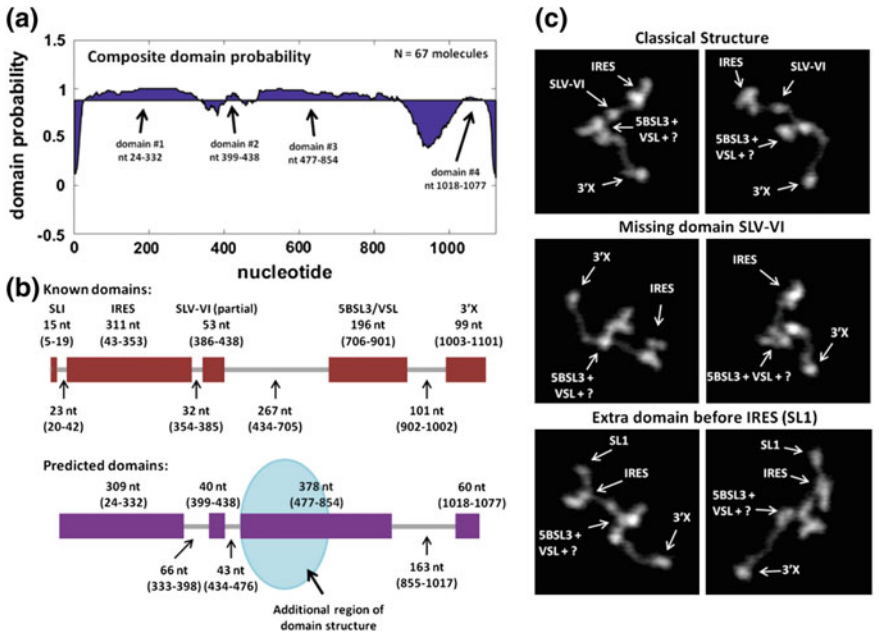

Fig. 3 Composite domain analysis in a JFH-1 HCV deletion mutant for N = 67 molecules. **a** The probability that a domain will be detected at each nucleotide value was calculated and plotted. The edges of the graph falsely have very low probabilities due to low height at the edge of the molecules. **b** Regions of expected structure based on previously described structures are shown on *top* and the predicted regions for each domain based on the domain probability in **a** are displayed below. **c** By sorting the molecules in the dataset, examples of conformational flexibility are observed. Image dimensions are 200 × 200 nm^2

By calculating the composite probability that a domain will be detected at each nucleotide for N = 67 molecules (Fig. 3a), another ~230 nt in addition to the 196 nt reported for the 5BSL and VSL regions (Fig. 3a, b) was predicted by our analysis, although this still remains to be confirmed for the full length HCV genome. Notably, this domain usually had an extended two-lobed structure suggesting that it is composed of 'subdomains'. The predicted nucleotides for each domain often vary from the predicted structures by as much as 50 nt, however there may be variations in the predicted value depending on how the domains may be oriented on the mica surface, and on the threshold used to define the region of domains. Additional sorting of the molecules also allows for alternative conformations of the molecules to be detected (Fig. 3c). For example, although many of the molecules in our dataset had the classical four domain structure, the second small domain (SLV-VI) was sometimes missing from a subset of the molecules. Additionally, in some molecules, the 15 nt SLI domain which precedes the IRES could be detected as a separate domain.