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Commentary: Can AFM Reveal Global Viral RNA Structure?

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A variety of structural motifs in single-stranded viral genomes have been shown to play important roles in guiding key steps in the lifecycle of many viruses [1]. However, most viral genomes are in excess of 1 kb in length, and available technologies generally lack the ability to study the global structural arrangement of long RNA molecules in a single experiment, causing the structures of many RNA molecules to be gradually pieced together over time. To address these issues, we have developed a new high-throughput method using Atomic Force Microscopy (AFM) imaging combined with automated analytic tools to extract information about the global secondary and tertiary structure of long single-stranded nucleic acids (>1 kb), mainly focusing on the 9.7 kb Hepatitis C virus (HCV) genome [2,3]. In recent years, the importance of gaining a detailed understanding of the viral lifecycle and the molecular structures guiding viral processes is reflected in the development of targeted antivirals against the NS3 protease, the NS5B polymerase, the NS5A proteins, cyclophilin A, and miRNA-122 [4]. Our method shows great promise for being able to further refine the molecular details of viral processes throughout the lifecycle of the virus, which can be instrumental in the refinement of antiviral strategies.

AFM Imaging and Analysis of Viral RNA Structure

Recently, we reported a method to reproducibly image the secondary or tertiary structure of single-stranded RNA molecules using AFM [2,3]. Of particular interest were the secondary structures, which exhibited readily observable reproducible features in the images. Since RNA folding is largely hierarchical [5,6], it is expected that the majority of secondary structures will be retained upon formation of the tertiary structure, so analyzing the arrangement of structural features in these molecules should yield structurally relevant information. Using this method, we have been able to obtain images of the secondary structures of a variety of RNA molecules ranging in size from 1.1 to 9.7 kb [2,3].

Following the development of this new methodology, new tools to analyze the structural features of the molecules in the images were needed. To address this issue, a series of automated MATLAB-based algorithms were developed for extracting information about the branched domain architecture of RNA molecules. Notably, after confirming that there is a linear dependence of the molecular volume on the number of nucleotides in the molecule using the Gwyddion software [2], we developed an algorithm in MATLAB to generate local volume profiles along the longest end-to-end chain identified from skeleton representations of the molecules as a way to detect domains in the molecule and estimate the number of nucleotides contained in each domain [3].

Identification of Domain Structure in HCV RNA

These methods have effectively been used to identify the well-known structural domains in a deletion mutant of the hepatitis C virus (HCV), including the internal ribosome entry site (IRES), a small domain corresponding to a partial SLV-VI domain, the 3'X RNA, and the poly(U) region **(Figure 1A).** Additionally, we identified a large bi-lobed domain comprising nucleotides corresponding to the 5BSL and VSL regions in addition to ~230 upstream nucleotides corresponding to the NS5B region, suggesting that these regions of the molecule may localize into a single domain structure [3]. These findings demonstrate the ability of AFM to identify the

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conserved domain structure present in RNA molecules and provide reasonable estimates of the number of nucleotides contained in each one. In addition, AFM has the ability to identify new domains or examine the general localization of a range of nucleotides into a single domain ultrastructure. Similar domains can also be observed in the full length HCV RNA **(Figure 1B).**

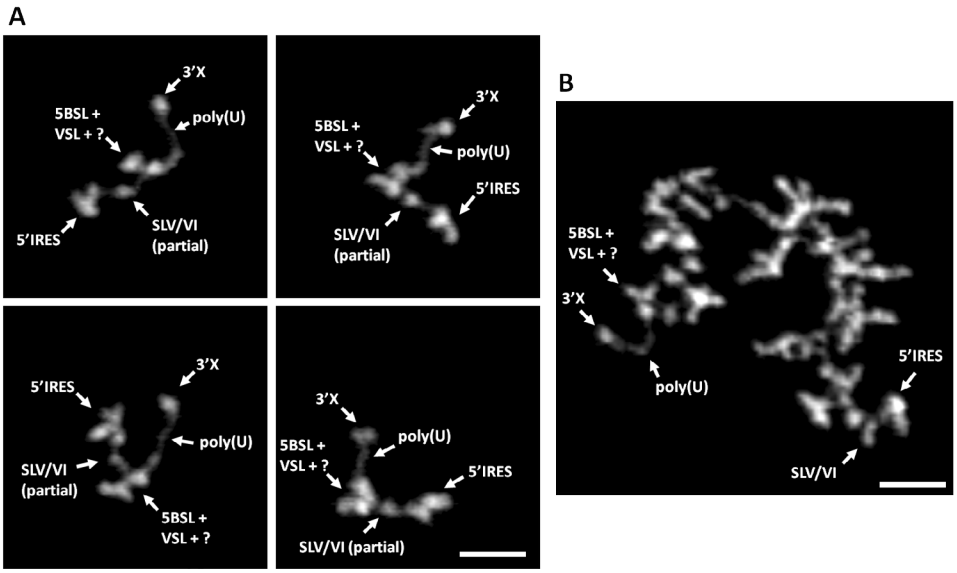

Figure 1. (A) HCV deletion mutant RNA (1.1 kb) containing the 5' UTR and 3' UTR of the HCV genome (8.6 kb of coding region deleted). Structures identified by local volume profiles are labeled. The 5' structures include the internal ribosome entry site (IRES) and a partial structure of stem loops V/VI (remaining part cut off by deletion). 3' structures include the 3'X, and poly(U). The large structure in the center contains regions corresponding to 5BSL and VSL structures with additional volume suggesting another ~230 nt of the NS5B coding region.
(B) HCV full length RNA (9.7 kb) with labeled structures corresponding to those in the deletion mutant. Scale bars=50 nm.

The ability to recognize the domain structure of RNA in our images opens up a number of exciting applications for how AFM technology can be used for RNA studies in the future. The long term goal of these methods is to reconstruct the various steps of the viral lifecycle in order to observe how the RNA domains are involved in those steps as a way to gather intelligence which can help us combat viral infections. In the short term, many improvements to the experimental methodologies and data analysis algorithms can help make this vision a reality.

Building an Automated Molecular Pattern Recognition Algorithm

The ultimate goal of our method is to automate the data analysis process as much as possible in order to create a high-throughput technique and to effectively perform pattern recognition on the molecules in our images. These pattern recognition procedures could be used to sort the molecules into groups based on their configurations in the images. It has been widely reported that RNA can fold into a diverse range of conformations which can influence the activity of the molecules [7]. This type of analysis can help to identify dominant and minor RNA conformations observed in the images. It is possible that certain structural transitions may direct a switch between different steps of the viral lifecycle, and thus targeting them might be a viable antiviral strategy. These pattern recognition procedures can be geared both towards analyzing the global domain architecture of RNA molecules in addition to assessing the conformational flexibility of individual domains. Towards this goal, processes to assess the shape and orientations of each individual domain should be added. In addition to characterizing the secondary structural domains, 3D algorithms to assess the compact tertiary structures of the folded molecules in Mg^{2+} buffer should also be developed. From this type of analysis composite models detailing the range of conformations that these molecules can adopt can be generated. These composite models can be refined by augmenting our models with additional RNA structure prediction methods. For example, the structural information obtained can be used as constraints when generating computational predictions of the secondary structure [8], and these predictions can be further confirmed by chemical mapping [9]. Then, the topology of the composite shape(s) of each domain can be used to predict how the secondary structures might be arranged in 3D space. Additionally, 3D structures from X-ray crystallography, NMR, cryo-EM, or small angle X-ray scattering, which are generally easier to obtain with smaller RNA fragments [1], can use structural models generated from AFM as a kind of outline into which the individual pieces can be fitted.

From Global Structure to Functional Role

Once the global structure of the molecules has been characterized, we can then turn to understanding the functional role that each structural component plays in regulating the viral lifecycle. One of the most straightforward ways to do this is to add various host and viral proteins or cofactors known to be involved in the various stages of the viral lifecycle in order to observe their mode of interaction with viral RNA domains. Using HCV as an example, the mechanisms by which various cellular factors bind to the IRES and alter its conformation to enhance or disrupt internal initiation of translation could be investigated [10]. In addition to characterizing the structures formed by these complexes, the dynamics of the binding events can also be visualized by imaging in buffer solution using high-speed AFM (hsAFM), with typical imaging rates of 1-2 frames/s [11,12]. Also, since AFM has also been

extensively used to investigate the structure of proteins in native or reconstituted membrane systems [13-15], this method can also be used to characterize the arrangement of components of the membrane-associated replicase complex in membrane fractions isolated from cells expressing HCV nonstructural proteins [16]. Since membrane fractions isolated from these cells have been shown to be able to synthesize RNA for *in vitro* replication assays, it should be possible to add RNA to these systems in order to directly visualize the steps involved in the synthesis of negative strand intermediates as well as new positive strand genomes. Technology such as recognition imaging [17,18] or the newly developed BIXAM confocal-hsAFM [19] can aid in identifying the proteins participating in the various activities during this process. Also, the steps of viral assembly can also be tracked by visualizing the interaction of the HCV core proteins with the RNA [20]. In addition to identifying the molecular details involved at various steps of the virus lifecycle, these methods can also be used as assays to screen for inhibitors of these processes which can greatly aid in the identification of new antivirals.

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