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Asymmetric packaging of polymerases within vesicular stomatitis virus ☆

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ABSTRACT

Vesicular stomatitis virus (VSV) is a prototypic negative sense single-stranded RNA virus. The bullet-shape appearance of the virion results from tightly wound helical turns of the nucleoprotein encapsidated RNA template (N-RNA) around a central cavity. Transcription and replication require polymerase complexes, which include a catalytic subunit L and a template-binding subunit P. L and P are inferred to be in the cavity, however lacking direct observation, their exact position has remained unclear. Using super-resolution fluorescence imaging and atomic force microscopy (AFM) on single VSV virions, we show that L and P are packaged asymmetrically towards the blunt end of the virus. The number of L and P proteins varies between individual virions and they occupy 57 ± 12 nm of the 150 nm central cavity of the virus. Our finding positions the polymerases at the opposite end of the genome with respect to the only transcriptional promoter.

1. Introduction

Vesicular stomatitis virus (VSV) possesses a single-stranded negative sense RNA genome and is the prototypic model for understanding transcription and replication of potent human pathogens including Ebola and rabies. VSV is also an effective oncolytic agent since some attenuated VSV strains replicate preferentially in malignant cells [1]. Taken together, VSV is emerging as a useful model and potent tool in the arsenal of modern medicine.

VSV virions are 180 nm long and 80 nm wide and resemble a bullet with one tapered and one blunt end. The VSV genome is tightly encapsidated by nucleoprotein (N), forming the N-RNA genome template. Recent CryoEM studies showed N-RNA forms a left-handed helix that winds around a cavity, with the 3′ end at the tapered end and the 5′ end at the blunt end [2–4] (please see corrections to ref 5 for the correct RNA orientation). Like all other non-segmented negative sense (NNS) RNA viruses, VSV packages multiple copies of a polymerase that consists of the L protein catalytic subunit (241 kDa) and a template-binding P protein subunit (30 kDa). The polymerases transcribe and replicate the N-RNA genome by engaging at promoter sites at or near the 3′ end of the genome template [5].

On average ~50 L and ~400 P proteins are packaged within each virion [6]. The position of the L and P proteins within the bullet shaped virion has not been established previously. L organizes itself into a ring structure with an approximate diameter of 10 nm and does not bind the template by itself [7–9]. P proteins form dimers through their central domains and also bind L and the N-RNA template [8,10,11]. In recent CryoEM studies of VSV [3], either symmetry mismatches between the N-RNA helix, L and P, or random positioning of L and P, resulted in averaging out of the L and P density within the central cavity of VSV. Therefore to resolve the position of L and P, single virion imaging techniques with sufficiently high resolution are required.

While the resolution of simultaneously imaged multiple fluorophores is limited by diffraction, the position of a single fluorescent

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molecule can be localized with nanometer precision. The precision of localization is inversely proportional to the square root of the number of photons collected from the single molecule [12]. It is therefore possible to reconstruct an image by successively photo-activating subsets of molecules that are further apart from each other than the diffraction limit and localizing their positions with nanometer precision. This principle was developed in photo-activatable localization microscopy (PALM, [13]) fluorescence photoactivatable localization microscopy (fPALM, [14]) and stochastic optical reconstruction microscopy (STORM, [15]) to achieve in plane resolutions of ~20 nm. The axial resolution of these techniques can be extended either through introduction of astigmatism associated with the out of plane images [16] or using Biplane imaging [17] both of which report an axial resolution of ~50 nm.

Although fluorescence is specific, it does not report the density of unlabeled protein molecules. Atomic force microscopy (AFM) is sensitive to the overall protein density, via changes in the mechanical properties of the sample: the protein density within an object can be probed by measuring the deformation of a sample in response to a force applied by the AFM cantilever. AFM has been used in this way to measure the stiffness of single virions [18–20]. In general, the non-enveloped viruses are very stiff with a Young's modulus in the range of GPa [20] while the enveloped viruses can be an order of magnitude softer in the range of 100 MPa [18].

Since the N-RNA is packaged with specific orientation of its 5′ end at the blunt end of the virion [3] and since the polymerases can only engage a promoter at the 3′ end [21,22], positioning of the polymerases within the bullet will inform our understanding of the early transcription of the N-RNA genome immediately upon delivery to the host cytoplasm and to some extent the final moments of packaging the genome in budding virions.

2. Materials and methods

2.1. Super-resolution imaging and fluorescence localization

Images were recorded with a SR 200 microscope (Vutara, Inc.) based on the Biplane approach [17]. The envelope of VSV virions was mapped by labeling ~75% of the VSV-G protein on the exterior of the virion with Alexa-Fluor®647 (Invitrogen A21245). Alexa fluorophores can be efficiently initialized to their dark state in imaging buffer [SI] and can then be photo-activated through application of 405 nm UV light. This method was used to generate the viral envelopes shown in Figs. 1 and 2.

To locate the internal proteins within individual virions, we used recombinant viruses encoding enhanced Green Fluorescent Protein (eGFP) linked to the viral proteins P or L. In these recombinant viruses, all of the P or L proteins are respectively replaced by P linked to eGFP (eGFP-P) or eGFP linked to L (L-eGFP) and these viruses have been previously characterized [23,24]. The center of mass of the eGFP labeled proteins was determined in each virion and its relative distance to the center of the high resolution envelope was measured. The eGFP proteins associated with these internal proteins were not photoswitched, rather their total fluorescence intensity and the position of their center of fluorescence with respect to the center of the envelope was determined.

Excitation and activation of single fluorescent molecules was achieved via a speckle-free illumination with an even intensity distribution, which was realized by a specialized beam homogenizer. Two color channels were detected sequentially at 50 frames/s. eGFP data was collected first over 500 frames at 30% power (1.2 kW/cm^2), then Alexa 647 was collected over 15,000 frames at 100% power (4 kW/cm^2). Data analysis was performed using the Vutara SRX software (Version 4.01).

2.2. Validation of super-resolution imaging method

First we have validated the resolution of the Biplane microscope by imaging beads on the microscope for 1000 imaging frames with an average fluorescence signal of 500 photons (comparable to our sample fluorescence). The analysis of this data as shown in detail in (SI) shows that we can localize these beads with less than 10 nm precision in *XY* and 25 nm precision along the optical axis. Resolution of the microscope is defined by the full width half maximum analysis and is 20 nm in plane and 50 nm along the optical axis.

Since the VSV G proteins are uniformly distributed on the surface of the envelope, we used them for our control experiments. VSV virions were pseudotyped with eGFP linked to the C terminus of VSV G protein (SI). Since virions are assembled on different cells within the population with varying levels of expression of VSV G-eGFP expression, the collected virions have a distribution of G-eGFP incorporation between virions. Each virion incorporates 1200 copies of G independent of whether the G has a GFP tag or not and G proteins are uniformly distributed on the surface of each virion [6]. We expect that the center of fluorescence of G-eGFP to

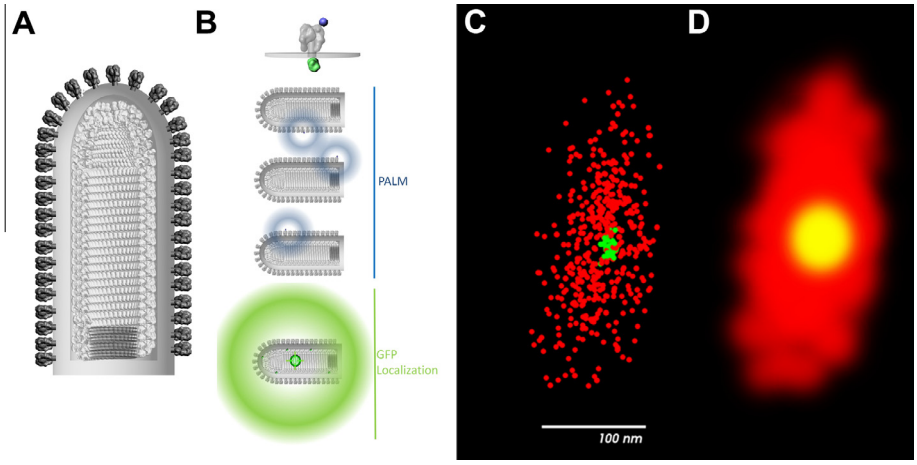

Fig. 1. Locating eGFP center of fluorescence within reconstruction of VSV envelope. (A) A model of VSV virion constructed partly from CryoEM data [3]. (B) VSV-G is tagged from the exterior with Alexa 647 and internally with eGFP, ensuring that the eGFP center of fluorescence coincides with the center of the virion. (C) Super-resolution fluorescence microscopy results in localization of VSV-G proteins (Red) and location of the center of fluorescence of eGFP (green). (D) Volumetric rendering of the VSV envelope from super-resolution fluorescence imaging data along with the location of the center of fluorescence of eGFP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)