Published in final edited form as:

Methods. 2013 February ; 59(2): 165–166. doi:10.1016/j.ymeth.2013.02.013.

“Methods to Study RNA Virus Molecular Biology”

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Introduction

Since the discovery of the first RNA-containing viruses near the end of the 19th century, researchers have been continuously developing biological systems and *in vitro* methodology to understand how these ubiquitous, highly-mutable pathogens are able to infect and replicate in nearly all living organisms. Beginning with studies that developed animal models and cell culture assays to allow the growth, isolation, and purification of many different RNA viruses, investigators in the field of RNA virology devised or implemented novel experimental approaches to understand the molecular underpinnings of virus-cell interactions, viral gene expression and RNA replication, viral modulation of host immune functions, and the pathogenic effects of RNA viral infections on cells, tissues, and organisms. In this special issue of *Methods*, we have highlighted a number of state-of-the-art research methodologies to address fundamental questions in the biology of positive strand RNA viruses, negative strand RNA viruses, and double strand RNA viruses. Although each article illustrates methodologies developed to study specific groups of RNA viruses, a number of these experimental approaches are broadly applicable to many different RNA viruses. Starting with methods used to study protein synthesis induced by positive strand RNA viruses that encode internal ribosome entry sites (IRESes), Jan and colleagues describe genetic constructs and sensitive reporter assays to assess the activities of viral IRES elements *in vitro* and in transfected cells. They also detail biochemical “toeprinting” approaches to identify translation initiation sites on viral mRNAs that contain IRES elements. Although their studies focused on an insect virus that employs IRES-mediated translation initiation, the methodologies described by Jan et al. can be readily applied to study IRES translation mechanisms of many other viral and cellular mRNAs. Continuing the thread of molecular approaches to study viral protein synthesis, Hodges and Connor present methods to study translational regulation of negative strand RNA viruses (e.g., rhabdoviruses and bunyaviruses) in cell-free assays as well as in metabolically-active animal cells in culture. They describe radioactive as well as non-radioactive experimental approaches to labeling proteins in uninfected and virus-infected cells, allowing a global analysis of newly synthesized proteins during infection as well as a single cell distribution of newly synthesized viral proteins. Hodges and Connor also outline techniques to analyze the effects of negative strand RNA viruses on the phosphorylation-dephosphorylation states of canonical translation initiation factors during infections of cultured cells, techniques that can be used to study such effects that might be induced by many different viruses.

Turning to steps in viral gene expression that occur prior to translation and its subsequent regulation, Ogino describes *in vitro* methodologies aimed at analyzing transcription and capping activities in the presence of recombinant forms of the large RNA-dependent RNA polymerase molecule (L) encoded in the negative strand genomic RNAs of rhabdoviruses like vesicular stomatitis virus and rabies virus. These sensitive *in vitro* assays have been invaluable in dissecting some of the mechanistic steps involved in generating 5' capped mRNAs and in determining the functional domains of the L polymerase that are responsible for this capping activity. Such experimental approaches should be readily adaptable to studies of the L polymerase functions of other non-segmented negative strand RNA viruses (e.g., paramyxoviruses).

For segmented RNA viruses that have a double-stranded genome (e.g., reoviruses and rotaviruses), experimental methodologies for introducing targeted genetic lesions into genomic RNAs have made great leaps forward during the past five years. As detailed in the article by Patton and colleagues, the different approaches that have been described for distinct members of this group of viruses involve transfection of multiple plasmid DNAs harboring cDNAs corresponding to individual dsRNA segments, transfection of multiple mRNAs derived from *in vitro* transcription of plasmid DNAs encoding sequences for each genome segment, or by transfection of plasmid DNA corresponding to a single dsRNA segment followed by infection with a helper virus. These so-called “reverse genetics” methods described by Patton and co-workers provide facile new approaches to generating mutant or recombinant viruses which can subsequently be used to analyze the functions of individual viral proteins and RNA sequences in the different steps of the viral replication cycle. Those advances now bring to all classes of RNA viruses an approach first established for the bacteriophage Q β by Charles Weissmann and colleagues in 1978. Such methods strongly complement the application of deep sequencing methodologies that are revitalizing more traditional forward genetic approaches to the study of RNA viruses.

A crucial aspect of RNA virus replication is the requirement for host cell functions that these viruses use to compensate for their relatively simple genetic complexities. As a result, viral genomes have evolved the capacity to hijack specific host cell activities, to utilize host RNA binding proteins for non-canonical functions, and to directly or indirectly impact host cell metabolic functions ranging from transcription to nuclear export/import to cytoplasmic signaling. To identify host genes and proteins impacted by virus infections, numerous large scale screening methodologies have been developed during the past two decades. Two such approaches are described in this volume. The first, by Nagy and co-workers, details the use of a temperature-sensitive library of essential yeast genes to screen for host factors that have roles in positive strand RNA virus replication. In this case, the virus studied is a plant virus (tomato bushy stunt virus) capable of replication in yeast. Given the common features of gene expression and RNA replication employed by all positive strand RNA viruses, it is clear that some of the host factors identified through high throughput screens of yeast will be important for viruses that infect plants or animals. In the second approach, described by Chin and Brass, an RNA interference-based screen of human cells infected with influenza virus is combined with high throughput imaging to identify host genes that affect replication of this negative-strand RNA virus. Here the authors systematically outline the steps for

setting up and executing the primary screen, for validating the screen and prioritizing putative candidate genes, and for validating candidate genes that will ultimately lead to mechanistic studies to determine how these gene products are used in influenza replication or how they down-modulate the viral infection. Chin and Brass also compare their experimental scheme with four other RNA interference-based screens to identify host factors required during influenza virus infections and suggest that such methodology may lead to the development of new anti-viral targets aimed at the influenza virus-host interface.

The innate immune response to RNA virus infections is a key host defense mechanism that is targeted by certain viruses during infection. A key player in orchestrating the innate immune response is interferon regulatory factor-3 (IRF-3), a transcription factor that is activated by phosphorylation which results in protein dimerization and transport to the nucleus. Methods have previously been developed to monitor the state of IRF-3 during viral infection, but a facile flow cytometry assay for this purpose has been lacking. Given that such an assay is dependent on highly specific antibodies to IRF-3, Gale and colleagues embarked on the production of monoclonal antibodies (mAbs) to recombinant human IRF-3 to be used in flow cytometry in concert with other experimental approaches. Using Sendai virus (a negative strand RNA virus) as an activator of IRF-3 in infected cells, Gale and co-workers characterize two different mAbs that have differential reactivities to native versus activated IRF-3 as well as to IRF-3 derived from non-human primates. These novel antibodies and the methodology described for their use should provide important tools for developing IRF-3 agonists in the context of antiviral drug development.

In the final two articles of this volume, we turn to new methods aimed at understanding the entry, replication, and pathogenesis of hepatitis C virus (HCV). Given the prevalence of HCV infections worldwide and the morbidity associated with these infections, the development of novel anti-viral therapeutics and possible vaccine strategies is a major focus of a large number of investigators. During the past 15 years, the study of HCV replication in cell culture and in animal models has undergone a remarkable methodological transformation. In the article by Vieyres and Pietschmann, several experimental breakthroughs are reviewed to set the stage for their detailed descriptions of cell culture assays for HCV entry and replication. The authors enumerate different virus-host systems and reporter assays to analyze specific steps in viral entry. Vieyres and Pietschmann also describe different inhibitors and fusion assays to reveal new mechanistic insights about the interaction of HCV virions with cell surface receptors and subsequent internalization to initiate the cytoplasmic phase of replication. In an effort to study HCV entry beyond cell culture models, Ploss and colleagues describe a genetically humanized mouse model that recapitulates HCV entry into liver cells. Using recombinant adenoviruses to express species-restrictive cell surface proteins derived from humans, the authors detail how to carry out intravenous infections with recombinant isolates of HCV that express the cre recombinase. Productive infection generates a reporter signal in infected hepatocytes that can be detected in the intact mouse or by flow cytometry. Ploss and co-workers suggest that genetically humanized mouse models for HCV provide valuable new tools for developing new HCV inhibitors to target viral entry and for testing vaccine candidates in animals other than chimpanzees.

Collectively, the experimental methods and biological systems described in this volume span a diverse array of RNA viruses to address molecular mechanisms used by these viruses to gain entry into cells, to carry out intracellular viral gene expression and RNA replication, and, ultimately, to generate progeny virions. It is our hope that readers will find this issue to be a valuable compendium of up-to-date, practical information for the study of RNA virus molecular biology that brings cutting-edge technology to address fundamental questions in RNA virus-host interactions.